Neural circuit mapping of orexigenic systems

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Cover illustration: Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) expressed in the neurones in the arcuate nucleus. Original image taken by Iris Stoltenborg.

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To my blood and chosen family

“The more you know, the more you know you don’t know”

Aristotle

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ABSTRACT

Orexigenic systems in the brain process appetite and/or hunger information and ultimately coordinate feeding responses. These brain circuits are critical for survival and originally evolved to ensure that we consume sufficient amounts of diverse nutrients to survive future famines. External and internal signals, such as food availability and energy state, converge on the orexigenic brain systems. Imbalances or faulty processing of these inputs can result in disordered eating behaviour. Examples are hormonal imbalances of satiety or hunger signals and aberrant responses to food-predicting cues in certain brain areas in people suffering from obesity on one hand of the body weight spectrum and anorexia nervosa on the other. Eating disorders impact on health and hence, place a huge financial burden on society. Currently, we lack effective treatments for the full spectrum of eating disorders. To impact, we need better non-invasive and effective treatments. The first step towards this goal is to better identify the brain targets, genes and mechanisms driving feeding behaviours. With this ultimate goal in mind, we explored the role of different orexigenic systems in feeding behaviour and aimed to identify the neuronal substrates involved.

Firstly, we found that olfactory food cues increase food intake and food seeking in rats. The cue engaged the hunger-signalling ghrelin system and activated a diverse population of cells in the arcuate nucleus of the hypothalamus, an important hub in the feeding circuitry. Secondly, we showed that *Ghsr-IRES-Cre* mice are a model for deficient ghrelin signalling (evidenced by the absence of ghrelin-induced food intake and decreased *Ghsr* mRNA expression in these mice) and that disrupted *Ghsr* expression rendered

compromised growth and metabolic response to fasting. Thirdly, chemogenetic activation of a ghrelin-responsive ensemble in the arcuate nucleus was sufficient to drive feeding-related behaviours (food intake, food motivation, food choice) and condition a place aversion in mice. Finally, we found that the lateral hypothalamus was potently activated in the activity-based anorexia (ABA) mouse model (in which mice are severely energy depleted), including cells expressing orexin. Inhibition of orexin signalling using the clinically approved dual orexin receptor antagonist suvorexant suppressed food anticipatory running wheel activity.

Altogether, the work presented in this thesis shows that the arcuate nucleus plays an important role in processing hunger and appetite signals. Our data highlight the importance of this brain area in relaying sensory (food cues) and hormonal (peripheral ghrelin) inputs, ultimately driving feeding-related behaviours and conveying ghrelin's negative valence. We also found that the orexigenic ghrelin signalling system plays a pivotal role in growth and in the metabolic response to a fast. Finally, data from our ABA experiment in mice suggest that suvorexant could potentially be used in anorexia nervosa patients to reduce orexin signalling and herewith aiding them to reduce hyperactivity and promote recovery.

Keywords: orexigenic, ghrelin, feeding, food-cue, arcuate nucleus, lateral hypothalamus, orexin, activity-based anorexia

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SAMMANFATTNING PÅ SVENSKA

Orexigena system i hjärnan bearbetar information om aptit och/eller hunger och koordinerar slutligen ätrespons. Dessa hjärnkretsar är avgörande för överlevnad, och utvecklades ursprungligen för att säkerställa att vi konsumerar tillräckliga mängder föda för att överleva en framtida hungersnöd. Externa och interna signaler såsom tillgänglighet på mat och energibehov signalerar till de orexigena hjärnsystemen. Obalans eller felaktig tolkning av dessa signaler kan resultera i en ätstörning från båda ändarna av viktspektrumet, från fetma till anorexi nervosa. Exempelvis kan det finnas obalans i hormonnivåer som styr mättnads- och hungersignaler, eller feltolkning i vissa hjärnområden som bearbetar matrelaterade signaler från omgivningen såsom till exempel lukter och synintryck. Ätstörningar orsakar inte bara lidande för individen utan också samhälleliga hälsoproblem med stora ekonomiska kostnader. Ökad kunskap kring aptitreglerande hjärnkretsar är nödvändigt för att upptäcka och utveckla nya effektiva icke-invasiva behandlingar som idag saknas. Med siktet inställt mot detta mål har vi i denna avhandling undersökt betydelsen av olika orexigena system för beteenden som relaterar till förändrat födointag och strävat efter att identifiera vilka hjärnkretsar som är inblandade.

Våra studier har visat att matlukter från omgivningen ökar både födointag och sökande efter föda hos råttor. Matlukter aktiverade både hungerhormonet ghrelin samt en grupp nervceller i hungercentrat arkuatuskärnan i hypotalamus. Vi har även funnit att *Ghsr-IRES-Cre*-möss saknar fungerande ghrelinsignalering och kan därmed användas som en modell för att studera ghrelinets funktion i bl.a. orexigena hjärnkretsar. *Ghsr-IRES-Cre*-mössen uppvisade en hämmad tillväxt och ett hämmad metabolt svar på fasta. Vi har vidare funnit att kemogenetisk aktivering av en population ghrelinkänsliga nervceller i arkuatuskärnan ensamt kan driva födointag, motivationen att äta samt påverka valet av mat. Dessutom leder denna aktivering till betingad aversion hos möss. Dessa fynd belyser vikten av arkuatuskärnan som en relästation som förmedlar vidare signaler från ghrelin i blodet till hjärnan för att bl.a. driva födointag anpassat till individens behov samt att förmedla känslomässigt obehag vid hunger. Slutligen har vi visat att laterala hypotalamus, inklusive orexinneuron, är kraftigt aktiverad hos anorektiska möss (anoreximodellen ABA), och att hämning av orexinsignalering med det kliniskt godkända läkemedlet suvorexant (dubbel orexinreceptorantagonist) minskade mössens normalt höga aktivitetsgrad innan måltid, vilket öppnar upp för att närmare studera orexin som en behandlingsmöjlighet av patienter med anorexia nervosa.

Sammantaget visar denna avhandling att arkuatuskärnan är viktig för att bearbeta hunger- och aptitsignaler. Denna del av hjärnan tycks förmedla sensoriska (matsignaler) och hormonella (ghrelin) signaler, vilket i slutändan driver på födointag, sökande efter mat samt motivation till att äta. Dessutom signalerar ghrelin känslomässigt obehag vid hunger. Vi fann också att ghrelinsignalering spelar en avgörande roll för tillväxt och metabolt svar på fasta. Slutligen tyder resultaten från våra anorektiska möss att suvorexant potentiellt skulle kunna användas som behandling av patienter med anorexia nervosa för att minska orexinsignalering och därmed fysisk aktivitet.

NEDERLANDSE SAMENVATTING

Orexigene systemen in de hersenen verwerken eetlust- en/of hongerinformatie en coördineren uiteindelijk ons eetgedrag. Deze hersencircuits zijn van cruciaal belang om te overleven. Ze zijn oorspronkelijk geëvolueerd om ervoor te zorgen dat we voldoende en gevarieerde voedingsstoffen binnenkrijgen om eventuele hongersnoden in de toekomst te overleven. Externe signalen (zoals verkrijgbaarheid van voedsel) en interne signalen (zoals energiereserves in ons lichaam; bijvoorbeeld vetreserves) convergeren op de orexigene hersensystemen. Het uit balans zijn of foutieve verwerking van deze inputs kunnen leiden tot verstoord eetgedrag. Twee voorbeelden hiervan zijn (i) hormonale afwijkingen van honger- of verzadigingssignalen (zoals ghreline of leptine) en (ii) afwijkende reacties van bepaalde hersengebieden op voedselvoorspellende signalen door bepaalde hersengebieden. Dit kan het geval zijn bij mensen met obesitas aan de ene kant van het gewichtspectrum, en anorexia nervosa aan de andere kant van het sprectrum. Eetstoornissen kunnen leiden tot gezondheidsproblemen die gepaard gaan met financiële kosten die de samenleving belasten. Dit benadrukt hoe belanrijk het is om te begrijpen welke hersencircuits honger en eetgedrag reguleren, zodat we in de toekomst effectieve, non-invasieve behandelingen kunnen ontwikkelen voor mensen die lijden aan een eestoornis. Met dat uiteindelijke doel voor ogen hebben we in dit proefschrift de rol van verschillende orexigene systemen in eetgedrag onderzocht. Daarnaast hebben we geprobeerd te identificeren wat voor soort neuronen en substraten hierbij betrokken zijn.

Ten eerste hebben we ontdekt dat bij ratten de geur van lekker eten (een “voedsel cue”; hier de geur van pindakaas) de consumptie van hun standaard, minder lekkere voedsel verhoogt. Daarnaast verhoogt blootstelling aan de cue zoekgedrag naar voedsel. De cue verhoogt ook het ghreline (een hongerhormoon) niveau in het bloed en activeert neuronen in een klein subgebied van de hypothalamus; de arcuate nucleus, een gebied dat een essentiële rol speelt in het coördineren van eetgedrag. Ten tweede hebben we aangetoond dat zogenoemde *Ghsr-IRES-Cre* (transgene) muizen een model zijn voor verstoorde ghreline signalering. We hebben geobserveerd dat in deze muizen de expressie van de ghreline receptor *Ghsr* verstoord is. Dit heeft tot gevolg dat deze muizen een groeiachterstand hebben en de hormonale reactie na een nacht vasten abnormaal is. Deze abnormale hormonale respons leidt bijvoorbeeld tot lage glucose bloedwaardes (hypoglykemie). Ten derde laten we zien dat bij muizen het activeren van een kleine groep ghreline-responsieve neuronen in de arcuate nucleus voldoende is om eetgedrag te beïnvloeden (voedselconsumptie, motivatie voor voedsel en voedselkeuze). Onze

observaties tonen ook aan dat het activeren van deze ghreline-responsieve cellen een onprettig gevoel opwekt, gezien dat muizen minder tijd doorbrengen in een ruimte die is geassocieerd met activatie van deze cellen. Ten slotte hebben we in een muismodel voor anorexia nervosa aangetoond dat het onderdrukken van orexine signalen met een drug (suvorexant) loopwielactiviteit onderdrukt. Aangezien orexine-onderdrukkende middelen in de kliniek gebruikt worden om slapeloosheid te behandelen bij anorexia nervosa patiënten, zou het interessant zijn om te testen of deze geneesmiddelen ook helpen bij het verminderen van hyperactiviteit.

Samengevat laat het werk in dit proefschrift zien dat de arcuate nucleus een belangrijke rol speelt bij het verwerken van honger- en eetlustsignalen. Dit hersengebied lijkt sensorische (voedsel cues) en hormonale (ghreline uit de periferie) inputs te verwerken om uiteindelijk eetgedrag aan te sturen. Daarbij speelt de arcuate nucleus een rol in het opwekken van het negatieve gevoel wat gepaard gaat met honger. We hebben ook ontdekt dat ghreline-signalering een cruciale rol speelt bij groei en bij de hormonale reactie op vasten. Ten slotte suggereren de uitkomsten van onze experimenten met het anorexia nervosa muis model dat suvorexant potentie heeft als medicijn bij anorexia nervosa patiënten. Het onderdrukken van orexine-signalering zou kunnen helpen hyperactiviteit te verminderen en zo herstel bevorderen.

LIST OF PAPERS

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

I **The orexigenic force of olfactory palatable food cues in rats**
Peris-Sampedro F, Stoltenborg I, Le May MV, Sole-Navais P, Adan RAH, Dickson SL
Nutrients, 2021; 13:3101

II **Genetic deletion of the ghrelin receptor (GHSR) impairs growth and blunts endocrine response to fasting in Ghsr-IRES-Cre mice**
Peris-Sampedro F, Stoltenborg I, Le May MV, Zigman JM, Adan RAH, Dickson SL
Molecular Metabolism, 2021; 51:101223

III **TRAPing ghrelin-activated circuits: A novel tool to identify, target and control hormone-responsive populations in TRAP2 mice**
Stoltenborg I, Peris-Sampedro F, Schéle E, Le May MV, Adan RAH, Dickson SL
International Journal of Molecular Sciences, 2022; 23:559

IV **Impact of chemogenetic activation of a ghrelin-responsive neuronal ensemble in the arcuate nucleus on food intake, hunger valence evaluation and food motivation in mice**
Stoltenborg I, Schéle E, Poelman R, Adan RAH, Dickson SL
Manuscript

V **Engagement of the brain orexin system in activity-based anorexia behaviour in mice**
Schéle E, Stoltenborg I, Xie A, Peris-Sampedro F, Adan RAH, Dickson SL
European Neuropsychopharmacology, 2023; 70:63-71

CONTENT

ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
ABA	activity-based anorexia
AEBSF	4-(2-aminoethyl)benzenesulfolyl fluoride hydrochloride
AgRP	agouti-related peptide
AMP	adenosine monophosphate
Arc	arcuate nucleus
CAG	chicken beta-actin
CEPR2	closed economy progressive ratio 2
CNO	clozapine-N-oxide
CPA	conditioned place aversion
CPP	conditioned place preference
Cre	cre recombinase
DAB	3,3´-diaminobenzidine
DREADD	designer receptor exclusively activated by designer drugs
DXA	dual-energy X-Ray absopriometry
ELISA	enzyme linked immunosorbent assay
FAA	food anticipatory activity
FED3	feeding experimentation device version 3
fMRI	functional magnetic resonance imaging
FR	fixed ratio

GH	growth hormone
GHRH	growth hormone releasing hormone
GHS	growth hormone secretagogues
GHSR	growth hormone secretagogue receptor
GOAT	ghrelin-O-acyltransferase
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IRES	internal ribosome entry site
LatH	lateral hypothalamus
LEAP2	liver-expressed antimicrobial protein 2
lPBN	lateral parabrachial nucleus
LSM	laser scanning microscope
MC4R	melanocortin-4 receptor
NAc	nucleus accumbens
NPY	neuropeptide Y
PB	phosphate buffer
PBS	phosphate buffered saline
PNOC	prepronociceptin
POMC	proopiomelanocortin
PR2h	classical progressive ratio task 2 hours
PVH	paraventricular nucleus of the hypothalamus

TH	tyrosine hydroxylase
TRAP	targeted recombination in active populations
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
α-MSH	α-melanocte stimulating hormone

INTRODUCTION

This thesis concerns orexigenic systems of the brain. These are brain circuits that process appetite and/or hunger information and that ultimately coordinate the feeding response. The term "orexigenic" is comprised of 2 Greek words, "orexis" which means "appetite" and "genes" which means "producing". Orexigenic systems are critical for survival, ensuring that we search out and consume available foods and over-eat to provide sufficient energy and nutrients to survive a future famine. Thus, orexigenic systems are engaged in the response to hunger when in energy deficit but also in appetite, which can be driven not only by negative energy balance but also by learned cues that predict the palatable and desirable qualities of foods. The brain's orexigenic systems are controlled by metabolic, hormonal and neuronal signals that inform them about the status of energy balance. My thesis work has been largely focused on elucidating orexigenic systems in the hypothalamus that respond to ghrelin, a circulating hunger hormone, olfactory food cues and to severe energy deficit in an animal model of anorexia nervosa.

Eating behaviour

Typically, food intake during the day is organized into several meals, triggered by hunger and/or by the desire to eat. Satiation (i.e., the sense of fullness at the end of a meal) causes meal termination, after which feelings of satiety removes our attention from food and suppresses food intake (i.e. delays subsequent initiation of a next meal). Eating behaviour can be split into three major components: meal initiation, food choice and meal termination, regulated by different, yet partially overlapping circuits. In the past decades we have learned a great deal about the mechanisms underlying feeding behaviour. In early animal studies, brain lesions experiments were undertaken to explore the role of different nuclei of the hypothalamus in feeding behaviour, such as a study in 1951 showing that lesion of the ventromedial hypothalamus (VMH) caused excessive overeating and obesity (Anand & Brobeck, 1951). The VMH was therefore designated the role of "satiety centre". It was also found that animals with a lesioned lateral hypothalamus (LatH) starved to death; this nucleus was therefore identified as a "hunger centre" (Anand & Brobeck, 1951). It remained unclear what neurons and signal molecules in the VMH were implicated in energy balance. In 1959, parabiosis studies, in which the circulation of ob/ob and intact mice was co-joined, evidenced the existence of circulating factor(s) that regulate feeding behaviours and body weight (Hervey, 1959). In 1994 the pioneering work of Jeffrey Friedman identified the mutation

that caused obesity in the ob/ob mouse and its gene product, an adipose-derived hormone they named "leptin", from the Greek word "leptos" for "thin" (Zhang et al., 1994). This fat hormone is secreted in proportion to fat mass; it promotes satiety and energy expenditure in animals and humans (review: Zhang & Chua, 2017). Importantly, the discovery of leptin was key to identify neural pathways and molecules involved in food intake and body weight regulation. It is well known now that leptin acts on, but is not limited to, several hypothalamic nuclei, an important one being the arcuate nucleus (Arc; now an established critical hub in the feeding circuitry). The Arc is located in close proximity to the ventromedial hypothalamus (a satiety centre) and, in all probability formed part of the lesion that caused obesity in the studies of Anand and Brobeck in 1951. Later research revealed the existence of many other satiety factors with an anorexigenic (feeding suppressive) role, although only one circulating "hunger hormone" has been discovered to date, namely "ghrelin" (further discussed in the section "Ghrelin").

Orexigenic systems

Hunger is essential for survival when in negative energy balance. Destruction of hunger (AgRP; agouti-related peptide) neurones makes it difficult to initiate feeding and results in starvation (Luquet et al., 2005). There are plenty of endogenous anorexigenic substances, some examples being liver-expressed antimicrobial protein 2 (LEAP2; a recently discovered GHSR inverse agonist; Ge et al., 2018; M'Kadmi et al., 2019), the fat-derived hormone leptin and various gut-derived satiety signals such as glucagon-like peptide 1. Arguably, orexigenic systems become activated when circulating levels of these hormones are low (Jéquier, 2002). On the other hand, there is the circulating stomach-derived orexigenic hormone ghrelin (see below). The brain can also directly sense nutrients such as glucose and indirectly sense fuel availability, for instance fat reserves through the fat-derived hormone leptin. When in negative energy balance, for short or long term, nutrients and energy stores can get depleted, and low levels of anorexigenic signals can drive food intake. Aditionally, the brain also receives electical inputs from the periphery from metabolic organs (e.g. liver, fat, gut) via the vagus nerve. Via this route, the brain receives information about the state of the gastrointestinal tract such as gastric distention upon a meal or ingestion of potential toxins. This is all part of the gut-brain axis; a bi-directional interaction between the brain and the gut (Almy, 1989). Gut to brain signals consist of hormones, nutrients, microbial products, cytokines, immune cells and metabolites, relayed by the blood circulation, vagal afferent or the spinal cord (review: Mayer, 2011). Regulation in the other direction, brain to gut, encompasses regulation of gastrointestinal function by aspects such as cognition (such as learned food cues, see below),

stress and emotion (Al Omran & Aziz, 2014). In this thesis, we focus on hunger rather that satiety signalling and we define pro-appetite stimuli and their underling neuronal networks as orexigenic systems (Figure 1). Insights into neuronal circuits underlying energy balance and feeding are required to better understand eating disorders such as anorexia nervosa, binge eating and obesity. A better understanding of the aetiology will hopefully, eventually, lead to the development of effective treatments.

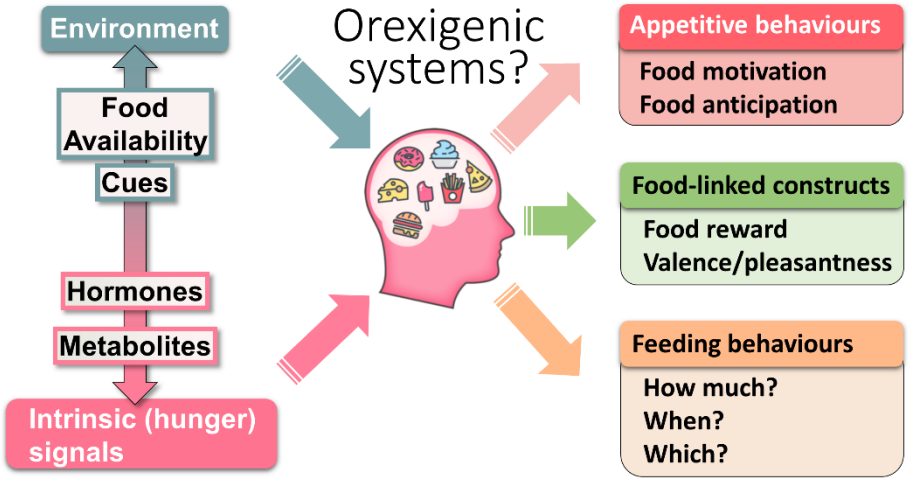


Figure 1. A schematic depicting the inputs and outputs of regulation of feeding behaviour. What are the underpinning orexigenic systems? *The following methods are examples of ways to measure the outcomes in rodents. Food motivation can be measured with an operant responding task and food anticipatory activity indicates food anticipation. Food reward and valence can be measured using conditioned place preference/aversion and feeding behaviours simply by monitoring food intake.*

A troubled orexigenic system in eating disorders?

A number of studies have explored associations between circulating hormones regulating food intake on meal initiation in those suffering from eating disorders – from obesity at one end of the body weight spectrum to anorexia nervosa at the other. There are indications, for example, that the physiological and psychological responses to food cues (Belfort-DeAguiar & Seo, 2018; Cleobury & Tapper, 2014; Epstein et al., 1996; Ferriday & Brunstrom, 2011) (a key trigger for meal initiation) and aberrant processing of food cues by the brain (review: Pursey et al., 2014) are accompanied with hormonal imbalance such as increased levels of satiety signals (leptin and insulin) and decreased levels of ghrelin (Bagdade et al., 1967; Considine et al., 1996; Soriano-Guillén et al., 2004; Tschöp et al., 2001). Additionally, studies in humans show that

there is an association between circulating leptin- and ghrelin-levels and food cue reactivity specific brain areas (Farr et al., 2016; Wever et al., 2021). At the opposite end of the body weight spectrum, patients suffering from anorexia nervosa have difficulty starting a meal and eating and they have elevated levels of ghrelin (Soriano-Guillén et al., 2004); brain processing of food cues is suppressed in a top-down manner in patients suffering from anorexia nervosa (Brooks et al., 2011; Sanders et al., 2015). These examples show that the hormones targeting systems in the brain controlling food intake (including orexigenic systems involved in food cue-reactivity) may play a role in the regulation of meal initiation. In order to go beyond association and prove causal involvement, manipulation of hormones (by injections) and neural pathways (using novel molecular neuroscience tools) is essential. It is important to better elucidate the orexigenic systems of the brain, that respond to food cues and to metabolic signals, since these systems might be defective in people suffering from eating disorders. Eating disorders cause major health issues and cause financial burden in our modern society, while effective and non-invasive treatments are lacking.

Anorexia nervosa

Orexigenic systems must be immensely activated in patients suffering from anorexia nervosa, since they are (severely) underweight. Anorexia nervosa is a psychiatric eating disorder primarily characterized by low body mass index due to self-starvation, accompanied by symptoms such as prominent restlessness and excessive physical activity, distorted body image and extreme fear of gaining weight (Dalle Grave et al., 2008; El Ghoch et al., 2013; Gorwood et al., 2016; Holtkamp et al., 2006; Mitchell & Peterson, 2020). It affects 0.9-4% of women and 0.3% of men (Hudson et al., 2007; Keski-Rahkonen & Mustelin, 2016; Micali et al., 2017) and mortality rates are higher than in other psychiatric disorders (Arcelus et al., 2011) and treatment outcomes are poor (Watson & Bulik, 2013). Since the biology of anorexia nervosa is largely unknown, aetiology-based treatments are lacking. There are several animal models for anorexia nervosa, that represent different components of the disorder and could facilitate research to understand the underpinnings of the disorder (Méquinion et al., 2015). One such model is the activity-based anorexia model (ABA).

Activity-based anorexia rodent model

ABA is currently the most well-known animal model of anorexia nervosa, in which animals have access to food only during a restricted period every day (only a few hours) and unlimited access to a running wheel. The protocol was

initially developed in rats (Routtenberg & Kuznesof, 1967) and later also applied on mice where results vary based on food restriction protocol, strain, age and sex (Beeler & Burghardt, 2021; Gelegen et al., 2007). In the original study of Routtenberg and Kusznesof in 1967, food access in this paradigm was limited to a fixed period, mostly for 1 hour a day. Besides developing excessive running, ABA rats also ate less compared to inactive (a.k.a. no running wheel) control rats on the same feeding schedule and lost weight rapidly (where controls lose but later on stabilise their weight), and eventually died within about a week (Routtenberg & Kuznesof, 1967). Later ABA studies revealed not only that total running activity increased, but also that animals started running excessively specifically in anticipation of their feeding time (food anticipatory activity) (Klenotich et al., 2012).

No animal model of psychiatric disease is perfect, however, and has limitations, where the ABA model is no exception. The biggest limitations are probably that (i) the ABA model is very acute compared to the human situation, lasting about 1 week while patients can be ill for years (Råstam et al., 2003; van Son et al., 2010; Wentz et al., 2009) and (ii) starvation is imposed by the researcher using scheduled feeding (although the animals appear to choose to run rather than eat), while anorexia nervosa patients self-starve. Nonetheless, it can still serve as a model to study the underpinnings of anorexia-like symptoms that ABA rodents develop. Young/adolescent rodents are more susceptible for developing ABA than adults. Also, in humans, nearly all anorexia nervosa cases begin before the age of 25 (Herpertz-Dahlmann, 2009). Similarly, about 90% of anorexia patients are female (Smink et al., 2012), which is reflected by female animals being more susceptible for developing ABA compared to males (Morgan et al., 2022). Finally, given that genetics play a major role in the development of anorexia nervosa (Watson et al., 2019), it might not be surprising that mouse strains respond differently to the ABA protocol.

Regardless of the limitations of the model, ABA-induced symptoms (self-starvation, excessive activity and severe weight loss) are observed in patients with anorexia nervosa, which makes the ABA model suitable to understand the underlying mechanisms that drive these behaviours, with a long-term view to identify druggable targets and develop treatments to reduce these symptoms and improve prospects for patients.

Feeding behaviours and constructs

Orexigenic systems can drive feeding (from food seeking through to how much and which food is eaten) and related behaviours (e.g. reward evaluation); these might seem simple on the surface, but orexigenic behaviours encompass many different components and start even before feeding begins. In the phase

preceding feeding, food is anticipated, for instance, due to negative energy balance, environmentally induced appetite (e.g. food cues, see below) or simply because of the time of day (e.g. thinking about expected dinner time). In rodents, food anticipation is clearly expressed in the form of food anticipatory activity. When animals are scheduled fed and hence, expect a meal at a fixed time, they increase physical activity (such as general locomotor activity, food-reinforced lever pressing and running wheel activity) in the period immediately before feeding (Bolles & Stokes, 1965; Boulos & Terman, 1980). Another important aspect of orexigenic behaviours is food motivation, expressed by how hard an animal is willing to work for food in, for example, an operant responding task, where nose poking or lever pressing are required to obtain a food reward. Studies showed that the willingness to lever press for food (a.k.a. food motivation) increases when orexigenic systems are activated, for instance, when animals are fasted (hungry), upon peripheral or central ghrelin injection and when cells established as "hunger neurones" (AgRP expressing neurones) are artificially activated (Aponte et al., 2011; Bake et al., 2019; Krashes et al., 2011; Perello et al., 2010; Skibicka et al., 2011). During feeding, orexigenic systems impact on how much food is consumed and which food is selected. Ghrelin and leptin signalling, for example, impact on food consumption in an opposite manner, where high ghrelin levels increase food intake and low leptin levels (e.g. due to fat loss) or reduced leptin signalling (in the case of disturbed leptin receptor function for example) stimulate long term anabolic processes (like body weight increase), such as by disinhibition of orexigenic systems (Jéquier, 2002). Food choice is also affected by ghrelin, where its injection drives rats on a high-fat-high-sugar diet to increase consumption of the "healthier" chow (Schéle et al., 2016), even in animals trained to binge on high fat diet (Bake et al., 2017).

Besides evoking feeding behaviours, activity of orexigenic systems also contribute to the way an orexigenic state is experienced. For example, hunger carries a negative valence. Valence coding can be unpleasant, as is the case for huger, or it can be positive when a pleasurable/reward stimulus is given. This is elegantly illustrated by studies showing that central injection of ghrelin conditions an aversion, where animals avoid a chamber or taste associated with these stimuli (Schéle et al., 2017). Thereby, optogenetic activation of AgRP "hunger neurones" conditions a taste avoidance, and chemogenetic inhibition of AgRP conditions a place preference (Betley et al., 2015).

When it comes to feeding, it is important to note the difference between homeostatic and hedonic motivations; eating according to metabolic need and eating for pleasure and beyond immediate energy need, respectively. Food seeking, for example, can be induced not only by physiological hunger (energy deficit) but also by the palatability of a food. When it comes to the neural substrates engaged, broadly peaking, physiological feeding is mainly driven by

hypothalamic brain areas, while the limbic system is involved in driving hedonic feeding (especially dopamine signalling), although the two are higly connected because of their functional and anatomical overlap (Rossi & Stuber, 2018). Regarding hedonic feeding, it is important to make the distinction between "reward seeking" and "hedonics", the "wanting" and "liking" aspects of reward coding. Whereas reward-seeking (also referred to as incentive motivation) engages the ventral tegmental area (VTA) to nucleus accumbens (NAc) dopamine pathway, hedonic evaluation involves small hedonic hotspots in different brain areas (a crucial one located in the ventral pallidum) that amplify "liking" reactions when stimulated by neurotransmitters such as opioids and endocannabinoids (Berridge & Robinson, 2016). The clear distinction between the "liking" and "wanting" is exemplified by studies showing that dopamine stimulations in hedonic hotspots fails to enhance "liking", thus the role of dopamine seems restricted to "wanting" (Berridge & Kringelbach, 2015; Smith et al., 2011).

Ghrelin

Ghrelin is a circulating hormone, first discovered in 1999 by the group of Kenji Kangawa (Kojima et al., 1999). It is predominantly produced by the X/A-like enteroendocrine cells in the stomach (Date et al., 2000). Ghrelin is recognized as a powerful orexigen, increasing food intake in most species studied including rodents and humans (Wren, Seal, et al., 2001; Wren, Small, et al., 2001; Wren et al., 2000). The discovery of the ghrelin signalling system has roots in the field studying the hypothalamic-pituitary growth hormone axis (growth axis). The development of growth hormone secretagogues (GHS) in the early 1980s (Bowers et al., 1984) preceded the discovery of the growth hormone secretagogue receptor (ghrelin receptor; GHSR) in 1996 (Howard et al., 1996), after which the endogenous ligand for this receptor was discovered 1999 (Kojima et al., 1999). Ghrelin exists in two forms, acylated and des-acylated, where the latter gets acylated by the enzyme ghrelin-O-acyltransferase (GOAT) to form acyl-ghrelin (Yang et al., 2008). Acyl-ghrelin is referred to as active ghrelin because it's the only form that can bind to and activate the ghrelin receptor. Ever since the discovery of GHSR and its endogenous agonist ghrelin, we have gained more insight into the various roles of ghrelin signalling and the underlying neural circuitries, for example those underpinning feeding-related behaviours. Based on observations in humans that circulating ghrelin levels peak right before meals and initiate voluntary meals in the absence of time- or food-related cues and drop postprandially (Cummings et al., 2004; Cummings, Weigle, et al., 2002), it is suggested to be involved in meal-initiation. Ghrelin levels correlate with feelings of hunger in humans (Cummings et al., 2004), rise upon fasting (Tschöp et al., 2000) in

rodents and increase with weight loss in humans (Cummings, Weigle, et al., 2002). Besides an acute orexigenic role, ghrelin also fulfils criteria for being involved in long-term body weight regulation since circulation levels change related to energy stores. Ghrelin levels are increased in patients with anorexia nervosa (that is directly linked to their malnourished state) and is decreased in individuals with obesity (Shiiya et al., 2002), with the exception of patients (with Prader-Willi syndrome (Cummings, Clement, et al., 2002)).

Ghrelin crosses the blood-brain barrier (Banks et al., 2008; Banks et al., 2002; Rhea et al., 2018; Schaeffer et al., 2013; Uriarte et al., 2019) and exerts its effects on the brain by binding to its cognate receptor GHSR. The receptor is a G-protein coupled receptor and can influence excitability (mostly increase it by recruitment of $G\alpha_{q/11}$ G-proteins intracellularly) of GHSR expressing cells (Hedegaard & Holst, 2020; Ringuet et al., 2022) resulting in activation of many brain pathways involved in feeding regulation (reviews: Chronwall, 1985; Morselli et al., 2018; Shan & Yeo, 2011).

Functional magnetic resonance imaging (fMRI) studies in healthy humans reveal that intravenous ghrelin administration increases the neural response to food pictures and enhances food odour conditioning. Exposure to food pictures after ghrelin administration shows increased activity in brain areas that are implicated in coding incentive value of food cues, including the striatum, orbitofrontal cortex, amygdala and anterior insula (Malik et al., 2008). Ghrelin-mediated improvement of classical conditioning learning upon food odour exposure (and increase of experienced pleasantness of the odour) shows involvement of various brain areas, including dopamine responsive brain areas such as the VTA, ventral striatum and substantia nigra. Furthermore, ventral striatum activation in ghrelin (and not saline) infused subjects correlates with pleasantness ratings of conditioned food cues (Han et al., 2018).

From animal studies, we have learned that the hypothalamus contains several nuclei that are important in ghrelin's effect on feeding regulation, a brain area that is, however, challenging to examine in living humans due to its position deep in the brain near to bone, where magnetic imaging gets blurred. Rodent studies revealed that hypothalamic signalling pathways (that also connect to feeding centres beyond the hypothalamus) play an important role in feeding behaviours. Many hypothalamic feeding-regulating brain areas express the ghrelin receptor (the growth hormone secretagogue receptor, GHSR), such as the Arc, VMH, dorsomedial hypothalamus and LatH (Guan et al., 1997; Mani et al., 2017; Zigman et al., 2006). Additionally, many brain areas outside the hypothalamus express GHSR, including the VTA (notably including a sub-population of dopaminergic cells; Abizaid et al., 2006), the hippocampus and amygdala, and in the brain stem in the area postrema, parabrachial nucleus and nucleus of the solitary tract (Guan et al., 1997; Mani et al., 2017; Zigman et al., 2006). GHSR expression implies ghrelin responsiveness and, indeed,

central infusion of ghrelin (intracerebroventricular or locally in many GHSR expressing brain areas) promotes feeding related behaviour, some examples of brain areas being the amygdala, LatH, paraventricular nucleus of the hypothalamus (PVH), lateral parabrachial nucleus (lPBN) and the supramamillary nucleus (Alvarez-Crespo et al., 2012; Bake et al., 2020; Le May et al., 2019; Olszewski, Grace, et al., 2003; Olszewski, Li, et al., 2003; Schéle et al., 2016). Ghrelin, however, is predominantly produced in the stomach and, based on fluorescent labelled ghrelin injection studies, may only access a limited number of brain areas (Cabral et al., 2014; Schaeffer et al., 2013). This raises the question why brain areas that are seemingly inaccessible to peripheral ghrelin express its receptor.

There are two characteristics of GHSR that give the receptor ligand-free functionality: its constitutive activity and its capacity to form heterodimers with other G-protein coupled receptors. Even without ghrelin binding, GHSR reaches 50% of its signalling capacity, meaning that GHSR expression itself can influence excitability of a cell (Holst et al., 2003). Then, GHSR forming heterodimers with other receptors (including the dopamine-1 and dopamine-2, serotonin 2C, orexin, oxytocin and melanocortin 3 receptors) can impact on signalling efficacy and change which intracellular signalling pathways are recruited, regarding both GHSR itself and of the other half of the dimer, which can alter the excitability or activity of a cell (Jiang et al., 2006; Kern et al., 2012; Kern et al., 2015; Ringuet et al., 2022; Schellekens et al., 2015; Schellekens et al., 2013). To give examples (based on *in vitro* studies), dimerization of GHSR and the serotonin 2C receptor attenuates calcium signalling (although only upon ghrelin exposure) and reduces ghrelin induced food intake in mice *in vivo* (Schellekens et al., 2015). Intracellular signalling of the dopamine-2 receptor upon agonist activation changes after dimerization with GHSR *in vitro*, in the complete absence of ghrelin, from coupling to $G\alpha_{i/o}$ and suppression of intracellular cyclic-AMP (adenosine monophosphate) accumulation without mobilization of intracellular Ca^{2+}, to mobilization of intracellular Ca^{2+} (Kern et al., 2015). Thus, the brain ghrelin system may not require ghrelin for its activation; conceivably, activation is possible through excitation of the heterodimerized receptor and/or constitutive activity of GHSR, making regulation of GHSR expression part of physiology.

Ghrelin and the growth axis

Ghrelin was initially discovered in the context of the growth axis research, as mentioned above, and was identified as a growth hormone secretagogue because of its ability to increase growth hormone (GH) release (Kojima et al., 1999). Regulated by GH releasing hormone (GHRH) and the inhibitory somatostatin, GH is released in a pulsatile manner to optimise growth through

its effector insulin-like growth factor 1 (IGF-1). Ghrelin has the capacity to amplify GH release by increasing the amplitude (but not the number of peaks) (Seoane et al., 2000; Wren et al., 2000), but the biological relevance for this amplification remains somewhat unclear. In a review looking at phenotypes in rodent models with aberrant ghrelin signalling (Peris-Sampedro et al., 2021) it is suggested that, since ghrelin plays an important role in preventing hypoglycaemia in periods of fasting (such as increasing glucose levels at least in part by enhancing GH release) (Zhao et al., 2010), the pattern of ghrelin secretion (high when hungry) links better to the diabetogenic effects of GH rather than to its growth-promoting role (which is suppressed in times of famine). Ghrelin does, however, seem to play a role in linear growth, as neatly substantiated in the aforementioned review; when explored, at least some animal models with ghrelin deficient signalling develop a clear growth phenotype (lower body weight or remain shorter compared to wild types). In this thesis, we had the opportunity to phenotype *Ghsr-IRES-Cre* mice (**Paper II**), including their growth phenotype, suspecting that GHSR signalling may be defective in these mice.

Food cues

Cues that signal food availability (food cues) are known to engage orexigenic systems and promote searching for and consumption of food (Holland & Petrovich, 2005). Neuronal underpinnings of cue-potentiated feeding are commonly studied using a classical Pavlovanian conditioning set-up, where a secondary non-food cue (e.g. a tone or light) is associated to food availability (Petrovich et al., 2012; Petrovich et al., 2005). In nature, however, animals and humans rely on primary food cues to locate food, such as the sight or smell of food, which can drive eating even beyond metabolic need (Joyner et al., 2017). In our modern society, food is always readily available and we are surrounded by food cues (such as commercials). Since food cues are a powerful trigger that can induce overeating, a food cue rich environment is an important risk factor for developing obesity (Belfort-DeAguiar & Seo, 2018).

It is well known that the sense of smell can drive appetite, food seeking and food preference in animals and humans (Fine & Riera, 2019; Palouzier-Paulignan et al., 2012), but a lot is still to be uncovered about the underlying neurocircuitries and substrates important for olfactory food cue detection and ensuing behavioural outcomes; something we aimed to explore in **Paper I**. Mapping studies found terminals of orexin cells in the olfactory bulb in rodents and described bi-directional connections between the olfactory bulb and Arc (Gascuel et al., 2012; Russo et al., 2018; Schneider et al., 2020), indicating that a hypothalamic-olfactory axis exist, with potential relevance for feeding control. More specifically, there are data suggesting the involvement of ghrelin

signalling in this pathway, namely that 1) the ghrelin receptor, GHSR, is abundantly expressed in the olfactory bulb (Mani et al., 2017) and 2) the detection of terminals from olfactory-ghrelin containing cells in the Arc (Russo et al., 2018). Behaviourally, ghrelin increases sensitivity to smell in rodents and humans (Tong et al., 2011).

Neural substrates in central feeding regulation

The arcuate nucleus

The hypothalamic Arc has emerged as a key brain area involved in the regulation of feeding behaviour. It contains cells that are regulators of homeostatic feeding (feeding according to energy need), including two important cell types with opposing roles: anorectic cells expressing proopiomelanocortin (POMC) and the orexigenic AgRP population expressing both AgRP and neuropeptide Y (NPY) (reviews: Cone et al., 2001; Joly-Amado et al., 2014). The AgRP cells also abundantly express GHSR (ghrelin receptor) (Willesen et al., 1999) and are activated by ghrelin (Schaeffer et al., 2013). Besides these neuronal populations, the Arc contains a multitude of cell types that may contribute to feeding behaviour such as neurones expressing tyroxine hydroxylase (TH), GH releasing hormone (GHRH), prepronociceptin (PNOC), serotonin 1B and 2C receptors (Jais et al., 2020; Osterstock et al., 2010; Pirnik et al., 2014; Voigt & Fink, 2015; Zhang & van den Pol, 2016). For detailed information about Arc cell types please refer to study that performed single cell RNA sequencing on cells in the Arc and medial eminence (Campbell et al., 2017).

The Arc is considered to be a circumventricular organ, meaning the blood-brain barrier is more permeable at this site, and therefore circulating compounds from the periphery can more easily reach and act on this brain area. This explains why the Arc is one of the few brain sites that gets activated by for example peripheral ghrelin injection (Cabral et al., 2014; Schaeffer et al., 2013). There are many papers showing Fos expression in the Arc after peripheral ghrelin injection, the first one in 2000 (Hewson & Dickson, 2000) and NPY neurones (that co-express AgRP) are among the activated cells, but are not the only cells engaged by peripheral ghrelin (Dickson & Luckman, 1997). Importantly, the elegant work of Patrice Mollard using florescent labelled ghrelin, has revealed, not only that ghrelin crosses the blood brain barrier at the median eminence – Arc interface but that, once in the brain, it directly binds to those cells activated (that express Fos protein) (Schaeffer et al., 2013). We may infer therefore, that Fos expression by ghrelin at the level of the Arc involves the direct passage of ghrelin into the brain at the level of the median eminence.

Beyond the arcuate nucleus

Feeding is a complex process engaging extensive networks throughout the brain that include those involved in motivation, energy sensing, stress-responsivity, memory, to name but a few (Berthoud, 2004). Indeed, neurones in the Arc, targeted by peripheral signals, feed information into these networks to regulate relevant behaviours and constructs. These areas may be targeted directly by ghrelin, as evidence by the presence of ghrelin receptors and by injection studies showing ghrelin-induced effects. These brain areas may also be indirectly activated from afferent projections of ghrelin-activated cells (for example, by those located in the Arc). One example is the PVH, one important brain area connected to the Arc and feeding regulation. Ghrelin injection to the PVH causes a feeding response (Olszewski, Grace, et al., 2003) but this area can also be indirectly targeted by ghrelin via the Arc. Melanocortin-4 receptor (MC4R) expressing cells in the PVH are inhibited by the MC4R antagonist AgRP released from Arc AgRP cells and get activated by α-melanocyte stimulating hormone (α-MSH; MC4R agonist), released from Arc POMC neurones. Activation of the PVH cells by α-MSH initiates the anorectic/catabolic melanocortin signalling cascade, while increased release of AgRP (upon AgRP cell activation) leads to increase in food intake and energy conserving effects (Adan et al., 2006).
Other important brain areas in this feeding circuitry include the LatH that contains, for example, orexin expressing cells with an established role in energy balance and arousal (Chemelli et al., 1999). In the brainstem, the lPBN, an anorexic brain centre, receives inhibitory inputs from the Arc AgRP neurones that seems critical for feeding (Wu et al., 2009). In the context of this thesis, it is important to point out that all these brain areas express GHSR and ghrelin injection at these sites is orexigenic.

Orexin in the lateral hypothalamus

Orexins (orexin-A and orexin-B), also called hypocretins, were initially discovered as hypothalamic regulators of food intake (Sakurai et al., 1998) but are now best known for their role in arousal (reviews: Girault et al., 2012; Jacobson et al., 2022; Sakurai, 2005). Orexin knockout (female) mice, paradoxically, gain weight (Fujiki et al., 2006; Ramanathan & Siegel, 2014) and it emerged that while orexins increase food intake, these effects are over-shadowed by their effects to increase energy expenditure. The central expression of orexin is limited to the hypothalamus, mostly the LatH but extending into the dorsomedial hypothalamus (Broberger et al., 1998; de Lecea et al., 1998; Sakurai et al., 1998). Despite their restricted distribution, orexin-producing cells project to areas throughout the brain and exert their effects through binding to their receptors, orexin receptor 1 and 2. The two orexins

bind to both receptors but with different affinities (Sakurai et al., 1998). The receptors are expressed in these target brain areas, where the Arc, the paraventricular nucleus of the thalamus, locus coeruleus and tuberomammillary nucleus are considered to be the major effective sites of orexin signalling, since they receive particularly dense orexin projections (Date et al., 1999; Nambu et al., 1999; Peyron et al., 1998).
Stimulation of central orexin signalling increases physical activity (Hagan et al., 1999; Kotz et al., 2002; Kotz et al., 2006) and impacts on food intake and energy expenditure, where acute orexin stimulation increases feeding while chronic orexin overexpression has catabolic effects; it reduces feeding and increases energy expenditure (Funato et al., 2009). Diminished orexin signalling has opposite, suppressing effects on physical activity, demonstrated by experiments lowering orexin signalling by orexin neuron ablation (Akiyama et al., 2004; España et al., 2007; Mieda et al., 2004; Yamanaka et al., 2003) or by administration of antagonizing agents (Winrow et al., 2011). Since orexin signalling plays a role in feeding regulation, it is interesting to note that the system is also activated by the pro-appetite hormone ghrelin, where central ghrelin administration induces Fos expression in orexin cells in the LatH (Scott et al., 2007).
Given their role in arousal, targeting the orexin system was promising for clinical application regarding treatment of insomnia and indeed there are clinically approved orexin suppressants available to treat insomnia, including the dual orexin receptor antagonist suvorexant (review: Jacobson et al., 2022). Recently it has been suggested that this drug could also be applied to patients suffering from anorexia nervosa (discussed above) to prevent excessive orexin signalling, with the potential to promote sleep and improve cognitive health (Toor et al., 2021).

Modern molecular neuroscience techniques

Mapping of neural circuits in the past was based on tools such as hormone delivery and lesion studies. The development of novel tools in neuroscience in the past years has greatly expanded our possibilities to target and explore the function of specific neuronal populations, advancing our search of underlying circuitries to a whole new level. Bryan Roth, for example, developed a chemogenetic tool that makes it possible to manipulate the activity of defined neuronal populations in freely moving mice in a non-invasive way, called designer receptors exclusively activated by designer drugs (DREADDs) (Armbruster et al., 2007). The DREADDs were created by directed mutation of human muscarinic receptors, resulting in designer receptors that are insensitive to their endogenous ligand (acetylcholine), but that are potently activated by the inert designer drug Clozapine-N-Oxide (CNO). Initially, two

DREADDs were developed, where one increases (hM3Dq) and the other decreases (hM4Di) cellular excitability upon activation of the receptor, by recruiting Gq- or Gi- proteins, respectively.
Viral vectors can be injected to specific brain targets, that deliver and express DREADDs and the Cre-lox system makes it possible to target defined neuronal populations. In this case, a vector is designed to Cre-dependently express the DREADD, by inserting the sequence coding for the DREADD in a double-floxed inverted open reading frame. Cre recombinase enzyme is then necessary to recombine, or flip, the sequence, so it is in frame and can be expressed. Animals do not express Cre recombinase endogenously but it can be introduced using transgenesis.
In the *TRAP2* mouse model (Allen et al., 2017; Guenthner et al., 2013), a tamoxifen-inducible form of Cre ($CreER^{T2}$) was inserted into the locus of the immediate early gene *fos*, which is a known neuronal activity marker (Bullitt, 1990; Cochran et al., 1984; Curran & Morgan, 1985), resulting in $CreER^{T2}$ being co-expressed with *Fos*. $CreER^{T2}$ makes it possible to control the time window in which recombination can occur. On its own, the enzyme is located in the cytoplasm, but when tamoxifen is present and binds to $CreER^{T2}$, the complex relocates to the nucleus where it can induce recombination (see Figure 2 in the methods section for further details of this technique).
In the Fos-TRAP (targeted recombination in active populations) system, and thus in the *TRAP2* mouse model, it possible to target transiently activated neuronal populations, since $CreER^{T2}$ is co-expressed in activated cells that express *fos*. When 4-hydroxytamoxifen (4-OHT; a tamoxifen metabolite with a relatively short effective time period of 4-6 hours) is (peripherally) delivered to *TRAP2* mice, the presence of 4-OHT defines a time window in which $CreER^{T2}$ is “active” and recombination can take place and activated cells can be targeted, or “TRAPed”.

AIM

The overall aim of this thesis was to identify neuronal populations involved in orexigenic signalling and gain more insight into their neurochemical identity and the role they play in regulating feeding-related behaviours.

Specific aims

Paper I — Investigate whether the smell of a familiar palatable food can increase intake of regular chow and induce food seeking behaviour in rats, and explore whether ghrelin signalling is engaged and the neurochemical identity of the neurones involved.

Paper II — Determine to what extent we can use *Ghsr-IRES-Cre* mice to investigate the function of neuronal populations expressing the ghrelin receptor as part of the orexigenic system. To this end we looked into the effect of insertion of the *IRES-Cre* cassette in these mice on *ghsr* (ghrelin receptor) mRNA expression in the Arc, metabolic and growth outputs, and ghrelin responsiveness.

Paper III — Establish the Fos-TRAP system in our laboratory using the *TRAP2* mouse model with the aim to visualise the ghrelin-activated Arc ensemble and apply chemogenetic tools to explore the role of ghrelin-activated cells in the Arc in food intake and food choice in male and female mice.

Paper IV — Identify and characterise an ensemble of candidate "hunger neurones" in the Arc using *TRAP2* mice. We chemogenetically targeted ghrelin-activated cells in the Arc in fasted (hungry) mice and examined food intake, food motivation and valence conveyance upon activation of the "hunger neurones" ensemble.

Paper V — Examine a neuronal ensemble in the LatH activated in a severely orexigenic state, namely in mice exposed to the ABA paradigm, a mouse model mimicking features of anorexia nervosa. The ensemble was visualised using *TRAP2* mice and we set out to determine whether orexin signalling plays a role in ABA behaviours. To this end we examined whether the ensemble in the LatH included orexin-containing cells with

immunohistochemical staining, and explored whether the dual orexin antagonist suvorexant could reduce running wheel activity during ABA.

METHODOLOGICIAL CONSIDERATIONS

Animals

For the work in this thesis male rats and mice, and female mice were used. In **Paper I**, male Sprague-Dawley rats were used. Male *Ghsr-IRES-Cre* mice on a C57BL/6N background (homozygotes, heterozygotes and wild type litter mates) were used in **Paper II.** For the final 3 papers *TRAP2* mice on a C57BL/6J background and wild type controls were used. Specifically, double transgenic *TRAP2::Ai14* mice (heterozygous for both transgenes) were used in **Paper III** (females) and **Paper V** (females), while heterozygous *TRAP2* mice were used in **Paper III** (males and females) and **Paper IV** (males). In **Paper V** C57BL/6J wild type females were used as well.

The *Ghsr-IRES-Cre* mouse line was created by Mani, Zigman and colleagues (Mani et al., 2017) as a tool to explore ghrelin function and signalling. These mice were obtained from Monash Research Platform at Monash University in Australia. The *TRAP2* and *Ai14* (tdTomato Cre-reporter) mice originated from the Jackson laboratory.

All studies were approved by the local Ethics Committee for animal care at the Institute of Experimental Biomedicine at the University of Gothenburg in Sweden. Ethical permit numbers are specified in each paper.

All personnel that worked with animals were highly trained and competent according to Swedish law. Experiments were designed with the 3R principle in mind (Replace, Reduce, Refine), and data collection and analysis were performed by a blinded person as much as possible to minimise bias.

Stereotaxic surgeries

In **Paper III** and **Paper IV** stereotaxic surgeries were performed to deliver a viral vector to a specific brain area to Cre-dependently express the activating DREADD hM3Dq in *TRAP2* mice. The following adeno-associated virus (AAV) based vectors were delivered bilaterally (200 nL per injection, 1*E13 molucules/mL) to specific coordinates relative to Bregma (written as mm; anterior/posterior AP, medial/lateral ML, dorsal/ventral DV): In **Paper III** pAAV-hSyn-DIO,HA,hM3D(Gq)-IRES-mCitrine to the arcuate nucleus (Arc; AP -1.05, ML ±1.25, DV -5.90 at an angle of degrees), in **Paper IV** pAAV-hSyn-DIO-hM3D(Gq)-IRES-mCherry to the Arc (AP -1.080, ML ±0.300, DV -5.850). The source of the viral vectors is given in the papers.

During surgical procedures, mice were anesthetised using isoflurane inhalation. They were placed in the stereotaxic frame and the skull was exposed. A local anaesthetic was applied (Xylocaine 10%, AstraZeneca, Cambridge, UK). Two holes were drilled in the skull, and the vector was injected into the brain at the designated coordinates. After completion of the surgery, the animals received an analgesic (Metacam, 5 mg/kg, Boehringer Ingelheim, Ingelheim am Rhein, Germany) and were returned to their home cage for recovery.

TRAPing

When targeting transiently activated populations in *TRAP2* mice (Figure 2), there are several considerations regarding 4-hydroxytamoxifen (4-OHT) delivery, three important factors being timing, dose and vehicle. To reach optimal TRAPing efficacy of neurones, the timing of 4-OHT injection should be such that the peak of $CreER^{T2}$ expression coincides with the peak 4-OHT availability in the brain. When injecting 4-OHT in an aqueous vehicle, the intracellular 4-OHT concentration are highest one hour after peripheral injection (Ye et al., 2016), and protein expression ($CreER^{T2}$) is expected to peak around four hours post stimulation (Kawashima et al., 2013) (results depicted in supplementary figures). When delivering 4-OHT in an oil vehicle, however, TRAPing efficacy is high when injecting tamoxifen at the time of stimulation delivery (Guenthner et al., 2013). The dose of 4-OHT delivered to animals can also influence the number TRAPed cells and how accurate TRAPed cells represent activation patterns related to a stimulus. With a higher dose, the time window for TRAPing is expected to be longer since the 4-OHT levels are above TRAPing threshold for a longer period. This could lead to unspecific TRAPing, while on the on the other hand a too low dose could result in insufficient 4-OHT levels and thus not all activated cells will be targeted.

Drugs

Ghrelin

Rat ghrelin (#1465, Tocris, Bristol, UK) was dissolved in saline (0.3 mg/mL) and subcutaneously injected to give a dose of 3 mg/kg in all cases. In **Paper II**, food intake was monitored after ghrelin injection in wild type and *Ghsr-IRES-Cre* (heterozygous and homozygous) mice. In **Paper III** and **Paper IV** ghrelin was injected in heterozygous *TRAP2* mice with the goal to target, or TRAP, ghrelin-responsive cells. Control mice received a comparable volume of saline.

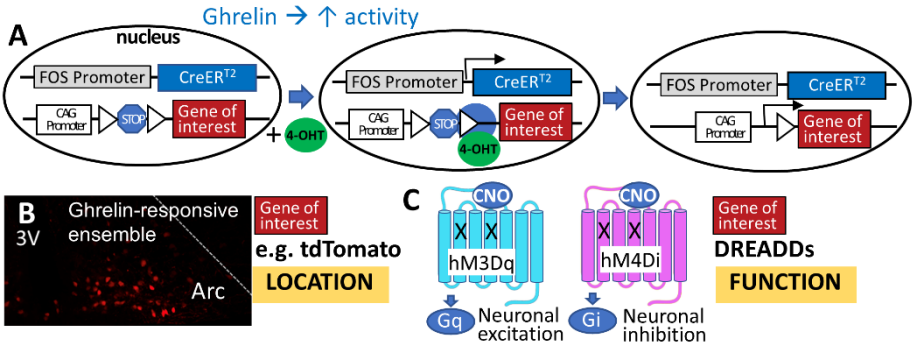


Figure 2. Schematic illustration of the TRAPing technique. *In TRAP2 (Targeted Recombination in ActivePopulations 2) mice, Fos expression results in the expression of CreER^{T2} (inducible Cre recombinase), which enters the nucleus in response to 4-hydroxy-tamoxifen (4-OHT) injection and causes recombination and permanent expression of a gene of interest [A]. It can be a marker gene (e.g. tdTomato), enabling their localization [B]. Alternatively, using a chemogenetic approach, the expressed gene (delivered by injection of a viral vector) can encode a designer (DREADD) receptor allowing the function of a given ensemble at a specific location to be explored. DREADDs are mutated human muscarinic receptors that can be activated by designer drugs such as CNO (clozapine-N-oxide) [C]. 3V: third ventricle, Arc: arcuate nucleus, CAG: chicken beta-actin*

Clozapine-N-Oxide (CNO)

The DREADD agonist CNO (#4936/10, Bio-Techne Ltd., Abingdon, UK) was used to achieve chemogenetic activation in **Paper III** and **Paper IV**. It was delivered by intraperitoneal injection at a dose of 1 mg/kg, 0.3 mg/kg or 0.1 mg/kg. Control injections consisted of a comparable volume of vehicle, consisting of saline and 1 %, 0.3 % or 0.1 % DMSO respectively.

4-hydroxytamoxifen (4-OHT)

IN OIL VEHICLE

The stock solution of 4-OHT (#H6278, Sigma-Aldrich, St. Louise, MO, USA) was made by dissolving it in ethanol at 37 °C to a concentration of 20 mg/mL. The stock solution was mixed with Chen oil (20 % castor oil and 80 % sunflower seed oil) and the ethanol evaporated by vacuum centrifugation. The final solution (10 mg/mL) was injected intraperitoneally in *TRAP2* mice (25 mg/kg) to induce TRAPing in **Paper V**.

IN AQUAOUS VEHICE

To obtain an aqueous solution in **Paper I** and **Paper II**, 4-OHT was dissolved in a vehicle comprising 2 % Tween 80 and 5 % DMSO in saline at a

concentration of 2 mg/mL and injected intraperitoneally in *TRAP2* mice (25 mg/kg) to induce TRAPing.

Suvorexant

The dual orexin receptor antagonist suvorexant (#A11500, Adooq Bioscience, Irvine, CA, USA) was dissolved in saline containing 10 % PEG 400, 10 % Tween 80 and 4 % DMSO and 20 mg/kg was injected intraperitoneally in mice undergoing the ABA paradigm. Controls underwent the same treatment but received vehicle instead of suvorexant. Running wheel activity was monitored in **Paper V**.

Food cue enriched environment

To explore the neuronal underpinnings and substrates driving overeating upon smell of a palatable food in **Paper I**, rats were exposed to a primary food cue enriched environment. Half of the rats were familiarized with the taste of the palatable food (i.e. peanut butter) while the control group remained naïve. During behavioural testing, rats in both groups were exposed to peanut butter odour or no smell (or both in a cross-over design), resulting in four experimental conditions per test. The control group that remained naïve to the taste of peanut butter was relevant to determine the effect of merely smelling food (as opposed to presenting the smell as a cue).
Familiarization to peanut butter taste was performed over 6 days, where every other day the rats had access to a limited amount of peanut butter, each exposure on a different time during the day to avoid anticipation.
During testing, the peanut butter odour was delivered to the home cage using a perforated metal ball (a tea strainer ball) dispensed from the lid of the cage, which was always present in their cage to avoid novelty. An open Eppendorf filled with peanut butter was placed in the perforated ball, while in the control situation an empty Eppendorf was used. Experimental outputs were compared between the groups (for food intake/meal patterns, FOS expression, food seeking and circulating ghrelin levels; see respective sections for details).

Behavioural testing

Baited open field

In **Paper I**, we explored primary food cue potentiated food seeking in a risky environment with a baited open field test, adapted from a previously used set-up (Lockie et al., 2017). A perforated metal ball (tea strainer) was secured in the middle of the open field arena, containing either an open Eppendorf filled with peanut butter or an empty one. The peanut butter taste familiar and naïve

rats were split into two groups (odour vs no odour) and tested in the set-up, while being recorded. By manual analysis of the videos, we assessed approach behaviour of the ball in the middle. We determined total contact time with the ball and first contact duration (to test approach behaviour) and latency to approach (measure for basal anxiety).

Food seeking (acoustic measurements)

In a food cue enriched environment (see section above), we tested food seeking behaviour upon exposure to a palatable food odour (peanut butter). The peanut butter was hidden inside the perforated metal ball, making sound when rats were attempting to get access to the food inside. Therefore, we measured the sound levels using a Sound Meter app (Crossley et al., 2021) and compared minimum, maximum and average sound intensity (in decibel) over a 15 minutes period between baseline and cue-enriched setting as a measure of food seeking in **Paper I**.

Food intake

MANUAL MEASUREMENTS

For individual housed mice in **Paper I, Paper II**, **Paper III** and in **Paper V**, food intake was measured manually. To get precise measurements, eight big chow pellets were placed on the designated place on the cage lid. Using an exact number and big pieces of chow ensured that no chow fell through the metal spirals into the cage, and the limited number of pellets made precise weighing of the food easier. The remaining food was measured at a fixed time point after treatment (CNO injection in this case) and food eaten calculated afterwards.

FED3 SYSTEM

Automated food intake measurements were performed in **Paper IV** using Feeding Experimentation Devices version 3 (FED3) (Matikainen-Ankney et al., 2021) (Figure 3) placed in the home cage of the mice. In the "free-feeding" mode used in this experiment, the device dispenses a 20 mg chow pellet and five seconds after the mouse takes one pellet, the next pellet is automatically dispensed. Before performing food intake measurements, the mice were habituated (i.e. "magazine training") to the FED3 over two days.

TSE SYSTEM

For automated food intake measurements in rats in **Paper I**, we used the automated feeding monitoring system from TSE systems (TSE LabMaster, Project 4261, TSE Systems, Bad Homburg, Germany). In this system, food

hoppers containing regular chow are suspended on sensors, collecting data every 10 seconds with a recording sensitivity of 1 mg.
The TSE system also allows recording of single meals, permitting meal pattern analysis. Meals were defined as: minimum consumption of 0.5 g of chow and meal termination when a rat did not eat for at least 10 minutes. The LabMaster software (TSE systems) was used to analyse meal patterns.

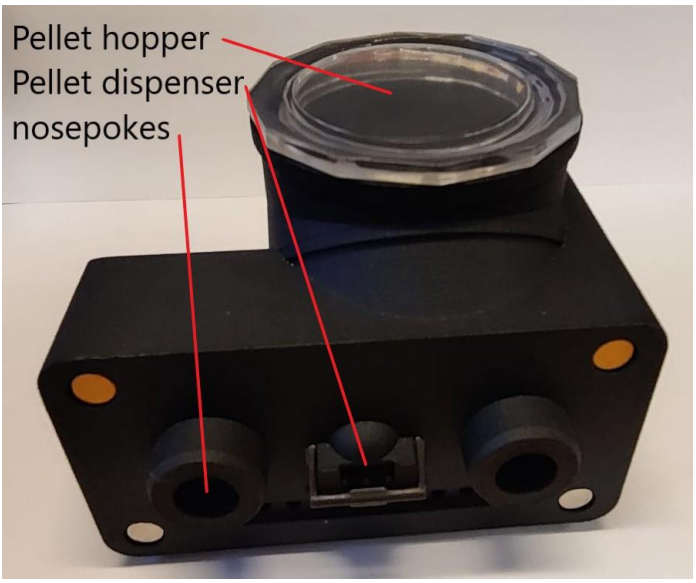


Figure 3. *Feeding experimentation device version 3 (FED3), used for food intake and food motivation experiments.*

Operant responding (food motivation)

FED3 devices were used in **Paper IV** to test food motivation in an operant responding task. After magazine training, the mice learned to nose poke for chow pellets. During training, the effort to obtain one pellet was increased over the consecutive training stages, starting with fixed ratio (FR) 1 where the mice had to nose poke once for a pellet, followed by FR3 and closed economy progressive ratio 2 (CEPR2). In the CEPR2 training, mice still obtained all their food from the device (hence "closed economy") and had to nose poke progressively more for each pellet earned, with a two-step increment (i.e. 1 – 3 – 5 – 7 nose pokes etc). When no nose pokes had been performed for a 30-minute period, the CEPR2 program reset and mice started again with 1 poke per pellet. In the final training stage, mice were habituated to intraperitoneal injections (with saline) for two days. Injection was followed by exposure to a classical progressive ratio task for two hours (PR2h), where the number of nose pokes required to earn a pellet increased exponentially (Richardson & Roberts,

1996). On testing days, the mice received treatment (in this case CNO or vehicle injection) and performed the PR2h task.

Conditioned place aversion (CPA)

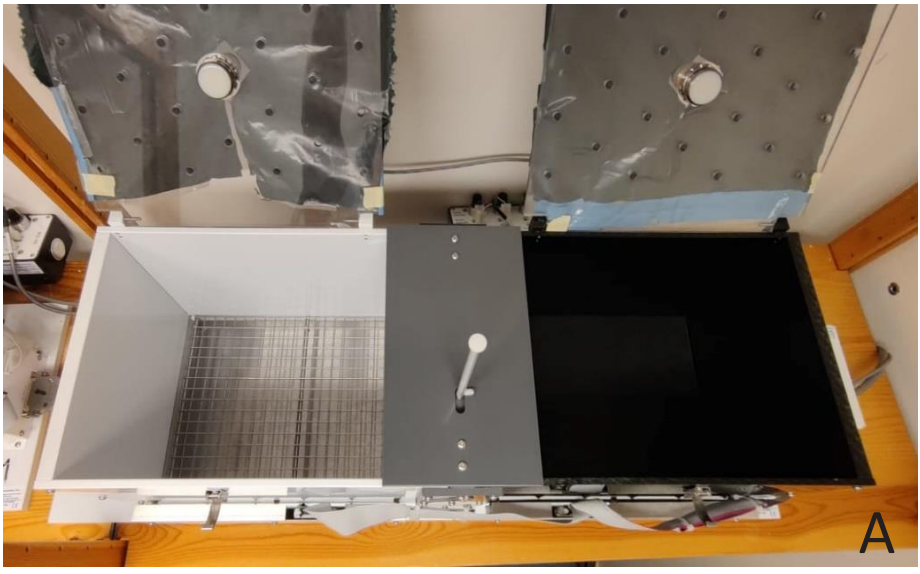
To examine the valence that chemogenetic activation of a neuronal ensemble in the arcuate nucleus conveys, we conditioned mice to associate the activation of the ensemble to one of the compartments in a CPA set-up (Figure 4) in **Paper IV**. To this end, mice were trained daily for ten consecutive days, where every other day chemogenetic activation (CNO injection) was paired to the black chamber, and on the other days saline injection was paired to the white chamber. On the day after the last training, mice had access to both chambers while the time spent in each chamber was recorded and was then compared to the chamber preference data from the pre-test (performed on the day before training onset). If animals experience unpleasantness (negative valence) upon chemogenetic activation, they will spend less time in the associated compartment after training, indicating that activated cells carry a negative valence. The opposite would be true in the case of positive valence.

Running wheel activity

Running wheel activity was automatically monitored throughout the experiment using Med associates running wheels (Figure 5; Low-profile wireless running wheel No. ENV-047, Med associates, Fairfax, VT, USA) in **Paper V**. Mice were subjected to the ABA model (see above) and underwent treatment (i.e. injection of vehicle or suvorexant; an orexin receptor antagonist) in the FAA period preceding ABA day five, aiming to suppress orexin signalling and reduce running.

Dual-energy X-Ray absorptiometry (DXA)

In **Paper II**, we examined the body composition of adult mice with DXA. First, mice were anaesthetised (with a mixture of Sedastart vet. ® 1 mg/kg, Produlab Pharma B.V., Raamsdonksveer, The Netherlands, and Ketalar ® 75 mg/kg, Pfizer AB, New York, NY, USA). When sedated, their nose-to-anus body lengths were measured with a digital calliper and mice were placed into the DXA chamber (Faxitron UltraFocus dual-energy X-ray absorptiometry, Faxitron Bioptics, Tuscon, AZ, USA). The following parameters were analysed: soft and lean tissue, fat mass, bone area, bone mineral content, and body mass index (refer to the paper for extensive details). After the procedure, mice were injected subcutaneously with SedaStop vet. ® (2.5 mg/kg, Produlab Pharma B.V.) to reverse the sedation.



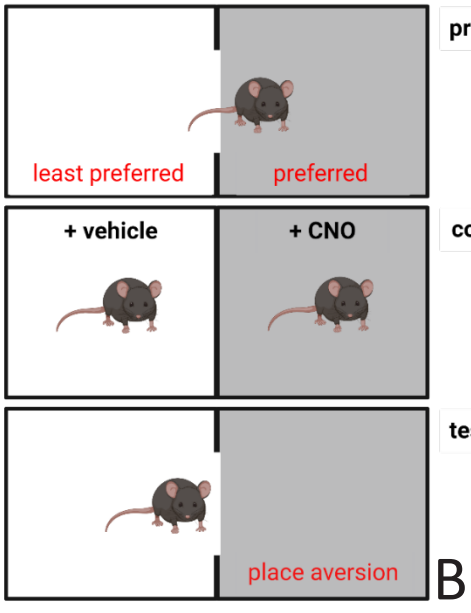


Figure 4. Standard two-chamber conditioned place preference/aversion test. *The two chambers of the apparatus differ in tactile and visual qualities [A]. There is a divider between the two chambers that is present during conditioning and absent during the place preference/aversion testing. Chemogenetic activation with clozapine-N-oxide (CNO) is paired with the chamber that was determined to be the preferred one in the pre-test [B].*

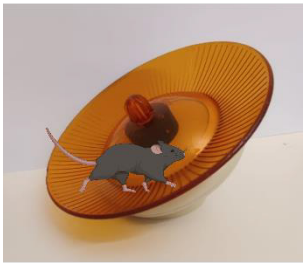


Figure 5. *Med associates low-profile running wheel, used in ABA mouse model in conjunction with scheduled feeding for 2 hours per day.*

Immunohistochemistry (IHC)

Brain tissue processing

In the case of Fos protein detection, animals were exposed to a stimulus (rats were exposed to a food-cue (**Paper I**) and mice were injected with ghrelin in **Paper II**) around 90 minutes prior to sacrifice, since FOS expression upon neuronal activation peaks around that time (Morgan et al., 1987). Perfusions were performed to obtain brain tissue that is suitable for IHC. To this end, mice (**Paper II** and **Paper V**) were deeply anaesthetised (with a mixture of Sedastart vet ® and Ketalar ®, similar as above), while rats (**Paper I**) were anaesthetised with another mixture (Rompun vet. ® 10 mg/kg, Bayer, Leverkusen, Germany and Ketaminol vet. ® 75 mg/kg, intervet, Boxmeer, The Netherlands). Subsequently, animals were transcardially perfused with heparinized 0.9 % saline followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were harvested, stored overnight in 4 % PFA containing 15 % sucrose at 4 °C and cryoprotected in 0.1 M PB containing 30 % sucrose at 4 °C until cryosectioning. Coronal sections (30 µm thick) at the level of the brain area of interest were cut using a cryostat and stored in an antifreeze solution (25 % glycerine, 25 % ethylene glycol, 50 % 0.1 M PB) at -20 °C until further processing.

Protein detection using 3,3'-diaminobenzidine (DAB)

In **Paper I** (rats) and **Paper II** (mice) Fos protein was detected in brain sections containing the arcuate nucleus with the DAB-hydrogen peroxidase method. Free-floating sections were briefly rinsed in 0.1 M PB, endogenous peroxidases deactivated. Sections were rinsed again (with 0.1 M PB containing 0.3 % Triton X-100) followed by blocking during 1 hour at room temperature in 0.1 M PB, 3 % normal goat serum, 0.25 % BSA and 0.3 % Triton X-100. Sections were then incubated over 3 nights at 4 °C with the primary antibody in blocking solution, rinsed and subsequently incubated for 2 hours at room temperature with the secondary antibody in blocking solution (see table 1). Incubation with a DAB, nickel and hydrogen peroxide solution made Fos expression visible. After rinsing, brain sections were mounted onto glass slides and coverslipped with Pertex mountant.

Protein detection using fluorescence

For fluorescent detection of Orexin-B in **Paper V**, sections containing the lateral hypothalamus were rinsed in 0.1 M phosphate buffered saline (PBS) and then blocked (0.1 M PBS containing 3 % normal donkey serum) for 1 hour at room temperature. Sections were then incubated with the primary antibody

for 2 days at 4 °C in blocking solution, rinsed in PBS and incubated with the secondary antibody for 1 hour at room temperature in blocking solution (see table 1). After the last rinse with 0.1 M PBS, sections were mounted on a glass slide and coverslipped with ProLong ® Diamond antifade mountant (Thermo Fisher, Waltham, MA, USA).

Table 1. List of antibodies used in this thesis

Paper	Primary antibody	Dilution	Secondary antibody	Dilution
I + II	Fos (Ab-5 (4-17) Rabbit pAB, PC38, Calbiochem, San Diego, CA, USA)	1:20 000	Peroxidase goat anti-rabbit immunoglobulin (Ig)G (PI1000, Vector Laboratories, Burlingame, CA, USA)	1:200
V	Orexin-B (SC8071, Santa Cruz, Dallas, TX. USA)	1:50	Donkey anti-goat Alexa 488 (A11055, Life Technologies, Waltham, MA, USA)	1:250

Imaging and quantification

Images of DAB IHC results (**Paper I** and **Paper II**) were acquired using a DMRB fluorescence microscope (10X/N.A. 0.30 objective; Leica Microsystems, Wetzlar, Germany). The number of Fos-positive nuclei per section in the arcuate nucleus was counted manually in ImageJ/Fiji (NIH, Bethesda, MD, USA) with the Cell counter plug-in. Cell counts were averaged per section per animal, and then per experimental group.
Images of the Orexin-B IHC (**Paper V**) were acquired using a confocal microscope (Zeiss, LSM 800, EC Plan-Neofluar 10X/N.A. 0.3 objective) to avoid overlap of cells in the Z-axis, since co-localization of tdTomato and Orexin-B was going to be determined. Every Orexin-B positive cell was counted in ImageJ/Fiji (NIH) with the Cell counter plug-in, and noted whether it was also tdTomato-positive (double positive). The number of Orexin-B expressing cells and double positive cells were averaged per section per animal, and then per experimental group.

RNAscope (fluorescent *in situ* hybridization)

Brain tissue processing

Brain tissue preparation for RNAscope in **Paper I**, **Paper III** and **Paper V** was performed in a similar manner as described in the section above (Immunohistochemistry), with some differences. Here, after brain collection,

they were incubated in in 4 % PFA overnight, followed by cryoprotection (sterile 0.1 M PBS with 25% sucrose). Thinner sections were cut with the cryostat (14 µm) and antifreeze solution was prepared with sterile PB.

mRNA detection

Expression of specific mRNAs was undertaken using fluorescent *in situ* hybridization with RNAscope in **Paper I** (*Ghsr*, P*omc*, *Agrp*, *Th*, *Fos*), **Paper II** (*ghsr*) and **Paper III** (*fos*, *ghsr*). For specifics about the probes used, see the respective papers.
On the day before the RNAscope assay, sections were mounted on SuperFrost Plus slides (631-9483, VWR, Radnor, PA, USA). After drying, they were rinsed in sterile Milli-Q purified water, air dried and placed in an oven overnight at 60 °C using the ACD HybEz II hybridization system (321462).
On the following day, the assay commenced with incubation of the slides for 7 minutes in hydrogen peroxide (322335) after which they were incubated in boiling Target Retrieval (322001) for 7 minutes. Sections were then briefly rinsed in sterile Milli-Q purified water, dehydrated in 100% ethanol and air dried for 5 minutes. A hydrophobic barrier was then drawn around the mounted sections with an ImmEdge hydrophobic barrier pen (310018) and sections incubated with Protease Plus (322331) for 30 minutes at 40 °C in the ACD HybEz II hybridization system. For the next steps (probe hybridization and signal amplification), the manfacturer's instructions for the tyramid-based RNAscope Multiplex Fluorescent v2 Assay (323100) were followed, using the tyramids Opal520 (1:500; PerkinElmer, Waltham, MA, USA), Cy3 (1:2000; Akoya Biosciences, Menlo Park, CA, USA) and Cy5 (1:2000; Akoya Biosciences, Menlo Park, CA, USA). All sections were counterstained with DAPI and coverslipped with ProLong ® Diamond antifade mountant (Thermo Fisher).
Besides the probes that were used to detect target mRNAs, every RNAscope essay included sections that were treated with Three-plex positive and negative control probes (recognizing PolR2A, cyclophilin and Ubiquitin (320881) or bacterial dihydrodipicolinate reductase, Dap B (329871), respectively) to ensure RNA integrity and good assay performance.

Imaging and quantification

RNAscope images were captured using a confocal laser scanning microscope (LSM 700 inverted, Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat 40X/1.3 Oil DIC objective, used at the Centre of Cellular Imaging at Gothenburg University. Optical sections of 1 µm (Z-stack) were obtained and tile scans were stitched to visualise the whole area of interest (images of the arcuate nucleus in **Paper I**, **Paper II**, and **Paper III**). For quantification of

mRNA expression, we used the maximum intensity projection of Z-stack images (created with Zen Black software from Zeiss). Counting was done in ImageJ/Fiji (NIH) using the Cell counter plugin.

Detection of circulation factors

Blood sample preparation

Trunk blood was collected from isoflurane anaesthetised and decapitated rats (**Paper I**) and from anaesthetised (with a mixture of Sedastart vet ® and Ketalar ®) mice (**Paper II**) blood was collected from the tail or by cardiac puncture.

All blood samples intended for detection of acyl-ghrelin levels (from mice and rats) were immediately collected into EDTA-coated tubes containing 4-(2-Aminoethyl)benzenesulfolyl fluoride hydrochloride (AEBSF) to a final concentration of 1 mg/mL. Samples collected to detect leptin were treated to obtain plasma by adding them to EDTA- coated tubes. Blood obtained to detect levels of GH, IGF-1 and insulin were collected into clot-activator serum tubes. Tubes were then centrifuged to obtain plasma or serum after which samples were aliquoted and stored at -80 °C until processing.

Hormones analyses

Commercially available enzyme linked immunosorbent assays (ELISAs) were used to examine the levels of acyl-ghrelin (#EZRGRA-90K, Merck Millipore, Darmstadt, Germany), leptin (#EZML-82K, Merck Millipore), GH (#EZRMGH-45K) and IGF-1 (#80574, Crystal Chem, Zaandam, The Netherlands) in the blood. The manufacturer's respective instructions were followed and samples were thawed only once to minimise degradation.

Blood glucose levels

In **Paper II** blood glucose levels after an overnight fast were determined using a glucometer (Accu-Check Performa, Roche Diagnistics Scandinavia AB, Bromma, Sweden). After mice were deeply anaesthetised, blood obtained from a tail nick was dropped on the glucometer and glucose levels measured.

Statistics and visualisation

Data presented in this thesis were analysed using the program IBM SPSS statistics (version 27 or 28; IBM Corp., Armonk, NY, USA). The most appropriate tests were selected based on experimental setup and outcome variables.

Figures and parts of figures included in this thesis were created with Biorender.com.

RESULTS

Paper I

Here we aimed to uncover whether merely the odour of a familiar palatable food could elicit feeding and food seeking, and what brain pathways and neuronal substrates are involved in cue-potentiated feeding behaviours.
We found that exposure to a palatable food cue (i.e. the smell of a familiar palatable food; peanut butter) affected feeding behaviour, prompted food seeking behaviour and over-consumption of chow, the only available food. These behavioural effects were not observed in peanut butter naïve controls. In peanut butter familiar rats (but not naïve rats), palatable food cue exposure caused activation of orexigenic systems, reflected by 1) an increase in plasma acyl-ghrelin, an orexigenic hormone with an established role in food-seeking, and 2) an increase in neuronal activation in the Arc, a key brain area involved in feeding behaviour, including in cells that express the orexigenic peptide AgRP. Thus, exposure to a palatable smell increased ghrelin and activated hunger neurones. The main results of this paper are depicted in Figure 6.

Figure 6. Olfactory food cue elicits feeding behaviours and activates neurones in the arcuate nucleus (Arc). *Upon detection of the odour of a familiar, palatable food, rats display increased food seeking behaviour and hyperphagia of their normal chow. Activated cells in the Arc includes cells that express agouti-related peptide (AgRP), the growth hormone secretagogue receptor (GHSR) and/or proopiomelanocortin (POMC).*

Paper II

With a view to utilizing neural circuit mapping technologies to target and characterize orexigenic (ghrelin-activated) systems, both functionally and neuroanatomically, we sought to characterise a relatively recently developed *Ghsr-IRES-Cre* mouse model (Mani et al., 2017), which was developed to enable specific targeting of cells expressing the ghrelin receptor (GHSR).

Thus, we explored the effects of the knock-in of the *IRES-Cre* cassette on the expression of the targeted gene (*ghsr)* and assessed functions related to GHSR, such as ghrelin-responsiveness, metabolic response to fasting and the growth and developmental phenotypes of *Ghsr-IRES-Cre* mice.
Surprisingly, we found a decrease in the number of cells expressing ghsr mRNA in the Arc in a gene-dose dependant manner, where more copies of the transgene resulted in less *ghsr* expression in the Arc. This indicates that the insertion of the *IRES-Cre c*assette interferes with the ability of GHSR-expressing cells to resond to ghrelin. In line with the disrupted expression of the ghrelin receptor, we observed that ghrelin did not stimulate food intake or activate Arc cells (i.e. lack of Fos expression) in homozygotes. Furthermore, we found that the genetic manipulation affected body weight of transgenic mice from early postnatal days and lasting to adulthood, but the transgenic pups did not display aberrant physical or motor development. Adult homozygous *Ghsr-IRES-Cre* mice were not only lighter compared to wild type littermates, but also shorter and had lower fat mass, while lean mass was unaffected. Finally, we observed that in both heterozygous and homozygous mice glucoregulatory and GH-releasing actions were disrupted when the mice were exposed to a metabolically challenging state, such as an overnight fast. In both genotypes a drop in GH, glucose and insulin levels was noted compared to wild types, and additionally, homozygotes had reduced IGF-1 levels compared to wild types upon an overnight fast, while acyl-ghrelin levels were increased. Thus, in Ghsr-IRES-Cre mice modification of the GHSR locus disrupted GHSR function resulting in a phenotype that resembles GHSR knockouts. The main results of this paper are depicted in Figure 7.

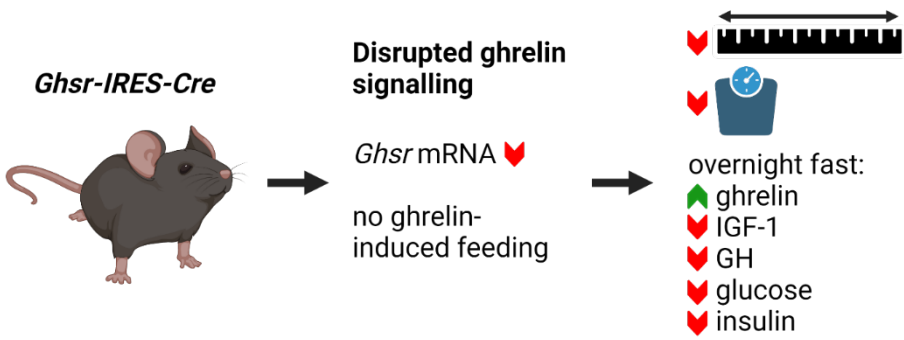


Figure 7. Disruption ghrelin signalling results in growth retardation and disturbed metabolic response. *Expression of Ghsr mRNA is reduced in Ghsr-IRES-Cre mice in a gene dose dependent manner and ghrelin-induced feeding is absent in homozygotes. The disrupted ghrelin receptor signalling resulted in reduced bodyweight, length, composition and aberrant metabolic response to an overnight fast. IGF-1: insulin-like growth factor 1; GH: growth hormone.*

Paper III

In paper III we targeted ghrelin-activated cells in the Arc using *ad libitum* fed heterozygous *TRAP2* mice, providing proof-of-concept that the Fos-TRAP system (i.e. *TRAP2* mouse; Allen et al., 2017) can be used to visualise and explore the function of the entire ghrelin (Fos-) activated ensemble in the Arc. We showed that chemogenetic excitation of ghrelin-activated ensemble of cells in the Arc drove mice to eat more, in a similar manner as observed after peripheral ghrelin administration. We also observed an orexigenic response, albeit to a lesser extent, by chemogenetic activation of the Arc ensemble TRAPed in the basal condition (saline), indicating that those activated cells in the basal state are also orexigenic. The location of the targeted ghrelin-activated cells resembled that of a ghrelin-induced Fos response and included AgRP cells (also in saline controls), suggesting that, together with the food intake data, we targeted a similar hormone (ghrelin)-responsive population in our TRAP mice. Besides establishing the Fos-TRAP system in our laboratory, we also found that chemogenetic activation of the ghrelin-activated Arc cells affected food choice in a sex-dependant manner, where only female mice increased their chow intake upon chemogenetic activation when presented with both chocolate and regular chow. The main results of this paper are depicted in Figure 8.

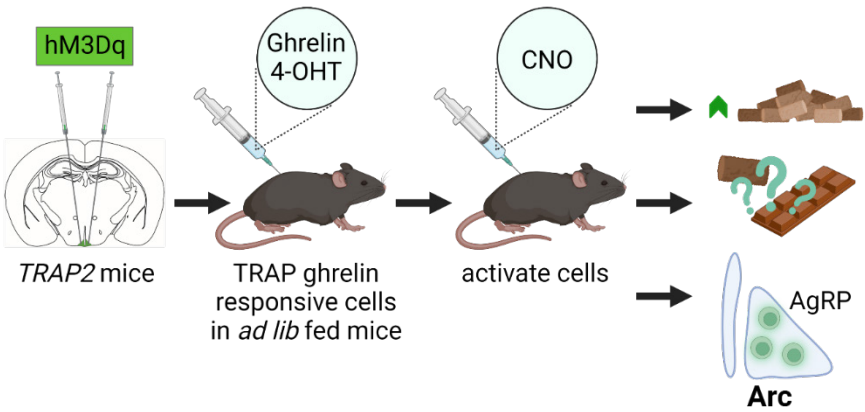


Figure 8. Ghrelin-responsive ensemble in the arcuate nucleus (Arc) affects food intake and food choice. *Chemogenetic activation of the ensemble was achieved by (i) viral vector delivery of a Cre-dependently expressed activating DREADD (designer receptor exclusively activated by designer drug; hM3Dq) to the Arc; (ii) targeted recombination of active populations (TRAP) upon ghrelin- and 4-hydroxytamoxifen (4-OHT) delivery and (iii) finally injecting the hM3Dq agonist clozapine-N-oxide (CNO). Activation of the ensemble resulted in increased food intake. Female mice also increased chow intake in a food-choice paradigm while controls did not. Agouti-related peptide (AgRP) expressing cells were amongst the chemogenetically activated cells. Ad lib: ad libitum*

Paper IV

Inspired by paper III, we used the *TRAP2* mouse model to identify and characterise an ensemble of candidate hunger neurons in the Arc. We targeted cells that were activated by ghrelin in fasted mice and explored their function in various feeding-related behaviours using a chemogenetic approach. We expected that a fasting state would facilitate an optimal yield of targeted ghrelin-activated cells, since it could be speculated that fasting "primes" the brain for orexigenic stimuli, based on the knowledge that the ghrelin-induced Fos response in the Arc (Hewson & Dickson, 2000) and expression of *Ghsr* and *Agrp* mRNAs (Cowley et al., 2003; Hahn et al., 1998; Kakava-Georgiadou et al., 2020) are increased by fasting. Using chemogenetic activation of the targeted "hunger ensemble" in the Arc we aimed to determine whether this ensemble is sufficient to induce food intake, drive food-motivated behaviour and convey the negative valence of hunger.

This study revealed that the targeted "hunger ensemble" in the Arc was sufficient to increase food intake in *ad libitum* fed mice. Thereby, the willingness to nose poke for food in a progressive ratio task (i.e. motivation) was increased after activation of the Arc ensemble. Finally, we found that activating the Arc ensemble was sufficient to condition a place avoidance, consistent with their role in conveying the negative valence of hunger. The main results of this paper are depicted in Figure 9.

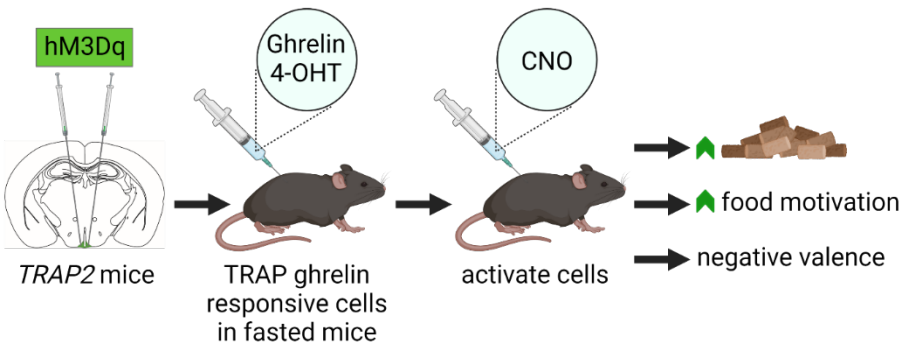


Figure 9. *Ghrelin-responsive ensemble in the arcuate nucleus (Arc) increases food choice and food motivation and conveys a negative valance. Chemogenetic activation of the ensemble was achieved by (i) viral vector delivery of a Cre-dependently expressed activating DREADD (designer receptor exclusively activated by designer drug; hM3Dq) to the Arc; (ii) targeted recombination of active populations (TRAP) upon ghrelin- and 4-hydroxytamoxifen (4-OHT) delivery and (iii) finally injecting the hM3Dq agonist clozapine-N-oxide (CNO). Activation of the ensemble resulted in increased food intake and food motivation and also conditioned a place aversion.*

Paper V

In this work, we applied the Fos-TRAP method to investigate cells activated in mice in a severely orexigenic state, specifically mice exposed to the ABA model. We observed that cells in the hypothalamus were Fos-activated in ABA mice, and immunohistochemical staining showed that a large proportion of these were orexin-containing. We then sought to determine the involvement of orexin signalling in running wheel behaviour in this mouse model mimicking features of anorexia nervosa. Given that orexin plays a role in arousal (Chemelli et al., 1999), we investigated whether the dual orexin receptor antagonist suvorexant could reduce running activity during ABA.
Additionally, acute peripheral administration of suvorexant reduced running wheel activity during the food anticipatory phase. The main results of this paper are depicted in Figure 10.

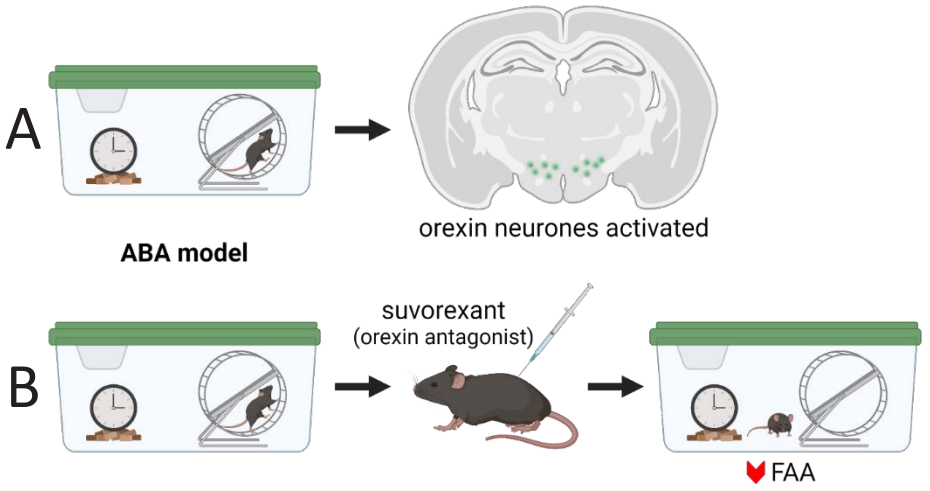


Figure 10. Orexin neurones play a role in food anticipatory activity (FAA) in the activity-based anorexia (ABA) model. *Orexin neurones in the hypothalamus are activated in mice exposed to the ABA model. In this model, mice have access to a running wheel and are scheduled fed for 2 hours per day [A]. Systemic injection of the orexin antagonist suvorexant suppresses FAA in ABA mice [B].*

DISCUSSION

In the past decades several neurocircuits, neuronal and peripheral signals were identified that regulate feeding behaviours, such as ghrelin and its effect on orexigenic neural circuits. We have learned that the hypothalamus plays a major role in feeding and mediating peripheral metabolic signals (review: Williams et al., 2001). Especially the arcuate nucleus (Arc) is an important hub in the feeding circuitry. It contains different sets of neurons, including AgRP and POMC neurones that are key in orexigenic and anorexigenic signalling, respectively, and are responsive to internal metabolic signals (review: Cone et al., 2001) but also to sensory input (review: (Chen & Knight, 2016). The lateral hypothalamus (LatH) also contains specific populations of cells implicated in metabolic regulation such as the orexin cells, that play a major role in arousal and physical activity as well (review: Sakurai, 2005). The studies included in this thesis demonstrated that (i) the Arc and ghrelin signalling are involved in primary food-cue induced feeding (**Paper I**), (ii) deficient ghrelin signalling leads to metabolic and growth phenotypes (**Paper II**), (iii) ghrelin responsive ensembles in the Arc are sufficient to drive food intake, food motivation, food choice and convey a negative valence and (**Paper III and Paper IV**) (iiii) antagonizing orexin signalling suppresses food anticipatory activity (but not food intake) in a mouse model of anorexia nervosa (**Paper V**).

Orexigenic signalling engaged in food-cue induced feeding

Food cues can potently drive feeding and, in this study, we demonstrated in rats that the smell of a familiar palatable food (primary food cue) induces overeating of the only available, less palatable regular chow. The cue also prompted food seeking and we found that various cell types in the Arc were activated upon exposure to the primary food cue and the ghrelin system was engaged (since blood levels of ghrelin were increased).

It is well-known that the sense of smell is a driver of appetite, food seeking and food preference in animals and humans (Fine & Riera, 2019; Palouzier-Paulignan et al., 2012), which our results confirmed (increased feeding and food seeking upon cue exposure) but the underpinning mechanisms remain largely elusive. Classical neuroanatomical studies and electrophysiological recordings have shown evidence of connectivity between the olfactory bulb and hypothalamic brain areas important for regulation of feeding behaviour, including the Arc and LatH (Russo et al., 2018; Schneider et al., 2020). Thereby, neurones in the Arc respond almost instantly to sensory detection of food and food odour cues (AgRP inhibited, POMC activated), an effect that depends on palatability and is transient if no food is available but sustained

when food is available (Chen et al., 2015). In line with this, we found that cells in the Arc are activated upon food odour exposure (measured by Fos expression) in a setting where no food is available. Interestingly, not only AgRP neurones were activated (as expected in hunger-promoting circumstances), but also POMC, tyrosine hydroxylase (dopaminergic) and some other unidentified Arc cells were recruited. A possible explanation why POMC neurones (known to signal satiety) were activated by an orexigenic cue is the acute activation of these cells upon sensory detection of a food smell (Chen et al., 2015). Since Fos expression takes time to dissipate, although transient, the acute activation of the POMC cells by the food cue can be the underlying reason for the Fos expression. Based on this, it could be speculated that, if that is the case, AgRP neurones should be inhibited by the cue and not express Fos, however, (i) this cue-potentiated inhibition is transient when food is not available as mentioned before and (ii) circulating ghrelin levels were increased upon primary cue exposure and AgRP neurones are known to be activated by this hormone (Seoane et al., 2003). That the ghrelin system is on board was not only evidenced by increased circulating ghrelin levels, but also by the activation of *Ghsr* expressing cells in the Arc. Dopaminergic Arc neurones (expressing tyrosine hydroxylase) are another orexigenic population and are activated by fasting and ghrelin (Zhang & van den Pol, 2016) but we found that only a small portion of these neurones was activated upon primary food cue exposure, suggesting that their role in food odour induced feeding is modest or negligible.

Using Fos mapping, we identified the Arc as a key brain area activated by olfactory food cues, while we did not observe prominent Fos expression in other hypothalamic or reward-linked areas. We noted Fos expression in areas involved in the relay of olfactory information (piriform and cingulate cortex, medial amygdaloid nucleus and the olfactory tubercle), however, those were also activated in control animals (naïve to the taste of the palatable food) exposed to the olfactory cue. It is important to note that the absence of Fos expression does not exclude involvement of non-Fos expressing neurones (Hudson, 2018; Johnstone et al., 2006). Neurones that are inhibited by an odour cue are unlikely to express Fos; we can only conclude that the Arc is involved.

To expand our options to further explore the cue-activated Arc population using transgenic animals, we tested mice in the same olfactory food-cue set-up as the rats. Against our expectation, cue-potentiated increase of chow intake did not emerge in mice. This was also shown in an earlier study with mice (Boone et al., 2021), however, mice eat small amounts over a day (which is the time the measurements took place), therefore manual food intake measurements might not be sensitive enough to detect a difference.

Altogether, this study shows that the smell of a palatable food can induce overeating of regular chow and ghrelin may mediate such cue-potentiated

feeding. Then, our data revealed that a heterogenous ensemble of Arc cells is engaged in the processing of primary food cues in the olfactory modality.

Disrupted ghrelin signalling in metabolism and growth

In the second study in this thesis we conducted experiments with *Ghsr-IRES-Cre* mice, initially with the goal to explore the role of ghrelin receptor (GHSR) expressing neuronal populations, for example using chemogenetics. To our surprise, however, we found that the insertion of the *IRES-Cre* cassette disrupted expression of G*hsr*, resulting in growth and metabolic phenotypes in a gene-dose dependent manner (a.k.a. both heterozygous and homozygous mice were affected, in a gene dose dependent manner). Compared to wild type litter mates, mice with reduced *ghsr* expression had a reduced body weight from early postnatal day onward and adults were shorter, had lower fat mass and disrupted glucoregulatory and GH-releasing actions after an overnight fast. To create the *Ghsr-IRES-Cre* mouse line, an *IRES-Cre* cassette was knocked-in to the 3'-untranslated region (3'-UTR) of the *Ghsr* gene (Mani et al., 2017). 3'-UTRs play a role in various aspects of post-transcriptional control of gene expression, such as mRNA stability and alternative splicing. Mutations in this region are implicated in several neurological disorders in humans, for instance substance use disorder and Parkinson's disease (review: Bae & Miura, 2020). Since 3'-UTRs are binding sites for microRNAs and RNA-binding proteins, insertions into the regions can lead to disruption of gene expression, where the *Ghsr-IRES-Cre* mouse line is not unique. It has been suggested before that in several Cre-lines, insertion of an *IRES-Cre* cassette in this region has led to disruption of gene expression, resulting in a phenotype (Bäckman et al., 2006; Joye et al., 2020; Rashid et al., 2017; Viollet et al., 2017). Much remains unknown about the roles of the 3'-UTR subregions and it is therefore not clear why in some Cre-lines generated in a similar manner these issues do not emerge (or maybe they are phenotyped less thoroughly) but speculatively it can be related to the specific location of the knock-in within the 3'-UTR.
Given the role of ghrelin signalling in energy homeostasis and GH release, it is maybe not surprising that reduced *Ghsr* expression in *Ghsr-IRES-Cre* mice resulted in impaired growth and disrupted endocrine response to an overnight fast. From the significant role that ghrelin plays in eliciting feeding behaviour it could even be expected that disruption would lead to more severe feeding-related phenotypes in our mice than the ones that emerged. However, feeding is so essential to life that it seems obvious that organisms would not rely on one system to drive this behaviour and other systems can compensate for the absence of ghrelin signalling. This is in line with the mild phenotypes observed in other models of aberrant ghrelin signalling, and there exist a multitude of rodent models (mainly mice) in which different components of the ghrelin

system are manipulated, from *Ghsr* or ghrelin expression, to expression of GOAT, the enzyme that is responsible for acylating ghrelin (a.k.a. activating ghrelin) (review: Peris-Sampedro et al., 2021).

Interestingly, we observed that metabolically challenging *Ghsr-IRES-Cre* mice with an overnight fast resulted in increased plasma ghrelin levels, while GH and IGF-1 were lower compared to wild type controls, especially in homozygotes. Ghrelin induces GH secretion mediated through GHSR (Sun et al., 2004), that in turn increases its effector IGF-1, which explains why GH and IGF-1 were not increased upon an overnight fast (associated with increased ghrelin) in our *Ghsr* deficient mice compared to controls. The amplified increase in ghrelin levels, however, was unexpected. One possible explanation is that the body tries to compensate for the disrupted ghrelin signalling or the low blood glucose levels (and note that ghrelin protects against hypoglycaemia). Another explanation could be the disruption of a negative feedback-loop between IGF-1 and ghrelin. There is evidence that supports a negative association between GH/IGF-1 and ghrelin in humans and animals, speculatively favouring a direct link between ghrelin and IGF-1 rather than GH based on for instance subjects with chronic liver disease that have elevated ghrelin levels and decreased IGF-1, despite of their high GH levels (Tacke et al., 2003). Corroborating the existence of a feedback loop, a study with a cohort of 1004 human subjects found a negative correlation between circulating levels of IGF-1 and ghrelin, where males, subjects with higher body mass index and insulin resistant patients showed an even stronger correlation (Pöykkö et al., 2005). Additionally, increased levels of GH and IGF-1 and reduced levels of ghrelin in patients with acute acromegaly are all restored after surgical removal of the adenoma in the pituitary responsible for excessive GH production (Freda et al., 2003). Thereby, acute administration of GH results in decreased levels of circulating ghrelin in GH deficient patients and GH sufficient subjects (Dall et al., 2002; Tarantini et al., 2009; Vestergaard et al., 2005). In rodents, circulating and gastric ghrelin are reduced by GH administration, while circulating ghrelin is increased after hypophysectomy (removal of the GH producing/releasing pituitary) (Lee et al., 2002; Tschöp et al., 2002). Chronic GH replacement therapy, however, does not seem to impact on ghrelin levels, although factors such as weight loss during the year of treatment and not matching controls based on sex and age could have obscured an effect, concerns raised by the authors (Janssen et al., 2001).

Thus, our study shows that GHSR signalling is disrupted in *Ghsr-IRES-Cre mice*, which affects growth form early age. It also emerged this disruption in *Ghsr-IRES-Cre* mice leads to a blunted response to a metabolically challenging state, and the levels of circulating hormones upon an overnight fast (and literature) hint towards an interplay between ghrelin and the growth axis via IGF-1. Together, these data show that many phenotypes expected in *Ghsr*

knock-outs emerge in our mice (regarding the two functions of GHSR; in growth and metabolism), evidencing that the *Ghsr-IRES-Cre* mouse model can serve as a GHSR knock-out model in future research.

Ghrelin responsive Arc ensembles drive feeding behaviours

The studies in **Paper III** and **Paper IV** together showed that chemogenetic activation of Arc cells responsive to peripheral ghrelin is sufficient to induce feeding, increase food motivation, impact on food choice (in females) and condition a place avoidance in mice. According to expectation, we also confirmed that AgRP neurones (an established target for ghrelin; Seoane et al., 2003) are part of the ghrelin-responsive Arc ensemble in **Paper III**.

Food intake

Since peripheral ghrelin is well known to induce feeding, reach the Arc (Schaeffer et al., 2013) and activate the potently orexigenic AgRP neurones embedded in this brain area, the increase in food intake after chemogenetic excitation of the ghrelin-responsive ensemble in *TRAP2* mice demonstrated that the used techniques (TRAPing and DREADDs) were implemented successfully. Interestingly, the difference in baseline condition between the two papers impacted on the behavioural output of the control mice (*ad libitum* fed in **Paper III**, fasted overnight in **Paper IV**), where excitation of Arc cells targeted in the fed state elicited feeding in the light phase, while excitation of Arc cells targeted in fasted mice did not. TRAPing (a.k.a. targeting cells by injecting 4-hydroxytamoxifen) was performed under otherwise similar conditions in the two papers, so factors such as the time of day are unlikely to have caused this difference in the feeding response. In the literature, there is no complete consensus about whether fasting induces Fos expression in the Arc; there is evidence that fasting does increase Fos in the Arc in mice (Engström Ruud et al., 2020), even specifically in AgRP cells (Liu et al., 2012), but in another study in rats the number of Fos positive cells in the Arc did not increase upon fasting (Fraga et al., 2005). Interestingly, the time of day at which we TRAPed cells (i.e. when Fos expression defines what cells are targeted) and the time at which the rat study examined fasting-induced Fos expression (Fraga et al., 2005) was comparable (half-way the light phase); it was chosen as a time of expected low spontaneous Fos expression in the Arc (Lamont et al., 2014; Ramirez-Plascencia et al., 2017). The studies mentioned above, where fasting did induce Fos in the Arc, examined Fos expression during the early light phase (Liu et al., 2012) or early dark phase (Engström Ruud et al., 2020). Together, these data indicate that whether fasting does or does not induce Fos expression in the Arc might depend on the time of day,

which can explain the absence of feeding after chemogenetic excitation in the overnight fasted control group. It remains curious, however, that our fed control group increased food intake upon chemogenetic excitation. Given that in our study with fed controls we found a correlation between the number of chemogenetically activated (Fos expressing) AgRP cells and food intake, the increase in food intake in the controls is probably driven by AgRP cells. Speculatively, the relatively long fasting period to which the other control mice were exposed before TRAPing could have exhausted the AgRP cells, leaving them inactive, not expressing Fos and therefore not TRAPed, so not activated during chemogenetic experiments explaining the absence of a feeding response.

Food choice

Not only total food intake, but also food choice was altered by chemogenetic activation of the ghrelin-responsive Arc ensemble in **Paper III**. In female mice, the activation led to an increase in total, chow and chocolate consumption, while controls only increased total caloric intake. That chow intake was higher upon chemogenetic activation of the ghrelin-responsive ensemble compared to the controls resonates with previous studies in rats where central ghrelin administration favours the consumption of "healthier" chow over sugar in a free choice paradigm (Schéle et al., 2016) and also chow consumption in rats trained to binge on a high fat diet while having chow access (Bake et al., 2017). Why we observed this shift in food choice only in females is not clear and more research is needed to elucidate the sex-dependant differences in feeding behaviours and underlying pathways involved. Notably, there exists evidence from studies with humans that food preference and craving, what foods are craved and the intensity of the craving are sexually dimorphic (Hallam et al., 2016).

Food motivation

Strikingly, data in **Paper IV** suggest that the ghrelin-responsive ensemble in the Arc is sufficient to drive food motivation. Chemogenetic excitation of the ensemble increased the number of nose pokes the mice performed to earn a chow pellet in a classical operant responding task. This resonates with data showing that (i) central ghrelin delivery increases lever pressing for chow and sucrose in rats (Bake et al., 2019), (ii) peripheral ghrelin administration increases nose poking for high fat food in mice (Perello et al., 2010) and (iii) chemogenetic and optogenetic activation of the ghrelin-responsive AgRP cells in the Arc increases food motivation (Aponte et al., 2011; Krashes et al., 2011). These effects to increase motivation can be driven by local injection of ghrelin into the ventral tegmental area (VTA) and appear to engage midbrain

dopaminergic neurones that project from the VTA to the nucleus accumbens (NAc) (Skibicka et al., 2011; Skibicka et al., 2013), a circuitry that is well-known for its involvement in motivation and reward. Food motivation is also increased by intra-LatH ghrelin injections (López-Ferreras et al., 2017). Peripheral ghrelin, however, does not directly activate cells in the VTA and LatH, but does so in the Arc (Schaeffer et al., 2013). Together with our data, this indicates that the ghrelin-responsive ensemble in the Arc might be of importance in relaying the effect of peripheral ghrelin on food motivation.
Little evidence exists for direct connectivity between the Arc (including AgRP neurones) and the VTA dopaminergic neurones (Mazzone et al., 2020; Watabe-Uchida et al., 2012). We only identified 2 published studies in which such connectivity has been identified, one showing that POMC neurones project to the VTA and another showing that AgRP neurones also project there (Dietrich et al., 2012; King & Hentges, 2011). Local VTA circuits might mediate an indirect effect from the Arc ensemble on the dopaminergic cells. There are studies, however, showing that AgRP activation does not directly change excitability of VTA dopaminergic neurones (Mazzone et al., 2020), suggesting that the connection between the Arc and VTA is more indirect. An alternative is that ghrelin's effect on food motivation might be mediated through the LatH and maybe even specifically the orexin cells, since orexin antagonist administration abolishes ghrelin's effect on food motivation (Perello et al., 2010). Orexin cells, in turn, project directly to midbrain dopaminergic neurones (Fadel & Deutch, 2002; Nakamura et al., 2000) and pose an alternative route through which ghrelin-responsive Arc cells could impact on VTA dopaminergic neurones and food motivation (López-Ferreras et al., 2017).

Valence

Besides driving feeding behaviours, ghrelin is also known to play a role in the negative valence-signalling that accompanies hunger. We found that activation of the ghrelin-responsive Arc ensemble resulted in a conditioned place aversion (CPA) in the absence of food, which resonates with two out of three studies regarding ghrelin's valence signalling. Paradoxically, one study in 2008 showed that peripheral ghrelin injection results in a conditioned place preference (CPP) (Jerlhag, 2008). The other two studies show that both central and peripheral ghrelin administration condition an avoidance in the absence of food (Lockie et al., 2015; Schéle et al., 2017). In the presence of food, however, the results are conflicting, which could be due to the different route of administration; central administration resulted in CPA (Schéle et al., 2017), while peripheral ghrelin induced a CPP (Lockie et al., 2015). Central delivery of ghrelin activates more brain areas compared to peripheral administration

(Cabral et al., 2014) and this difference in pathway activation could underly the opposing results.
In our study, we show that ghrelin-responsive cells, specifically in the Arc, are sufficient to condition an avoidance. This resonates with previous results which show that optogenetic activation of the ghrelin-responsive AgRP cells in the Arc condition an avoidance for place and taste (Betley et al., 2015).

In summary, the studies in **Paper III** and **Paper IV** highlight the potential importance of ghrelin-responsive cells in the Arc in relaying peripheral ghrelin information. This is evidenced by the involvement of the ghrelin-responsive Arc ensemble in food intake, food motivation, food choice and conveying valence, together with the knowledge that peripheral ghrelin can reach and directly activate Arc cells (Schaeffer et al., 2013). These observations give room to speculate that the Arc is a relay centre for peripheral ghrelin action on the brain, through which signalling to higher order neurones takes place to regulate the different aspects of feeding and hunger.

Orexin signalling and food anticipatory activity in an anorexia model

In the last study included in this thesis, we identified the neuronal orexin population in the hypothalamus as a potential target to reduce hyperactivity in anorexia nervosa. Over half of the orexin neurones were activated in mice exposed to ABA model (i.e. mice in an extremely orexigenic state). ABA mice are known to exhibit FAA in the period preceding scheduled feeding time (Klenotich et al., 2012), which we could suppress in our mice by inhibiting orexin signalling by administration of the dual orexin receptor antagonist suvorexant. We did not observe any signs of general physical immobility due to administration of this drug, suggesting that the dose given is below the threshold for any sedative effects. Previous studies are in line with our results, showing that locomotor activity in rats decreases upon oral administration of suvorexant (Winrow et al., 2011) and in mice locomotor activity is reduced by administration of the dual orexin receptor antagonist lemborexant (Beuckmann et al., 2019). Additionally, *ad libitum* fed orexin knockout mice exhibited reduced running wheel activity (España et al., 2007) and ablation of orexin neurones in fasted mice led to reduced exploratory activity and FAA (Akiyama et al., 2004; Mieda et al., 2004; Yamanaka et al., 2003). Collectively, these studies demonstrate that disrupted orexin signalling reduces locomotor activity, also physical activity specifically in the context of low nutritional status such as FAA.
Orexins are also known to be strong regulators of the sleep-wake cycle in animals and humans. Narcolepsy patients suffer from disturbed wakefulness and have very few orexin cells (Thannickal et al., 2000) and mouse models

where orexin signalling is disrupted all develop symptoms similar to human narcolepsy (Chemelli et al., 1999; Hara et al., 2001). In anorexia nervosa patients, elevated orexin levels possibly contribute to their insomnia. High orexin-A blood levels in patients suffering from anorexia nervosa are associated with poorer sleep according to one study (Sauchelli et al., 2016). Interesting to note is that patients suffering from anorexia nervosa who suffer more from sleep disturbances exhibit more severe symptoms overall independent of body mass index (Malcolm et al., 2022), suggesting that poor sleep might not merely be a consequence of weight loss but could be caused by anomalies related to other causes related to anorexia nervosa aetiology, possibly disturbances in orexin signalling. Some studies showed that untreated AN patients have elevated orexin-A blood levels and/or decrease their orexin-A levels upon treatment (Bronsky et al., 2011; Janas-Kozik et al., 2011), while others could not repeat this and even showed decreased levels in patients suffering from anorexia nervosa compared to controls (Janas-Kozik et al., 2011; Steward et al., 2019). Circulating orexin levels, however, need to be interpreted with caution since they are in general very low in humans, do not follow the same circadian rhythms as in cerebrospinal fluid and circulating levels of orexin might not reflect elevated orexin release in the brain (Mäkelä et al., 2018).

Together, our data and existing literature point out the potential of targeting the orexin system to aid recovery in patients suffering from anorexia nervosa by improving sleep and reducing hyperactivity. In fact, several orexin antagonists are being used in the clinic to treat insomnia in patients suffering from anorexia nervosa, making it even more appealing to explore the potential of these drugs in reducing hyperactivity; especially in restricting-type anorexia nervosa from whom as much as 80% are compulsive exercisers (Dalle Grave et al., 2008). Suvorexant, specifically, has a significantly longer half-life in humans (9-13 hours; Sun et al., 2013) compared to rodents (0.6 hours; Winrow et al., 2011), making chronic treatment of anorexia nervosa patients feasible despite the short-lived effect of about 2 hours we observed in our mice.

CONCLUSION

This thesis provides novel information regarding the importance and chemical identity of orexigenic ensembles in the Arc as a conductor of orexigenic signals from the periphery (ghrelin) and external sensory information (olfactory food cues). Ghrelin-responsive ensembles in the Arc turn out to be sufficient to drive several orexigenic behaviours and convey negative valence accompanying increased ghrelin levels. Thereby, we showed that the orexigenic GHSR signalling system plays a role in both growth and metabolic regulation using the *Ghsr-IRES-Cre* mouse model in which *Ghsr* expression is disrupted. Finally, orexin signalling turned out to be a promising target for treatment of patients suffering from anorexia nervosa. The dual orexin receptor antagonist suvorexant reduced FAA in ABA mice and orexin antagonists are used in the clinic to treat insomnia in patients suffering from anorexia nervosa. Thus, antagonizing orexin might improve treatment prospects by aiding to improved sleep and possibly containment of hyperactivity in these patients.

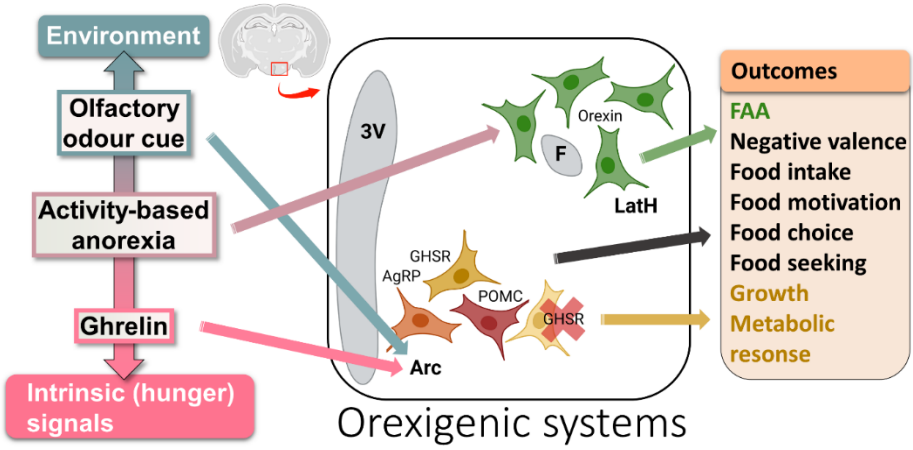


Figure 11. Schematic of orexigenic systems engaged by various hunger and/or appetite increasing inputs. *The results presented in this thesis show that ensembles of cells the arcuate nucleus (Arc) are activated by olfactory food cues and peripheral ghrelin. The cue-activated ensemble included cells expressing growth hormone secretagogue receptor (GHSR), agouti-related peptide (AgRP) and/or proopiomelanocortin (POMC). Orexigenic inputs to the Arc increase feeding-related behaviours and the ensemble conveys a negative valence. Orexin cells in the lateral hypothalamus (LatH) are activated in the activity-based anorexia mouse model. Orexin antagonist decreases food anticipatory activity (FAA) in this mouse model. Disrupted Ghsr expression results absence of ghrelin-induced food intake, growth retardation and a blunted metabolic response to fasting. 3V: third ventricle; F: fornix*

FUTURE PERSPECTIVES

Reflecting on the results from this thesis work, there are a number of ways in which this work can be advanced.

In **Paper I**, it would be interesting to discover more about the neurocircuitry underpinning the dramatic behavioural response in rats exposed to the olfactory food cue, in which they try to access the hidden food for 30-40 minutes. We anticipated engagement of many brain areas in this response yet, using Fos mapping, only the Arc was specifically activated by the cue. If indeed dopaminergic signalling is engaged, we could perform fiber photometry measurements to determine whether dopamine binding to its receptors (and thus dopamine release) is increased upon cue exposure in for example the NAc. This is possible using GRAB (Sun et al., 2018) or dLight1 (Patriarchi et al., 2018), two independently developed G-protein-coupled receptor-based dopamine sensors. We would expect the dopamine signal to be greater in the presence of the food odour cue in odour familiar rather than naïve rats, and in those exposed to the food cue rather than in its absence. More specifically, it would be interesting to explore the necessity and/or sufficiency of the VTA to NAc (VTA → NAc) dopaminergic pathway in cue-potentiated feeding behaviours. To this end we could apply a chemogenetic strategy that has been used before (Kakava-Georgiadou et al., 2019) to express DREADDs exclusively in VTA → NAc dopaminergic cells. Briefly, this can be achieved by injecting TH-Cre rats with two viral vectors; a retrograde canine adenovirus that Cre-dependently expresses Flp recombinase in the NAc and in the VTA an anterograde adeno-associated virus that expresses DREADDs in a Flp-dependent way. We could choose to apply either an excitatory or inhibitory DREADD and explore whether (i) activating dopaminergic VTA → NAc can elicit or amplify cue-induced feeding behaviours and (ii) inhibiting this pathway abolishes or attenuates those behaviours. Additionally, it would be interesting to determine whether delivery of GHSR antagonists can suppress the impact of food cue exposure on behaviour and on the activation of pathways involved. Then, establishing the olfactory food cue model in mice would open up a range of opportunities to further study the underlying neurocircuitry, since there is a wide variety of transgenic mouse models (way fewer transgenic rats are available). Measuring food intake with the FED3 system will facilitate the detection of small differences in food intake. Thereby we could possibly prime the brain for orexigenic inputs by fasting the mice before cue exposure to enhance the feeding effect and cell activation. This would enable us, for example, to use *TRAP2* mice to investigate the cue-activated neuronal ensembles in a similar way as in papers included in this thesis.

In **Paper II**, we demonstrated that homozygote *Ghsr-IRES-Cre* mice have loss of function of the ghrelin signalling system, leading to phenotypes surprisingly including growth retardation. Further studies could explore more extensively the impact on the hypothalamo-pituitary growth axis, including whether *Ghsr-IRES-Cre* mice also lose their ability to respond to GHRH. Consistent with this, one early study showed that little (lit/lit) mice that lack a functional GHRH receptor do not respond to GHS (Jansson, 1986). Thus, although GHRH and ghrelin bind to different receptors, the GHRH signalling system needs to be intact in order for GHS and ghrelin (Obal et al., 2003) to exert their GH-releasing effects. The reverse may also be true and follow-up studies could explore whether GHSR signalling needs to be intact for animals to response to GHRH. Gene expression studies in *Ghsr-IRES-Cre* mice could determine mRNA levels of GHRH and somatostatin (the inhibitory hypothalamic peptide controlling GH release) in the hypothalamus and also mRNA levels of GH at the level of the pituitary. In rodents, GH release is highly pulsatile (Tannenbaum & Martin, 1976) and sexually dimorphic (Clark et al., 1987; Edén, 1979; Jansson et al., 1985; Saunders et al., 1976). In mice, it is difficult to study the pattern of secretion since they need to be conscious and it is difficult, if not impossible, to perform repetitive sampling in ways that do not stress the animals (and note that stress interferes with GH release). However, it could be possible to measure mouse urinary protein in urine samples or mRNA levels in the liver; this liver-derived protein is known to be higher in male mice than in females, reflecting the pulsatility of the GH-secretory pattern (Husman et al., 1985; Norstedt & Palmiter, 1984). Higher pulsatility is linked to heightened growth (Albertsson-Wikland & Rosberg, 1988; Albertsson-Wikland et al., 1994; Hindmarsh et al., 1988) and so mouse urinary protein measurement could provide an indication of whether the secretory pattern is less pulsatile in the *Ghsr-IRES-Cre* mice.

In **Paper III** and **Paper IV**, we found that chemogenetic activation of ghrelin-responsive cells in the Arc drives a variety of orexigenic behaviours. Since, in these studies, we utilized *TRAP2* mice, it would be possible to perform RNA single cell sequencing on the TRAPed cells and discover their genetic landscape. Additional populations of activated (ghrelin responsive) neurones could be detected in other brain areas, most notably in the lateral parabrachial nucleus (lPBN), a brain area that paradoxically is profoundly anorexigenic. Given previous work of our group implicating the lPBN is ghrelin's orexigenic effects (Bake et al., 2020; Le May et al., 2021), it would be interesting to further explore their neurochemical identity and functional role, using techniques identical to those of the current paper. We expect to find that this population is involved in food intake (Bake et al., 2020) and that they may also contribute to negative valence coding (Carter et al., 2015). Although a sub-population of lPBN cells express GHSR (Le May et al., 2021), the possibility

exists that ghrelin's primary action is exerted upstream, at the levels of the Arc AgRP cells since these cells project to the lPBN (Atasoy et al., 2012; Betley et al., 2013; Campos et al., 2016; Carter et al., 2013; Wu et al., 2009) and activating this projection puts a break on this anorexigenic centre (Campos et al., 2016; Essner et al., 2017). Further neurocircuit mapping studies, therefore, could elucidate the relative importance of the GHSR expressing lPBN population in ghrelin's behavioural effects. Further elucidating the connectivity of the ghrelin-responsive ensemble in the Arc would also enable us to investigate the possibly different function of divergent projections, maybe teasing out which is important for different feeding behaviours. Thereby, the sexually dimorphic effect we observed on food choice is interesting to further investigate. Sex differences in ghrelin responsiveness have been pointed out before (López-Ferreras et al., 2017) but knowledge is limited. A first step would be to reproduce our results with a bigger cohort and investigate ghrelin's effect on feeding behaviours in female and male mice.

In **Paper V**, we demonstrate engagement of LatH orexin neurones in anorexia-like behaviour. A more chronic study would shed light on the long-term effects of orexin antagonist on feeding, body weight and running wheel activity. It would also be interesting to explore what other cell types were activated in this brain area in the ABA mouse model and what their function is related to anorexia-like behaviour. Thereby, in these studies, we noted engagement of several other brain areas in the response to ABA. After fully mapping these areas, we aim to select one or two prioritized areas and undertake further chemogenetic studies to explore their contribution to anorexia-like behaviours. An important advancement of this project would be to undertake a study in patients suffering from anorexia nervosa and determine whether treatment with an orexin antagonist has benefits for various manifestations of their disease, including sleep disturbance, anorexia, hyperactivity but also features not explored here such as food anxiety.

The introduction of the Fos-TRAP technique in our lab emerges as an extremely powerful tool in our future studies, with many possibilities to explore the identity and function of populations activated in states of altered energy imbalance. Knowledge obtained by such studies would help us further unravel the underpinnings of orexigenic signalling and can eventually lead to a better understanding of hunger circuits and contribute to the identification of neuronal targets for effective future treatments of disordered eating.

ACKNOWLEDGEMENTS

"Shall I refuse my dinner because I do not fully understand the process of digestion?"

Oliver Heaviside
A quote in reference to Heaviside using mathematical operators that were not yet clearly defined by the mathematics community.

Finally, everyone's favourite chapter! I would like to express my gratitude to all the wonderful people who were involved in the work in this thesis and everyone else who has made this journey possible and joyful:

My main supervisor, **Roger A. H. Adan**, thank you for giving me the opportunity to do research in a field that I am passionate about and providing me with guidance and motivation when needed. Onze wekelijke zooms heb ik altijd gewaardeerd. Ze waren efficiënt, nuttig en met een vleugje droge humor, zoals het Nederlandsers beaamt...

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REFERENCES

Abizaid, A., Liu, Z. W., Andrews, Z. B., Shanabrough, M., Borok, E., Elsworth, J. D., Roth, R. H., Sleeman, M. W., Picciotto, M. R., Tschöp, M. H., Gao, X. B., & Horvath, T. L. (2006). Ghrelin modulates the activity and synaptic input organization of midbrain dopamine neurons while promoting appetite. *J Clin Invest*, *116*(12), 3229-3239.

Adan, R. A., Tiesjema, B., Hillebrand, J. J., la Fleur, S. E., Kas, M. J., & de Krom, M. (2006). The MC4 receptor and control of appetite. *Br J Pharmacol*, *149*(7), 815-827.

Akiyama, M., Yuasa, T., Hayasaka, N., Horikawa, K., Sakurai, T., & Shibata, S. (2004). Reduced food anticipatory activity in genetically orexin (hypocretin) neuron-ablated mice. *Eur J Neurosci*, *20*(11), 3054-3062.

Al Omran, Y., & Aziz, Q. (2014). The Brain-Gut Axis in Health and Disease. In M. Lyte & J. F. Cryan (Eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease* (pp. 135-153). Springer New York

Albertsson-Wikland, K., & Rosberg, S. (1988). Analyses of 24-hour growth hormone profiles in children: relation to growth. *J Clin Endocrinol Metab*, *67*(3), 493-500.

Albertsson-Wikland, K., Rosberg, S., Karlberg, J., & Groth, T. (1994). Analysis of 24-hour growth hormone profiles in healthy boys and girls of normal stature: relation to puberty. *J Clin Endocrinol Metab*, *78*(5), 1195-1201.

Allen, W. E., DeNardo, L. A., Chen, M. Z., Liu, C. D., Loh, K. M., Fenno, L. E., Ramakrishnan, C., Deisseroth, K., & Luo, L. (2017). Thirst-associated preoptic neurons encode an aversive motivational drive. *Science*, *357*(6356), 1149-1155.

Almy, T. P. (1989). Historical Perspectives of Functional Bowel Disease. In W. J. Snape (Ed.), *Pathogenesis of Functional Bowel Disease* (pp. 1-11). Springer US

Alvarez-Crespo, M., Skibicka, K. P., Farkas, I., Molnár, C. S., Egecioglu, E., Hrabovszky, E., Liposits, Z., & Dickson, S. L. (2012). The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. *PLoS One*, *7*(10), e46321.

Anand, B. K., & Brobeck, J. R. (1951). Hypothalamic control of food intake in rats and cats. *Yale J Biol Med*, *24*(2), 123-140.

Aponte, Y., Atasoy, D., & Sternson, S. M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat Neurosci*, *14*(3), 351-355.

Arcelus, J., Mitchell, A. J., Wales, J., & Nielsen, S. (2011). Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies. *Arch Gen Psychiatry*, *68*(7), 724-731.

Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., & Roth, B. L. (2007). Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A*, *104*(12), 5163-5168.

Atasoy, D., Betley, J. N., Su, H. H., & Sternson, S. M. (2012). Deconstruction of a neural circuit for hunger. *Nature*, *488*(7410), 172-177.

Bäckman, C. M., Malik, N., Zhang, Y., Shan, L., Grinberg, A., Hoffer, B. J., Westphal, H., & Tomac, A. C. (2006). Characterization of a mouse strain expressing Cre recombinase from the 3' untranslated region of the dopamine transporter locus. *Genesis*, *44*(8), 383-390.

Bae, B., & Miura, P. (2020). Emerging Roles for 3' UTRs in Neurons. *Int J Mol Sci*, *21*(10).

Bagdade, J. D., Bierman, E. L., & Porte, D., Jr. (1967). The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. *J Clin Invest*, *46*(10), 1549-1557.

Bake, T., Edvardsson, C. E., Cummings, C. J., & Dickson, S. L. (2019). Ghrelin's effects on food motivation in rats are not limited to palatable foods. *J Neuroendocrinol*, *31*(7), e12665.

Bake, T., Hellgren, K. T., & Dickson, S. L. (2017). Acute ghrelin changes food preference from a high-fat diet to chow during binge-like eating in rodents. *J Neuroendocrinol*, *29*(4).

Bake, T., Le May, M. V., Edvardsson, C. E., Vogel, H., Bergström, U., Albers, M. N., Skibicka, K. P., Farkas, I., Liposits, Z., & Dickson, S. L. (2020). Ghrelin Receptor Stimulation of the Lateral Parabrachial Nucleus in Rats Increases Food Intake but not Food Motivation. *Obesity (Silver Spring)*, *28*(8), 1503-1511.

Banks, W. A., Burney, B. O., & Robinson, S. M. (2008). Effects of triglycerides, obesity, and starvation on ghrelin transport across the blood-brain barrier. *Peptides*, *29*(11), 2061-2065.

Banks, W. A., Tschöp, M., Robinson, S. M., & Heiman, M. L. (2002). Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J Pharmacol Exp Ther*, *302*(2), 822-827.

Beeler, J. A., & Burghardt, N. S. (2021). Activity-based Anorexia for Modeling Vulnerability and Resilience in Mice. *Bio Protoc*, *11*(9), e4009.

Belfort-DeAguiar, R., & Seo, D. (2018). Food Cues and Obesity: Overpowering Hormones and Energy Balance Regulation. *Curr Obes Rep*, *7*(2), 122-129.

Berridge, K. C., & Kringelbach, M. L. (2015). Pleasure systems in the brain. *Neuron*, *86*(3), 646-664.

Berridge, K. C., & Robinson, T. E. (2016). Liking, wanting, and the incentive-sensitization theory of addiction. *Am Psychol*, *71*(8), 670-679.

Berthoud, H. R. (2004). Mind versus metabolism in the control of food intake and energy balance. *Physiol Behav*, *81*(5), 781-793.

Betley, J. N., Cao, Z. F., Ritola, K. D., & Sternson, S. M. (2013). Parallel, redundant circuit organization for homeostatic control of feeding behavior. *Cell*, *155*(6), 1337-1350.

Betley, J. N., Xu, S., Cao, Z. F. H., Gong, R., Magnus, C. J., Yu, Y., & Sternson, S. M. (2015). Neurons for hunger and thirst transmit a negative-valence teaching signal. *Nature*, *521*(7551), 180-185.

Beuckmann, C. T., Ueno, T., Nakagawa, M., Suzuki, M., & Akasofu, S. (2019). Preclinical in vivo characterization of lemborexant (E2006), a novel dual orexin receptor antagonist for sleep/wake regulation. *Sleep*, *42*(6).

Bolles, R. C., & Stokes, L. W. (1965). Rat's anticipation of diurnal and a-diurnal feeding. *J Comp Physiol Psychol*, *60*(2), 290-294.

Boone, M. H., Liang-Guallpa, J., & Krashes, M. J. (2021). Examining the role of olfaction in dietary choice. *Cell Rep*, *34*(7), 108755.

Boulos, Z., & Terman, M. (1980). Food availability and daily biological rhythms. *Neurosci Biobehav Rev*, *4*(2), 119-131.

Bowers, C. Y., Momany, F. A., Reynolds, G. A., & Hong, A. (1984). On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology*, *114*(5), 1537-1545.

Broberger, C., De Lecea, L., Sutcliffe, J. G., & Hökfelt, T. (1998). Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. *J Comp Neurol*, *402*(4), 460-474.

Bronsky, J., Nedvidkova, J., Krasnicanova, H., Vesela, M., Schmidtova, J., Koutek, J., Kellermayer, R., Chada, M., Kabelka, Z., Hrdlicka, M., Nevoral, J., & Prusa, R. (2011). Changes of orexin A plasma levels in girls with anorexia nervosa during eight weeks of realimentation. *Int J Eat Disord*, *44*(6), 547-552.

Brooks, S. J., O'Daly, O. G., Uher, R., Friederich, H. C., Giampietro, V., Brammer, M., Williams, S. C., Schiöth, H. B., Treasure, J., & Campbell, I. C. (2011). Differential neural responses to food images in women with bulimia versus anorexia nervosa. *PLoS One*, *6*(7), e22259.

Bullitt, E. (1990). Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *J Comp Neurol*, *296*(4), 517-530.

Cabral, A., Valdivia, S., Fernandez, G., Reynaldo, M., & Perello, M. (2014). Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility. *J Neuroendocrinol*, *26*(8), 542-554.

Campbell, J. N., Macosko, E. Z., Fenselau, H., Pers, T. H., Lyubetskaya, A., Tenen, D., Goldman, M., Verstegen, A. M., Resch, J. M., McCarroll, S. A., Rosen, E. D., Lowell, B. B., & Tsai, L. T. (2017). A molecular census of arcuate hypothalamus and median eminence cell types. *Nat Neurosci*, *20*(3), 484-496.

Campos, C. A., Bowen, A. J., Schwartz, M. W., & Palmiter, R. D. (2016). Parabrachial CGRP Neurons Control Meal Termination. *Cell Metab*, *23*(5), 811-820.

Carter, M. E., Han, S., & Palmiter, R. D. (2015). Parabrachial calcitonin gene-related peptide neurons mediate conditioned taste aversion. *J Neurosci*, *35*(11), 4582-4586.

Carter, M. E., Soden, M. E., Zweifel, L. S., & Palmiter, R. D. (2013). Genetic identification of a neural circuit that suppresses appetite. *Nature*, *503*(7474), 111-114.

Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., & Yanagisawa, M. (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell*, *98*(4), 437-451.

Chen, Y., & Knight, Z. A. (2016). Making sense of the sensory regulation of hunger neurons. *Bioessays*, *38*(4), 316-324.

Chen, Y., Lin, Y. C., Kuo, T. W., & Knight, Z. A. (2015). Sensory detection of food rapidly modulates arcuate feeding circuits. *Cell*, *160*(5), 829-841.

Chronwall, B. M. (1985). Anatomy and physiology of the neuroendocrine arcuate nucleus. *Peptides*, *6 Suppl 2*, 1-11.

Clark, R. G., Carlsson, L. M., & Robinson, I. C. (1987). Growth hormone secretory profiles in conscious female rats. *J Endocrinol*, *114*(3), 399-407.

Cleobury, L., & Tapper, K. (2014). Reasons for eating 'unhealthy' snacks in overweight and obese males and females. *J Hum Nutr Diet*, *27*(4), 333-341.

Cochran, B. H., Zullo, J., Verma, I. M., & Stiles, C. D. (1984). Expression of the c-fos gene and of an fos-related gene is stimulated by platelet-derived growth factor. *Science*, *226*(4678), 1080-1082.

Cone, R. D., Cowley, M. A., Butler, A. A., Fan, W., Marks, D. L., & Low, M. J. (2001). The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord*, *25 Suppl 5*, S63-67.

Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L., & et al. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med*, *334*(5), 292-295.

Cowley, M. A., Smith, R. G., Diano, S., Tschöp, M., Pronchuk, N., Grove, K. L., Strasburger, C. J., Bidlingmaier, M., Esterman, M., Heiman, M. L., Garcia-Segura, L. M., Nillni, E. A., Mendez, P., Low, M. J., Sotonyi, P., Friedman, J. M., Liu, H., Pinto, S., Colmers, W. F., . . . Horvath, T. L. (2003). The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron*, *37*(4), 649-661.

Crossley, E., Biggs, T., Brown, P., & Singh, T. (2021). The Accuracy of iPhone Applications to Monitor Environmental Noise Levels. *Laryngoscope*, *131*(1), E59-e62.

Cummings, D. E., Clement, K., Purnell, J. Q., Vaisse, C., Foster, K. E., Frayo, R. S., Schwartz, M. W., Basdevant, A., & Weigle, D. S. (2002). Elevated plasma ghrelin levels in Prader Willi syndrome. *Nat Med*, *8*(7), 643-644.

Cummings, D. E., Frayo, R. S., Marmonier, C., Aubert, R., & Chapelot, D. (2004). Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *Am J Physiol Endocrinol Metab*, *287*(2), E297-304.

Cummings, D. E., Weigle, D. S., Frayo, R. S., Breen, P. A., Ma, M. K., Dellinger, E. P., & Purnell, J. Q. (2002). Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med*, *346*(21), 1623-1630.

Curran, T., & Morgan, J. I. (1985). Superinduction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepines. *Science*, *229*(4719), 1265-1268.

Dall, R., Kanaley, J., Hansen, T. K., Møller, N., Christiansen, J. S., Hosoda, H., Kangawa, K., & Jørgensen, J. O. (2002). Plasma ghrelin levels during exercise in healthy subjects and in growth hormone-deficient patients. *Eur J Endocrinol*, *147*(1), 65-70.

Dalle Grave, R., Calugi, S., & Marchesini, G. (2008). Compulsive exercise to control shape or weight in eating disorders: prevalence, associated features, and treatment outcome. *Compr Psychiatry*, *49*(4), 346-352.

Date, Y., Kojima, M., Hosoda, H., Sawaguchi, A., Mondal, M. S., Suganuma, T., Matsukura, S., Kangawa, K., & Nakazato, M. (2000). Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology*, *141*(11), 4255-4261.

Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., & Nakazato, M. (1999). Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proc Natl Acad Sci U S A*, *96*(2), 748-753.

de Lecea, L., Kilduff, T. S., Peyron, C., Gao, X., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautvik, V. T., Bartlett, F. S., 2nd, Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., & Sutcliffe, J. G. (1998). The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A*, *95*(1), 322-327.

Dickson, S. L., & Luckman, S. M. (1997). Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. *Endocrinology*, *138*(2), 771-777.

Dietrich, M. O., Bober, J., Ferreira, J. G., Tellez, L. A., Mineur, Y. S., Souza, D. O., Gao, X. B., Picciotto, M. R., Araújo, I., Liu, Z. W., & Horvath, T. L. (2012). AgRP neurons regulate development of dopamine neuronal plasticity and nonfood-associated behaviors. *Nat Neurosci*, *15*(8), 1108-1110.

Edén, S. (1979). Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology*, *105*(2), 555-560.

El Ghoch, M., Calugi, S., Pellegrini, M., Milanese, C., Busacchi, M., Battistini, N. C., Bernabè, J., & Dalle Grave, R. (2013). Measured physical activity in anorexia nervosa: features and treatment outcome. *Int J Eat Disord*, *46*(7), 709-712.

Engström Ruud, L., Pereira, M. M. A., de Solis, A. J., Fenselau, H., & Brüning, J. C. (2020). NPY mediates the rapid feeding and glucose metabolism regulatory functions of AgRP neurons. *Nat Commun*, *11*(1), 442.

Epstein, L. H., Paluch, R., & Coleman, K. J. (1996). Differences in salivation to repeated food cues in obese and nonobese women. *Psychosom Med*, *58*(2), 160-164.

España, R. A., McCormack, S. L., Mochizuki, T., & Scammell, T. E. (2007). Running promotes wakefulness and increases cataplexy in orexin knockout mice. *Sleep*, *30*(11), 1417-1425.

Essner, R. A., Smith, A. G., Jamnik, A. A., Ryba, A. R., Trutner, Z. D., & Carter, M. E. (2017). AgRP Neurons Can Increase Food Intake during Conditions of Appetite Suppression and Inhibit Anorexigenic Parabrachial Neurons. *J Neurosci*, *37*(36), 8678-8687.

Fadel, J., & Deutch, A. Y. (2002). Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. *Neuroscience*, *111*(2), 379-387.

Farr, O. M., Tsoukas, M. A., Triantafyllou, G., Dincer, F., Filippaios, A., Ko, B. J., & Mantzoros, C. S. (2016). Short-term administration of the GLP-1 analog liraglutide decreases circulating leptin and increases GIP levels and these changes are associated with alterations in CNS responses to food cues: A randomized, placebo-controlled, crossover study. *Metabolism*, *65*(7), 945-953.

Ferriday, D., & Brunstrom, J. M. (2011). 'I just can't help myself': effects of food-cue exposure in overweight and lean individuals. *Int J Obes (Lond)*, *35*(1), 142-149.

Fine, L. G., & Riera, C. E. (2019). Sense of Smell as the Central Driver of Pavlovian Appetite Behavior in Mammals. *Front Physiol*, *10*, 1151.

Fraga, I. C., Fregoneze, J. B., Carvalho, F. L., Dantas, K. B., Azevedo, C. S., Pinho, C. B., & de Castro, E. S. E. (2005). Acute fluoxetine administration differentially affects brain C-Fos expression in fasted and refed rats. *Neuroscience*, *134*(1), 327-334.

Freda, P. U., Reyes, C. M., Conwell, I. M., Sundeen, R. E., & Wardlaw, S. L. (2003). Serum ghrelin levels in acromegaly: effects of surgical and long-acting octreotide therapy. *J Clin Endocrinol Metab*, *88*(5), 2037-2044.

Fujiki, N., Yoshida, Y., Zhang, S., Sakurai, T., Yanagisawa, M., & Nishino, S. (2006). Sex difference in body weight gain and leptin signaling in hypocretin/orexin deficient mouse models. *Peptides*, *27*(9), 2326-2331.

Funato, H., Tsai, A. L., Willie, J. T., Kisanuki, Y., Williams, S. C., Sakurai, T., & Yanagisawa, M. (2009). Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metab*, *9*(1), 64-76.

Gascuel, J., Lemoine, A., Rigault, C., Datiche, F., Benani, A., Penicaud, L., & Lopez-Mascaraque, L. (2012). Hypothalamus-olfactory system crosstalk: orexin a immunostaining in mice. *Front Neuroanat*, *6*, 44.

Ge, X., Yang, H., Bednarek, M. A., Galon-Tilleman, H., Chen, P., Chen, M., Lichtman, J. S., Wang, Y., Dalmas, O., Yin, Y., Tian, H., Jermutus, L., Grimsby, J., Rondinone, C. M., Konkar, A., & Kaplan, D. D. (2018). LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor. *Cell Metab*, *27*(2), 461-469.e466.

Gelegen, C., Collier, D. A., Campbell, I. C., Oppelaar, H., van den Heuvel, J., Adan, R. A., & Kas, M. J. (2007). Difference in susceptibility to activity-based anorexia in two inbred strains of mice. *Eur Neuropsychopharmacol*, *17*(3), 199-205.

Girault, E. M., Yi, C. X., Fliers, E., & Kalsbeek, A. (2012). Orexins, feeding, and energy balance. *Prog Brain Res*, *198*, 47-64.

Gorwood, P., Blanchet-Collet, C., Chartrel, N., Duclos, J., Dechelotte, P., Hanachi, M., Fetissov, S., Godart, N., Melchior, J. C., Ramoz, N., Rovere-Jovene, C., Tolle, V., Viltart, O., & Epelbaum, J. (2016). New Insights in Anorexia Nervosa. *Front Neurosci*, *10*, 256.

Guan, X. M., Yu, H., Palyha, O. C., McKee, K. K., Feighner, S. D., Sirinathsinghji, D. J., Smith, R. G., Van der Ploeg, L. H., & Howard, A. D. (1997). Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res*, *48*(1), 23-29.

Guenthner, C. J., Miyamichi, K., Yang, H. H., Heller, H. C., & Luo, L. (2013). Permanent genetic access to transiently active neurons via TRAP: targeted recombination in active populations. *Neuron*, *78*(5), 773-784.

Hagan, J. J., Leslie, R. A., Patel, S., Evans, M. L., Wattam, T. A., Holmes, S., Benham, C. D., Taylor, S. G., Routledge, C., Hemmati, P., Munton, R. P., Ashmeade, T. E., Shah, A. S., Hatcher, J. P., Hatcher, P. D., Jones, D. N., Smith, M. I., Piper, D. C., Hunter, A. J., . . . Upton, N. (1999). Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci U S A*, *96*(19), 10911-10916.

Hahn, T. M., Breininger, J. F., Baskin, D. G., & Schwartz, M. W. (1998). Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci*, *1*(4), 271-272.

Hallam, J., Boswell, R. G., DeVito, E. E., & Kober, H. (2016). Gender-related Differences in Food Craving and Obesity. *Yale J Biol Med*, *89*(2), 161-173.

Han, J. E., Frasnelli, J., Zeighami, Y., Larcher, K., Boyle, J., McConnell, T., Malik, S., Jones-Gotman, M., & Dagher, A. (2018). Ghrelin Enhances Food Odor Conditioning in Healthy Humans: An fMRI Study. *Cell Rep*, *25*(10), 2643-2652.e2644.

Hara, J., Beuckmann, C. T., Nambu, T., Willie, J. T., Chemelli, R. M., Sinton, C. M., Sugiyama, F., Yagami, K., Goto, K., Yanagisawa, M., & Sakurai, T. (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron*, *30*(2), 345-354.

Hedegaard, M. A., & Holst, B. (2020). The Complex Signaling Pathways of the Ghrelin Receptor. *Endocrinology*, *161*(4).

Herpertz-Dahlmann, B. (2009). Adolescent eating disorders: definitions, symptomatology, epidemiology and comorbidity. *Child Adolesc Psychiatr Clin N Am*, *18*(1), 31-47.

Hervey, G. R. (1959). The effects of lesions in the hypothalamus in parabiotic rats. *J Physiol*, *145*(2), 336-352.

Hewson, A. K., & Dickson, S. L. (2000). Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol*, *12*(11), 1047-1049.

Hindmarsh, P. C., Matthews, D. R., & Brook, C. G. (1988). Growth hormone secretion in children determined by time series analysis. *Clin Endocrinol (Oxf)*, *29*(1), 35-44.

Holland, P. C., & Petrovich, G. D. (2005). A neural systems analysis of the potentiation of feeding by conditioned stimuli. *Physiol Behav*, *86*(5), 747-761.

Holst, B., Cygankiewicz, A., Jensen, T. H., Ankersen, M., & Schwartz, T. W. (2003). High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol*, *17*(11), 2201-2210.

Holtkamp, K., Herpertz-Dahlmann, B., Hebebrand, K., Mika, C., Kratzsch, J., & Hebebrand, J. (2006). Physical activity and restlessness correlate with leptin levels in patients with adolescent anorexia nervosa. *Biol Psychiatry*, *60*(3), 311-313.

Howard, A. D., Feighner, S. D., Cully, D. F., Arena, J. P., Liberator, P. A., Rosenblum, C. I., Hamelin, M., Hreniuk, D. L., Palyha, O. C., Anderson, J., Paress, P. S., Diaz, C., Chou, M., Liu, K. K., McKee, K. K., Pong, S. S., Chaung, L. Y., Elbrecht, A., Dashkevicz, M., . . . Van der Ploeg, L. H. (1996). A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science*, *273*(5277), 974-977.

Hudson, A. E. (2018). Genetic Reporters of Neuronal Activity: c-Fos and G-CaMP6. *Methods Enzymol*, *603*, 197-220.

Hudson, J. I., Hiripi, E., Pope, H. G., Jr., & Kessler, R. C. (2007). The prevalence and correlates of eating disorders in the National Comorbidity Survey Replication. *Biol Psychiatry*, *61*(3), 348-358.

Husman, B., Norstedt, G., Mode, A., & Gustafsson, J. A. (1985). The mode of growth hormone administration is of major importance for the excretion of the major male rat urinary proteins. *Mol Cell Endocrinol*, *40*(2-3), 205-210.

Jacobson, L. H., Hoyer, D., & de Lecea, L. (2022). Hypocretins (orexins): The ultimate translational neuropeptides. *J Intern Med*, *291*(5), 533-556.

Jais, A., Paeger, L., Sotelo-Hitschfeld, T., Bremser, S., Prinzensteiner, M., Klemm, P., Mykytiuk, V., Widdershooven, P. J. M., Vesting, A. J., Grzelka, K., Minère, M., Cremer, A. L., Xu, J., Korotkova, T., Lowell, B. B., Zeilhofer, H. U., Backes, H., Fenselau, H., Wunderlich, F. T., . . . Brüning, J. C. (2020). PNOC(ARC) Neurons Promote Hyperphagia and Obesity upon High-Fat-Diet Feeding. *Neuron*, *106*(6), 1009-1025.e1010.

Janas-Kozik, M., Stachowicz, M., Krupka-Matuszczyk, I., Szymszal, J., Krysta, K., Janas, A., & Rybakowski, J. K. (2011). Plasma levels of leptin and orexin A in the restrictive type of anorexia nervosa. *Regul Pept*, *168*(1-3), 5-9.

Janssen, J. A., van der Toorn, F. M., Hofland, L. J., van Koetsveld, P., Broglio, F., Ghigo, E., Lamberts, S. W., & Jan van der Lely, A. (2001). Systemic ghrelin levels in subjects with growth hormone deficiency are not modified by one year of growth hormone replacement therapy. *Eur J Endocrinol*, *145*(6), 711-716.

Jansson, J. O. (1986). The dwarf "little" (LIT/LIT) mouse is resistant to growth hormone releasing peptide (GHRP-6) as well as to GH-releasing hormone (GRH). *Endocrinology*, *118*(0), 397.

Jansson, J. O., Edén, S., & Isaksson, O. (1985). Sexual dimorphism in the control of growth hormone secretion. *Endocr Rev*, *6*(2), 128-150.

Jéquier, E. (2002). Leptin signaling, adiposity, and energy balance. *Ann N Y Acad Sci*, *967*, 379-388.

Jerlhag, E. (2008). Systemic administration of ghrelin induces conditioned place preference and stimulates accumbal dopamine. *Addict Biol*, *13*(3-4), 358-363.

Jiang, H., Betancourt, L., & Smith, R. G. (2006). Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. *Mol Endocrinol*, *20*(8), 1772-1785.

Johnstone, L. E., Fong, T. M., & Leng, G. (2006). Neuronal activation in the hypothalamus and brainstem during feeding in rats. *Cell Metab*, *4*(4), 313-321.

Joly-Amado, A., Cansell, C., Denis, R. G., Delbes, A. S., Castel, J., Martinez, S., & Luquet, S. (2014). The hypothalamic arcuate nucleus and the control of peripheral substrates. *Best Pract Res Clin Endocrinol Metab*, *28*(5), 725-737.

Joye, D. A. M., Rohr, K. E., Keller, D., Inda, T., Telega, A., Pancholi, H., Carmona-Alcocer, V., & Evans, J. A. (2020). Reduced VIP Expression Affects Circadian Clock Function in VIP-IRES-CRE Mice (JAX 010908). *J Biol Rhythms*, *35*(4), 340-352.

Joyner, M. A., Kim, S., & Gearhardt, A. N. (2017). Investigating an Incentive-Sensitization Model of Eating Behavior: Impact of a Simulated Fast-Food Laboratory. *Clinical Psychological Science*, *5*(6), 1014-1026.

Kakava-Georgiadou, N., Severens, J. F., Jørgensen, A. M., Stoltenborg, I., Garner, K. M., Luijendijk, M. C. M., Drkelic, V., van Dijk, R., Dickson, S. L., Pers, T. H., Basak, O., & Adan, R. A. H. (2020). Single-cell analysis reveals cellular heterogeneity and molecular determinants of hypothalamic leptin-receptor cells. *bioRxiv*, 2020.2007.2023.217729.

Kakava-Georgiadou, N., Zwartkruis, M. M., Bullich-Vilarrubias, C., Luijendijk, M. C. M., Garner, K. M., van der Plasse, G., & Adan, R. A. H. (2019). An Intersectional Approach to Target Neural Circuits With Cell- and Projection-Type Specificity: Validation in the Mesolimbic Dopamine System. *Front Mol Neurosci*, *12*, 49.

Kawashima, T., Kitamura, K., Suzuki, K., Nonaka, M., Kamijo, S., Takemoto-Kimura, S., Kano, M., Okuno, H., Ohki, K., & Bito, H. (2013). Functional labeling of neurons and their projections using the synthetic activity-dependent promoter E-SARE. *Nat Methods*, *10*(9), 889-895.

Kern, A., Albarran-Zeckler, R., Walsh, H. E., & Smith, R. G. (2012). Apo-ghrelin receptor forms heteromers with DRD2 in hypothalamic neurons and is essential for anorexigenic effects of DRD2 agonism. *Neuron*, *73*(2), 317-332.

Kern, A., Mavrikaki, M., Ullrich, C., Albarran-Zeckler, R., Brantley, A. F., & Smith, R. G. (2015). Hippocampal Dopamine/DRD1 Signaling Dependent on the Ghrelin Receptor. *Cell*, *163*(5), 1176-1190.

Keski-Rahkonen, A., & Mustelin, L. (2016). Epidemiology of eating disorders in Europe: prevalence, incidence, comorbidity, course, consequences, and risk factors. *Curr Opin Psychiatry*, *29*(6), 340-345.

King, C. M., & Hentges, S. T. (2011). Relative number and distribution of murine hypothalamic proopiomelanocortin neurons innervating distinct target sites. *PLoS One*, *6*(10), e25864.

Klenotich, S. J., Seiglie, M. P., McMurray, M. S., Roitman, J. D., Le Grange, D., Dugad, P., & Dulawa, S. C. (2012). Olanzapine, but not fluoxetine, treatment increases survival in activity-based anorexia in mice. *Neuropsychopharmacology*, *37*(7), 1620-1631.

Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., & Kangawa, K. (1999). Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*, *402*(6762), 656-660.

Kotz, C. M., Teske, J. A., Levine, J. A., & Wang, C. (2002). Feeding and activity induced by orexin A in the lateral hypothalamus in rats. *Regul Pept*, *104*(1-3), 27-32.

Kotz, C. M., Wang, C., Teske, J. A., Thorpe, A. J., Novak, C. M., Kiwaki, K., & Levine, J. A. (2006). Orexin A mediation of time spent moving in rats: neural mechanisms. *Neuroscience*, *142*(1), 29-36.

Krashes, M. J., Koda, S., Ye, C., Rogan, S. C., Adams, A. C., Cusher, D. S., Maratos-Flier, E., Roth, B. L., & Lowell, B. B. (2011). Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J Clin Invest*, *121*(4), 1424-1428.

Lamont, E. W., Bruton, J., Blum, I. D., & Abizaid, A. (2014). Ghrelin receptor-knockout mice display alterations in circadian rhythms of activity and feeding under constant lighting conditions. *Eur J Neurosci*, *39*(2), 207-217.

Le May, M. V., Hume, C., Sabatier, N., Schéle, E., Bake, T., Bergström, U., Menzies, J., & Dickson, S. L. (2019). Activation of the rat hypothalamic supramammillary nucleus by food anticipation, food restriction or ghrelin administration. *J Neuroendocrinol*, *31*(7), e12676.

Le May, M. V., Peris-Sampedro, F., Stoltenborg, I., Schéle, E., Bake, T., Adan, R. A. H., & Dickson, S. L. (2021). Functional and Neurochemical Identification of Ghrelin Receptor (GHSR)-Expressing Cells of the Lateral Parabrachial Nucleus in Mice. *Front Neurosci*, *15*, 633018.

Lee, H. M., Wang, G., Englander, E. W., Kojima, M., & Greeley, G. H., Jr. (2002). Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology*, *143*(1), 185-190.

Liu, T., Kong, D., Shah, B. P., Ye, C., Koda, S., Saunders, A., Ding, J. B., Yang, Z., Sabatini, B. L., & Lowell, B. B. (2012). Fasting activation of AgRP neurons requires NMDA receptors and involves spinogenesis and increased excitatory tone. *Neuron*, *73*(3), 511-522.

Lockie, S. H., Dinan, T., Lawrence, A. J., Spencer, S. J., & Andrews, Z. B. (2015). Diet-induced obesity causes ghrelin resistance in reward processing tasks. *Psychoneuroendocrinology*, *62*, 114-120.

Lockie, S. H., McAuley, C. V., Rawlinson, S., Guiney, N., & Andrews, Z. B. (2017). Food Seeking in a Risky Environment: A Method for Evaluating Risk and Reward Value in Food Seeking and Consumption in Mice. *Front Neurosci*, *11*, 24.

López-Ferreras, L., Richard, J. E., Anderberg, R. H., Nilsson, F. H., Olandersson, K., Kanoski, S. E., & Skibicka, K. P. (2017). Ghrelin's control of food reward and body weight in the lateral hypothalamic area is sexually dimorphic. *Physiol Behav*, *176*, 40-49.

Luquet, S., Perez, F. A., Hnasko, T. S., & Palmiter, R. D. (2005). NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science*, *310*(5748), 683-685.

M'Kadmi, C., Cabral, A., Barrile, F., Giribaldi, J., Cantel, S., Damian, M., Mary, S., Denoyelle, S., Dutertre, S., Péraldi-Roux, S., Neasta, J., Oiry, C., Banères, J. L., Marie, J., Perello, M., & Fehrentz, J. A. (2019). N-Terminal Liver-Expressed Antimicrobial Peptide 2 (LEAP2) Region Exhibits Inverse Agonist Activity toward the Ghrelin Receptor. *J Med Chem*, *62*(2), 965-973.

Mäkelä, K. A., Karhu, T., Jurado Acosta, A., Vakkuri, O., Leppäluoto, J., & Herzig, K. H. (2018). Plasma Orexin-A Levels Do Not Undergo Circadian Rhythm in Young Healthy Male Subjects. *Front Endocrinol (Lausanne)*, *9*, 710.

Malcolm, A., Toh, W. L., Crocker, K., & Phillipou, A. (2022). The contribution of sleep to anorexia nervosa severity. *Eat Weight Disord*, *27*(4), 1563-1568.

Malik, S., McGlone, F., Bedrossian, D., & Dagher, A. (2008). Ghrelin modulates brain activity in areas that control appetitive behavior. *Cell Metab*, *7*(5), 400-409.

Mani, B. K., Osborne-Lawrence, S., Mequinion, M., Lawrence, S., Gautron, L., Andrews, Z. B., & Zigman, J. M. (2017). The role of ghrelin-responsive mediobasal hypothalamic neurons in mediating feeding responses to fasting. *Mol Metab*, *6*(8), 882-896.

Matikainen-Ankney, B. A., Earnest, T., Ali, M., Casey, E., Wang, J. G., Sutton, A. K., Legaria, A. A., Barclay, K. M., Murdaugh, L. B., Norris, M. R., Chang, Y. H., Nguyen, K. P., Lin, E., Reichenbach, A., Clarke, R. E., Stark, R., Conway, S. M., Carvalho, F., Al-Hasani, R., . . . Kravitz, A. V. (2021). An open-source device for measuring food intake and operant behavior in rodent home-cages. *Elife*, *10*.

Mayer, E. A. (2011). Gut feelings: the emerging biology of gut-brain communication. *Nat Rev Neurosci*, *12*(8), 453-466.

Mazzone, C. M., Liang-Guallpa, J., Li, C., Wolcott, N. S., Boone, M. H., Southern, M., Kobzar, N. P., Salgado, I. A., Reddy, D. M., Sun, F., Zhang, Y., Li, Y., Cui, G., & Krashes, M. J. (2020). High-fat food biases hypothalamic and mesolimbic expression of consummatory drives. *Nat Neurosci*, *23*(10), 1253-1266.

Méquinion, M., Chauveau, C., & Viltart, O. (2015). The use of animal models to decipher physiological and neurobiological alterations of anorexia nervosa patients. *Front Endocrinol (Lausanne)*, *6*, 68.

Micali, N., Martini, M. G., Thomas, J. J., Eddy, K. T., Kothari, R., Russell, E., Bulik, C. M., & Treasure, J. (2017). Lifetime and 12-month prevalence of eating disorders amongst women in mid-life: a population-based study of diagnoses and risk factors. *BMC Med*, *15*(1), 12.

Mieda, M., Williams, S. C., Sinton, C. M., Richardson, J. A., Sakurai, T., & Yanagisawa, M. (2004). Orexin neurons function in an efferent pathway of a food-entrainable circadian oscillator in eliciting food-anticipatory activity and wakefulness. *J Neurosci*, *24*(46), 10493-10501.

Mitchell, J. E., & Peterson, C. B. (2020). Anorexia Nervosa. *N Engl J Med*, *382*(14), 1343-1351.

Morgan, G. S. K., Mata, Y., Carrillo, B., Pellón, R., Collado, P., Gotti, S., & Pinos, H. (2022). Influence of early maternal separation on susceptibility to the activity-based anorexia model in male and female Sprague Dawley rats. *Neurosci Res*, *184*, 54-61.

Morgan, J. I., Cohen, D. R., Hempstead, J. L., & Curran, T. (1987). Mapping patterns of c-fos expression in the central nervous system after seizure. *Science*, *237*(4811), 192-197.

Morselli, L. L., Claflin, K. E., Cui, H., & Grobe, J. L. (2018). Control of Energy Expenditure by AgRP Neurons of the Arcuate Nucleus: Neurocircuitry, Signaling Pathways, and Angiotensin. *Curr Hypertens Rep*, *20*(3), 25.

Nakamura, T., Uramura, K., Nambu, T., Yada, T., Goto, K., Yanagisawa, M., & Sakurai, T. (2000). Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. *Brain Res*, *873*(1), 181-187.

Nambu, T., Sakurai, T., Mizukami, K., Hosoya, Y., Yanagisawa, M., & Goto, K. (1999). Distribution of orexin neurons in the adult rat brain. *Brain Res*, *827*(1-2), 243-260.

Norstedt, G., & Palmiter, R. (1984). Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell*, *36*(4), 805-812.

Obal, F., Jr., Alt, J., Taishi, P., Gardi, J., & Krueger, J. M. (2003). Sleep in mice with nonfunctional growth hormone-releasing hormone receptors. *Am J Physiol Regul Integr Comp Physiol*, *284*(1), R131-139.

Olszewski, P. K., Grace, M. K., Billington, C. J., & Levine, A. S. (2003). Hypothalamic paraventricular injections of ghrelin: effect on feeding and c-Fos immunoreactivity. *Peptides*, *24*(6), 919-923.

Olszewski, P. K., Li, D., Grace, M. K., Billington, C. J., Kotz, C. M., & Levine, A. S. (2003). Neural basis of orexigenic effects of ghrelin acting within lateral hypothalamus. *Peptides*, *24*(4), 597-602.

Osterstock, G., Escobar, P., Mitutsova, V., Gouty-Colomer, L. A., Fontanaud, P., Molino, F., Fehrentz, J. A., Carmignac, D., Martinez, J., Guerineau, N. C., Robinson, I. C., Mollard, P., & Méry, P. F. (2010). Ghrelin stimulation of growth hormone-releasing hormone neurons is direct in the arcuate nucleus. *PLoS One*, *5*(2), e9159.

Palouzier-Paulignan, B., Lacroix, M. C., Aimé, P., Baly, C., Caillol, M., Congar, P., Julliard, A. K., Tucker, K., & Fadool, D. A. (2012). Olfaction under metabolic influences. *Chem Senses*, *37*(9), 769-797.

Patriarchi, T., Cho, J. R., Merten, K., Howe, M. W., Marley, A., Xiong, W. H., Folk, R. W., Broussard, G. J., Liang, R., Jang, M. J., Zhong, H., Dombeck, D., von Zastrow, M., Nimmerjahn, A., Gradinaru, V., Williams, J. T., & Tian, L. (2018). Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors. *Science*, *360*(6396).

Perello, M., Sakata, I., Birnbaum, S., Chuang, J. C., Osborne-Lawrence, S., Rovinsky, S. A., Woloszyn, J., Yanagisawa, M., Lutter, M., & Zigman, J. M. (2010). Ghrelin increases the rewarding value of high-fat diet in an orexin-dependent manner. *Biol Psychiatry*, *67*(9), 880-886.

Peris-Sampedro, F., Le May, M. V., Stoltenborg, I., Schéle, E., & Dickson, S. L. (2021). A skeleton in the cupboard in ghrelin research: Where are the skinny dwarfs? *J Neuroendocrinol*, *33*(11), e13025.

Petrovich, G. D., Hobin, M. P., & Reppucci, C. J. (2012). Selective Fos induction in hypothalamic orexin/hypocretin, but not melanin-concentrating hormone neurons, by a learned food-cue that stimulates feeding in sated rats. *Neuroscience*, *224*, 70-80.

Petrovich, G. D., Holland, P. C., & Gallagher, M. (2005). Amygdalar and prefrontal pathways to the lateral hypothalamus are activated by a learned cue that stimulates eating. *J Neurosci*, *25*(36), 8295-8302.

Peyron, C., Tighe, D. K., van den Pol, A. N., de Lecea, L., Heller, H. C., Sutcliffe, J. G., & Kilduff, T. S. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci*, *18*(23), 9996-10015.

Pirnik, Z., Majercikova, Z., Holubova, M., Pirnik, R., Zelezna, B., Maletinska, L., & Kiss, A. (2014). Effect of ghrelin receptor agonist and antagonist on the activity of arcuate nucleus tyrosine hydroxylase containing neurons in C57BL/6 male mice exposed to normal or high fat diet. *J Physiol Pharmacol*, *65*(4), 477-486.

Pöykkö, S. M., Ukkola, O., Kauma, H., Kellokoski, E., Hörkkö, S., & Kesäniemi, Y. A. (2005). The negative association between plasma ghrelin and IGF-I is modified by obesity, insulin resistance and type 2 diabetes. *Diabetologia*, *48*(2), 309-316.

Pursey, K. M., Stanwell, P., Callister, R. J., Brain, K., Collins, C. E., & Burrows, T. L. (2014). Neural responses to visual food cues according to weight status: a systematic review of functional magnetic resonance imaging studies. *Front Nutr*, *1*, 7.

Ramanathan, L., & Siegel, J. M. (2014). Gender differences between hypocretin/orexin knockout and wild type mice: age, body weight, body composition, metabolic markers, leptin and insulin resistance. *J Neurochem*, *131*(5), 615-624.

Ramirez-Plascencia, O. D., Saderi, N., Escobar, C., & Salgado-Delgado, R. C. (2017). Feeding during the rest phase promotes circadian conflict in nuclei that

control energy homeostasis and sleep-wake cycle in rats. *Eur J Neurosci*, *45*(10), 1325-1332.

Rashid, H., Chen, H., Hassan, Q., & Javed, A. (2017). Dwarfism in homozygous Agc1(CreERT) mice is associated with decreased expression of aggrecan. *Genesis*, *55*(10).

Råstam, M., Gillberg, C., & Wentz, E. (2003). Outcome of teenage-onset anorexia nervosa in a Swedish community-based sample. *Eur Child Adolesc Psychiatry*, *12 Suppl 1*, I78-90.

Rhea, E. M., Salameh, T. S., Gray, S., Niu, J., Banks, W. A., & Tong, J. (2018). Ghrelin transport across the blood-brain barrier can occur independently of the growth hormone secretagogue receptor. *Mol Metab*, *18*, 88-96.

Richardson, N. R., & Roberts, D. C. (1996). Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods*, *66*(1), 1-11.

Ringuet, M. T., Furness, J. B., & Furness, S. G. B. (2022). G protein-coupled receptor interactions and modification of signalling involving the ghrelin receptor, GHSR1a. *J Neuroendocrinol*, *34*(9), e13077.

Rossi, M. A., & Stuber, G. D. (2018). Overlapping Brain Circuits for Homeostatic and Hedonic Feeding. *Cell Metab*, *27*(1), 42-56.

Routtenberg, A., & Kuznesof, A. W. (1967). Self-starvation of rats living in activity wheels on a restricted feeding schedule. *J Comp Physiol Psychol*, *64*(3), 414-421.

Russo, C., Russo, A., Pellitteri, R., & Stanzani, S. (2018). Ghrelin-containing neurons in the olfactory bulb send collateralized projections into medial amygdaloid and arcuate hypothalamic nuclei: neuroanatomical study. *Exp Brain Res*, *236*(8), 2223-2229.

Sakurai, T. (2005). Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis. *Sleep Med Rev*, *9*(4), 231-241.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski, G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., . . . Yanagisawa, M. (1998). Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*, *92*(4), 573-585.

Sanders, N., Smeets, P. A., van Elburg, A. A., Danner, U. N., van Meer, F., Hoek, H. W., & Adan, R. A. (2015). Altered food-cue processing in chronically ill and recovered women with anorexia nervosa. *Front Behav Neurosci*, *9*, 46.

Sauchelli, S., Jiménez-Murcia, S., Sánchez, I., Riesco, N., Custal, N., Fernández-García, J. C., Garrido-Sánchez, L., Tinahones, F. J., Steiger, H., Israel, M., Baños, R. M., Botella, C., de la Torre, R., Fernández-Real, J. M., Ortega, F. J., Frühbeck, G., Granero, R., Tárrega, S., Crujeiras, A. B., . . . Fernández-Aranda, F. (2016). Orexin and sleep quality in anorexia nervosa: Clinical relevance and influence on treatment outcome. *Psychoneuroendocrinology*, *65*, 102-108.

Saunders, A., Terry, L. C., Audet, J., Brazeau, P., & Martin, J. B. (1976). Dynamic studies of growth hormone and prolactin secretion in the female rat. *Neuroendocrinology*, *21*(3), 193-203.

Schaeffer, M., Langlet, F., Lafont, C., Molino, F., Hodson, D. J., Roux, T., Lamarque, L., Verdié, P., Bourrier, E., Dehouck, B., Banères, J. L., Martinez, J., Méry, P. F., Marie, J., Trinquet, E., Fehrentz, J. A., Prévot, V., & Mollard, P. (2013). Rapid sensing of circulating ghrelin by hypothalamic appetite-modifying neurons. *Proc Natl Acad Sci U S A*, *110*(4), 1512-1517.

Schéle, E., Bake, T., Rabasa, C., & Dickson, S. L. (2016). Centrally Administered Ghrelin Acutely Influences Food Choice in Rodents. *PLoS One*, *11*(2), e0149456.

Schéle, E., Cook, C., Le May, M., Bake, T., Luckman, S. M., & Dickson, S. L. (2017). Central administration of ghrelin induces conditioned avoidance in rodents. *Eur Neuropsychopharmacol*, *27*(8), 809-815.

Schellekens, H., De Francesco, P. N., Kandil, D., Theeuwes, W. F., McCarthy, T., van Oeffelen, W. E., Perelló, M., Giblin, L., Dinan, T. G., & Cryan, J. F. (2015). Ghrelin's Orexigenic Effect Is Modulated via a Serotonin 2C Receptor Interaction. *ACS Chem Neurosci*, *6*(7), 1186-1197.

Schellekens, H., van Oeffelen, W. E., Dinan, T. G., & Cryan, J. F. (2013). Promiscuous dimerization of the growth hormone secretagogue receptor (GHS-R1a) attenuates ghrelin-mediated signaling. *J Biol Chem*, *288*(1), 181-191.

Schneider, N. Y., Chaudy, S., Epstein, A. L., Viollet, C., Benani, A., Pénicaud, L., Grosmaître, X., Datiche, F., & Gascuel, J. (2020). Centrifugal projections to the main olfactory bulb revealed by transsynaptic retrograde tracing in mice. *J Comp Neurol*, *528*(11), 1805-1819.

Scott, V., McDade, D. M., & Luckman, S. M. (2007). Rapid changes in the sensitivity of arcuate nucleus neurons to central ghrelin in relation to feeding status. *Physiol Behav*, *90*(1), 180-185.

Seoane, L. M., López, M., Tovar, S., Casanueva, F. F., Señarís, R., & Diéguez, C. (2003). Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. *Endocrinology*, *144*(2), 544-551.

Seoane, L. M., Tovar, S., Baldelli, R., Arvat, E., Ghigo, E., Casanueva, F. F., & Dieguez, C. (2000). Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. *Eur J Endocrinol*, *143*(5), R7-9.

Shan, X., & Yeo, G. S. (2011). Central leptin and ghrelin signalling: comparing and contrasting their mechanisms of action in the brain. *Rev Endocr Metab Disord*, *12*(3), 197-209.

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M. S., Tanaka, M., Nozoe, S., Hosoda, H., Kangawa, K., & Matsukura, S. (2002). Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab*, *87*(1), 240-244.

Skibicka, K. P., Hansson, C., Alvarez-Crespo, M., Friberg, P. A., & Dickson, S. L. (2011). Ghrelin directly targets the ventral tegmental area to increase food motivation. *Neuroscience*, *180*, 129-137.

Skibicka, K. P., Shirazi, R. H., Rabasa-Papio, C., Alvarez-Crespo, M., Neuber, C., Vogel, H., & Dickson, S. L. (2013). Divergent circuitry underlying food reward and intake effects of ghrelin: dopaminergic VTA-accumbens projection mediates ghrelin's effect on food reward but not food intake. *Neuropharmacology*, *73*, 274-283.

Smink, F. R., van Hoeken, D., & Hoek, H. W. (2012). Epidemiology of eating disorders: incidence, prevalence and mortality rates. *Curr Psychiatry Rep*, *14*(4), 406-414.

Smith, K. S., Berridge, K. C., & Aldridge, J. W. (2011). Disentangling pleasure from incentive salience and learning signals in brain reward circuitry. *Proc Natl Acad Sci U S A*, *108*(27), E255-264.

Soriano-Guillén, L., Barrios, V., Campos-Barros, A., & Argente, J. (2004). Ghrelin levels in obesity and anorexia nervosa: effect of weight reduction or recuperation. *J Pediatr*, *144*(1), 36-42.

Steward, T., Mestre-Bach, G., Granero, R., Sánchez, I., Riesco, N., Vintró-Alcaraz, C., Sauchelli, S., Jiménez-Murcia, S., Agüera, Z., Fernández-García, J. C., Garrido-Sánchez, L., Tinahones, F. J., Casanueva, F. F., Baños, R. M., Botella, C., Crujeiras, A. B., Torre, R., Fernández-Real, J. M., Frühbeck, G., . . . Fernández-Aranda, F. (2019). Reduced Plasma Orexin-A Concentrations are Associated with Cognitive Deficits in Anorexia Nervosa. *Sci Rep*, *9*(1), 7910.

Sun, F., Zeng, J., Jing, M., Zhou, J., Feng, J., Owen, S. F., Luo, Y., Li, F., Wang, H., Yamaguchi, T., Yong, Z., Gao, Y., Peng, W., Wang, L., Zhang, S., Du, J., Lin, D., Xu, M., Kreitzer, A. C., . . . Li, Y. (2018). A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell*, *174*(2), 481-496.e419.

Sun, H., Kennedy, W. P., Wilbraham, D., Lewis, N., Calder, N., Li, X., Ma, J., Yee, K. L., Ermlich, S., Mangin, E., Lines, C., Rosen, L., Chodakewitz, J., & Murphy, G. M. (2013). Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep*, *36*(2), 259-267.

Sun, Y., Wang, P., Zheng, H., & Smith, R. G. (2004). Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A*, *101*(13), 4679-4684.

Tacke, F., Brabant, G., Kruck, E., Horn, R., Schöffski, P., Hecker, H., Manns, M. P., & Trautwein, C. (2003). Ghrelin in chronic liver disease. *J Hepatol*, *38*(4), 447-454.

Tannenbaum, G. S., & Martin, J. B. (1976). Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology*, *98*(3), 562-570.

Tarantini, B., Ciuoli, C., Checchi, S., Montanaro, A., Bonato, V., Theodoropoulou, A., Pasqui, L., & Pacini, F. (2009). Serum ghrelin levels in growth hormone-sufficient and growth hormone-deficient patients during growth hormone-releasing hormone plus arginine test. *J Endocrinol Invest*, *32*(4), 335-337.

Thannickal, T. C., Moore, R. Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., & Siegel, J. M. (2000). Reduced number of hypocretin neurons in human narcolepsy. *Neuron*, *27*(3), 469-474.

Tong, J., Mannea, E., Aimé, P., Pfluger, P. T., Yi, C. X., Castaneda, T. R., Davis, H. W., Ren, X., Pixley, S., Benoit, S., Julliard, K., Woods, S. C., Horvath, T. L., Sleeman, M. M., D'Alessio, D., Obici, S., Frank, R., & Tschöp, M. H. (2011). Ghrelin enhances olfactory sensitivity and exploratory sniffing in rodents and humans. *J Neurosci*, *31*(15), 5841-5846.

Toor, B., Ray, L. B., Pozzobon, A., & Fogel, S. M. (2021). Sleep, Orexin and Cognition. *Front Neurol Neurosci*, *45*, 38-51.

Tschöp, M., Flora, D. B., Mayer, J. P., & Heiman, M. L. (2002). Hypophysectomy prevents ghrelin-induced adiposity and increases gastric ghrelin secretion in rats. *Obes Res*, *10*(10), 991-999.

Tschöp, M., Smiley, D. L., & Heiman, M. L. (2000). Ghrelin induces adiposity in rodents. *Nature*, *407*(6806), 908-913.

Tschöp, M., Weyer, C., Tataranni, P. A., Devanarayan, V., Ravussin, E., & Heiman, M. L. (2001). Circulating ghrelin levels are decreased in human obesity. *Diabetes*, *50*(4), 707-709.

Uriarte, M., De Francesco, P. N., Fernandez, G., Cabral, A., Castrogiovanni, D., Lalonde, T., Luyt, L. G., Trejo, S., & Perello, M. (2019). Evidence Supporting a Role for the Blood-Cerebrospinal Fluid Barrier Transporting Circulating Ghrelin into the Brain. *Mol Neurobiol*, *56*(6), 4120-4134.

van Son, G. E., van Hoeken, D., van Furth, E. F., Donker, G. A., & Hoek, H. W. (2010). Course and outcome of eating disorders in a primary care-based cohort. *Int J Eat Disord*, *43*(2), 130-138.

Vestergaard, E. T., Hansen, T. K., Nielsen, S., Moller, N., Christiansen, J. S., & Jorgensen, J. O. (2005). Effects of GH replacement therapy in adults on serum levels of leptin and ghrelin: the role of lipolysis. *Eur J Endocrinol*, *153*(4), 545-549.

Viollet, C., Simon, A., Tolle, V., Labarthe, A., Grouselle, D., Loe-Mie, Y., Simonneau, M., Martel, G., & Epelbaum, J. (2017). Somatostatin-IRES-Cre Mice: Between Knockout and Wild-Type? *Front Endocrinol (Lausanne)*, *8*, 131.

Voigt, J. P., & Fink, H. (2015). Serotonin controlling feeding and satiety. *Behav Brain Res*, *277*, 14-31.

Watabe-Uchida, M., Zhu, L., Ogawa, S. K., Vamanrao, A., & Uchida, N. (2012). Whole-brain mapping of direct inputs to midbrain dopamine neurons. *Neuron*, *74*(5), 858-873.

Watson, H. J., & Bulik, C. M. (2013). Update on the treatment of anorexia nervosa: review of clinical trials, practice guidelines and emerging interventions. *Psychol Med*, *43*(12), 2477-2500.

Watson, H. J., Yilmaz, Z., Thornton, L. M., Hübel, C., Coleman, J. R. I., Gaspar, H. A., Bryois, J., Hinney, A., Leppä, V. M., Mattheisen, M., Medland, S. E., Ripke, S., Yao, S., Giusti-Rodríguez, P., Hanscombe, K. B., Purves, K. L., Adan, R. A. H., Alfredsson, L., Ando, T., . . . Bulik, C. M. (2019). Genome-wide association

study identifies eight risk loci and implicates metabo-psychiatric origins for anorexia nervosa. *Nat Genet*, *51*(8), 1207-1214.

Wentz, E., Gillberg, I. C., Anckarsäter, H., Gillberg, C., & Råstam, M. (2009). Adolescent-onset anorexia nervosa: 18-year outcome. *Br J Psychiatry*, *194*(2), 168-174.

Wever, M. C. M., van Meer, F., Charbonnier, L., Crabtree, D. R., Buosi, W., Giannopoulou, A., Androutsos, O., Johnstone, A. M., Manios, Y., Meek, C. L., Holst, J. J., & Smeets, P. A. M. (2021). Associations between ghrelin and leptin and neural food cue reactivity in a fasted and sated state. *Neuroimage*, *240*, 118374.

Willesen, M. G., Kristensen, P., & Rømer, J. (1999). Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology*, *70*(5), 306-316.

Williams, G., Bing, C., Cai, X. J., Harrold, J. A., King, P. J., & Liu, X. H. (2001). The hypothalamus and the control of energy homeostasis: different circuits, different purposes. *Physiol Behav*, *74*(4-5), 683-701.

Winrow, C. J., Gotter, A. L., Cox, C. D., Doran, S. M., Tannenbaum, P. L., Breslin, M. J., Garson, S. L., Fox, S. V., Harrell, C. M., Stevens, J., Reiss, D. R., Cui, D., Coleman, P. J., & Renger, J. J. (2011). Promotion of sleep by suvorexant-a novel dual orexin receptor antagonist. *J Neurogenet*, *25*(1-2), 52-61.

Wren, A. M., Seal, L. J., Cohen, M. A., Brynes, A. E., Frost, G. S., Murphy, K. G., Dhillo, W. S., Ghatei, M. A., & Bloom, S. R. (2001). Ghrelin enhances appetite and increases food intake in humans. *J Clin Endocrinol Metab*, *86*(12), 5992.

Wren, A. M., Small, C. J., Abbott, C. R., Dhillo, W. S., Seal, L. J., Cohen, M. A., Batterham, R. L., Taheri, S., Stanley, S. A., Ghatei, M. A., & Bloom, S. R. (2001). Ghrelin causes hyperphagia and obesity in rats. *Diabetes*, *50*(11), 2540-2547.

Wren, A. M., Small, C. J., Ward, H. L., Murphy, K. G., Dakin, C. L., Taheri, S., Kennedy, A. R., Roberts, G. H., Morgan, D. G., Ghatei, M. A., & Bloom, S. R. (2000). The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology*, *141*(11), 4325-4328.

Wu, Q., Boyle, M. P., & Palmiter, R. D. (2009). Loss of GABAergic signaling by AgRP neurons to the parabrachial nucleus leads to starvation. *Cell*, *137*(7), 1225-1234.

Yamanaka, A., Beuckmann, C. T., Willie, J. T., Hara, J., Tsujino, N., Mieda, M., Tominaga, M., Yagami, K., Sugiyama, F., Goto, K., Yanagisawa, M., & Sakurai, T. (2003). Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron*, *38*(5), 701-713.

Yang, J., Brown, M. S., Liang, G., Grishin, N. V., & Goldstein, J. L. (2008). Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell*, *132*(3), 387-396.

Ye, L., Allen, W. E., Thompson, K. R., Tian, Q., Hsueh, B., Ramakrishnan, C., Wang, A. C., Jennings, J. H., Adhikari, A., Halpern, C. H., Witten, I. B., Barth, A. L., Luo, L., McNab, J. A., & Deisseroth, K. (2016). Wiring and Molecular Features of

Prefrontal Ensembles Representing Distinct Experiences. *Cell*, *165*(7), 1776-1788.

Zhang, X., & van den Pol, A. N. (2016). Hypothalamic arcuate nucleus tyrosine hydroxylase neurons play orexigenic role in energy homeostasis. *Nat Neurosci*, *19*(10), 1341-1347.

Zhang, Y., & Chua, S., Jr. (2017). Leptin Function and Regulation. *Compr Physiol*, *8*(1), 351-369.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, *372*(6505), 425-432.

Zhao, T. J., Liang, G., Li, R. L., Xie, X., Sleeman, M. W., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Goldstein, J. L., & Brown, M. S. (2010). Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc Natl Acad Sci U S A*, *107*(16), 7467-7472.

Zigman, J. M., Jones, J. E., Lee, C. E., Saper, C. B., & Elmquist, J. K. (2006). Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol*, *494*(3), 528-548.

APPENDIX

SCIENTIFIC CONTRIBUTIONS BYOND THIS THESIS

Other scientific publications Iris Stoltenborg has contributed to during her PhD are listed here.

Single-cell analysis reveals cellular heterogeneity and molecular determinants of hypothalamic leptin-receptor cells
Kavaka-Georgiadou N, Severens JF, Jørgensen AM, Stoltenborg I, Garner KM, Luijendijk, MCM, Drkelic V, van Dijk R, Dickson SL, Pers TH, Basak O, Adan RAH
BioRxiv, 2020

A skeleton in the cupboard in ghrelin research: Where are the skinny dwarfs?
Peris-Sampedro F, Le May MV, Stoltenborg I, Schéle E, Dickson SL
Journal of Neuroendocrinology, 2021; 33:e13025

Neurocircuitry Through Which Leptin Targets Multiple Inputs to the Dopamine System to Reduce Food Reward Seeking
Omrani A, de Vrind VAJ, Lodder B, Stoltenborg I, Kooij K, Wolterink-Donselaar IG, Luijendijk-Berg MCM, Garner KM, Van't Sant LJ, Rozeboom A, Dickson SL, Meye FJ, Adan RAH
Biological Phychiatry, 2021; 90:843-852