Addressing knowledge gaps in the brain ghrelin signalling system– its neural circuitry and role in food-linked behaviours

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To the women who made me, by blood and by example

“The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory”

Paraphrased from Ramón Cajal’s autobiography, Recuerdos de mi vida, 1937

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Abstract

The drive to seek out and consume food is a fundamental survival mechanism, orchestrated by the brain through a range of behaviours in response to both intrinsic signals of energy status and external food-related cues. Disruption or faulty processing of these signals can lead to disordered eating behaviour across the body weight spectrum. For example, the availability of high-calorie, palatable foods and abundance of environmental cues are thought to stimulate overconsumption and contribute to the global obesity epidemic or, in contrast to this, loss of appetite in conditions such as anorexia nervosa, cancer cachexia or frailty can be detrimental. A better understanding of the brain mechanisms controlling food-linked behaviours is essential for developing effective, non-invasive therapies. Among the many signalling molecules that modulate networks controlling feeding behaviour, the hormone ghrelin stands alone as the only orexigenic (pro-feeding) hormone and represents a powerful tool to access and modulate brain networks involved in feeding control. This thesis addresses important gaps in our understanding of the brain ghrelin signalling system, focussing on its neural circuitry and its role in food-linked behaviours, using advanced neural circuit mapping techniques to explore both mechanistic and translational aspects.

First, we demonstrate that the ghrelin mimetic GHRP-6 can stimulate the brain ghrelin signalling system through non-invasive intranasal application, reproducing many of the known effects of peripheral ghrelin administration in mice, including increased food intake, growth hormone release and activation of cells in the arcuate nucleus (Arc) including AgRP and GHRH neurones. In the second and third studies of this thesis, we used chemogenetic re-activation to functionally characterize hunger-responsive neuronal ensembles in the dorsomedial hypothalamus and Arc, demonstrating that re-activation of these ensembles stimulated food intake, and was able to drive food motivated behaviour. In the fourth and final study, we show that ghrelin's role in hunger extends to increase attention-linked behaviours and food intake in the presence of a food cue in a novel behavioural task adapted for mice.

Together, the studies in this thesis expand our understanding of the brain ghrelin signalling system. They identify novel functional circuits involved in hunger and food-motivated behaviour and obtained new insights into how ghrelin modulates behaviour towards environmental cues. Finally, a new, non-invasive strategy to stimulate the brain ghrelin signalling system has been proposed, which holds translational value.

Keywords: ghrelin, feeding, food motivation, food-cue, arcuate nucleus, dorsomedial hypothalamus, GHRP-6, growth hormone, intranasal administration, chemogenetics

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Sammanfattning på Svenska

Drivkraften att leta efter föda och äta är en fundamental överlevnadsmekanism som styrs av hjärnan. Den påverkas både av kroppens inre signaler om energibehov och av yttre faktorer, som dofter, synintryck och tillgång till mat. När balansen i dessa signaler rubbas kan det leda till ätstörda beteenden – både hos personer med övervikt och hos dem med undervikt. Exempelvis kan ständig tillgång på kaloririk och välsmakande mat tillsammans med alla visuella och luktmässiga signaler i vår omgivning bidra till att vi äter mer än vi behöver och kan vara en bidragande orsak till ökad förekomst av obesitas världen över. Å andra sidan kan aptitlöshet, som ofta ses vid anorexi, cancer eller hos sköra äldre också bero på störningar i hjärnans reglering av hunger och få alvarliga hälsokonsekvenser. En större förståelse för hur hjärnan styr beteenden kopplade till mat och ätande är grundläggande för att utveckla bättre icke-invasiva behandlingsmetoder.

Det finns en rad signalmolekyler som hjälper hjärnan att styra vårt ätande. Hormonet ghrelin har en unik roll som orexigen (främjar ätande) signalmolekyl och verkar kraftfullt på hjärnans signalsystem som styr vårt ätande. I denna avhandling används avancerad ny teknik för att undersöka en rad viktiga kunskapsluckor kring hur ghrelin påverkar hjärnan med särskilt fokus på att studera signalvägar som är viktiga för hjärnans reglering av matrelaterande beteenden.

Första studien visar att substansen GHRP-6 som efterliknar ghrelin kan stimulera hjärnans ghrelinsystem genom icke-invasiv administration via näsan och kan därigenom reproducera många av de kända effekter som ghrelin injektioner ger hos möss såsom ökat födointag, frisättning av tillväxthormon samt aktivering av neuron i arkuatuskärnan (Arc), inklusive AgRP och GHRH positiva neuron. I andra och tredje studien använde vi s.k. kemogenetik för att experimentellt identifiera och återaktivera specifika neuronansamlingar som är funktionellt aktiva under perioder av hunger och finns i dorsomediala hypotalamus och Arc. Återaktivering av dessa neuron ökade födointaget och kunde framkalla matmotiverat beteende. I fjärde och sista studien använder vi en ny metod för att utöka förståelsen kring hur ghrelin påverkar hunger och födointag genom ökad uppmärksamhetsgrad.

Studierna i avhandlingen utökar vår förståelse kring hjärnans ghrelinsignalering. Fynden identifierar nya funktionella signalvägar som styr hunger och matmotiverade beteenden och ger nya insikter om hur ghrelin påverkar beteenden som svar på omgivande signaler. Slutligen föreslår avhandlingen en ny icke-invasiv strategi för att stimulera hjärnans ghrelinsignalering med potential för framtida kliniska tillämpningar.

Nederlandse samenvatting

De drang om voedsel te zoeken en te consumeren is een fundamenteel overlevingsmechanisme, en omvat een scala aan gedragingen, aangestuurd door de hersenen die reageren op zowel interne signalen over de energiebalans als externe voedsel gerelateerde prikkels. Wanneer deze signalen verstoord raken of verkeerd worden verwerkt, kan dit leiden tot afwijkend eetgedrag, wat grote gevolgen kan hebben ongeacht waar op het gewicht spectrum iemand zich bevindt. Zo kan bijvoorbeeld de constante beschikbaarheid van calorierijk, smakelijk voedsel en de overvloed aan voedselgereateerde omgevingsprikkels, zoals reclames, bijdragen aan overconsumptie en daarmee aan de wereldwijde obesitasepidemie. Daartegenover staat dat een onderdrukte eetlust bij aandoeningen zoals anorexia nervosa, kankergerelateerde cachexie of bij kwetsbare ouderen ernstige gevolgen kan hebben. Het beter begrijpen van de hersenmechanismen die eetgedrag aansturen is essentieel voor het ontwikkelen van effectieve en niet-invasieve behandelmethoden voor verstoord eetgedrag. Binnen de complexe netwerken die eetgedrag reguleren, heeft het hormoon ghreline een bijzondere positie: het is het enige bekende orexigene (eetlustopwekkende) hormoon dat circuleert in het bloed en biedt een krachtig handvat dat aangegrepen kan worden om hersennetwerken te beinvoelden die betrokken zijn bij eetgedrag. Dit proefschrift richt zich op het beantwoorden van belangrijke gaten in onze kennis over ghreline-signalering in de hersenen, met een focus op de onderliggende hersencircuits en de rol van ghreline in voedselgerelateerd gedrag. Daarbij maken we gebruik van geavanceerde technieken om zowel de onderliggende mechanismen als de klinisch toepasbaarheid te bestuderen.

In de eerste studie tonen we aan dat het ghreline mimeticum GHRP-6 via een niet-invasieve, intranasale toediening het ghrelinesysteem in de hersenen kan activeren. Dit bootst verschillende bekende effecten van perifere ghrelinespiegels na bij muizen, waaronder verhoogde voedselinname, toename van groeihormoon in het bloed en activatie van neuronen in de arcuate nucleus (Arc), waaronder AgRP- en GHRH-neuronen. In de tweede en derde studie hebben we gebruikgemaakt van chemogenetische tools om honger-gevoelige neurale netwerken in de dorsomediale hypothalamus en de Arc functioneel te karakteriseren. We toonden aan dat reactivatie van deze netwerken niet alleen

de voedselinname verhoogt, maar ook voedselgemotiveerd gedrag stimuleert. In de vierde en laatste studie laten we zien dat ghreline de aandacht voor voedselprikkels beïnvloedt, binnen een nieuw gedragsparadigma ontwikkeld voor muizen.

Samenvattend dragen de studies in dit proefschrift bij aan een verdieping van onze kennis over het ghreline-singaleringssysteem van de hersenen. We brengen nieuwe functionele hersennetwerken in kaart die betrokken zijn bij honger en motivatie voor voedsel, en bieden waardevolle inzichten over hoe ghreline gedrag beïnvloedt als reactie op omgevingssignalen. Ook introduceren we een nieuwe, niet-invasieve methode om dit ghreline-signaleringssysteem te stimuleren, wat translationele waarde heeft voor toekomstige toepassingen in de kliniek en belangrijke mogelijkheden biedt voor toekomstige therapeutische toepassingen.

List of papers

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

I. **Intranasal delivery of a ghrelin mimetic engages the brain ghrelin signalling system in mice**.
Poelman R, Le May MV, Schéle E, Stoltenborg I, Dickson SL. *Endocrinology, 2025; 5:166*

II. **Activation of a ghrelin-responsive hunger ensemble in the dorsomedial hypothalamus is sufficient to drive food-related behaviours in male and female mice.**
Stoltenborg I, Poelman R, Vecchi E, Schéle E, Dickson SL. *Manuscript*

III. **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, Poelman R, Vecchi E, Schéle E, Dickson SL. *Manuscript*

IV. **Impact of ghrelin on behavioural outcomes linked to attentiveness to a conditioned food cue.**
Poelman R, Le May MV, Stoltenborg I, Vecchi E, Schéle E, Adan RAH, Dickson SL.
Manuscript

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Abbreviations

4-OHT	4-hydroxytamoxifen
AgRP	growth hormone
Arc	arcuate nucleus
BMI	body mass index
CEPR2	closed economy progressive ratio 2
CNO	clozapine N-oxide
CPA	conditioned place aversion
Cre	cre recombinase
Da	dalton (molecular weight)
DAB	3,3'-diaminobenzidine
DREADD	designer receptor exclusively activated by designer drugs
ELISA	enzyme linked immunosorbent assay
FED3	feeding experimentation device version 3
fMRI	functional magnetic resonance imaging
FR	fixed ratio
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GHSR	growth hormone Secretagogue Receptor

GHS	growth hormone Secretagogue
GLP-1	glucagon-like peptide 1
GOAT	ghrelin-O-acyltransferase
IHC	immunohistochemistry
i.p.	intraperitoneal
ITI	inter trial interval
LatH	lateral hypothalamus
LH	limited hold
LSM	laser scanning microscope
NPY	neuropeptide Y
PB	phosphate buffer
PFA	paraformaldehyde
PBS	phosphate buffered saline
POMC	proopiomelanocortin
PR2h	classical progressive ratio task 2 hours
PT	pretraining
PVH	paraventricular nucleus of the hypothalamus
RNA	ribonucleic acid
s.c.	subcutaneous
SD	stimulus duration

TRAP	targeted recombination of active populations
VMH	ventromedial hypothalamus
VTA	ventral tegmental area
ZT	zeitgeber time

Introduction

"Calories in – calories out" is often cited as the golden rule for maintaining energy balance, recognizing that body weight reflects a delicate balance between the amount and composition of food ingested and energy expenditure. For body weight to increase, food intake must exceed energy needs. From an evolutionary perspective, body weight gain offers a survival advantage, ensuring not only that nutritional needs are met but also that energy reserves exist to survive when food is less available, during winter or in a period of famine. With the ultimate goal of food ingestion, the brain orchestrates a diverse set of behaviours, driven both by physiological signals of nutritional status and by environmental cues signalling food availability.

Hunger is a powerful physiological drive that is experienced when in negative energy balance. In Swedish, there is a saying - hungern är den bästa kryddan - indicating that foods increase in desirability when hungry, irrespective of their palatability. Hunger carries a negative valence – an uncomfortable feeling – that is only resolved by eating. The brain receives information about hunger from metabolic signals, such as low blood glucose, and by endocrine signals from fat stores (e.g., leptin, reflecting on long-term energy reserves), the pancreas (e.g., insulin and glucagon, acutely signalling information about circulating nutrients) and the gut (e.g., ghrelin, glucagon-like peptide 1, peptide YY, signalling hunger, satiety and satiation). The term 'appetite' extends beyond hunger to encompass the desire for and pleasure from eating specific foods. Unlike hunger, experiencing an appetite for specific foods does not require that we are in a negative energy balance; we can have an appetite for palatable foods, even when we do not feel hungry.

Over the past couple of decades, we have learned that many of the hormonal signals driving hunger also impact on appetite, ensuring that we seek out and consume a variety of foods that contain essential nutrients, and palatable foods that are most energy dense. While seeking out calorie-dense foods is essential for survival, the Westernized environment has changed dramatically over a relatively short time period and high-calorie, palatable foods are now readily available and over-consumed. In addition, environmental cues that signal food availability (such as the sight or smell of food) are abundant, often in the shape of food advertisements. Indeed, exposure to pictures of palatable foods or food

commercials on TV has been shown to increase food intake in both hungry and sated individuals (Cornell et al., 1989; Harris et al., 2009). Increased exposure to food predicting cues is only one of the many factors (environmental, genetic and social) that are thought to contribute to overconsumption and subsequent overweight and obesity. Understanding how hunger and appetite hormones signal to the brain, and how this is impacted by environmental food-predicting cues remains an important research focus for therapeutic developments to help those suffering from obesity and eating disorders.

Since 1990, the prevalence of adult obesity worldwide has more than doubled, and adolescent obesity has increased from 2% to 8%. As of 2022, 43% of adults were overweight and 16% were living with obesity (WHO). Obesity is associated with serious health risks that reduce lifespan, including stroke, hypertension, diabetes, heart disease and certain forms of cancer (Global Burden of Disease, 2021) and all of this carries a huge burden on healthcare and society (Nagi et al., 2024; Okunogbe et al., 2022). Disruptions in energy balance are not limited to individuals with overweight. At the other end of the body weight spectrum are people who suffer from a loss of appetite such as those with anorexia nervosa, cancer cachexia or frailty. Although the prevalence of anorexia nervosa is low relative to obesity (0.9-4% in women and 0.3% in men), the mortality rate is high (5.9%; van Hoeken & Hoek, 2020) with high relapse rates and poor treatment outcomes (Watson & Bulik, 2013). These challenges can, in part, be attributed to an incomplete understanding of the biological mechanisms underlying the disorder. These contrasting examples underscore the need to better understand the mechanisms driving food-linked behaviour, as a first step towards the goal of finding aetiology-based treatment targets.

We have learned a great deal about the neuronal circuits responding to intrinsic and external signals that drive feeding behaviours. Key brain regions involved include the hypothalamus and brainstem, which are critical for energy sensing and homeostatic control, as well as areas linked to reward, cognition and emotion. Among the many signalling molecules that modulate these networks, the hormone ghrelin plays a central role in driving orexigenic responses. The work in this thesis addresses gaps of knowledge in understanding the ghrelin signalling system, regarding both its neural circuitry and its role in food-linked behaviours.

The brain ghrelin signalling system

Ghrelin and its receptor

Given the strong evolutionary drive to eat for survival, it is surprising that almost all feeding-related hormones identified to date - whether produced by the gut, adipose tissue or pancreas - suppresses food intake. Ghrelin, released from the empty stomach, stands alone as the only orexigenic (pro-feeding) hormone. Indeed, after its discovery in 1999 (Kojima et al., 1999), it rapidly gained status as a hunger hormone, based on studies showing that circulating levels rise when fasting (Tschöp et al., 2000), before meals (Cummings et al., 2001) and in association with hunger (Cummings et al., 2004) and also by the fact that ghrelin administration robustly stimulates food intake in humans and rodents (Wren, Seal, et al., 2001; Wren, Small, et al., 2001; Wren et al., 2000). Consistent with this role, ghrelin's effects extend beyond mere food intake to affect other consummatory behaviours, namely food choice (Schéle et al., 2016) and meal patterns (Grill, 2006), and it also drives behaviours that precede food intake, such as food anticipation (Merkestein et al., 2012), food motivation (Egecioglu et al., 2010; Perello et al., 2010; Skibicka, Hansson, et al., 2012; Skibicka, Shirazi, et al., 2012) and the evaluation of food reward (Schéle et al., 2016). Taken together with the fact that ghrelin release can occur in a sated state, when anticipating food (Merkestein et al., 2012) and when exposed to food cues (Peris-Sampedro, Stoltenborg, Le May, Sole-Navais, et al., 2021; Schüssler et al., 2012) it would appear that ghrelin can be released in the absence of hunger and its physiological role extends to appetite.

Curiously, by far the majority of ghrelin is produced in the oxyntic glands of the gastric mucosa, specifically in X/A-like enteroendocrine cells (Date et al., 2000), since this is also the site of production of gastric acid. Ghrelin is synthesised as a 28 amino acid peptide that undergoes acylation by the enzyme ghrelin O-acyltransferase (GOAT, also present in the X/A-like cells). It is only this acetylated form that is biologically active and can bind its dedicated receptor, the growth hormone secretagogue receptor (GHSR; Yang et al., 2008). [From here on, I use the term ghrelin to indicate this acetylated form]. GHSR is a G-protein coupled receptor that was cloned in 1996 (Howard et al., 1996), a few years prior to ghrelin's discovery, its name reflecting the fact that it binds synthetic compounds called growth hormone secretagogues (GHS; see

below), which are now known to be ghrelin mimetics. GHSR activation predominantly recruits intracellular Gαq/11 G-proteins, enhancing excitability of GHSR-expressing cells. The receptor is expressed in the central nervous system (Zigman et al., 2006; Guan et al., 1997), notably in regions involved in the regulation of feeding control (Skibicka & Dickson, 2011).

Ghrelin signalling in the arcuate nucleus

The hypothalamic arcuate nucleus (Arc) was first identified as an important node within the brain ghrelin signalling system by studies in rodents showing increased neuronal activation following peripheral GHS administration (Dickson et al., 1993) and later by ghrelin (Hewson & Dickson, 2000). Situated in the mediobasal hypothalamus, the Arc sits conveniently adjacent to the third ventricle and median eminence, the latter serving as a gateway for the release and entry of circulating signals, such as ghrelin, into the brain (Banks et al., 2002; Rhea et al., 2018; Schaeffer et al., 2013). Although the mechanisms by which and if (un-)acylated ghrelin crosses the blood brain barrier remain debated, studies using fluorescently labelled ghrelin have demonstrated its entry via the median eminence and binding to those cells activated in the Arc (Schaeffer et al., 2013), suggesting a direct route for ghrelin to reach and activate cells this region.

Among the neurones activated by GHS and ghrelin in the Arc, a major subset are those expressing neuropeptide Y (NPY), that co-express agouti-related peptide (AgRP; Dickson & Luckman, 1997), both of which are orexigenic peptides. These AgRP/NPY neurones constitute as much as 50% of the GHS-activated Arc population (Dickson & Luckman, 1997). AgRP neurone activity is heightened during fasting and is suppressed rapidly upon feeding (Betley et al., 2015). Their essential role in feeding is evidenced by studies showing that artificial activation of AgRP neurones, elicits robust feeding responses (Aponte et al., 2011; Krashes et al., 2011), while their ablation leads to reduced feeding and starvation (Luquet et al., 2005). Optogenetic studies have further shown that mice avoid environments associated with AgRP activation (Betley et al., 2015), suggesting that AgRP neurones convey the negative valence (unpleasantness) associated with hunger. Together, these findings have led to the recognition of AgRP neurones as the quintessential “hunger neurones”, a key component of the orexigenic network.

The neuronal populations of the Arc are heterogenous (Campbell et al., 2017) with diverse roles that include energy balance, fertility and growth. For feeding control, in addition to the orexigenic NPY/AgRP population, the Arc harbours proopiomelanocortin (POMC) neurones that are activated by the hormone leptin and inhibited by energy deficit associated with elevated ghrelin levels (Mizuno et al., 1998; Tschöp et al., 2000). Indeed, there are indications that NPY/AgRP neurones directly inhibit this POMC population via GABAergic release (Cowley et al., 2003). While unlikely that ghrelin recruits anorexigenic POMC neurones during hunger, a small number of POMC neurones express GHSR, and ~10% of the GHS-activated Arc population co-expresses POMC (Dickson & Luckman, 1997; Willesen et al., 1999). Other Arc populations recruited by GHS in the study by Dickson et al include the growth hormone-releasing hormone (GHRH) neurones regulating the growth axis and, to a lesser extent, tyrosine-hydroxylase-containing neurones that control prolactin secretion and somatostatin neurones.

Relevance of additional hypothalamic areas for ghrelin action

The Arc and, in particular, the NPY/AgRP neurones is without doubt the most important hypothalamic area for ghrelin's orexigenic effects and also an area of highest expression of GHSR (Zigman et al., 2006). Many additional hypothalamic areas are also involved in food intake and energy balance regulation and may be directly or indirectly (via the Arc) targetted by ghrelin. For example, in the mouse brain, GHSR is expressed in the paraventricular nucleus (PVH), the lateral hypothalamus (LatH) and dorsomedial hypothalamus (DMH) (Zigman et al., 2006) and ghrelin injection to these brain areas induces a feeding response (Hyland et al., 2020; Olszewski, Grace, et al., 2003; Olszewski, Li, et al., 2003).

The DMH (the focus of **Paper II**) is a less explored orexigenic region of the hypothalamus that expresses the ghrelin receptor (GHSR) (Zigman et al., 2006). Both central and peripheral ghrelin injections increase expression of *Fos*, an immediate early gene widely used as a marker for neuronal activity (Bullitt, 1990; Cochran et al., 1984; Curran & Morgan, 1985), in the DMH (Lawrence et al., 2002), and direct ghrelin infusion into this region triggers a feeding response (Hyland et al., 2020). Tracing studies have shown that

ghrelin-activated Arc neurones project strongest to the DMH, and that the activated DMH neurones are surrounded by a dense network of AgRP-positive fibers, suggesting that AgRP/NPY signalling plays a role in the DMH's response to ghrelin (Bouret et al., 2004; Kobelt et al., 2008). Supporting this, NPY expression in the DMH is elevated in animal models of obesity and during food deprivation, though not during acute fasting (Bi et al., 2003; Kesterson et al., 1997). The obesogenic role of DMH NPY signalling is further demonstrated by findings that DMH-specific overexpression of NPY increases food intake and leads to weight gain in mice (Yang et al., 2009), while NPY knockout in the DMH prevents and even reverses diet-induced obesity (Kim & Bi, 2016; Chap et al., 2011). Despite its clear role in modulating food intake, there is surprisingly little known about the contribution of the DMH to other food-linked behaviours. Evidence suggests that the DMH is part of a network that regulates food anticipation, a hyperactive state that precedes expected food access, and thought to reflect the drive to eat. DMH *Fos* expression is heightened during food anticipation (Angeles-Castellanos et al., 2004; Gooley et al., 2006), and lesions of the DMH attenuate food anticipatory activity (Gooley et al., 2006; Tahara et al., 2010). Moreover, a DMH sub-population seems to specifically respond to pro-feeding stimuli, such as food cue exposure and ghrelin, that drive food anticipatory activity at the level of the DMH (van der Plasse et al., 2013). However, our understanding of how ghrelin- and hunger-activated neuronal ensembles in the DMH influence broader food-linked behaviours remains incomplete – highlighting a key gap in the current literature.

Beyond the hypothalamus: Ghrelin and the reward system in food-linked behaviours

The brain ghrelin signalling system extends beyond the hypothalamus to include areas involved in reward processing, such as the ventral tegmental area (VTA). The VTA contains dopamine neurones that are involved in incentive salience and that drive the motivation for food. GHSR has been co-localized on a subpopulation of dopamine neurones in this area (Abizaid et al., 2006) and ghrelin delivery (peripherally or to this site) induces dopamine release in the nucleus accumbens (Jerlhag et al., 2006; Jerlhag et al., 2007; Jerlhag et al., 2011), indicating that ghrelin activates the mesoaccumbal dopamine pathway. Ghrelin delivery, centrally or intra-VTA has been shown to increase

motivation for sucrose in a lever-pressing progressive ratio task (Bake et al., 2019; Skibicka 2011). Ghrelin's effects on food motivation appear to engage the VTA-accumbens (mesoaccumbal) pathway since this effect was blocked by delivery of dopamine receptor 1 and 2 antagonists into the nucleus accumbens (Skibicka, Shirazi, et al., 2012). While ghrelin directly activates the VTA dopamine neurones for food-motivated behaviour, it remains unexplored the extent to which this behaviour can be driven from afferent ghrelin-responsive targets, such as the Arc and DMH, as addressed in **Papers II** and **III**.

Environmental food cue reactivity

In addition to intrinsic signals such as ghrelin, the mesoaccumbal dopamine system is also activated by environmental food cues. The sight and smell of food itself provides a primary food cue, but food availability can also be signalled by a secondary, conditioned food cue in the form of a stimulus other than the food itself, such as a light or tone stimulus. A secondary food cue is more commonly used in research and early rodent studies demonstrated that exposure to a conditioned food cue led to faster initiation of feeding and increased food intake (Weingarten 1983). Additionally, exposing mice to a conditioned light/sound (secondary) cue increases the activity of VTA dopamine neurones, measured by fiber photometry (Tang et al., 2012; van der Plasse et al., 2015) and can also increase dopamine release in the nucleus accumbens (Cone et al., 2015; Day et al., 2007), implicating the mesoaccumbal dopamine pathway in the neural response to food cues. Engagement of relevant brain areas such as the dopamine signalling system, together with physiological responses (salivation, hormone secretion) and behavioural responses that ultimately lead to food ingestion are conditioned responses to our environment and is often referred to as "food cue reactivity". It is important to understand how food cue reactivity and the obesogenic environment relate to and interact with each other, since it has become apparent that heightened food cue reactivity can lead to increased feeding and subsequent weight gain (reviewed by (Boswell & Kober, 2016)).

Human functional magnetic resonance imaging studies have consistently shown that, relative to neutral non-food images, exposure to images depicting food increases activity in brain areas that are implicated in visual processing,

taste, reward, impulsivity (meta-analysis of 17 studies; (van der Laan et al., 2011) and, of relevance to **Paper IV**, also attention (Yokum et al., 2011).

There are indications, however, that an individual's attentional focus could play a significant role in the neural response to visual food cues (Franssen et al., 2020; Pimpini et al., 2022). Food-related attentional bias can be assessed in humans by a Stroop colour naming task using food-related words or in a visual probe task, where two images, food and neutral, are shown and then replaced by a dot to focus on while eye movement is followed, allowing researchers to quantify the tendency to preferentially attend to food-related versus neutral images. For this thesis, we will concentrate on studies using the visual probe task to assess attentional bias. Several intrinsic and external factors have been identified that influence this attentional bias to visual food cues, including the energy density and hedonic value of the foods depicted (di Pellegrino et al., 2011; Doolan et al., 2014; van der Laan et al., 2011) or individual differences in impulsivity traits (Hou et al., 2011) and a general predisposition toward heightened food cue responsivity (Brignell et al., 2009; Hou et al., 2011). Additionally, BMI is indicated to play a role in attentional bias towards food images. Studies have shown that BMI correlated positively with neuronal activation of brain areas involved in attention and attentional bias towards food images (Yokum et al., 2011). Moreover, women with obesity exhibited increased attentional bias to food images even in a sated state (Castellanos et al., 2009). However, studies have also suggested that physiological states, such as fasting or satiety, rather than body weight or BMI, appear to modulate the attentional bias to food, with studies demonstrating increased attentional allocation to food stimuli in the fasted state (Castellanos et al., 2009; Nijs et al., 2010; Schlezingerová et al., 2024). These findings suggest that food cue reactivity is shaped not only by stable traits such as BMI or impulsivity, but also by dynamic metabolic signals that reflect current energy needs, such as ghrelin.

Indeed, exposure to food cues triggers ghrelin release in both rodents (Peris-Sampedro, Stoltenborg, Le May, Sole-Navais, et al., 2021) and humans (Schüssler et al., 2012) and may be part of a feedforward hunger response that is interrupted once food is eaten. Consistent with this, fasting ghrelin correlates positively with activation of brain areas related to visual attention, reward and taste (Kroemer et al., 2013; Wever et al., 2021) and peripheral ghrelin administration increases brain activity to food versus scenery images (Malik et

al., 2008). While these studies demonstrate a role for ghrelin in food cue reactivity, the effect of ghrelin on attentional bias to food cues has not yet been addressed, providing a key issue addressed in **Paper IV**.

Figure 1 depicts a schematic overview of the neuronal circuits and behavioural functions modulated by the brain ghrelin signalling system discussed in this thesis.

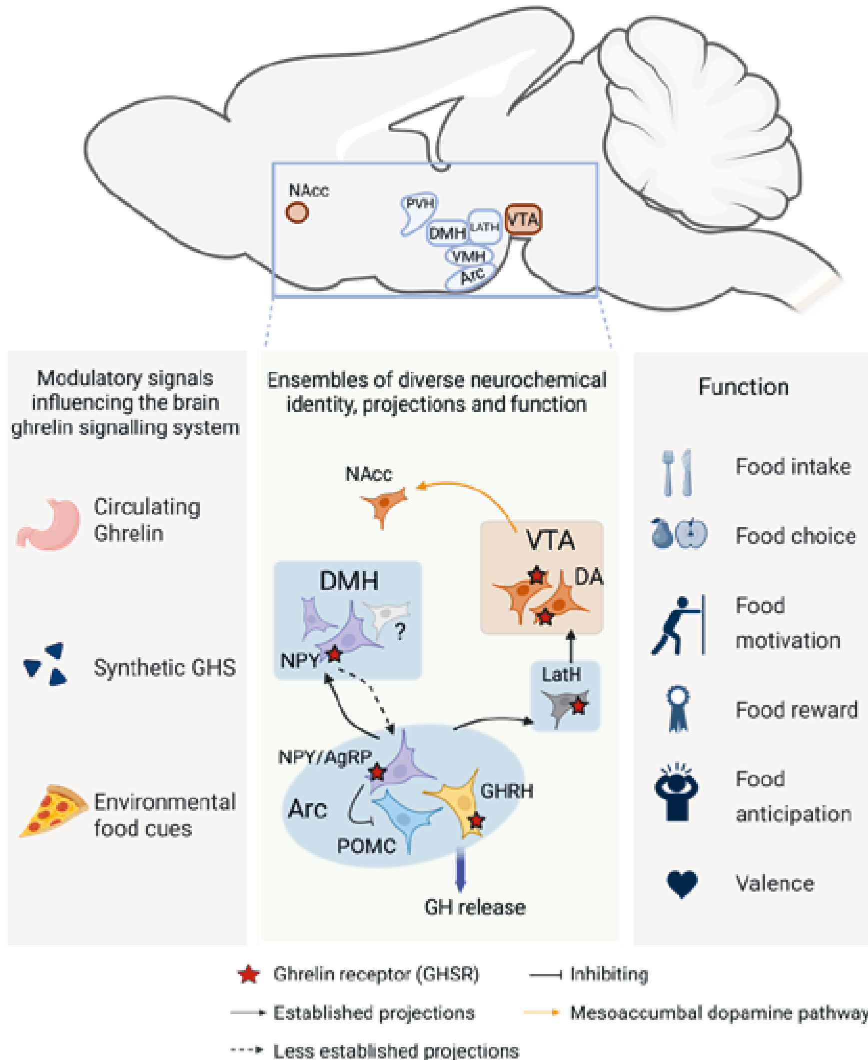


Figure 1: Simplified representation of neuronal circuits and behavioural functions modulated by the brain ghrelin signalling system relevant for this thesis. *This illustration highlights the key brain regions responsive to peripheral ghrelin (endogenous ghrelin released from the stomach or ghrelin that has been administered) or synthetic ghrelin mimetics (GHS). Ghrelin binds to its receptor (the Growth Hormone Secretagogue Receptor; GHSR) that is expressed in discrete sub-populations of neurones in a number of relevant brain regions to increase neuronal activation and drives a diverse set of food-linked behaviours. NPY/AgRP neurones in the hypothalamic arcuate nucleus (Arc), that express GHSR, are known to drive food intake, food choice and activation of these neurones carries a negative valence that is associated with hunger. AgRP/NPY neurones inhibit the satiety-promoting POMC neurones, and project to the dorsomedial hypothalamus (DMH), that also contains Ghsr-expressing neurones. Ghrelin administration into the DMH can also drive food intake and food anticipatory behaviour. Ghsr is also expressed in the ventral tegmental area (VTA), that is thought to receive projections from the Arc via the Lateral Hypothalamus (LatH), and projects to the nucleus accumbens (NAcc). Ghrelin signalling in the mesoaccumbal (VTA → NAcc) dopamine pathway is thought to play a role in food motivation and reward. Synthetic GHS also act on the brain ghrelin signalling system and were originally used to stimulate growth hormone (GH) release. Environmental food cues also modulate food-linked behaviours, as exposure stimulates food intake and food anticipatory behaviour and ghrelin is thought to enhance food cue reactivity.*

GHSR and the growth axis

Beyond its now well-established role in driving hunger and food-linked behaviours, the brain ghrelin signalling system also plays a role in the regulation of growth hormone (GH) secretion – a role that was its earliest identified function. Ghrelin's discovery in 1999 followed years of research into a group of synthetic GHS compounds used to stimulate GH release. GHS include both peptides, such as growth hormone releasing peptide-6 (GHRP-6) (Bowers et al., 1984) and non-peptides, such as MK-0677 (Smith et al., 1993), molecules that bind to GHSR with high affinity and in a competitive manner with ghrelin (Bennett et al., 2009).

As reviewed (Robinson, 2000), pulsatile GH release is tightly controlled by two opposing populations of neuroendocrine cells, the GHRH neurones originating in the Arc and the inhibitory somatostatin neurones of the periventricular nucleus. Both send projections to the median eminence where they release their peptides into a portal capillary system for transport to the anterior pituitary, targeting the somatotroph (GH-releasing) cells. Somatostatin neurones additionally send projections to GHRH neurones, inhibiting GHRH release. There is much evidence to suggest that acute "somatostatin withdrawal" is critical for generating a GH pulse both at the hypothalamic level (by triggering GHRH release) and at the pituitary levels (leaving GHRH action unopposed). Pulsatile GH release is also controlled by

feedback inhibition when circulating GH levels are high (Clark et al., 1988) that appears to operate at the level of the GHRH and somatostatin neurones. Once released, GH promotes anabolic processes throughout the body, including growth, protein synthesis, and metabolic adaptations such as lipolysis and glucose regulation, several of which are mediated via its downstream effector, insulin-like growth factor 1.

GHSR is highly expressed in the anterior pituitary (Howard et al., 1996; Guan et al., 1997), where ghrelin and GHS can directly stimulate GH release from somatotroph cells (Kojima et al., 1999). Additionally, ghrelin and GHS stimulate pituitary GH release indirectly by activating GHRH neurones in the Arc (Dickson et al., 1993; Dickson & Luckman, 1997; Osterstock et al., 2010), a neuronal population of which ~20% also express GHSR (Tannenbaum et al., 1998; Willesen et al., 1999).

Interest in stimulating the GH axis using GHS has mostly been driven by their potential in clinical applications such as diagnostic tests for GH deficiency (Petersenn et al., 2002; Pombo et al., 1996; Popovic et al., 2000), treatment for certain types of GH deficiency (Bowers et al., 1992) and rejuvenation of the GH axis in aging individuals (Thorner et al., 1997). However, a major limitation of current GHS therapies is the reliance on daily injections, which poses a challenge for long-term adherence (Aydın et al., 2014; Cutfield et al., 2011; Loftus et al., 2022) - particularly in children. Oral administration has been explored as a less invasive alternative, but peptide GHS such as GHRP-6 have low oral bioavailability (0.3%; (Aline Moulin & Fehrentz, 2013). The small molecule, non-peptide MK-0677 demonstrates considerably higher oral bioavailability compared to peptide compounds, and the development of a similar, orally active compound, LUM-201 recently renewed therapeutic interest in GHS therapy in children with short stature (Bright et al., 2021; Bright & Thorner, 2022). Surprisingly, despite the well-established orexigenic effects of GHS in rodents (Wren et al., 2000; Wren, Small et al., 2001; Peris-Sampedro, Le May, et al., 2021), their clinical application as appetite stimulants has received limited attention. This gap in translational research sparked our interest to explore whether ghrelin and GHS could effectively stimulate the brain ghrelin signalling system via a less invasive administration route, namely intranasal delivery, and is addressed in **Paper I**.

Gaining access to the brain ghrelin signalling system

To study the neuronal networks regulated by the brain ghrelin signalling system, we need to be able to access and target these systems. This thesis addresses this in two ways, by administering ghrelin and ghrelin mimetics through a new route: the intranasal route (**Paper I**) and additionally by targeting and re-activating ghrelin-activated ensembles in the Arc (**Paper III**) and DMH (**Paper II**)

Intranasal ghrelin and GHS administration

A persistent challenge in neuroscience research is the effective delivery of compounds to the central nervous system. The blood brain barrier prevents most foreign substances from entering the brain from the circulating blood, permitting only small, lipophilic molecules to cross passively, while active transport requires the presence of specific transport mechanisms. Exceptions to this barrier exist at circumventricular organs, such as the median eminence, where the vasculature of the blood brain barrier is relatively permeable ('leaky'). Intranasal delivery has gained interest as an alternative, non-invasive, delivery method directly targeting the central nervous system (Dhuria et al., 2010).

The uptake of intranasally delivered compounds can occur through several distinct mechanisms; one involves internalization by either the olfactory or trigeminal neurones (based on the position of administration in the nose), followed by axonal transport (Illum, 2002; Kristensson & Olsson, 1971; Thorne et al., 1995). This takes several hours for the compound to reach the brain and therefore requires the compound to be stable over an extended period. Another route of uptake would be the paracellular route (between the neurones) and is proposed to be suitable for transporting lipophilic, smaller molecules (McMartin et al., 1987). Intranasally delivered compounds can also enter the circulation through uptake into the rich vascular network lining the nasal cavity, allowing for rapid and less invasive systemic uptake in comparison to injections, but does not bypass the blood brain barrier. A key limitation of the intranasal route, especially for peptide-based compounds, is rapid removal through the mucociliary clearance system and the enzymatic environment of the nasal mucosa degrading the compound before reaching its

target (Illum, 2002). The lower molecular weight of GHS compared to ghrelin – GHRP-6 is 873 and MK-0677 625 compared to the 3370 Da ghrelin) might make these compounds suitable for intranasal administration.

Interestingly, there is very little published on intranasal treatment with ghrelin or GHS in the clinic. Peptide GHSs have been applied intranasally to children with short stature and healthy adult male subjects to stimulate IGF-1 and GH release respectively (Hayashi et al., 1991; Laron et al., 1995), but orexigenic effects were not assessed in these studies. One case study has been published where an anorexia nervosa patient was treated intranasally with the GHS GHRP-2 for one year resulting in increased feeling of hunger, food intake and body weight after 14 months (Haruta et al., 2015). Additionally, recent efforts have explored the use of liposome carriers to protect ghrelin from enzymatic degradation in the nasal cavity, highlighting the growing interest in novel delivery systems for ghrelin and ghrelin mimetics (Salade et al., 2017; Salade et al., 2018).

Chemogenetic re-activation of active populations

As mentioned previously, hypothalamic circuits involved in food-linked behaviour such as the Arc or DMH contain distinct neuronal populations with a variety of molecular profiles. To study how specific neuronal populations respond to hunger (here, ghrelin delivery in the fasted state) (and contribute to food-linked behaviours, it would be of interest to take a more holistic approach and study populations of neurones not by their anatomical location, projections or neurochemical identity, but also by their functional relevance. The TRAP2 (Targeted Recombination of Active Populations) technique offers such an approach, enabling the permanent genetic recombination of neurones based on activity (via *Fos* expression) in response to defined stimuli.

In TRAP2 transgenic mice (Allen et al., 2017; Guenthner et al., 2013), a tamoxifen-inducible form of Cre (CreER^{T2}) is inserted into the *Fos* gene locus, resulting in in the expression of CreER^{T2} in active (*Fos*-expressing) neurones. On its own, CreET^{T2} remains in the cytoplasm, and Cre-dependent recombination can only occur in the presence of injectable 4-hydroxitamoxifen (4-OHT), which binds to CreER^{T2} and transports it into the nucleus (illustrated in **Figure 2**). 4-OHT

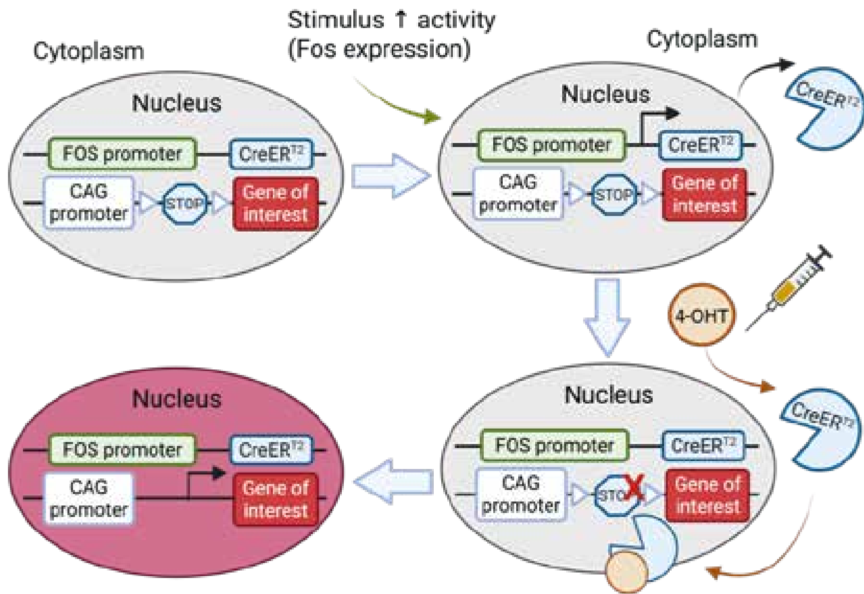


Figure 2. Schematic illustration of the TRAP2 technique. In TRAP2 mice (Targeted Recombination of Active Populations 2), *Fos expression when a neuron is activated – for example, by a stimulus – results in the expression of CreER^{T2} (an inducible Cre recombinase). CreERT2 by itself remains in the cytoplasm and only enters the nucleus in response to the injectable 4-hydroxy-tamoxifen (4-OHT), where it causes recombination and permanent expression of a gene of interest, in this example a fluorescent marker gene (e.g. tdTomato). CAG: chicken beta-actin.*

is a tamoxifen metabolite that has a relative short effective time period of 4-6 hours. This way, the window for "TRAPing" active neurones is time locked to the presence of injected 4-OHT. One application of the TRAP2 mouse model is to Cre-dependently express a reporter gene of choice, such as the fluorescent mCherry, to locate and identify a previously active neuronal ensemble weeks after exposure to the stimulus. Furthermore, the TRAP2 technique can also be used for chemogenetic re-activation studies involving Cre-dependent expression of designer receptors (DREADDS) in activated populations that are potently reactivated by the designer drug such as Clozapine-N-Oxide (CNO) (Armbruster et al., 2007). Viral vectors carrying (Cre-dependent) DREADDs can be injected into specific brain areas to express receptors that can either increase (excitatory DREADD; hM3Dq) or decrease (inhibitory DREADD; hM4Di) cellular excitability upon activation by CNO injection. When applied

in the TRAP2 mouse model, CNO injections can be used to re-activate TRAPed neuronal ensembles previously activated by a defined stimulus. This elegant technique allowed us to study the behavioural response when re-activating hunger-TRAPed neurones in the DMH and Arc, assessed respectively in **Paper II** and **III**, and allows for further identification of the ensemble.

Aims

The overall aims of this thesis were to address knowledge gaps in the brain ghrelin signalling system, specifically in (i) gaining access to it via intranasal delivery and (ii) understanding its neural circuitry and (iii) its role in food-linked behaviours.

The specific aims were:

Paper I	To determine whether the brain ghrelin signalling system is activated by intranasal delivery of ghrelin and/or peptide and non-peptide GHSs.
Paper II	To validate the role of a "hunger" activated neuronal ensemble in the DMH on food-linked behaviours.
Paper III	To validate the role of a 'hunger' activated neuronal ensemble in the Arc on food-linked behaviours.
Paper IV	To establish a behavioural paradigm to assess attentiveness to a conditioned food cue in mice and investigate how ghrelin or fasting affects attention-linked behaviour to a conditioned food cue.

Methodological considerations

Animals

The work in this thesis used female and male mice. All experiments in **Paper I** and **Paper IV** were carried out on adult male C57BL/6J mice (Charles River Laboratories), except for the GH measurements in **Paper I**, which were carried out on female C57B6/6J mice. For **Paper II** both male and female heterogenous TRAP2 mice on a C57BL6/6J background and wild type controls were used, while experiments in **Paper III** were performed on male TRAP2 and wild type mice (Jackson Laboratory).

The decision to use female or male mice was carefully considered in the study design, with the aim of balancing the need to reduce animal use and the importance of representation of both sexes in biomedical research. In **Paper I**, our goal was to explore whether intranasal delivery of GHSR agonist could reproduce ghrelin's known effect on food intake and FOS expression. As most prior studies used male mice to validate such effects, and considering the suppressive effects of oestrogen on food intake (Asarian & Geary, 2006), including ghrelin-induced feeding (Clegg et al., 2007), we restricted these initial experiments to males. However, we did use females for GH measurements due to their more consistent GH pulse amplitude (Jansson et al., 1985; MacLeod et al., 1991; Norstedt & Palmiter, 1984). It should be noted that these sex differences should be a reason to include rather than exclude females, and while studying sex difference was not the focus of this paper, we recommend future studies building on this work to include both sexes. Similarly, in **Paper IV**, where we developed and validated a novel attention task for food cues, we focussed on male mice due to the exploratory nature of the work. It is still debated whether females differ in behavioural responses, possibly depending on the aspect of behaviour measured and test used (Levy et al., 2023; Tsao et al., 2023), and it would be valuable to assess the task's applicability in females in future studies. In contrast, **Paper II** included both sexes to assess the role of the DMH in food-related behaviours. Given the novelty of these findings, including both sexes strengthened reproducibility of our findings as well as representation. **Paper III** focussed on the more extensively studied Arc. Since prior work from our lab using the same mouse model and techniques found no effect of sex on food intake upon re-activation

of a ghrelin-TRAPed population (Stoltenborg et al., 2022), we only used male mice in that study.

All studies included in this thesis were approved by the local Ethics Committee for animal care at the Institute of Experimental Biomedicine, University of Gothenburg, Sweden. Ethical permit numbers are provided in each respective paper. All personnel involved in animal work were trained and certified in accordance with Swedish legislation. Experiments were designed in adherence to the 3Rs principles with the aim to Replace, Reduce and Refine, and carried out by the researcher with Responsibility towards the animals. Whenever possible, data collection and analysis were performed by a blinded experimenter minimize bias.

Chemogenetic re-activation of active populations

Surgical procedures for viral vector delivery

In **Paper II** and **III**, stereotaxic surgeries were performed to deliver a viral vector to the Arc or DMH to Cre-dependently express the activating DREADD hM3Dq or express a control vector mCherry in TRAP2 mice. Mice were anesthetised via isoflurane inhalation and secured in a stereotaxic frame. After exposing the skull, a local anaesthetic was applied (Xylocaine 10%, AstraZeneca, Cambridge, UK). Using predetermined coordinates relative to Bregma, bilateral injections were made into the Arc or DMH. Specific coordinates and source of the viral vectors are provided in the respective Papers. Post-surgical care included subcutaneous (s.c.) administration of the analgesic Metacam® (5 mg/kg; Boehringer Ingelheim, Ingelheim am Rhein, Germany) and saline, followed by recovery inside a heated cage. Male mice were single housed post-surgery to prevent fighting, whereas female mice were group housed for one week to recover from the surgery, before single housing. TRAPing procedures were performed three weeks after the surgery to ensure expression of the viral vector (Aschauer et al., 2013; Nectow & Nestler, 2020).

TRAPing

When using the TRAP2 mouse model in **Paper II** and **III**, Cre-dependent recombination of transiently activated populations is dependent on the

presence of injectable 4-OHT (**Figure 2**). Efficient TRAPing depends on the timing, dose and vehicle used for 4-OHT delivery. For the studies included in this thesis, 4-OHT (#H6278-50MG; Sigma-Aldrich, Schnelldorf, Germany) was dissolved in an aqueous solution (2 mg/mL dissolved in vehicle, comprising 2% tween 80 and 5% DMSO in saline). 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}) was expected to peak around four hours post stimulation (Kawashima et al., 2013). To minimize stress-induced background *Fos* expression, mice were habituated to s.c. saline injections prior to TRAPing experiments. A dose of 25 mg/kg was chosen to balance effective targeting of active neurones while limiting background targeting and the time window for TRAPing. Experiments in which the TRAPed population was chemogeneticallly re-activated commenced two weeks after TRAPing to ensure DREADD or mCherry expression.

Re-activation using CNO

In order to chemogenetically re-activate previously active (TRAPed- and activating DREADD-expressing) neurones, mice received an intraperitoneal injection of the DREADD agonist clozapine-N-oxide (CNO; #4936/10, Bio-Techne Ltd., Abingdon, UK) at a 1 mg/kg dose, dissolved in vehicle (1% DMSO in saline). Control mice received an equal dose of vehicle.

"Hunger" paradigms

Fasting

In **Paper IV**, a 'hunger' state was induced either by ghrelin injection or fasting. In order to generate a fasted state chow was removed from the cage 22 hours prior to the experiment. In **Paper II** and **III**, mice were fasted over night from dark onset the evening before until TRAPing with 4-OHT at ZT (Zeitgeber Time) 5.

Subcutaneous ghrelin injections

In **Paper II** and **III** rat ghrelin (#1465, Tocris, Bristol, UK) was dissolved in saline and subcutaneously (s.c.) injected in TRAP2 mice in order to target ghrelin-responsive cells in the DMH and Arc respectively, and in **Paper IV** to study ghrelin's effect on attentiveness to a conditioned food cue. Control mice received a similar volume of saline vehicle. Mice were habituated to s.c. injections using saline prior to the start of the experiment.

Intranasal administration of ghrelin, GHRP-6 and MK-0677

In **Paper I** ghrelin (#1465, Tocris), GHRP-6 (#G4535, Sigma-Aldrich Darmstadt, Germany), or MK-0677 (#SML0993, Sigma-Aldrich, Darmstadt, Germany) were dissolved in saline and tested for initial intranasal delivery in a dose-dependent manner: ghrelin was tested in 0.1 and 1 mg/kg doses, GHRP-6 in 0.5 and 5 mg/kg doses and MK-0677 in 3, 10, and 30 mg/kg doses. The 5 mg/kg dose of GHRP-6 robustly induced food intake without an aversive effect and was therefore selected for the remainder of experiments. Mice were gradually habituated to handling and to intranasal administration over 21 days, as described previously (Hanson et al., 2013). For intranasal administration of the compounds, awake mice were restrained in a horizontal, upside-down position. A micropipette was then used to administer half of the dose in 5 µl drops, placed on one nostril with 1-minute intervals. Ater a 5-minute break where the mouse was placed in the home cage, the remainder of the dose was administered drop-wise through the other nostril. Control mice received a similar volume of saline.

Food intake measurements

Manual food intake measurements

Food intake was measured manually for initial testing of the orexigenic properties of intranasal ghrelin, GHRP-6 and MK-0677 in **Paper I**. Each individually housed mouse received one pre-weighed chow pellet placed on the designated place on the cage lid, big enough to ensure it did not fall through the grid into the cage. The food that remained was weighed at fixed time points after treatment on a precision scale, and the amount of food consumed was

calculated afterward. Chow pellets were kept in an open container in the same room as the individually housed mice for 24 hours in advance, to ensure that a sudden change in environmental humidity did not affect the measurements.

FED3 automized food intake measurements and meal pattern analysis

The Feeding Experimentation Device 3 (FED3; Open Ephys Production Site, Lisbon, Portugal) was used to measure food intake (**Papers I-IV**) and operant behaviour (**Papers II-IV**) inside the familiar environment of the home cage (Matikainen-Ankney et al., 2021). The FED3 has a programmable output, and is provided with example programs, such as the "FreeFeeding" program, where the FED3 dispenses a 20 mg chow food pellet into the magazine well (**Figure 3**) each time a pellet is removed, therefore providing a continuous supply of food. The timing of each pellet removal from the well was logged via an internal microSD for later analysis, allowing for precise food intake measurements. Chow was always removed from the cage when the FED3 was present and, prior to experimental measurements, mice learned to feed from the FED3 over a 48 hour period, after which the FED3 was removed and regular chow returned until the experiment. Food intake was calculated from dispensed pellets per hour using the graphical user interface provided with the FED3, Fed3Viz (Python Software Foundation, Beaverton, OR, USA. Python Language Reference, version 3.12, http://www.python.org). The same time-stamped pellet data was used to perform additional meal pattern analysis in **Paper I-III** using our own custom graphical user interface (Python Software Foundation, Python Language Reference, version 3.12, http://www.python.org). One meal was defined as a minimum of 2 pellets (40 mg) eaten with an interval of less than 5 minutes (Rathod & Di Fulvio, 2021). During habituation and after each experiment, each cage was checked for hoarded pellets.

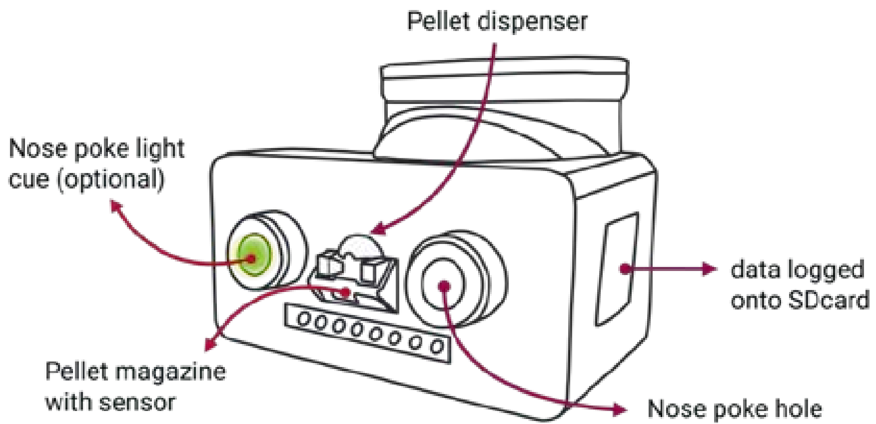


***Figure 3: Illustration of the Feeding Experimentation Device 3 (FED3), used for automized food intake measurements and operant responding tasks inside the home cage**. Chow pellets are dispensed from the container on top into a pellet magazine for retrieval. A sensor in the magazine logs the time of dispensing and removal of the pellet from the magazine, and logs pokes into the nose poke holes on either side of the magazine onto a SD card. The device can additionally be programmed to make pellet dispensing contingent on nose pokes into one of the two holes. Additionally, the nose poke holes contain a LED light that can be programmed to light up, which we used in **Paper IV** to cue food availability.*

Operant responding behavioural testing

Food motivation

In addition to food intake measurements, the FED3 device was also used to perform several different operant responding tasks. In **Paper II** and **III**, the FED3 was used to explore motivation for food in a progressive ratio (PR) nose-poking task, in which it becomes exponentially more difficult (i.e. they must perform more nose pokes) to obtain one pellet of food which each chow pellet earned. The FED3 device was the only source of food during training and testing. During motivation training (training scheme showed in Table 1), mice first learned to nose poke once (fixed ratio 1, FR1) inside either the left (half of the cohort) or the right (remaining cohort) nose poke hole located on either side of the magazine well (**Figure 3**), whereafter mice moved through two more training stages: through FR3 and trough a Closed Economy progressive ratio 2 (CEPR2), where the nose poking requirement after each pellet earned increased with two pokes. Finally, mice were exposed for 2 hours on two consecutive days to an Exponential Progressive Ratio task (PR2h), where the

required number of pokes for each pellet increased according to a formula (Richardson & Roberts, 1996) and were then placed back on CEPR2 for the remainder of the day. To move on to the next training stage, mice had to stabilize their body weight, which took maximum of 4 days per training step (see **Table 1**). On the experimental days in **Papers II** and **III**, mice were exposed to the PR2 protocol for 2 hours directly after 1 mg/kg CNO (a Gq agonist) or vehicle injection in crossover fashion.

Training stage	Pokes to earn one pellet	Training duration
Fixed Ratio 1	1	Max. 4 x 25 hours
Fixed Ratio 3	3	Max. 4 x 25 hours
Closed Economy Progressive Ratio 2 (CEPR2)	2 poke increase (1-3-5-7 etc.) Reset to 1 when > 30 min inactive	Max. 4 x 25 hours
Exponential PR2 Then CEPR2	Exponential increase/pellet	2 days (2h) (CEPR2 22h)

Table 1: Overview of the training stages for the motivation task, where mice work more for each pellet after one pellet earned.

Analysis of the FED3 data was performed in the graphical user interface Fed3Viz (Python Software Foundation. Python Language Reference, version 3.12, http://www.python.org). The breakpoint, determined as the number of pokes or pellets earned before giving up (no poking for 30 min or longer) or at the end of the 2 hour test, was used as the indicator of motivation to work for food.

The attention paradigm

To assess attention-linked behaviour to a food cue in mice (**Paper IV**), we designed a novel training and testing paradigm, utilizing the programmable output of the FED3 and the FED3 Arduino library resource (FED3 library version 1.16.2) to write a customized code for cue-potentiated feeding in the Arduino software (Arduino IDE 1.8.16, Arduino S.r.l., Monza, Italy), where a nose poke in the same location as a light stimulus leads to dispensing of a pellet into the magazine well. This attention paradigm existed of several training phases and 'attention challenges' on experimental days.

The attention protocol – Training procedures

For all training phases, the FED3 was placed in the home cage during the light cycle. During the training period, pellets from the FED3 were the only food source mice had access to. Training sessions started at ZT12 unless otherwise specified, both to ensure mice had access to food when it is most naturally to eat (Possidente & Birnbaum, 1979), and that the cue light would be visible in the dark. Training in the home cage did allow for minimal disturbance by the researcher and environmental changes, as well as for longer daily training sessions that lasted 12 or 6 hours. A trial started when the light cue inside the nose poke hole automatically lit up, such that mice did not initiate a trial. There was no limit to how many trials could be completed within a training session. Food intake and body weight were monitored daily and mice that completed <100 trials (2 grams of food) or reached <90% of baseline bodyweight received an extra pellet of regular chow (~1.5 grams). However, this was only necessary on a few training days over the whole training period since mice generally managed to reach their food intake goals from the FED3 within 4-6 hours of training. Pellet hoarding was not observed during daily cage checks.

During the pretraining (PT) stages, mice were habituated to feeding from the FED3 and nose poking for a pellet, while simultaneously associating a light cue inside the nose poke hole with the availability of food. In PT0, a pellet was dispensed into the magazine well and, if there was a pellet available, there was a continuous light on (stimulus duration, SD) in both nose poke holes (**Figure 4a**). Removal of the pellet resulted in immediate dispensing of a new pellet, and the light cue was off briefly (1-3 seconds) while a pellet was being dispensed. In PT1, both nose poke holes were illuminated, and a pellet was dispensed upon nose poking in one of the two holes, followed by a 30 second inter trial interval (ITI) where there was no light and during this time a nose poke was registered but did not have any programmed consequences (**Figure 4b**).

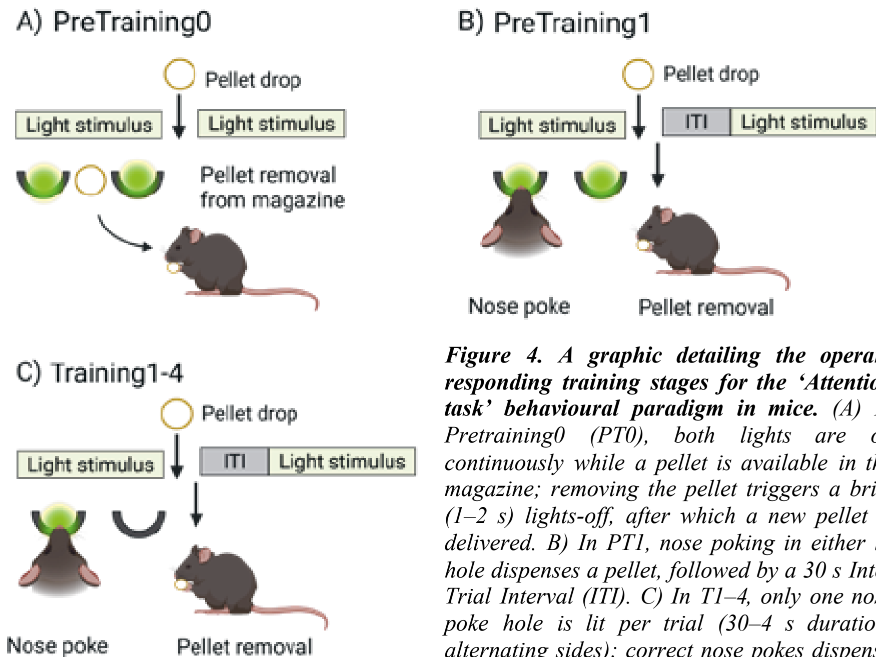


Figure 4. A graphic detailing the operant responding training stages for the 'Attention task' behavioural paradigm in mice. *(A) In Pretraining0 (PT0), both lights are on continuously while a pellet is available in the magazine; removing the pellet triggers a brief (1–2 s) lights-off, after which a new pellet is delivered. B) In PT1, nose poking in either lit hole dispenses a pellet, followed by a 30 s Inter Trial Interval (ITI). C) In T1–4, only one nose poke hole is lit per trial (30–4 s duration, alternating sides); correct nose pokes dispense a pellet and trigger a 30 s ITI.*

To train mice to respond to a shorter cue, the SD was reduced in steps during training stages T1-T4 from 30 seconds to 4 seconds (**Table 2**). For each trial only one nose poke hole would illuminate, alternating between left and right in an unpredictable manner generated by a pseudorandom binary sequence (**Figure 4c**). Mice could respond to the light cue in several ways, illustrated in **Figure 5a**. A 'correct response' (nose poke within the SD in the illuminated nose poke hole) was logged and resulted in the dispensing of a pellet, and removal of the pellet initiated a 30 second ITI. In order for the mice to have enough time to respond with a nose poke in the illuminated hole, a 4 second limited hold (LH) was introduced during T3 and T4, and a nose poke during the LH window would also be logged as a correct response and result in dispensing of a pellet. Nose pokes at the non-illuminated location were registered as 'incorrect responses', nose pokes during the ITI as 'premature responses' and if a mouse was not interacting with the FED3 for < 2 minutes, missed cues would be registered as a 'training omission response'. During training, logged omissions (when a mouse was not interacting > 2 minutes with

	Parameters used			
Training stage	**Stimulus Duration (SD)**	**Limited Hold (LH)**	**Inter Trial Interval (ITI)**	**Progression criteria**
PT0	Continuous (with pellet)	-	-	> 120 pellets eaten
PT1	Continuous (poke for pellet)	-	30 seconds	> 100 pellets eaten
T1	30 seconds	-	30 seconds	> 80% accuracy < 20% training omissions < 30% premature responses > 100 correct trials
T2	16 seconds	-	30 seconds	
T3	8 seconds	4 seconds	30 seconds	
T4	4 seconds	4 seconds	30 seconds	

Table 2: Schedule of operant responding training stages for the attention task behavioural paradigm*. Stimulus parameters (stimulus duration, SD; Inter Trial Interval, ITI; Limited Hold, LH), progression criteria and final criteria for inclusion into the experiment.*

the FED3) were excluded, due to the long training sessions and mice not feeding continuously. Together, these parameters were used to calculate the progression criteria for each training stage (**Table 2**), which made sure mice understood the task presented in the current stage prior to increasing the difficultness. Accuracy was calculated as #correct response / (# correct + incorrect response) * 100%, training omissions as #training omissions / (#trials*100) and premature responses as #premature pokes / #total pokes * 100%. Finally, mice that showed stable performance on T4 for 2-4 days were included in the experiment. In order to minimize over-training while starting the experiment for the entire cohort on the same day, mice that reached inclusion criteria were temporarily removed from training and received regular chow, where after they were re-trained for two daily sessions on T4 until reaching inclusion criteria once more.

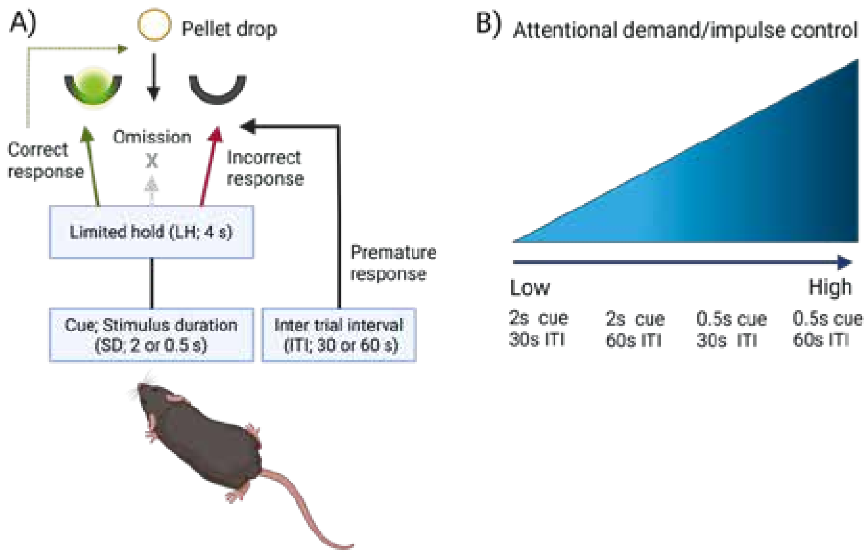


Figure 5: Graphic containing details about the attention challenge task, used to assess attentional performance towards a conditioned food cue*. A) Detailing a correct, incorrect, omission or premature response during a training session in the home cage. A nose poke into the illuminated poke hole is registered as a correct response, a nose poke into the non-illuminated poke is registered as an incorrect response, no response as an omission and a poke when none of the nose pokes are illuminated (during the Inter Trial Interval; ITI) as a premature response. A correct response results in dispensing of a 20 mg chow pellet, other responses had no further programmed consequences. B) Illustrating the range in attentional demand/impulse control required from the mice to respond to varying cue and ITI length. SD; stimulus duration, LH; Limited Hold.*

The attention challenge and manipulation of test parameters

After completing the training, FED3s were removed from the cage and mice were kept on regular chow until exposure to the attention challenge task, where chow was removed two hours before starting. The attention challenge task was designed to modify the attentional demand and impulse control required from the mice to respond correctly to the conditioned food cue during each trial by 1) shortening the SD and 2) prolonging the ITI, as illustrated in **Figure 5**. Pseudo-randomization of the different trials, and between the left and right nose poke holes ensured unpredictability of the task. Mice were presented with a short (0.5 second) or long (2 second) light cue in the left or right nose poke hole, followed by a 4 second LH window for mice to respond and either a 30 second or 60 second ITI upon pellet removal. This way, the attention task

provided us with information such as correct and incorrect responses, omissions and premature responses that were used to interpret behavioural patterns linked to attention, impulsivity and motivation towards the food-predicting cue.

Conditioned placed aversion (CPA)

In **Paper II,** we examined the valence that is conveyed by chemogenetic re-activation of the ‘hunger’-TRAPed neuronal ensemble in in the DMH. Mice were conditioned to associate the re-activation of these neurones with one of the two compartments in a CPA setup, which is composed of two chambers with distinct visual and tactile qualities that are separated by a guillotine door (Med Associates Inc, Fairfax, VT, USA; **Figure 6a**). One of the chambers was white with a smooth-surface plastic floor and the other chamber is black with a rugged-surface plastic floor. The time spent in each of the chambers was recorded using infrared beams. Mice were habituated to injection with saline and freely exploring the chambers for 30 minutes on two consecutive days, and on the third day initial chamber preference was recorded for 15 minutes (**Figure 6b**). After this, mice were exposed to 6-10 daily 30-minute sessions of which half of the sessions were used to pair re-activation by CNO injection of the neuronal ensemble with the presence in the most preferred compartment and the other half to pair the vehicle injection with the presence in the least preferred compartment of the CPA setup. On the final test day, mice were placed in the CPA setup and allowed to move freely in both compartments for 15 minutes while time spent in each chamber was being recorded. This way, if mice experience unpleasantness (negative valence) when the DMH neuronal ensemble is re-activated, they will spend less time in the compartment associated to the re-activation, which indicates that these cells carry a negative valence, or a positive valence if they spent time in the opposite compartment.

A)

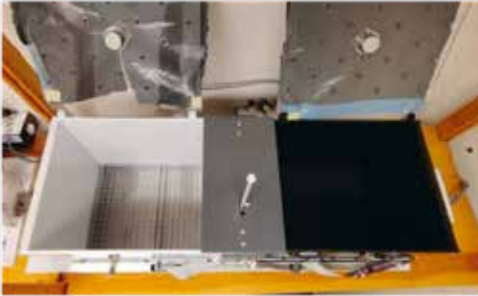
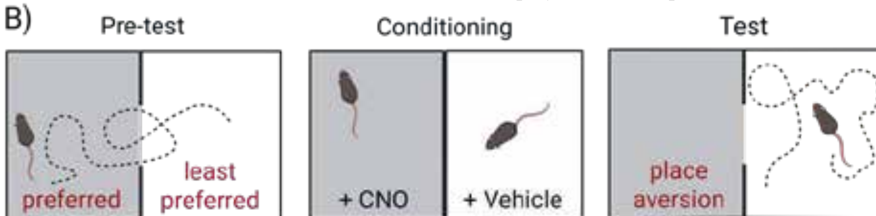


__Figure 6: standard two-chamber conditioned place preference/aversion test. The two chambers of the apparatus are different in tactile and visual qualities__. A) there is a divider between the chambers that is present during conditioning and removed during the preference/aversion testing. B) Chemo-genetic re-activation with clozapine-N-oxide (CNO) is paired with the chamber that was determined to be preferred in the pre-test

B)



Biochemical and imaging techniques

Brain tissue processing

In order to assess *Fos* mRNA- or protein expression, mice were exposed to a stimulus such as intranasal GHRP-6 (**Paper I**) or CNO i.p. injection (**Papers II** and **III**) or vehicle ~90 minutes prior to sacrifice, corresponding to a time point of optimal *Fos* expression upon neuronal activation (Morgan et al., 1987). In order to obtain brain tissue for immunohistochemistry (IHC), mice were deeply anaesthesized 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) and transcardially perfused with heparinized 0.9 % saline followed by 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). 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 for 2-3 days. Brains were promptly frozen on dry ice and stored at -80°C until cryosection. Coronal sections (20 µm) were collected from the whole brain or region of interest using a cryostat and stored in tissue storage solution at -20°C until further processing (25% glycerin, 25% ethylene glycol, 50% sterile 0.1 M PB).

Immunohistochemistry

To visualize FOS protein expression in the Arc upon intranasal treatment with GHRP-6 (**Paper I**), an immunohistochemical staining protocol using nickel-enhanced 3,3'- diaminobenzidine (DAB) was performed as described previously (Le May et al., 2019). Free-floating brain sections containing the Arc were rinsed briefly in 0.1 M PB and treated for deactivation of endogenous peroxidases. After rinsing with 0.1M PB/0.3% Triton X-100 and blocking for 1 hour at room temperature in 0.1M PB with normal goat serum (3%), bovine serum albumin (0.25%) and Triton X-100 (0.3%) sections were incubated with an anti-c-FOS rabbit primary antibody (dilution 1:1000; No. 226003, RRID: AB_2231974, Synaptic Systems) overnight at room temperature. To detect the binding of the primary antibody to the epitope, sections were rinsed and subsequently incubated with a peroxidase goat-anti-rabbit immunoglobulin (Ig)G secondary antibody (dilution 1:1000; No. A-11032, RRID: AB_2534091, Thermo Fisher Scientific), followed by treatment with a DAB, nickel and hydrogen peroxide solution. After the last washing step, sections were mounted onto glass slides, coverslipped with Pertex mountant (Histolab) and kept in the fridge until imaging.

mRNA detection with RNAscope in situ hybridization

The DAB IHC staining is a robust tool to visualise FOS protein expression, a marker of neuronal activation. However, the work included in this thesis extended beyond this by exploring the neurochemical identity of the *Fos* mRNA-expressing cells. In **Paper I** we sought to do this upon intranasal administration of GHRP-6 in the Arc and in **Paper II** we were interested in the neurochemical identify of re-activated hunger-responsive neurones in the DMH. To this end, we utilized the RNAscope technique, where multiple probes were used to perform triple fluorescent *in situ* hybridization to assess co-localization of different mRNAs on the same brain section. In **Paper I**, we performed two sets of RNAscope stainings: the first for co-staining of *Fos* with *Ghsr* and *Agrp*, and the other with *Ghsr* and *Ghrh* probes. In **Paper II** we used probes against *Fos*, *Ghsr* and *Npy* mRNAs. More specific information about the probes used can be found in the respective papers.

All reagents were purchased from Advanced Cell Diagnostics (ACD, Newark, CA, USA), unless stated otherwise. Twenty µm thick sections were mounted on SuperFrost plus slides (631-9483, VWR, Radnor, PA, USA) and dried at 60°C overnight in a HybEz oven (No. 321462). On the day of the assay, slides were first incubated in 4% PFA for 15 minutes at 4°C and washed in demineralized water, wherafter the RNAscope protocol was performed as described previously (Peris-Sampedro, Stoltenborg, Le May, Sole-Navais, et al., 2021; Peris-Sampedro, Stoltenborg, Le May, Zigman, et al., 2021; Stoltenborg et al., 2022), including sequential incubation in hydrogen peroxide, target retrieval buffer and Protease Plus enzyme to optimize probe penetration and signal detection. In **Paper I**, in the first assay, *Ghsr*, *Agrp*, and *Fos* mRNA probes were labeled with Opal 570 (1:2000, No. FP1488001KT, Akoya Biosciences, Malborough, MA, USA), Opal 520 (1:500, No. FP1487001KT, Akoya Biosciences) and Opal 650 (1:2000, No. FP1496001KT, Akoya Biosciences) respectively, while in the second assay *Fos*, *Ghrh*, and *Ghsr* mRNA probes were labeled with the same Opal 570, Opal 520 and Opal 650, respectively. In **Paper II**, *Fos*, *Npy* and *Ghsr* mRNA probes were labeled with Opal 570, Opal 520 and Opal 650 respectively. All sections were counterstained with DAPI, coverslipped with Prolong Diamond Antifade mountant (#P36970; Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4°C in the dark until imaging.

Imaging and quantification

To quantify the number of cells in the Arc expressing *Fos* protein upon intranasal treatment with GHRP-6 (**Paper I**), unilateral images were acquired using a Leica DMRB fluorescence microscope (10X/N.A. 0.30; Leica Microsystems, Wetzlar, Germany). The number of *Fos*-positive neurones per hemisection was counted manually and calculated as the average from 3 blind countings, averaged for each brain and, ultimately, averaged for each experimental group. The RNAscope data in **Paper I-III** was quantified from images captured using a laser scanning confocal microscope (LSM700 inverted, Zeiss, Oberkochen, Germany) equipped with a Plan-Apochromat 40/1.3 oil DIC objective. Optical sections of 90 µm were obtained and tile scans were stitched to visualize the whole area of interest (3 X 3 tile scans for the Arc and 4 x 4 tile scans for the DMH). A maximum intensity projection of the Z-stack images was generated with the Zen Black software (Zeiss) and

neurones were automatically counted using QuPath software (version 0.3.0). Neurones were identified by DAPI staining (a cell nucleus staining) and defined as being positive for a given peptide mRNA when more than 2 fluorescent dots/cell were detected (Peris-Sampedro, Stoltenborg, Le May, Sole-Navais, et al., 2021). The quantification for co-expression per hemisection was averaged for each brain and experimental group.

Detection of circulating factors

To assess whether intranasal GHRP-6 treatment stimulates GH release, whole blood samples were collected from the lateral saphenous vein from awake mice at 10 to 20 minutes following intranasal administration. Whole blood samples were clotted for 30 minutes and centrifuged to obtain serum, which was promptly frozen and stored at -80 °C. Serum GH was measured in duplicate with a commercial enzyme-linked immunosorbent assay kit (No. EZRMGH-45K, RRID: AB_2892711, EMD Millipore, Burlington, MA, USA) following the manufacturer's instructions. Samples were thawed only once.

Statistics and visualisation

Data 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 were created with Inkscape (version 1.4), https://BioRender.com and https://Canva.com.

Results

Paper I

In this paper, we aimed to assess whether intranasal administration of GHSR agonists could activate the brain ghrelin signalling system in a manner comparable to systemic delivery, particularly with respect to stimulating food intake, neuronal activity in the Arc and GH release. To this end, we screened three different GHSR agonists - ghrelin, the nonpeptide MK-0677 and the peptide GHRP-6 - using food intake as the primary outcome measure. Among these, GHRP-6 and MK-0677 induced orexigenic response following intranasal application compared to saline-treated mice. However, only GHRP-6 was well-tolerated by the mice and there were signs of a stress response by intranasal MK-0677 delivery.

We found that the feeding response to intranasal administration of GHRP-6 was driven by increases in both meal size and frequency. This behavioural response was accompanied by an increase in the number of cells expressing FOS in the Arc. Further characterization using RNAscope revealed that the majority of *Fos*-expressing neurones co-expressed *Agrp* (59.7%), consistent with the observed increase in food intake in our study and with the known targets of peripherally delivered GHSR agonists. Although co-expression of *Fos* with *Ghrh* (9.2%) was smaller than expected (Kamegai et al., 1996; Dickson & Luckman 1997), serum GH levels nevertheless rose significantly following intranasal GHRP-6 administration.

Paper II

In this study, we demonstrate that chemogenetic re-activation of a neuronal ensemble within the DMH originally activated during an acute hunger state (i.e. ghrelin administration to fasted mice) is sufficient to drive feeding-related behaviours. Utilizing the Fos-TRAP strategy in combination with a Cre-dependent viral vector delivering an excitatory DREADD to the DMH, we captured (TRAPed) neurones active during this hunger state and later reactivated this population via CNO administration.

CNO injection led to increased food intake (both cumulatively and hour-by-hour) which was reflected by increases in both meal size and meal frequency compared to saline-TRAPed controls, in male and female mice. Furthermore, CNO-injected mice of both sexes displayed increased motivation to obtain food in an operant responding task. Re-activation of this ensemble also conditioned a place aversion, indicating that the hunger-associated signal carries negative valence.

To further characterise the identity of this DMH ensemble, we performed RNAscope and found that in ghrelin-TRAP mice, roughly one-fifth of *Fos*-expressing cells in the anterior DMH co-expressed *Npy* and a similar fraction expressed *Ghsr*. In contrast, these proportions were more than twice as high in the saline-TRAPed controls. Notably, a significantly larger subset of the *Fos*-expressing population in the ghrelin-TRAP group did not co-express *Npy* or *Ghsr*, possibly indicating that an additional, unidentified population may contribute to the observed behavioural response.

Paper III

Here, we used the same chemogenetic approach as in **Paper II** to functionally identify a neuronal ensemble in the Arc that is involved in hunger. With the Fos-TRAP approach in combination with a Cre-dependent viral vector delivering an excitatory DREADD to the Arc, we targeted Arc neurones previously activated in mice experiencing hunger (overnight fast with and without ghrelin supplementation).

Re-activation of this population increased cumulative and hourly food intake in both fasting-TRAP and ghrelin-TRAP groups relative to controls. This increase was more pronounced in ghrelin-TRAP mice. Corresponding analyses of meal patterns showed that both TRAP groups increased meal frequency upon re-activation, while meal size was modestly elevated in ghrelin-TRAPed mice.

Food-motivated behaviour was assessed using an operant responding task. Upon chemogenetic re-activation, both ghrelin-TRAP and fasting-TRAP mice increased their number of nose pokes to breakpoint, pellets earned in the task and correct nose pokes, demonstrating an increased motivation to obtain food.

Paper IV

In the final study of this thesis, we investigated the extent to which ghrelin replicates the effects of fasting on attentional responses to food cues (Castellanos et al., 2009; Nijs et al., 2010; Schlezingerová et al., 2024). To this end, we developed a novel home-cage operant conditioning task designed to assess attention-related behaviours towards a conditioned food cue in mice. Mice underwent staged training and were subsequently tested for 30 minutes following an overnight fast or peripheral ghrelin injection.

In this experimental paradigm, both fasting and ghrelin modulated a set of inter-connected behavioural outcomes indicative of attentional performance. Specifically, both conditions increased correct responding and reduced omissions, suggesting enhanced attentional performance. Interestingly, we found that fasting, but not ghrelin, also increased incorrect responding, suggesting a potentially stronger attentional engagement under fasting conditions. However, further comparison with saline-treated controls revealed that fasting and ghrelin elicited broadly similar effects across all behavioural outcomes. Additionally, both fasting and ghrelin led to an increase in premature responding which, taken together with the observed increase in incorrect responding, may reflect impaired inhibitory control rather than selective attention alone. Notably, we found that both fasting and ghrelin delivery led to a greater increase in cued food intake relative to non-cued feeding.

Figure 7 provides a schematic representation that summarizes the key novel findings from **Paper I** (top left, in pink), **Paper II** and **III** (top right, in blue) and **Paper IV** (bottom, in yellow).

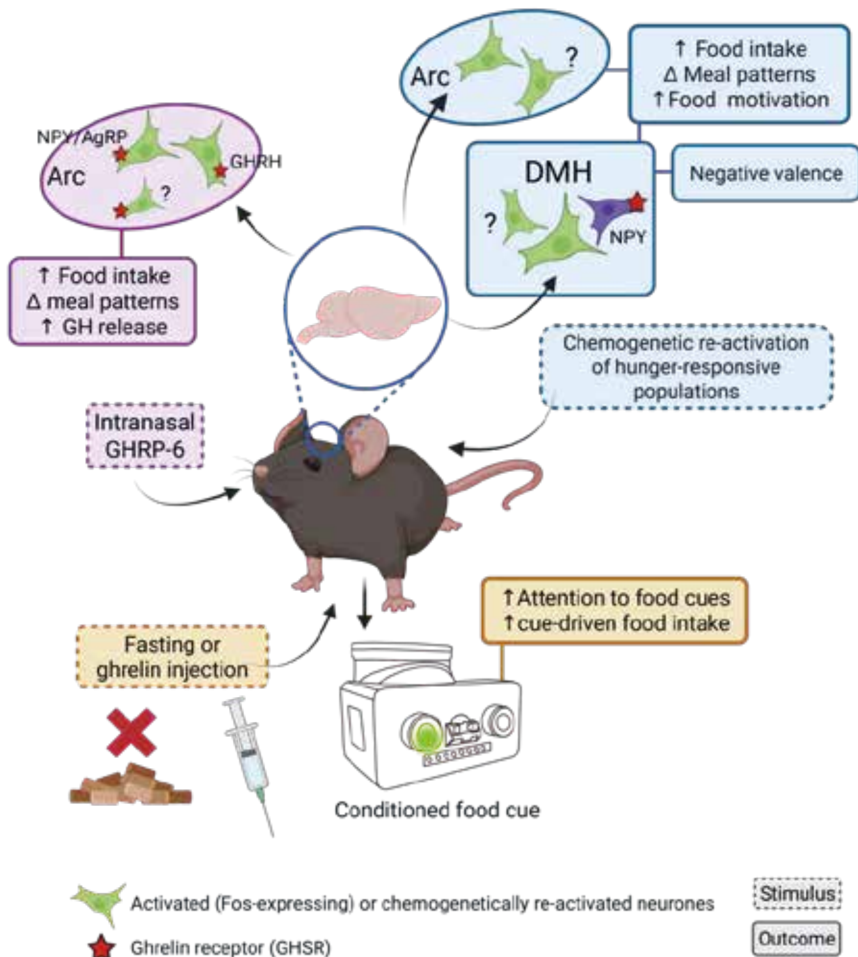


Figure 10: Schematic representation illustrating the main novel findings presented in this thesis. *Intranasal administration of the synthetic ghrelin mimetic GHRP-6 (Top left, in pink) reproduced many of the previously reported effects of peripheral ghrelin (mimetics), namely increasing the number of activated neurones in the Arcuate nucleus (Arc), engaging NPY/AgRP and GHRH neuronal populations, stimulating food intake and growth hormone (GH) release. Chemogenetic re-activation by CNO injection of a hunger-responsive ensemble in the Arc and in the Dorsomedial hypothalamus (DMH) stimulates food intake and food motivation (top right, light blue). Moreover, re-activation of the DMH ensemble conditioned a negative valence, which is associated with the feeling of hunger. The behavioural effects of re-activation of the DMH population are likely independent of NPY neurones, although the specific neurochemical identity of the Arc and DMH hunger-responsive ensembles remains to be elucidated (indicated with question marks). Finally, both fasting and ghrelin injection increased attention-linked behaviours towards a conditioned food cue, and enhanced cue-driven food intake (bottom, yellow). GHRP-6 = Growth Hormone Releasing Peptide 6; NPY = Neuropeptide Y; AgRP = Agouti Related Peptide; CNO = clozapine N-oxide*

Discussion

This thesis builds on current knowledge of the brain ghrelin signalling system by addressing specific gaps of knowledge in the field. By applying state-of-the-art techniques, it offers invaluable insights into the neural circuits and behavioural roles of ghrelin. Across the included studies, novel tools in neural circuit mapping, behavioural neuroscience and pharmacological delivery route were employed to dissect the mechanisms by which ghrelin influences feeding and food-linked behaviours. In **Papers II** and **III**, we used chemogenetic re-activation to functionally characterize hunger-activated neuronal ensembles (induced by ghrelin delivery to fasted mice) in the Arc and DMH. In addition to using a chemogenetic approach to reproduce ghrelin's best characterized behavioural effect - food intake - we demonstrate, surprisingly, that food motivated behaviour can also be driven from both of these sites. **Paper IV** is built upon the assumption that hungry mice will be more attentive to food cues, and we sought to discover whether ghrelin's role in hunger extends to increasing attention for a food cue. Using a novel behavioural task adapted to mice, we demonstrate that fasting and ghrelin both enhance attention-linked behaviour towards a conditioned food cue and increases cued food intake. In **Paper I**, we explored an alternative, less invasive route for targeting the brain ghrelin signalling system. Intranasal delivery of a synthetic ghrelin mimetic faithfully reproduced many of the known effects of peripheral GHSR agonist administration, including increased food intake, GH release and activation of a neuronal ensemble in the Arc, including AgRP and GHRH neurones (Lall et al., 2001; Bowers et al., 1984; Dickson & Luckman 1997). This approach may represent a promising delivery strategy for accessing the brain ghrelin signalling system, with potential benefit for improving food intake in individuals suffering from loss of appetite (such as those with anorexia nervosa or frail elderly) or for GH release.

Engagement of the brain ghrelin signalling system through intranasal application of a ghrelin mimetic

In **Paper I**, we tested 3 different GHSR agonists, namely ghrelin, the non-peptide GHS, MK-0677, or the peptide GHS, GHRP-6, using food intake as a primary screen. Of these compounds, only GHRP-6 reliably and robustly

increased food intake when delivered intranasally including without any adverse effects, demonstrating orexigenic properties that were comparable to those observed with subcutaneous injection, whilst being well tolerated by the animals.

The intranasal delivery of GHRP-6 significantly enhanced cumulative food intake for up to six hours post-administration compared to saline-treated controls, consistent with earlier reports on its orexigenic effects in rodents (Lall et al., 2001). A closer analysis of meal patterns during the first two hours post-administration, when the effect on food intake was the strongest, revealed that this increase was primarily driven by a higher meal frequency during the first 2 hours, with a modest increase in meal size observed only during the first hour. This suggests that the increase in food consumption after intranasal GHRP-6 is mostly due to the mouse eating more meals while failing to decrease meal size. These behavioural effects are informative of the brain regions involved since those controlling meal frequency more closely associated with hypothalamic hunger/satiety systems, while meal termination (and therefore control of meal size) is driven by hindbrain circuits (Grill, 2006, 2010).

In addition to its effects on food intake, intranasal GHRP-6 also elicited a potent rise of circulating GH levels, demonstrating the classical GH-releasing effect associated with GHS administration (Bowers et al., 1984; Bowers et al., 1990; Chapman et al., 1996). This experiment was performed in female mice to reduce variability arising from pulsatile GH secretion, which is less pronounced in females than in males (Edén, 1979; Jansson et al., 1985; MacLeod et al., 1991). Indeed, GH levels were more stable in the saline-treated group, but some variability remained in the GHRP-6 treated group. Interestingly, although all mice responded to GHRP-6, the magnitude of the GH response varied across individuals. One possible explanation is that GHRP-6 amplifies the amplitude of physiological GH pulses in female mice, and it becomes easier to detect pulsatility, as has been shown in studies using male mice (Bowers et al., 2004; Veldhuis et al., 2008). Therefore, it may be that the GH response is amplified but still pulsatile, which explains the greater variability in GH response to intranasal GHRP-6 in our study. Furthermore, residual variability may reflect external factors such as stress-induced suppression of GH secretion (Xu et al., 2011), despite efforts to habituate the animals to handling procedures.

To further demonstrate engagement of the brain ghrelin system by intranasal GHRP-6, we assessed neuronal activation in the Arc using FOS immunohistochemistry. The Arc was chosen as a primary site of interest given its central role in feeding regulation and also because previous studies found robust activation of this region following peripheral administration of ghrelin and GHS (Dickson et al., 1995; Dickson et al., 1993; Dickson & Luckman, 1997; Hewson & Dickson, 2000; Pirnik et al., 2011). In line with this, intranasal GHRP-6 increased the number of cells expressing *Fos* mRNA in the Arc and, further identification using RNAscope, revealed that the majority of activated neurones co-expressed *Agrp* (59.7%), a key orexigenic marker, which is similar to the observed 50% of *Fos*-positive cells co-expressing *Npy* (that also contain AgRP) in the Arc of rats that received systemic GHRP-6 (Dickson & Luckman, 1997). A smaller population (9.2%) of the activated ensemble co-expressed *Ghrh*, which is considerably lower than the 25% reported in earlier studies with systemic injections of GHRP-6 in rats (Tannenbaum et al., 1998; Willesen et al., 1999), which possibly reflects species differences or a technical variation in *in situ* hybridization.

Interestingly, our data highlight the existence of a population of neurones in the Arc activated by intranasal GHRP-6 but that do not express *Ghsr*, *Agrp* or *Ghrh*; these may be activated downstream via GHSR-expressing cells, or be activated independently of the GHRP-6 stimulus. Nonetheless, the overall profile of activated neurones aligned well with known mechanisms of GHSR agonist action, supporting the validity of intranasal delivery as a means to engage physiologically relevant components of the brain's orexigenic and GH-regulatory network.

In the current study, the route by which intranasal GHRP-6 accesses the brain ghrelin signalling system remains unknown. As introduced earlier, compounds delivered intranasally can reach the brain via several pathways, of which two routes, proposed for peptide uptake, bypass the blood brain barrier. The first is the intracellular route, which involves endocytosis of the compound into olfactory or trigeminal neurones. However, this process is relatively slow, taking several hours for the compound to reach central targets (Illum, 2002; Kristensson & Olsson, 1971; Thorne et al., 1995). Indeed, this mechanism has been largely dismissed as physiologically relevant for intranasal delivery of certain peptides such as oxytocin (Leng & Ludwig, 2016). While initially of interest – particularly because olfactory neurones express GHSR, and ghrelin

is known to reach the olfactory bulb rapidly from the circulation (Stark, 2024) - the time course of our observed effects makes this route unlikely. Specifically, we detected an increase in food intake within 30 minutes following intranasal GHRP-6 administration, a latency inconsistent with the intracellular transport hypothesis. Additionally, although ghrelin's actions in the olfactory bulb may influence aspects of feeding behaviour (e.g., sniffing, foraging and affecting meal patterns), these effects appear distinct from acute modulation of food consumption itself and remain to be fully characterized (Stark, 2024). Another proposed route is the paracellular route, in which the compound diffuses between cells lining the nasal epithelium to access the brain. This route is generally more permissive to small, lipophilic molecules with a molecular weight below ~1000 Da. GHRP-6, at only 872 Da, falls within this range, although it is not particularly lipophilic. An alternative possibility is that intranasal GHRP-6 does not bypass the blood brain barrier but instead enters the systemic circulation via uptake into the dense vascular bed of the nasal cavity. From there, it may act on the brain by accessing circumventricular organs such as the median eminence as has been shown for peripheral delivery of ghrelin (Schaeffer et al., 2013). This is consistent with previous evidence showing that peripherally administered GHRP-6 activates the Arc (Dickson et al., 1993) and would explain the rapid onset of the behavioural effect seen in our study. In this scenario, intranasal delivery may improve efficacy by avoiding first-pass metabolism in the gastrointestinal tract and liver, thus improving bioavailability relative to oral routes. Nonetheless, the route by which intranasal GHRP-6 accesses the brain ghrelin signalling system remains elusive, and would require delivery of labelled GHRP-6, as has been done previously for systemic ghrelin (Cabral et al., 2014; Schaeffer et al., 2013). At present, no such molecule is available.

In summary, the main novel finding of **Paper I** is the demonstration that intranasal GHRP-6, but not ghrelin, engages the brain ghrelin system, faithfully reproducing known effects of GHSR agonists on brain pathways controlling food intake and GH release. Arguably, intranasal delivery provides a less invasive route than peripheral injection for accessing the brain ghrelin signalling system. Additionally, we provide a robust analysis of meal patterns following intranasal GHRP-6 delivery, that favours effects on meal frequency over meal size, although both were impacted.

"Hunger" ensembles drive food intake and food-linked behaviour from the Arc and DMH

In **Papers II** and **III**, we sought to identify hunger-activated populations, building upon the knowledge that fasting enhances the neural response to ghrelin, at least at the level of the Arc (Hewson & Dickson, 2000). In other words, by injecting ghrelin to overnight fasted mice, we maximize the hunger response and recruitment of ensembles responding to huger. In addition to the Arc, we also targeted the DMH, whose role in driving the response to hunger had not yet been explored Thus, utilizing the TRAP2 technique, we "TRAPed" ensembles in the DMH (**Paper II**) and Arc (**Paper III**) after ghrelin injection to fasted mice and, some weeks later, chemogenetically re-activated them in order to discover their role in food-linked behaviours. When re-activated, both neuronal ensembles robustly increased not only food intake and meal frequency but also increased motivation to work for food. Additionally, we showed that re-activation of the hunger ensemble in the DMH conditioned a place aversion, which we interpret to reflect the unpleasant feeling of hunger.

The observed effect on food intake in both studies is in line with previous studies where food intake could be driven from the DMH (Bellinger & Bernardis, 2002; Hyland et al., 2020) by ghrelin and from ghrelin-TRAPed neurones in the Arc (Stoltenborg et al., 2022), and we expanded to this knowledge by reporting on the modulation of meal patterns, which were affected in a similar manner as after intranasal GHRP-6 administration in **Paper I** of this thesis.

In **Paper II** we demonstrate, for the first time, that the DMH harbours a hunger ensemble that can drive food-linked behaviours beyond what has been reported previously, namely food intake and anticipation. Specifically, we show that re-activation of this ensemble conveys a negative valence associated with hunger, which has previously been shown for intracerebroventricular ghrelin administration (Schéle et al., 2017). Moreover, we found that re-activation drives food motivated behaviour, in line with studies in rats showing that ghrelin increases lever pressing for sucrose (Skibicka et al., 2011; Skibicka, Hansson, et al., 2012) and for chow (Bake et al., 2019). In **Paper III** we also demonstrated that motivation could be driven from a hunger ensemble in the Arc. Increased food motivation, including that induced by ghrelin, is known to

engage the mesoaccumbal dopamine pathway (Skibicka, Shirazi, et al., 2012; Jerlhag et al., 2006; Jerlhag et al., 2007; Jerlhag et al., 2011). Our finding that re-activation of the hunger-activated DMH and Arc ensembles is sufficient to drive food motivation, suggests that these ensembles likely communicate with the midbrain dopamine system to elicit these effects. It will be important to explore how the hunger signal that we found originating from the DMH or Arc reaches the VTA dopamine neurones.

Paper II also provides more clarification regarding the extent to which peripheral ghrelin causes *Fos* expression in the DMH. While this has been reported in rats (Kobelt et al., 2008), a previous study did not detect *Fos* in the DMH following ghrelin injection in fed mice (Cabral et al., 2014). In the TRAP2 model, only cells that express *Fos* are targeted; thus, *Fos* expression is a prerequisite for the chemogenetic work, indirectly evidencing their activation. Moreover, in the RNAscope studies we found that neurochemically defined sub-populations of activated neurones differed from control mice. There are several possible explanations for the differences in *Fos* expression between our study and previous studies. It could be that fasting enhances *Fos* expression by ghrelin in the DMH, as is the case for the Arc (Hewson & Dickson, 2000). Fasting induces a 4-fold increase of *Ghsr* mRNA in tissue taken from whole hypothalami, and the difference we observed could be explained by fasting priming the brain, making it more sensitive to ghrelin (Hewson & Dickson, 2000; Scott et al., 2007). Alternatively, studies have shown that ghrelin has deeper penetration into the brain during stress and in particular, social defeat stress; given that fasting is also a form of stress (Smith et al., 2024) and could similarly increase brain penetration. The brain shows greater permeability for peripheral signals around the Arc (Jiang et al., 2020; Langlet et al., 2013; Myers, 2013; Schaeffer et al., 2013) and it could be that the activation of this DMH hunger ensemble is indirect, originating from the Arc. While fasting might explain the difference in *Fos* expression in the DMH, we found similar behavioural outcomes when re-activating the Arc hunger ensemble in **Paper III**. It might be that ghrelin levels are sufficiently high during fasting to elicit the behavioural response without supplementation of ghrelin. Still, ghrelin may be important for recruiting this hunger ensemble, as was shown in a previous study where re-activation of a ghrelin-recruited population in non-fasted mice could drive food intake (Stoltenborg et al., 2022).

Given that chemogenetic re-activation of the DMH ensemble only induced behavioural responses in the Ghrelin-TRAP group but not in the saline-TRAP group, we were surprised by the outcome of the RNAscope studies that revealed a similar number of *Fos*-expressing cells in both groups upon re-activation. It may be that there are differences in the identity of the sub-populations reactivated between groups or that the hunger ensemble was indirectly activated, for example from the Arc (Bouret et al., 2004; Hewson & Dickson, 2000). Upon triple staining with RNAscope, we found that that while approximately 20% of the *Fos*-expressing cells in the DMH could be identified as *Npy*-expressing and a further 20% as *Ghsr*-expressing in the Ghrelin-TRAP group, more than double this number expressed these genes in the Saline-TRAP group. These findings make it unlikely that *Npy*- or *Ghsr*-expressing neurones in the DMH drive the behavioural response upon re-activation. Moreover, quantification of gene expression revealed a reduction in *Npy* and *Ghsr* expression in the anterior DMH in the ghrelin-TRAP group that was combined with a trend towards a higher number of unidentified *Fos*-expressing cells, compared to saline-TRAP mice; taken together, it would appear that we have targeted an important DMH population other than *Npy* or *Ghsr* neurones that drive the observed food-linked behaviours. Therefore, it will be interesting to further explore the identity of the neuronal ensemble. Because this population is most likely orexigenic, interesting candidates to explore would be DMH cholinergic and melanocortin 4-expressing neurones, since they have been shown to induce hyperphagia (Chen et al., 2004). Although we have not yet neurochemically identified the hunger ensemble in the Arc in **Paper III**, it is well established that the neurones in the Arc recruited by ghrelin and fasting are predominantly AgRP, shown both by electrophysiology and *Fos* expression (Andrews et al., 2008; Takahashi & Cone, 2005), and that 40% of the neurones recruited by ghrelin in a previous TRAP2 experiment were *Agrp*-expressig (Stoltenborg et al., 2022). Because of this, together with the knowledge of the role of AgRP in conveying the negative valence of hunger (Betley et al., 2015), which is also seen after ghrelin delivery (Schéle et al., 2017), we would expect to find AgRP neurones as part of this population. We can confirm this by RNAscope experiments in the future.

In summary, the novel findings of **Papers II** and **III** in this thesis are that neuronal ensembles in both the DMH and the Arc, that are recruited by hunger, can drive food-linked behaviours beyond mere food intake; they affect meal

patterns and increase motivation to work for food. Additionally, we demonstrate that this neuronal ensemble in the DMH conveys a negative valence associated with hunger. The neurones driving this behavioural effect from the DMH are most likely not the *Npy*- or *Ghsr*- expressing population, and the exact neurochemical identity of the hunger ensembles in the DMH and Arc remains to be elucidated.

A role for ghrelin in attention to environmental food cues

In the final study of this thesis, **Paper IV**, we developed a novel home-cage operant task for mice to assess attention-linked behaviour towards a conditioned food cue and explored whether ghrelin or fasting modulates attention to food cues. Ghrelin release is part of the physiological response to hunger, making this an interesting comparison. Based on previous work in humans where fasting enhances attentional bias to food images and words where fasting ghrelin corresponds positively with activation of brain areas related to visual attention (Nijs et al., 2010; Schlezingerová et al., 2024), we expected attentiveness to be heightened in fasted animals and used this to describe and validate a novel paradigm for exploring attention to food cues. In our novel paradigm, both fasting and ghrelin increased attentional engagement, that was reflected by inter-connected behavioural outcomes, including increased correct responses, reduced omissions and elevated premature responses during the task. This was accompanied by a greater increase of cued food intake relative to non-cued feeding, which suggests that the increased attention elicited by fasting or ghrelin translates to enhanced cue-driven consumption. These findings support findings from human studies showing that elevated ghrelin levels enhance neuronal activation when exposed to visual food cues (Kroemer et al., 2013; Malik et al., 2008; Wever et al., 2021) and extend this prior work by showing that ghrelin enhances not only general food cue reactivity, but specifically attentional performance toward food-predictive stimuli in mice.

It is important for interpretation of the observations in our paradigm to understand which aspects of attention we are measuring. Broadly speaking, “attention” refers to a variety of theoretical constructs by which the central nervous system apprehends, organizes and selects sensory input and generates

coordinated behaviours. Therefore, attention is not a unitary phenomenon, but rather a term that encompasses a variety of attentional processes. The complexity of this makes it a challenge to realistically capture and assess all aspects of attentiveness. In our paradigm, the attention task aims to reflect on the visual temporal and spatial allocation of attention to environmental food-predicting cues (i.e., initial orientation towards the "attention-grabbing" light stimulus) as well as sustained attention over time (i.e., maintain attention to the nose poke holes during the ITI in order to detect the signal). A number of cognitive processes have to be integrated for optimal performance in our task; for a detailed discussion on interpretation of the behavioural outcomes of an operant task similar to ours see (Robbins, 2002). By modifying task parameters – such as alternating the location of the cue, lengthening the ITI and shortening the cue duration – our task required mice to maintain attention across both nose poke holes and sustain focus over time to avoid missing the cue. Under these conditions, a high number of correct responses and few omissions reflect strong attentional performance. As expected, increasing the attentional demand through reduced stimulus duration and extended ITIs led to clear declines in accuracy and rises in both incorrect and missed responses. This is consistent with prior reports using similar attention testing protocols in C5BL/6 mice (de Bruin et al., 2006; Humby et al., 1999).

The novel attention paradigm introduced in this thesis offers potential for uptake by the research community. While training mice to perform complex operant tasks remains challenging, resulting in many researchers to favour rats for such studies, mouse models offer distinct advantages, particularly with the availability of a greater variety of transgenic models compared to rats. Moreover, our task can be implemented in the home cage environment, enabling longer training sessions, testing in a familiar setting and minimal experimenter interference – factors that are especially important on test days to reduce external influences on task performance. Nonetheless, it should be noted that our attention paradigm has a few limitations. One limitation is that our task does not report on response latency to the light cue, which is an important measure of attentional performance, and also a parameter that translates to human tests of visual attention. However, response latency can not only be decreased when attentional performance is worse, but also by heightened motivation or impulsivity (Robbins, 2002). Response latencies also slow down before omission errors and are thought to reflect lapses in sustained

attention due to disengagement with the task. Therefore, while communicating important information about attentional performance, response latency changes should be considered in the context of other test parameters such as premature responses and omissions. While adding the ability to assess response latency would elevate our attention task in the future, we consider behavioural outcomes such as correct and incorrect responses and omission errors to be sufficient reflections on attentive behaviour. An additional limitation of our current paradigm is that we did not control for impairments in sensory processing, which can be done by increasing and decreasing the strength of the light cue.

The increase in correct responses and reduction in omissions following fasting and ghrelin administration in our experimental paradigm reflect increased attentional performance. A potential mechanism underlying increased attentional performance could be the modulation of neural circuits that are involved in attentional control and reward evaluation. Ghrelin administration is known to engage dopaminergic pathways in the mesolimbic system, which is known for its involvement in reward and food motivation (Abizaid et al., 2006; Jerlhag et al., 2006; Jerlhag et al., 2007). Additionally, the mesolimbic system is indicated to also be involved in attentional processes, for example lesions of the medial striatum or the prefrontal cortical-dorsal striatal system in rats resulted in deficits in attentional performance in an operant responding paradigm (Christakou et al., 2001; Rogers et al., 2001). Future studies are needed to clarify the specific neural mechanisms linking ghrelin action with attentional engagement; this could be achieved, for example, by pharmacological blockade of ghrelin receptors or real-time neural recordings in relevant brain areas during the attentional task.

While our task primarily targeted attentional processes, performance was likely influenced by motivation and impulsivity, since ghrelin levels are known to increase motivation (Egecioglu et al., 2010; Perello & Dickson, 2015; Perello et al., 2010; Skibicka & Dickson, 2011; Skibicka et al., 2011) and impulsive behaviour in rats (Anderberg et al., 2016). Indeed, fasting and ghrelin administration reduced omission errors – which is often interpreted as increased motivation in similar operant responding tasks – and elevated premature responses, indicative of inhibitory control. However, in these operant responding tasks mice commonly have initiated the start of a trial. As mice in our paradigm did not self-initiate trials, a behavioural change in

omission responses may reflect a combination of attentional and motivational components rather than pure measures of each domain. In addition, when inhibitory control is decreased, incorrect responding is expected to increase simultaneously. We did observe an increase of incorrect responses following an overnight fast but, interestingly, not after ghrelin administration.

In summary, the novelty of **Paper IV** lies in establishing an innovative attention paradigm in the home cage setting, offering a new approach to study attentional bias to food cues in mice, and demonstrating that both overnight fasting and ghrelin administration influence behavioural responses in a manner indicative of heightened attentional engagement. These findings support the notion that hunger-driven attentional bias toward food cues involves the brain ghrelin signalling system, a concept that warrants further investigation, particularly through studies in which attention for a food cue is explored in the context of ghrelin inhibition. Overall, this work underscores the relevance of cue-driven attention in driving excessive food intake.

Conclusion

This thesis advances our understanding of the brain ghrelin signalling system by uncovering new aspects of its neural circuitry and role in food-linked behaviours. We identified novel hunger- (including ghrelin-) responsive neuronal ensembles in both the Arc and DMH and showed that their re-activation has a role in stimulating feeding behaviours beyond mere food intake, such as altering feeding patterns and enhancing motivation to work for food. Notably, re-activation of the DMH ensemble appears to encode the negative feeling associated with hunger, thereby extending ghrelin's known influence in the DMH on food-linked behaviours. While the precise molecular identity of these ensembles remained to be elucidated, our data suggest that the DMH hunger-responsive population drives the observed behavioural effect independently of *Npy*- or *Ghsr*-expressing neurones. Additionally, we obtained new insights into how ghrelin heightens attention to food-related cues and enhances cue-driven overconsumption in mice, providing insight into how metabolic signals interact with the environment to influence feeding behaviour. This interplay between internal and external signals may be particularly relevant in understanding how modern environments encourage overeating.

Finally, this thesis proposes a new, non-invasive approach to engage the brain ghrelin signalling system via intranasal delivery of the GHSR agonist GHRP-6, which faithfully replicates the known effects of peripheral administration on brain pathways that control food intake and GH release, offering translational potential for conditions involving reduced appetite or GH release.

Future perspectives

Understanding how the brain integrates internal and external signals related to hunger and food availability continues to be a focus of ongoing research in neuroscience, with broad implications for both healthy and disordered eating behaviours. This thesis has contributed novel insights into the brain ghrelin signalling system and its role in regulating food-linked behaviours. As with any scientific work, these findings prompt new questions and reveal several promising avenues for future research, which are outlined below.

In **Paper I**, we demonstrated that intranasal administration of the GHSR agonist GHRP-6 stimulates food intake and GH release in mice. Our findings suggest that these effects are centrally mediated through engagement of the brain ghrelin signalling system, as supported by an increased number of FOS-expressing cells in the Arc, of which the majority were identified as *Agrp*- and *Ghsr*-expressing neurones. One key next step would be to confirm the necessity of GHSR signalling in these effects, which we could do in our lab by injecting a viral vector that Cre-dependently expresses hM4Di in the Arc of transgenic *Ghsr-Cre* mice and chemogenetically inhibit *Ghsr*-expressing neurons in this area. Enhancing the dose-response analysis using automated food intake monitoring (e.g., the FED3 system) and including female mice would strengthen the translational relevance of our findings. Additionally, it would be exiting to explore encapsulating GHRP-6 in lipid-based vesicles for improved delivery, as has been proposed for ghrelin (Salade et al., 2017; Salade et al., 2018). Because GHRP-6 is a smaller and more stable synthetic compound, it may prove more efficient and cost-effective than ghrelin. This could also re-enable the use of MK-0677 for intranasal delivery, as lipid vesicles would protect the nasal mucosa from irritation.

An important translational question for future studies building onto the findings presented in **Paper I** concerns the clinical applicability of intranasal GHSR agonists. The GH response to chronic administration of GHS in humans dramatically wanes (Ragim et al., 1998) which makes intranasal GHRP-6 more suitable as a provocative test for GH deficiency rather than a chronic treatment for GH deficiency. However, the appetite-stimulating effects of GHRP-6 may prove more persistent. Haruta et al. (2015) reported sustained increases in hunger following intranasal GHRP-6 administration over a year in an

individual with anorexia nervosa, even as the GH response waned. It would thus be valuable to explore whether the acute orexigenic effects we observed persist with repeated administration.

Papers II and **III** revealed that hunger-responsive neuronal ensembles in the Arc and DMH promote feeding and motivational behaviours, and that the DMH ensemble conveys negative valence. These findings expand our understanding of ghrelin-responsive circuitry beyond the well-established Arc and raise new questions about the identity and projections of the DMH population. Additional RNAscope analyses could help define the neurochemical identity of these neurones, with candidate markers for the DMH ensemble including cholinergic or MC4R-expressing populations, both implicated in hyperphagia (Chen et al., 2004), and an RNAscope analysis for *Agrp* in the Arc (Andrews et al., 2008; Betley et al., 2015; Schéle et al., 2017; Stoltenborg et al., 2022; Takahashi & Cone, 2005). To further map the broader circuitry underlying these behaviours, FOS immunostaining could be used on existing brain sections to screen for downstream activation patterns following ensemble re-activation. This approach could reveal other brain regions that are indirectly recruited by re-activation of the hunger-ensemble. Additionally, projection mapping studies using Cre-dependent viral tracers in TRAP2 mice subjected to hunger protocols would help identify the direct targets of Arc and DMH hunger ensembles. We also observed that reactivating these hunger-TRAPed ensembles increases motivation to work for food, raising the question of whether this occurs via dopamine release in the mesolimbic system. Previous studies show that peripheral ghrelin enhances dopaminergic signalling from the ventral tegmental area (VTA) to the nucleus accumbens (Jerlhag et al., 2008). Future studies could use microdialysis to test whether ensemble re-activation in the DMH or Arc similarly increases dopamine release.

Paper IV introduced a novel home cage-based attention paradigm for mice, demonstrating that both fasting and ghrelin administration increase attention-linked behaviours towards a conditioned food cue and promote cue-driven food intake. Several technical improvements could enhance this paradigm, such as implementing response latency measurements via Arduino code updates, tracking motor activity to control for motor impairments via video monitoring, and validating the paradigm in female mice. Since mice eat

primarily during the dark phase (when they train), it would be of interest to assess whether *ad libitum* chow access during non-training light phases compromises task engagement, in order to refine our protocol with regard to improving animal welfare.

Our attention task opens new doors to investigate how internal signals and environmental food cues interact. One future study expanding on our findings that ghrelin and fasting increase attention to a cue predicting availability of chow, would be to test if attentiveness towards an appetitive, rewarding cue, such as one predicting sucrose pellets, is affected in a similar manner. Conversely, appetite-suppressing compounds such as GLP-1 agonists could be tested for their ability to reduce attention to food cues. As reviewed by Hayashi et al (2023), there have been anecdotal reports from users of GLP-1 agonists that they reduce “food noise”, which refers to a heightened and/or persistent food cue reactivity, often leading to food-related intrusive thoughts and maladaptive eating behaviours. GLP-1 agonists have been shown to affect food-linked behaviours beyond food intake. In mice, GLP-1 agonists reduce the motivation to work for food (Ghidewon et al., 2022) and central administration of a GLP-1 agonist reduced the dopamine response in the VTA to a food-predicting cue (Konanur et al., 2020). In contrast, peripheral administration of GLP-1 does not appear to impact on the rewarding value of food cues (Kooij et al., 2024), and the role for GLP-1 agonists in food cue reactivity remains to be further elucidated. Therefore, it would be interesting to assess its effects on attentional bias towards food cues in the future.

To further dissect the neural mechanisms behind these behaviours, the TRAP2 system could be applied to label and manipulate cue-responsive neurones engaged during attention tasks in the fasted or ghrelin-treated state. Combining this with chemogenetic inhibition or activation and projection mapping could clarify whether ghrelin-enhanced attentional engagement depends on specific circuits (e.g., Arc, VTA, prefrontal cortex). Moreover, delivery of a viral vector that Cre-dependently expresses hM4Di in the Arc of transgenic *Ghsr-Cre* mice and chemogenetically inhibit *Ghsr*-expressing neurons in this area could be used to help determine the necessity of local ghrelin receptor signalling in modulating cue-driven attention.

Together, the research outlined in this thesis highlights how integrating behavioural paradigms with molecular tools in transgenic mice can unravel the

complex interplay between internal states like hunger and external food-related cues. The development of new tools, such as the home cage attention task, and the application of powerful models like TRAP2 mice, provide a robust foundation for future work. Expanding on these findings may contribute meaningfully to our understanding of feeding behaviours and support the development of targeted interventions for disordered eating.

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"Nothing in life is to be feared; it is only to be understood"
- Marie Curie

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The brain is wider than the sky,
For, put them side by side,
The one the other will contain
With ease, and you beside.

The brain is deeper than the sea,
For, hold them, blue to blue,
The one the other will absorb.
As sponges, buckets do.

The brain is just the weight of god,
For, lift them, pound for pound,
And they will differ, if they do,
As syllable from sound.

Emily Dickinson, ca. 1862

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