The Individual and Combined Anorexigenic Effects of Oxytocin and Naltrindole in Rodents

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Abstract

Obesity is a characterized by excess body mass, and it reflects a failure to match energy intake to energy expenditure. Energy intake is regulated by a plethora of mechanisms, including the oxytocin (OT) and opioid systems, making both attractive targets for obesity pharmacotherapy. Initial studies utilizing OT and mu-opioid receptor antagonist naltrexone (NTX) suggest that combined targeting of OT and opioid signaling may enhance hypophagia. Thus far, there have no attempts to examine potential synergy between OT and delta opioid receptor blockers. The work presented in the current thesis was conceptualized to bridge this gap in knowledge by evaluating the anorexigenic potential of OT and naltrindole (NTI) when administered both individually and in combination.

The literature describing OT effects on fat intake remains inconsistent. Macronutrient-focused studies that assess the anorexigenic effect of OT on fat consumption using liquid emulsions, most commonly Intralipid, report no suppression of intake. Conversely, studies which use solid high-fat foods more often report OT-induced hypophagia, despite not being designed to systematically test the influence of fat content. Therefore, in Specific Aim 1 of this thesis I systematically determined whether OT suppresses the consumption of solid fatty foods. Adult male rats received intraperitoneal (i.p.) OT (0.1–10.0 mg/kg) and were tested in no-choice and two-choice paradigms using solid mash diets providing 62%, 79%, or 91% of energy from fat; chow diets providing 10% or 60% of energy from fat; or a matrix-matched, cornstarch-rich control chow. The effect of fatty food consumption on neuronal activity was measured using c-Fos immunohistochemistry and c-Fos-OT double staining, where sated rats were either given small meals of 10% fat chow, 60% fat chow, or no food. In the single choice tests, OT reduced the consumption of the solid diets across a range of fat contents, and in the two-choice paradigm, OT-treated rats consumed less 60% fat chow than 10% fat chow compared to control animals. Consumption of 60% fat chow, but not 10% fat chow, increased activation of OT neurons in the PVN and SON and increased c-Fos immunoreactivity in multiple feeding-related regions. These findings demonstrate that OT suppresses intake of solid high-fat foods and help reconcile the discrepancy between Intralipid-based and solid-diet paradigms. In the context of the overarching aim of this thesis, our findings also indicate that conclusions about the anorexigenic effect of OT drawn from Intralipid intake data should be

interpreted as specific to Intralipid, rather than assumed to generalise to dietary fat consumption more broadly.

The literature lacks a consensus on how NTI affects sucrose intake. Prior reports frequently describe mixed or null effects of NTI on sucrose consumption, but these findings are commonly derived from paradigms in which animals are not habituated to sucrose and instead receive sucrose access in acute, ad hoc test sessions. For this reason, Specific Aim 2 of this thesis was to determine the effect of NTI, and by extension the role of DOR, on sucrose intake in rats that habitually consume sucrose solution. Adult male rats were habituated to daily 1 h access to sucrose solution for three weeks. Following habituation, rats received i.p. NTI (5 or 10 mg/kg) prior to their regular sucrose access period and sucrose intake was measured. We also assessed whether NTI altered deprivation-induced water intake or produced a conditioned taste aversion (CTA). Additionally, c-Fos immunoreactivity was assessed in rats with habitual sucrose access and in sucrose-naïve controls, with and without NTI treatment. NTI at 10 mg/kg reduced sucrose solution consumption independent of changes in thirst and without evidence of a CTA. Moreover, NTI induced changes in neuronal activity across a broader set of feeding-related regions in sucrose-habituated rats than in sucrose non-naïve rats. Together, these findings demonstrate that NTI can suppress sucrose intake once consumption is habitual, and that habituation is associated with a broader recruitment of central feeding-related mechanisms following NTI treatment. Of relevance to the overarching aim of this thesis, these results indicate that null effects of NTI observed under ad hoc sucrose access conditions should not be assumed to reflect NTI efficacy once sucrose intake is predictable and habitual.

Finally, having established foundational knowledge of the individual effects of OT and NTI on intake in Specific Aims 1 and 2, we next examined their combined administration. Based on prior reports indicating that simultaneous targeting of OT and opioid signalling can produce stronger anorexigenic effects than either system alone, we tested OT and NTI in combination. We aimed to determine whether co-administration could overcome context-dependent limitations observed with each drug individually, including OT's failure to suppress Intralipid intake and the dependence of NTI effects on habitual sucrose exposure. Specific Aim 3 of this thesis was to test whether the simultaneous targeting of OT receptors and DOR, through combined administration of OT and NTI, synergistically suppresses the consumption of liquid diets. Adult male mice received i.p. OT (0.1–3.0 mg/kg), NTI (3.0–10 mg/kg), or OT+NTI prior to 2 h access to saccharin, Intralipid, or

sucrose solutions, and intake was measured. We also assessed post–water deprivation water consumption and characterised treatment-induced changes in neuronal activation in feeding-related regions using c-Fos immunohistochemistry. NTI, but not OT, reduced saccharin consumption, whereas OT+NTI produced a stronger reduction than NTI alone, consistent with a synergistic anorexigenic effect. In contrast, OT, but not NTI, reduced sucrose consumption, and neither drug alone nor the combination altered Intralipid intake. These intake outcomes occurred independent of changes in thirst. At the neuronal activation level, OT × NTI interactions were observed in the nucleus accumbens core and shell and in hypothalamic nuclei including the ventromedial and suprachiasmatic nuclei. Although combined treatment did not overcome the individual limitations observed for sucrose and Intralipid intake, the synergistic suppression of saccharin consumption is notable and extends evidence that combined targeting of OT and opioid signalling may hold promise for development of anorexigenic treatments.

In summary, this thesis provides novel insights into the context-dependent anorexigenic actions of both OT and NTI, demonstrated by the efficacy of OT as an inhibitor of solid fatty food consumption but not Intralipid, and by the dependence of NTI-induced sucrose suppression on habitual consumption. Furthermore, this thesis provides additional evidence that simultaneous targeting of OT and delta opioid signalling can produce anorexigenic effects which extend beyond those achieved with single-drug treatment, as demonstrated by the synergistic suppression of saccharin intake observed with OT and NTI co-administration.

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List of Abbreviations

AcbC	Nucleus accumbens core
AcbS	Nucleus accumbens shell
AgRP	Agouti-related peptide
AP	Area postrema
ARC	Arcuate nucleus of the hypothalamus
ASD	Autism spectrum disorder
BBB	Blood brain barrier
beta-FNA	Beta-funaltrexamine
BLA	Basolateral Amygdala
BMI	Body mass index
CART	Cocaine- and amphetamine-related transcript
CCK	Cholecystokinin
CEA	Central Amygdala
CNS	Central nervous system
CS	Cornstarch
CSF	Cerebral spinal fluid
CTA	Conditioned taste aversion
CTOP	Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2
DAB	Diaminobenzidine
DAMGO	D-Ala^2-Met-enkephalinamide-Gly-ol
DMH	Dorsomedial nucleus of the hypothalamus
DMV	Dorsal motor nucleus of the vagus
DOR	Delta-opioid receptor
DOR1	[D-Pen^2,D-Pen^5]enkephalin sensitive delta opioid receptor
DOR2	Deltorphin-II sensitive delta opioid receptor
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
fMRI	Functional magnetic resonance imaging
GKR	Translocation signal peptide sequence
GLP-1	Glucagon-like peptide 1
GPCR	G protein coupled receptor
HFHS	High-fat high-sugar
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal

i.v.	Intravenous
IR	Immunoreactivity
KO	Knockout
KOR	Kappa-opioid receptor
LH	Lateral hypothalamus
MOR	Mu-opioid receptor
NAc	Nucleus accumbens
nor-BNI	Nor-binaltorphamine
NPY	Neuropeptide Y
NTI	Naltrindole
NTS	Nucleus of the solitary tract
NTX	Naltrexone
OT	Oxytocin
OTKO	Oxytocin knock out
OTR	Oxytocin receptor
OTRKO	Oxytocin receptor knock out
PFA	Paraformaldehyde
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
PYY	Peptide YY
RAGE	Receptor for advanced glycation end-products
SON	Supraoptic nucleus of the hypothalamus
VMH	Ventromedial nucleus of the hypothalamus
VP	Ventral Pallidum
VTA	Ventral tegmental area
WT	Wild type

Chapter 1
Introduction

1.1 Obesity and overconsumption of food

Overconsumption of food is a contributor to a range of deleterious health outcomes, including metabolic diseases such as obesity. By examining trends in diets and human health, we can begin to understand the origins of dysregulated food consumption, and in turn gain insight into the neural mechanisms that influence this behaviour. The first section of this introduction begins with a description of the change in human diets over time and how this leads to behaviours such as delayed meal termination. From here, obesity as an epidemic is described with particular focus given to the disruption of satiety signalling. Finally, artificial sweeteners and their contribution to dysregulated feeding behaviour are discussed.

1.1.1 The emergence of increased fats, sugars, and portion sizes in the human diet

On the scale of human evolution, the foods and eating schedules we experience today have only very recently emerged. Advances in agriculture, food processing, and industrialisation have caused substantial changes to human diets in a relatively short time. This rapid shift far outpaces our physiological ability to adapt through evolution. As a result, there is a growing mismatch between the feeding schedules our bodies are adapted for and the portion sizes and eating behaviours that are common in the current food environment (Cordain et al., 2005).

Over the last 5 to 7 million years, since the emergence of hominins, typical diets have varied drastically depending on environmental conditions and food availability. For this reason, it is difficult to determine exact ancestral diets. However, pre-agricultural hominins likely depended on wild plant and animal sources obtained through foraging, scavenging, and hunting (Cordain et al., 2005; Konner & Eaton, 2010). These diets consisted entirely of unprocessed or minimally processed foods and lacked the caloric density and palatability common in modern diets. Portion sizes were also likely smaller than those typical today (Bragazzi et al., 2024; Cordain et al., 2005).

Since the development of early agriculture and, particularly with the acceleration of modern industrialised food production, human diets have changed dramatically. Food groups such as dairy products, refined sugars, refined vegetable oils, cereals, alcohol, and

high-fat domestic meats, which were all previously inaccessible, are now common staples. Modern humans tend to consume foods with greater glycaemic loads, more saturated and trans fatty acids, and lower protein-to-carbohydrate ratios. There has also been a reduction in micronutrient density, a shift from net base-yielding to net acid-yielding diets, an increased sodium-to-potassium ratio, and a decline in dietary fibre intake (Cordain et al., 2005).

In addition to macronutrient and micronutrient shifts, modern diets have also introduced a wide array of additives into the human diet. This includes synthetic compounds used to improve appearance and palatability, extend shelf life, or increase agricultural yield. Many of these compounds do not occur naturally and have only recently become part of the human diet (Bragazzi et al., 2024). Artificial sweeteners are one example of modern synthetic food additives.

In combination, these changes have led to a substantial increase in the energy density and palatability of the human diet, while simultaneously reducing bulk. Ancestral diets typically promoted earlier physiological meal termination through high fibre and water content, coupled with low caloric density, while modern diets delay satiety signals, enabling larger meal sizes (Konner & Eaton, 2010) (Drewnowski, 1998). The combination of high palatability, rapid consumption, and increased portion sizes contributes to extended eating episodes and the sustained overconsumption of food (Popkin & Ng, 2022) (Hall et al., 2019).

While changes in the hominin diet have been outlined, to understand how this impacts human health we must consider how these changes affect food intake regulation.

The energy density of food describes the amount of energy per unit mass of food. It is most commonly expressed in the imperial unit of kilocalories per gram (kcal/g); the metric unit is kilojoules per gram (kJ/g). Because the mass of food consumed is a driver of satiety, people tend to eat a relatively consistent weight of food each day. Any mismatch between caloric content and satiety cues can result in meals delivering more energy before termination signals are triggered. As a result, when the energy density of the diet increases, total caloric intake tends to increase as well (Rolls, 2009).

Food composition plays a central role in determining energy density. Water and fibre, both non-caloric components, reduce energy density, whereas fats and sugars increase it. As previously discussed, modern diets tend to be lower in fibre than ancestral diets (Cordain et al., 2005), and ultra-processed, hyperpalatable foods often contain less water. At the same time, fats and sugars have become increasingly prevalent in the food supply,

driving up the energy density of commonly consumed foods (Fazzino et al., 2019; Monteiro et al., 2019).

Among the macronutrients, fat provides the highest caloric content per gram, while sugar is particularly effective at stimulating reward pathways in the central nervous system (CNS). Importantly, the combination of fat and sugar presentation activates these reward systems more strongly than either nutrient alone (DiFeliceantonio et al., 2018). As a result, modern diets are not only more energy-dense, but also more rewarding and less satiating, both of which are factors that together contribute to the consumption of more calories per meal (Small, 2009). This increase in calorie consumption per meal influences obesity risk directly through excessive caloric intake relative to energy expenditure, and also indirectly through various mechanisms such as metabolic disruption, as described later in this introduction.

In addition to the mentioned changes in caloric density and macronutrient profiles of food, the relatively recent addition of synthetic additives also contributes to the mismatch between the modern diet and what we are evolutionarily adapted to consume. Artificial sweeteners which provide sweetness in the absence of calories are one such additive.

1.1.2 Artificial sweetener overconsumption

Artificial sweeteners, such as saccharin, are non-caloric food additives that provide sweetness and enhance palatability without any significant energy contribution. They were first introduced into the diet in the late 19th century and rose in popularity in the second half of the 20th century in response to increasing public health concerns about sugar consumption, obesity, and diabetes. Artificial sweeteners are marketed as a healthier alternative to sugar and are now common across a wide range of easily accessible diet and "sugar-free" products (Sylvetsky & Rother, 2016).

Ironically, although artificial sweeteners are often considered to be tools to reduce caloric intake and combat rising rates of obesity and diabetes, their use has also raised new health concerns. Epidemiological studies report associations between regular artificial sweetener consumption and increased risk of weight gain, metabolic syndrome, and type 2 diabetes (Azad et al., 2017). While the underlying mechanisms remain debated, proposed explanations include gut hormone signalling disruption and gut microbiome alteration (Brown & Rother, 2012; Palmnäs et al., 2014; Swithers & Davidson, 2008). As a result, artificial sweeteners are no longer viewed as universally beneficial and may themselves play a role in diet-induced metabolic dysfunction.

In addition to their potential metabolic effects, artificial sweeteners may also influence health more indirectly through disruption of learned relationships between sweetness and caloric content. Typically, the perception of sweetness is closely linked to the presence of caloric content and reinforces the association between taste and energy. Artificial sweeteners disrupt this association by providing a sweet taste without delivering calories. This may impair accurate prediction of the energetic value of foods. The decoupling of taste from caloric content can interfere with learned satiety signals and promote maladaptive feeding behaviours, particularly when energy-dense foods are readily available. By weakening the association between sweetness and caloric feedback, artificial sweeteners may impair within-meal regulation and disrupt the signals which typically regulate meal termination even in meals without artificial sweeteners consumed after their intake (Davidson & Swithers, 2004; Swithers & Davidson, 2008).

Saccharin is one of the earliest and most widely studied artificial sweeteners and is especially relevant in the context of this thesis. Its palatability to rodents and non-caloric nature make it a useful tool for investigating feeding behaviour and reward-driven intake. Understanding how artificial sweeteners like saccharin affect these systems is essential for developing targeted strategies to address their overconsumption and contribution to wider health issues.

1.1.3 Obesity epidemiology

Obesity is primarily characterised by an excessive accumulation of body fat and is associated with a range of adverse health outcomes. Although simple in appearance, obesity reflects neurochemical disruption in the regulation of energy balance, appetite, and food intake (Schwartz et al., 2017). Obesity prevalence globally has increased dramatically in recent decades (Devajit & Haradhan Kumar, 2023; Jaacks et al., 2019; Popkin & Doak, 1998; World Obesity Federation, 2022). To better understand the scope and nature of this condition, both the population-level rise in obesity and the individual-level features that characterise it must be considered. In this review, particular attention is given to the behavioural and neurobiological components of food consumption that are central to the aims of this thesis.

Obesity is defined as having a body mass index (BMI) greater than or equal to 30 kg/m^2 (Schwartz et al., 2017). While the use of BMI as a metric of health has been criticised, particularly due to its inability to distinguish between lean and fat mass, it remains a widely used indicator of excess adiposity in population health studies. Currently, more than one billion people worldwide are classified as obese, and prevalence rates have increased dramatically in just the past few decades, particularly since the 1980s (Devajit & Haradhan Kumar, 2023; Jaacks et al., 2019; Popkin & Doak, 1998). By 2030, it is projected that one in five women and one in seven men globally will meet the criteria for obesity (World Obesity Federation, 2022).

While no single cause currently explains this rise, changes in energy density, portion size, macronutrient composition, and palatability of foods are commonly regarded as major contributing factors (Blüher, 2019; Schwartz et al., 2017). In particular, the increased intake of highly palatable, energy-dense foods high in sugar and fat is thought to play a central role in driving a chronic positive energy balance and delayed satiety signalling. This, in turn, enables larger meal sizes and subsequent weight gain (Monteiro et al., 2019) (Rolls, 2009).

The relationship between diet and obesity reflects alterations in the brain systems that govern feeding behaviour, including those regulating hunger, satiety, reward, and termination of feeding (Berthoud, 2012; Blüher, 2019). These neurobiological mechanisms, including those influenced by OT and opioid signalling, are a focus of this thesis.

While epidemiology describes the scale and potential origins of the obesity epidemic, its symptomatology offers insight into the mechanisms through which weight gain and dysregulated feeding behaviours develop.

Although obesity is defined by excess body weight or fat mass, it is accompanied by a range of physiological and behavioural changes that extend beyond physical appearance. Metabolically, obesity is commonly associated with insulin resistance, systemic inflammation, and impaired hormonal signalling related to appetite and energy balance. These changes contribute to a higher risk of developing conditions such as type 2 diabetes, cardiovascular disease, and non-alcoholic fatty liver disease (Blüher, 2019; Hotamisligil, 2006; Yki-Järvinen, 2014).

In addition to these metabolic consequences, obesity is also characterised by alterations in the experience of hunger, fullness, and food-related reward. Many individuals with obesity exhibit a blunted satiety response, increased reactivity to food cues, and a strong preference for energy-dense foods high in fat and sugar (Berthoud, 2011; Mela, 2006; Small, 2009). These behaviours suggest a disruption in the internal regulatory mechanisms that typically help balance energy intake with physiological need (Lutter & Nestler, 2009; Volkow & Baler, 2015). This disruption is often characterised by increased portion sizes and delayed meal termination, indicating a shift in the internal thresholds that typically signal satiety. This failure to terminate meals at appropriate points may reflect diminished sensitivity to satiety signals or increased hedonic drive to continue eating (Amin & Mercer, 2016; Cassidy & Tong, 2017; Hall et al., 2014).

Rather than reflecting a simple failure of self-control, these symptoms indicate dysregulation in the systems that govern appetite, motivation, metabolism, and food choice (Lutter & Nestler, 2009; Volkow & Baler, 2015). Understanding the nature of these systems and how they influence food intake behaviour allows for the identification of both solutions to and causes of food intake issues. The OT system and endogenous opioid system are both examples of neurological regulators of these processes.

The first-line treatment for obesity remains lifestyle modification, typically involving changes to diet and increased physical activity. However, for many individuals, lifestyle changes alone are insufficient to produce sustained weight loss. As a result, pharmacological treatments are increasingly used as adjuncts to lifestyle changes, particularly in individuals who are resistant to or have not achieved success with behavioural interventions alone (Bray et al., 2016). One example of an approved pharmacological treatment is Contrave, a combination oral therapy containing naltrexone (NTX) and bupropion, which is used to reduce appetite and support weight loss (New Zealand Formulary, 2025). The clinical availability of such combination therapies highlights the potential utility of targeting multiple regulatory systems involved in food intake simultaneously.

While current pharmacological options remain limited, the broader strategy of combining agents to engage distinct feeding-related pathways represents an area of ongoing development. Understanding how different neuromodulatory systems interact to regulate food intake is therefore critical for informing the design of future combination treatments and is directly relevant to the aims of this thesis.

1.2 Neurobiology of food intake regulation

Food intake is regulated concertedly by homeostatic, autonomic, and reward-based systems distributed throughout the CNS. These systems maintain energy homeostasis and shape feeding behaviours through the integration of internal signals with external sensory and motivational cues. The hypothalamus and brainstem sense energy availability and initiate hunger or satiety responses, while mesolimbic structures such as the nucleus accumbens (NAc) and amygdala regulate the motivational and hedonic aspects of eating, as depicted in Figure 1.1(Berthoud, 2011). These systems work concurrently during feeding to induce meal termination. In this regard, and of importance to this thesis, disruption of or imbalance between these systems can lead to altered meal termination signalling. Understanding how systems such as OT and endogenous opioids contribute to feeding behaviour is therefore essential to identifying mechanisms that underlie dysregulated eating and to developing novel therapeutic strategies.

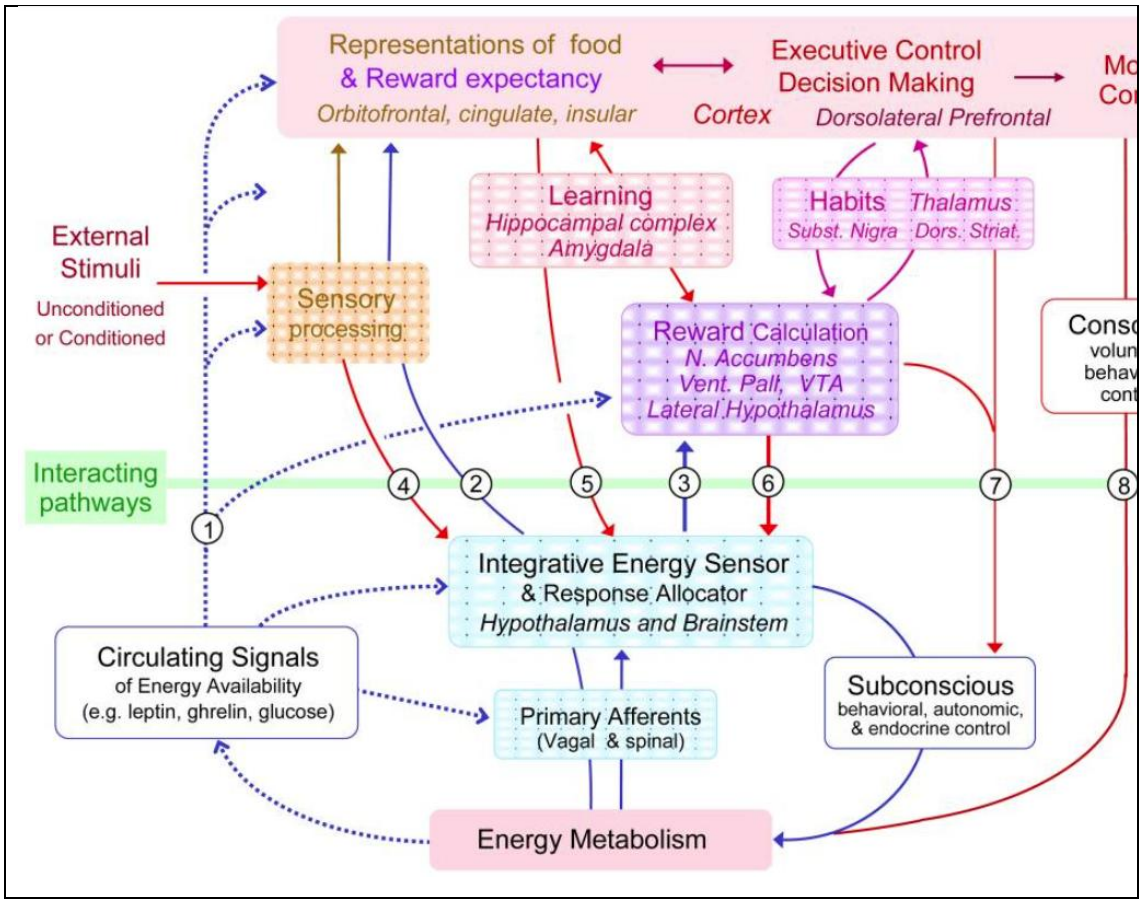

Figure 1.1 Schematic Diagram of interactions between food intake regulatory systems. Homeostatic systems are represented in blue. External sensory systems in yellow, reward processing in purple, and cognitive and executive functions in red, all of which are involved in hedonic food intake regulation. Blue arrows represent the influence of hedonic systems by homeostatic ones, with broken blue lines indicating circulating metabolites, hormones, or other factors being responsible. Solid blue lines represent direct neural pathways. Red arrows

represent the influence of homeostatic systems by hedonic ones. Reproduced with permission from Berthoud 2011(Berthoud, 2011).

Figure 1.1 illustrates that food intake does not arise from a single regulatory pathway, but from reciprocal interactions between circulating metabolic signals, visceral afferent input, hypothalamic and brainstem integration, and higher-order processes involved in sensory evaluation, learning, reward, and executive control. Information about energy availability can influence the perceived value of food and subsequent behavioural decisions, while cognitive and reward-related processes can in turn modify autonomic and homeostatic responses (Berthoud, 2011). This framework provides the basis for the following discussion of homeostatic, autonomic, and reward-driven food intake regulation, with particular attention given to the OT and endogenous opioid systems.

1.2.1 Homeostatic-driven food intake regulation

Homeostatic food intake regulation is the process through which energy balance is maintained by adjusting hunger and satiety in response to internal physiological signals. Unlike reward-driven feeding, which is influenced by palatability and other external cues, homeostatic feeding is primarily driven by the body's energetic needs. In healthy individuals, the process of homeostatic food intake regulation adjusts feeding behaviours to be appropriate to energy expenditure, preventing both starvation and excessive weight gain (Berthoud, 2011; Morton et al., 2014; Saper et al., 2002).

The hypothalamus is the centre for homeostatic food intake regulation. Various circuits both within and influenced by this region respond to signals of energy availability and process adaptive feeding responses to maintain metabolic stability (Berthoud, 2006; Morton et al., 2006; Schwartz et al., 2000). The paraventricular nucleus of the hypothalamus (PVN) is one such hypothalamic region involved in homeostatic feeding regulation. The PVN integrates signals from multiple hypothalamic regions such as the arcuate (ARC), ventromedial (VMH), and dorsomedial (DMH) nuclei, as well as peripheral signals to coordinate energy balance. The PVN is one of two regions in the CNS containing populations of OTergic neurons, with the other being the supraoptic nucleus of the hypothalamus (SON). The general activation of OTergic neurons is strongly linked to the suppression of food intake. Projections of both OTergic and non-OTergic neurons from the PVN extend to both autonomic and neuroendocrine targets, allowing it to influence feeding behaviour, energy expenditure, and gastrointestinal function. Through these outputs, the PVN responds to homeostatic cues and contributes to behavioural and physiological responses that maintain energy homeostasis (Kerem & Lawson, 2021; Romano et al., 2020; Sabatier et al., 2013; Williams et al., 2001). The OT-mediated regulation of food intake is a central topic of this thesis and is covered in greater detail later in this chapter.

The ARC is another hypothalamic subregion involved in homeostatic food intake regulation. This region is particularly important for integrating circulating metabolic signals such as leptin, insulin, ghrelin, and glucose. A classical framework describes two well-characterised neuronal populations with broadly opposing effects on feeding: orexigenic neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), and anorexigenic neurons expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These populations project to the PVN, as well as the lateral hypothalamus (LH) and VMH, where they contribute to the regulation of hunger and satiety (Morton et al., 2006; Schwartz et al., 2000). This framework remains

useful for describing the organisation of ARC feeding circuitry, although it does not encompass the full cellular and functional diversity now recognised within the region (Romano et al., 2020).

Recent studies using cell-type-specific recording and transcriptomic approaches have refined this classical framework. Fibre photometry recordings in awake mice demonstrated that the detection of food rapidly decreased AgRP neuron activity and increased POMC neuron activity before consumption began. These responses varied according to nutritional state and the properties of the food presented, indicating that ARC neurons integrate real-time information about food availability with slower signals of energy status (Chen et al., 2015). In parallel, single-cell RNA sequencing of the adult mouse ARC–median eminence identified multiple transcriptionally distinct AgRP and POMC populations, together with additional neuronal populations potentially involved in energy balance (Campbell et al., 2017). Together, these findings show that AgRP and POMC neurons are not uniform populations responding only to circulating metabolic signals, but are transcriptionally diverse and dynamically regulated in relation to both internal state and food availability.

Homeostatic systems, which normally signal meal termination once energy needs are met, can be disrupted when satiety signalling fails. For example, in models of obesity, desensitisation to the anorexigenic hormones leptin and insulin is common. These hormones are used to communicate the state of energy stores in the body. Leptin resistance, in particular, leads to lower activation of anorexigenic POMC neurons and fails to suppress orexigenic AgRP neurons in the ARC. This impairs hunger-driven meal termination(Timper & Brüning, 2017). In parallel, nutrient-driven neuroinflammation and hormonal dysregulation may also affect PVN function by contributing to altered OT signalling and further disruption of satiety pathways (Thaler et al., 2012). These changes collectively impair the brain's ability to regulate food intake based on internal energy needs, creating a state of homeostatic imbalance that contributes to sustained overeating (Timper & Brüning, 2017).

1.2.2 Autonomic food intake regulation

Similar to how the hypothalamus is the centre of homeostatic food intake regulation, the brainstem is the centre of autonomic food intake regulation. Autonomic regulation of food intake is primarily driven by signals from the gut that communicate information about stomach distension, meal size, and nutrient content. The brainstem is responsible for immediate and short-term control of feeding behaviours such as meal initiation and termination, as well as the mediation of food aversion behaviours (Berthoud & Neuhuber, 2000; Grill & Hayes, 2012).

In the medulla, the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus (DMV) are regions involved in the regulation of food intake. The NTS receives vagal afferent input from the gastrointestinal tract to regulate feeding-related behaviours (Berthoud, 2008; Grill & Hayes, 2009). It works in close association with the neighbouring AP which lies outside of the blood-brain-barrier (BBB) and detects circulating signals, and the DMV, which influences autonomic output to the gut. Together, these nuclei coordinate satiety signalling, gastric motility, and digestive function on an autonomic basis and communicate this information to other food intake-related brain regions such as the hypothalamus (Rinaman, 2010).

These brainstem circuits are influenced by neurochemical signals that convey the physiological state of the gut during and after a meal. Some examples of signalling compounds include cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), and ghrelin. These signals act either via the vagus nerve or directly on the brainstem, including in the NTS and AP. CCK promotes meal termination by activating vagal afferents that project to the NTS in response to fat and protein ingestion. GLP-1 and PYY are secreted following food intake and similarly signal satiety through neural and endocrine pathways. Ghrelin, however, is secreted primarily by the stomach during fasting and stimulates feeding by acting on both the hypothalamus and brainstem. These hormones interact with the brainstem to contribute to the regulation of meal duration and frequency of feeding through interpretation of real-time information about meal composition and size (Berthoud, 2008; Cummings & Overduin, 2007). However, in the presence of highly palatable or energy-dense foods, this regulatory signalling may be overridden, resulting in impaired early satiation responses and excessive food intake (Sclafani, 2013).

1.2.3 Reward-driven food intake regulation

While homeostatic and autonomic systems regulate food intake based on internal energy needs and gastrointestinal feedback, feeding behaviour is also strongly influenced by reward. These systems are particularly relevant in environments where palatable, energy-dense foods are available, as they can drive consumption beyond metabolic requirements (Berthoud, 2011). Reward-driven food intake is governed primarily by the mesolimbic dopamine system, which evaluates the salience and hedonic value of food-related stimuli and promotes motivated behaviours such as food seeking, preference formation, and overconsumption (Volkow & Baler, 2015; Volkow & Wise, 2005). Key regions involved in this system are the NAc, ventral tegmental area (VTA), and amygdala. These regions process the reinforcing properties of food and interact with homeostatic centres to shape feeding decisions (Kenny, 2011).

The NAc is responsible for mediating the motivational aspects of food intake. This region receives both dopaminergic input from the VTA and glutamatergic input from regions such as the prefrontal cortex, amygdala, and hippocampus. These inputs influence reward-related processing based on salience signalling from the other regions, as outlined in Figure 1.2 (Russo & Nestler, 2013). In the context of feeding, NAc activation is linked to increased approach behaviour toward palatable foods and greater responsiveness to food-associated cues (Caref & Nicola, 2018; Kenny, 2011). This region also receives some input from the hypothalamus, allowing for some homeostatic influence on reward-driven motivation (Kerem & Lawson, 2021). Dysregulation of hedonic signalling in the NAc may contribute to compulsive eating and sustained overconsumption of food, particularly in the presence of highly palatable foods (Kenny, 2011; Volkow et al., 2013).

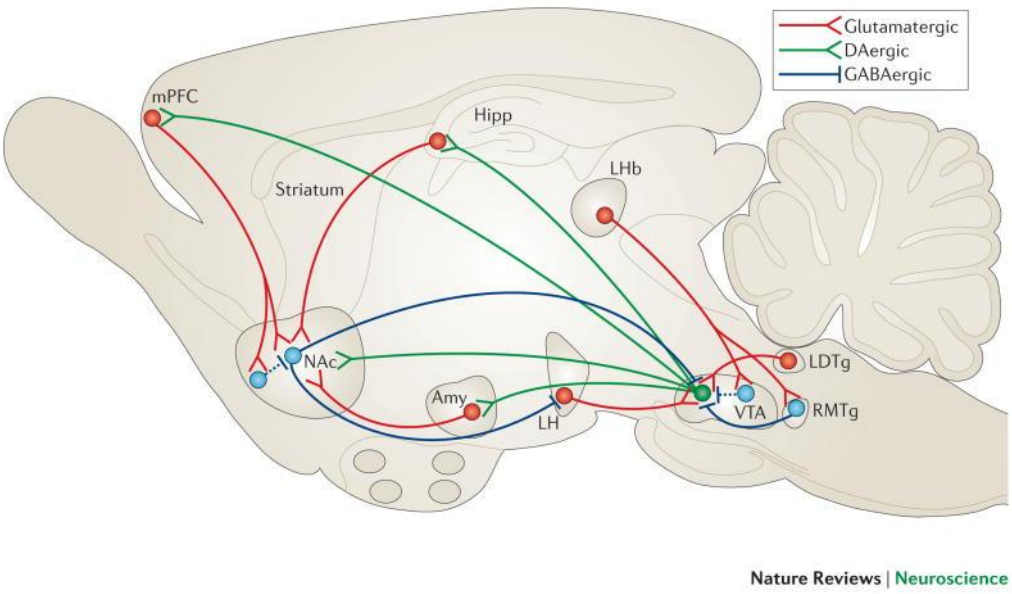

Figure 1.2 A simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the ventral tegmental area (VTA) and nucleus accumbens (NAc) (dopaminergic=green; glutamatergic=red; and GABAergic=blue) in the rodent brain. (Reproduced with permission from Russo and Nestler 2014 (Russo & Nestler, 2013))

The amygdala processes the emotional and motivational salience of food-related cues, which in turn guide responses to palatable and rewarding foods. It encodes the emotional valence of food, assigning emotional meaning to specific flavours, textures, and contexts (Baxter & Murray, 2002; Janak & Tye, 2015). This hedonic evaluation influences the subjective experience of pleasure during consumption. The basolateral amygdala (BLA) processes sensory and contextual information with reward signals, and projects the central amygdala (CeA) and downstream structures such as the NAc (Stuber et al., 2011). The CeA regulates behavioural responses to food cues by enhancing consumption through hedonic and learned associations (Douglass et al., 2017). Both the BLA and CeA are connected to hypothalamic nuclei, enabling interaction between hedonic and homeostatic systems (Petrovich, 2011). Under conditions of heightened palatability or emotional arousal, enhanced amygdala signalling may bias behaviour toward overconsumption, even in the absence of caloric need.

The VTA is a midbrain structure that serves as the primary source of dopaminergic input to other components of the reward system, including the NAc, prefrontal cortex, and amygdala (Morales & Margolis, 2017). VTA dopamine neurons encode reward prediction and motivational salience, firing in response to both unexpected rewards and cues that predict reward availability (Schultz, 1998). The VTA reinforces food-seeking behaviour and strengthens learned associations between palatable foods and environmental cues (Volkow et al., 2011). These dopaminergic signals shape decision-making and contribute

to the persistence of food-motivated behaviour, even in the absence of homeostatic need. VTA hyper-responsivity to palatable food cues may contribute to heightened craving, compulsive consumption, and difficulty disengaging from food-related stimuli (Kenny, 2011).

1.3 Oxytocin

While food intake is regulated by a range of neural circuits, the function of these regions is critically shaped by neuroregulatory signalling. This thesis focuses on two signalling systems that influence feeding behaviour across multiple brain regions: oxytocin (OT) and the opioid system. The following section introduces OT, which is a highly evolutionarily conserved neuropeptide involved in the regulation of food intake.

1.3.1 Structure, genetics and history of oxytocin

The uterine-stimulating properties of posterior pituitary extracts were described by Dale in 1906, although OT was not isolated and chemically characterised until several decades later (Dale, 1906; Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018). From this effect, the name "oxytocin" was derived: "oxy" from the Greek oxus (sharp or sudden) and "tocin" from tokos (childbirth) (Howell, 1898) (Dale, 1906) (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018). These early pituitary extracts are now known to contain both OT and arginine-vasopressin and to have induced uterine contractions, raised blood pressure, and stimulated milk ejection. In the mid-20th century, OT was the first biologically active peptide hormone to be chemically synthesised. This synthesis earned Vincent du Vigneaud the 1955 Nobel Prize in Chemistry (du Vigneaud et al., 1954; Jurek & Neumann, 2018). Since then, the understanding of OT has expanded substantially.

OT is a neuropeptide synthesised by neurons in the PVN and SON of the hypothalamus, many of which project to the posterior pituitary to mediate peripheral hormone release as part of the hypothalamic–pituitary axis (Brown et al., 2020; Gimpl & Fahrenholz, 2001). Pituitary hormones are divided into two groups according to their structure and function: the arginine-vasopressin-like group and the OT-like group (Grigor'eva & Golubeva, 2010). These two hormones are structurally very similar and could not be isolated in much of the early work on pituitary hormones (du Vigneaud et al., 1954). Both are nonapeptides composed of a six-amino-acid ring closed by a disulfide bond, with a three-amino-acid long side chain. These two molecules differ only by which amino acids are present in positions 3 and 8 (Figure 1.3) (Baribeau & Anagnostou, 2015; Bordt et al., 2019; Grigor'eva & Golubeva, 2010).

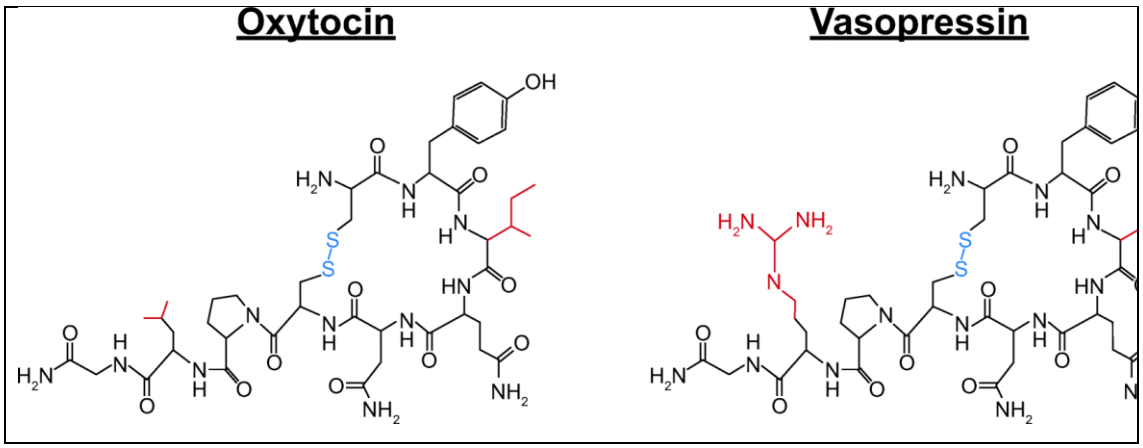

Figure 1.3 Structure of Oxytocin and Vasopressin. Disulfide ring closure bonds are highlighted in blue, the two amino acids which differentiate oxytocin and vasopressin are highlighted in red. (reproduced with permission from(Bordt et al., 2019)).)

Due to its relatively large structure and hydrophilicity, OT crosses the BBB only to a very limited extent through passive mechanisms (Higashida et al., 2022; Wu et al., 2023). Early work investigating the permeability of the BBB to OT found that only ~0.002% of intravenously (*i.v)* administered OT reaches the cerebrospinal fluid (CSF) within 10 minutes of administration in rats. For this reason, it was initially considered that peripheral administration of OT would be ineffective for modulating the CNS (Mens et al., 1983). However, later work found that peripheral administration can increase measured extracellular OT concentrations within the brain over longer time frames. For example, intraperitoneal (*i.p)* administration of OT in mice substantially increases OT concentrations in dialysates of various brain regions in mice 30 minutes after administration (Neumann et al., 2013). *i.p* administration of OT is now a common method of delivery in *in vivo* animal model research. While peripheral OT administration is effective, the mechanisms through which it produces central and behavioural effects remain incompletely understood.

However, direct transport of OT across the BBB is not the only mechanism through which peripheral administration may produce central and behavioural effects. i.p. OT has been shown to activate OT-responsive vagal afferent neurons and increase c-Fos IR in the NTS, with both NTS activation and suppression of food intake attenuated by subdiaphragmatic vagotomy or chemical disruption of sensory afferents (Iwasaki et al., 2015). Follow-up work found that i.p. OT also increased c-Fos expression in the PVN, predominantly in OTergic neurons, and that both this response and the associated reduction in food intake were attenuated following vagotomy. The anorexigenic effect was also reduced in mice lacking the OT gene and following central OTR antagonism, suggesting that peripheral OT can recruit endogenous central OT signalling through a vagal relay (Iwasaki et al.,

2019). Consistent with this, a peripherally restricted OT conjugate retained the ability to suppress food intake and food-motivated behaviour despite limited CNS penetrance (Asker et al., 2023). Together, these findings indicate that peripheral OT may act through both direct blood-to-brain transport and indirect peripheral-to-central neural pathways. These mechanisms are not mutually exclusive, and their relative contributions may vary according to the route and dose of administration, time course, and behavioural outcome. There are two methods of peripheral OT delivery which are frequently used in research: blood-to-brain delivery methods such as *i.p.* or *i.v.* injections, or intranasal administration. Uptake of OT from the bloodstream appears to occur in a largely unidirectional manner towards the CNS via brain capillary endothelial cells through the receptor for advanced glycation end-products (RAGE). Knockouts of *Ager*, the mouse gene for RAGE, demonstrate impaired uptake of peripheral OT into the CNS and display behavioural abnormalities consistent with disruption of the OT system (Higashida et al., 2022; Yamamoto et al., 2019). Intranasal administration has been shown to increase OT concentrations in behaviourally relevant brain regions, both when administered alone and when co-administered with nasal vasoconstrictors. Again, the exact nature of this transport remains unknown. This intranasal efficacy is likely due to the convergence of multiple uptake mechanisms involving both nose-to-brain and nose-to-blood-to brain routes (Yao et al., 2023). Intranasal administration is common in human trials as it is non-invasive and easily self-administered.

The *OXT* gene, located on chromosome 20p13, spanning approximately 800 bp, and composed of 3 exons and 2 introns, encodes OT in humans. The first of these exons encodes the nonapeptide itself, the translocation signal peptide sequence (GKR), a tripeptide processing signal, and the first nine amino acids of neurophysin I. The second exon encodes the majority of neurophysin I carrier protein, while the third exon encodes its C-terminal region (Figure 1.4) (Gimpl & Fahrenholz, 2001; National Center for Biotechnology Information (NCBI), 2025a).

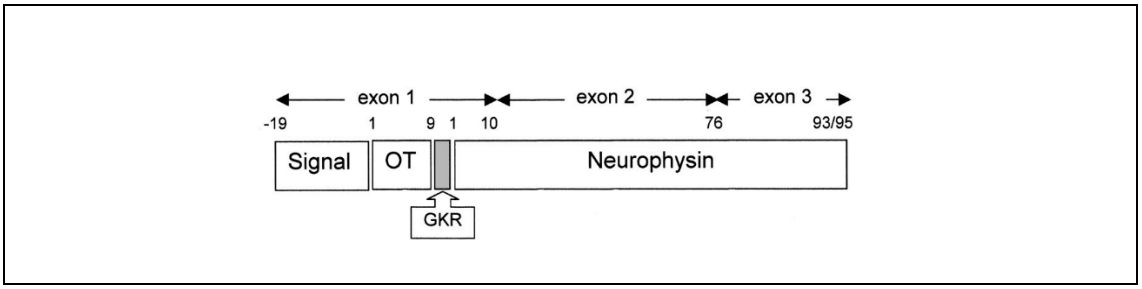

Figure 1.4 Domain organisation of prepro-oxytocin-neurophysin I including the processing sites. (Reproduced with permission from Gimpl and Farnholz, 2001(Gimpl & Fahrenholz, 2001))

OT is first produced in an inactive form and requires post-translational processing before it reaches its final active form. Translation of the *OXT* gene yields a single large inactive precursor protein called prepro-oxytocin-neurophysin I. This precursor protein contains a signal peptide, the OT nonapeptide sequence, and the carrier protein neurophysin I. Within the endoplasmic reticulum, the signal peptide is cleaved, producing pro-oxytocin-neurophysin I. As the protein progresses through the secretory pathway, further structural modifications occur. A disulfide bond forms between cysteine residues at positions 1 and 6 of the OT sequence, and the peptide is cleaved from neurophysin I (Brakch et al., 1989; Landgraf & Neumann, 2004). Finally, the OT precursor undergoes C-terminal α-amidation by peptidylglycine α-amidating monooxygenase to generate the mature form of the hormone. OT is then stored alongside neurophysin I in vesicles before being released into the circulation or into other brain regions (Florea et al., 2022; Yonekura et al., 2023).

OT and arginine-vasopressin, along with their homologues, are present in all vertebrate species. Arginine-vasotocin is considered the most probable ancestral peptide and is expressed in all vertebrates except mammals. This hormone combines the functional properties of both OT and arginine-vasopressin-like proteins. From vasotocin, two distinct lineages of separate hormones emerge: the isotocin-mesotocin-OT line which is associated with social and reproductive functions, and the vasotocin-vasopressin line which is primarily involved in water homeostasis (Gimpl & Fahrenholz, 2001; Grigor'eva & Golubeva, 2010).

Mesotocin is the OT-like peptide found in the majority of non-mammalian terrestrial vertebrates, while isotocin is found in the majority of bony fish. Humans and other placental mammals, by contrast, express OT, not mesotocin or isotocin. These peptides are all nonapeptides and differ by only one or two amino acids, reflecting their high degree of sequence conservation and shared evolutionary origin. The broad conservation of the presence and sequence similarity of OT and OT homologues across species is indicative of the fundamental biological roles these hormones play.(Gimpl & Fahrenholz, 2001; Grigor'eva & Golubeva, 2010).

1.3.2 Non-food intake related roles of oxytocin

In addition to its role in feeding regulation, OT is primarily known for its involvement in other physiological and behavioural functions, most notably in social bonding and lactation. In this section, the role of OT in these functions is briefly described.

OT is colloquially referred to as the "love hormone". This moniker is in many regards well earned. Social cognition and behaviour encompass a broad set of processes and are inherent to and necessary for the functioning of human society; OT is critical in the regulation and mediation of social cognition and behaviour (Sue Carter, 1998). OT is involved in many beneficial social behaviours such as enabling animals to overcome their natural avoidance of close proximity to others, and inhibiting defensive social behaviour (Heinrichs et al., 2009). Although social and affiliative behaviours are highly complex, OT is understood to be critical for these processes (Sue Carter, 1998). Intranasal administration of OT has been shown to increase interpersonal trust among humans, with trust being an essential prerequisite of social affiliation. This same administration of intranasal OT did not increase the general willingness to bear risk, but did increase willingness to bear social risks through interpersonal interactions (Heinrichs et al., 2009; Kosfeld et al., 2005). Intranasal OT also enhances social cognition, including the ability to recognise emotional states from facial expressions (Domes et al., 2007).

The link between OT and social behaviour and cognition can be seen further when considering autism spectrum disorders (ASD). Aberrant social behaviour is a characteristic trait of ASD; this includes but is not limited to altered social cognition, social withdrawal, and atypical communication (American Psychiatric Association, 2013; Heinrichs et al., 2009; Young & Barrett, 2015). Individuals with ASD have been shown to have improved social cognition when assigning emotional significance to speech intonation following i.v. infusion of OT (Hollander et al., 2007).

Milk ejection is a neuroendocrine reflex primarily driven by OT and represents another non-feeding-related function of this hormone. Milk ejection is most commonly initiated by mechanical stimulation of pressure-sensitive receptors in the dermis of the areolae, nipple, or teat. Activation of these receptors generates nerve impulses which travel to the PVN and SON of the hypothalamus, which in turn initiate the release of OT from the posterior pituitary into the bloodstream. Once in circulation, OT travels to the mammary glands and binds to receptors on myoepithelial cells. This binding triggers contraction of the myoepithelium surrounding the alveoli and ducts, raising intra-alveolar pressure and initiating milk ejection into the larger ducts (Goodman & Grosvenor, 1983; Moberg & Prime, 2013; Yukinaga & Miyamichi, 2025).

1.3.3 Distribution of oxytocin and oxytocin receptors

OT and its receptor (OTR) are distributed broadly across the CNS, particularly in regions involved in feeding regulation, stress, and social behaviour. In this section, the distribution of OTergic neurons and OTR expression in the brain is discussed. Particular focus is given to areas implicated in food intake. Peripheral expression of OT and its receptor is also briefly considered.

Understanding the distribution of OTergic neurons in the CNS provides anatomic context and insight into the role that OT plays in the CNS. OT is produced in the hypothalamus, a region strongly involved in homeostatic regulation of food intake. OTergic neurons are classified into two types: magnocellular and parvocellular. This classification is based on their size, location, and projection targets.

Magnocellular neurons are responsible for the majority of OT production and are located in both the SON and PVN. These neurons project to the posterior pituitary, where they release OT into circulation to reach peripheral targets, as shown in Figure 1.5 (Jurek & Neumann, 2018; Kerem & Lawson, 2021; Sabatier et al., 2013). This action is responsible for OT release during the milk ejection reflex.

In addition to peripheral OT delivery, magnocellular neurons of the PVN and SON are highly dendritic. In addition to neurohypophysial and axonal release, magnocellular neurons release OT from their somata and dendrites within the PVN and SON. Somatodendritic OT release can be regulated independently of secretion from posterior-pituitary terminals and may influence neighbouring hypothalamic neurons through local or volume transmission. This allows selective targeting of both central and peripheral sites (Ludwig & Leng, 2006; Sabatier et al., 2003; Sabatier et al., 2013) (Kerem & Lawson, 2021).

Alongside pituitary and dendritic OT release, a subset of magnocellular neurons has recently been identified that project OT beyond the hypothalamus to other central regions such as the amygdala, caudate putamen, lateral septum, and NAc. Notably, these neurons do not project to the VTA or NTS. These regions are instead targeted by parvocellular neurons (Kerem & Lawson, 2021; Zhang et al., 2021).

Parvocellular OTergic neurons are generally described as being located within the PVN and project to central targets including the NTS, VTA, amygdala, and NAc (Kerem & Lawson, 2021; Sabatier et al., 2013; Sofroniew, 1980). OTergic projections from both the PVN and SON to the ARC have also been reported, as summarised in Figure 1.5. Although projections of this type are commonly associated with parvocellular neurons,

parvocellular OT neurons are not typically identified within the SON. The cellular origin of the SON-to-ARC projection therefore remains unresolved (Maejima et al., 2014)..

Although the magnocellular–parvocellular distinction provides a useful framework for describing OTergic projection patterns, recent cell-specific work has identified further diversity among PVN OT neurons. By combining genetic labelling, electrophysiological recording, morphological reconstruction, and unsupervised clustering, Chen et al. identified at least six morpho-electrical subtypes of PVN OT neurons. Of these, only one subtype, located in the posterior PVN and displaying both magnocellular and parvocellular characteristics, showed altered neuronal activity across feeding conditions. These findings suggest that feeding-related responses within the OT system may involve particular PVN OTergic subpopulations rather than uniform recruitment of either the magnocellular or parvocellular population (Chen et al., 2022).

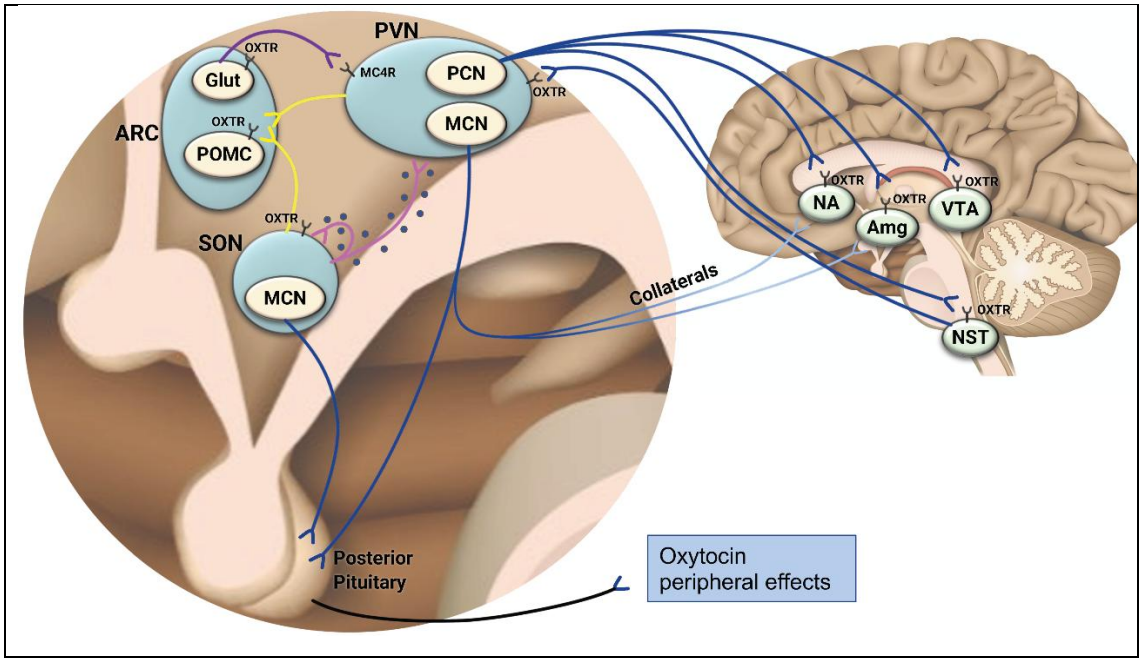

Figure 1.5 Neural circuitry of OT in the CNS. Blue lines represent parvocellular or magnocellular OTergic projections from the PVN and SON. Yellow lines represent OTergic projections from the PVN and SON towards the ARC. The Pink line represents somato-dendritic release of OT from magnocellular neurons. The purple line represents MC4R and OXTR expressing neurons from the ARC which project to the PVN. (Reproduced from Kerem and Lawson. 2021(Kerem & Lawson, 2021))

Similar to the anatomic context provided by understanding the distribution of OTergic neurons, understanding the function and distribution of OT receptors in the CNS offers additional insight into the systems influenced by OT.

The OT receptor is a rhodopsin-like (class A/1) G-protein-coupled receptor (GPCR). A schematic model of the OT receptor can be seen in Figure 1.6. OT receptors are coupled

to the $G_{q/11}\alpha$ pathway, which leads to the generation of inositol triphosphate and 1,2-diacylglycerol. 1,2-diacylglycerol stimulates protein kinase C, which sets off phosphorylation cascades to other proteins. Inositol triphosphate, however, triggers the release of Ca^{2+} ions from intracellular stores. This cytoplasmic Ca^{2+} elevation regulates neuronal excitability, enhances neurotransmitter release, and influences gene transcription. In the periphery, increases in cytoplasmic Ca^{2+} can initiate smooth muscle contraction, contributing to the uterine-stimulating effect that OT is known for (Gimpl & Fahrenholz, 2001; Jurek & Neumann, 2018; Vrachnis et al., 2011).

Due to the high structural similarity between OT and arginine-vasopressin, their receptors display considerable pharmacological overlap. Arginine-vasopressin is at least a partial agonist of the OT receptor. The binding affinity of OT receptors for OT is only approximately 10-fold higher than it is for arginine-vasopressin. The structural similarity and relationship of these two neuropeptides have already been described in this thesis. However, it is worth noting that these similarities extend to receptor activation. Furthermore, arginine-vasopressin becomes a full agonist of the OT receptor with substitution of only two amino acids present at equivalent positions on the arginine-vasopressin receptor (Chini et al., 1996; Gimpl & Fahrenholz, 2001; Kimura et al., 1994).

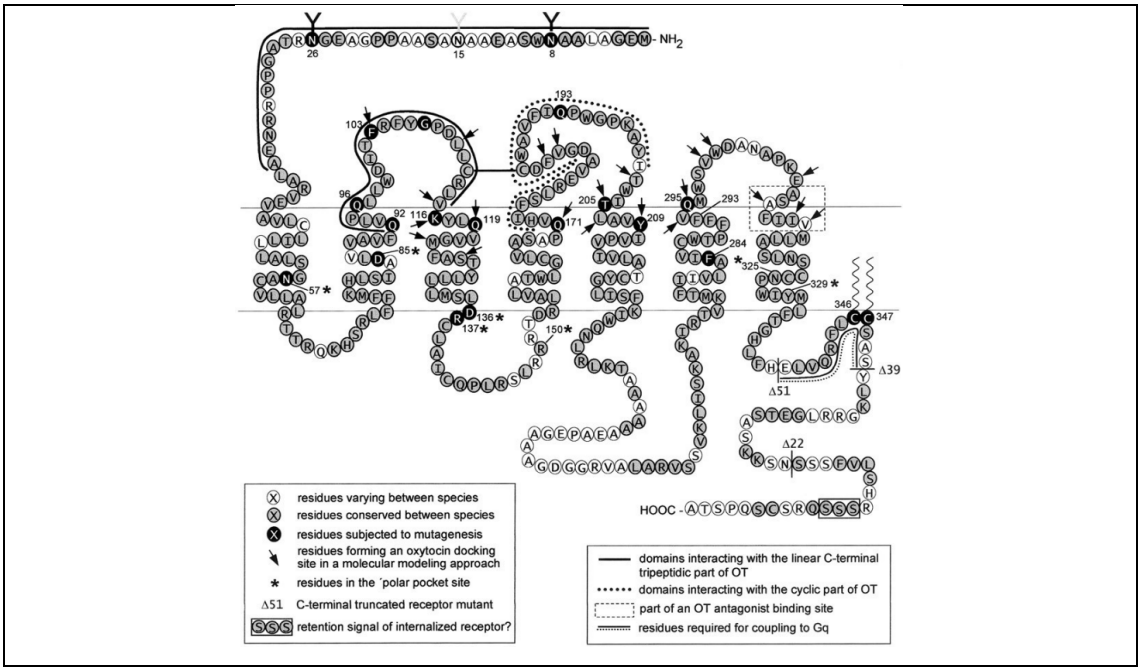

Figure 1.6 Schematic model of the human OT receptor. (Reproduced with permission from Gimpl and Fahrenholz, 2001 (Gimpl & Fahrenholz, 2001))

OT receptors are broadly expressed throughout the CNS; however, their distribution is not ubiquitous. Rather, it is restricted to areas implicated in the functional roles of OT, particularly those related to feeding and social behaviour. The distribution of the OTR can be inferred by spatially mapping OXTR mRNA expression. Seminal studies mapping

OT binding and OXTR mRNA expression have identified receptor presence in the olfactory system, cortex, basal ganglia, limbic system, thalamus, hypothalamus, brainstem, and pituitary gland. Of particular relevance to this thesis is the presence of OT receptors in structures involved in homeostatic and reward regulation such as the hypothalamus, amygdala, and NAc (Gimpl & Fahrenholz, 2001; TRIBOLLET et al., 1992).

In the hypothalamus, OT receptors can be found in both the PVN and SON, the sites of OT production. The presence of OT receptors in these regions suggests the presence of local autoregulatory feedback. OT receptors are also found in the ARC, a centre for coordination of energy homeostasis (Gimpl & Fahrenholz, 2001; TRIBOLLET et al., 1992). This region contains two functionally opposing neuronal populations: orexigenic NPY/AgRP-expressing neurons, and anorexigenic POMC/CART-expressing neurons. OT receptors are also expressed within the ARC, including on a population of POMC neurons. Intra-ARC administration of OT reduces food intake, while OT application increases intracellular Ca2+ in isolated POMC neurons, supporting a direct excitatory effect on this population (Maejima et al., 2014). Additional OT receptors have also been identified in the VMH, another region related to satiety signalling (Gimpl & Fahrenholz, 2001; Morton et al., 2006; Schwartz et al., 2000; TRIBOLLET et al., 1992). Various other hypothalamic regions also express OT receptors; however, the regions discussed have been highlighted in particular for their significant roles in feeding regulation.

In the amygdala, OT receptors and OXTR mRNA have been identified in several nuclei, most notably the CeA and BLA (Gimpl & Fahrenholz, 2001; TRIBOLLET et al., 1992). Both of these regions are involved in regulating feeding behaviour through the coordination of emotional salience and learned associations related to food (Janak & Tye, 2015; Stuber et al., 2011). Activation of OT receptors in both regions has been shown to reduce standard chow consumption in rats, and activation of OT receptors in the BLA has been shown to suppress consumption of palatable chow (Klockars et al., 2018).

OT receptors have also been identified in the NAc (Gimpl & Fahrenholz, 2001). Traditionally, the interaction between OT and the NAc has been viewed as indirect, with OTergic neurons from the hypothalamus enhancing the activity of dopaminergic mesolimbic neurons in the VTA that, in turn, project to the NAc (Sabatier et al., 2013). However, recent findings described earlier in this section revealed a subset of magnocellular OT neurons with direct projections to the NAc (Kerem & Lawson, 2021; Zhang et al., 2021).

Beyond the CNS, OT is also involved in a range of physiological processes in the periphery. For example, OT plays a major role in the female reproductive system. As previously mentioned, the uterotonic effects of OT were recognised before the hormone was chemically isolated and identified. Activation of OT receptors present in the myometrium results in contractions of uterine smooth muscle cells. During pregnancy, elevated oestrogen levels upregulate OTR expression, resulting in up to a 100-fold increase in receptor concentrations at term (FUCHS et al., 1983; Uvnäs-Moberg, 2024). OTR antagonists have been investigated as tocolytic agents for the prevention of preterm birth, but they have generally proven ineffective and have sometimes been associated with adverse effects (Flenady et al., 2014). Additionally, OT and OTR mRNA are both expressed in human cumulus cells surrounding oocytes, and this expression may contribute to fertilisation and early embryonic development (Gimpl & Fahrenholz, 2001). OT also plays a role in the male reproductive system. It is produced by both Leydig and Sertoli cells and is involved in testicular and prostate function (Gimpl & Fahrenholz, 2001; Stadler et al., 2020).

The central role of OT in the milk ejection reflex has been described earlier in Section 0. To elaborate on the peripheral component of this system, OT receptors are expressed in the myoepithelial cells of the mammary glands. Activation of these receptors by OT released from the posterior pituitary into the circulation triggers milk ejection(Goodman & Grosvenor, 1983; Grigor'eva & Golubeva, 2010).

While arginine-vasopressin plays a more prominent role in the kidneys, OT also contributes to renal function. OT has been shown to exert natriuretic and kaliuretic effects, consistent with the presence of OT receptors in the kidneys (Gimpl & Fahrenholz, 2001; Haanwinckel et al., 1995; Japundžić-Žigon et al., 2020). Beyond the reproductive system, mammary glands, and kidneys, OT or its receptors have also been identified in the pancreas, adrenal glands, and adipose tissue (Gimpl & Fahrenholz, 2001).

1.3.4 Regulation of food intake by oxytocin

Beyond its established roles in reproduction and social behaviour, and of primary interest to this thesis, OT plays a critical role in the regulation of food intake. Studies across a range of species and experimental models have shown that OT exerts an anorexigenic effect and can reduce food intake under a variety of experimental conditions (Arletti et al., 1989; Arletti et al., 1990; Jonaidi et al., 2003; Olson et al., 1991) (Morton et al., 2012) (Mullis et al., 2013) (Klockars et al., 2018) (O. A. Klockars et al., 2017) (Liyanagamage et al., 2023) (A. Klockars et al., 2017). These effects are mediated through both energy-driven and reward-related circuits, located across various brain regions including the hypothalamus, NAc, and brainstem (reviewed in (Kerem & Lawson, 2021; Olszewski et al., 2022; Sabatier et al., 2013)). This section summarises our current understanding of OT's role in the regulation of energy intake. The discovery of OT as a hypophagic agent is described, followed by an expansion on the seemingly macronutrient selective nature of this effect. The regulation of homeostatic- and hedonic-driven feeding by OT is then discussed.

The anorexigenic properties of OT were first described in the late 1980s by Arletti and colleagues *(Arletti et al., 1989; Arletti et al., 1990)*. In their initial study, they reported that both i.p. and intracerebroventricular (i.c.v.) administration of OT in rats reduced food intake, shortened meal duration, and increased the latency to initiate feeding. Pre-treatment with an OT receptor antagonist via i.c.v. infusion blocked these effects (Arletti et al., 1989). This study was followed by work exploring OT's effects on both food and fluid intake under varying conditions, including scheduled versus ad libitum access, and food- or water-deprived versus non-deprived states. These findings further identified OT as a broad regulator of ingestive behaviours, suppressing both food and water intake across various experimental paradigms (Arletti et al., 1990). Since these original discoveries, interest in and knowledge of the anorexigenic properties of OT has grown substantially, particularly in the context of its potential translational relevance to human feeding behaviour. In the early 2010s, the first human studies examining the role of OT in food intake and satiety began to emerge. Borg and colleagues (Borg et al., 2011) were the first to experimentally investigate the effects of OT on feeding-related processes in humans. In this work, they delivered i.v. OT and observed decreased subjective satiety ratings without altering nutrient intake or gastric emptying. While these findings contrasted with earlier preclinical work and have been challenged by subsequent studies showing that OT is anorexigenic in humans(Lawson et al., 2015; Ott et al., 2013), this marked the first use of OT in humans in the context of food intake regulation. Following

this, human studies were carried out a year later by two other groups: Zhang and colleagues and Ott and colleagues. Ott *et al.* found that intranasal OT administration reduced reward-driven snack intake in healthy young men who were already in states of satiety (Ott et al., 2013). Zhang et al. reported that eight weeks of intranasal OT treatment reduced body weight in a small group of adults with obesity. In parallel mouse experiments, OT and two related analogues improved insulin resistance and glucose tolerance (Zhang et al., 2013). Alongside these clinical studies, later preclinical work has further demonstrated that peripheral OT can suppress food intake, although this effect appears to depend on the food presented and the experimental conditions. Acute i.p. OT reduced food intake in rats maintained on both low- and high-fat diets (Morton et al., 2012), whereas i.v. OT reduced deprivation-induced chow intake but did not alter the consumption of sucrose, saccharin, or Intralipid solutions (A. Klockars et al., 2017). These findings support an anorexigenic effect of peripheral OT while indicating that its efficacy is not uniform across feeding paradigms. Consistent with the mechanisms discussed earlier, this effect can involve vagal afferent signalling and may persist even when OT is largely restricted to the periphery (Asker et al., 2023; Iwasaki et al., 2015). Research in both preclinical and clinical settings continues to explore OT's potential as an anorexigenic treatment. These efforts are particularly focused on its application in obesity and other feeding-related disorders. However, several challenges must be addressed before the use of OT as a treatment for obesity. These include the current lack of data on the long-term safety and efficacy of OT treatment, as well as limited understanding of its effects on reward-based eating behaviours in females (Olszewski et al., 2022).

By the mid-2000s, the early foundational work by Arletti and other pioneers had been significantly expanded. A pivotal 2005 study by Amico et al. began a shift in how OT's anorexigenic effects were understood. They investigated sucrose solution intake in mice lacking the gene for OT (OTKO mice). OTKO mice consumed more sucrose solution than their wild type (WT) counterparts on first exposure and continued to do so over time. These findings led the authors to suggest that central OT pathways play a role in limiting the intake of sweet, highly palatable sucrose solutions (Amico et al., 2005). Two follow-up studies explored whether OT's effects extended to other carbohydrate solutions, both sweet and non-sweet. They investigated whether these effects applied to fat-rich solutions. Sclafani et al. examined the intake of carbohydrate and fat solutions by OTKO mice, along with behavioural responses such as motivation to obtain these nutrients. Using a progressive ratio operant licking procedure, they observed no difference in motivational

drive for sucrose between OTKO and WT mice. This suggests that motivation and intake may be differentially affected by OT signalling. In two-bottle choice tests, OTKO mice consumed more of both sweet and non-sweet carbohydrate solutions than WT mice, with the strongest effects seen for sucrose solution. Notably, there was no difference in the consumption of a fatty liquid emulsion between OTKO and WT mice (Sclafani et al., 2007). Miedlar et al. tested the consumption of both sucrose and Intralipid, a soy-based emulsion of lipids, by OTKO mice. Aside from an initial spike in intake during first exposure, OTKO mice consumed intralipid at levels comparable to WT controls. However, the same OTKO mice consumed significantly more sucrose solution than WT controls (Miedlar et al., 2007). From this work, the theory emerged that OT functions as a carbohydrate-selective inhibitor of food intake. However, this model was based almost entirely on studies using OTKO mice and was not consistently supported by findings from OT receptor knockout (OTRKO) models (Lee et al., 2008).

At this point, Olszewski and colleagues offered new evidence supporting OT's carbohydrate-selective anorexigenic effects using non-transgenic C57BL/6 mice. Their work found that sucrose exposure induced a two-fold greater activation of OT neurons in the PVN compared to intralipid exposure. Sucrose intake was also associated with a greater increase in OT mRNA expression in the hypothalamus. Furthermore, administration of L-368,899, an OT receptor antagonist, increased sucrose intake but had no effect on Intralipid consumption. It also altered neuronal activity in regions involved in satiety, reward, and aversion (Olszewski et al., 2010). These findings reinforced the idea that OT does not reliably inhibit the intake of lipid emulsions, even in the absence of knockout-dependent models. However, the notion that OT does not inhibit fat consumption is largely based on studies using Intralipid as a representative high-fat liquid diet(Liyanagamage et al., 2023; Miedlar et al., 2007; Olszewski et al., 2010; Sclafani et al., 2007). The suitability of using Intralipid as a high-fat liquid diet in animal studies has already been questioned, particularly due to its atypical gastric emptying kinetics relative to solid, fat-rich foods (Friedman et al., 1996). This limitation is addressed directly in the present thesis.

While the macronutrient-specific nature of OT as an anorexigenic agent has been explored, it is only one of the avenues of investigation into OT's role in feeding regulation. A separate but overlapping area of research explores whether OT acts through homeostatic or hedonic regulatory mechanisms for feeding. It remains uncertain which of these pathways is the primary driver of OT's effects, or if a combination of both contributes to its action. These perspectives on homeostatic and hedonic control of

feeding are not mutually exclusive and are possibly influenced by broader contextual conditions. Both potential mechanisms and the supporting evidence are explored below, beginning with homeostatic regulation of food intake.

As previously described, the hypothalamus is central to homeostatic food intake regulation. This is consistent with the principal populations of OT-producing neurons in the CNS being located within the hypothalamus, particularly in the PVN and SON(Chen et al., 2022; Zhang et al., 2021). The PVN is well known for its role in homeostatic food intake regulation. Some of the earliest indirect evidence supporting OT as being involved with satiety comes from studies investigating the effects of PVN ablation. Ablation of the PVN led to increased meal size in rats, which, despite being very early in this field, was interpreted as being potentially caused by satiety signalling disruption (Leibowitz et al., 1981). Neuronal activity across the hypothalamus, including in the PVN and, notably, OTergic neurons in the SON, have been shown to increase over the course of a meal as satiety is approached (Johnstone et al., 2006). The homeostatic nature of OT's role in food regulation becomes very apparent when the relationship between the PVN and the ARC is considered. As previously mentioned, the ARC contributes to metabolic homeostasis via two opposing populations of first-order neurons: orexigenic NPY/AgRP neurons, and anorexigenic POMC/CART neurons. Projections between the PVN and ARC, including OTergic projections to OTR-expressing POMC neurons, may contribute to this regulatory interaction (Maejima et al., 2014; Sabatier et al., 2013). In addition to direct projections, OT released dendritically from PVN neurons can diffuse to influence neighbouring hypothalamic regions through volume transmission (Brown et al., 2020; Ludwig & Leng, 2006). One such neighbouring region is the VMH, One such region is the VMH, where direct administration of OT reduces intake driven by energy need rather than palatability (O. A. Klockars et al., 2017).

There is substantial evidence that OT's role in food intake is not isolated to energy homeostasis and includes involvement in the reward-related components of feeding. Consumption of food also activates reward pathways in the brain. The hedonic value of food is typically linked to the palatability of what is consumed. Broadly, some evidence suggests that OT may reduce the intake of palatable foods by dampening reward-related neural activity.

Anatomically, OTergic neurons project both directly and indirectly to various mesolimbic structures. Parvocellular OTergic neurons are known to project directly to the NAc, amygdala, and VTA (Kerem & Lawson, 2021; Sabatier et al., 2013; Sofroniew, 1980). Some magnocellular neurons have also been identified that project directly to the NAc

and amygdala, among other regions (Kerem & Lawson, 2021; Zhang et al., 2021). Parvocellular OTergic neurons also indirectly stimulate reward-related structures. For example, OTR-expressing neurons in the VTA project to the NAc, amygdala, and other forebrain regions. Many of these neurons are glutamatergic, while a smaller proportion are dopaminergic, indicating that OT may influence mesolimbic circuitry through several neuronal populations (Peris et al., 2017).

In many of these regions, functional studies have been conducted to extend the evidence beyond anatomical correlation. Direct administration of OT into the VTA reduces palatable sucrose consumption, while administration of OTR antagonists increases it (Mullis et al., 2013). Administration of OT to the NAc core, an important mesolimbic region, decreases consumption of both palatable sucrose and saccharin solutions. This effect is abolished by pretreatment with an OTR antagonist (Herisson et al., 2016). Delivery of OT into the BLA reduces the consumption of both sucrose and saccharin solutions. This effect is also abolished by pre-treatment with an OTR antagonist (Klockars et al., 2018). In humans, intranasal OT administration has been shown via functional magnetic resonance imaging (fMRI) to reduce functional connectivity between the VTA and other food motivation–related brain regions when presented with images of high-calorie foods (Kerem et al., 2020).

Additionally, OT has been shown to more strongly influence the regulation of palatable food intake compared to less palatable foods. In humans, intranasal OT reduced the consumption of palatable snacks presented after a meal, but does not affect the hunger-driven consumption of the meal itself (Ott et al., 2013). Intranasal OT also decreased the consumption of both palatable sweet chocolate chip biscuits and salty crackers by young men. However, it had no effect on the intake of oatcakes, which were rated as less palatable in a snacking context (Burmester et al., 2018). A similar effect was observed in a follow-up study, where intranasal OT decreased snacking by women on palatable chocolate chip biscuits but not oatcakes or crackers (Burmester et al., 2019). In a non-caloric paradigm, OTKO but not WT mice overconsumed palatable saccharin solutions to the extent that they exceeded their daily fluid requirements (Billings et al., 2006).

Together, the broad central and peripheral actions of OT position it as a critical hormone capable of influencing feeding behaviour across multiple regulatory domains, many of which overlap with other reward- and motivation-related signalling systems.

1.4 Opioid system

The opioid system consists of small signalling peptides that regulate pain, reward processing, and social and emotional behaviours. It comprises neurons synthesising opioids and three types of opioid receptors: mu (MOR), kappa (KOR), and delta (DOR) opioid receptors. Each of these receptor types has distinct functions, ligands, and distributions (Kieffer & Evans, 2009). In addition to its well-established roles in nociception and reward, the opioid system has been implicated in the regulation of food intake, particularly in the modulation of hedonic feeding and meal size. While much of the existing literature has focused on MOR-mediated effects, growing evidence suggests that DOR signalling also contributes to feeding regulation, although its role remains less well defined. In this thesis, the role of DOR in food intake regulation is explored. The following section provides an overview of the three opioid receptor subtypes, with particular emphasis on DOR. This is followed by a review of current evidence linking opioid signalling to food intake, focusing on mechanisms relevant to feeding behaviour and meal termination. Naltrindole (NTI), a selective antagonist of DOR, is then introduced. Finally, to provide broader context, non-food intake-related functions of the opioid system are briefly addressed.

1.4.1 Mu, kappa, and delta opioids

MOR has received the greatest attention within the opioid literature, largely due to its well-established roles in analgesia and reward (Le Merrer et al., 2009) (Kieffer & Evans, 2009). For example, MOR agonists increase dopamine release in the striatum (Di Chiara & Imperato, 1988). In addition to these roles, MOR has also been implicated in the regulation of hedonic food intake; this will be explored in later sections of the thesis. MOR is widely distributed throughout the CNS. Of particular relevance to feeding behaviour, MOR is widely distributed throughout the CNS, including mesolimbic and hypothalamic regions (Erbs et al., 2015; George et al., 1994; Mansour et al., 1994; Mansour et al., 1988). Exogenous agonists of MOR include morphine, fentanyl, and D-Ala2-Met-enkephalinamide-Gly-ol (DAMGO). Endomorphins and beta endorphins are endogenous agonists of MOR. NTX and Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) are antagonists of MOR, with CTOP being a selective MOR inhibitor and NTX being a non-selective antagonist with a high affinity for MOR (Stein, 2016).

KOR is often viewed as the functional counterpart to MOR. Where MOR activation is associated with euphoria and reward, KOR activation is linked to dysphoria, anhedonia, and stress states (Bruijnzeel, 2009). Among many other mechanisms, KOR influences

mood and reward by inhibiting dopamine release in the striatum (Di Chiara & Imperato, 1988; Maisonneuve et al., 1994). Additionally, administration of KOR agonists has been shown to increase food intake, while KOR antagonists are hypophagic in certain rodent paradigms (Badiani et al., 2001; Beczkowska et al., 1992; Levine et al., 1990; Morley & Levine, 1981; Ruegg et al., 1997). KORs are widely distributed across the CNS, including the mesolimbic system, hypothalamus, and numerous cortical and subcortical regions (Mansour et al., 1994; Mansour et al., 1988; Meng et al., 1993; Vijay et al., 2016). U-69593, U50,488, and bremazocine are commonly used exogenous agonists of KOR. The primary endogenous ligands for KOR are dynorphins; these are derived from the precursor prodynorphin. Norbinaltorphimine is a selective antagonist of KOR, while NTX is also a non-selective KOR antagonist (Stein, 2016).

DORs are the last of the three classical opioid receptor subtypes, and are of particular interest to this thesis. The broad role of DOR remains relatively undercharacterised compared to MOR and KOR. Nonetheless, DORs are involved in emotional processing, reward, and motor control. They may also be relevant to the treatment of conditions such as epilepsy and hypoxia (Chu Sin Chung & Kieffer, 2013). There is evidence of the existence of two pharmacologically defined subtypes of DORs, [D-Pen^2,D-Pen^5]enkephalin (DPDPE)-sensitive DOR (DOR1) and deltorphin II-sensitive DOR (DOR2). These receptor subtypes function independently, with some agonists and antagonists exhibiting preferential affinity for one over the other (Margolis et al., 2017; Zhou et al., 2021). However, there is argument as to whether these two subtypes are appropriately identified as separate receptors given that they are not genetically distinct and knockout of a single gene results in loss of all function associated with both subtypes (Dietis et al., 2011). DOR is broadly distributed throughout the CNS, including cortical areas, limbic structures, and the striatum. Early studies reported DOR to be absent from most of the mesencephalon and brainstem. However, more recent work has identified its presence in these regions (Erbs et al., 2015; Mansour et al., 1994; Mansour et al., 1988). DPDPE and SNC80 are common exogenous agonists of DOR, while enkephalins, deltorphins, and beta endorphins are endogenous agonists of DOR. DOR, much like other opioid receptors, is susceptible to antagonistic binding by NTX. Both ICI 174,864 and NTI are selective antagonists of DOR, with the latter being a focus of this thesis (Stein, 2016).

1.4.2 The role of the opioid system in food intake regulation

The endogenous opioid system plays established roles in both the homeostatic and hedonic regulation of food intake. Of the three classical opioid receptor subtypes, MOR is most strongly implicated in the regulation of feeding behaviour (Nogueiras et al., 2012). The influence of opioid signalling on food intake has been recognised for several decades. In the late 1970s, the an early study demonstrated an anorexigenic effect following administration of an opioid receptor antagonist. Naloxone, a non-selective opioid receptor antagonist, was shown to suppress post-deprivation food intake in rats (Holtzman, 1979). Since then, numerous studies have demonstrated that administration of opioid receptor antagonists generally decreases food intake, whereas agonist treatment tends to promote feeding (Giuliano et al., 2012; Gosnell et al., 1986; Sandoval-Caballero et al., 2023; Zhang et al., 1998). Similar to OT, the opioid system contributes to both hypothalamic homeostatic control and mesolimbic hedonic regulation of food intake. The homeostatic regulation of food intake by the opioid system will be described below, followed by hedonic regulation. Additionally, the role of DOR, in particular, in food intake regulation will be explored.

Opioid receptors are differentially expressed across key hypothalamic nuclei involved in homeostatic feeding, including the ARC, PVN, VMH, and LHA (Erbs et al., 2015; Mansour et al., 1994; Mansour et al., 1988; Vijay et al., 2016). Evidence for the homeostatic role of opioid receptors is further supported by their interactions with neuropeptides involved in energy balance, as shown in Figure 1.7. For example, MORs are expressed postsynaptically on POMC neurons, where their activation inhibits neuronal firing (Pennock & Hentges, 2011). This localisation suggests that MOR activation suppresses anorexigenic signalling via inhibition of POMC neuron activity. Central opioid receptor antagonism has also been shown to attenuate feeding induced by AgRP and NPY. Combined MOR and KOR blockade reduced AgRP-induced intake, while centrally administered MOR and KOR antagonists reduced NPY-induced feeding (Brugman et al., 2002; Kotz et al., 1993). Together, these findings support interactions between opioid signalling and neuropeptide systems involved in homeostatic feeding regulation.

The role of the opioid system in the hedonic aspects of food intake has been especially well studied. The consumption of palatable food represents a form of natural reward. This reward process is largely mediated by the mesolimbic system, particularly the NAc and the VTA, as depicted in Figure 1.7 (Nogueiras et al., 2012; Spanagel et al., 1992). Although all three classical opioid receptor subtypes are expressed in these regions, MOR

is the most consistently linked to hedonic enhancement of palatable food intake (Erbs et al., 2015; Mansour et al., 1994; Mansour et al., 1988; Nogueiras et al., 2012; Vijay et al., 2016). The hedonically driven regulation of food intake by the opioid system has typically been explored using MOR agonists or antagonists by investigating food motivation or the consumption of palatable foods. In rats, administration of DAMGO into the VTA induced feeding despite satiety, an effect reversed by simultaneous injection of NTX into the shell of the NAc (MacDonald et al., 2003). This induction of feeding in the absence of energy need is highly indicative of a reward-driven motivation. Direct administration of DAMGO into the NAc also increases the consumption of palatable saccharin solutions without increasing water consumption (Zhang & Kelley, 2002). Administration of MOR antagonists suppresses binge-like eating behaviours in rats. Binge-like eating reflects a shift toward palatability-driven consumption, where the hedonic value of food overrides energy homeostasis (Giuliano et al., 2012). In humans, MOR antagonists reduce sensory hedonic ratings of highly palatable sucrose and fat-rich snacks (Nathan et al., 2012). Collectively, these findings suggest that MOR activation enhances the hedonic value of palatable foods, promoting intake beyond homeostatic need. Conversely, MOR antagonism diminishes food reward and suppresses excessive consumption.

Figure 1.7 summarises the conceptual overlap between the homeostatic and hedonic actions of the endogenous opioid system. Opioid receptors are represented within hypothalamic regions involved in energy-related control of feeding and mesolimbic regions involved in food reward, illustrating how opioid signalling may influence intake through several interacting processes rather than through a single pathway(Nogueiras et al., 2012). Of particular relevance to this thesis, DOR is represented within both hypothalamic and mesolimbic components of this system, although its functional contribution to food intake remains less clearly defined than that of MOR.

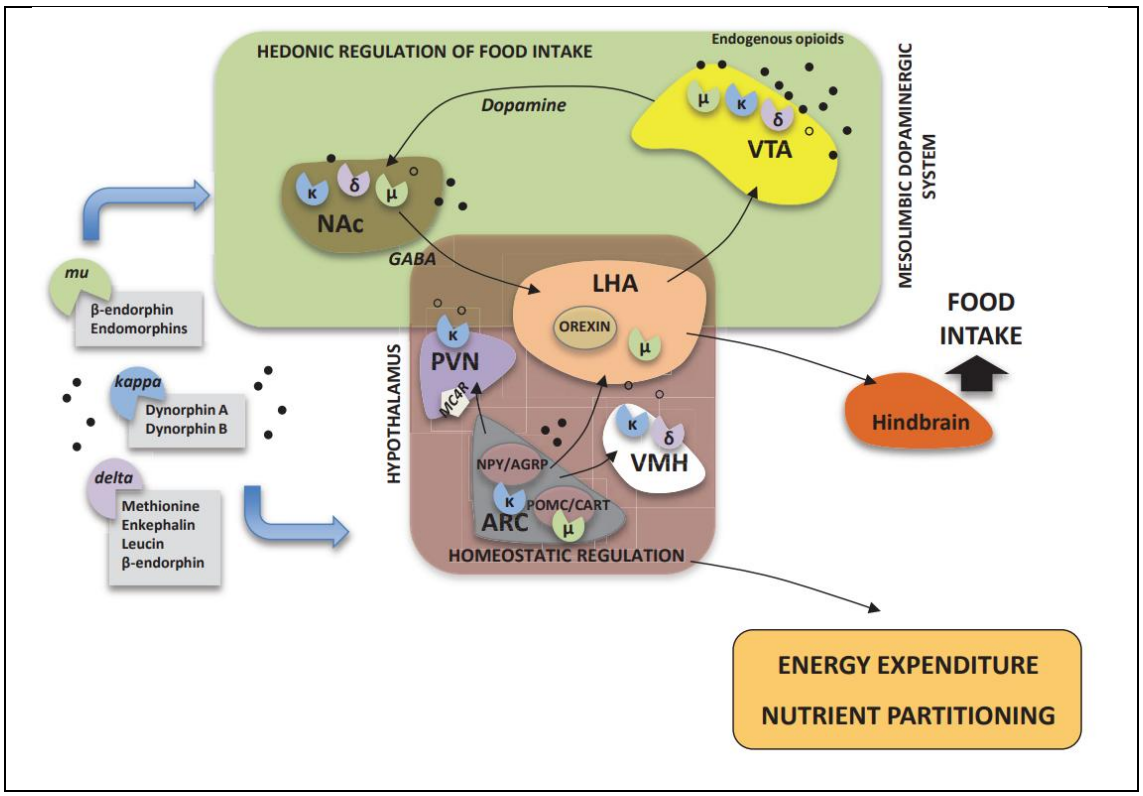

Figure 1.7 Homeostatic and Hedonic Centers of Endogenous Opioid System Action on Food Intake Regulation (Reproduced with permission from Nogueiras et al (Nogueiras et al., 2012))

Of the three classical opioid receptor subtypes, DOR is the least studied, particularly in relation to its involvement in feeding behaviour. The limited available literature is often conflicting and does not provide a clear or consistent narrative regarding the role of DOR in food intake regulation(Beczkowska et al., 1992; Beczkowska et al., 1993; Chow et al., 1997; Davis et al., 2009; Hutchinson et al., 2000; Inui & Shimura, 2014; Kaneko et al., 2014; Krishnan-Sarin et al., 1995; Nascimento et al., 2014; Sakamoto et al., 2015). Central administration of the DOR agonist DPDPE increases consumption of standard chow in sated mice but decreases consumption of a high-fat diet within the same paradigm (Kaneko et al., 2014). Central administration of the DOR1 antagonist DALCE fails to reduce maltodextrin intake and only slightly decreases saccharin consumption. In contrast, administration of a non-subtype-selective DOR antagonist, NTI, significantly reduces saccharin intake but has no effect on maltodextrin consumption (Beczkowska et al., 1993). Central administration of the DOR agonist DSLET stimulates food consumption, although to a lesser extent than selective agonists of other opioid receptor subtypes (Gosnell et al., 1986). Other studies have found that NTI decreases saccharin intake and suppresses conditioned anticipatory behaviour, but does not affect drinking-related behaviours (Chow et al., 1997). Additionally, administration of NTI into the ventral pallidum increases both the perceived palatability and the consumption of saccharin

solution in rats (Inui & Shimura, 2014). The inconsistent effects of DOR agonists and antagonists on food intake suggest that the role of DOR in feeding regulation is context-dependent. While a relationship between DOR signalling and food intake is evident, its precise nature remains unclear.

Notably, the role of DOR in the regulation of sucrose consumption remains unclear. Some studies suggest that NTI administration fails to alter the intake of sucrose (Beczkowska et al., 1992; Sakamoto et al., 2015), while other work has demonstrated that central NTI injection can potentiate sucrose consumption (Kelley et al., 1996). The studies to date which have investigated the influence of NTI on sucrose consumption have typically used paradigms involving the use of ad hoc sucrose meals, rather than habitual consumption. This gap in the literature is later addressed in the current thesis.

1.4.3 Naltrindole

NTI is a non-peptide antagonist with high selectivity for DOR over MOR and KOR (Portoghese et al., 1988). It does not distinguish between the pharmacologically defined DOR1 and DOR2 profiles, allowing it to be used to examine the broader contribution of DOR signalling (Dietis et al., 2011).

NTI is a synthetic indolomorphinan with the IUPAC name 17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy -3,14-dihydroxy-6,7,2',3'-indolomorphinan (Figure 1.8) (National Center for Biotechnology Information (NCBI), 2025b). NTI was rationally developed as a DOR antagonist based on the structure-activity relationships observed in enkephalin peptides. The phenylalanine residue at position four of enkephalins is critical to binding DOR. To mimic this interaction, a rigid benzene moiety was incorporated into the morphinan scaffold, with an indole system added to impose conformational restraint. NTI is a selective non-peptide DOR antagonist (Portoghese et al., 1988) that has been shown to suppress saccharin intake following systemic administration in rats(Krishnan-Sarin et al., 1995).

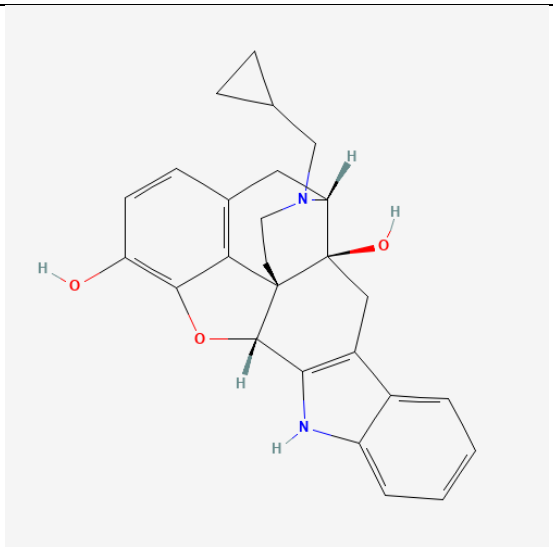

Figure 1.8 2 Dimensional Structure of NTI (National Center for Biotechnology Information (NCBI), 2025b)

The crystal structure of DOR bound to NTI shows that NTI occupies the orthosteric ligand-binding pocket and interacts with receptor residues that contribute to opioid-receptor subtype selectivity (Granier et al., 2012). As DOR is a GPCR, binding of NTI inhibits the conformational changes required for G protein coupling. This inhibition prevents activation of downstream signalling cascades and consequently alters synaptic transmission (Quirion et al., 2020).

1.4.4 Non-food intake-related roles of the opioid system

The opioid system contributes to a wide range of physiological processes, of which the regulation of food intake is a relatively niche topic of discussion. While the role of the opioid system most relevant to this thesis is its regulation of food intake, it is worth noting its other physiological roles for completeness. This is particularly relevant as many of these other functions overlap with the mechanisms involved in food intake regulation.

The opioid system is central to the processing of pleasure. Within the mesolimbic system, activation of MOR in hedonically critical subregions of structures such as the NAc and the ventral pallidum results in hedonically-driven "liking" of stimuli. Antagonism of MOR in these regions instead suppresses this pleasure response. In the context of food intake, this occurs in response to palatable foods, but it is not limited to feeding. Other forms of natural reward, including social interaction, sexual activity, and even music and art, can also activate pleasure through this system. Opioid signalling throughout the mesolimbic system interacts with and influences dopaminergic signalling to produce this effect. Dopamine is linked to wanting; opioids are linked to liking (Berridge & Kringelbach, 2015; Le Merrer et al., 2009).

The opioid system is also critical to the regulation of nociception in both the central and peripheral nervous systems. MOR activation produces analgesia, particularly in acute pain. DOR activation, to a lesser extent, produces analgesia in chronic and inflammatory pain. KOR also reduces pain perception but simultaneously induces dysphoria. Opioid-based treatments are a critical component of modern medicine for the management of pain (Gavériaux-Ruff & Kieffer, 2011; Kieffer & Evans, 2009; Stein, 2016).

Alongside its natural physiological functions and medical applications, the opioid system is also central to the effects of many substances of abuse. Opioid agonists, particularly MOR agonists, have long been substances of abuse. MOR activation in the mesolimbic system enhances dopamine release in the NAc, driving feelings of pleasure and reward. Other non-opioid drugs, such as nicotine and alcohol, also affect this pathway, though via more indirect mechanisms. KOR activation produces aversive and dysphoric effects that can counteract drug reward, and the regulation of KOR activation is known to be altered through chronic exposure to opioid drugs. DOR is implicated in the regulation of emotional states which influence drug-taking behaviour. Often, these same reward-related circuits are activated by palatable foods (Le Merrer et al., 2009; Trigo et al., 2010).

1.5 Overarching and specific aims of this thesis

The regulation of food intake is shaped by multiple neuronal systems which operate concertedly. Among these, the OT system and DOR have both been shown to influence food consumption; however, gaps remain in our understanding of these systems, including their dependence on food composition and the effects of habitual exposure compared with ad hoc consumption. Moreover, while both systems act on overlapping neural circuits involved in feeding regulation, they are typically examined in isolation, and the conditions under which OTR agonism and DOR antagonism interact remain poorly defined. This thesis therefore focuses on exploring the anorexigenic effects of OT and NTI, both independently and in combination.

The **overarching aim** of this thesis is to explore both the individual effects of OT and NTI on food intake and the efficacy of their combined administration in suppressing food consumption. This is achieved through observations of neural activity and food intake behaviour in animal models. This work aims to contribute to the body of knowledge that may inform the development of new pharmacological options for individuals with obesity and dysregulated eating.

Specific Aim 1: To systematically determine whether OT suppresses the consumption of solid fatty foods.

This aim seeks to reconcile reports of OT suppressing fatty food intake but not fat emulsion intake and to provide clearer interpretation of feeding data obtained using intralipid-based paradigms.

Specific Aim 2: To determine the effect of NTI, and by extension the role of DOR, on sucrose intake in rats that habitually consume sucrose solution.

This aim clarifies the effects of NTI treatment on sucrose consumption outside of ad hoc consumption paradigms commonly used in the literature and provides foundational context for subsequent studies examining NTI effects on sucrose intake.

Specific Aim 3: To test whether the simultaneous targeting of OT receptors and DOR, through combined administration of OT and NTI, synergistically suppresses the consumption of liquid diets.

This aim provides insight into the efficacy of combined OT receptor and DOR targeting for suppressing food intake and is informed by the findings of Specific Aims 1 and 2.

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Chapter 2

Oxytocin Suppresses Consumption of Solid High-Fat Foods in Rats

2.1 Abstract

Oxytocin (OT) is a neuropeptide with well-established anorexigenic properties. While macronutrient-focused studies using liquid diets have shown that OT reliably reduces carbohydrate intake, particularly sucrose, it appears ineffective at suppressing intake of Intralipid, a soybean oil emulsion commonly used as a high-fat liquid diet. In contrast, OT reduces consumption of solid high-fat diets, suggesting that diet form may influence responsiveness to OT. In the current study, male rats received intraperitoneal OT (0.1–1.0 mg/kg) and were presented with solid mash diets containing 62 %, 79 %, or 91 % of energy from fat; chow diets containing 10 % or 60 % fat-derived energy; or a control chow rich in cornstarch (CS). Intake was assessed under both no-choice and two-choice conditions. Neural activation was examined through c-Fos immunoreactivity in several feeding-related brain regions, and c-Fos-OT co-labelling in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. OT administration reduced the consumption of solid diets differing in fat content and, in two-choice tests, led to a lower intake of 60 % fat chow relative to 10 % fat chow diet. Consumption of small amounts of 60% fat chow, but not 10% fat chow, increased c-Fos immunoreactivity in feeding-related brain regions and activated OT neurons in the PVN and SON in sated rats. These findings suggest that OT suppresses the intake of solid foods across a range of fat contents and support its role in regulating the consumption of energy-dense foods, including those high in fat.

2.2 Introduction

Oxytocin (OT), a neuropeptide classically associated with reproduction and social behaviour, is also a key regulator of food intake. OT acts through both homeostatic and reward-related circuits involved in feeding regulation [1, 2].

OT was first identified as an anorexigenic agent in the late 1980s, when central and peripheral administration were shown to suppress food intake in rats through an OTR-mediated mechanism [3, 4]. Subsequent studies have established that OT can reduce food intake across a range of experimental paradigms, routes of administration, and species [5-11]. OT has a particularly pronounced effect on the consumption of palatable, energy-dense foods, including solid foods containing high levels of fat and sugar [12, 13].

A number of studies have explored the involvement of this neuropeptide in suppressing the consumption of specific macronutrients. In order to assess the effect of OT on single macronutrient intake, simple liquid diets have typically been used. In a 2005 study, Amico et al. reported that OT knockout (OTKO) mice overconsumed a 10% sucrose solution relative to WT controls [14]. Subsequent work has repeatedly demonstrated that OT preferentially suppresses consumption of the carbohydrates, including cornstarch (CS), fructose, glucose, and polycose, with a pronounced effect on sucrose consumption [15-18].

Remarkably, macronutrient-targeted studies consistently show that OT has little to no effect on fat consumption. In these experiments, Intralipid, a soybean oil emulsion, is typically used to represent dietary fat in liquid form. Sclafani et al. reported that OTKO mice consumed more carbohydrate solutions than WT controls, but their Intralipid intake did not differ [15]. Miedlar et al. similarly found that OTKO mice persistently overconsumed sucrose solution compared to WT controls but did not show greater cumulative Intralipid intake, apart from a transient increase during the first exposure [16].. Olszewski et al. showed that pharmacological OT receptor blockade with L-368,899 increased sucrose solution intake but did not alter Intralipid intake; in the same study, consumption of sucrose, but not Intralipid elevated c-Fos immunoreactivity in the PVN [17].

The apparent insensitivity of Intralipid intake to OT is unexpected given that studies using solid foods typically show suppression of fatty food intake. In a study by Lawson et al., an analysis of macronutrient intake during a menu-style breakfast meal showed that intranasal OT selectively reduced fat consumption in fasted healthy men without altering appetite or the intake of other macronutrients [19]. Other human studies using complex HFHS foods report similar outcomes. For example, Ott et al. and Burmester et al. found

that OT repeatedly decreased chocolate chip cookie intake in both men and women [13, 20, 21]. In rodents, OT has been shown to reduce high-fat diet intake across several paradigms. Morton et al. reported that acute OT administration suppressed consumption of high-fat chow in diet-induced obese rats [7]. Likewise, Maejima et al. found that subcutaneous OT reduced high-fat chow intake both acutely and over multiple days, although the chronic effect waned after six days of treatment [22].

The use of Intralipid as a high-fat liquid diet may contribute to the observed discrepancy in OT's effect on fatty food intake. Intralipid is associated with unusual gastric emptying kinetics, as it separates into a rapidly emptied aqueous phase and a slowly emptied lipid phase. It has also been shown to have diminished satiating properties, as its consumption does not suppress subsequent Intralipid intake in rats [23]. Additional gustatory and sensory factors may further confound feeding studies employing Intralipid. Accordingly, clarifying the efficacy of OT in suppressing solid high-fat food consumption in the present chapter will provide insight relevant to later experiments in this thesis that assess the effect of OT and opioid receptor antagonism on liquid-diet intake.

Given the discrepancy between studies using Intralipid and those employing solid diets to assess OT's effect on fat intake, we hypothesised that i.p. OT would suppress the consumption of solid high-fat foods. Mash diets containing 62%, 79%, or 91% of energy from fat were used to determine whether this effect was evident across progressively higher fat concentrations. Commercial 10% and 60% fat chow diets provided a comparison between low- and high-fat foods within a matrix-matched formulation, while a cornstarch-rich control chow was included to examine whether carbohydrate composition influenced the effect of OT on intake. No-choice tests assessed the effect of OT on intake of each diet, whereas two-choice tests examined whether OT also altered relative selection between diets. In feeding studies, rats were deprived overnight to stimulate intake and minimise the influence of palatability. c-Fos immunoreactivity was subsequently assessed in feeding-related brain regions of sated rats following limited consumption of 10% or 60% fat chow diets and in unfed controls. Using sated animals diminished the influence of hunger on neuronal activation.

2.3 Materials and methods

2.3.1 Animals

Age-matched adult male rats (Sprague-Dawley, AgResearch Hamilton New Zealand, 500 ± 50 g and ~180 days post-natal at study onset) were single-housed in clear plexiglass cages. The cages were located in a light- and temperature-controlled vivarium (22 ºC,

L:D 12:12, lights on at 07:00 h). Rats were maintained on and had ad libitum access to standard chow (Sharpes, Carterton New Zealand) and water except where experimental protocols dictated otherwise.

All procedures adhered to the NIH Guide for the Care and Use of Laboratory Animals guidelines and were approved by the University of Waikato Animal Ethics Committee (protocol number: 1179).

2.3.2 Feeding studies

The effect of i.p. OT on the consumption of solid foods with varying fat content was assessed using two diet types. First, simple mash diets composed solely of vegetable shortening (Kremelta, Australia) and flour (Pams, New Zealand) were tested. Then, commercially available rodent chow diets were used. The inclusion of multiple diet types served to minimise potential confounding from gustatory and sensory properties.

2.3.2.1 Effect of i.p oxytocin in no-choice acceptance paradigms

We assessed the episodic consumption of solid foods in mice treated with i.p OT (Sigma, St Louis, U.S.A.) or saline. Three solid mash diets containing 62%, 79%, or 91% of energy from fat were prepared using vegetable shortening and all-purpose flour. These diets were made at shortening:flour ratios of 2:3, 3:2, or 4:1, and had energy densities of 5.6, 6.72, and 7.78 kcal/g respectively, with no added water. The remaining energy was primarily derived from carbohydrates in the flour.

Rats were given access to mash diets for 1 h/ day on 3 days prior to the experiment to avoid neophobia. Standard chow was removed from the home cages 16 h prior to the experiment to stimulate intake and diminish the role of palatability. On experiment day (11:00 am), rats received an i.p injection of OT (0.1, 0.3, or 1.0 mg/kg) or vehicle (n=6/group). Forty-five minutes following injection, rats were given pre-weighed mash diet in their standard hoppers. After 1 hour, the weight of mash diet remaining was measured and corrected for spillage. Data were collected as g food and normalised to g food/ kg body weight. For each diet the same cohort of rats were used. The animals were randomly assigned to groups, and the body weights did not differ between groups; 3 days of washout period elapsed between each experiment.

The same protocol was used to assess the effect of i.p. OT on the consumption of three commercial chow diets: a 60% fat diet (D12492), a 10% fat diet (D12450J), and a cornstarch-rich control diet (D12450K; Research Diets Inc., USA). The nutritional composition of these diets is summarised in Table 2-1.

Table 2-1

Nutritional composition of the commercial chow diets used in the feeding studies

Characteristic	60% fat chow	10% fat chow	Cornstarch-rich chow
Product code	D12492	D12450J	D12450K
Protein (% energy)	20	20	20
Carbohydrate (% energy)	20	70	70
Fat (% energy)	60	10	10
Energy density (kcal/g)	5.24	3.85	3.85
Cornstarch (g/100 g diet)	0	48	52.1
Maltodextrin/Lodex 10 (g/100 g diet)	16.2	11.8	14.2
Sucrose (g/100 g diet)	8.9	6.9	0.4
Soybean oil (g/100 g diet)	3.2	2.4	2.4
Lard (g/100 g diet)	31.7	1.9	1.9

D12492 and D12450J provided the principal high-fat and low-fat comparison. D12450J and D12450K had the same proportions of energy derived from protein, carbohydrate, and fat, but differed in their relative cornstarch, maltodextrin, and sucrose contents.

Although the 62% fat mash and 60% fat chow contained similar proportions of energy derived from fat, they were not compositionally equivalent. The mash was a simple mixture of vegetable shortening and flour, whereas the commercial chow was a complete formulated diet with a different food matrix, ingredient composition, and energy density. Intake values for these diets were therefore not treated as a direct comparison between equivalent formulations.

2.3.2.2 Effect of I.P oxytocin on two-choice diet preference

To investigate the influence of i.p OT on the preference of solid mash diets, we utilised a two-choice preference paradigm. Rats (the same cohort that had been used in the no-choice acceptance studies) were deprived of food 16 h prior to experiment to stimulate intake and diminish the role of palatability. On experiment day, rats received an injection of OT (1.0 mg/kg) or vehicle (n=12/group). Forty-five minutes following injection, pre-weighed servings of 62% and 79% mash diets were placed in a subdivided hopper. After 1 hour the weight of mash diet remaining was measured and corrected for spillage. Data were collected as g food and normalised to g food/ kg body weight.

The same protocol was used to assess the effect of i.p OT on the preference of 10% fat and 60% fat chow diets. It should be noted that the 91% fat mash diet and CS diet were excluded from testing in the choice paradigms due to low-baseline consumption in the no-choice paradigms.

2.3.3 Neuronal activation after chow diet intake

Neuronal activation following consumption of diets differing in fat content was assessed using OT-c-Fos immunohistochemistry. The same cohort of rats used in the feeding studies was employed, following a 14-day washout period. On the test day, standard chow was removed three hours into the light phase (10:00 a.m.). Rats were not deprived overnight prior to the experiment to mitigate any overlapping effects of hunger on neuronal activation. After 2 h, rats were given access to 5 g servings of either 60% fat chow, 10% fat chow, or no food for 1 h in their home cages (n=8/group). Visual inspection at the end of the feeding period confirmed that the rats consumed all or most of the food served. Rats given no food were controls.

One hour following the end of the meal, rats were deeply anesthetized with 35% urethane before they were transcardially perfused with 50 mL of saline followed by 500 mL of 4% paraformaldehyde (PFA) in TBS. The excised brains were postfixed for 48 h in PFA at 4 ^{o}C. Coronal 60-µm sections were sliced using a vibratome (Leica, Germany) and processed as free-floating sections.

Sections were incubated in 10% methanol and 3% H_2O_2 (in TBS; 10 min). Following a TBS rinse, they were incubated in the rabbit-anti-Fos antibody (Synaptic Systems, Australia, 1:1500) in 0.25% gelatine and 0.5% Triton X-100 (Sigma, USA) in TBS overnight at 4°C. The sections were rinsed in TBS and incubated for 1 h in the goat-anti-rabbit antibody (Vector, USA, 1:400). After TBS rinses, they were incubated in the avidin-biotin complex (Vector, USA) for 1 h. The colour reaction was initiated with 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, and 10% nickel sulphate in TBS (15 min). Sections were mounted on gelatine-coated slides, air dried, dehydrated in ethanol, soaked in xylene, and coverslipped in Entellan (Sigma, Germany).

To visualize c-Fos and OT, Fos-stained sections underwent the protocol utilizing the primary rabbit-anti-OT antibody (Chemicon, Temecula, USA, 1:13000). Nickel sulphate was omitted from the DAB step to achieve brown staining.

Images were captured under a Nikon H55OS (Nikon Instruments, Melville, USA) microscope equipped with an OMAX camera (Omax, Kent, USA). The regions of interest, outlined using the Allen Brain Atlas (ABA; anterior: posterior bregma ranges in the parentheses), were: AcbC – nucleus accumbens core (1.28 : 0.96); AcbS – Acb shell (as AcbC); ARC – arcuate nucleus (−2.14: −2.52); BLA – basolateral amygdala (−2.64: −2.92); CEA – central nucleus of the amygdala (−2.64: −2.92); DMH – dorsomedial nucleus (−2.80: −3.24); DMNV – dorsal motor nucleus of the vagus (−13.76: −14.16); NTS – nucleus of the solitary tract (−13.70: −14.16); PVN – paraventricular nucleus

(−1.56: −1.92); and SON – supraoptic nucleus (−0.96: −1.20); VMH – ventromedial nucleus (−2.80: −3.24)

Each region was outlined as per ABA and its area measured in mm^2 (ImageJ, NIH, Bethesda, USA). c-Fos-immunoreactive nuclei were counted on four to eight sections per region per animal. The numbers were added for each region in each animal and densities of nuclei per mm^2 were calculated.

In c-Fos-OT analysis, five PVN and five SON sections containing OT cells were used in each animal. We assessed the number of OT neurons and the number of OT cells colocalizing with c-Fos. The % of c-Fos-immunoreactive OT cells was calculated per animal and averaged for each experimental group.

2.3.4 Statistical analysis

All statistical analyses, except for the two-choice preference test involving the mash diets, were conducted using one-way ANOVA with Dunnett's post hoc test. The two-choice mash preference data were analysed using unpaired two-tailed t-tests. Values are presented as means ± SEM and were deemed significantly different when $p \leq 0.05$.

2.4 Results

2.4.1 No-choice acceptance tests

In rats given mash diets, saline-treated control animals consumed 3 g/kg b wt of the 62% fat mash and 6 g/kg b wt of the 79% fat mash. At 1.0 mg/kg, OT reduced intake of the 62% fat mash to 0.8 g/kg b w and the 79% fat mash to 2 g/kg b w (62%: $p = 0.0074$, $F(3,20) = 0.6131$; 79%: $p = 0.0005$, $F(3,20) = 0.3422$) (Figure 2.1;Figure 2.2). Intake of the 79% fat mash was also reduced by 0.3 mg/kg OT, from 6 to 2.8 g/kg b w ($p = 0.003$, $F(3,20) = 0.3422$). No significant effect of OT was observed on consumption of the 91% fat diet. However, it should be noted that baseline consumption of this diet was low at >1 g/kg b wt (Figure 2.3).

For chow diets, rats given saline consumed approximately 25 g/kg b wt of 60% fat chow and 17 g/kg b wt of 10% fat chow. At 1.0 mg/kg, OT reduced consumption of the 60% fat chow to 10 g/kg b w and the 10% fat chow to 7 g/kg b w (60%: $p = 0.0195$, $F(3,20) = 0.3468$; 10%: $p = 0.0390$, $F(3,20) = 0.4580$) (Figure 2.4;Figure 2.5). No effect on food intake was observed at 0.1 or 0.3 mg/kg OT. The baseline consumption of the CS chow was approximately 9 g/kg b w; OT did not affect the intake of CS chow (Figure 2.6).

2.4.2 Two-choice preference tests

In saline-treated rats, the 79% fat mash diet accounted for approximately 22% of total mash diet intake, whereas almost all chow intake was derived from the 60% fat chow diet. OT administration did not significantly alter the relative intake of the 62% and 79% fat mash diets (Figure 2.7). However, with a larger difference in fat content (10% vs 60% fat chow), 1.0 mg/kg OT administration significantly decreased 60% fat chow consumption relative to 10% fat chow ($p = 0.0406$, $F(3,31) = 3.970$). Lower doses (0.1 and 0.3 mg/kg OT) had no significant effect on relative chow intake (Figure 2.8).

2.4.3 c-Fos immunoreactivity

60% fat chow consumption significantly increased c-Fos immunoreactivity in the AcbS ($p = 0.0157$, $F(2,15) = 1.155$), AcbC ($p = 0.012$, $F(2,15) = 1.033$), CEA ($p = 0.0104$, $F(2,18) = 2.613$), and DMH ($p = 0.0411$, $F(2,13) = 1.874$). c-Fos activation in rats given 10% fat chow did not significantly differ from unfed controls in any brain region examined (Figure 2.9).

In the PVN, 60% fat chow consumption increased OT neuronal activation compared to both unfed controls ($p < 0.0001$, $F(2,19) = 0.5427$) and rats fed 10% fat chow ($p < 0.0001$, $F(2,19) = 0.5427$). Additionally, OT activation was higher in rats given 10% fat chow than in unfed controls ($p = 0.0004$, $F(2,19) = 0.5427$) (Figure 2.10).

In the SON, 60% fat chow consumption also increased OT activation relative to both unfed controls ($p = 0.0002$, $F(2,18) = 0.9712$) and rats fed 10% fat chow ($p = 0.0256$, $F(2,18) = 0.9712$). No significant differences were observed between rats given10% fat chow and unfed controls (Figure 2.11).

2.5 Discussion

Reports describing the effect of OT on high-fat food intake have yielded inconsistent outcomes. While OT reliably suppresses intake of solid high-fat diets, it fails to reduce consumption of Intralipid, a lipid emulsion. Deblon et al. found that chronic i.c.v. infusion of OT decreased cumulative intake of solid high-fat chow in diet-induced obese mice over 14 days [24]. In contrast, Liyanagamage et al. recently reported that intranasal OT reduced episodic sucrose solution intake, but had no effect on Intralipid consumption [10]. These findings indicate that OT's anorexigenic effects depend on fat presentation, with intake reduced when fat is embedded in a solid matrix (chow) but not when delivered as an emulsified liquid (Intralipid).

To address this discrepancy, we examined the effects of i.p. OT on intake of solid diets differing in fat content. At 1.0 mg/kg, OT reduced consumption of the 62 % and 79 % fat mash diets from 3 to 0.8 g/kg b wt and from 6 to 2 g/kg b wt, respectively. Intake of the 60% fat chow was also suppressed at this dose, decreasing from 25 to 10 g/kg b wt. Additionally, intake of the 79% fat mash diet was significantly reduced by 0.3 mg/kg OT (from 6 to 2.8 g/kg b w)t. These findings mirror earlier work in which OT reduced the consumption of HFHS chow: Head et al. reported that 1.0 mg/kg i.p. OT reduced HFHS chow intake from 35 to 25 g/kg b wt following 22 h food deprivation [12]. The lower baseline intake in the present study likely reflects differences in the diet matrix and palatability. In contrast, consumption of the 91% fat mash diet was unaffected by OT at any dose, which may be attributable to its already low acceptance under control conditions.

OT also reduced consumption of the 10 % fat chow, which differed from the 60 % fat chow only in fat and CS content. To determine whether CS content influenced this reduction, we tested a matrix-matched chow lacking sucrose and enriched in CS. Baseline intake of this CS-rich diet was markedly lower than that of the other chows, with control animals consuming an average of 9 g/kg b wt, compared to 17 and 25 g/kg b wt for the 10 % and 60 % chow diets, respectively. OT treatment did not further reduce intake of the CS diet at any dose tested. The low baseline acceptance relative to the other chow diets suggests that CS is unlikely to contribute to OT's sensory-driven anorexigenic effects and does not underlie the reduced consumption of the 60 % fat chow. Nonetheless, CS content may still affect satiety-related mechanisms such as gastric distension.

Previously published work has shown that central OT receptor blockade with L-368,899 increases intake of sucrose over Intralipid in a two-choice paradigm without affecting

total consumption [17]. In contrast, i.p. OT-treated animals in the current study consumed proportionally less of the higher fat option when given a choice between 10 % and 60 % fat chow. However, OT treatment did not influence the relative consumption of the mash diets when given a choice, likely because the difference in fat content between the mash diets tested (62 % vs 79 % fat) was smaller than that between the chow diets (10% vs 60% fat). These observations align with the results from the no-choice conditions, where OT suppressed food intake regardless of fat content, and further highlight the discrepancy between outcomes obtained with solid diets versus those using emulsified lipid solutions. These findings indicate that OT inhibits the consumption of fatty foods and that this effect is not diminished by increases in dietary fat content. This aligns with previous studies using solid high-fat diets [7, 13, 19-22, 24], and contrasts with reports based on Intralipid consumption, where OT fails to suppress intake [10, 15-17]. Overall, these data support the broad notion that OT suppresses the consumption of food. Exceptions to this were limited to the CS chow and 91 % fat mash diets, both of which were consumed at low levels even in untreated animals. These outcomes did not contradict OT involvement in meal termination. OT constrained intake when baseline acceptance was sufficient (62–79 % mash; 10 % and 60 % chow) and showed little effect when acceptance was low (91 % fat mash; CS-rich chow). The limited intake of the 91 % mash may reflect a decline in acceptance at extreme fat concentrations. Mindell et al. observed that rats showed the highest acceptance for emulsions containing 25–50 % fat, with substantially reduced intake of pure fat [25]. The inverted-U profile of fatty food acceptance previously reported is consistent with the present data, although differences in diet matrix likely influence the specific concentration of peak acceptance. Additional evidence suggests that fat-carbohydrate mixtures are consumed more avidly by rats than fat alone [26]. While the 91 % mash retained some carbohydrate content, it may have been below the threshold required to enhance intake.

The outcome of the c-Fos-OT double-immunoreactivity analysis indicates that activation of OT-expressing neurons is most pronounced following the consumption of high-fat food. In both the PVN and SON, consumption of the 60% fat chow diet produced the highest levels of OT neuron activation, whereas intake of the 10% fat chow resulted in a moderate increase in PVN OT activation relative to unfed controls. These results align with our feeding data and are consistent with previous reports showing that OT neuron recruitment accompanies meal termination (e.g., [27]; further reviewed in [2, 28]).

Consumption-induced c-Fos immunoreactivity further supported the engagement identified in the c-Fos-OT analysis. Consumption of 60% fat chow, but not 10% fat chow,

increased c-Fos immunoreactivity in brain regions implicated in the regulation of feeding and known to be influenced by oxytocinergic signalling. Elevated neuronal activity was observed in the central amygdala (CEA) and nucleus accumbens (AcbC, AcbS), mesolimbic sites that are targeted by PVN-derived oxytocinergic projections [2, 29-32]. c-Fos activation was also increased in the dorsomedial hypothalamus (DMH), a site that integrates energy balance and hedonic input, and may receive dendritically released OT from magnocellular neurons in the PVN and SON [33-37]. Under sated conditions, these changes likely reflect nutrient-specific signalling rather than effects of energy deprivation. The activity observed in these regions, in conjunction with increased OT neuron activation in the PVN and SON, may suggest engagement of an OT-dependent network coordinating responses to high-fat intake. This network activation aligns with the observed reduction in intake of 60 fat chow relative to the 10 % fat chow in the two-choice test, indicating a potential role in OT-mediated macronutrient-specific suppression of food selection. Animals fed 10% chow exhibited non-significant trends toward increased activity in several of the same regions, suggesting that these circuits may still be engaged to a lesser extent. The absence of significant c-Fos induction may reflect slower or weaker activation by low-fat diets under sated conditions, or that activation may occur below the detection threshold of this assay. In contrast, the presence of higher fat content in the 60% chow was sufficient to elicit robust activation, indicating that high-fat foods may be more effective in recruiting these feeding-related circuits. It remains possible that with a larger or longer feeding bout, these regions would also be activated by lower fat diets.

We conclude that OT suppresses consumption across a range of solid diets differing in fat content and that intake of relatively higher-fat foods engages brain regions implicated in OT-mediated feeding regulation, including the PVN, SON, CEA, Acb, and DMH. These findings suggest that conclusions drawn from prior studies which have used emulsion-based fat diets may need to be revisited and that OT may be more effective at suppressing the consumption of high-fat foods than previously thought. Of relevance to the overarching aim of this thesis, these findings indicate that conclusions regarding the efficacy of OT, or OT-containing combination treatments, based on Intralipid intake in later chapters should not be assumed to generalise to suppression of fatty foods more broadly.

2.6 Figures

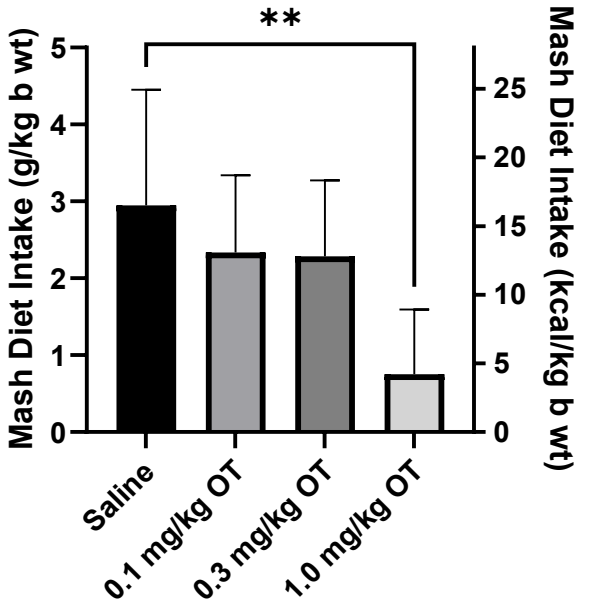

Figure 2.1 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of mash diet in which 62% of the energy is derived from fat. Data are means ± SEM; **p<0.01

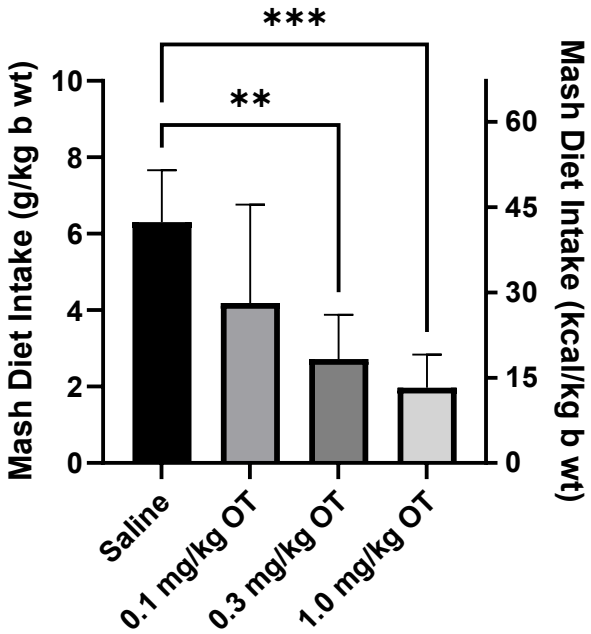

Figure 2.2 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of mash diet in which 79% of the energy is derived from fat. Data are means ± SEM; **p<0.01.; ***p<0.001

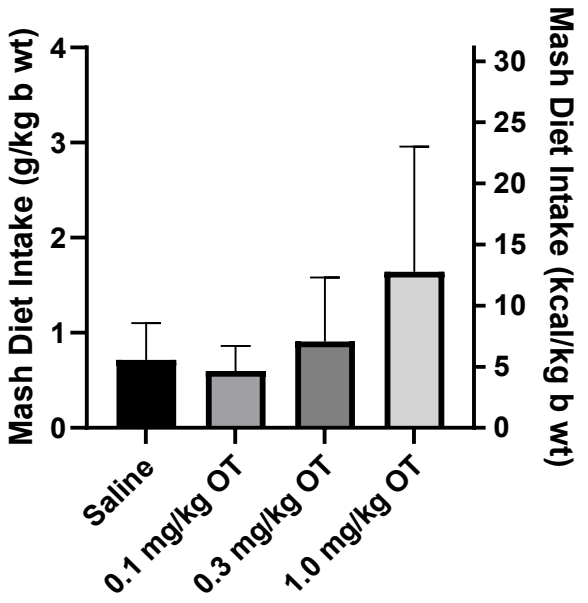

Figure 2.3 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of mash diet in which 91% of the energy is derived from fat. Data are means ± SEM

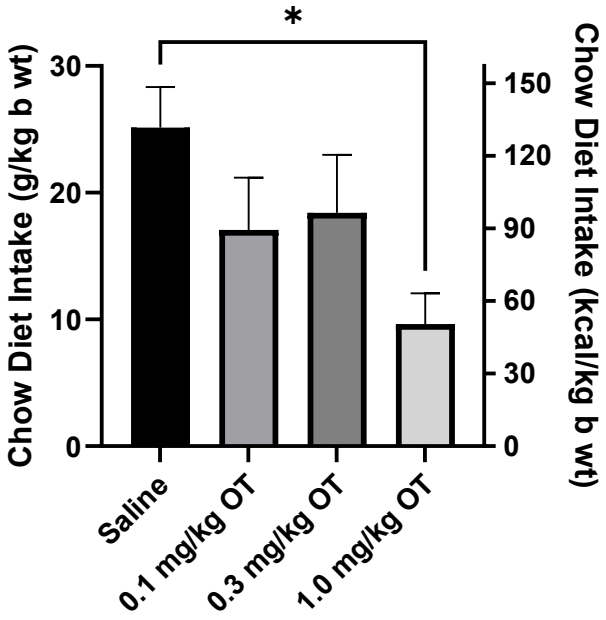

Figure 2.4 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of chow diet in which 60% of the energy is derived from fat. Data are means ± SEM; *$p \leq 0.05$

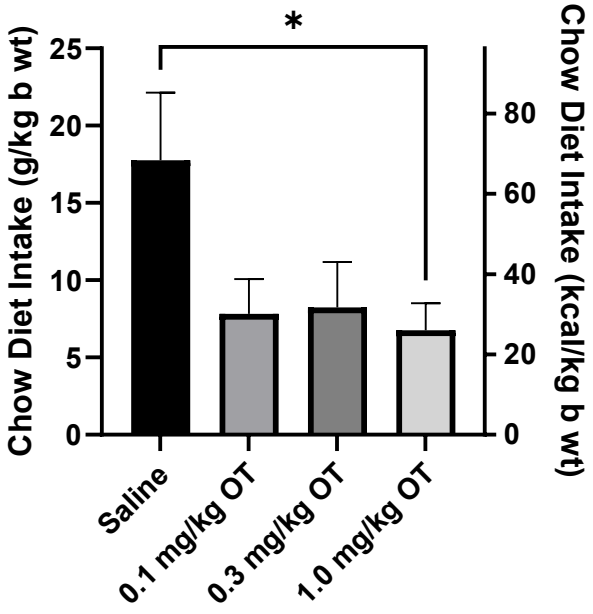

Figure 2.5 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of chow diet in which 10% of the energy is derived from fat. Data are means ± SEM; *p ≤ 0.05

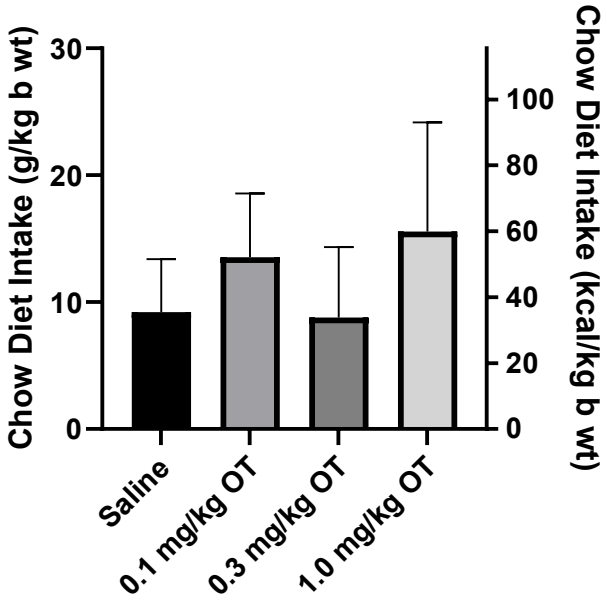

Figure 2.6 Effect of intraperitoneal saline, 0.1 mg/kg, 0.3mg/kg, or 1.0mg/kg OT on 1-h intake of a CS rich chow diet in which 10% of the energy is derived from fat. Data are means ± SEM

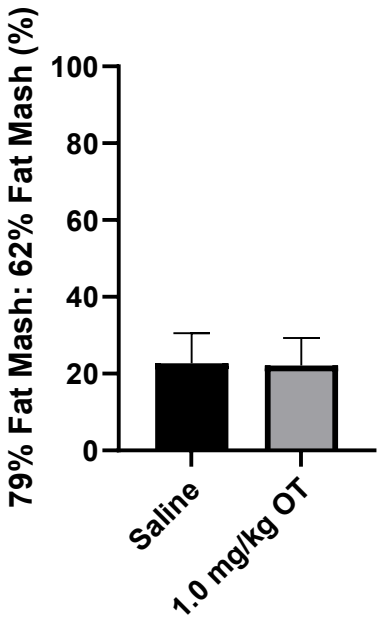

Figure 2.7 Effect of intraperitoneal saline or 1.0mg/kg OT on 1-h intake of mash diets of differing fat contents in a two-choice preference test. Values represent intake of the 79% fat mash as a percentage of total intake of the 79% and 62% fat mash diets. Data are means ± SEM

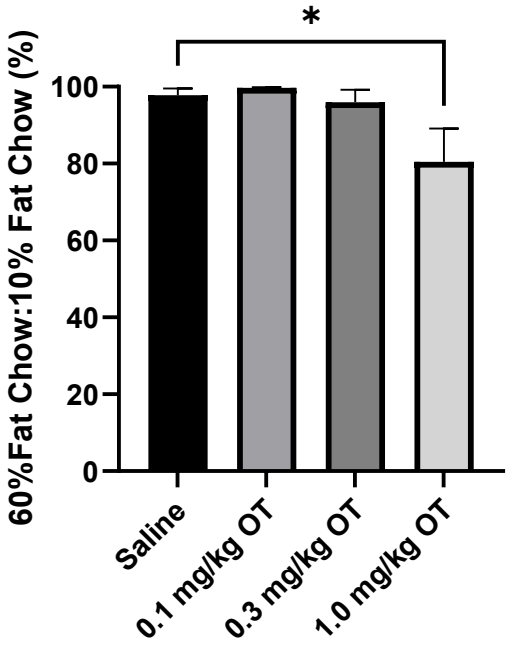

Figure 2.8 Effect of intraperitoneal saline, 0.1mg/kg, 0.3 mg/kg, or 1.0mg/kg OT on 1-h intake of chow diets of differing fat content in a two-choice preference test. Data are means ± SEM; *p ≤ 0.05

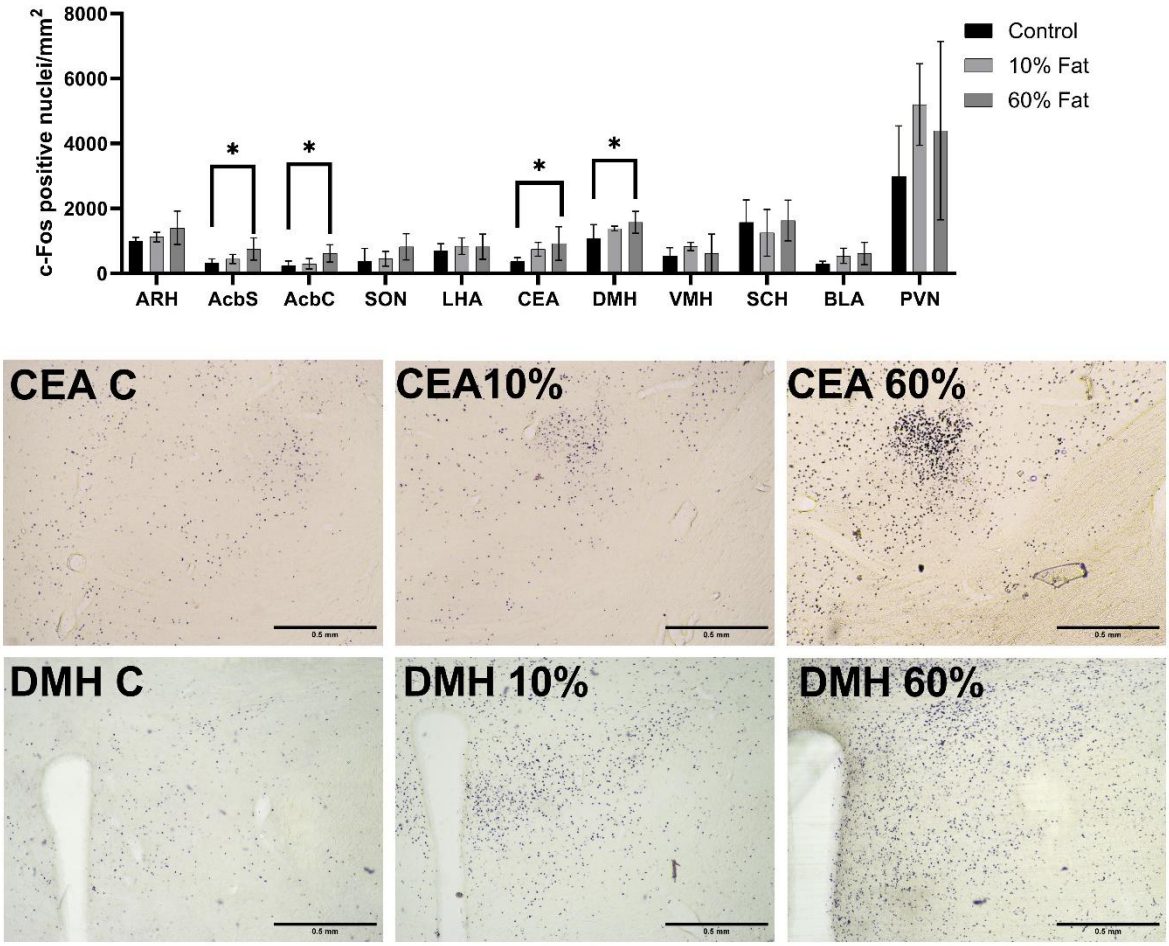

Figure 2.9 Effect on c-Fos immunoreactivity in feeding related brain areas following consumption of 60% fat chow diet, 10% fat chow diet, or no food. Photomicrographs depict staining in the CEA and DMH. Data are means ± SEM; *$p \leq 0.05$

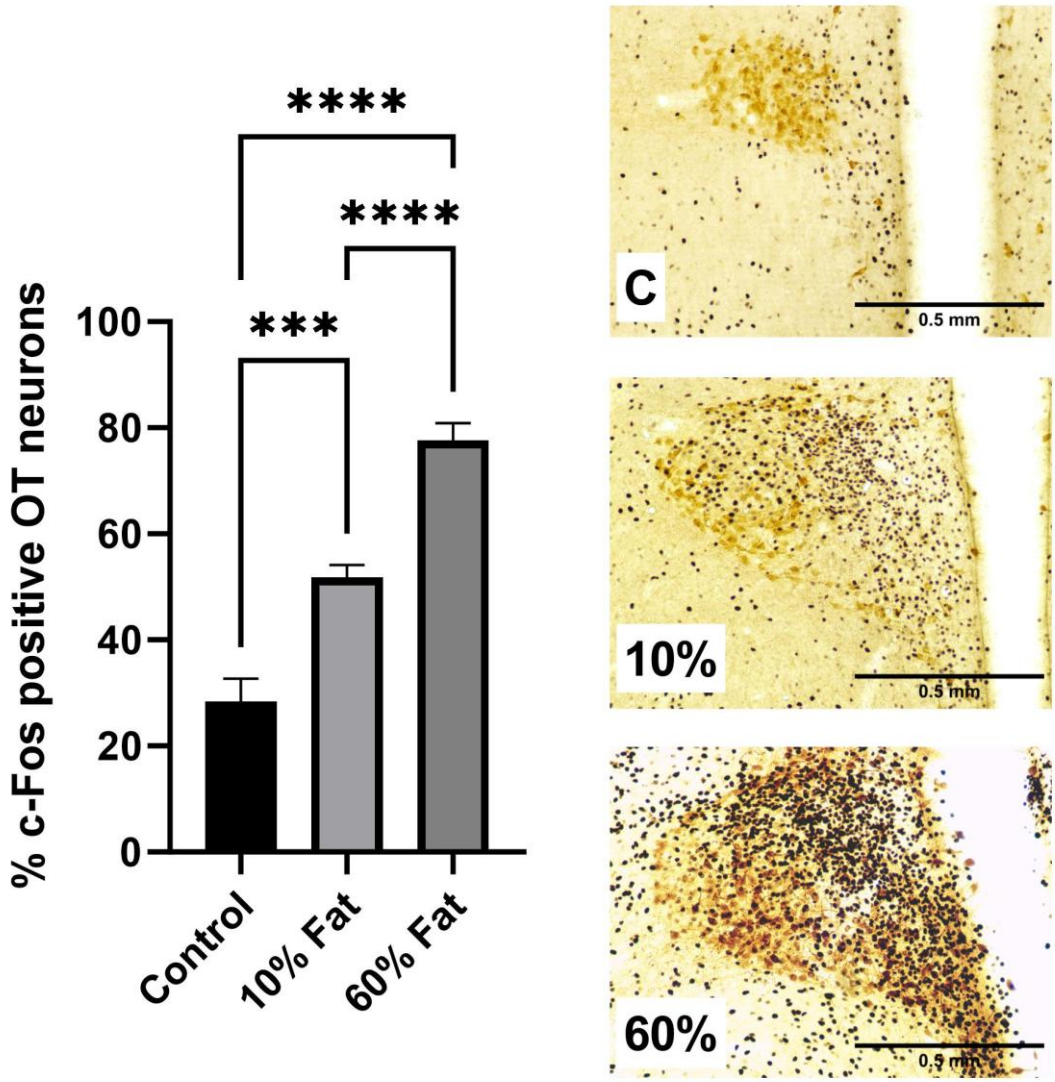

Figure 2.10 Percentages of OT positive neurons co expressing c-Fos in the PVN following consumption of 10% fat chow diet, 60% fat chow diet, or no food. Photomicrographs depict staining in the PVN. Data are means ± SEM; ***p<0.001.; ***p<0.0001

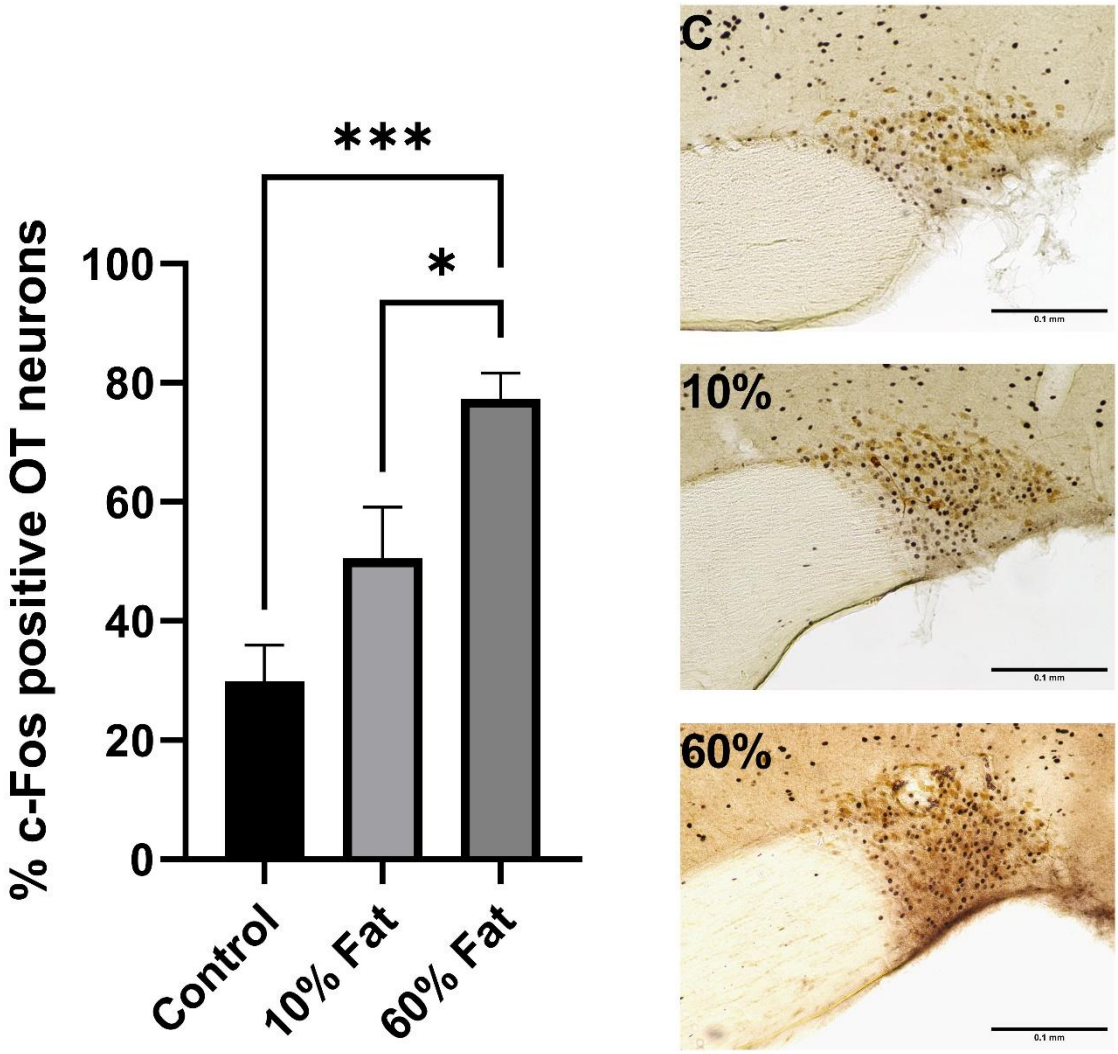

Figure 2.11 Percentages of OT positive neurons co expressing c-Fos in the SON following consumption of 10% fat chow diet, 60% fat chow diet, or no food. Data are means ± SEM; *p ≤ 0.05.; ***p<0.001

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Chapter 3

Delta Opioid Receptor Antagonist Naltrindole Acutely Suppresses Habitual Sucrose Solution Consumption Without Causing Aversive Consequences

3.1 Abstract

The previous reports attempting to define the role of the delta opioid receptor (DOR) and its antagonist, naltrindole (NTI) in the regulation of sugar intake have produced contradictory results. We have investigated whether acute peripheral NTI administration in rats affects intake of a sucrose solution consumed daily and whether the effect might be secondary to a conditioned taste aversion (CTA) or thirst. We have assessed c-Fos immunoreactivity induced by NTI in sucrose-fed vs sucrose-naïve rats. Rats had access to a 10% sucrose solution for 1 h/day for 3 weeks. At 3 weeks, they were injected intraperitoneally with NTI prior to the sucrose meal to establish the lowest dose that reduces sugar intake. We examined whether NTI generates a CTA or affects deprivation-induced water intake. c-Fos was assessed in rats consuming sucrose habitually vs sucrose-naïve controls injected with NTI. 10 mg/kg NTI produced a 22 % reduction in sucrose intake. It had no effect on water consumption after overnight water deprivation. It failed to generate a CTA. NTI injection in sucrose-naïve rats produced limited c-Fos changes; however, in animals habitually consuming sucrose, NTI vastly affected activation of sites involved in reward and energy homeostasis, with a significant drug x diet interaction in the nucleus of the solitary tract and the supraoptic nucleus. Acute intraperitoneal injection of NTI reduces habitual sucrose intake without a CTA or thirst consequences, and affects activity of dispersed brain circuits involved in feeding control. This supports the notion of DOR involvement in the appetite for sugar.

3.2 Introduction

A considerable body of evidence shows that opioid peptides mediate the rewarding aspects of feeding behavior [1, 2]. This is particularly well documented for ligands that bind either selectively or predominantly to the mu receptor (MOR), and the kappa receptor (KOR). MOR agonists, such as morphine and [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO), increase consumption of preferred diets, whereas antagonists, e.g., beta-funaltrexamine (beta-FNA), naloxone and naltrexone, are highly effective at suppressing intake of palatable foods, including those high in sucrose (e.g., [1] [3], [2]). Similarly, activation of the KOR with dynorphin increases palatability-driven feeding, whereas blockade with nor-binaltorphamine (nor-BNI) reduces it [3]. The results of the injection experiments are in line with the outcomes of other studies that have shown, among other findings, changes in MOR and KOR transcript levels in response to palatable food consumption [4] as well as diminished preference for sweetened solutions in MOR-deficient and MOR-knockout (KO) mice, though not in KOR KOs [5, 6].

Unlike the MOR and KOR-related experimental work, attempts to define the role of the delta receptor (DOR) in feeding control and the effects of its highly selective and potent antagonist, naltrindole (NTI) [7], have produced contradictory results. The outcomes appear to depend on the route of drug administration, diet type, and diet presentation paradigms, among other factors. For example, DOR activation increased consumption of standard chow, but decreased intake of a high-fat diet in rodents [8]. Intracerebroventricular NTI reduced saccharin intake, but had no effect on maltodextrin consumption [9]. Krishnan-Sarin reported that peripheral NTI at 10 mg/kg suppressed consumption of saccharin (as well as of ethanol) in rats bred for alcohol preference [10]. Chow et al. found that peripheral NTI reduced consumption of saccharin, but not presentation-induced anticipatory behavior toward this sweetener, in rats [11]. Conversely, Inui and Shimura [12] found that NTI injection into the ventral pallidum (VP), a basal ganglia structure involved in reward and motivation, increased rather than decreased the consumption and the perceived palatability of saccharin. In a conditioned taste aversion (CTA) test, NTI VP administration resulted in a longer latency to the CTA response [12].

Evidence concerning sucrose consumption is more limited and similarly inconsistent. Intraventricular NTI did not significantly alter intake of a 10% sucrose solution under ad libitum conditions [13], whereas peripheral NTI reduced sucrose-reinforced responding in an operant paradigm [14]. These studies differed substantially in route of

administration and the conditions under which sucrose was presented, leaving it unclear how DOR blockade affects sucrose consumed as an established, scheduled daily meal.

In a seminal paper linking central opioid receptors with palatability-driven consumption, Pomonis and colleagues studied brain c-Fos activation in rats given 3-week daily access to a 10% sucrose solution [15]. They found that daily sucrose intake generated c-Fos immunoreactivity (IR) in several opioid receptor-expressing areas associated with reward, including the nucleus accumbens and the amygdala. Opioid receptor blockade with an anorexigenic dose of naloxone (mainly MOR blockade considering the drug's selectivity) in daily sucrose solution-fed vs water-only animals produced a significant drug x diet interaction in c-Fos IR in the central nucleus of the amygdala (CEA). However, because naloxone blocks multiple opioid receptor subtypes, this work did not establish the specific contribution of DOR signalling to either habitual sucrose intake or the associated pattern of neuronal activation.

Considering the conflicting effects of DOR blockade across sucrose paradigms, we adapted the paradigm described by Pomonis et al. [15] with minor modifications and gave animals daily access to a 10% sucrose solution for 1 h per day for three weeks. We then treated them acutely with NTI just prior to a sucrose meal to establish the dose at which this drug affects sugar intake. We also examined whether this NTI dose produces a CTA or affects deprivation-induced water intake. Finally, we studied c-Fos IR in animals consuming sucrose habitually vs sucrose-naïve water controls injected acutely with NTI or vehicle.

3.3 Methods

3.3.1 Animals

Adult male Sprague-Dawley rats were single-housed in wire-topped clear polycarbonate cages in a temperature-regulated (22 °C) vivarium maintained on a 12:12 h light-dark cycle (lights on at 07:00 h). Animals had ad libitum access to tap water and standard laboratory chow (Sharpes, Carterton, New Zealand; 3.6 kcal/g), except where experimental protocols dictated otherwise.

The experiments adhered to the NIH Guide for the Care and Use of Laboratory Animals guidelines and were approved by the Institutional Animal Ethics Committee.

3.3.2 Experiment 1: Effect of acute NTI injection in rats habituated to daily sucrose solution intake

Rats were given daily access to a sucrose solution (10 % sucrose in water w/w) for 1 h from 11:00 till 12:00 for 3 weeks. Sucrose was presented to rats via the replacement of their water bottles with identical bottles filled with a 10% sugar solution. Chow was removed during sucrose presentation. On the final day, the animals were randomly divided into groups and received an IP injection of vehicle, 5 mg/kg or 10 mg/kg NTI (Sigma-Aldrich, Burlington, MA, USA) at 11:00 (n=10/group). Ten minutes later, they received 1-h access to a pre-weighed bottle containing a 10% sucrose solution. The amount of consumed sucrose solution was established by weighing bottles. Data were analyzed with one-way ANOVA followed by Dunnett's test. Values were considered significantly different for $P \leq 0.05$

3.3.3 Experiment 2: Effect of NTI on deprivation-induced water intake

Water was removed at 17:00 on the day preceding the injection; chow was available during the hours of water deprivation. On the experimental day, rats were injected IP with vehicle or 10 mg/kg NTI (the dose that produced a significant effect in Experiment 1; n=8/group) and pre-weighed water bottles were returned to cages. Water intake was measured 1 hour later. During the 1 h period of water intake measurement, chow was taken away. Data were compared with a t-test. Differences were considered significant for $P \leq 0.05$.

3.3.4 Experiment 3: Effect of NTI on acquisition of a conditioned taste aversion to a novel saccharin solution

Rats were deprived of water at 17:00. On the subsequent day at 11:00, chow was removed from the cages and rats received a bottle containing a novel 0.1% saccharin solution instead of water. After 1 h of drinking, rats were injected IP with vehicle, 3 mEq LiCl (a positive control for CTA) or 10 mg/kg NTI (n = 7/group). During the next 2 days after the injection, animals had unrestricted access to water and chow. They were then again deprived of water overnight and, on the next day (at 11:00), chow was removed from the cages and a 1-h two-bottle (water vs. saccharin) preference test was performed to assess a development of a CTA to saccharin (water and saccharin were given in identical bottles placed side by side and the left-right position of the bottles was reversed for every other animal). Chow was available at all times except for the two-bottle test and the initial saccharin presentation.

Bottles were weighed before and after the CTA test. The amount of consumed saccharin solution was expressed as the percentage of total fluid (water + saccharin) intake during the preference test. The data were analyzed with ANOVA followed by Dunnett's post-hoc test (significant for $P \leq 0.05$).

3.3.5 Experiment 4: NTI-induced neuronal activation

We used the 10% sucrose solution meal protocol as described in Experiment 1 (i.e., sugar water given daily from 11:00 to 12:00). In addition, there was an age/weight-matched sucrose-naïve cohort of animals that instead of receiving sucrose, had a new bottle filled with water placed in the cage for 1 h from 11:00 to 12:00 for the same number of days as the sucrose-fed rats. On the experimental day, the animals belonging to each diet sub-cohorts (sucrose-fed or sucrose-naïve) were randomly divided into two groups (n=5-7/group): one of them was treated with vehicle and the other with 10 mg/kg NTI at 11:00. After the drug treatment they had no access to sucrose, water or chow until being sacrificed 1 h later.

One hour after the injection, rats were anesthetized with urethane and perfused through the aorta with 50 ml saline followed by 500 ml 4% paraformaldehyde (PFA) in 0.1 phosphate buffer (pH 7.4). Brains were excised and post-fixed overnight in PFA at 4°C. 60-µm-thick coronal sections were cut with a vibratome (Leica, Frankfurt, Germany) and processed as free-floating sections for c-Fos.

Sections were rinsed in 50 nM TBS (pH 7.4) and treated for 10 min in 3% H_2O_2 and 10% methanol (in TBS). After rinsing in TBS they were incubated overnight at 4 C in the rabbit-anti-Fos antibody (1:4000; Synaptic Systems, Murarrie, Australia) washed in TBS and incubated for 1 h in the goat-anti-rabbit antibody (1:400; Vector Laboratories, Burlingame, CA, USA). After washes in TBS, sections were incubated for 1 h in the avidin–biotin complex (1:800; Elite Kit, Vector Laboratories, Burlingame, CA, USA). The vehicle for all incubations was a TBS-based solution of 0.25% gelatin and 0.5% Triton X-100. The peroxidase in the sections was visualized with 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, and 0.3% nickel sulfate in TBS. Sections were mounted onto gelatin-coated slides, air-dried, dehydrated in ethanol, soaked in xylene (Merck KGaA, Darmstadt, Germany), and embedded in Entellan (Merck KGaA, Darmstadt, Germany). The number of Fos-IR nuclei per 1 mm^2 was counted bilaterally for each area of interest using ImageJ software. The regions included: the arcuate nucleus (ARC), area postrema (AP), basolateral amygdala (BLA), central nucleus of the amygdala (CEA), dorsal motor nucleus of the vagus (DMX), dorsomedial hypothalamic nucleus (DMH), nucleus accumbens core (AcbC), nucleus accumbens shell (AcbSh), nucleus of the solitary tract (NTS), paraventricular nucleus (PVN), suprachiasmatic nucleus (SCN), supraoptic nucleus (SON), and ventromedial hypothalamus (VMH). The boundaries were determined according to the Paxinos and Watson atlas on 2–4 sections per animal. Images captured via a CCD camera attached to a Nikon Eclipse 400 microscope were analyzed using Nikon NIS Elements software. Group means of densities of Fos-IR nuclei were compared with two-way ANOVA and values were considered significantly different for $P \leq 0.05$.

3.4 Results

Rats given daily access to a 10 % sucrose solution for 1 h readily drank the palatable fluid. On the injection day, which occurred at 3 weeks of the regimen, vehicle-treated rats drank an average of 63.9 g/kg of the sucrose solution. The IP injection of 5 mg/kg NTI had no effect on sucrose consumption; however, 10 mg/kg NTI produced a significant reduction in sucrose intake by 22 % ($P = 0.022$;Figure 3.1).

The 10-mg/kg dose of NTI that acutely suppressed sucrose solution intake had no effect on the amount of water drunk by animals deprived of water overnight (Figure 3.2). It also failed to generate a CTA to a saccharin solution, unlike an aversive agent, LiCl ($P=0.002$;Figure 3.3).

The c-Fos study (Figure 3.4) revealed that at the time of anticipated sucrose meal, vehicle-injected sucrose-fed rats had higher c-Fos IR levels in the BLA ($P = 0.018$), DMH ($P = 0.016$), VMH ($P = 0.031$) and ARC ($P = 0.012$), compared to sucrose-naïve vehicle controls (Fig. 4). In sucrose-naïve animals, NTI produced a trend toward a change in c-Fos IR in the DMH ($P = 0.17$), BLA ($P = 0.076$) and CeA ($P = 0.13$). However, in sucrose-fed rats, NTI injection generated a significant difference in the density of c-Fos-positive nuclei in the DMH ($P < 0.001$), SON ($P < 0.001$), PVN ($P = 0.041$), CEA ($p = 0.014$), AP ($P = 0.010$), NTS ($P < 0.001$) and DMX ($P = 0.007$). A significant drug treatment x diet interaction was found in the SON ($P = 0.002$, $F(1, 21) = 12.23$) and NTS ($P = 0.021$, $F(1, 17) = 6.460$).

3.5 Discussion

The reports describing the effects of DOR ligands on feeding, including on sugar consumption, have produced remarkably disparate outcomes. This contrasts with the consensus regarding drugs that target the MOR and KOR, which have been found to primarily affect eating for pleasure. For example, Kelley et al. found that intra-accumbal NTI markedly potentiated both sucrose drinking and the duration of chow feeding [16]. On the other hand, Beczkowska et al reported no effect of intraventricular NTI on sugar water consumption under ad libitum conditions [13]. Henderson-Redmon and Czachowski studied the effects of peripheral NTI in alcohol-preferring and non-selected rats given sucrose and ethanol in an operant setting. They reported that NTI suppressed sucrose-associated lever responses in both groups, and that ethanol-reinforced preferring rats were the most sensitive to the effects of NTI on intake and seeking [14].

Our experiments show, for the first time, that i.p. injection of NTI just prior to a daily meal of palatable sugar solution acutely suppresses sucrose solution intake. The NTI-induced 22 % reduction in the amount of sugar consumed is moderate compared to that induced by antagonists of other opioid receptors. For example, Head et al. found that naltrexone at 1 mg/kg b wt almost halved the episodic intermittent 10 % sugar solution intake in rats, whereas a low 0.3-mg dose generated a 33 % reduction [17]. Ward and colleagues reported that AcbSh infusion of beta-FNA decreased sucrose solution intake by 40% [18], whereas Leventhal et al. showed that 20 % sucrose intake in sham-fed rats was diminished by beta-FNA by 44 % and by nor-BNI by 62 % [19].

It should also be noted that NTI at the high 10-mg/kg dose was effective, whereas 5 mg/kg NTI – the dose that had been earlier shown to reduce ethanol intake [14] - did not affect sucrose solution consumption. Consequently, this raises a question as to whether the anorexigenic effect of 10 mg/kg NTI in our study might have been caused by sickness or malaise induced by NTI at this relatively high dose. Our CTA experiment determined that 10 mg/kg NTI administered just after the presentation of a novel saccharin solution did not generate an aversion to saccharin, whereas positive control rats treated with a standard aversive agent, LiCl, acquired a CTA. The lack of a CTA response to NTI is in agreement with the outcome of the previous NTI VP study, which showed a longer rather than shorter latency to the CTA response in VP NTI-treated animals [12].

It is also unlikely that the suppression of sucrose solution consumption in NTI-treated rats was secondary to a reduction in thirst. Although dorsal striatal infusions of NTI decrease water intake [20], peripheral NTI has not been found to affect water-drinking behavior (even at doses that reduce ethanol and saccharin consumption) [10]. Similarly, in our

experiment, NTI-injected rats that had been deprived of water overnight did not consume a different amount of water in the post-deprivation drinking session.

Overall, the results of our behavioral studies support the notion that NTI (and, thus, the DOR) affects intake of a palatable sucrose solution. They are in line with the earlier findings that NTI (similar to naloxone) enhances suppression of sugar solution intake after a shift from 32% to 6% sucrose concentration relative to unshifted controls, suggesting the involvement of the DOR in modulating sucrose-driven reward [21].

Our data corroborated earlier findings from studies using sweet solutions and solid palatable diets [2, 15, 22], showing that habitual access to a palatable diet is associated with altered activation across brain regions involved in reward and energy homeostasis. In the present study, vehicle-treated rats with a history of habitual sucrose access showed greater c-Fos IR in the BLA, DMH, VMH and ARC than sucrose-naïve vehicle controls. Importantly, sucrose was not presented on the experimental day, meaning that these differences cannot be attributed to the immediate sensory or postingestive effects of sucrose consumption. Instead, they may reflect adaptations associated with repeated sucrose exposure and/or anticipation of the scheduled sucrose meal. Consistent with this interpretation, neuronal activation within the ARC and particularly the DMH has been associated with anticipation of food presented on a predictable schedule [23]. The BLA is also involved in encoding and retrieving the incentive value of anticipated food rewards [24].

In sucrose-naïve rats, comparisons between vehicle- and NTI-treated animals showed no significant effect of NTI in any region, although non-significant trends were observed in the DMH, BLA and CEA. In animals habitually exposed to sucrose, corresponding comparisons between vehicle- and NTI-treated groups identified significant differences in c-Fos IR in the DMH, SON, PVN, CEA, AP, NTS and DMX. Although this pattern suggests a broader response to NTI following habitual sucrose exposure, a significant drug treatment × diet interaction was detected only in the SON and NTS. These two regions therefore provide the clearest statistical evidence that the neuronal response to NTI differed according to previous sucrose exposure. Thus, significant effects of NTI were observed across a broader set of regions in animals with a history of habitual sucrose exposure than in sucrose-naïve animals, similar to what has previously been described following MOR-predominant antagonism with naloxone [15] and following treatment with the non-opioid anorexigen oxytocin [22]. The anatomical distribution of DORs provides some context for these effects, although it does not map directly onto the pattern of NTI-induced c-Fos IR. Relatively high DOR densities have been reported in regions

including the BLA and NAc, whereas DOR binding is comparatively sparse throughout much of the hypothalamus, with moderate binding reported in the VMH [25] [26]. Consequently, changes following systemic NTI in regions such as the PVN, SON and DMH should not necessarily be interpreted as evidence of direct DOR blockade within these nuclei, but may instead reflect downstream recruitment of interconnected feeding and neuroendocrine pathways.

The significant treatment × diet interaction in the NTS is particularly notable given the role of this region in processing gustatory and visceral information. Functional DOR signalling has been demonstrated in gustatory NTS neurons, where DOR activation reduced excitatory synaptic input and this effect was prevented by naltrindole [27]. Although this work was conducted in hamsters rather than rats, it demonstrates that DOR signalling can directly modify gustatory processing within the NTS. The present interaction therefore raises the possibility that habitual sucrose exposure alters the contribution of opioid signalling to processing within this pathway. In contrast, the SON interaction may reflect downstream recruitment of neuroendocrine circuitry rather than local DOR blockade, particularly given the relatively sparse DOR distribution reported across much of the hypothalamus [26].

The absence of a significant c-Fos response in the NAc is noteworthy given the relatively high DOR expression in this region [25] and evidence that local DOR antagonism can alter sucrose consumption. Intra-accumbal NTI has been reported to increase sucrose drinking [16], while DOR antagonism within the NAc shell has also altered sucrose licking in an anticipatory contrast paradigm [28]. However, the absence of a c-Fos difference does not necessarily indicate that the NAc or its DORs were uninvolved. Neuronal responses within the NAc during sucrose-related behaviour are heterogeneous, and substantial populations of NAc neurons decrease rather than increase their firing during sucrose consumption [29]. Such cell-specific or inhibitory responses may not produce a detectable change in overall c-Fos IR. Furthermore, the present experiment assessed neuronal activation at the anticipated time of a sucrose meal without sucrose presentation, whereas the local pharmacological studies examined responses associated with sucrose consumption.

These findings exemplify the complexity of the neural pathways affected by habitual sugar intake and suggest that previous sucrose exposure alters the neural context in which DOR blockade acts. These data further substantiate the claim that there is a functional relationship between the DOR and consumption of sucrose and suggest that the use of

DOR ligands should be studied in the context of their ability to modify palatability-driven food intake, including appetite for sugar.

Overall, we conclude that NTI suppresses sucrose solution intake without causing a CTA or affecting thirst and that its effect on consumption is associated with changes in neuronal activation in brain areas involved in homeostatic and reward-related control of feeding behavior. Of relevance to the overarching aim of this thesis, these findings indicate that any null effects of NTI on sucrose intake reported in ad hoc feeding paradigms should not be assumed to reflect a failure to suppress sucrose consumption once sucrose intake is habitual.

3.6 Figures

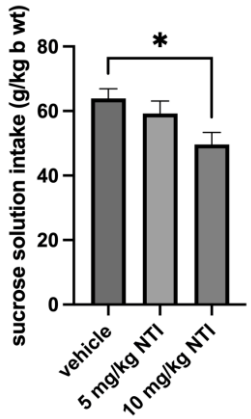

Figure 3.1 Effect of intraperitoneal vehicle, 5 mg/kg or 10 mg/kg NTI on 1-h intake of a 10% sucrose solution. Non-deprived rats were accustomed to getting daily 1-hour access to sucrose for three weeks prior to the IP treatment. Data are means ± SEM; * - $p \leq 0.05$

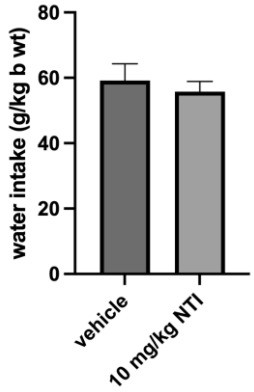

Figure 3.2 Effect of intraperitoneal vehicle vs 10 mg/kg NTI on water consumption after overnight water deprivation. Data are means ± SEM.

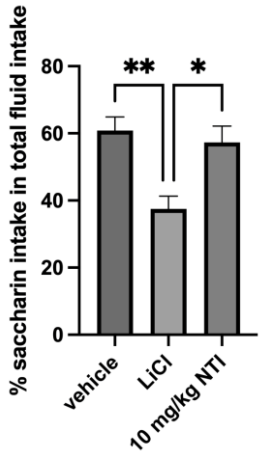

Figure 3.3 Conditioned taste aversion (CTA) acquisition in rats treated with LiCl (positive CTA control) or 10 mg/kg NTI versus vehicle-treated controls. Data are means ± SEM; * $p<0.05$; **$p<0.01$

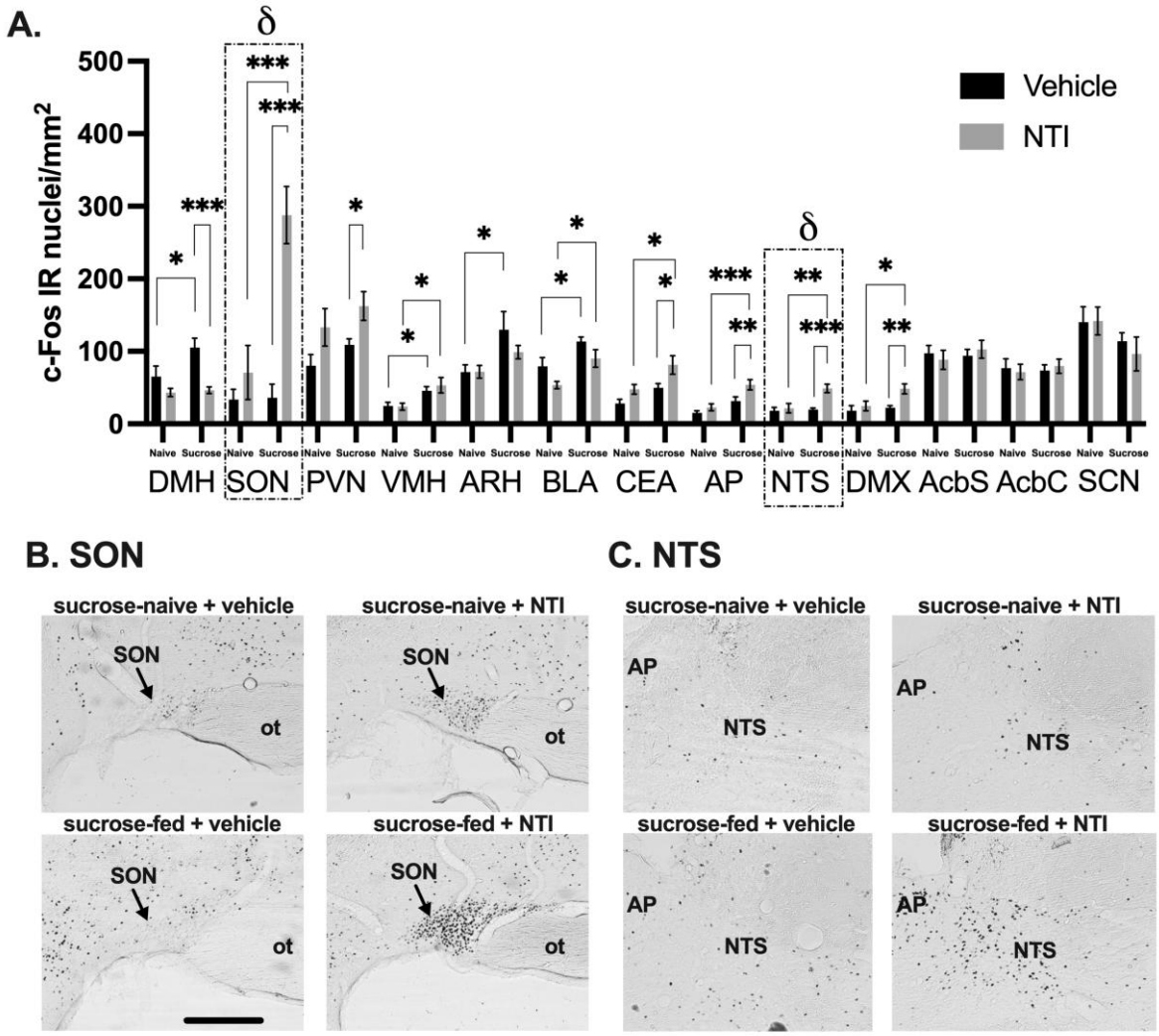

Figure 3.4 Effect of an intraperitoneal injection of vehicle vs 10 mg/kg NTI in rats that were sucrose-naïve ("Naïve") and in rats that received habitual daily 1-h access to 10% sucrose solution ("Sucrose") on c-Fos in feeding-related brain areas (A). Sucrose-naïve animals were given a new water bottle at the time corresponding to the presentation of sucrose in sucrose-fed groups. The boxes (δ) around the SON and NTS define a significant treatment x diet interaction and the photomicrographs depict staining in those two areas (B: SON and C: NTS) * $p<0.05$; ** $p<0.01$, scale bar corresponds to 0.2 mm (B) and 0.1 mm (C).

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Chapter 4

Effect of Simultaneous Peripheral Oxytocin and Naltrindole Injection at a Subthreshold Dose on Saccharin Solution Intake and Neuronal Activation in Mice

4.1 Abstract

Oxytocin (OT) and endogenous opioids contribute to central control of food intake, with OT influencing energy- and reward-driven feeding and opioids primarily influencing reward-motivated food intake. Prior reports indicate that simultaneous targeting of these receptors can synergistically reduce food intake; combined administration of OT and the opioid receptor antagonist naltrexone (NTX) has been shown to reduce food intake and eating behaviours in both a clinical case of hypothalamic obesity and in rodents at doses of drugs that were ineffective alone. Here we tested whether co-administration of OT with naltrindole (NTI), a selective δ-opioid receptor (DOR) antagonist, reduces liquid-diet intake in contexts which single-drug treatments have produced mixed results. Adult male C57BL/6 mice received intraperitoneal OT (0.1–3.0 mg/kg), NTI (3.0–10 mg/kg), or OT+NTI, and the 2-h consumption of saccharin, sucrose, or Intralipid was measured. To determine whether any effects on food intake were reflective of changes to thirst, we also assessed water intake after a15-h water deprivation period. Finally, the effect of OT+NTI on neuronal activity in feeding-related brain regions was measured using c-Fos immunohistochemistry. NTI, but not OT, reduced saccharin consumption when administered individually. OT+NTI treatment resulted in a further reduction in saccharin intake relative to NTI treatment alone. OT decreased sucrose intake, while NTI did not, and the combination treatment did not exceed the effect of OT alone. None of the treatments altered Intralipid intake. Reductions in liquid-diet intake occurred independently of changes in thirst. c-Fos analysis revealed an interactive effect of OT × NTI on neuronal activation in the nucleus accumbens shell and core as well as in the ventromedial and suprachiasmatic nuclei of the hypothalamus. OT increased c-Fos immunoreactivity in the paraventricular and dorsomedial hypothalamic nuclei. Treatment with each drug also influenced neuronal activity in the basolateral and central nuclei of the amygdala. The failure of NTI to reduce sucrose intake in this experiment, compared

with the earlier chapter, may stem from ad hoc access in this study rather than the habitual access used previously. The present results align with prior chapters indicating that OT does not curb Intralipid intake. We conclude that co-administration of OT and NTI synergistically decreases saccharin, but not sucrose or Intralipid consumption, and that the change in intake is accompanied by alterations in neuronal activity across a range of feeding-related brain regions.

4.2 Introduction

Central control of food intake involves oxytocin (OT) and endogenous opioids. OT affects both energy- and reward-driven feeding (reviewed in [1]), while the opioid system primarily regulates reward-motivated intake (reviewed in [2, 3]). Recent work indicates that targeting these systems simultaneously is particularly effective at reducing food consumption, suggesting a functional interaction. This interaction has been demonstrated in a case study involving an adolescent with hypothalamic obesity and uncontrolled food-seeking after craniopharyngioma resection. Co-administration of OT and naltrexone (NTX), a non-selective opioid receptor antagonist with high affinity for the μ-opioid receptor (MOR), was more effective than OT alone at improving management of energy intake [4]. To further explore this clinical finding, Head et al. demonstrated that combined administration of OT+NTX decreased intake of 10% sucrose in rats at a dose that was ineffective when administered individually [5]. This synergistic effect of simultaneous OT and opioid receptor antagonism, which enabled subthreshold inhibition of food intake, suggests potential for targeting consumption of foods not effectively suppressed by single-drug administration.

The opioid system is implicated in the regulation of food intake. In addition to MOR, two more classes of opioid receptor exist: kappa-opioid receptor (KOR), and delta-opioid receptor (DOR). Of these, MOR is the most well-understood and the effects of MOR ligands on food consumption have been widely explored in the literature. Generally, MOR agonists increase consumption of palatable foods [6-8], whereas MOR antagonists decrease palatable food consumption [7, 8]. In contrast, the relationship between DOR and food intake is the least well-understood, with inconsistent reports of its effects. Naltrindole (NTI), a selective DOR antagonist, reduces saccharin consumption in some studies [9-13], whereas other reports show that NTI either fails to reduce saccharin or increases it [14-16]. Previous studies describing the role of DORs in fat intake regulation are likewise mixed: central DOR agonism suppresses consumption of a solid high-fat diet [17], whereas NTI does not alter intake of a lipid emulsion [18]. The effects of NTI treatment on sucrose consumption are also inconsistent: NTI was ineffective in mice familiar with sucrose but not consuming it habitually [18], whereas in Chapter 3 we showed that NTI suppressed sucrose solution intake in rats that habitually consumed it. Because DOR antagonism shows mixed effects on saccharin, fat, and sucrose intake, we hypothesised that concurrent targeting of DOR and OT receptors would suppress the intake of these liquid diets more than DOR antagonism alone.

OT is also a potent anorexigen, with a pronounced suppressive effect on the consumption of palatable, energy-dense foods [19-21] and on sucrose [22, 23]. However, OT's anorexigenic effects vary by diet and remain incompletely characterised. OT often reduces intake of sweet non-caloric solutions, although findings are mixed [24-28]. Similarly, the effects of OT on fat intake remain unclear: solid high-fat foods are suppressed by OT [19-21, 29-32], whereas liquid fat emulsions, such as Intralipid, a soybean oil-based fat emulsion used as a liquid source of dietary fat, are not [33-36]. In Chapter 2, we systematically showed that OT suppresses fatty food intake when the foods are not lipid emulsions. Because OT alone is insufficient to curb the consumption of some liquid solutions, including saccharin and emulsified fat, we hypothesised that combining OT with an opioid receptor antagonist would improve suppression.

Given the mixed single-drug effects of OT and NTI on saccharin, sucrose, and Intralipid intake, we tested whether co-administration would synergistically reduce consumption of these liquid diets. Because prior reports on the effects of OT and NTI on saccharin consumption are inconsistent, we first established each drug's effects on saccharin intake across a range of doses. We then measured the effect of simultaneous injection of OT and NTI on saccharin, sucrose, and Intralipid consumption. Intralipid was included to determine whether concurrent targeting of the OT and DOR systems could overcome the resistance of emulsified fat intake to suppression by either drug alone. To determine whether any observed effects reflected changes in thirst, we quantified water intake after a 15-h period of water deprivation. Finally, we examined c-Fos immunoreactivity in feeding-related brain regions after i.p. saline, OT, NTI, or the combination.

4.3 Materials and methods

4.3.1 Animals and Drugs

Adult male age-matched mice (C57BL/6, AgResearch Hamilton New Zealand, 22 ± 5 g and ~50 days post-natal at study onset) were single-housed in clear plexiglass cages. The cages were located in a light- and temperature-controlled vivarium (22 ºC, L:D 12:12, lights on at 07:00 h). Mice were maintained on standard chow (Sharpes, Carterton New Zealand) and water and had ad libitum access except where experimental protocols dictated otherwise.

All procedures adhered to the NIH Guide for the Care and Use of Laboratory Animals guidelines and were approved by the University of Waikato Animal Ethics Committee (protocol number: 1211).

4.3.2 Feeding Studies

4.3.2.1 Effect of i.p. OT, NTI, or simultaneous administration on saccharin solution consumption

Because the literature describing the effect of OT and NTI on saccharin solution intake was mixed, we first needed to determine the individual effects of these drugs on saccharin consumption across a range of doses. Animals in this study were not deprived of food or water prior to testing to diminish the influence of thirst and hunger on feeding.

Mice were accustomed to consuming 0.1% saccharin solution for 2 h/day on 3 days prior to experimentation to prevent neophobia. To determine the influence of OT on saccharin consumption, on experiment day (10:00 am), mice were given an i.p. injection of OT (Sigma, St Louis, U.S.A.; 0.1, 0.3, or 3.0 mg/kg) or vehicle (0.9% saline for OT) (n=15/group). Food and water were removed immediately after injection. Thirty minutes after injection, pre-weighed bottles containing saccharin solution were given to the mice in place of their standard water bottle. After 2 h, the bottle of saccharin was removed and weighed to assess consumption, correcting for spillage.

The same protocol was used to determine the effect of NTI and simultaneous NTI and OT on saccharin consumption. The same cohort of mice was used in each experiment. The mice were randomly assigned to groups each test, and the body weights did not differ between groups; a washout period of 3 days elapsed between each experiment. To assess the influence of NTI on saccharin solution intake, mice were injected with either NTI (Sigma-Aldrich, Burlington, MA, U.S.A.; 3.0, or 10 mg/kg in 5% DMSO) or vehicle (5%

DMSO for NTI)(n=15/group). The effect of simultaneous NTI and OT was tested by giving mice either vehicle+NTI (saline + 10 mg/kg NTI), or OT+NTI (0.3 or 3.0 mg/kg OT + 10 mg/kg NTI) (n=20/group). The use of a 10 mg/kg dose of NTI to test the simultaneous effect of OT+NTI was selected because it was the only dose which reduced saccharin consumption, 0.3 and 3.0 mg/kg OT were used because they were the highest doses of OT tested and no dose approached a significant reduction in saccharin consumption in individual testing.

Data were collected as mass of solution consumed and normalised to volume of solution consumed per unit mass of body weight (ml/kg b wt) and analysed by one-way ANOVA with Dunnett's post hoc tests. Values are presented as means ± SEM and were deemed significantly different when $p \leq 0.05$.

4.3.2.2 Effect of i.p. OT, NTI, or simultaneous administration on sucrose or Intralipid solution consumption

To determine if the observed synergistic effect of simultaneous i.p. OT+NTI on saccharin solution consumption would also influence the intake of other liquid diets of interest, we assessed the consumption of sucrose and Intralipid solutions following treatment. The same cohort used for the saccharin tests was employed here. Animals were pre-exposed to 15% sucrose and 4.1% Intralipid solution, and consumption was measured using the protocol described above. Mice received i.p. injections of vehicle+vehicle, vehicle+NTI (10 mg/kg), OT (3.0 mg/kg)+vehicle, or OT (3.0 mg/kg)+NTI (10 mg/kg) (n = 15/group) prior to access to either sucrose or Intralipid.

Data were analysed by two-way ANOVA with Šidák-corrected post hoc tests. Values are presented as means ± SEM and were deemed significantly different when $p \leq 0.05$.

4.3.3 Influence of i.p. OT and NTI on post-deprivation water acceptance

To examine whether the effects of simultaneous i.p. OT+NTI on liquid diet consumption were caused by a general reduction in water intake, we measured post-deprivation water acceptance following treatment.

Mice were water-deprived for 15 h overnight. On the test day, animals were randomly assigned to the same treatment groups as described in the sucrose and Intralipid acceptance tests and received i.p. injections. Thirty minutes later, water was returned for 2 h and intake was measured.

Data were analysed by two-way ANOVA with Šidák-corrected post hoc tests. Values are presented as means ± SEM and were deemed significantly different when $p \leq 0.05$.

4.3.4 Neuronal activation after i.p. OT and NTI injection

The effect of i.p. OT+NTI on neuronal activity was investigated using c-Fos immunohistochemistry. The same cohort of mice used in the solution acceptance and water deprivation tests was employed, following a 2-week washout period.

At 10:00 am (3 h into the light phase), mice received i.p. injections of either vehicle+vehicle, vehicle+NTI (10 mg/kg), OT (3.0 mg/kg)+vehicle, or OT (3.0 mg/kg)+NTI (10 mg/kg) (n=8/group). Food and water were removed immediately following injection and was not returned to the animals. Ninety minutes later, mice were anesthetized with 35% urethane, and they were transcardially perfused with saline followed by 50 ml of 4% paraformaldehyde (PFA) in TBS. The excised brains were postfixed for 48 h in PFA at 4 ºC. Coronal 60-µm sections were sliced using a vibratome (Leica, Germany) and processed as free-floating sections.

Sections were incubated in 10% methanol and 3% H_2O_2 (in TBS; 10 min). Following a TBS rinse, they were incubated in the rabbit-anti-Fos antibody (Synaptic Systems, Australia) in 0.25% gelatine and 0.5% Triton X-100 (Sigma, USA) in TBS overnight at 4°C. The sections were rinsed in TBS and incubated for 1 h in the goat-anti-rabbit antibody (Vector, USA). After TBS rinses, they were incubated in the avidin-biotin complex (Vector, USA) for 1 h. The colour reaction was initiated with 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, and 10% nickel sulphate in TBS (15 min). Sections were mounted on gelatine-coated slides, air dried, dehydrated in ethanol, soaked in xylene, and cover slipped in Entellan (Sigma, Germany).

Images were captured under a Nikon H55OS (Nikon Instruments, Melville, USA) microscope equipped with an OMAX camera (Omax, Kent, USA). The regions of interest, outlined using the Allen Brain Atlas (ABA; anterior: posterior bregma ranges in the parentheses), were: AcbC – NAc core (1.28 : 0.96); AcbS – NAc shell (as AcbC); ARC – arcuate nucleus (−2.14: −2.52); BLA – basolateral amygdala (−2.64: −2.92); CEA – central nucleus of the amygdala (−2.64: −2.92); DMH – dorsomedial nucleus (−2.80: −3.24); DMV – dorsal motor nucleus of the vagus (−13.76: −14.16); NTS – nucleus of the solitary tract (−13.70: −14.16); PVN – paraventricular nucleus (−1.56: −1.92); and SON – supraoptic nucleus (−0.96: −1.20); VMH – ventromedial nucleus (−2.80: −3.24)

Each region was outlined as per ABA and its area measured in mm^2 (ImageJ, NIH, Bethesda, USA). Fos-immunoreactivity nuclei were counted on four to eight sections per region per animal. The numbers were added for each region in each animal and densities of activated nuclei per mm2 were calculated.

Data were analysed by two-way ANOVA with Šidák-corrected post hoc tests. Values are presented as means ± SEM and were deemed significantly different when $p \leq 0.05$.

4.4 Results

Intraperitoneal OT (0.1, 0.3, and 3.0 mg/kg) did not decrease saccharin intake (Figure 4.1). NTI decreased saccharin consumption at a dose of 10 mg/kg ($p = 0.0417$) (Figure 4.2). When co-administered, 0.3 mg/kg OT + 10 mg/kg NTI did not reduce saccharin intake relative to 10 mg/kg NTI alone, whereas 3.0 mg/kg OT + 10 mg/kg NTI significantly decreased consumption relative to treatment with 10 mg/kg NTI alone ($p = 0.0478$) (Figure 4.3).

A two-way ANOVA revealed a main effect of OT on sucrose intake ($F(1,52) = 15.34$, $p < 0.001$). Post-hoc tests showed reduced sucrose consumption in OT+vehicle treated animals versus vehicle+vehicle ($p = 0.027$) and after OT+NTI injection versus vehicle+NTI ($p = 0.009$) (Figure 4.4). In contrast, NTI, OT, or OT+NTI did not affect Intralipid consumption (Figure 4.5).

Post-deprivation water intake was not affected by OT, NTI, or their combination. Although a two-way ANOVA detected an OT × NTI interaction ($F(1,55) = 4.800$, $p = 0.033$), Šidák-corrected post-hoc tests confirmed no differences from vehicle+vehicle controls in any group (all $p > 0.05$) (Figure 4.6).

c-Fos immunoreactivity was examined across mesolimbic, hypothalamic, and brainstem regions following i.p. vehicle+vehicle, OT+vehicle, vehicle+NTI, or OT+NTI injection (Figure 4.7). Overall, treatment effects were region-dependent, with OT × NTI interactions occurring in reward-related and hypothalamic regions, while other regions showed effects attributable to individual drug treatments or no significant treatment effects.

In the nucleus accumbens, significant OT×NTI interactions on neuronal activation were detected in both the shell ($F(1,21) = 4.979$, $p = 0.037$) and the core ($F(1,21) = 17.09$, $p < 0.001$).

OT increased c-Fos immunoreactivity in the CEA ($F(1,23) = 105.9$, $p < 0.001$); activation was higher in OT+vehicle treated rats than in vehicle+vehicle treated rats ($p < 0.001$) and following NTI+OT treatment vs vehicle+NTI treatment ($p < 0.001$). In the BLA, NTI decreased activation ($F(1,23) = 8.446$, $p = 0.008$), whereas OT increased it ($F(1,23) = 74.44$, $p < 0.001$); activation in OT+NTI treated animals was lower than in OT+vehicle treated mice ($p = 0.005$).

OT × NTI drug interactions on c-Fos immunoreactivity were observed in the VMH ($F(1,22) = 5.927$, $p = 0.023$) and SCH ($F(1,20) = 10.08$, $p = 0.005$). Both OT ($F(1,20) = 11.77$, $p = 0.003$) and NTI ($F(1,20) = 28.00$, $p < 0.001$) increased neuronal activity in the SCH; post hoc tests showed higher activation in vehicle+NTI and OT+vehicle treated rats

versus vehicle+vehicle treated animals (both $p < 0.001$). OT also increased activation in the DMH ($F(1,22) = 5.742$, $p = 0.026$) and PVN ($F(1,20) = 66.11$, $p < 0.001$), with greater activation in the PVN following OT+vehicle treatment than vehicle+vehicle treatment ($p < 0.001$) and in NTI+OT than vehicle+NTI ($p < 0.001$). No significant effects were observed in the ARH, LHA, or SON.

In the brainstem, no effects of OT, NTI, or their combination were detected in the NTS or DMX.

4.5 Discussion

To our knowledge, this is the first study to examine co-administration of OT and NTI for its effects on food intake. Prior work demonstrating co-administration of OT with opioid receptor antagonists is limited. Hsu et al. [4] and Head et al. [5] reported that a combination treatment of OT and NTX synergistically reduces food intake. Here we extend that approach using the less well-understood DOR antagonist, NTI. Outside of feeding, interactions between DOR and OT have been observed, including the involvement of DOR in OT-induced antinociception [37]. Considering both the reported OT+NTX synergy and the interactions between DOR and OT described in the literature, we tested whether simultaneous injection of OT and a DOR antagonist would suppress food intake, with particular interest in liquid diets that were modestly or inconsistently suppressed by either drug alone.

Among the tastants tested, the literature describing the effect of single-drug administration on saccharin consumption was the most conflicting. NTI has been reported to increase, decrease, or have no effect on saccharin solution intake [9-16]. OT is likewise an inconsistent suppressor of the intake of non-caloric sweet solutions [24-28]. Because the effects of OT and NTI on saccharin had not yet been established in our own work, we first assessed the influence of both drugs individually across doses. OT did not alter saccharin intake at any dose tested. NTI reduced saccharin intake at 10 mg/kg, a dose shown in Chapter 3 to be below the aversive threshold and not to induce conditioned taste aversion. We then evaluated whether co-administration of OT and NTI would produce a stronger effect than NTI treatment alone. The combination of 3.0 mg/kg OT with 10 mg/kg NTI suppressed saccharin intake relative to treatment with vehicle and NTI. Although the tastant and the opioid receptor target differ, this observation mirrors interactions described in the literature where co-administration of OT with NTX is more effective at reducing food intake than either drug alone [4, 5].

Because OT reliably inhibits sucrose consumption [33, 34, 36, 38], we did not establish an OT dose-response for sucrose. Likewise, because in Chapter 3 we showed that 10 mg/kg NTI suppressed sucrose intake in rats accustomed to scheduled sucrose, we did not develop a dose-response for NTI. We therefore tested four conditions: vehicle+vehicle (V+V), OT 3.0 mg/kg + vehicle (OT+V), vehicle + NTI 10 mg/kg (V+NTI), and OT+NTI. Only OT reduced sucrose intake. This reduction was expected and concordant with prior literature. NTI had no effect, as V+V did not differ from V+NTI and OT+V did not differ from OT+NTI. The lack of an NTI effect in the present study compared to Chapter 3 likely reflects the ad hoc, rather than habitual, sucrose access used

in this experiment; these findings are consistent with other work that has described the effect of NTI on ad hoc sucrose consumption [18]. No synergistic effect of OT+NTI on sucrose was observed, indicating that the combined effect seen with saccharin may be diminished in the presence of caloric feedback.

In Chapter 2 we demonstrated that OT suppresses the intake of solid high-fat foods despite existing literature reporting no effect on fat emulsions [33-36]. To build on this knowledge, we tested whether concurrent OT receptor and DOR targeting would overcome the single-agent limitations in suppressing Intralipid consumption. In the present study, neither OT, NTI, nor OT+NTI reduced Intralipid intake. This outcome reinforces that OT does not suppress the intake of fatty emulsions [33-36] and adds to the evidence that NTI also fails to curb Intralipid consumption [18]. Among other possibilities, such as a general lack of OT+NTI synergy for fat consumption or a persistent resistance of Intralipid to intake inhibition, the absence of an OT+NTI effect on Intralipid may suggest that the synergistic effect of these drugs is attenuated in the presence of caloric feedback.

The observed effects on tastant consumption did not appear to reflect a general reduction in water intake. After 15-h water deprivation, the consumption of water was not reduced by OT, NTI, or a combination of the two drugs. An OT × NTI interaction was detected for water intake, but no treatment caused a significant change in water consumption relative to vehicle-treated controls. This supports previously published results describing the effects of OT and NTI on water intake: NTI reduces water consumption when infused into the dorsal striatum [39], but generally does not reduce water intake after i.p. or i.c.v. administration [12, 14]. OT was reported to reduce water consumption in some early studies [40], but more recent work typically finds no effect on water intake [26] [36].

c-Fos analysis showed that OT and NTI altered neuronal activation in feeding-related brain regions. The nucleus accumbens is a centre for the regulation of reward-driven feeding. In the shell, opioid signalling affects hedonic aspects of food intake [41, 42], whereas in the core, opioids increase reward value and attenuate satiety signals that limit consumption [43]. OT injected into the AcbC, but not the AcbS, decreases sucrose and saccharin consumption in non-deprived rats [25]. The observed OT × NTI interactions in accumbens c-Fos may reflect the concerted actions of the opioid and OT systems within reward-focused feeding-related circuitry.

c-Fos immunoreactivity was also altered in the amygdala. The amygdala includes the basolateral (BLA) and central (CEA) nuclei. The BLA integrates the reward value of stimuli (reviewed in [44]). For example, learning a positive post-training shift in a food's

incentive value requires an intact BLA and relies on a MOR-dependent mechanism [45, 46]. The CEA also participates in the regulation of food intake. Intra-CEA MOR agonism is orexigenic, and concurrent MC3R/MC4R activation in the PVN attenuates this effect, indicating a functional link between the regions in intake regulation [47]. In the present study, OT increased neuronal activity in both the CEA and BLA, whereas NTI decreased activity in the BLA. OT is known to act in both of these regions: injections into the BLA or CEA reduce deprivation-induced food intake, and OT in the BLA reduces palatable solution consumption [22]. NTI's reduction of BLA activity is notable given the abundant DOR expression and the receptor's unclear role in feeding, and the anxiolytic effect of intra-BLA DOR agonism points to a possible anxiety-related mechanism [48, 49].

Administration of OT, NTI, or OT+NTI increased c-Fos immunoreactivity in several hypothalamic nuclei involved in homeostatic energy regulation [50]. OT × NTI interactions were observed in the ventromedial (VMH) and suprachiasmatic (SCH) nuclei of the hypothalamus. The VMH contributes to energy-driven intake regulation [51, 52]; intra-VMH OT decreases chow intake in deprived and scheduled-feeding paradigms, but not saccharin in rats [52]. If the VMH interaction relates to the observed synergistic effect of OT+NTI on saccharin intake, it may indicate that VMH-mediated suppression of food intake is influenced by the opioid system. The SCH interaction suggests a circadian component to treatment sensitivity during the test window [53]. The presence of interactions in these homeostatic hypothalamic sites is interesting given that synergistic inhibition of solution intake was only observed with saccharin, which is non-caloric. It has been previously proposed that, owing to generalisation of sweet taste, non-caloric sweeteners may be treated as pseudo-caloric stimuli that engage carbohydrate-related homeostatic circuits [33]. The observed increase in c-Fos immunoreactivity in the PVN after treatment with OT was expected, as the PVN synthesizes OT and expresses abundant receptors consistent with local feedback [54]. OT also increased neuronal activity in the dorsomedial nucleus of the hypothalamus (DMH). The DMH is known to receive oxytocinergic input [54] and participate in energy- and reward-related control of food intake through other neurotransmitters, such as neuropeptide-Y and cholecystokinin [55, 56].

Considering the c-Fos findings across the nucleus accumbens, amygdala, and hypothalamus, we cannot specify a single mechanism of action. Nonetheless, the activation of a range of feeding-related regions by OT, NTI, and OT+NTI is reasonable given the observed effects on solution consumption. The immunoreactivity data suggest a multi-site response to treatment rather than a single locus of effect. Notably, interactions

in reward- and energy-related regions may underlie the synergistic effect of OT+NTI on saccharin consumption, a highly palatable but non-caloric tastant. No synergistic effect was observed when caloric feedback was present with sucrose or Intralipid.

We conclude that simultaneous administration of OT and NTI reduced saccharin consumption to a greater extent than treatment with either drug alone. This synergistic effect appeared to be specific to saccharin consumption, with combination administration failing to produce any effect beyond what was observed with individual drug administration for both sucrose and Intralipid solutions. Furthermore, treatment with OT, NTI, or a combination resulted in changes in c-Fos immunoreactivity in a range of feeding-related circuits involved in both reward- and energy-driven food intake regulation.

4.6 Figures

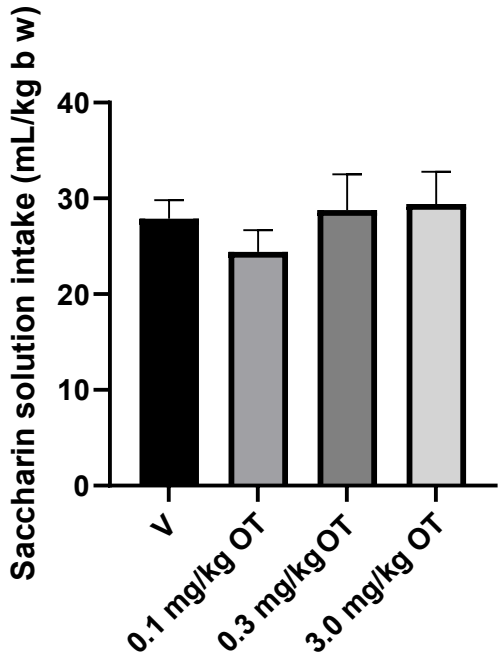

Figure 4.1 Effect of intraperitoneal vehicle, 0.1 mg/kg. 0.3 mg/kg, or 3 mg/kg OT on 2 h intake of a 0.1% saccharin solution. Data are means ± SEM.

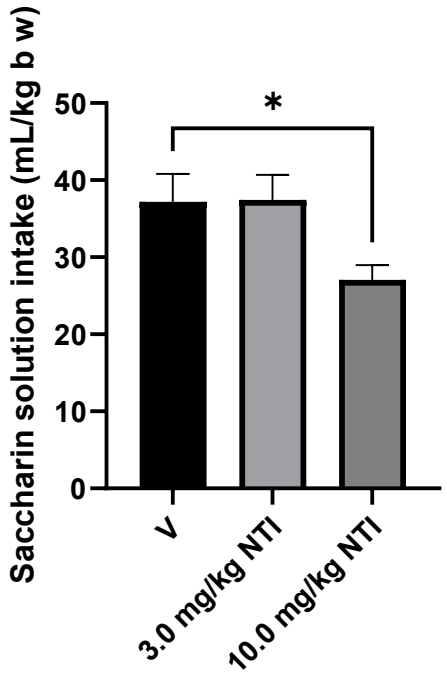

Figure 4.2 Effect of intraperitoneal vehicle, 3.0 mg/kg, or 10.0 mg/kg NTI on 2 h intake of a 0.1% saccharin solution. Data are means ± SEM.

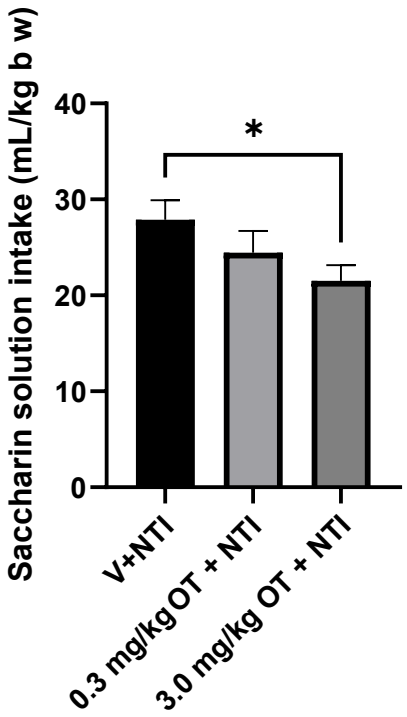

Figure 4.3 Effect of simultaneous intraperitoneal vehicle, 0.3 mg/kg, or 3.0 mg/kg OT and 10 mg/kg NTI on 2 h intake of a 0.1% saccharin solution. Data are means ± SEM; * $p<0.05$.

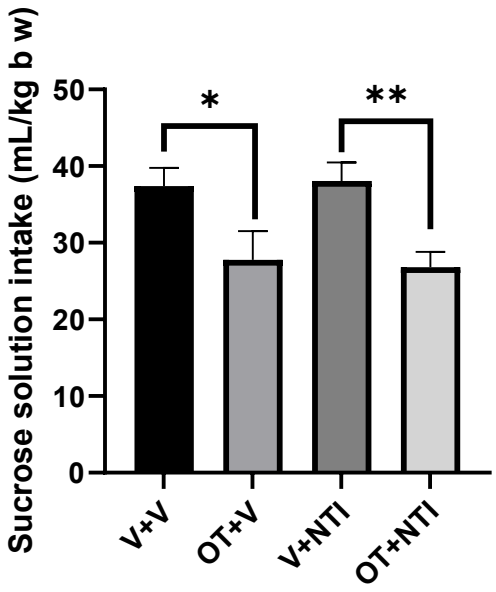

Figure 4.4 Effect of simultaneous intraperitoneal vehicle+vehicle, vehicle+10 mg/kg NTI, 3.0 mg/kg OT+vehicle, or 3.0 mg/kg OT and 10 mg/kg NTI on 2 h intake of a 15% sucrose solution. Data are means ± SEM; * $p<0.05$; ** $p<0.01$.

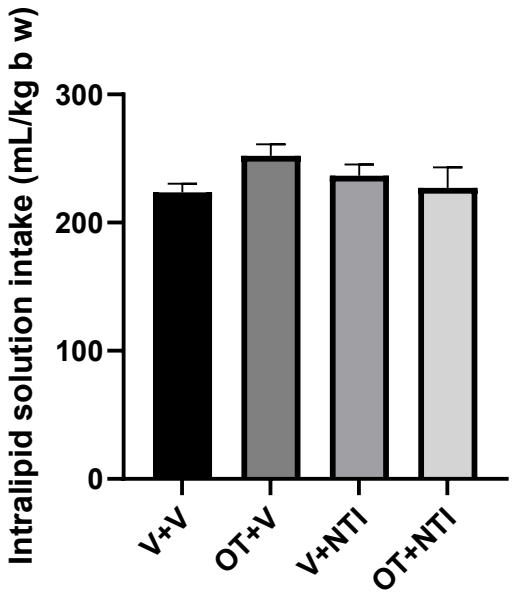

Figure 4.5 Effect of simultaneous intraperitoneal vehicle+vehicle, vehicle+10 mg/kg NTI, 3.0 mg/kg OT+vehicle, or 3.0 mg/kg OT and 10 mg/kg NTI on 2 h intake of a 4% Intralipid solution. Data are means ± SEM.

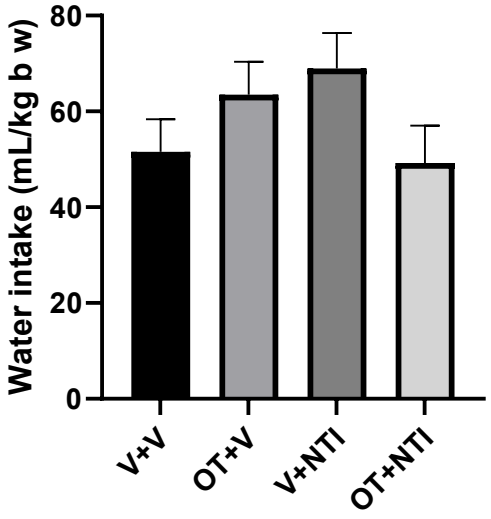

Figure 4.6 Effect of simultaneous intraperitoneal vehicle+vehicle, vehicle+10 mg/kg NTI, 3.0 mg/kg OT+vehicle, or 3.0 mg/kg OT and 10 mg/kg NTI on 2 h water intake following 15 h water deprivation. Data are means ± SEM.

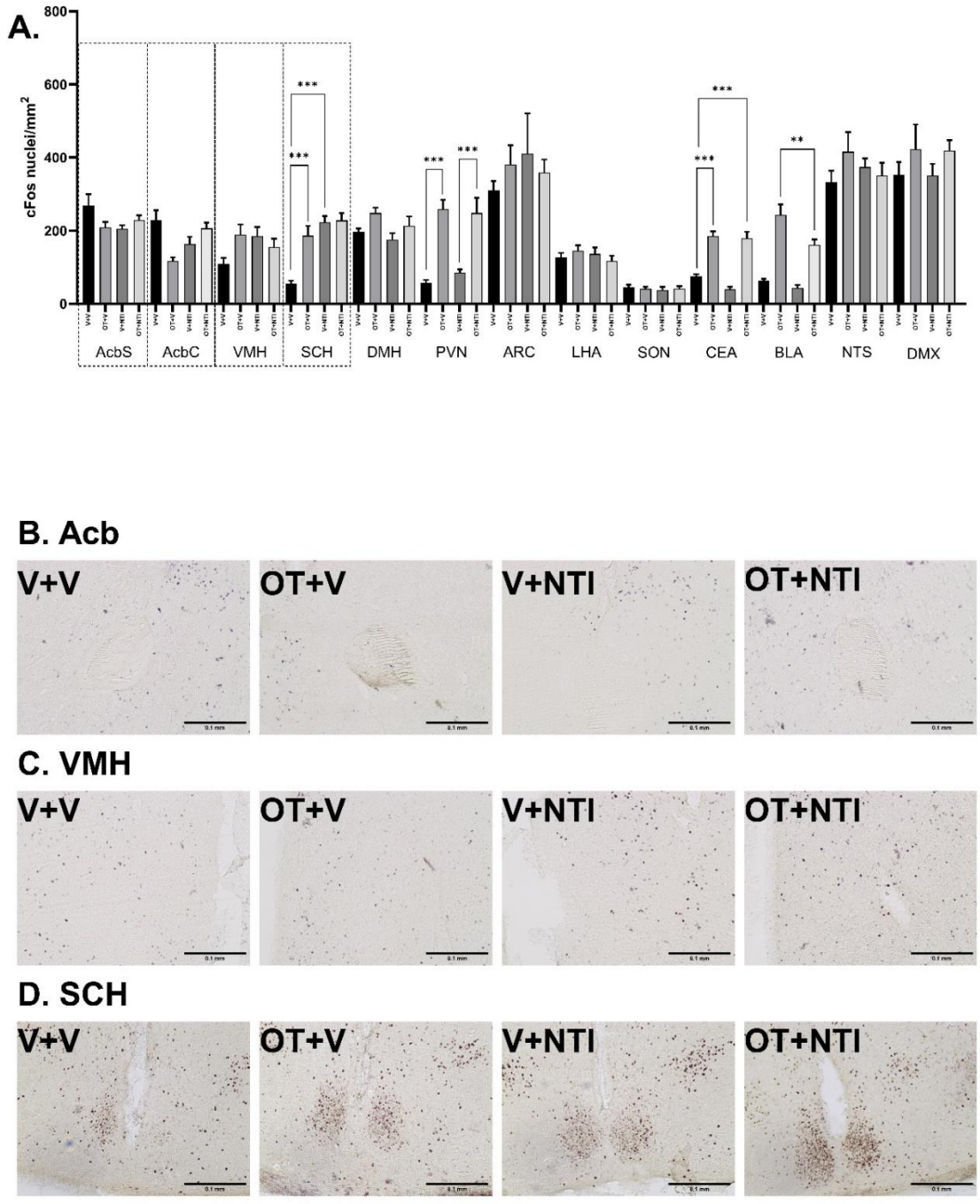

Figure 4.7 Effect of simultaneous intraperitoneal vehicle+vehicle, vehicle+10 mg/kg NTI, 3.0 mg/kg OT+vehicle, or 3.0 mg/kg OT+10 mg/kg NTI on c-Fos immunoreactivity in feeding related brain areas (A). Dotted boxes around the AcbS, AcbC (B), VMH (C), and SCH (D) represent significant OT x NTI interaction where $p<0.05$. Data are means ± SEM; **$p<0.01$; ***$p<0.001$.

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Chapter 5 Discussion and Conclusions

5.1 Discussion

Homeostatic control of food intake is essential for survival, linking consumption to physiological demand in order to defend against both inadequate intake and sustained positive energy balance. Overconsumption emerges when this central regulation fails to constrain intake relative to homeostatic requirements. Excessive meal size is one example of this dysregulation, wherein termination signalling fails to appropriately constrain portion size. Larger meals can contribute to obesity and further destabilise control of food intake, a risk amplified when meals are rich in fats and/or carbohydrates, or when they include synthetic additives that maintain palatability in the absence of calories (e.g., saccharin). For most of human evolutionary history, diets were comparatively high in water and fibre content and therefore lower in energy density, the neural circuitry present today developed to regulate consumption of these diets [1]. However, the rise in both the availability and energy density of foods today challenges the central control of intake, resulting in the consumption of larger portion sizes, causing sustained positive energy balance.

The central control of food intake can be broadly divided into homeostatic, hedonic, and autonomic components; of these, homeostatic and hedonic regulation are most relevant to the present thesis. Homeostatic regulation links feeding to physiological demand, promoting intake when energy availability is low and constraining intake as requirements are met, thereby defending against both insufficient consumption (starvation) and sustained excess intake (obesity). In parallel, hedonic regulation influences feeding via reward processing, biasing behaviour toward foods that are highly palatable and positively reinforcing, thus increasing the likelihood that such foods are sought out and consumed [2]. In practice these processes operate together to shape meal initiation and, critically, meal size and termination: consumption of an appropriately sized meal following hunger should restore the individual toward homeostatic balance while also being experienced as rewarding, reinforcing that behavioural sequence in the future. Both forms of regulation are driven by a range of neurological systems that function in concert.

The oxytocin (OT) system is the first of the two food-intake regulatory systems that are the focus of this thesis. Anatomically, this system is centred in the hypothalamus, a region of the central nervous system (CNS) implicated in a broad range of homeostatic processes. OT was first demonstrated to exert an anorexigenic effect in the late 1980s [3]. Subsequent studies have extended this finding, demonstrating anorexigenic efficacy

across routes of administration, species, and on the consumption of a variety of foods [4, 5] [6] [7] [8] [9] [10] [11]. In general, OT is regarded as a potent inhibitor of palatable food consumption, acting primarily via homeostatic control mechanisms to initiate satiety and reduce meal size, while also exerting effects on hedonic aspects of feeding (reviewed in [12-17]). Accordingly, OT has been widely investigated as a potential pharmacological intervention for obesity; however, several challenges remain before clinical translation is feasible (reviewed in [15]). For example, the effect of OT administration on the consumption of some foods remains unclear. Namely, OT suppresses the consumption of solid high-fat foods [6, 18-23], but not liquid emulsions of fat [10, 24-26]. Another key concern is the relatively high doses often required to reliably inhibit food intake, which raises the possibility of off-target effects [27, 28].

The other system involved in the central control of food intake that is relevant to this thesis is the opioid system. Comprising three opioid receptor subtypes, μ-opioid receptor (MOR), κ-opioid receptor (KOR), and δ-opioid receptor (DOR), the opioid system is best characterised as a regulator of reward-driven feeding (reviewed in [29, 30]). Of the three receptors, MOR has been most thoroughly examined in the context of food intake: MOR agonism is generally understood to increase the reward value of feeding and, in turn, promote larger meal sizes [31-33], whereas MOR antagonism typically reduces food reward and produces anorexigenic effects [32, 33]. In contrast, the contribution of the other opioid receptors, particularly DOR, to feeding regulation remains comparatively less well defined (reviewed in [29, 34, 35]). Where DOR antagonism has been examined, findings are mixed: naltrindole (NTI), a selective DOR antagonist, reduces saccharin consumption in some studies [36-40], whereas other reports show that NTI either fails to reduce saccharin intake or increases it [41-43]. Notably, unlike OT, opioid receptor antagonism has already been translated into approved obesity pharmacotherapy, although the existing approach primarily targets MOR.

Contrave is a prescription-only, orally administered weight-management medicine approved for use in New Zealand that combines naltrexone (NTX) and bupropion [44]. NTX is a non-selective opioid receptor antagonist and, in fixed-dose co-administration with bupropion, is used clinically to reduce appetite and support weight loss in obesity. While Contrave provides a clinically available example of leveraging opioid receptor antagonism for weight management, the broader strategy of pairing opioid antagonists with additional anorexigenic mechanisms remains an active area of investigation.

One investigation into combination pharmacotherapy for overeating and obesity was a case report describing co-administration of OT and naltrexone (NTX) to treat

hypothalamic obesity in an adolescent male following craniopharyngioma resection. In this study, Hsu and colleagues administered intranasal OT for 10 weeks, producing a modest reduction in body weight and hyperphagia. When OT was paired with intranasal NTX, the intervention produced larger reductions in body weight and hyperphagia, alongside improved satiety, relative to OT alone [45].

These clinical observations were subsequently expanded upon by Head and colleagues, who examined the effects of IV OT and NTX co-administration in rodents feeding. Co-administration of OT and NTX suppressed episodic sucrose consumption at doses that were ineffective when administered alone. In contrast, daily treatment with combined OT and NTX prior to scheduled high-fat high-sugar meals did not reduce body weight, consistent with compensatory increases in standard chow intake later in the day. Nonetheless, the interaction observed in episodic feeding suggests that combined treatment with OT and opioid receptor antagonism may allow anorexigenic effects to be achieved at lower individual doses, supporting continued investigation of this combination of drugs as an anti-obesity treatment [46].

While Hsu et al [45] and Head et al [46] examined OT and NTX co-administration, no studies to date have tested simultaneous OT receptor agonism alongside selective DOR antagonism. In the present thesis, we therefore evaluated combined administration of OT and NTI across multiple food types and provide the first evidence that this pairing can produce synergistic suppression of intake. To link behavioural effects to mechanism, we assessed both food intake and neuronal activation following drug treatment and/or feeding. This approach allowed us to characterise the anorexigenic efficacy of the combination while also identifying candidate neural mechanisms that may mediate its effects.

However, interpreting combined OT and NTI effects first required clarification of several unresolved single-drug effects. A prominent example is OT's action on fatty foods, which remains inconsistent across the literature. OT has been reported to reliably, and in some cases selectively, reduce intake of solid high-fat foods [6, 18-23], yet multiple studies consistently report a lack of effect on fat emulsions [10, 24-26]. Accordingly, prior to testing OT effects in liquid-diet paradigms, we first needed to systematically determine whether OT suppresses intake of solid fatty foods and to directly compare these outcomes with reports using emulsions. Establishing whether Intralipid is inhibited differently from other fatty foods was necessary to interpret any findings acquired using Intralipid as being reflective of either the general effect of OT on fats, or if they were indeed Intralipid specific.

To address this, Chapter 1 employed both no-choice and two-choice feeding paradigms in rats using solid mash and chow diets spanning a range of fat contents, while maintaining minimal variability in food matrix. This design allowed us to systematically evaluate how dietary fat content influences OT-induced intake suppression. In the no-choice experiments, OT reduced consumption across a broad range of fat contents, with the principal exceptions occurring at the extremes. Taken together, these data support the conclusion that OT can suppress intake of solid fatty foods. This interpretation was further supported by the two-choice preference tests, in which OT reduced the relative intake of 60% fat chow compared with 10% fat chow, consistent with greater suppression of the higher-fat option.

To support our behavioural data, and gain insight into neural activity engaged by fat consumption, we examined c-Fos immunohistochemistry in sated rats following consumption of no food, 10% fat chow, or 60% fat chow. Consumption of 60% fat chow, but not 10% fat chow, increased activation of OT neurons within the PVN and increased neuronal activity in the AcbC, AcbS, CEA and DMH. These regions span circuitry involved in both reward-related and homeostatic control of feeding, and several are also responsive to OT. For example, direct OT administration into the AcbC reduces sucrose and saccharin intake, while OT administration into the BLA and CEA suppresses feeding [8, 47]. The co-occurrence of activation across these regions and OT neurons therefore identifies plausible OT-sensitive circuitry recruited during high-fat feeding, although it does not establish that the regional c-Fos responses were caused directly by OT release. These findings suggest that OT-linked mechanisms engaged during feeding may be preferentially recruited by higher-fat intake and are particularly consistent with our behavioural observation that OT reduced the relative consumption of 60% fat chow compared with 10% fat chow in the two-choice preference test.

Our findings help reconcile two commonly reported trends in the OT–fat intake literature. Macronutrient-focused studies often report little or no effect of OT on the consumption of fats, but these paradigms typically use liquid diets for compositional simplicity and therefore rely almost exclusively on Intralipid as a liquid diet as a complete representative of fatty foods [10, 24-26]. In parallel, multiple studies using solid high-fat foods, which were not designed to parametrically test fat content, report an OT-induced suppression of fat intake [6, 18-23]. By systematically comparing solid diets across fat contents, we confirm that OT can suppress intake of solid high-fat foods, which leads us to infer that outcomes obtained using Intralipid should not be assumed to represent OT's effects on fatty foods more broadly. Accordingly, in chapters where Intralipid is used as a diet

representative of fatty foods, any observed effects should be interpreted as effects on Intralipid consumption specifically, rather than as definitive evidence for OT's efficacy, or lack thereof, on dietary fat consumption. This does not diminish the translational relevance of suppressing intake of fatty emulsions, but it does indicate that such findings may not generalise to suppression of solid high-fat food consumption. Establishing how OT influences intake of solid high-fat foods, and how Intralipid-based paradigms should be interpreted in this context, provided some of the foundational knowledge required for our final goal of examining combined OT and NTX treatment.

The use of NTX in combination with OT by Hsu [45] and Head [46] was grounded in a substantial literature describing the individual anorexigenic effects of both compounds. While Chapter 1, together with prior work, provided a clearer basis for interpreting OT's effects on intake, the literature describing NTI and, by extension, the role of DOR in feeding regulation remained comparatively sparse, particularly relative to the extensive descriptions of the roles and effects of NTX and MOR present in the literature.

Uncertainty regarding NTI and, by extension, DOR function is well illustrated in the literature on sucrose intake. Several studies report that NTI fails to alter consumption of sucrose solutions [41, 48], whereas other work suggests that central NTI administration can potentiate sucrose intake [49]. This inconsistency is notable given that NTI has also been reported to, albeit inconsistently, reduce intake of the similarly sweet, but non-caloric, tastant saccharin [36-40]. A common feature of many of the paradigms used to test the effect of NTI on sucrose intake is that consumption is assessed in ad hoc or acute tests, rather than under conditions where intake is habitual and predictable. To clarify whether NTI can suppress sucrose intake under habitual consumption conditions, we tested its effects in rats with established sucrose drinking.

To test this, we used a no-choice sucrose acceptance test following i.p. NTI administration, assessed episodic post–water deprivation water intake to exclude nonspecific effects on thirst, and tested whether NTI induced a conditioned taste aversion. These experiments were conducted in rats habituated to daily 1 h access to sucrose solution for three weeks. Under these conditions, NTI (10 mg/kg) reduced sucrose consumption independent of any reduction in thirst, and this dose did not produce a conditioned taste aversion. These findings corroborate and extend those reported by Pellegrini and colleagues [50], potentially reflecting the shared feature that both studies assessed NTI under paradigms involving repeated daily sucrose access (including their successive negative contrast design).

To elucidate how NTI affects neuronal activity in feeding-related CNS regions, and whether this differs between rats accustomed and unaccustomed to sucrose consumption, we used c-Fos immunohistochemistry. In sucrose-naïve rats, comparisons between vehicle- and NTI-treated animals showed no significant effects of NTI, although trends were observed in the DMH, BLA and CEA. In animals with habitual sucrose access, NTI was associated with significant differences in c-Fos immunoreactivity in the DMH, SON, PVN, CEA, AP, NTS and DMX. However, significant treatment × sucrose exposure interactions were detected only in the SON and NTS, providing the clearest evidence that previous sucrose exposure altered the neuronal response to NTI. The NTS is particularly relevant because DOR activation has been shown to reduce excitatory input to gustatory NTS neurons, an effect prevented by naltrindole [51]. In contrast, DOR binding is relatively sparse throughout much of the rat hypothalamus, suggesting that changes observed in regions such as the SON, PVN and DMH may reflect downstream network recruitment rather than direct local DOR blockade [52]. Together, these findings suggest that habitual sucrose consumption alters the neural context in which DOR blockade acts, with significant NTI-associated changes occurring across a broader set of feeding-related regions under habitual-access conditions and treatment × sucrose exposure interactions in the SON and NTS. This context dependence may help explain why NTI suppressed sucrose intake under habitual-access conditions in the present study but has been ineffective in paradigms using ad hoc sucrose exposure, and highlights the importance of feeding history when interpreting the behavioural effects of DOR blockade.

In our final set of experiments, we tested whether simultaneous targeting of OT receptors and DOR, via combined administration of OT and NTI, would be more effective than either drug alone. We first established the effects of each drug on saccharin, the only tastant not examined elsewhere in this thesis. Reports describing NTI effects on saccharin intake are highly mixed [36-43], and the literature on OT effects on non-caloric sweet solutions is similarly conflicting [11, 47, 53-55]. Given this inconsistency, and because we had not systematically characterised the effects of either OT or NTI on saccharin intake, we began by testing each compound individually in a no-choice acceptance paradigm. Mice received OT (0.1–3.0 mg/kg) or NTI (3.0–10 mg/kg), and saccharin consumption was measured. OT did not reduce saccharin intake at any dose tested, whereas NTI suppressed saccharin consumption at 10 mg/kg. Notably, when this same dose of NTI was administered in combination with OT, saccharin intake was further reduced beyond NTI alone, consistent with a synergistic effect of the combined treatment. This interaction did not appear to reflect nonspecific effects on thirst, as OT, NTI, and

OT+NTI did not alter water consumption following a 15 h water deprivation period. In contrast, we observed no synergistic suppression of sucrose or Intralipid intake: Intralipid consumption was not altered by either drug or the combination, and sucrose consumption was reduced by treatment with OT and OT+NTI, but not NTI.

To better characterise the neural effects of the same drug combination, we again used c-Fos immunohistochemistry, with neuronal activity assessed following drug treatment in the absence of a test diet. This analysis identified OT × NTI (drug x drug) interactions in the nucleus accumbens (core and shell) and in hypothalamic nuclei including the ventromedial hypothalamus and the suprachiasmatic nucleus. In addition, both OT and NTI individually altered neuronal activity within the basolateral and central nuclei of the amygdala, and OT increased neuronal activity in the paraventricular and dorsomedial hypothalamic nuclei. Overall, the distribution of single-drug effects and OT × NTI interactions encompassed regions implicated in reward processing and homeostatic control of intake that are modulated by both OT and opioid signalling (reviewed in [13, 29, 56]). This breadth argues against a single locus mediating the behavioural effects of treatment, and instead suggests that the response to OT, NTI, and their combination reflects recruitment of a distributed network of nuclei. The OT × NTI interactions in the nucleus accumbens are particularly relevant given the established involvement of this region in palatable food consumption and its sensitivity to both receptor systems. OTRs are expressed in both the AcbC and AcbS, and direct AcbC OT administration reduces sucrose and saccharin intake through an OTR-dependent mechanism [47]. DOR signalling within the accumbens can also influence sucrose-directed behaviour; local naltrindole administration in the AcbS alters sucrose licking in a learned anticipatory contrast paradigm [57]. The accumbal interactions observed here are therefore consistent with convergence between OT- and DOR-sensitive reward circuitry, although they do not establish that the receptors interact directly within the same cells or that these c-Fos effects specifically mediate the reduction in saccharin intake.

With the behavioural outcomes and accompanying changes in neural activation following OT and NTI co-administration established, we can now interpret these findings in the context of the overarching thesis aim: to determine the effects of combined OT and NTI treatment on intake. Although the absence of synergistic suppression for sucrose and Intralipid is disappointing, these null effects remain informative when considered alongside the conditional single-drug effects identified in Chapters 1 and 2. First, the failure of OT+NTI to reduce Intralipid intake should not be taken as evidence that the combination is ineffective for suppressing fat consumption more broadly. In Chapter 1,

we showed that OT suppresses intake of solid high-fat foods despite consistent literature reports that it does not suppress Intralipid consumption, indicating that Intralipid and solid fatty foods are not equivalent readouts of OT's effects on dietary fat intake. Intralipid is useful due to its compositional simplicity; however, outcomes obtained in Intralipid-based paradigms may not generalise to other forms of fat intake. Intralipid exhibits atypical gastric emptying and distinct orosensory properties, which may contribute to its different intake profile relative to solid high-fat foods [58]. It therefore remains plausible that repeating the combined OT+NTI experiment using solid high-fat diets would yield a different outcome, including the emergence of a synergistic anorexigenic effect.

More broadly, the physical form of the foods used across these experiments may also have contributed to some of the context-dependent effects observed. Solid chow, mash diets, and liquid solutions differ not only in nutrient composition, but also in texture, oral processing, and gastric handling. Food texture can influence satiation and the amount consumed, with greater textural complexity or harder foods associated with reduced intake [59, 60. Intralipid may represent a particularly distinct case, as ingested Intralipid separates within the stomach into aqueous and lipid phases that empty at different rates {Friedman, 1996 #54]. Consequently, differences in the effects of OT and NTI across liquid, mash, and solid foods may not reflect macronutrient composition alone. Differences in physical form may alter the sensory and postingestive signals generated during feeding and, in turn, influence the neural regulation of intake. However, because food form was not manipulated independently of composition in the present experiments, its contribution to the observed treatment effects cannot be determined directly.

A similar context dependence may apply to sucrose. NTI suppressed sucrose intake under habitual-access conditions, whereas it was ineffective when sucrose was presented under ad hoc conditions in the combined-treatment experiments. This is consistent with previous studies using ad hoc sucrose access, in which NTI alone also failed to suppress sucrose intake [41, 48]. These differences raise the possibility that previous sucrose experience influences the behavioural efficacy of DOR blockade and, consequently, the likelihood of observing an interaction with OT. The accompanying c-Fos findings are consistent with feeding history influencing the response to NTI, with significant treatment × sucrose exposure interactions observed in the SON and NTS. Although these findings do not establish that habitual access accounts for the different behavioural outcomes across experiments, they identify feeding history as an important variable for future combination studies. Testing OT+NTI following established sucrose access would

directly determine whether combined treatment is more effective under conditions in which NTI alone suppresses sucrose consumption.

The observed synergistic effect of combined OT and NTI treatment on saccharin intake was particularly notable. The selectivity of this interaction, present for saccharin consumption but not for sucrose or Intralipid, could reflect differences in post-ingestive caloric feedback such that caloric consequences attenuate synergy that is otherwise evident for a non-caloric sweet stimulus. However, an alternative interpretation is that this trend reflects methodological context rather than caloric feedback, consistent with the context-dependent effects identified in Chapters 1 and 2. In the present experiments, fat intake was modelled using Intralipid rather than solid high-fat foods, and sucrose was assessed under ad hoc rather than habitual access conditions. Distinguishing between these possibilities will require further work. Nonetheless, despite differences in both the diets assessed and the opioid receptor target relative to prior reports [45, 46], the present findings build upon the notion that simultaneous OT administration and opioid receptor antagonism can produce synergistic anorexigenic effects.

A substantial body of work is still required before these approaches could be translated into routine clinical use. Nonetheless, the present thesis contributes to the growing evidence that both OT signalling and opioid receptor antagonism may hold potential as anti-obesity strategies. Importantly, the observation of a synergistic interaction, including suppression of intake at doses that were ineffective when administered alone, supports the broader premise that combination therapies may be a productive direction for obesity pharmacotherapy. This may be particularly relevant for candidates such as OT, where a key translational concern is the relatively high doses often required to reliably reduce intake, raising the risk of off-target effects (reviewed in [15]). In this context, combination approaches may offer a route to efficacy at lower individual doses.

Our work provides insight into both the individual effects of OT and NTI on intake, and the consequences of their combined administration, which together warrant further investigation of their anorexigenic potential. First, our systematic evaluation of OT effects on solid high-fat foods helps bridge the gap between studies reporting robust suppression of solid high-fat intake [6, 18-23] and macronutrient-focused paradigms reporting little or no effect on fat intake which utilised Intralipid as a fatty tastant [10, 24-26]. On this basis, it may be worthwhile to revisit conclusions about the effects of OT on the consumption of fats using solid high-fat foods rather than Intralipid as the primary fat stimulus. Second, we show that NTI suppresses sucrose intake in rats with habitual sucrose access, and that sucrose habituation is associated with recruitment of a broader

network of feeding-related nuclei by NTI. These findings suggest that further work examining NTI effects under habitual-access conditions may be particularly informative. With respect to combined OT and opioid antagonism, further experiments could test whether synergy emerges when intake is assessed using solid high-fat foods, or when sucrose is assessed in animals habituated to its consumption. More broadly, Head and colleagues reported that daily OT and NTX administered prior to scheduled access to a highly palatable meal did not reduce body weight due to compensatory increases in standard chow intake [46]. The present work focused primarily on single-meal outcomes; therefore, an important next step will be to determine how combined OT and NTI influences longer time scales of feeding, including total daily intake and compensation across subsequent meals.

5.2 Conclusions

While previous research has shown that both OT and δ-opioid receptor antagonism can influence or reduce food intake, the present thesis demonstrates that their combined administration may, in some cases, produce additional benefits for suppressing consumption of specific diets. The following specific conclusions were reached in this study:

The specific findings of this study are:

- Unlike studies that represent dietary fat using Intralipid, the present study shows that OT suppresses intake of solid fat-containing foods, including high-fat diets. OT neuronal activation was also proportionally greater following consumption of higher-fat foods.
- NTI reduces sucrose solution consumption in rats habituated to sucrose access, independent of changes in thirst or induction of a conditioned taste aversion. NTI also engages a broader network of feeding-related regions in sucrose-habituated animals than in sucrose-naïve animals.
- Combined OT and NTI treatment did not produce a synergistic effect on the consumption of Intralipid or sucrose solutions.
- Combined OT and NTI treatment produced a synergistic anorexigenic effect on saccharin solution consumption, independent of changes in thirst, and also induced OT x NTI interaction effects in the nucleus accumbens core and shell and hypothalamic nuclei including the ventromedial and suprachiasmatic nuclei.

5.3 References

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