Oxytocin as a pharmacological tool to curb overeating: Exploring synergy with opioid receptor blockade

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Abstract

The key factors shaping consumption involve a feeling of hunger (determines search for calories), satiation (underpins termination of ingestive behaviour), and reward (adjusts consumption to the 'pleasantness' of food, regardless of actual energy needs). Though each of the many feeding-related neural systems affects the select facets of appetite to a different degree, most basic research (and subsequently, also clinical) studies exploring anorexigenic potential of neuroactive molecules, focus on targeting only one system. This single-system approach has been the case for one of the most potent anorexigens identified to-date, oxytocin (OT). OT is known to promote satiation and its release coincides with, e.g., excessive stomach distension and osmolality. OT's effects on feeding reward are weaker as they are mitigated by macronutrient composition, flavour and energy density. Its potential influence on perceived hunger is unknown. Therefore, in this thesis I wished to examine whether by combining OT with a ligand that targets those facet(s) of feeding largely unaffected by OT, a synergistic effect on feeding can be achieved. Considering that opioid receptor antagonists mainly reduce reward-driven feeding, but do not promote satiety (notably, their effect on hunger remains undefined), I focused on investigating the simultaneous effects of OT receptor stimulation and opioid receptor blockade on food intake.

As potential impact of either opioid antagonists or OT alone on hunger perception has never been examined, prior to studying simultaneous effects of the two types of molecules on feeding, I investigated whether either OT or opioid ligands individually reduce a feeling of hunger. The first set of experiments explored whether hypophagic doses of OT would alter hunger responsiveness in rats trained to discriminate between 22 h (hunger) vs 2 h (no hunger) food deprivation in a two-lever, operant discrimination procedure. Intraperitoneal (i.p.) OT did not decrease 22-h deprivation-appropriate responding to

match that following 2-h food deprivation, thus, it did not reduce the perceived level of hunger. In order to understand mechanisms behind this ineffectiveness of OT, I used c-Fos immunohistochemistry to determine whether OT activates a different subset of brain sites under 22- vs. 2-h deprivation. I found that in sated animals, OT induces c-Fos changes in a broader network of hypothalamic and brain stem sites compared to the hungry state. Finally, by employing qPCR, I found that fasted animals had lower OT receptor mRNA levels in the brain stem, a CNS "entry" region for peripheral OT, than their ad libitum-fed counterparts. It can be concluded that OT does not diminish a feeling of hunger and that, therefore, OT's anorexigenic properties are manifested once consumption has already begun which is—at least to some extent—driven by changes in brain responsiveness to OT treatment in the hungry vs. fed state.

In the second set of experiments, I explored whether opioid receptor ligands, acting primarily on reward-related drivers of consumption and not on satiation, might also expand onto the third facet of feeding, i.e., hunger. I subsequently utilized the aforementioned hunger discrimination paradigm to measure behavioural responses associated with hunger to explore whether modulating the opioid receptor network will alter the perception of energy deficit (hunger). While a hunger-inducer, neuropeptide Y (NPY), shifted hunger-associated operant responses in 2-hr-deprived (sated) animals, which is reflective of an increase in hunger, opioid receptor agonists (DAMGO, DSLET, orphanin FQ and butorphanol) did not induce such changes. In line with that, hypophagic doses of an opioid antagonist, NTX, did not reduce the hunger discriminative stimuli induced by either 22 h deprivation, or by NPY administration in 2 h food-restricted rats. I therefore conclude that the opioid system, similarly to OT, does not affect perceived hunger.

The subsequent experiments relied on the hypothesis that by combining the effect of opioid receptor blockade on diminishing feeding reward and of OT on promoting early

satiation, a synergistic effect can be achieved. This hypothesis was strengthened by a recent case report involving a male with hypothalamic obesity, concurrent administration of OT and NTX, synergistically affected energy balance. In the third set of studies performed in adolescent rats I indeed found that, while OT reduces deprivation-induced chow intake, and NTX reduces palatable food intake, that the combination of these drugs at subthreshold (ineffective in the context of feeding) doses produces a hypophagic effect on acute food intake. Furthermore, using c-Fos immunochemistry, I found robust changes in feeding-related brain regions within the brain stem-hypothalamic network as a result of this drug combination. I conclude that administration of combined OT and NTX acutely suppresses food intake, likely by modulating activity of brain regions associated with both satiety and reward.

Importantly, the OT-NTX synergy described above pertained to adolescent animals and to short-term food intake. Thus, in the fourth set of studies, I examined both acute feeding responses and longer-term feeding and body weight changes in adult rats treated with OT-NTX. I found that in adult animals, OT-NTX did not appear to produce a significant acute effect on standard chow intake as in adolescents, but I still observed the acute hypophagic effect in palatable feeding scenarios. Furthermore, while the drug combination indeed had a cumulative effect on reducing palatable food intake with chronic administration, the beneficial effects on caloric intake and body weight were offset by compensatory elevated intake of standard chow in the remainder of the day. The results of PCR gene expression analysis across three brain regions (hypothalamus, brain stem and nucleus accumbens) showed that the OT-NTX combination produced unique mRNA level changes in brain stem-hypothalamic networks.

Overall, evidence gathered throughout this project shows that co-administration of two molecules that do not diminish hunger, but that do affect distinct facets of feeding: OT (early satiation) and NTX (eating for pleasure), generates a synergistic effect in reducing

food intake. This effect can be attained with doses of drugs that individually would be too low to mitigate feeding, most likely thanks to the unique action of the combined drugs at feeding-relevant brain circuits.

Table of Contents

List of Figures

Chapter 1
Introduction

Obesity is a prevalent issue in Western society and treatments to reduce unhealthy consumption are in high demand. Treatments that target multiple aspects of the drive to consume may be promising pharmacological strategies to reduce food intake. While it has been shown beyond reasonable doubt that oxytocin (OT) decreases feeding for calories and for sweet/carbohydrate-driven reward by signalling early satiation, it is unclear why this seemingly potent anorexigenic system does not guard against overeating (especially, of palatable tastants). A case study using a combination of OT to target satiation, and naltrexone (NTX) to address reward-related drives to consume, has shown promise in reducing food intake, resulting in significant body-weight loss. This may be the result of a potentiating effect of these drugs when co-administered.

A recent case study of a child suffering from craniopharyngioma leading to uncontrollable overeating and unhealthy increases in body-weight, trialled treatment by co-administration of OT and NTX, and reported a significant decrease in the intake of palatable foods, and in the subject's body weight [1]. This study may outline a potentiating effect of OT and NTX when combined. Therefore, in the following studies, I investigate the effects of OT and NTX administered systemically (intravenously and intraperitoneally), and observe changes in food intake and body weight, and the associated neuromolecular effects.

Firstly, I will here explore the fundamentals of the OT system and how this may be utilized to regulate one component of addiction, the physiological drive from the homeostatic system.

1.1 OT and its receptor

Oxytocin (OT) is an evolutionarily ancient and biologically fundamental peptide for vertebrates. OT-like molecules have been detected in virtually all vertebrates, and the nonapeptide sequence of OT (CYIQNCPLG) is well conserved (Figure 1.1.). This is in line with the involvement of this peptide in the most basic mechanisms (such as osmoregulation, reproduction or feeding) common for organisms, regardless of their level of organizational complexity [2]. OT-like precursor polypeptides in invertebrate species also display a high level of homology to the corresponding vertebrate molecule as documented, for example, in the preproannetocin sequence analysis of the earthworm, Eisenia foetida. Interestingly, infusion of OT homologues in invertebrates affects similar processes as those regulated by OT in mammals, e.g., gut motility and reproductive functions [7; 8].

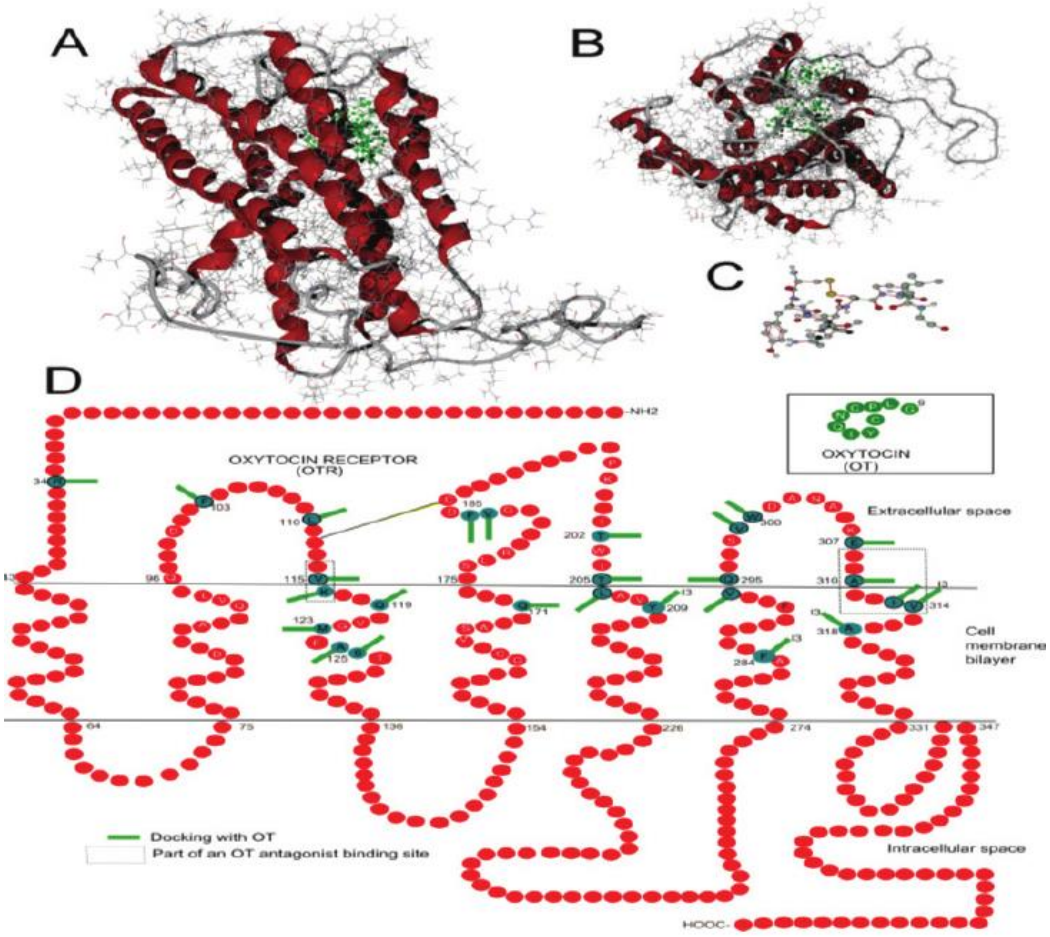


Figure 1.1. Oxytocin and binding sites on oxytocin receptor. This figure illustrates molecular docking of the three-dimensional models of activated human oxytocin receptor with oxytocin obtained by MolDock Optimizer algorithm from Molegro Virtual Docker software. (A) The front upright view position (side view) of the receptor structure with oxytocin. (B) Panel B shows an intracellular view (i.e. rotation by 90˚ out of plane). (C) Conformational view of oxytocin molecule. (D) The schematic model of the human oxytocin receptor with marked amino-acid residues that are putatively involved in ligand-binding. From [3]

OT binds to its receptor, a member of the Rhodopsin-type G protein-coupled receptor family [19; 30], and the cyclic rather than linear part of the neuropeptide's molecule determines binding selectivity [4] (Figure 1.2.). The OT receptor is also bound by vasopressin (VP) with only 10 times less affinity than for OT, and the concentration of VP necessary to induce a response comparable to that elicited by OT is approximately 100 times higher [4; 5]. VP is regarded as a partial agonist at the OT receptor and many studies have revealed that the VP receptor agonists have a dual OT-VP receptor binding profile [5]. However, OT and VP receptors' affinities for their respective antagonists have much less overlap since antagonists target a different and more sequence-specific protein fragment than the one recognized by agonists [2].

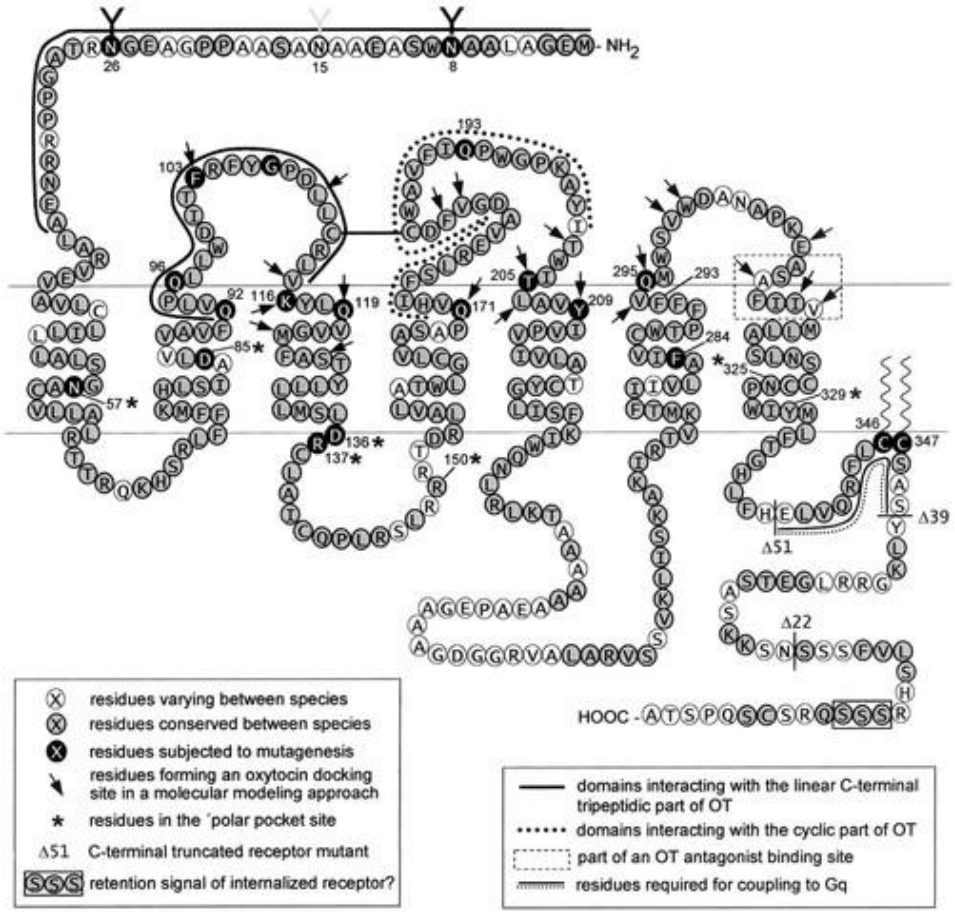


Figure 1.2. Structure of the human oxytocin receptor showing the 7 trans-membrane domains, the N terminal extracellular hormone binding domain and the C terminal intracellular domain. Each circle represents an amino acid identified by their standard one-letter codes. (Modified from [10])

As first documented in a study identifying the most prevalent hypothalamus-specific mRNAs, OT was found to be the most abundant of 43 neuropeptide transcripts [4]. Overall, the cyclic nonapeptide OT and its structurally related peptides facilitate reproduction in all vertebrates at several levels [5].

1.2 Genes encoding elements of the OT system

The OT precursor protein gene is estimated to date back at least 500 million years [6]. Human physiological and pathophysiological studies supported by rodent and cell culture experiments have identified three genes as the functional core of the OT system: *OXT* (structural gene for oxytocin), *OXTR* (oxytocin receptor), and *CD38* [13]. CD38 is involved in OT secretion via Ca2+ release , and oxytocin-

neurophysin I (OXT) encodes the OT prepropeptide containing the nonapeptide OT and the carrier protein neurophysin-I [13; 14].

The human gene for OT-neurophysin I encoding the OT prepropeptide is mapped to chromosome 20p13 [9] and consists of three exons (Figure 1.3). The first exon encodes a translocator signal, the nonapeptide hormone, the tripeptide processing signal (GKR), and the first nine residues of neurophysin; the second exon encodes the central part of neurophysin (residues 10–76); and the third exon encodes the COOH-terminal region of neurophysin (residues 77–93/95) [10].

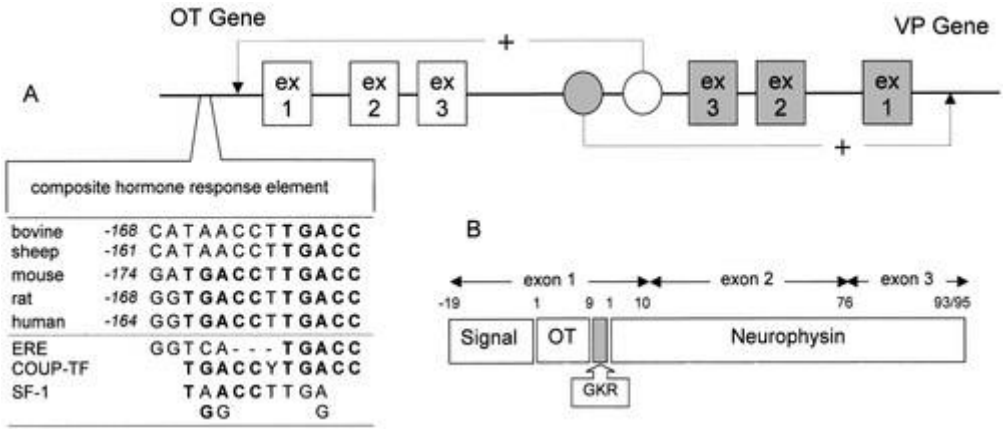


Figure 1.3. Organization of the oxytocin (OT) and vasopressin (VP) gene structure including schematic depiction of the putative cell-specific enhancers (open circle, enhancer of OT gene; shaded circle, enhancer of VP gene). [Modified from Gainer et al. [11].] A: details of the approximately −160-bp region (composite hormone response element) of the upstream OT gene promoter conserved across five species including the sequences of the response elements estrogen response element (ERE), chicken ovalbumin upstream promoter transcription factor I (COUP-TF), and steroidogenic factor-1 (SF-1) are indicated. [Modified from Ivell et al. [12].] B: domain organization of preprooxytocin including the processing sites. The precursor is split into the indicated fragments by enzymatic cleavages, one involving a glycyl-lysyl-arginine (GKR) sequence and leaving a carboxamide group at the COOH-terminal end of OT. [10]

The OXTR gene is present in a single copy in the human genome and it was mapped to the gene locus 3p25–3p26.2 [15-17]. The gene spans 17 kb and contains 3 introns and 4 exons. Exons 1 and 2 correspond to the 5′ noncoding region. Exons 3 and 4 encode the amino acids of the OT receptor. Intron 3, which is the largest at 12 kb, separates the coding region immediately after the putative transmembrane domain 6. Exon 4 contains the sequence encoding the seventh transmembrane domain, the COOH terminus, and the entire 3′-noncoding region, including the polyadenylation signals (Figure 1.3). Although many GPCRs have an intron-less gene structure, the genes for some other members of the GPCR family including the human vasopressin V2 receptor [15; 18] contain an intron at the same location after transmembrane domain 6. The transcription start sites lie 618 and 621 bp upstream of the initiation codon as demonstrated by primer extension analysis. Nearby, a TATA-like motif and a potential SP-1 binding site is found in the human OT receptor gene. The 5′-flanking region also contains invert GATA-1 motifs, one c-Myb binding site, one AP-2 site, two AP-1 sites, but no complete estrogen response element (ERE). Instead, there are two half-palindromic 5′-GGTCA-3′ motifs and one half-palindromic 5′-TGACC-3′ motif of ERE. Moreover, there are two nucleofactor interleukin-6 (NF IL-6) binding consensus sequences and two binding site sequences for an acute phase reactant-responsive element at the 5′-flanking region [19].

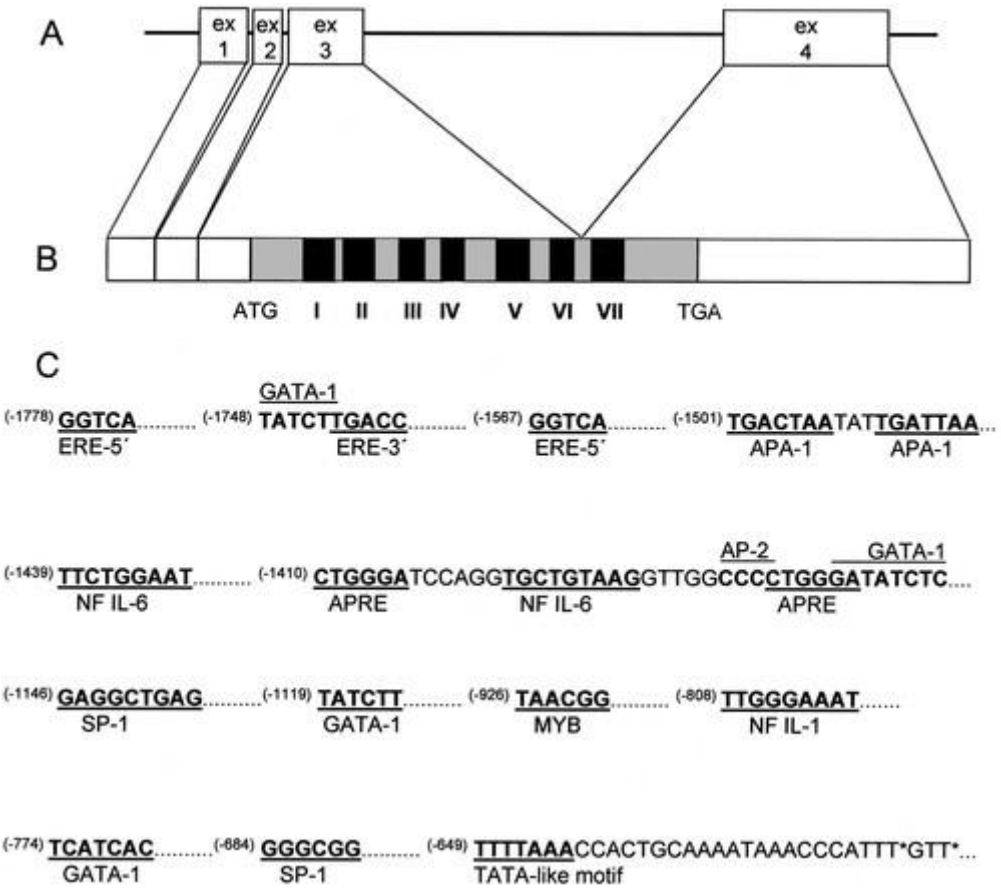


Figure 1.2. Organization of the human OXTR gene including the localization of consensus sequences for transcription factors. The human OXTR gene consists of four exons. Exons 3 and 4 encode the amino acid sequence for the OT receptor. The start (ATG) and stop (TGA) codons of the receptor cDNA are indicated. The DNA sequences encoding for transmembrane regions I–VII are indicated by black areas. [Modified from Inoue et al. [15].]

One fundamental question concerns the possible existence of OT receptor subtypes [20; 21]. Such subtypes have been suggested to be possibly present, e.g., in the rat uterus [22; 23], kidney [24], or brain [25; 26], and – if true – this could explain differential pharmacological profiles or immunoreactivity patterns occasionally reported in conjunction with some organs. However, application of polymerase chain reaction (PCR) methods and Southern analysis in several tissues known to possess OT binding activity failed to identify a gene encoding a further OT receptor subtype [21; 27].

1.3 Structure

OT was the first peptide hormone to have its structure determined, and the first to be chemically synthesized in a biologically active form [10; 28]. OT belongs to the family

of neurohypophyseal nonapeptide hormones, which are characterized by a cyclic hexapeptidic part with a disulfide bridge between Cys1 and Cys6 and a C-terminal linear tripeptidic extension [4] (Figure 1.4).

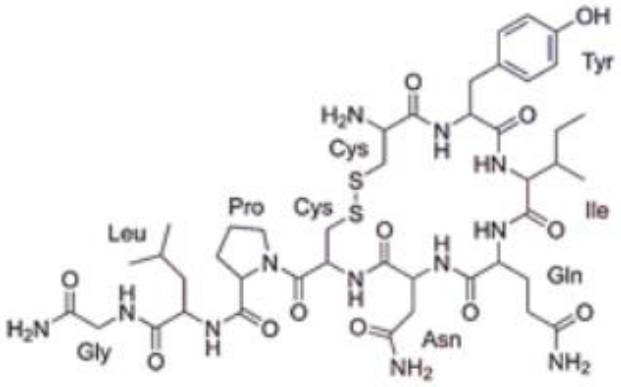


Figure 1.3. Molecular structure of oxytocin

This results in a peptide constituted of a six-amino acid cyclic part and a COOH-terminal α-amidated three-residue tail. Based on the amino acid at position 8, these peptides are classified into vasopressin and OT families: the vasopressin family contains a basic amino acid (Lys, Arg), and the OT family contains a neutral amino acid at this locus (Figure 1.5). Isoleucine in position 3 is essential for binding the OT receptor, and Arg or Lys in position 8 for acting on vasopressin receptors. The difference in the polarity of these amino acid residues is thought to enable the vasopressin and OT peptides to interact with the respective receptors [10; 29].

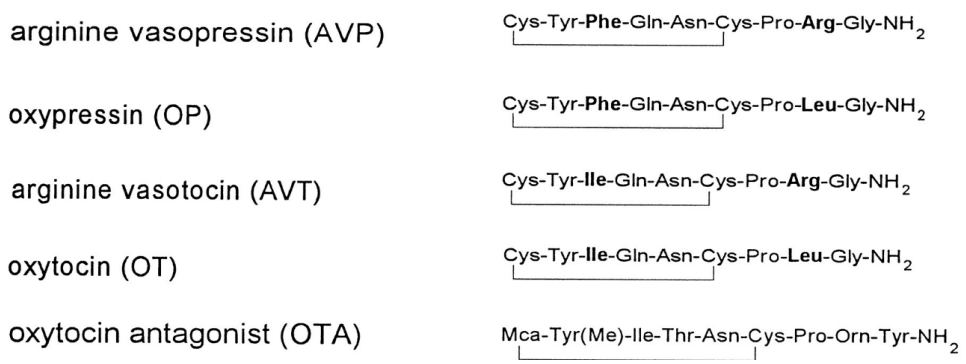


Figure 1.4. Schematic representation of amino acid composition of vasopressin, oxytocin, two related hormones, and an oxytocin antagonist. All four agonists are cyclic nonapeptides, which differ in their amino acid positions 3 and/or 8. Arginine vasopressin (AVP) and oxypressin (OP) are identical in their cyclic hormone part; this is also the case for arginine vasotocin (AVT) and oxytocin (OT). With regard to the acyclic portion, AVP and AVT are identical on the one hand and OP and OT on the other. The oxytocin antagonist (OTA) has some non-natural amino acids: Mca, 1-(β-mercapto-β,β-cyclopentamethylene propionic acid); Tyr (Me), methyl ether of tyrosine; Orn, ornithine [4]

1.4 Basic regulatory function at cell level

The OT prepropeptide is cleaved while being transported down the axon to terminals located in the posterior pituitary [31]. The mature peptide products, OT and its carrier molecule neurophysin, are stored in axon terminals until neuronal membrane depolarization elicits their release [32]. The main function of neurophysin, a small (93–95 residues) disulfide-rich protein, appears to be related to the proper targeting, packaging, and storage of OT within the granules before release into the bloodstream. OT is found in high concentrations (>0.1 M) in the neurosecretory granules of the posterior pituitary, in a 1:1 ratio complex with neurophysin. In such complexes, OT-neurophysin dimers are the basic functional units as suggested by the crystal structure of the neurophysin-OT complex [33]. Cys-1 and Tyr-2 in the OT molecule are the principal neurophysin binding residues. In particular, the protonated α-amino group (Cys-1) in OT forms an essential contact site to neurophysin via electrostatic and multiple hydrogen bonding interactions. Due to its dependence on amino group protonation (pKa ~6.4), the binding strength

between OT and neurophysin is much higher in an acidic compartment, like the neurosecretory granules (pH ~5.5). Conversely, the dissociation of the complex is facilitated as the complex is released from the neurosecretory granules and enters the plasma (pH 7.4) [10]. The specific morphology of OT neurons allows OT to have a dual role as both a hormone and neurotransmitter. Neurosecretory granules containing OT are widely distributed in Purkinje fibres and distributed along neurons.

OT receptors can be coupled to subunits such as Gq, Gi1, Gi2, Gi3, GoA, and GoB, causing an increase in cytosolic calcium concentration (coupling to the Gq subunit) or inhibition of adenylate cyclase activity (coupling with the Gi subunit). OT promotes uterine contraction through the activation of calcium channels associated with receptors and the release of sarcoplasmic reticulum calcium. OT binds to the receptor where its effect is mediated by a second messenger, which is regulated by voltage or hormone regulation on the muscle cell membrane and by contractor-mediated extracellular calcium influx. OT increases the production of inositol 1,4,5-triphosphate, and the mobilization of 5-trisphosphate inositol stores intracellular calcium release in the endoplasmic reticulum and sarcoplasmic reticulum. In addition, OT causes cells to produce inward currents through receptor-activated, non-selective cation channels that depolarize cell membranes, producing action potentials and muscle contractions. A recent study showed that OT synthesized in the brain and secreted into the circulation binds to receptor for advanced glycation end-products (RAGE) molecules on capillary endothelial cells, a component of the blood brain barrier (BBB), and is thus transported across the blood brain barrier. By being transported into the brain, OT can there exert central nervous functions [34]. Prostaglandins are mediators of OT actions, such as during excitation of neurons in the supraoptic nuclei, where they are released in response to OXTR agonists [35] (Figure 1.7).

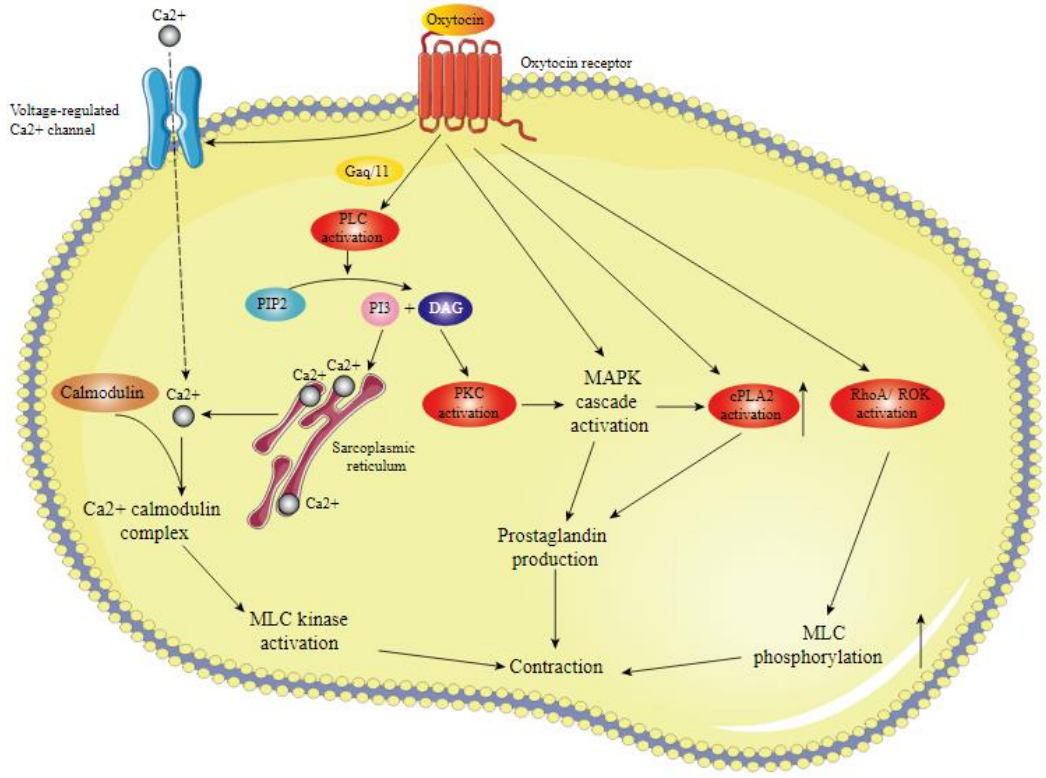


Figure 1.5. Intracellular Oxytocin signalling pathway. PLC: phospholipase C, RhoA: Ras homolog family member A, MLC: megalencephalic leukoencephalopathy with subcortical cysts, PKC: Protein kinase C, DAG: dystrophin-associated glycoprotein, PIP2: Phosphatidylinositol 4, 5-biphosphate, cPLA2: calcium-dependent phospholipase A2. (Modified from [35])

It should be emphasized that the intake of a sufficient amount of energy does not appear to be the main or the necessary factor that induces OT neuronal activity underlying termination of ingestive behaviour. In fact, OT neuronal activity and release coinciding with termination of feeding occur upon changes in calorie-independent parameters associated with consumption. Those parameters include excessive stomach distension and elevated plasma osmolality [36-38]. In addition, central OT inhibits consumption of toxin-tainted foods and supports long-term avoidance of those by acting through not only the brain stem but also the amygdala [39; 40].

It has been speculated that, under basic physiological conditions, activation of the OT system by orexigenic factors, serves as the feedback mechanism that does not allow dangerously excessive food intake to occur [2]. Activity of the OT system is elicited by administration of anorexigenic peptides and it reflects a general increase in activation of

circuitry that mediates satiation. However, an elevated response of OT cells has been shown also upon injection of powerful orexigens, such as NPY and ghrelin, in both males and females [41].

A powerful bioinformatics study, derived from a review of over 1800 PubMed articles, has recently characterized the entire array of OT signalling pathways (Figure 1.8.) [42]. Findings showed that OXTR stimulation is primarily mediated through Gαi/Gαq/Gαo protein activation. OT induces pro-inflammatory cytokine overexpression through mitogen-activated protein kinase (MAPK)/Nuclear factor kappa B (NFκB) pathway, which in turn is associated with onset of labour [43]. Prostaglandins (PGE2 and PGF2 alpha) production through arachidonic acid cleavage is one of the effective mediators in OT-induced processes especially to those related to onset of labour [44; 45]. OT also triggers osteoclastogenesis and osteoblast differentiation through MAPK1/3 activation and, thus, it may play a significant role as a therapeutic agent in osteoporosis [46]. It also affects functions associated with the CNS such as regulation of nociception, which is mediated by activation of potassium ion channel (KCJN11) [47], and anxiolytic effects mediated through expression of regulator of G-protein signalling 2 (RGS2) [48]. In the heart, OT elicits cardioprotective function by reducing infarct size and postischemic recovery, and this recovery is mediated through PI3K-AKT/ NOS/natriuretic peptide A (NPPA) expression and p38 MAPK/heat shock 27 kDa protein 1 (HSPB1) phosphorylation [49].

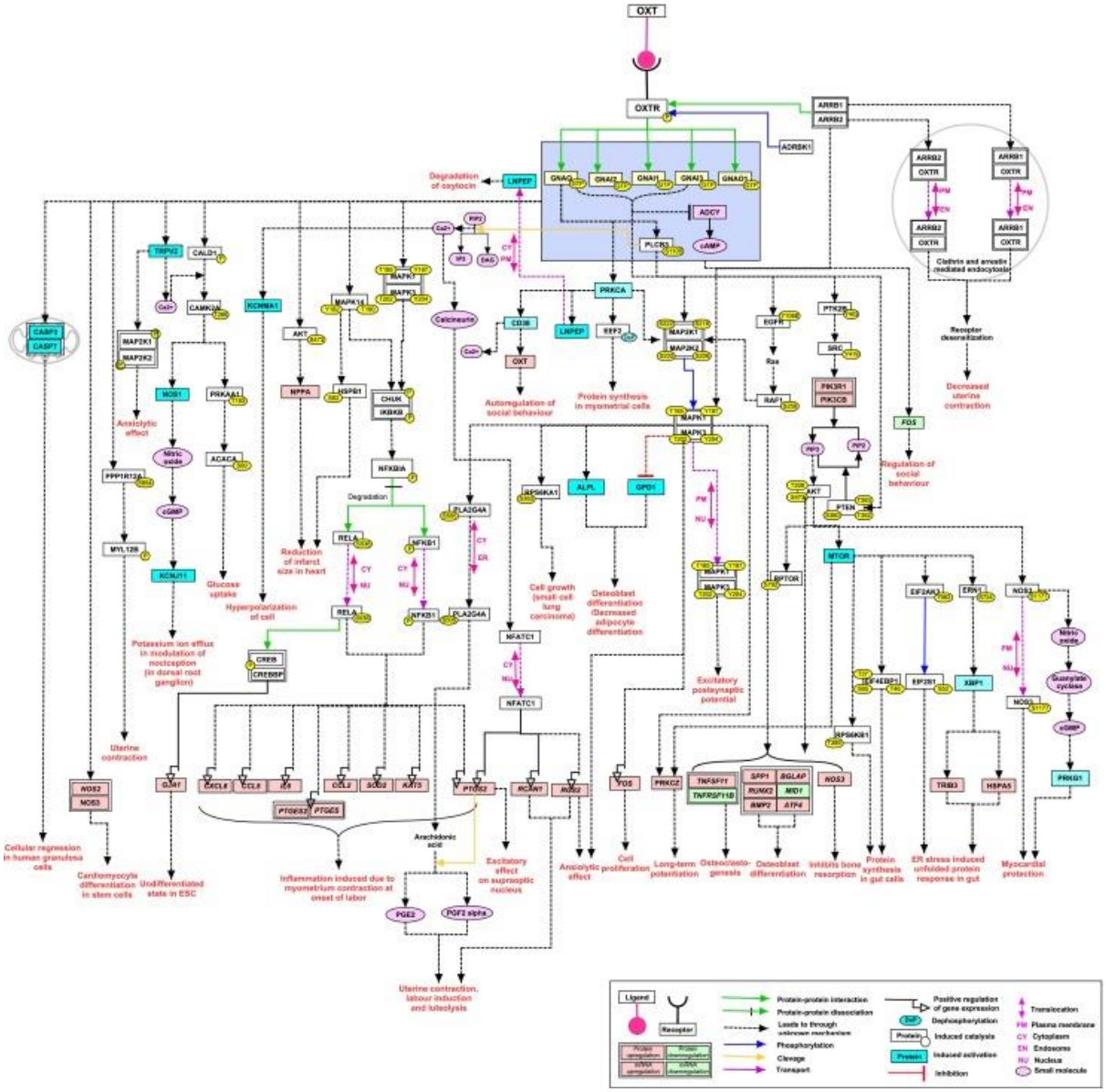


Figure 1.6. A schematic representation of intracellular reactions induced by oxytocin. The pathway map depicts reactions induced by stimulatory action of OXT through OXTR. Reactions represented in map are protein-protein interaction, post-translational modification, activation/inhibition and gene/ protein expression. Sites and residues for post-translational modifications are also shown. Legend is provided for identification of different pathway reactions. From [42]

OT affects peripheral tissues directly by binding its G protein-coupled receptor localized in, to name a few, the mammary gland, ovary, uterus and bone [50; 51].

At the genetic level, deletion of the gene encoding OT or OT receptor in mice indeed sheds more light on feeding-related mechanisms regulated by the OT system. Nishimori et al. raised mice deficient in the OT receptor gene and found that despite similar food intake as seen in wild-type controls, the KO animals started developing obesity in week

12 after birth [52]. They had a much higher mass of both white and brown fat. The impairment of the adrenergic receptor system in these animals may also contribute to the elevated adipose tissue weight. Takayanagi et al. provided further characterization of the OT receptor −/− obese phenotype [53]. They reported an increase in the mass of abdominal fat pads and elevated triglycerides in the blood in the KO strain. This high adiposity was not associated with an altered feeding or locomotor activity profiles, though thermogenesis was impaired in these mice [2].

Data pertaining to obesity in OT-deficient mice are somewhat conflicting with some authors reporting no body weight differences between KO and wild-type animals, whereas others have shown an increase in adiposity whose onset occurs as late as at 4–6 months of age. Camerino found that OT KO mice develop hyperleptinemia, a decreased insulin sensitivity and glucose intolerance as well as lower adrenaline levels [54], which led to the hypothesis that the metabolic changes accompanying OT deficiency stem from a decreased sympathetic nervous tone. They exhibit altered macronutrient preference profiles and eating patterns. For example, OT KO mice display enhanced preference for carbohydrates, particularly sweet ones [55]. In line with the KO data, recent real-time PCR studies in wild-type rats showed the effect of scheduled volume-unrestricted consumption of high-sugar versus regular diet on OT gene expression levels in the hypothalamus [56].

1.5 Distribution in periphery and brain:

1.5.1 OT neurons

OT is a nonapeptide hormone produced primarily by the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus. After synthesis as an inactive precursor, it is conveyed axonally to the posterior pituitary where it can be released into peripheral tissue [10]. The PVN also contains smaller parvocellular OT neurons. Forty percent of these terminate in the pituitary, whereas the rest send axons throughout the

brain [10; 57; 58]. The topography of this central innervation parallels OT's involvement in food intake [59; 60]. The PVN OT fibres innervate central targets, most prominently, the dorsal vagal complex in the brain stem [61; 62]. The brainstem dorsal vagal complex (DVC) is the first site for integration of visceral synaptic and hormonal cues that act to inhibit food intake [63]. The DVC consists of three nuclei: the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus (DMNV) and the area postrema (AP). The NTS serves as a "relay" station for a number of peripheral signals, including those related to gut functioning; the DMNV is part of the efferent and afferent vagal innervation of the stomach; and, finally, the AP mediates gastric responses triggered by high osmolality or presence of toxins in the blood [59; 61]. Neurons of the sensory branch of the vagus nerve make glutamatergic synapses onto NTS neurons to relay information from the periphery, including the stomach and upper intestine [64; 65]. Targeted chemogenetic activation of appetite‐responsive NTS neuronal populations causes short term decreases in food intake [66-69]. Importantly, the OT-brainstem innervation is reciprocal, i.e., brainstem neurons terminate in the proximity of OT perikarya in the hypothalamus and OT terminals are found in the hindbrain, such as in the case of a powerful anorexigen, glucagon-like peptide-1 (GLP-1) [70]. However, OT terminals as well as the OT-receptor detected with in situ hybridization or autoradiography, are also present outside this basic brainstem-hypothalamus pathway [71].

1.5.2 OT receptor

The distribution of the OT receptor (OXTR) mRNA across the brain provides a proxy for the distribution of OT binding [72], allowing for a detailed mapping of the anatomical geography of the OT system in the brain. Seminal animal work using histochemistry and immunohistochemistry revealed high concentrations of OXTR mRNA in the hypothalamus, amygdala, olfactory bulb, ventral pallidum, and the dorsal vagal nucleus in rodents [25; 73]. Further, experimentally increasing [74] or decreasing [75] OXTR

expression in the prairie vole nucleus accumbens modulated partner preference behaviour, suggesting a correspondence between the spatial distribution of OXTR mRNA, its functional neuroanatomy, and behavioural relevance [13]. In addition, OT fibres terminate at OT perikarya expressing OT receptors, which suggests a positive feedback loop as the OT receptor has excitatory properties [76]. This auto-amplification of OT release plays a role in lactation [77; 78], but it has not been examined in association with other processes [2]. A schematic representation of the OT system in the brain in relation to feeding is presented in Figure 1.9.

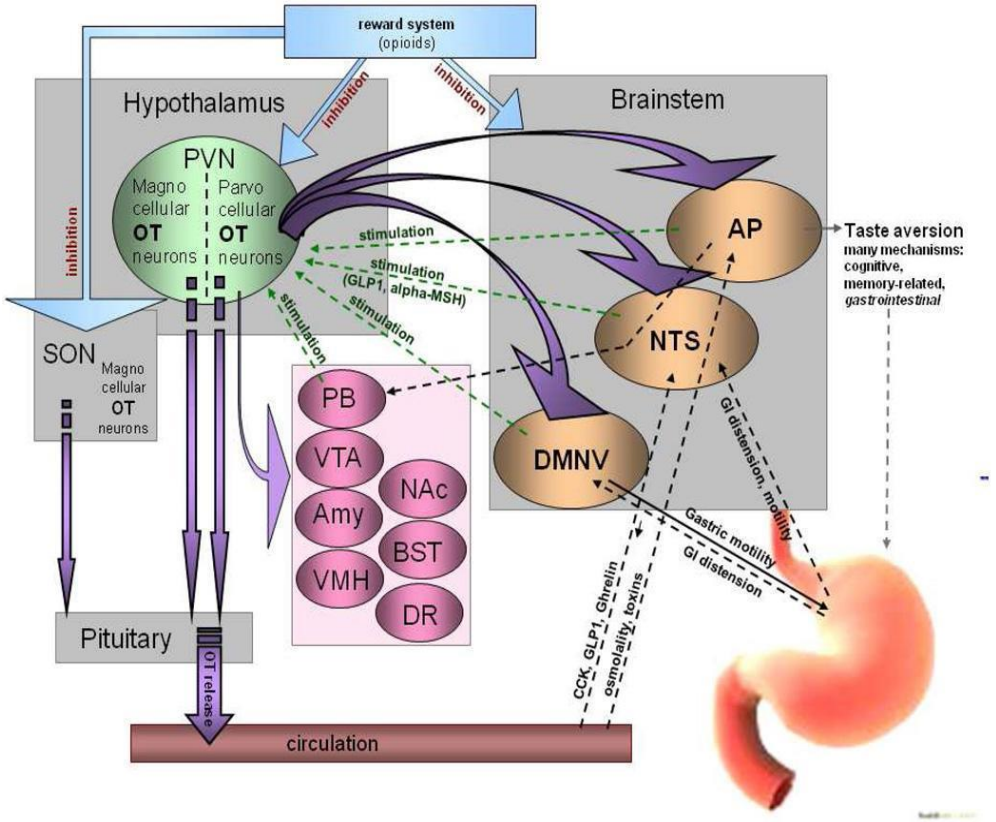


Figure 1.7. Topography of central OT pathways involved in food intake regulation with special emphasis on functional significance of the circuits. OT neurons project from parvocellular PVN neurons in the hypothalamus to brainstem sites known to regulate feeding (NTS, AP, DMNV) and to the pituitary, where OT is released to the general circulation. While the brainstem-hypothalamus pathways have been extensively studied in relation to OT's involvement in anorexigenic responses stemming from peripheral parameters, such as GI tract distension, osmolality of the blood, etc., many other feeding-related sites that contain OT terminals or OT receptors have not been comprehensively evaluated in relation to anorexigenic action of OT. These include areas involved in reward (ventral tegmental area, VTA; nucleus accumbens, NAc; bed nucleus of the stria terminalis, BST), affect (dorsal raphe nucleus, DR), energy homeostasis (ventromedial hypothalamic nucleus, VMH) and stress (amygdala, Amy; and parabrachial nucleus; PB) [2; 79-82].

1.6 Feeding-unrelated roles of the OT peptide system

While OT maintains homeostasis, these homeostatic signals must rapidly change as demand increases, especially during pregnancy, and this may occur by altered responsiveness of the OT system. Such altered sensitivity of the homeostatic system may also occur from overstimulation of opioid receptors responsible for hedonic signalling. OT is known to control parturition and maternal behaviour. It is released after dilation of the cervix during labour; and in lactating animals, it stimulates milk ejection [83; 84].

Data from human and laboratory animal studies link OT receptor activation/availability with modifications in non-feeding rewards (from natural rewards, such as social and reproductive behaviours to administration of drugs of abuse). For example, Jarrett and colleagues found that cocaine treatment changes OT receptor binding density in the bed nucleus of the stria terminalis in female rats [87]. Baracz et al. reported that direct intraparenchymal administration of OT in the core of the nucleus accumbens dose-dependently decreases methamphetamine-seeking behaviour [88]. The same group of investigators found also that intra-accumbens core OT attenuates methamphetamine-induced conditioned place preference in rats [89]. In another set of experiments employing OT receptor ligand injections in the nucleus accumbens and lentiviral-mediated overexpression of the OT receptor in this site, Bahi showed that OT attenuates the development, maintenance, and primed reinstatement of ethanol-induced conditioned place preference [90]. Intracranial infusions of OT in female mice promote the development of a conditioned social preference [40; 91].

Damiano et al. showed by using functional magnetic resonance imaging (fMRI) that certain single nucleotide polymorphisms in the OT receptor gene are associated with a differential response of the mesolimbic system during anticipation of monetary rewards in healthy human subjects [92]. Furthermore, neurochemical studies have pointed to a relationship between OT and dopamine in modification of perceived rewards. In one such

study in mice, central administration of OT has been found to reduce methamphetamine-elicited dopamine release in the striatum and nucleus accumbens [93] and promote a concomitant decrease in glutamate release and increase in extracellular presence of γ-aminobutyric acid (GABA) in the medial prefrontal cortex [40; 94].

Research on humans has further shown beneficial effects of intranasal OT on performance on tests assessing social cognition [95] and gaze to the eye region [13; 96]. Importantly, one of the most fundamental roles of OT is promoting various facets of social behaviour. OT has prosocial effects and is essential for initiating social interactions in rats [97]. It plays a key role in parent–infant bonding in rats, prairie voles, sheep, and primates including humans [98-100], and it reduces the occurrence of maternal neglect [101]. Pharmacological manipulations of the OT system alter partner-directed activities during pair interactions in marmosets [102]. In same-sex meadow voles, OT injection enhanced non-reproductive affiliative preferences beyond baseline levels, and this effect was counteracted by blockade of the OT receptor [103]. OT facilitates altruistic behaviours, such as sharing resources to benefit others. OT in humans increases cooperation directed toward in-group functions, defending and strengthening the in-group by modulating parochial altruism, and increasing in-group trust (e.g. expectation of self-sacrifice to promote group welfare)[104]. OT promotes generosity: OT-treated subjects are more inclined to help a stranger by splitting a set amount of money more generously [105] and they donate more to charity [106]. Empathy toward strangers is associated with OT release and it leads to subsequent acts of generosity [107]. Finally, intranasal OT increases the ease of imagining compassionate qualities [108].

The complexity of interactions between an animal and its social environment depends on many factors, such as a species, sex, (patho)physiological status, availability of resources, stressors as well as hierarchy within a group [109]. This is governed by a host of neuroregulators, including OT, which aside from controlling social behaviours, acts as an

anorexigen. Interestingly, Olszewski et al. showed that dominant mice consuming sugar have a significantly higher level of OT mRNA in hypothalami than their subordinate conspecifics. Thus, it is not just the pharmacological treatment with the OT ligand that differentially affects sugar consumption in dominant versus subordinate animals, but also the endogenous OT system is affected differently by sugar consumption in animals subjected to in-group hierarchy [110].

This relation between social interactions and feeding may stem from the fact that some forms of social interaction include an anxiogenic component, which is further exacerbated by the challenge of food availability, palatability and preference [110]. In this context, it is particularly crucial that OT exerts anxiolytic effects [111]. A number of studies examining the link between a social status and feeding/energy metabolism have suggested that social environment-driven modifications in animals' anxiety profile affect appetite, metabolism and endocrine parameters in seemingly related experimental paradigms. For example, subordinate mice given a cafeteria diet and subjected to several days of sensory contact increased their body weight and shifted their preference towards fatty foods, whereas dominant mice increased their carbohydrate intake [112]. It should also be noted that OT promotes “altruistic” behaviours whose occurrence benefits only one’s own social group [113]. Olszewski et al. surmises that the interaction might partially stem from the OT's presumed effect on the propensity to share resources (food), and in the dominant-submissive stable dyads, the dominant animal exhibits greater sensitivity to the OT receptor blockade [110].

While OT has been known to increase prosocial behaviours, recently it has also been linked to tribal behaviours and aggression [114]. This finding may reinforce the “social salience” hypothesis. This is the idea that what OT really does, is make us more aware of social cues. Therefore, one may notice more social signals when the drug is in its system than when it is not. Further studies using connectivity/imaging data showing both activity

in the salience network, and heightened pattern recognition would solidify this hypothesis, and might help elucidate if this effect begins in the thalamus, or is perhaps the result of a more top down filtering from the medial prefrontal cortex. These findings may reinforce the idea that OT strengthens in-group bonds, and exacerbates division toward out-groups. This could serve the purpose of maintaining tribes, or small in-groups, at the exclusion of others in an effort to retain resources.

An interesting note about OT is that this molecule has also been proposed to participate in the regulation of learning and memory performance, and its concentration may increase during flow states, or optimum performance cognitive states[115]. This state of peak performance results in reduction in self-referential thinking and amplified focus, that can increase task performance, and may involve a component of OT release. If this is indeed the case, OT's social effects may fit better with the social salience hypothesis than simply the love/trust hypothesis [114]. In line with this, a 2020 study proposes that OT could help to treat cognitive disorders, including Alzheimer's disease. Researchers demonstrated that OT reversed the effects of amyloid-beta on hippocampal long-term potentiation (LTP) in mice. These effects were blocked by pre-treatment with the selective OT receptor antagonist L-368,899. Furthermore, the treatment with the extracellular signal-regulated kinase (ERK) inhibitor U0126 and selective Ca2+-permeable AMPA receptor antagonist NASPM completely antagonized the effects of OT. The findings suggest OT could be used as a therapeutic for the treatment of Alzheimer's disease and other dementias, and that ERK phosphorylation and Ca2+-permeable AMPA receptors are involved in this effect of OT [116].

Cardiac activity of OT involves maintenance of blood pressure, increased angiogenesis and anti-inflammatory activity [117]. Further, OT has a significant role in bone development and is implicated in skeletal remodelling and osteoblast maturation [118].

In addition to this, it is involved in the pathophysiology of various disorders such as diabetes, osteoporosis and neuropsychiatric disorders [119; 120].

1.7 Involvement of OT in regulation of consumption

By the start of the 1980's, several research groups showed that lesioning of the PVN and disruption of the PVN-hindbrain pathways resulted in hyperphagia and significant body weight gain in rats [121-123]. Lesions that extended beyond the PVN did not generate a greater effect on ingestive behaviour or obese phenotype [124]. Knife cuts leading to the disruption of the PVN-hindbrain pathways were shown to be crucial in the development of hyperphagia and obesity [81; 125]. The search was then undertaken for neuropeptides derived from the PVN whose lack precipitated this effect. Release of OT and increased activity of OT neurons was shown to coincide with satiation-associated termination of feeding in laboratory animals [50]. Injection studies provided preliminary evidence linking OT with feeding termination. Arletti et al. were first to report that intracerebroventricular (ICV) injection of OT causes a marked reduction in deprivation-induced food intake in rats [126]. Many authors have confirmed the finding and, by using intraparenchymal OT receptor ligand injections or employing OT receptor-specific cytotoxins, have identified the hindbrain (particularly the dorsal vagal complex) as the area through which OT-driven feeding inhibitory mechanisms are executed [40; 127]. Importantly, hyperphagia and obesity occur in mutations that lead to insufficiencies in OT PVN neuronal population development, such as that observed in the single-minded-1 (SIM-1) mouse model, and these negative symptoms can be reversed by OT treatment [128]. ICV injections of OT and OT receptor agonists dose-dependently suppressed chow intake in male and female rats stimulated to eat by scheduled feeding or by food deprivation [50; 129; 130]. This effect was reversible by administration of OT receptor antagonists, although OT receptor antagonism by itself did not increase energy intake [50]. Peripheral administration of only high doses of OT generated hypophagia, which

strongly suggested that central OT affects feeding [129; 130]. Interestingly, OT homologue injection experiments in several other species have shown that this feeding inhibitory function seems to be as well conserved as the OT molecule itself. For example, ICV infusions of OT in birds caused a dose-dependent decrease in feed intake, feeding time and pecking frequency [131]. The observed hypophagic outcome of central treatment with OT agents produced little explanation as to what mechanisms underlie this effect. Subsequent studies that focused on defining the physiological basis of OT's effect on feeding generated substantial evidence indicating that OT acts as a "homeostatic" inhibitor of ingestive behaviour. The OT system is also involved in gastric motility. As it responds to significant stomach distention, to elevated plasma osmolality that accompanies food intake, blocks consumption of toxic tastants, and promotes avoidance of such tastants upon subsequent presentations, it was concluded that OT prevents the animal from maintaining consumption that could potentially jeopardize the internal milieu (Figure 1.10) [132].

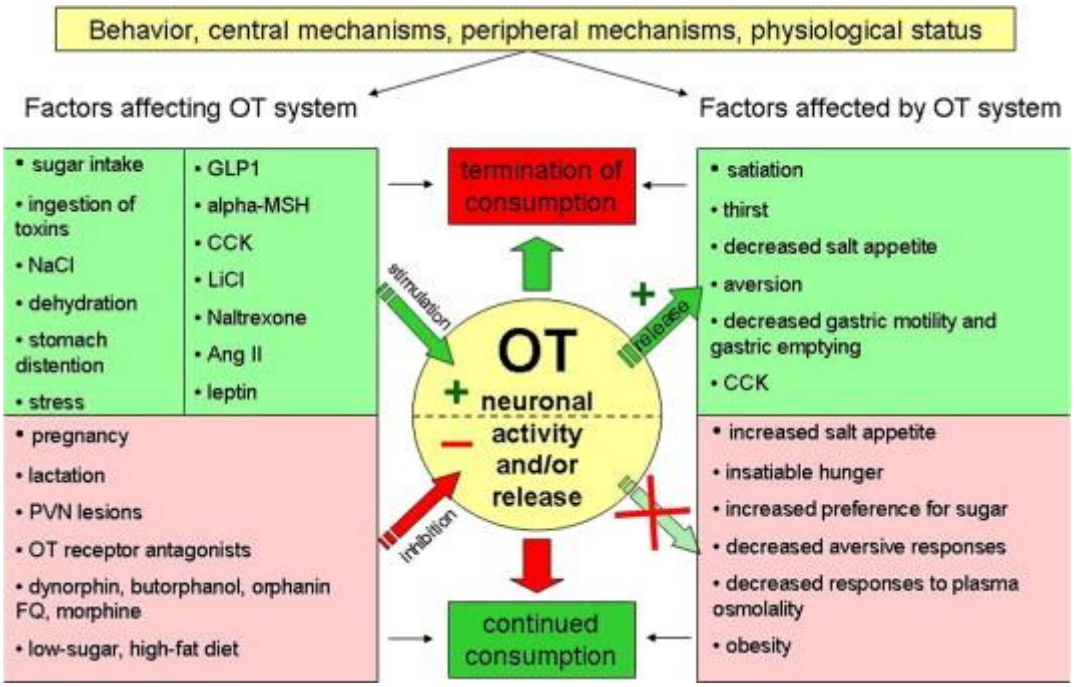


Figure 1.8. A schematic representation of functional relationship between OT neuronal activity/release and feeding-related behaviours, processes and physiological conditions. Modified from [2]

In individually housed rodents, injections of OT receptor antagonists elevate consumption of carbohydrates [133-135], OT preferentially decreases appetite for sugar [134], and consumption of high-sugar diets modifies OT gene expression [135].

1.7.1 Osmolality

Increases in plasma osmolality signify dehydration, improper fluid homeostasis or ingestion of highly osmotic foods. Changes in the osmotic status activate OT neurons projecting within the CNS and to the pituitary. Hyperosmolality resulting from water deprivation induced expression of an immediate-early gene, c-Fos, in magnocellular OT neurons in the SON and PVN [136-138]. Van Tol reported that prolonged osmotic stimulation through long-term exposure to 2% NaCl instead of drinking water increased OT mRNA levels of OT-immunoreactive neurons in these sites [139]. Consequently, intraperitoneal (IP) injections of NaCl solutions as well as hypovolemia due to dehydration produced an elevated plasma OT profile [140]. Importantly, dehydration by itself suppresses food intake: mice deprived of both food and water ingested less chow during a 60-min re-feeding period than animals deprived of only food [141]. Conversely, administration of hypertonic NaCl inhibited food intake and stimulated concurrent OT release [141-143]. Puryear et al. found that in OT knockout (KO) mice, the hypophagic effect of water restriction was attenuated [144]. This group additionally showed that animals deficient in OT displayed an increased consumption of NaCl compared to wild-type controls. Furthermore, genetic deletion of the OT receptor decreased salt appetite [144; 145]. It is noteworthy that stimulatory effects of intravenous (IV) infusions of NaCl on plasma OT levels were blunted in rats with lesions of the AP, which suggested that AP neurons facilitate OT responses to osmotic challenge. It should be noted, however, that once an IV infusion of 0.5 M NaCl was combined with that of 1 M mannitol in AP-lesioned animals, plasma OT levels increased normally [59; 146]. Moreover, no effect of AP lesions on the increases of plasma OT levels after plasma volume deficits was

observed, indicating that the AP is important only for secretion of OT in response to hypernatremia [59]. Interestingly, some authors argued that the involvement of OT in the regulation of osmotic balance does not serve as a proof of this peptide's involvement in the control of consummatory behaviour, but rather links OT merely to an improper water balance. Nevertheless, it was later noted that the role of OT as an osmotic regulator in some species (e.g., in sheep) is taken over by VP; in these animals, OT retains its anorexigenic properties [147].

1.7.2 Taste Aversion

OT has been proposed to inhibit food intake in order to prevent the organism from ingestion of toxic substances. This notion is supported by conditioned taste aversion (CTA) studies. In natural conditions, aversion develops upon ingestion of food that causes unpleasant gastrointestinal sensation (sickness, malaise and/or nausea) driven by toxicity. The set of behavioural responses follows and it includes termination of consummatory behaviour and subsequent avoidance of tastants of a similar flavour recognized as "tainted". In the laboratory setting, a CTA, as an associative phenomenon, is generated in a paradigm in which presentation of a novel food is paired with an injection of a toxin [148-150]. Consumption of this tastant upon subsequent presentations is significantly reduced [2]. Central mechanisms responsible for the development and maintenance of aversion-based hypophagia are complex. The brainstem sites: the NTS, AP, DMNV and parabrachial nucleus (PBN), take part in the recognition and integration of peripheral aversive signals, including the presence of toxins in the circulation and changes in the gastrointestinal (GI) tract motility parameters. Among these sites, the AP seems to play a particularly crucial role as its lesions prevent animals from acquiring an aversive response. This was shown by Curtis et al. who reported that while sham-operated and non-operated control animals developed a CTA to the novel tastant whose initial presentation was paired with a single LiCl injection, animals with AP lesions failed to show any signs of

aversion [151]. In line with those findings, AP as well as the NTS and PBN display an increased Fos immunoreactivity following CTA inducing treatments, such as LiCl or copper sulfate injections [152]. This signifies the importance of not only the hypothalamic OT system, but also the relay sites that allow the OT neurons to act at central target sites and, in the case of aversion which involves also the pituitary OT release, at peripheral tissues. The CTA-dependent activity of OT neurons most likely diminishes a drive to consume a tastant associated with sickness and favours an abrupt inhibition of consumption that has already been undertaken. One should note however that there is a significant magnocellular (thus, pituitary/peripheral) component of the OT system's response to aversive stimulation. Since peripheral OT generally does not inhibit consummatory behaviour, the role of OT in the periphery in aversion is likely unrelated to consumption, but it may rather be associated with facilitation of mechanisms preparing peripheral tissues and organs for consequences of toxicity. In fact, involvement of circulating OT in cardiovascular or natriuretic responses supports this hypothesis [153-157]. OT signalling is observed regardless of the nature of agents that induces sickness; those include chemicals, such as LiCl or copper sulfate, as well as natural or synthetic ligands of central receptors, e.g., high doses of CCK and naloxone [152; 158; 159]. This indicates that OT is the final component of many pathways involved in the mediation of CTAs rather than being limited to treatment-selective mechanisms that activate a specific group of receptors and engage specific mechanisms. Although OT is thought to be the final component of neural circuitry supporting CTA responsiveness, it is not a sole or necessary one: OT administration has not been shown to elicit aversive consequences, whereas LiCl-treated animals, incapable of developing CTAs due to the AP lesion, still display a surge in OT release [151]. It seems particularly interesting however that, while intraperitoneal LiCl given in rats with AP lesions does not induce a CTA, it produces anorexia accompanied by increased OT levels [151]. Therefore, OT may play an auxiliary

role in the CTA process, being one of the factors ensuring inhibition of consummatory behaviour [2].

One should note that agents that mediate satiation (such as the aforementioned CCK and naloxone), within a certain range of doses, terminate feeding without aversive consequences. However, once injected at higher doses, they precipitate a CTA. This brings about an interesting hypothesis that perhaps extremely robust, uncontrollable eating that could potentially disturb homeostasis has such a profound effect on feeding-related peptide plasma and central profiles that it is eventually curbed by activated aversive feeding termination mechanisms that encompass OT neurons [2]. This could explain temporary avoidance of foods that had been greatly overconsumed during a single meal. Aversion-like eating restrictive behaviours have been observed in humans who interspace binge eating episodes with periods of avoidance of diets that were part of a binging event [160; 161].

A reduction in the number of OT neurons has been reported for Prader–Willi syndrome patients exhibiting extreme overeating [162], which may lead to the hypothesis that administration of OT, paired with the inhibition of reward pathways, may mitigate such behaviours. Notably, the ventromedial hypothalamic (VMH) nucleus has been identified as a hypothalamic site through which OT causes early meal termination in free feeding and fasted rats [163]. In addition, central OT inhibits consumption of toxin-tainted foods and supports long-term avoidance of those by acting through not only the brain stem but also the amygdala[39], implying that OT receptor antagonism blunts responsiveness of the amygdala to aversive foods.

According to histological analyses, sites that integrate OT signalling include components of the reward network that mediate the portion of feeding driven by pleasure (e.g., the ventral tegmental area, nucleus accumbens and bed nucleus of the stria terminalis), areas that alter food intake under stressful conditions (e.g. the amygdala) as well as those that

modify affective functions and can consequently change various aspects of eating behaviour (the dorsal raphe nucleus) [73; 164; 165].

1.8 OT and reward

OT mRNA has been shown to be highly expressed in human paraventricular nucleus of the hypothalamus, the lateral hypothalamic area, and the supraoptic nucleus, and there is evidence of co-expression with OXTR mRNA and the μ and κ types opioid receptor mRNA [166], providing a putative avenue for interactions between the OT and opioid pathways [13].

Quintana et al. showed that expression of three selected OT pathway genes was enriched in subcortical and olfactory regions and there was high co-expression with several dopaminergic and muscarinic acetylcholine genes, reflecting an anatomical basis for critical gene pathway interactions. fMRI meta-analysis revealed that the OT pathway gene maps correspond with the processing of anticipatory, appetitive, and aversive cognitive states. The OT signalling system may therefore interact with dopaminergic and muscarinic acetylcholine signalling to modulate cognitive state processes involved in complex human behaviours [13].

1.9 Beyond oxytocin & homeostasis

An interesting issue arises regarding what other neural factors mediating sugar reward might act to diminish the activity of the OT system, either directly (via synaptic connectivity) or indirectly (as part of a larger network). Opioids are likely candidates for this interaction. Opioid receptor agonists are particularly effective at inducing intake of palatable foods, including those high in sugar; conversely, antagonists block reward-driven consumption. Regular exposure to high sucrose foods evokes changes in expression of genes coding opioid peptides and receptors [172]. Interestingly, opioids do

not seem just to stimulate feeding, but they have been suggested to maintain consummatory behaviour by inhibiting activity of neural systems associated with termination of feeding, such as via OT. Preliminary studies have suggested that activation of OT neurons at the end of a meal may be particularly vulnerable to modification by opioids. Administration of a wide-spectrum opioid receptor agonist, butorphanol tartrate, inhibits activity of PVN OT cells upon feeding termination; this effect is particularly pronounced when a high-sugar diet is given [167]. NTX is a commonly used opioid-receptor antagonist, and has been shown to reduce food intake, especially of palatable foods [168]. Opioids mediate rewarding aspects of consumption also in schedule-fed rats. Elevated intake of high-sugar foods during scheduled meals is associated with upregulation of genes encoding for orexin and OT in the hypothalamus and NPY in the brainstem [56].

Release of the anorexigenic neuropeptide OT is linked with satiation and has been associated with the end of a meal. It has been reported that OT plasma levels rise at feeding termination and elevated activity of OT neurons occurs at that time [169]. The activity of OT neurons can be determined immunohistochemically based on the level of colocalization of an immediate-early gene transcription factor, c-Fos, that indicates neuronal activity in OT neurons. Moreover, administration of OT directly in the brain dose-dependently decreases food intake in free-feeding rats, as well as in schedule-fed food-deprived rats [129]. Injections of agents that induce early satiety, such as alpha-melanocyte stimulating hormone and glucagon-like peptide-1 activate OT neuronal firing [170]. Finally, stimulation of OT neurons has been linked with ingestion of substances that jeopardize homeostasis, including toxins and ions [169]. Under such conditions, OT seems to serve as a signalling molecule that precipitates an immediate termination of ingestive behaviour in order to minimize the consumption of tainted food. Overconsumption of preferred tastants is thought to be strongly dependent on the activity

of the reward system; opioid peptides and receptors constitute one of its main components. For example, studies have shown that blocking of opioid receptors with selective (binding preferentially to opioid receptors of a certain subtype) and non-selective antagonists, such as naloxone or NTX; leads to decreased intake of a high-sucrose diet [171], and that opioid receptor agonists are particularly effective at increasing consumption of preferred foods. Prolonged intake of palatable foods leads to changes in mRNA levels of genes encoding components of the opioid system in several feeding-related brain sites [172]. One of the most puzzling questions is how palatability can possibly be capable of leading to significant food overconsumption that goes beyond energy needs, that subjects the organism to dangerously high levels of ions in the blood or food volume in the stomach. In fact, it has been conceptualized that the release of opioids occurring at the time of anticipated or actual consumption of palatable food causes inhibition of central pathways that mediate satiety. It has been suggested that endogenous opioids target, among others, the anorexigenic OT system. Antagonism of opioid receptors leads to an increase in activation of OT neurons in the paraventricular nucleus of the hypothalamus (PVN) [56], the site hosting the largest population of these cells in the brain and known for its role in feeding control [172]. Previous experiments have shown that rats injected with an opioid receptor agonist had significantly lower OT neuronal activity at the end of a tasty meal than control animals did [173]. Aside from the PVN, opioid receptors are present in nearly all of the central sites that are related to food intake regulation. They have been found in sites that are mainly associated with reward, such as the nucleus accumbens (NAcc), ventral tegmental area (VTA), bed nucleus of the stria terminalis (BST) and caudate putamen (CP), as well as in the hypothalamic arcuate nucleus and brainstem nucleus of the solitary tract, classically thought of as mainly regulating consumption for energy [174].

1.10 Reward system: Principles of central processing of consumption driven by palatability

Feeding behaviour is controlled by a complex set of interconnected brain circuits that control different aspects of this behaviour, including hedonic or "reward" based feeding. Feeding evolved to initiate in response to hunger (which corresponds to the conscious perception of an energy deficit) and to stop once satiated, even if food remains available. This behaviour is commonly described as homeostatic intake and is controlled by a brain circuit with prominent roles of several hypothalamic subdivisions (arcuate (ARC) and paraventricular nucleus of the hypothalamus (PVN), dorsomedial nucleus of the hypothalamus (DMH)) and the nucleus of the solitary tract (NTS). The function of these brain nuclei is to integrate information about the nutritional status and energy state of the organism to initiate or stop feeding behaviour [175]. The nuclei controlling homoeostatic intake relay information to the reward circuit, which is necessary for the assignment of incentive salience to the food and environmental cues that are present during food seeking and intake [176; 177].

Feeding reward can be understood as two different processes, which have different neurobiological underpinnings involving several brain circuits and neurotransmitters. One brain circuit regulates liking (the hedonic response to food intake) and another regulates wanting (the motivation to seek and obtain food) [178]. In rodents, liking is measured through analysis of facial expressions in response to food exposure and wanting is operationalized as the amount of work an individual is willing to perform to obtain food [179]. The regulation of liking involves several brain sites located both in the midbrain and cortical areas, and in these circuits there is a prominent function of opioid and orexin peptides [180]. The neural circuit that regulates wanting is built around the mesolimbic dopaminergic (DA) system, in which DA neurons located in the ventral tegmental area (VTA) project to different forebrain regions, including the nucleus accumbens (NAcc)

and the prefrontal cortex (PFC) [181]. The separation between liking and wanting in the context of food intake is well reflected in classic experiments showing that elimination of VTA dopaminergic neurons does not alter facial expressions in response to intake of a sucrose solution [182], and that hyper-dopaminergic mice have higher motivation to obtain a chocolate pellet without altering facial responses to sucrose [183].

The neurobiological regulation of liking is a distributed process over several brain regions [179]. Despite the definition of liking as the hedonic consequences of food intake, it is important to notice that liking does not describe the sensory experience associated to food intake and thus sensory brain regions are not considered part of the neurobiological substrate of liking [179]. The distributed processing of liking is reflected in evidence indicating the presence of brain regions called hedonic "hot-spots" or "cold-spots", in which activation of opioid (mu and delta) or orexin neuropeptides receptors has a large positive or negative influence in liking, respectively [178]. Hedonic hot-spots have been identified in the NAcc, ventral pallidum (VP), medial orbitofrontal cortex (OFC) while cold-spots have been observed in the posterior OFC and the insula [180]. In addition to opioid peptides, endocannabinoids can also increase liking for sucrose by their actions in the NAcc [184]. Thus, neuronal regulation of liking involves endocannabinoids, opioid and orexin peptides acting in a distributed brain network.

The neuronal control of "wanting" also involves different brain regions, but with a central role of the mesolimbic dopaminergic circuit [176]. The specific role of DA release in response to food intake is debated, but there is now a larger consensus that activation of dopaminergic VTA neurons is key for the attribution of incentive salience to food and environmental cues that occur during eating [181]. For example, activation of dopaminergic VTA neurons during intake is sufficient to change choice from a sweet sucralose solution to water [185]. Activation of the dopaminergic VTA neurons leads to

dopamine release in several brain regions, including NAcc, PFC and dorsal striatum in an experience and context-dependent manner [186]. Intake of palatable food increases DA concentration in NAcc, which is related to flavour and not caloric food content [187] but this effect decreases over repeated exposure [188]. In the context of operant or Pavlovian conditioning, the release of DA shifts from the act of intake to the act or cue that predicts reward, suggesting the ability of dopamine to predict reward-delivery and serve as an error-signal [189; 190]. This dopaminergic circuit does not operate independently and is regulated by input from several brain areas. For example, DA action in the NAcc is modulated by excitatory input from the basolateral amygdala [191] and activity of dopaminergic VTA neurons is regulated by excitatory and inhibitory input from the lateral hypothalamus (LH) [192], including orexin neurons targeting the VTA [193]. The mesolimbic dopaminergic circuit is also regulated by endocannabinoids, as activation of the CB1 receptor in the NAcc increases food intake and regulates synaptic plasticity in VTA, NAcc and PFC neurons with the overall effect of increasing activity in this brain circuit [194]. Thus, neuronal regulation of wanting is organised around the mesolimbic DA system, which is modulated by input from several brain regions. These pathways work in conjunction with homeostatic signalling pathways such as via OT, sending information from the periphery regarding an animal's physiological status, and can in this way modify sensitivity to homeostatic and hedonic pressures for consumption. These networks collectively comprise the structures regulating both the homeostatic and hedonic components of food intake, which can become out of balance during addiction (Figure 1.11.)

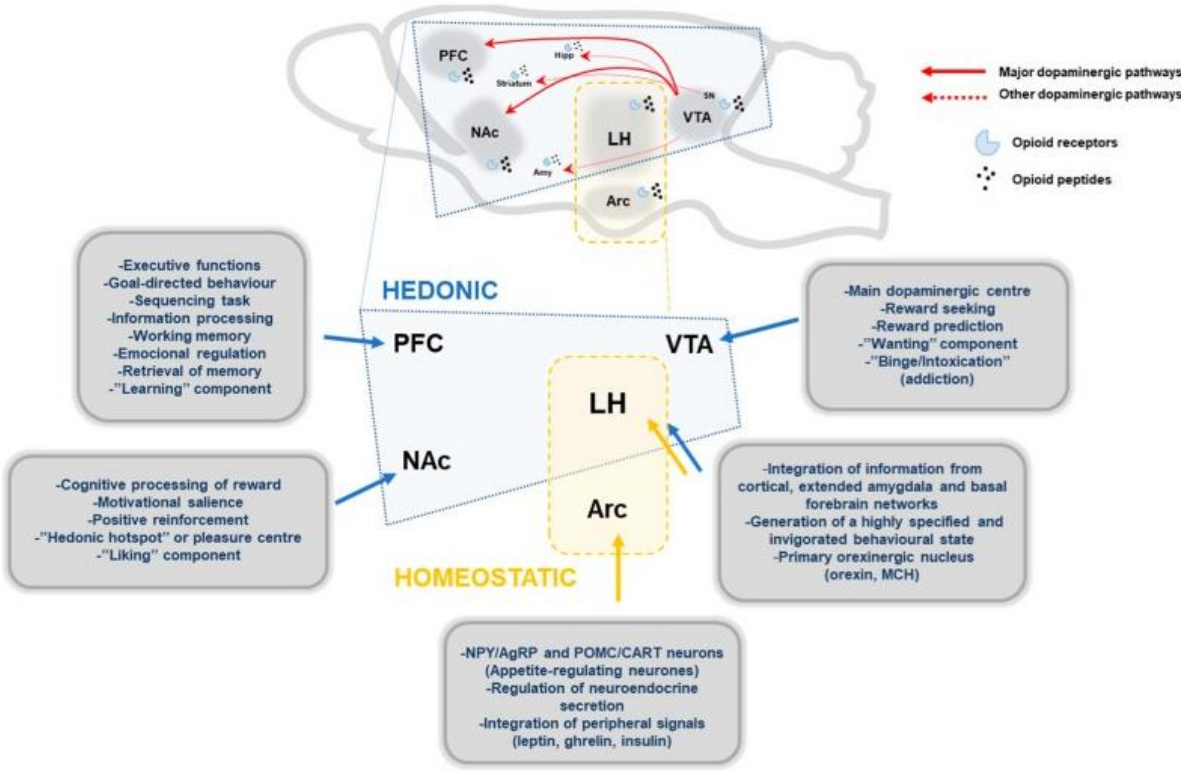


Figure 1.9. Schematic representation of main brain structures implicated in hedonic and homeostatic food intake regulation. Main dopaminergic pathways, mesolimbic and mesocortical pathway, are represented with red lines, and other minor dopaminergic connections with broken red lines. Endogenous opioids peptides modulate the dopaminergic pathways through opioid receptors (μ, κ, δ). Hedonic pathways: PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmentum area; LH, lateral hypothalamus; Amy, Amygdala; Hipp, Hippocampus; SN, substantia nigra. Homeostatic pathways: Arc, arcuate nucleus; MCH, melanin concentrating-hormone [From Novelle et al., 2018 [195]].

Overall, feeding reward is regulated by distinct brain circuits that control the behaviours of "liking" and "wanting" food, which act together to integrate the hedonic response to food and the motivation to seek and obtain foods. These brain circuits operate in the context of a larger brain network that controls higher order contributions to food intake (i.e. learning, cue-induced feeding, food choice, preference) [196].

Interestingly, a 2020 study by the Chinese Academy of Sciences proposes that they have been successful in preventing relapse of addiction by interrupting the brain pathways responsible for morphine-associated memories in mice, "erasing" the drug-associated memory and stopping relapse of addiction. Optogenetic stimulation of the PVT was able to block relapse. Keyes et al. describes how the paraventricular nucleus of the thalamus (PVT) orchestrates the acquisition and maintenance of opiate-associated memories via

projections to the central nucleus of the amygdala (CeA) and nucleus accumbens (NAc). PVT→CeA activity associates morphine reward to the environment, whereas using optogenetic transient inhibition of the PVT→NAc pathway during retrieval causes enduring protection against opiate-primed relapse. They revealed distributed network activities that are altered in non-relapsing mice, which guided them to find that activating the downstream NAc→lateral hypothalamus (LH) pathway also prevents relapse [197].

1.11 Specific aims

Feeding behaviour is therefore determined by a variety of intrinsic and extrinsic factors, though the ones that appear to be in the forefront of shaping the consummatory response involve: a feeling of hunger (which determines the motivation to seek calories and food choices), satiation (which underpins the process of termination of ingestive behaviour), and reward processing (which can to a large extent shift the hunger-satiety continuum, adjusting consumption to the 'pleasantness' of food instead of to the actual energy needs of the organism). In our search for neuroactive agents to curb excessive food consumption, those that affect more than one facet of feeding control are of particular interest, as they target a broader range of appetite regulating processes.

After more than a century of search for pharmacological treatments that combat obesity, our choice of FDA-approved pharmaceuticals is currently limited to five. Two of those drugs, Qsymia (phentermine + topiramate) and Contrave (bupropion + NTX), are combination medicines: this fact reflects the need to simultaneously target many neural and neuroendocrine systems in order to increase the likelihood of effectively treating this multifactorial condition.

The combination medicine approach served as the basis of the recent case report in which hypophagic properties of OT, a molecule being subject of several clinical trials related to disordered appetite and obesity, were successfully augmented by co-administration of NTX in a patient with hypothalamic obesity caused by craniopharyngioma resection [1]. The recent years have brought a tremendous interest in anorexigenic properties of OT. OT has been shown beyond reasonable doubt to promote satiation and early termination of food intake. Though OT also decreases feeding for palatability, its effectiveness is limited as it is vastly mitigated by the flavour and composition of tastants and by the context (e.g., social environment, novelty) in which palatable foods are offered. Thus far, there has been no direct evidence suggesting a link between OT and the third facet of

appetite control: a feeling of hunger (in other words, whether OT can delay a drive to search for energy). In fact, molecules that produce early satiation or affect eating for pleasure do not necessarily alter a self-perceived level of hunger. Though it seems unlikely, however, one has to consider that OT has been found to slightly increase latency to begin a meal, and thus a possible role of OT in hunger control certainly needs to be addressed.

Therefore, in the **first experimental aim** of my thesis, I used a unique hunger discrimination paradigm, in which rats were trained to generate an operant response indicating a hungry versus sated state, and I studied whether OT treatment makes them less likely to recognize being energy-deprived. This study was accompanied by the assessment of neuronal activation in response to the OT treatment in hungry versus non-deprived animals and by the analysis of OXTR gene expression. I also looked into whether blockade of opioid receptors with a non-selective antagonist, NTX, a compound known to affect predominantly eating for reward, but not through the change in the energy-driven consumption, has a similar effect to OT in the hunger discrimination operant paradigm.

The results of the first experimental aim of my thesis showed the lack of change in perceived hunger levels in animals treated by OT. This strengthens the notion of OT acting mainly as an early satiation signal, while being ineffective in reducing a feeling of hunger (as per the first aim) and partially ineffective in decreasing feeding reward (as shown in earlier studies). Considering the recent surge in experimental and clinical studies exploring usability of OT in managing food intake and body weight, the question arises whether OT, the molecule that affects primarily only one of the three key aspects of feeding behaviour (early satiation), is in fact sufficient as a lone pharmacological tool to control food intake, or it should be used in combination with another neuroactive molecule which targets a different facet of feeding control that the one affected by OT.

Since OT's effects on feeding reward are weakened by a variety of factors, I stipulated that effectiveness of OT on energy balance can be potentiated by using it in a combination therapy with a molecule that suppresses palatability-driven consumption. Opioid receptor antagonists, agents that based on the first experimental aim have no effect on hunger discrimination (similarly to OT), powerfully diminish feeding reward. Therefore, I examined the effectiveness of this combination therapy in the **second** and **third experimental aims**:

In the **second experimental aim**, I determined whether adolescent rats injected with subthreshold doses of OT and a non-selective opioid receptor antagonist, NTX, show a more robust reduction in short-term meal responsiveness. It was studied in the context of diets that differ in palatability/energy density. I also examined differences in brain activation in feeding-related sites in response to the combination treatment.

In the **third experimental aim**, I evaluated long-term feeding and body weight consequences of combined OT-NTX daily injections in adult rats. The effects of the treatment on expression of genes relevant to feeding in key brain areas were assessed.

Specific Aims

Overarching goal: Investigate the effect of combining OT and NTX at subthreshold doses, on food intake driven by homeostatic pressures and palatability, and the corresponding neuromolecular changes.

1. Determine the effect of OT and NTX on hunger discrimination (Chapter 2 & 3).
2. Determine the acute effects of a combination of OT and NTX at subthreshold doses on food intake in adolescent rats, and the corresponding changes in neuronal activation (Chapter 4).
3. Determine the chronic effects of a combination of OT and NTX at subthreshold doses on food intake and body weight in adult rats, and the corresponding changes in gene expression in feeding-related brain regions (Chapter 5).

Specific Aim 1: Determine the effect of OT and NTX on hunger discrimination.

I used a unique hunger discrimination paradigm, in which rats were trained to generate an operant response indicating a hungry versus sated state, and I studied whether OT treatment makes them less likely to recognize being energy-deprived. This study was accompanied by the assessment of neuronal activation in response to the OT treatment in hungry versus non-deprived animals and by the analysis of OXTR gene expression. I also looked into whether blockade of opioid receptors with a non-selective antagonist, NTX, a compound known to affect predominantly eating for reward, but not through the change in the energy-driven consumption, has a similar effect to OT in the hunger discrimination operant paradigm.

Specific Aim 2: Determine the acute effects of a combination of OT and NTX at subthreshold doses on food intake in adolescent rats, and the corresponding changes in neuronal activation.

I determined whether adolescent rats injected with subthreshold doses of OT and a non-selective opioid receptor antagonist, NTX, show a more robust reduction in short-term meal responsiveness. It was studied in the context of diets that differ in palatability/energy density. I then utilised immunohistochemistry to examine differences in brain activation in feeding-related sites in response to the combination treatment.

Specific Aim 3: Determine the chronic effects of a combination of OT and NTX at subthreshold doses on food intake and body weight in adult rats, and the corresponding changes in gene expression.

I evaluated long-term feeding and body weight consequences of combined OT-NTX daily injections in adult rats. The effects of the treatment on expression of genes relevant to feeding in key brain areas were also assessed using rt-PCR analysis.

1.12 References

[1] Hsu, E. A., Miller, J. L., Perez, F. A., & Roth, C. L. (2018). Oxytocin and naltrexone successfully treat hypothalamic obesity in a boy post-craniopharyngioma resection. *The Journal of Clinical Endocrinology & Metabolism, 103*(2), 370-375.

[2] Olszewski, P. K., Klockars, A., Schiöth, H. B., & Levine, A. S. (2010). Oxytocin as feeding inhibitor: Maintaining homeostasis in consummatory behavior. *Pharmacology Biochemistry and Behavior, 97*(1), 47-54.

[3] Gutkowska, J., & Jankowski, M. (2011). Oxytocin as an Inducer of Cardiomyogenesis. In.

[4] Postina, R., Kojro, E., & Fahrenholz, F. (1996). Separate agonist and peptide antagonist binding sites of the oxytocin receptor defined by their transfer into the V2 vasopressin receptor. *Journal of Biological Chemistry, 271*(49), 31593-31601.

[5] Chini, B., Mouillac, B., Balestre, M.-N., Trumpp-Kallmeyer, S., Hoflack, J., Hibert, M., Andriolo, M., Pupier, S., Jard, S., & Barberis, C. (1996). Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin. *FEBS letters, 397*(2-3), 201-206.

[6] Acher, R., Chauvet, J., & Chauvet, M. (1995). Man and the chimaera. Selective versus neutral oxytocin evolution. *Advances in experimental medicine and biology, 395*, 615.

[7] Oumi, T., Ukena, K., Matsushima, O., Ikeda, T., Fujita, T., Minakata, H., & Nomoto, K. (1994). Annetocin: an oxytocin-related peptide isolated from the earthworm, Eisenia foetida. *Biochemical and biophysical research communications, 198*(1), 393-399.

[8] Ukena, K., Oumi, T., Matsushima, O., Ikeda, T., Fujita, T., Minakata, H., & Nomoto, K. (1995). Effects of annetocin, an oxytocin-related peptide isolated from the earthworm Eisenia foetida, and some putative neurotransmitters on gut motility of the earthworm. *Journal of Experimental Zoology, 272*(3), 184-193.

[9] Rao, V. G., Löffler, C., Battey, J., & Hansmann, I. (1992). The human gene for oxytocin-neurophysin I (OXT) is physically mapped to chromosome 20p13 by in situ hybridization. *Cytogenetic and Genome Research, 61*(4), 271-273.

[10] Gimpl, G., & Fahrenholz, F. (2001). The oxytocin receptor system: structure, function, and regulation. *Physiological reviews, 81*(2), 629-683.

[11] Gainer, H. (1998). Cell-specific gene expression in oxytocin and vasopressin magnocellular neurons. In *Vasopressin and Oxytocin* (pp. 15-27). Springer.

[12] Ivell, R., Walther, N., Wehrenberg, U., McArdle, C., & Ungefroren, H. (1993). The regulation of neurohypophyseal peptide gene expression in gonadal tissues. *Regulatory peptides, 45*(1-2), 263-267.

[13] Quintana, D. S., Rokicki, J., van der Meer, D., Alnæs, D., Kaufmann, T., Córdova-Palomera, A., Dieset, I., Andreassen, O. A., & Westlye, L. T. (2019). Oxytocin pathway gene networks in the human brain. *Nature communications, 10*(1), 1-12.

[14] Sausville, E., Carney, D., & Battey, J. (1985). The human vasopressin gene is linked to the oxytocin gene and is selectively expressed in a cultured lung cancer cell line. *Journal of Biological Chemistry, 260*(18), 10236-10241.

[15] Inoue, T., Kimura, T., Azuma, C., Inazawa, J., Takemura, M., Kikuchi, T., Kubota, Y., Ogita, K., & Saji, F. (1994). Structural organization of the human oxytocin receptor gene. *Journal of Biological Chemistry, 269*(51), 32451-32456.

[16] Michelini, S., Urbanek, M., Dean, M., & Goldman, D. (1995). Polymorphism and genetic mapping of the human oxytocin receptor gene on chromosome 3. *American journal of medical genetics, 60*(3), 183-187.

[17] Simmons Jr, C. F., Clancy, T. E., Quan, R., & Knoll, J. H. (1995). The oxytocin receptor gene (OXTR) localizes to human chromosome 3p25 by fluorescence in situ hybridization and PCR analysis of somatic cell hybrids. *Genomics, 26*(3), 623-625.

[18] Seibold, A., Brabet, P., Rosenthal, W., & Birnbaumer, M. (1992). Structure and chromosomal localization of the human antidiuretic hormone receptor gene. *Am J Hum Genet, 51*(5), 1078-83.

[19] Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., & Okayama, H. (1992). Structure and expression of a human oxytocin receptor. *Nature, 356*(6369), 526-529.

[20] Peter, J., Burbach, H., Adan, R. A., Lolait, S. J., van Leeuwen, F. W., Mezey, E., Palkovits, M., & Barberis, C. (1995). Molecular neurobiology and pharmacology of the vasopressin/oxytocin receptor family. *Cellular and molecular neurobiology, 15*(5), 573-595.

[21] Verbalis, J. G. (1999). The brain oxytocin receptor (s)? *Frontiers in neuroendocrinology, 20*(2), 146-156.

[22] Chan, W., Chen, D.-L., & Manning, M. (1993). Oxytocin receptor subtypes in the pregnant rat myometrium and decidua: pharmacological differentiations. *Endocrinology, 132*(3), 1381-1386.

[23] Chen, D., Chan, W., & Manning, M. (1994). Agonist and antagonist specificities of decidual prostaglandin-releasing oxytocin receptors and myometrial uterotonic oxytocin receptors in pregnant rats. *Reproduction, 102*(2), 337-343.

[24] Arpin-Bott, M., Waltisperger, E., Freund-Mercier, M., & Stoeckel, M. (1997). Two oxytocin-binding site subtypes in rat kidney: pharmacological characterization, ontogeny and localization by in vitro and in vivo autoradiography. *Journal of endocrinology, 153*(1), 49-59.

[25] Adan, R., Van Leeuwen, F., Sonnemans, M., Brouns, M., Hoffman, G., Verbalis, J., & Burbach, J. (1995). Rat oxytocin receptor in brain, pituitary, mammary gland, and uterus: partial sequence and immunocytochemical localization. *Endocrinology, 136*(9), 4022-4028.

[26] de Wied, D., Diamant, M., & Fodor, M. (1993). Central nervous system effects of the neurohypophyseal hormones and related peptides. *Frontiers in neuroendocrinology, 14*(4), 251-302.

[27] Barberis, C., & Tribollet, E. (1996). Vasopressin and oxytocin receptors in the central nervous system. *Critical Reviews™ in Neurobiology, 10*(1).

[28] Du Vigneaud, V., Ressler, C., & Trippett, S. (1953). The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. *J Biol Chem, 205*(2), 949-57.

[29] Barberis, C., Mouillac, B., & Durroux, T. (1998). Structural bases of vasopressin/oxytocin receptor function. *Journal of Endocrinology, 156*(2), 223-229.

[30] Fredriksson, R., Lagerström, M. C., Lundin, L.-G., & Schiöth, H. B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular pharmacology, 63*(6), 1256-1272.

[31] Brownstein, M. J., Russell, J. T., & Gainer, H. (1980). Synthesis, transport, and release of posterior pituitary hormones. *Science, 207*(4429), 373-378.

[32] Renaud, L. P., & Bourquet, C. W. (1991). Neurophysiology and neuropharmacology of hypothalamic magnocellular neurons secreting vasopressin and oxytocin. *Progress in neurobiology, 36*(2), 131-169.

[33] Rose, J. P., Wu, C.-K., Hsiao, C.-D., Breslow, E., & Wang, B.-C. (1996). Crystal structure of the neurophysin—oxytocin complex. *Nature structural biology, 3*(2), 163-169.

[34] Yamamoto, Y., Liang, M., Munesue, S., Deguchi, K., Harashima, A., Furuhara, K., Yuhi, T., Zhong, J., Akther, S., & Goto, H. (2019). Vascular RAGE transports oxytocin into the brain to elicit its maternal bonding behaviour in mice. *Communications biology, 2*(1), 1-13.

[35] Wang, Y.-F., & Hatton, G. I. (2006). Mechanisms underlying oxytocin-induced excitation of supraoptic neurons: prostaglandin mediation of actin polymerization. *Journal of neurophysiology, 95*(6), 3933-3947.

[36] Calatayud, S., Quintana, E., Esplugues, J., & Barrachina, M. D. (1999). Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion. *Naunyn-Schmiedeberg's archives of pharmacology, 360*(6), 676-682.

[37] Renaud, L., Tang, M., McCann, M., Stricker, E., & Verbalis, J. (1987). Cholecystokinin and gastric distension activate oxytocinergic cells in rat hypothalamus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 253*(4), R661-R665.

[38] Nelson, E. E., Alberts, J. R., Tian, Y., & Verbalis, J. G. (1998). Oxytocin is elevated in plasma of 10-day-old rats following gastric distension. *Developmental brain research, 111*(2), 301-303.

[39] Olszewski, P. K., Waas, J. R., Brooks, L. L., Herisson, F., & Levine, A. S. (2013). Oxytocin receptor blockade reduces acquisition but not retrieval of taste aversion and blunts responsiveness of amygdala neurons to an aversive stimulus. *Peptides, 50*, 36-41.

[40] Klockars, A., Levine, A. S., & Olszewski, P. K. (2015). Central Oxytocin and Food Intake: Focus on Macronutrient-Driven Reward. *Frontiers in Endocrinology, 6*(65).

[41] Olszewski, P. K., Bomberg, E. M., Martell, A., Grace, M. K., & Levine, A. S. (2007). Intraventricular ghrelin activates oxytocin neurons: implications in feeding behavior. *Neuroreport, 18*(5), 499-503.

[42] Chatterjee, O., Patil, K., Sahu, A., Gopalakrishnan, L., Mol, P., Advani, J., Mukherjee, S., Christopher, R., & Prasad, T. K. (2016). An overview of the oxytocin-oxytocin receptor signaling network. *Journal of cell communication and signaling, 10*(4), 355-360.

[43] Kim, S. H., MacIntyre, D. A., Da Silva, M. F., Blanks, A. M., Lee, Y. S., Thornton, S., Bennett, P. R., & Terzidou, V. (2015). Oxytocin activates NF-κB-mediated inflammatory pathways in human gestational tissues. *Molecular and cellular endocrinology, 403*, 64-77.

[44] Terzidou, V., Blanks, A. M., Kim, S. H., Thornton, S., & Bennett, P. R. (2011). Labor and inflammation increase the expression of oxytocin receptor in human amnion. *Biology of reproduction, 84*(3), 546-552.

[45] Jeng, Y.-J., Liebenthal, D., Strakova, Z., Ives, K. L., Hellmich, M. R., & Soloff, M. S. (2000). Complementary mechanisms of enhanced oxytocin-stimulated prostaglandin E2 synthesis in rabbit amnion at the end of gestation. *Endocrinology, 141*(11), 4136-4145.

[46] Tamma, R., Colaianni, G., Zhu, L.-l., DiBenedetto, A., Greco, G., Montemurro, G., Patano, N., Strippoli, M., Vergari, R., & Mancini, L. (2009). Oxytocin is an anabolic bone hormone. *Proceedings of the National Academy of Sciences, 106*(17), 7149-7154.

[47] Gong, L., Gao, F., Li, J., Yu, X., Ma, X., Zheng, W., Cui, S., Liu, K., Zhang, M., & Kunze, W. (2015). Oxytocin-induced membrane hyperpolarization in pain-sensitive dorsal root ganglia neurons mediated by Ca2+/nNOS/NO/KATP pathway. *Neuroscience, 289*, 417-428.

[48] Okimoto, N., Bosch, O. J., Slattery, D. A., Pflaum, K., Matsushita, H., Wei, F.-Y., Ohmori, M., Nishiki, T.-i., Ohmori, I., & Hiramatsu, Y. (2012). RGS2 mediates the anxiolytic effect of oxytocin. *Brain research, 1453*, 26-33.

[49] Ondrejcakova, M., Barancik, M., Bartekova, M., Ravingerova, T., & Jezova, D. (2012). Prolonged oxytocin treatment in rats affects intracellular signaling and induces myocardial protection against infarction. *General physiology and biophysics, 31*(3), 261-270.

[50] Olson, B. R., Drutarosky, M. D., Chow, M.-S., Hruby, V. J., Stricker, E. M., & Verbalis, J. G. (1991). Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats. *Peptides, 12*(1), 113-118.

[51] Tom, N., & Assinder, S. J. (2010). Oxytocin in health and disease. *The International Journal of Biochemistry & Cell Biology, 42*(2), 202-205.

[52] Nishimori, K., Takayanagi, Y., Yoshida, M., Kasahara, Y., Young, L. J., & Kawamata, M. (2008). New aspects of oxytocin receptor function revealed by knockout mice: sociosexual behaviour and control of energy balance. *Progress in brain research, 170*, 79-90.

[53] Takayanagi, Y., Kasahara, Y., Onaka, T., Takahashi, N., Kawada, T., & Nishimori, K. (2008). Oxytocin receptor-deficient mice developed late-onset obesity. *Neuroreport, 19*(9), 951-955.

[54] Camerino, C. (2009). Low sympathetic tone and obese phenotype in oxytocin-deficient mice. *Obesity, 17*(5), 980-984.

[55] Sclafani, A., Rinaman, L., Vollmer, R. R., & Amico, J. A. (2007). Oxytocin knockout mice demonstrate enhanced intake of sweet and nonsweet carbohydrate solutions. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 292*(5), R1828-R1833.

[56] Olszewski, P. K., Shaw, T. J., Grace, M. K., Höglund, C. E., Fredriksson, R., Schiöth, H. B., & Levine, A. S. (2009). Complexity of neural mechanisms underlying overconsumption of sugar in scheduled feeding: involvement of opioids, orexin, oxytocin and NPY. *Peptides, 30*(2), 226-233.

[57] Pelletier, G. (1991). Anatomy of the hypothalamic-pituitary axis. *Methods and achievements in experimental pathology, 14*, 1-22.

[58] Swanson, L., & Sawchenko, P. (1980). Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology, 31*(6), 410-417.

[59] Huang, W., Sved, A. F., & Stricker, E. M. (2000). Vasopressin and oxytocin release evoked by NaCl loads are selectively blunted by area postrema lesions. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 278*(3), R732-R740.

[60] McCANN, M. J., & Rogers, R. C. (1990). Oxytocin excites gastric-related neurones in rat dorsal vagal complex. *The Journal of physiology, 428*(1), 95-108.

[61] Sagar, S. M., Price, K. J., Kasting, N. W., & Sharp, F. R. (1995). Anatomic patterns of FOS immunostaining in rat brain following systemic endotoxin administration. *Brain Research Bulletin, 36*(4), 381-392.

[62] Charpak, S., Armstrong, W. E., Mühlethaler, M., & Dreifuss, J. J. (1984). Stimulatory action of oxytocin on neurones of the dorsal motor nucleus of the vagus nerve. *Brain Research, 300*(1), 83-89.

[63] Grill, H. J., & Hayes, M. R. (2012). Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. *Cell metabolism, 16*(3), 296-309.

[64] Doyle, M. W., & Andresen, M. C. (2001). Reliability of monosynaptic sensory transmission in brain stem neurons in vitro. *Journal of neurophysiology, 85*(5), 2213-2223.

[65] Williams, E. K., Chang, R. B., Strochlic, D. E., Umans, B. D., Lowell, B. B., & Liberles, S. D. (2016). Sensory neurons that detect stretch and nutrients in the digestive system. *Cell, 166*(1), 209-221.

[66] Cerritelli, S., Hirschberg, S., Hill, R., Balthasar, N., & Pickering, A. E. (2016). Activation of brainstem pro-opiomelanocortin neurons produces opioidergic analgesia, bradycardia and bradypnoea. *PLoS One, 11*(4), e0153187.

[67] D'Agostino, G., Lyons, D. J., Cristiano, C., Burke, L. K., Madara, J. C., Campbell, J. N., Garcia, A. P., Land, B. B., Lowell, B. B., & Dileone, R. J. (2016). Appetite controlled by a cholecystokinin nucleus of the solitary tract to hypothalamus neurocircuit. *Elife, 5*, e12225.

[68] MacDonald, A. J., Holmes, F. E., Beall, C., Pickering, A. E., & Ellacott, K. L. (2020). Regulation of food intake by astrocytes in the brainstem dorsal vagal complex. *Glia, 68*(6), 1241-1254.

[69] Pow, D. V., & Morris, J. F. (1989). Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis. *Neuroscience, 32*(2), 435-439.

[70] Shughrue, P. J., Lane, M. V., & Merchenthaler, I. (1996). Glucagon-like peptide-1 receptor (GLP1-R) mRNA in the rat hypothalamus. *Endocrinology, 137*(11), 5159-5162.

[71] Leng, G., Onaka, T., Caquineau, C., Sabatier, N., Tobin, V. A., & Takayanagi, Y. (2008). Oxytocin and appetite. *Progress in brain research, 170*, 137-151.

[72] Young, L. J., Muns, S., Wang, Z., & Insel, T. R. (1997). Changes in oxytocin receptor mRNA in rat brain during pregnancy and the effects of estrogen and interleukin-6. *Journal of neuroendocrinology, 9*(11), 859-865.

[73] Yoshimura, R., Kiyama, H., Kimura, T., Araki, T., Maeno, H., Tanizawa, O., & Tohyama, M. (1993). Localization of oxytocin receptor messenger ribonucleic acid in the rat brain. *Endocrinology, 133*(3), 1239-1246.

[74] Keebaugh, A. C., & Young, L. J. (2011). Increasing oxytocin receptor expression in the nucleus accumbens of pre-pubertal female prairie voles enhances alloparental responsiveness and partner preference formation as adults. *Hormones and behavior, 60*(5), 498-504.

[75] Keebaugh, A. C., Barrett, C. E., Laprairie, J. L., Jenkins, J. J., & Young, L. J. (2015). RNAi knockdown of oxytocin receptor in the nucleus accumbens inhibits social attachment and parental care in monogamous female prairie voles. *Social neuroscience, 10*(5), 561-570.

[76] Neumann, I., Douglas, A. J., Pittman, Q. J., Russell, J. A., & Landgraf, R. (1996). Oxytocin released within the supraoptic nucleus of the rat brain by positive feedback action is involved in parturition-related events. *Journal of neuroendocrinology, 8*(3), 227-233.

[77] Moos, F., Poulain, D., Rodriguez, F., Guerne, Y., Vincent, J.-D., & Richard, P. (1989). Release of oxytocin within the supraoptic nucleus during the milk ejection reflex in rats. *Experimental brain research, 76*(3), 593-602.

[78] Neumann, I., Russell, J., & Landgraf, R. (1993). Oxytocin and vasopressin release within the supraoptic and paraventricular nuclei of pregnant, parturient and lactating rats: a microdialysis study. *Neuroscience, 53*(1), 65-75.

[79] Buijs, R., De Vries, G., Van Leeuwen, F., & Swaab, D. (1983). Vasopressin and oxytocin: distribution and putative functions in the brain. In *Progress in brain research* (Vol. 60, pp. 115-122). Elsevier.

[80] De Vries, G., & Buijs, R. (1983). The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum. *Brain research, 273*(2), 307-317.

[81] Kirchgessner, A. L., & Sclafani, A. (1988). PVN-hindbrain pathway involved in the hypothalamic hyperphagia-obesity syndrome. *Physiology & behavior, 42*(6), 517-528.

[82] Olson, B. R., Freilino, M., Hoffman, G. E., Stricker, E. M., Sved, A. F., & Verbalis, J. G. (1993). c-Fos expression in rat brain and brainstem nuclei in response to treatments that alter food intake and gastric motility. *Molecular and Cellular Neuroscience, 4*(1), 93-106.

[83] Crowley, W., Parker, S., STRONG, W. A., Spinolo, L., & Grosvenor, C. (1992). Neurotransmitter and Neurohormonal Regulation of Oxytocin Secretion in Lactation a. *Annals of the New York Academy of Sciences, 652*(1), 286-302.

[84] SOLOFF, M. S., CHAKRABORTY, J., SADHUKHAN, P., SENITZER, D., WIEDER, M., FERNSTROM, M. A., & SWEET, P. (1980). Purification and characterization of mammary

myoepithelial and secretory cells from the lactating rat. *Endocrinology, 106*(3), 887-897.

[85] Douglas, A. J., Johnstone, L. E., & Leng, G. (2007). Neuroendocrine mechanisms of change in food intake during pregnancy: a potential role for brain oxytocin. *Physiology & behavior, 91*(4), 352-365.

[86] Brunton, P. J., Arunachalam, S., & Russel, J. (2008). Control of neurohypophysial hormone secretion, blood osmolality and volume in pregnancy. *J Physiol Pharmacol, 59*(Suppl 8), 27-45.

[87] Jarrett, T., McMurray, M., Walker, C., & Johns, J. (2006). Cocaine treatment alters oxytocin receptor binding but not mRNA production in postpartum rat dams. *Neuropeptides, 40*(3), 161-167.

[88] Baracz, S. J., Everett, N. A., McGregor, I. S., & Cornish, J. L. (2016). Oxytocin in the nucleus accumbens core reduces reinstatement of methamphetamine-seeking behaviour in rats. *Addiction biology, 21*(2), 316-325.

[89] Baracz, S. J., Rourke, P. I., Pardey, M. C., Hunt, G. E., McGregor, I. S., & Cornish, J. L. (2012). Oxytocin directly administered into the nucleus accumbens core or subthalamic nucleus attenuates methamphetamine-induced conditioned place preference. *Behavioural brain research, 228*(1), 185-193.

[90] Bahi, A. (2015). The oxytocin receptor impairs ethanol reward in mice. *Physiology & behavior, 139*, 321-327.

[91] Kent, K., Arientyl, V., Khachatryan, M. M., & Wood, R. I. (2013). Oxytocin induces a conditioned social preference in female mice. *Journal of neuroendocrinology, 25*(9), 803-810.

[92] Damiano, C. R., Aloi, J., Dunlap, K., Burrus, C. J., Mosner, M. G., Kozink, R. V., McLaurin, R. E., O'Dhaniel, A., Carter, R. M., & Huettel, S. A. (2014). Association between the oxytocin receptor (OXTR) gene and mesolimbic responses to rewards. *Molecular autism, 5*(1), 7.

[93] Qi, J., Yang, J.-Y., Song, M., Li, Y., Wang, F., & Wu, C.-F. (2008). Inhibition by oxytocin of methamphetamine-induced hyperactivity related to dopamine turnover in the mesolimbic region in mice. *Naunyn-Schmiedeberg's archives of pharmacology, 376*(6), 441-448.

[94] Qi, J., Han, W. Y., Yang, J. Y., Wang, L. H., Dong, Y. X., Wang, F., Song, M., & Wu, C. F. (2012). Oxytocin regulates changes of extracellular glutamate and GABA levels induced by methamphetamine in the mouse brain. *Addiction biology, 17*(4), 758-769.

[95] Domes, G., Heinrichs, M., Michel, A., Berger, C., & Herpertz, S. C. (2007). Oxytocin improves "mind-reading" in humans. *Biological psychiatry, 61*(6), 731-733.

[96] Guastella, A. J., Mitchell, P. B., & Dadds, M. R. (2008). Oxytocin increases gaze to the eye region of human faces. *Biological psychiatry, 63*(1), 3-5.

[97] Insel, T. R. (1992). Oxytocin—a neuropeptide for affiliation: evidence from behavioral, receptor autoradiographic, and comparative studies. *Psychoneuroendocrinology, 17*(1), 3-35.

[98] Feldman, R., Gordon, I., Schneiderman, I., Weisman, O., & Zagoory-Sharon, O. (2010). Natural variations in maternal and paternal care are associated with systematic changes in oxytocin following parent–infant contact. *Psychoneuroendocrinology, 35*(8), 1133-1141.

[99] Kendrick, K. M., Keverne, E. B., & Baldwin, B. A. (1987). Intracerebroventricular oxytocin stimulates maternal behaviour in the sheep. *Neuroendocrinology, 46*(1), 56-61.

[100] Neumann, I. D. (2008). Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. *Journal of neuroendocrinology, 20*(6), 858-865.

[101] Strathearn, L. (2011). Maternal neglect: oxytocin, dopamine and the neurobiology of attachment. *Journal of neuroendocrinology, 23*(11), 1054-1065.

[102] Smith, A. S., Ågmo, A., Birnie, A. K., & French, J. A. (2010). Manipulation of the oxytocin system alters social behavior and attraction in pair-bonding primates, Callithrix penicillata. *Hormones and behavior, 57*(2), 255-262.

[103] Beery, A., & Zucker, I. (2010). Oxytocin and same-sex social behavior in female meadow voles. *Neuroscience, 169*(2), 665-673.

[104] De Dreu, C. K., Greer, L. L., Handgraaf, M. J., Shalvi, S., Van Kleef, G. A., Baas, M., Ten Velden, F. S., Van Dijk, E., & Feith, S. W. (2010). The neuropeptide oxytocin regulates parochial altruism in intergroup conflict among humans. *Science, 328*(5984), 1408-1411.

[105] Zak, P. J., Stanton, A. A., & Ahmadi, S. (2007). Oxytocin increases generosity in humans. *PloS one, 2*(11), e1128.

[106] Barraza, J. A., McCullough, M. E., Ahmadi, S., & Zak, P. J. (2011). Oxytocin infusion increases charitable donations regardless of monetary resources. *Hormones and behavior, 60*(2), 148-151.

[107] Barraza, J., & Zak, P. (2009). Empathy toward strangers triggers oxytocin release and subsequent generosity. *Annals of the New York Academy of Sciences, 1167*(1), 182-189.

[108] Rockliff, H., Karl, A., McEwan, K., Gilbert, J., Matos, M., & Gilbert, P. (2011). Effects of intranasal oxytocin on'compassion focused imagery'. *Emotion, 11*(6), 1388.

[109] Tamashiro, K. L., Hegeman, M. A., & Sakai, R. R. (2006). Chronic social stress in a changing dietary environment. *Physiology & behavior, 89*(4), 536-542.

[110] Olszewski, P. K., Allen, K., & Levine, A. S. (2015). Effect of oxytocin receptor blockade on appetite for sugar is modified by social context. *Appetite, 86*, 81-87.

[111] Manning, M., Misicka, A., Olma, A., Bankowski, K., Stoev, S., Chini, B., Durroux, T., Mouillac, B., Corbani, M., & Guillon, G. (2012). Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics. *Journal of neuroendocrinology, 24*(4), 609-628.

[112] Moles, A., Bartolomucci, A., Garbugino, L., Conti, R., Caprioli, A., Coccurello, R., Rizzi, R., Ciani, B., & D'amato, F. (2006). Psychosocial stress affects energy balance in mice: modulation by social status. *Psychoneuroendocrinology, 31*(5), 623-633.

[113] Israel, S., Weisel, O., Ebstein, R. P., & Bornstein, G. (2012). Oxytocin, but not vasopressin, increases both parochial and universal altruism. *Psychoneuroendocrinology, 37*(8), 1341-1344.

[114] Anpilov, S., Shemesh, Y., Eren, N., Harony-Nicolas, H., Benjamin, A., Dine, J., Oliveira, V. E., Forkosh, O., Karamihalev, S., & Hüttl, R.-E. (2020). Wireless Optogenetic Stimulation of Oxytocin Neurons in a Semi-natural Setup Dynamically Elevates Both Pro-social and Agonistic Behaviors. *Neuron*.

[115] Keeler, J. R., Roth, E. A., Neuser, B. L., Spitsbergen, J. M., Waters, D. J. M., & Vianney, J.-M. (2015). The neurochemistry and social flow of singing: bonding and oxytocin. *Frontiers in human neuroscience, 9*, 518.

[116] Takahashi, J., Yamada, D., Ueta, Y., Iwai, T., Koga, E., Tanabe, M., Oka, J.-I., & Saitoh, A. (2020). Oxytocin reverses Aβ-induced impairment of hippocampal synaptic plasticity in mice. *Biochemical and Biophysical Research Communications*.

[117] Gutkowska, J., & Jankowski, M. (2012). Oxytocin revisited: its role in cardiovascular regulation. *Journal of neuroendocrinology, 24*(4), 599-608.

[118] Joydeb, M., Rahul, L., & Agarwala, B. (2013). Butterfly species richness and diversity in the Trishna Wildlife Sanctuary in South Asia. *Journal of Insect Science (Madison), 13*(79).

[119] Elabd, S., & Sabry, I. (2015). Two birds with one stone: possible dual-role of oxytocin in the treatment of diabetes and osteoporosis. *Frontiers in endocrinology, 6*, 121.

[120] Rozek, L. S., Dolinoy, D. C., Sartor, M. A., & Omenn, G. S. (2014). Epigenetics: relevance and implications for public health. *Annual review of public health, 35*, 105-122.

[121] Leibowitz, S. F., Hammer, N. J., & Chang, K. (1981). Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat. *Physiology & Behavior, 27*(6), 1031-1040.

[122] Shor-Posner, G., Azar, A. P., Insinga, S., & Leibowitz, S. F. (1985). Deficits in the control of food intake after hypothalamic paraventricular nucleus lesions. *Physiology & Behavior, 35*(6), 883-890.

[123] Gold, R. M., Ieni, J. R., & Simson, E. L. (1977). Delayed or precocious hyperphagia after symmetrical or asymmetrical hypothalamic knife cuts in male and female weanling rats. *Physiology & behavior, 18*(2), 275-281.

[124] Leibowitz, S. F., Hammer, N. J., & Chang, K. (1983). Feeding behavior induced by central norepinephrine injection is attenuated by discrete lesions in the hypothalamic paraventricular nucleus. *Pharmacology Biochemistry and Behavior, 19*(6), 945-950.

[125] Kirchgessner, A. L., Sclafani, A., & Nilaver, G. (1988). Histochemical identification of a PVN-hindbrain feeding pathway. *Physiology & behavior, 42*(6), 529-543.

[126] Arletti, R., Benelli, A., & Bertolini, A. (1989). Influence of oxytocin on feeding behavior in the rat. *Peptides, 10*(1), 89-93.

[127] Baskin, D. G., Kim, F., Gelling, R. W., Russell, B. J., Schwartz, M. W., Morton, G. J., Simhan, H. N., Moralejo, D. H., & Blevins, J. E. (2010). A New Oxytocin-Saporin

Cytotoxin for Lesioning Oxytocin-Receptive Neurons in the Rat Hindbrain. *Endocrinology, 151*(9), 4207-4213.

[128] Kublaoui, B. M., Gemelli, T., Tolson, K. P., Wang, Y., & Zinn, A. R. (2008). Oxytocin Deficiency Mediates Hyperphagic Obesity of Sim1 Haploinsufficient Mice. *Molecular Endocrinology, 22*(7), 1723-1734.

[129] Arletti, R., Benelli, A., & Bertolini, A. (1990). Oxytocin inhibits food and fluid intake in rats. *Physiology & behavior, 48*(6), 825-830.

[130] Benelli, A., Bertolini, A., & Arletti, R. (1991). Oxytocin-induced inhibition of feeding and drinking: no sexual dimorphism in rats. *Neuropeptides, 20*(1), 57-62.

[131] Jonaidi, H., Oloumi, M., & Denbow, D. (2003). Behavioral effects of intracerebroventricular injection of oxytocin in birds. *Physiology & behavior, 79*(4-5), 725-729.

[132] Flanagan, L. M., Olson, B. R., Sved, A. F., Verbalis, J. G., & Stricker, E. M. (1992). Gastric motility in consious rats given oxytocin and an oxytocin antagonist centrally. *Brain research, 578*(1-2), 256-260.

[133] Herisson, F. M., Brooks, L. L., Waas, J. R., Levine, A. S., & Olszewski, P. K. (2014). Functional relationship between oxytocin and appetite for carbohydrates versus saccharin. *Neuroreport, 25*(12), 909-914.

[134] Mullis, K., Kay, K., & Williams, D. L. (2013). Oxytocin action in the ventral tegmental area affects sucrose intake. *Brain research, 1513*, 85-91.

[135] Olszewski, P. K., Klockars, A., Olszewska, A. M., Fredriksson, R., Schioth, H. B., & Levine, A. S. (2010). Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake. *Endocrinology, 151*(10), 4736-4744.

[136] Fenelon, V., Poulain, D., & Theodosis, D. (1993). Oxytocin neuron activation and Fos expression: a quantitative immunocytochemical analysis of the effect of lactation, parturition, osmotic and cardiovascular stimulation. *Neuroscience, 53*(1), 77-89.

[137] Rinaman, L., Stricker, E., Hoffman, G., & Verbalis, J. (1997). Central c-Fos expression in neonatal and adult rats after subcutaneous injection of hypertonic saline. *Neuroscience, 79*(4), 1165-1175.

[138] Stricker, E. M., & Verbalis, J. G. (1996). Central inhibition of salt appetite by oxytocin in rats. *Regulatory peptides, 66*(1-2), 83-85.

[139] VAN TOL, H. H., VOORHUIS, D. T. A., & BURBACH, J. P. H. (1987). Oxytocin gene expression in discrete hypothalamic magnocellular cell groups is stimulated by prolonged salt loading. *Endocrinology, 120*(1), 71-76.

[140] Ludwig, M., Callahan, M. F., Neumann, I., Landgraf, R., & Morris, M. (1994). Systemic osmotic stimulation increases vasopressin and oxytocin release within the supraoptic nucleus. *Journal of neuroendocrinology, 6*(4), 369-373.

[141] Rinaman, L., Vollmer, R. R., Karam, J., Phillips, D., Li, X., & Amico, J. A. (2005). Dehydration anorexia is attenuated in oxytocin-deficient mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 288*(6), R1791-R1799.

[142] Chen, H., Morris, M., Key, M. P., & Chen, Y. (2004). Rapid Neurosecretory and Cardiovascular Response to Osmotic Stimulation in Conscious Mice. *Neuroendocrinology, 80*(4), 225-232.

[143] Flynn, F. W., Curtis, K. S., Verbalis, J. G., & Stricker, E. M. (1995). Dehydration Anorexia in Decerebrate Rats. *Behavioral Neuroscience, 109*(5), 1009-1012.

[144] Puryear, R., Rigatto, K. V., Amico, J. A., & Morris, M. (2001). Enhanced salt intake in oxytocin deficient mice. *Experimental Neurology, 171*(2), 323-328.

[145] Rigatto, K., Puryear, R., Bernatova, I., & Morris, M. (2003). Salt appetite and the renin-angiotensin system: Effect of oxytocin deficiency. *Hypertension, 42*(4), 793-797.

[146] Curtis, K. S., Huang, W., Sved, A. F., Verbalis, J. G., & Stricker, E. M. (1999). Impaired osmoregulatory responses in rats with area postrema lesions. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology, 277*(1 46-1), R209-R219.

[147] Svennersten-Sjaunja, K., & Olsson, K. (2005). Endocrinology of milk production. *Domestic Animal Endocrinology, 29*(2), 241-258.

[148] Thiele, T. E., Roitman, M. F., & Bernstein, I. L. (1996). c-Fos induction in rat brainstem in response to ethanol- and lithium chloride-induced conditioned taste aversions. *Alcoholism: Clinical and Experimental Research, 20*(6), 1023-1028.

[149] Yamamoto, T., Shimura, T., Sako, N., Azuma, S., Bai, W. Z. H., & Wakisaka, S. (1992). C-fos expression in the rat brain after intraperitoneal injection of lithium chloride. *NeuroReport, 3*(12), 1049-1052.

[150] Yamamoto, T., Shimura, T., Sako, N., Yasoshima, Y., & Sakai, N. (1994). Neural substrates for conditioned taste aversion in the rat. *Behavioural Brain Research, 65*(2), 123-137.

[151] Curtis, K. S., Sved, A. F., Verbalis, J. G., & Stricker, E. M. (1994). Lithium chloride-induced anorexia, but not conditioned taste aversions, in rats with area postrema lesions. *Brain Research, 663*(1), 30-37.

[152] Olszewski, P. K., Shi, Q., Billington, C. J., & Levine, A. S. (2000). Opioids affect acquisition of LiCl-induced conditioned taste aversion: Involvement of OT and VP systems. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology, 279*(4 48-4), R1504-R1511.

[153] Forsling, M. L., Judah, J. M., & Windle, R. J. (1994). The effect of vasopressin and oxytocin on glomerular filtration rate in the conscious rat: Contribution to the natriuretic response. *Journal of Endocrinology, 141*(1), 59-67.

[154] Houshmand, F., Faghihi, M., & Zahediasl, S. (2009). Biphasic protective effect of oxytocin on cardiac ischemia/reperfusion injury in anaesthetized rats. *Peptides, 30*(12), 2301-2308.

[155] Loichot, C., Grima, M., De Jong, W., Helwig, J.-J., Imbs, J.-L., & Barthelmebs, M. (2002). Oxytocin-induced renin secretion by denervated kidney in anaesthetized rat. *European journal of pharmacology, 454*(2-3), 241-247.

[156] Ondrejcakova, M., Ravingerova, T., Bakos, J., Pancza, D., & Jezova, D. (2009). Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion. *Canadian journal of physiology and pharmacology, 87*(2), 137-142.

[157] Petersson, M., & Uvnäs-Moberg, K. (2008). Postnatal oxytocin treatment of spontaneously hypertensive male rats decreases blood pressure and body weight in adulthood. *Neuroscience letters, 440*(2), 166-169.

[158] Flanagan, L. M., Verbalis, J. G., & Strieker, E. M. (1988). Naloxone potentiation of effects of cholecystokinin and lithium chloride on oxytocin secretion, gastric motility and feeding. *Neuroendocrinology, 48*(6), 668-673.

[159] Olszewski, P. K., Wirth, M. M., Shaw, T. J., Grace, M. K., Billington, C. J., Giraudo, S. Q., & Levine, A. S. (2001). Role of α-MSH in the regulation of consummatory behavior: immunohistochemical evidence. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 281*(2), R673-R680.

[160] Neumark-Sztainer, D., Wall, M., Story, M., & Fulkerson, J. A. (2004). Are family meal patterns associated with disordered eating behaviors among adolescents? *Journal of adolescent health, 35*(5), 350-359.

[161] Baigrie, S. S. (2008). Examining the relationship between binge eating and coping strategies and the definition of binge eating in a sample of Spanish adolescents. *The Spanish Journal of Psychology, 11*(1), 172-180.

[162] Swaab, D. F., Purba, J. S., & Hofman, M. A. (1995). Alterations in the hypothalamic paraventricular nucleus and its oxytocin neurons (putative satiety cells) in Prader-Willi syndrome: a study of five cases. *The Journal of Clinical Endocrinology & Metabolism, 80*(2), 573-579.

[163] Noble, E. E., Billington, C. J., Kotz, C. M., & Wang, C. (2014). Oxytocin in the ventromedial hypothalamic nucleus reduces feeding and acutely increases energy expenditure. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 307*(6), R737-R745.

[164] Ostrowski, N. L. (1998). Oxytocin receptor mRNA expression in rat brain: implications for behavioral integration and reproductive success. *Psychoneuroendocrinology, 23*(8), 989-1004.

[165] Van Leeuwen, F. W., Van Heerikhuize, J., Van Der Meulen, G., & Wolters, P. (1985). Light microscopic autoradiographic localization of [3H] oxytocin binding sites in the rat brain, pituitary and mammary gland. *Brain research, 359*(1-2), 320-325.

[166] Dal Monte, O., Piva, M., Anderson, K. M., Tringides, M., Holmes, A. J., & Chang, S. W. (2017). Oxytocin under opioid antagonism leads to supralinear enhancement of social attention. *Proceedings of the National Academy of Sciences, 114*(20), 5247-5252.

[167] Mitra, A., Gosnell, B. A., Schiöth, H. B., Grace, M. K., Klockars, A., Olszewski, P. K., & Levine, A. S. (2010). Chronic sugar intake dampens feeding-related activity of neurons synthesizing a satiety mediator, oxytocin. *Peptides, 31*(7), 1346-1352.

[168] Gosnell, B., & Levine, A. (2009). Reward systems and food intake: role of opioids. *International journal of obesity, 33*(S2), S54.

[169] Stricker, E. M., & Verbalis, J. G. (1991). Caloric and noncaloric controls of food intake. *Brain research bulletin, 27*(3-4), 299-303.

[170] Tauchi, M., Zhang, R., D'Alessio, D. A., Stern, J. E., & Herman, J. P. (2008). Distribution of glucagon-like peptide-1 immunoreactivity in the hypothalamic paraventricular and supraoptic nuclei. *Journal of chemical neuroanatomy, 36*(3-4), 144-149.

[171] Cleary, J., Weldon, D. T., O'Hare, E., Billington, C., & Levine, A. S. (1996). Naloxone effects on sucrose-motivated behavior. *Psychopharmacology, 126*(2), 110-114.

[172] Spangler, R., Wittkowski, K. M., Goddard, N. L., Avena, N. M., Hoebel, B. G., & Leibowitz, S. F. (2004). Opiate-like effects of sugar on gene expression in reward areas of the rat brain. *Molecular Brain Research, 124*(2), 134-142.

[173] Olszewski, P. K., & Levine, A. S. (2007). Central opioids and consumption of sweet tastants: when reward outweighs homeostasis. *Physiology & behavior, 91*(5), 506-512.

[174] Eriksson, J. Binge eating of sugar: from behavioral models to neurochemical and molecular mechanisms of overeating.

[175] Morton, G. J., Meek, T. H., & Schwartz, M. W. (2014). Neurobiology of food intake in health and disease. *Nature Reviews Neuroscience, 15*(6), 367-378.

[176] Rossi, M. A., & Stuber, G. D. (2018). Overlapping brain circuits for homeostatic and hedonic feeding. *Cell metabolism, 27*(1), 42-56.

[177] Stuber, G. D., & Wise, R. A. (2016). Lateral hypothalamic circuits for feeding and reward. *Nature neuroscience, 19*(2), 198-205.

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

[179] Berridge, K. C., Ho, C.-Y., Richard, J. M., & DiFeliceantonio, A. G. (2010). The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. *Brain research, 1350*, 43-64.

[180] Castro, D. C., & Berridge, K. C. (2017). Opioid and orexin hedonic hotspots in rat orbitofrontal cortex and insula. *Proceedings of the National Academy of Sciences, 114*(43), E9125-E9134.

[181] Cameron, J. D., Chaput, J.-P., Sjödin, A. M., & Goldfield, G. S. (2017). Brain on fire: Incentive salience, hedonic hot spots, dopamine, obesity, and other hunger games. *Annual Review of Nutrition, 37*, 183-205.

[182] Berridge, K. C., & Robinson, T. E. (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain research reviews, 28*(3), 309-369.

[183] Pecina, S., Cagniard, B., Berridge, K. C., Aldridge, J. W., & Zhuang, X. (2003). Hyperdopaminergic mutant mice have higher "wanting" but not "liking" for sweet rewards. *Journal of Neuroscience, 23*(28), 9395-9402.

[184] Mahler, S. V., Smith, K. S., & Berridge, K. C. (2007). Endocannabinoid hedonic hotspot for sensory pleasure: anandamide in nucleus accumbens shell enhances 'liking'of a sweet reward. *Neuropsychopharmacology, 32*(11), 2267-2278.

[185] Domingos, A. I., Vaynshteyn, J., Voss, H. U., Ren, X., Gradinaru, V., Zang, F., Deisseroth, K., De Araujo, I. E., & Friedman, J. (2011). Leptin regulates the reward value of nutrient. *Nature neuroscience, 14*(12), 1562-1568.

[186] Brown, H. D., McCutcheon, J. E., Cone, J. J., Ragozzino, M. E., & Roitman, M. F. (2011). Primary food reward and reward-predictive stimuli evoke different patterns of phasic dopamine signaling throughout the striatum. *European journal of neuroscience, 34*(12), 1997-2006.

[187] Tellez, L. A., Han, W., Zhang, X., Ferreira, T. L., Perez, I. O., Shammah-Lagnado, S. J., Van Den Pol, A. N., & De Araujo, I. E. (2016). Separate circuitries encode the hedonic and nutritional values of sugar. *Nature neuroscience, 19*(3), 465-470.

[188] Bassareo, V., & Di Chiara, G. (1997). Differential influence of associative and nonassociative learning mechanisms on the responsiveness of prefrontal and accumbal dopamine transmission to food stimuli in rats fed ad libitum. *Journal of Neuroscience, 17*(2), 851-861.

[189] Roitman, M. F., Stuber, G. D., Phillips, P. E., Wightman, R. M., & Carelli, R. M. (2004). Dopamine operates as a subsecond modulator of food seeking. *Journal of Neuroscience, 24*(6), 1265-1271.

[190] Volkow, N. D., Wang, G.-J., & Baler, R. D. (2011). Reward, dopamine and the control of food intake: implications for obesity. *Trends in cognitive sciences, 15*(1), 37-46.

[191] Stuber, G. D., Sparta, D. R., Stamatakis, A. M., van Leeuwen, W. A., Hardjoprajitno, J. E., Cho, S., Tye, K. M., Kempadoo, K. A., Zhang, F., & Deisseroth, K. (2011). Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *nature, 475*(7356), 377-380.

[192] Nieh, E. H., Vander Weele, C. M., Matthews, G. A., Presbrey, K. N., Wichmann, R., Leppla, C. A., Izadmehr, E. M., & Tye, K. M. (2016). Inhibitory input from the lateral hypothalamus to the ventral tegmental area disinhibits dopamine neurons and promotes behavioral activation. *Neuron, 90*(6), 1286-1298.

[193] Baimel, C., Lau, B. K., Qiao, M., & Borgland, S. L. (2017). Projection-target-defined effects of orexin and dynorphin on VTA dopamine neurons. *Cell reports, 18*(6), 1346-1355.

[194] Lau, B. K., Cota, D., Cristino, L., & Borgland, S. L. (2017). Endocannabinoid modulation of homeostatic and non-homeostatic feeding circuits. *Neuropharmacology, 124*, 38-51.

[195] Novelle, M. G., & Diéguez, C. (2018). Food addiction and binge eating: lessons learned from animal models. *Nutrients, 10*(1), 71.

[196] Uribe-Cerda, S., Morselli, E., & Perez-Leighton, C. (2018). Updates on the neurobiology of food reward and their relation to the obesogenic environment. *Current Opinion in Endocrinology, Diabetes and Obesity, 25*(5), 292-297.

[197] Keyes, P. C., Adams, E. L., Chen, Z., Bi, L., Nachtrab, G., Wang, V. J., Tessier-Lavigne, M., Zhu, Y., & Chen, X. (2020). Orchestrating Opiate-Associated Memories in Thalamic Circuits. *Neuron*.

Chapter 2

Effect of oxytocin on perception of hunger

2.1 Abstract

Centrally and peripherally administered OT decreases food intake and activation of the endogenous OT systems, which is associated with termination of feeding. Evidence gathered thus far points to OT as a facilitator of early satiation, a peptide that reduces the need for a meal that has already begun. It is not known, however, whether OT can diminish a feeling of hunger, thereby decreasing a perceived need to seek calories. Therefore, in the current project, I first confirmed that intraperitoneal (i.p.) OT at 0.3–1 mg/kg reduces food intake in deprived and non-deprived rats. I then used those OT doses in a unique hunger discrimination protocol. First, rats were trained to discriminate between 22- and 2-h food deprivation (hungry vs. sated state) in a two-lever operant procedure. After rats acquired the discrimination, they were food-restricted for 22 h and given i.p. OT before a generalization test session. OT did not decrease 22-h deprivation-appropriate responding to match that following 2-h food deprivation, thus, it did not reduce the perceived level of hunger. In order to better understand the mechanisms behind this ineffectiveness of OT, I used c-Fos immunohistochemistry to determine whether i.p. OT activates a different subset of feeding-related brain sites under 22- vs. 2-h deprivation. I found that in sated animals, OT induces c-Fos changes in a broader network of hypothalamic and brain stem sites compared to those affected in the hungry state. Finally, by employing qPCR analysis, I asked whether food deprivation vs. sated state have an impact on OT receptor expression in the brain stem, a CNS “entry” region for peripheral OT. Fasted animals had significantly lower OT receptor mRNA levels than their ad libitum-fed counterparts. I conclude that OT does not diminish a feeling of hunger before a start of a meal. Instead, OT's anorexigenic properties are manifested once consumption

has already begun which is—at least to some extent—driven by changes in brain responsiveness to OT treatment in the hungry vs. fed state. OT should be viewed as a mediator of early satiation rather than as a molecule that diminishes perceived hunger.

2.2 Introduction

A nine amino acid neuropeptide OT, synthesized primarily in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, released throughout the CNS and, via the neurohypophysis, into general circulation, has been known to regulate a number of functions, including parturition, lactation, and social behaviours. In 1989, Arletti et al. reported for the first time that intracerebral and intraperitoneal (i.p.) administration of OT in rats causes a marked reduction in food intake [1]. Since then, a plethora of evidence has emerged that supports the involvement of OT in termination of feeding.

Injections of OT in the third and fourth cerebral ventricles as well as in numerous brain areas, including the hypothalamic ventromedial nucleus (VMH), dorsal vagal complex, central (CEA), and basolateral (BLA) amygdala, ventral tegmental area (VTA) and nucleus accumbens core (AcbC), and the limbic system, produce cessation of ingestive behaviour [2-10]. This early termination of feeding after OT treatment pertains to relatively bland laboratory chow, as well as those that are highly palatable [2; 3; 10; 11]. Despite its limited ability to cross the blood-brain barrier (BBB), peripherally administered OT (including via intraperitoneal (i.p.), subcutaneous, and intravenous routes) also potently decreases food intake, most likely by engaging the brainstem where the BBB protection is weak [1; 2; 9; 12-14].

Increased c-Fos immunoreactivity in OT neurons and elevated OT plasma levels coincide with meal cessation [15]. Hypothalamic OT mRNA expression is downregulated during fasting and restored by re-feeding [16]. Administration of molecules that support satiation, such as cholecystokinin (CCK), alpha-melanocyte stimulating hormone, and glucagon-like peptide-1 (GLP-1), increases activity of the OT system [17-21]. Furthermore, OT

release has been associated with peripheral changes that typically occur as ingestive behaviour nears its end, such as elevated osmolality, an increase in select nutrient levels, and excessive stomach distension [9; 22-25].

Overall, those findings strongly point to the role of OT in promoting satiety, facilitating early termination of consumption, and reducing meal size. On the other hand, one aspect of OT's involvement in feeding control that has not been investigated in detail is whether OT has a capacity to reduce a feeling of hunger. That, e.g., peripherally administered OT increases latency to begin a meal, might suggest it to some extent, but direct evidence is lacking. Scarcity of data also stems from methodological difficulties in assessing hunger in laboratory animals.

There is a unique protocol, however, that relies on rats reporting their hunger status using an operant behaviour. Animals are trained to discriminate between acute food deprivation lasting 22 h (hunger) or 2 h (no actual energy depletion). In one such study, Corwin et al. trained rats maintained at 80% of their free-feeding body weight to discriminate between food consumed 22 or 3 h before experimental sessions [26]. The anorexigen CCK or ingestion of sweetened condensed milk induced effects similar to chow consumption occurring 3 h before a test session, in contrast to fenfluramine which did not reliably produce effects similar to 3-h food ingestion. Thus, CCK produced effects that resembled a lack of hunger. Similar outcomes to those induced by CCK were seen in response to an anti-obesity drug, sibutramine [27; 28].

In the current project, I employed this unique hunger discrimination protocol (employing 22-h vs. 2-h deprivation) to examine whether OT administered i.p. prior to discrimination testing in rats reduces a feeling of hunger. I used OT doses based on previous reports and on the results of two additional feeding studies in hungry and sated animals performed here. In order to better understand whether i.p. OT activates a different subset of feeding-related brain sites depending on the lack of access to food for 22 or 2 h, I conducted an

analysis of c-Fos immunoreactivity. Finally, since i.p. OT is thought to directly act at the brain stem, I asked whether food deprivation vs. a sated state has an impact on OT receptor expression established with real-time PCR.

2.3 Materials and Methods

2.3.1 Animals

Male Sprague-Dawley rats aged 12-weeks old (average b. wt. 400 g) were housed individually in standard plastic cages with wire tops in a temperature-controlled (22°C) animal facility with a 12:12 light:dark cycle (lights on at 08:30 in the discrimination studies and 07:00 in the remaining experiments). Water and standard laboratory chow (Sharpes Stock Feed, Diet 86; 3.6kcal/g) were available ad libitum unless stated otherwise. Animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The University of Waikato Animal Ethics Committee and the University of Wisconsin-Eau Claire Institutional Animal Care and Use Committee approved all procedures described here.

2.3.2 Behavioural Studies

2.3.2.1 Establishing Effective Doses of OT That Reduce Feeding in Animals Deprived for 22 h and 2 h

As I sought to investigate effects of OT on consumption of the same kind of diet in both 22-h deprived (thus, driven to eat by energy needs) and 2-h deprived (thus, not motivated to eat by energy needs) animals, I chose to give them episodic access to palatable high-fat high-sugar (HFHS; Research Diets #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) chow. Rodents avidly consume HFHS diet even in the absence of hunger. This was done to test whether i.p. OT retained its anorexigenic properties in the context of 2 and 22 h deprivation scenarios as they were slightly different from previously described protocols that typically relied either on depriving animals overnight, 16 or 24

h (hunger) or on giving animals episodic 2–4 h access to palatable food without depriving them even for 2 h (relative satiety).

2.3.2.1.1 Effect of OT on palatable food intake in rats deprived for 2 h

In order to examine whether OT decreases the consumption of HFHS food, I adjusted our previously published protocol [14; 29]. Rats maintained on ad libitum food and water had standard chow taken away at 10:00 (water remained in the cage). Two hours later, HFHS chow was placed in the cages for 2 h. Fifteen minutes prior to the HFHS food presentation, animals received an i.p. injection of isotonic saline or 0.1, 0.3, or 1 mg/kg OT Sigma, St. Louis, MO, USA (n = 9/group). The animals had had previous episodic (2 h per day, 5 and 10 days before the study) exposure to this HFHS chow to avoid neophobia. Data were analysed with a one-way ANOVA followed by Dunnett's post-hoc analysis, with a significance level set at $p \leqslant 0.05$.

2.3.2.1.2 Effect of OT on palatable food intake in rats deprived for 22 h

The cohort of rats used in the previous experiment (2.2.1.1) was studied here. A 2-week "washout" period elapsed between the experiments. Animals that had had access to standard chow were food-deprived for 22 h (deprivation ending at 12:00). They were then given access for 4 h to the HFHS chow. Fifteen minutes prior to the HFHS food presentation, animals received an i.p. injection of isotonic saline or 0.1, 0.3, or 1 mg/kg OT. HFHS chow intake after 22 h of deprivation was measured at 1 and 4 h. Data were analysed with a one-way ANOVA followed by Dunnett's post-hoc analysis, with a significance level set at $p \leqslant 0.05$.

2.3.2.2 Establishing effects of OT on discrimination between 22 and 2 h of food deprivation

Experimentally naïve male Sprague Dawley rats (Harlan, Madison, WI) were ~12-weeks old at the beginning of the procedures. Food (Harlan Teklad chow, Madison, WI) and water were continuously available unless otherwise stated.

2.3.2.2.1 Apparatus

Daily discrimination sessions were conducted in standard operant chambers equipped with two response levers (Med-Associates, St. Albans, VT), placed in ventilated, sound-attenuating cubicles. Forty-five mg food pellets (Bio-Serve F#0021, Frenchtown, NJ) reinforced lever pressing and were delivered by a pellet dispenser into a food pellet trough located between the two levers. A house light in the back wall of the operant chamber illuminated the chambers during sessions. Experimental contingencies and data recording were performed with Med Associates software and a computer located in an adjacent room.

2.3.2.2.2 Discrimination training

Rats were initially food deprived to ~85% of their free feeding body weight and trained to press a level via the method of successive approximations. First, a single lever press was reinforced with a 45 mg food pellet (Bio-Serv F#0021, Frenchtown NJ). Response requirements were increased gradually until 15 presses (fixed ratio (FR) 15) were needed to generate food. When reliable responding to both levers was achieved, rats were given free access to food for 305 days before subsequent discrimination training began. Rats were trained to discriminate between 22 and 2 h of acute food deprivation using multiple cycle training. Under 22-h conditions, food was removed 22 h before the training session. Rats were placed into the operant chamber 5 min before the first training cycle. When the first training cycle started, the house light was turned on and 15 left lever presses were

reinforced with 45 mg food pellet delivery (FR 15 reinforcement schedule). Incorrect (right) lever presses were punished with 8 s of darkness under an FR 15 schedule. Training continued until 5 reinforcers were earned or 5 min elapsed. At least one more additional training cycle, identical to the first, was conducted 30–120 min after the previous cycle. Under 2-h conditions, the contingencies were reversed: right lever presses were reinforced and left lever presses were punished under the FR 15 schedule. Conditions were quasi-random with the provision that the same training condition (22 or 2 h of food deprivation) could not be given for more than two consecutive sessions.

Discrimination training continued until the subject emitted 80% or greater condition-appropriate responses prior to delivery of the first reinforcer and for the entire training session during all training cycles for 8 of 10 consecutive daily sessions.

2.3.2.2.3 Generalization test: evaluation of the ability of OT to reduce the discriminative stimulus effects of 22-h food deprivation

The final discrimination tests assessed the ability of OT to reduce the discriminative stimulus effects of 22-h food deprivation. These tests were conducted under 22-h deprivation conditions. During the first response period, only left lever presses were reinforced. Following the first response period, rats were injected i.p. with isotonic saline or OT (0.01–1 mg/kg range). After injections, rats were placed in stainless steel cages without food or water. During the next response period occurring 30 min after the injection, responses toward both levers were reinforced. Generalization tests lasted until the subject earned 5 reinforcers or until 5 min elapsed, whichever occurred first. Appropriate discriminative performance for at least 2 training days (one preceded by 22-h deprivation, one preceded by 2-h deprivation) was required between generalization tests. Immediately after generalization tests, subjects were placed in stainless steel cages and had access to a pre-weighed amount of regular food (~25 g of Teklad rat chow) placed on

the floor of the cage, and water available in a bottle attached to the cage. Food intake was measured at the end of 1 h. Afterwards, rats were returned to their home cage and had free access to food and water until 2 h before the next training session.

2.3.2.3 Data analysis

One-way ANOVA was calculated (SPSS, Chicago, IL, USA) by assessing the effects of OT versus control conditions on the discriminative stimulus effects of 22-h food deprivation, lever pressing rate, and food intake. Tukey HSD post hoc tests were performed following significant ANOVA values to determine pairwise differences among conditions. Significance was set at $p \leqslant 0.05$.

2.3.3 Establishing OT-induced c-Fos immunoreactivity in feeding-related brain sites in rats deprived for 2 and 22 h

For practical reasons, including the transfer of rats between cages and behavioural manipulations that could have affected baseline Fos expression, I chose a different cohort of animals here than those used in behavioural studies. Experimentally naive, age-matched male Sprague Dawley rats were divided into two cohorts (n = 12 per cohort) which were subjected to either 2 or 22 h of food deprivation (in both cases, deprivation period ended at 12:00). At the end of the deprivation, half of the animals in each cohort received an i.p. injection of isotonic saline, and the other half, 1 mg/kg OT. An hour after drug administration, animals were deeply anesthetized with urethane (35% dissolved in 0.9% saline, i.p.), and perfused through the aorta with 50 ml of saline followed by 500 ml of 4% paraformaldehyde in 0.1 phosphate buffer (pH 7.4). Brains were excised and post-fixed overnight in the same fixative at 4°C. 60 μm-thick coronal sections were cut with a vibratome (Leica, Germany) and later processed as free-floating sections for standard single antigen immunostaining of c-Fos.

2.3.3.1 Immunohistochemistry

Sections were rinsed in 50 nM TBS (pH 7.4–7.6), and then pre-treated for 10 min in 3% H2O2, 10% methanol (diluted in TBS). After rinsing in TBS they were incubated overnight at 4°C in the primary rabbit-anti-Fos antibody (diluted 1:3000; Synaptic Systems, Australia) washed in TBS, and subsequently incubated for 1 h at room temperature in the secondary goat-anti-rabbit antibody (1:400; Vector Laboratories). Following four washes in TBS, sections were incubated for 1 h with the avidin–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the tissue was visualized with 0.05% diaminobenzidine (DAB), 0.01% H2O2 and 0.3% nickel sulfate (12-min incubation). Sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried, dehydrated in ascending concentrations of ethanol, soaked in xylene (Merck KGaA, Germany) and embedded in Entellan (Merck KGaA, Germany). The number of Fos-positive nuclei per 1 mm2 was counted bilaterally for each neuroanatomical region of interest using ImageJ Software, with boundaries defined according to the Paxinos and Watson brain atlas, on 2–4 sections per animal. Images provided by a CCD camera attached to a Nikon Eclipse 400 microscope were analysed using Nikon NIS Elements image software. The following areas were analysed (in the parentheses, anterior-posterior ranges of bregma levels of sections used to analyse each site are shown): AcbC—nucleus accumbens core (1.28–0.96); AcbS—nucleus accumbens shell (1.28–0.96); AP—area postrema (−13.92 to −14.16); ARC—arcuate nucleus (−2.16 to −2.52); BLA—basolateral amygdala (−2.64 to −2.92); CEA—central nucleus of the amygdala (−2.64 to −2.92); DMH—dorsomedial nucleus of the hypothalamus (−3.00 to −3.24); DMV—dorsal motor nucleus of the vagus (−13.76 to −14.16); NTS—nucleus of the solitary tract (−13.76 to −14.16); PVN—paraventricular nucleus of the hypothalamus (−1.56 to −1.92); SON—supraoptic nucleus

(−0.96 to −1.2); VMH—ventromedial nucleus (−3.00 to −3.24); VTA—ventral tegmental area (−6.72 to −6.84).

2.3.3.2 Data analysis

Densities of Fos-positive nuclear profiles (per 1 mm^2) were averaged per individual, and then per group. Data between the two groups (saline vs. OT) in each cohort were compared using a t-test. Values were considered significantly different for $p \leqslant 0.05$.

2.3.4 Establishing the effect of food deprivation on brainstem expression of the OT receptor gene

2.3.4.1 Deprivation and brain stem collection

The rats were divided to two groups. One group (n = 9) had unlimited access to standard chow and water, whereas the other had food taken away ~24 h before the animals were sacrificed by decapitation (n = 13). The brain stem was dissected and put in RNAlater (Ambion) for 2 h at room temperature and the samples were then frozen at −80C until further preparation.

2.3.4.2 rtPCR protocol and data analysis

A standard protocol of sample preparation and rtPCR was used, as developed by our lab [30]. Samples were homogenized in TRIzol (Ambion); RNA was extracted with chloroform and precipitated in isopropanol. After centrifuging, the pellet was washed, air-dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (Merck) and the absence of genomic DNA was established by PCR of a 5% template. 100 ng/μl genomic DNA served as a positive control, whereas MilliQ H2O as a negative one. The product was analysed by electrophoresis. 5 μg RNA samples were diluted with MilliQ H2O. RNA was reverse-transcribed in the master mix (Promega; 20 μl). Samples were incubated for 1 h (37°C), followed by PCR to confirm cDNA synthesis. RtPCR reactions were performed in duplicates. Sample cDNA template (25 ng)

was used per primer [OT receptor primer sequences: ttcttctgctgctctgctcgt (fwd) and tcatgctgaagatggctgaga (rev)]. Expression of three housekeeping genes (glyceraldehyde-3-phosphate- dehydrogenase, β-actin, and β-tubulin) was used to calculate normalization factors (GeNorm). Primer efficiencies were calculated with LinRegPCR (HFRC) and Ct values were corrected for differences in primer efficiencies. rtPCR results were analysed with a Student's t-test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leqslant 0.05$.

2.4 Results

The effects of 0.1, 0.3, and 1.0 mg/kg OT i.p. on HFHS palatable chow intake were investigated after 2 h and after 22 h of food deprivation. Control animals that had standard food taken away for 2 h and subsequently gained short-term access to the HFHS chow (thus, these rats were in effect sated) ate approximately 6 grams of the HFHS diet. OT at 0.3 mg/kg and 1 mg/kg decreased HFHS food consumption [$F(3, 32) = 6.44$; 0.3 mg, $p = 0.042$; 1 mg, $p = 0.016$] during the 2-h access period by approximately 33% (Figure 2.1A). In animals subjected to 22-h food deprivation (which is a much more challenging energy deprivation scenario and it promotes search of and intake of caloric tastants) after which they gained access to the HFHS chow, 1 mg/kg OT decreased consumption by approximately 25% at 2 h [$F(3, 32) = 3.38$; $p = 0.015$] and 4 h [$F(3, 32) = 5.12$; $p = 0.004$) of re-feeding (Figure 2.1B). There was a trend toward a decrease for 0.3 mg/kg OT at 4 h ($p = 0.073$).

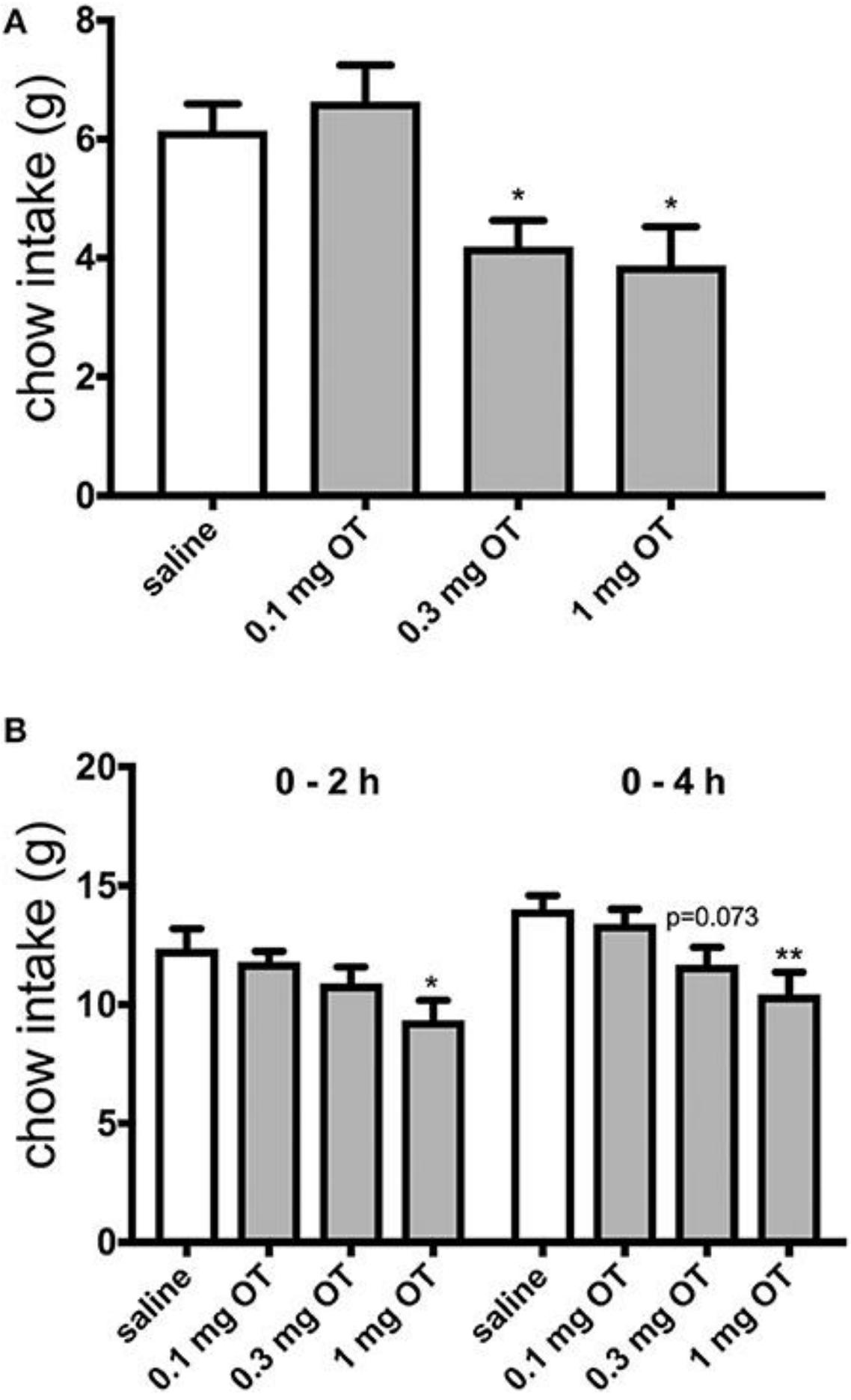
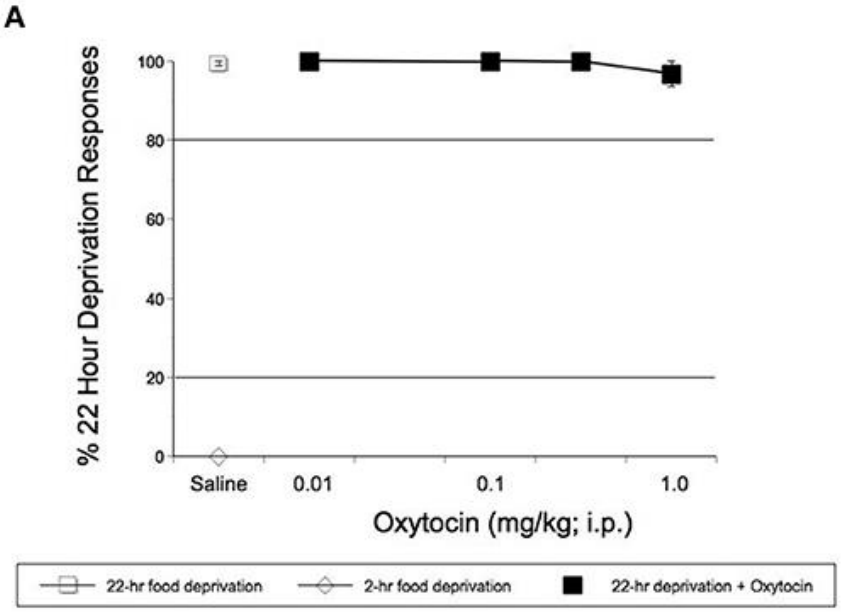
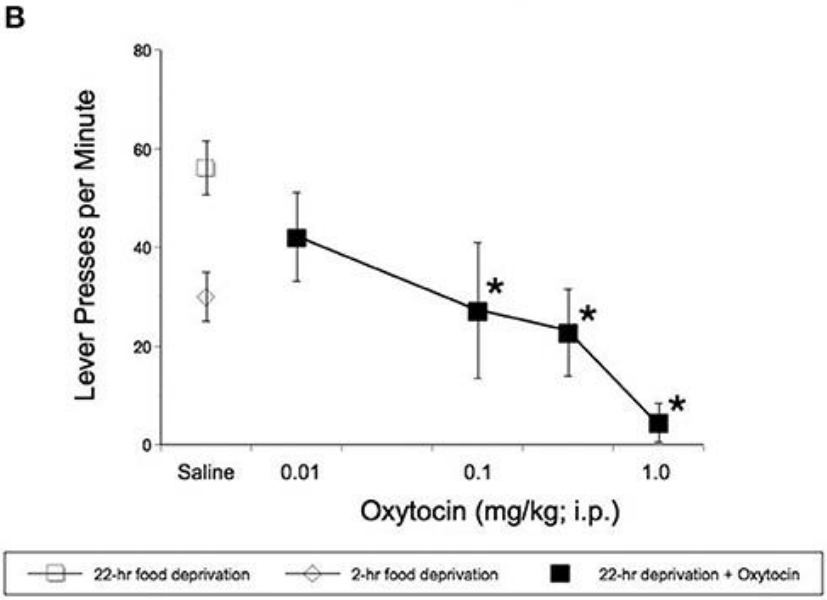

A
chow intake (g)
8
6
4
2
0
*
*
saline
0.1 mg OT
0.3 mg OT
1 mg OT
B
chow intake (g)
20
15
10
5
0
0 - 2 h
0 - 4 h
*
p=0.073
**
saline
0.1 mg OT
0.3 mg OT
1 mg OT
saline
0.1 mg OT
0.3 mg OT
1 mg OT

Figure 2.1: Effect of i.p. OT injection (0–1.0 mg/kg) on HFHS chow intake after a period of having no access to food for 2 h (A) or 22 h (B). Saline was the vehicle. HFHS availability period was 2 h in the sated (2-h deprived) rats and 4 h in the 22-h deprived animals. Water was available ad libitum. OT at 0.3 mg and 1 mg/kg decreased HFHS food consumption [F(3, 32) = 6.44; 0.3 mg, p = 0.042; 1 mg, p = 0.016] during the 2-h access period (A). In animals subjected to 22-h food deprivation after which they gained access to the HFHS chow, 1 mg/kg OT decreased consumption at 2 h [F(3, 32) = 3.38; p = 0.015] and 4 h [F(3, 32) = 5.12; p = 0.004] of re-feeding (B). There was a trend toward a decrease for 0.3 mg/kg OT at 4 h (p = 0.073). *p < 0.05; **p < 0.01. Saline was the vehicle. HFHS availability period was 2 h in the sated (2-h deprived) rats and 4 h in the 22-h deprived animals. Water was available ad libitum. OT at 0.3 mg and 1 mg/kg decreased HFHS food consumption [$F_{(3, 32)}$ = 6.44; 0.3 mg, p = 0.042; 1 mg, p = 0.016] during the 2-h access period (A). In animals subjected to 22-h food deprivation after which they gained access to the HFHS chow, 1 mg/kg OT decreased consumption at 2 h [$F_{(3, 32)}$ = 3.38; p = 0.015] and 4 h [$F_{(3, 32)}$ = 5.12; p = 0.004] of re-feeding (B). There was a trend toward a decrease for 0.3 mg/kg OT at 4 h (p = 0.073). *p < 0.05; **p < 0.01.

Rats learned to discriminate between 22- and 2-h food deprivation in a mean of 90 sessions. Operant studies revealed that OT even at doses that reduced HFHS diet intake in the experiments described above, did not alter the discriminative stimulus effects of 22-h food deprivation [F(4, 16) = 1.00, p = 0.436, Figure 2.2A]. OT did significantly alter response rates in rats [F(4, 31) = 8.08, p = 0.0001, Figure 2.2B]. OT significantly reduced rates of lever pressing following 0.1 mg/kg OT (p = 0.044), 0.32 (p = 0.001) and 1 mg/kg OT i.p. (p < 0.001) (Figure 2.2B). As shown in Figure 2C, OT-treated animals deprived for 22 h and subjected to the hunger discrimination paradigm, did not show significantly reduced consumption of regular chow when they were transferred to a transition cage for 1 h after the operant test was concluded.


A
% 22 Hour Deprivation Responses
100
80
60
40
20
0
Saline
0.01
0.1
1.0
Oxytocin (mg/kg; i.p.)
22-hr food deprivation
2-hr food deprivation
22-hr deprivation + Oxytocin


B
Lever Presses per Minute
80
60
40
20
0
*
*
*
Saline
0.01
0.1
1.0
Oxytocin (mg/kg; i.p.)
22-hr food deprivation
2-hr food deprivation
22-hr deprivation + Oxytocin

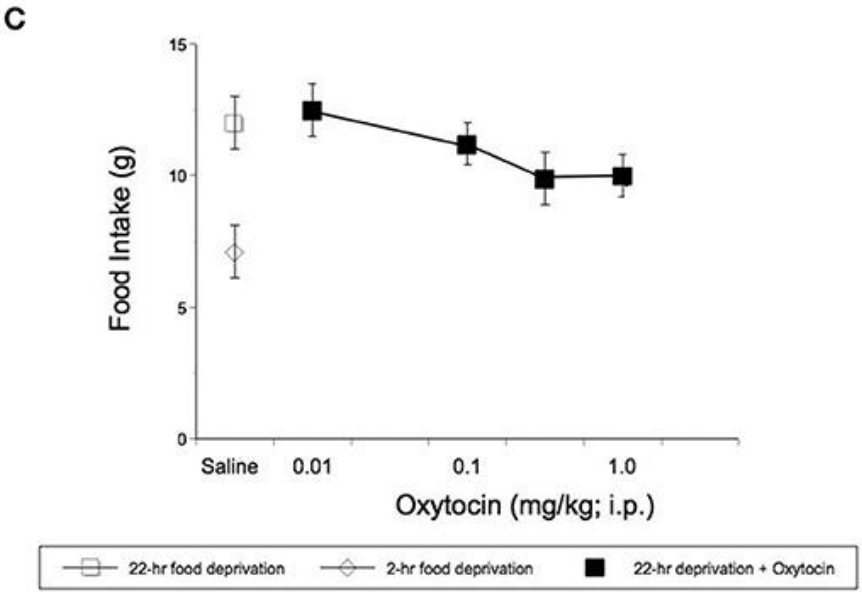

C
Food Intake (g)
15
10
5
0
Saline
0.01
0.1
1.0
Oxytocin (mg/kg; i.p.)
22-hr food deprivation
2-hr food deprivation
22-hr deprivation + Oxytocin

Figure 2.2: Effect of i.p. OT on the stimulus effects of 22-h food deprivation (A), lever pressing response rates (B), and regular laboratory chow intake in 1 h immediately following the completion of the discrimination test (C). Animals had access to regular chow ad lib, before chow was withheld for 22 h and animals were injected. Saline was the vehicle. Operant studies revealed that OT did not alter the discriminative stimulus effects of 22-h food deprivation [$F(4, 16) = 1.00$, $p = 0.436$ (A)]. OT did significantly alter response rates in rats [$F(4, 31) = 8.08$, $p = 0.0001$ (B)]. OT significantly reduced rates of lever pressing following 0.1 ($p = 0.044$), 0.32 ($p = 0.001$), and 1 mg/kg OT i.p.($p < 0.0001$) (B). (C) Shows that animals deprived for 22 h and treated with OT, did not show significantly reduced consumption of regular chow in 1hr after the completion of the operant test. *$p < 0.05$.

In a separate set of studies, I sought to investigate the effects of i.p. OT on neuronal activation in animals that are hungry and in animals that are sated. Intraperitoneal injection of OT at 1 mg/kg in rats deprived for 2 h affected c-Fos immunoreactivity in eight of the 13 feeding-related brain sites studied here (Figure 2.3). The number of c-Fos positive nuclei per mm2 in response to OT was elevated in the PVN ($p = 0.011$), SON (***$p < 0.001$), NTS ($p = 0.003$), DMV (***$p < 0.001$), and CEA (***$p < 0.001$). A decrease was noted in the ARC ($p = 0.019$), VMH (***$p < 0.001$), and DMH (***$p < 0.001$). On the other hand, in animals deprived for 22 h (Figure 2.4), six areas showed differences in c-Fos levels: an increase was noted in the PVN ($p = 0.042$), SON (***$p < 0.001$), VMH ($p = 0.013$), CEA (***$p < 0.001$), and BLA ($p = 0.012$), whereas a decrease, in the AP (***$p < 0.001$).

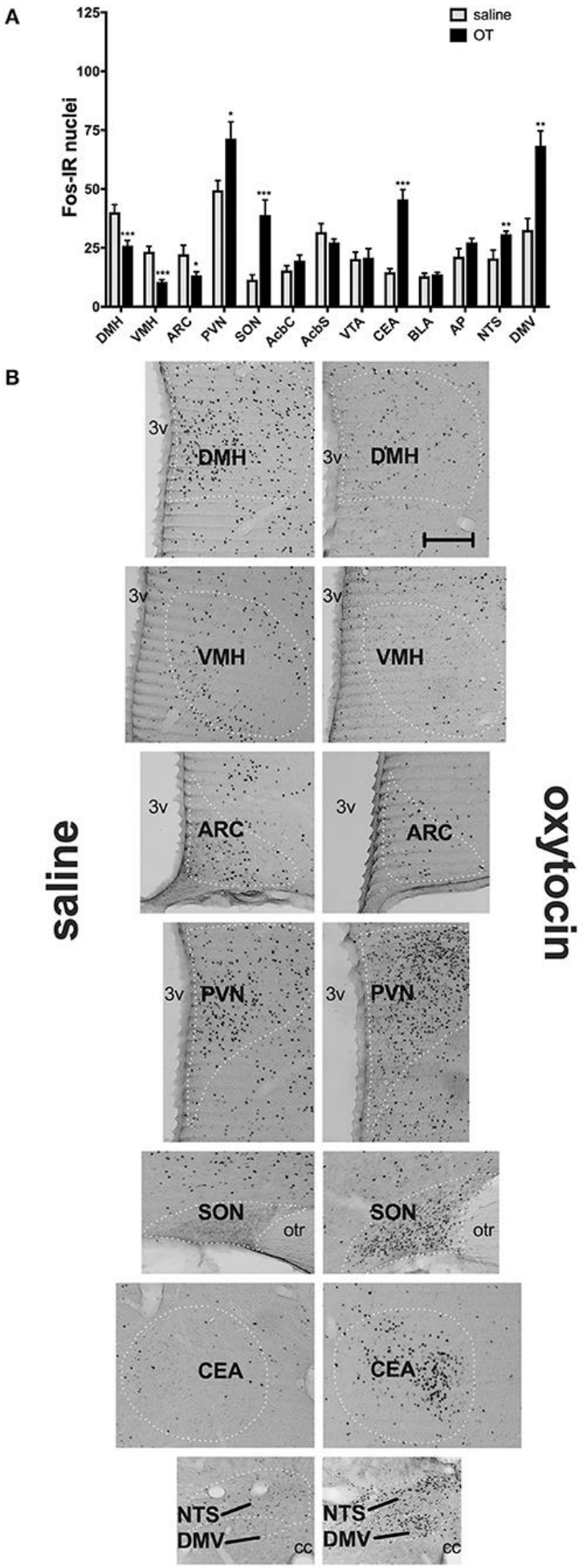

A
saline
OT
Fos-IR nuclei
125
100
75
50
25
0
DMH
VMH
ARC
PVN
SON
AcbC
AcbS
VTA
CEA
BLA
AP
NTS
DMV
B
saline
oxytocin
3v
DMH
VMH
ARC
PVN
SON
otr
CEA
NTS
DMV
cc

Figure 2.3: c-Fos immunoreactivity in feeding-related brain sites following i.p. administration of saline or OT (1 mg/kg) in animals that had no access to food for 2 h (A). Panel (B) presents photomicrographs depicting sites that showed a significant difference in c-Fos levels (saline-treated rats—left side; OT-treated rats—right side). Densities of Fos-positive nuclear profiles (per 1 mm2 of a site) were averaged per individual, and then per group. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; AP, area postrema; ARC, arcuate nucleus; BLA, basolateral amygdala; CEA, central nucleus of the amygdala; DMH, dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; VMH, ventromedial nucleus; VTA, ventral tegmental area. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

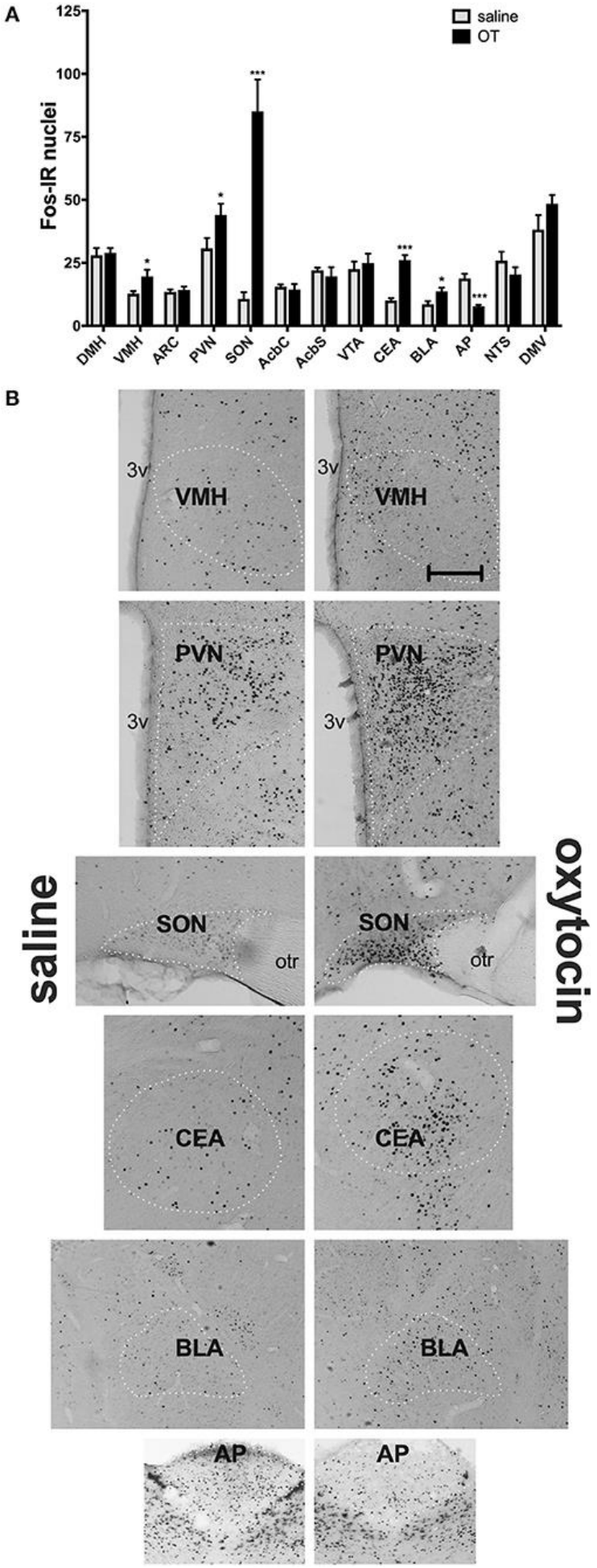

A
saline
OT
Fos-IR nuclei
125
100
75
50
25
0
DMH
VMH
ARC
PVN
SON
AcbC
AcbS
VTA
CEA
BLA
AP
NTS
DMV
B
saline
oxytocin
3v
VMH
PVN
SON
otr
CEA
BLA
AP

Figure 2.4: c-Fos immunoreactivity in feeding-related brain sites following i.p. administration of saline or OT (1 mg/kg) in animals that had no access to food for 22 h (A). Panel (B) presents photomicrographs depicting sites that showed a significant difference in c-Fos levels (saline-treated rats—left side; OT-treated rats—right side). Densities of Fos-positive nuclear profiles (per 1 mm^2 of a site) were averaged per individual, and then per group. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; AP, area postrema; ARC, arcuate nucleus; BLA, basolateral amygdala; CEA, central nucleus of the amygdala; DMH, dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; VMH, ventromedial nucleus; VTA, ventral tegmental area. *$p < 0.05$; ***$p < 0.001$.

Finally, by applying rtPCR analysis, I showed a downregulation of the OT receptor gene in the brain stem of rats that underwent deprivation compared to their sated counterparts ($p = 0.0024$; Figure 2.5).

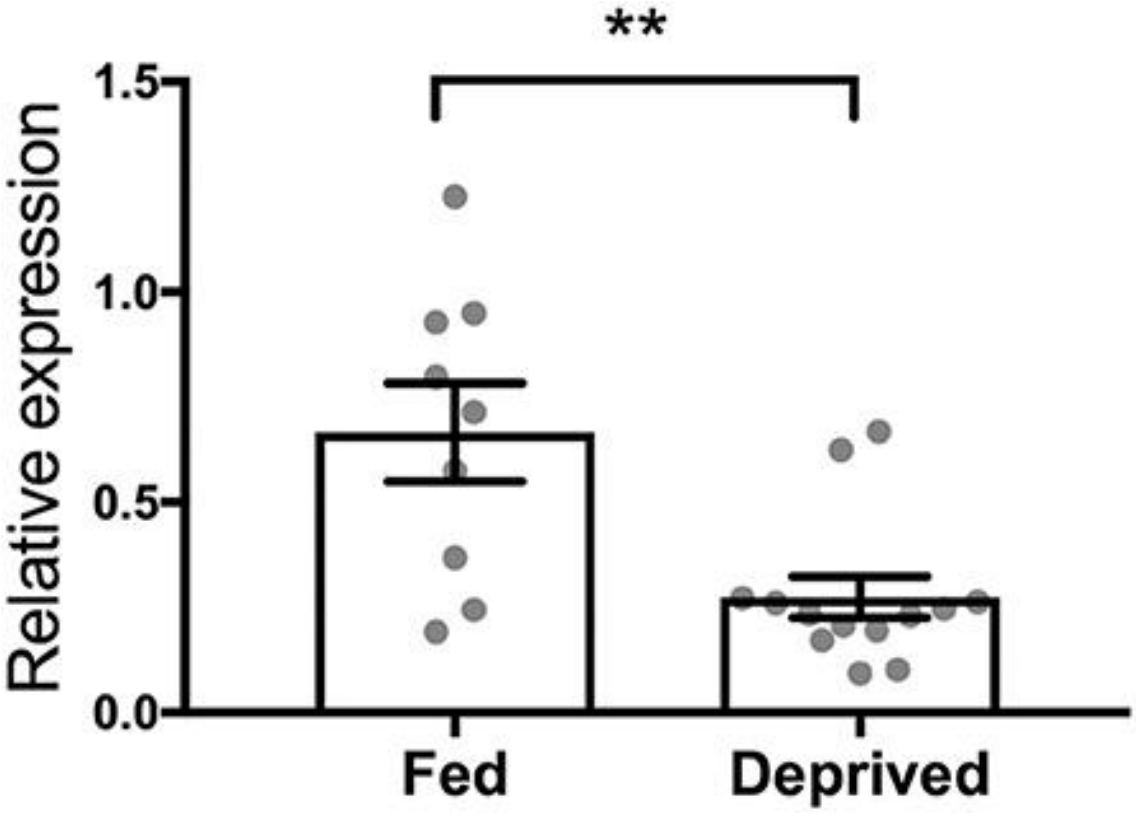


Figure 2.5: Effect of 24-h food deprivation on expression of the OT receptor gene established with real-time PCR in the brain stem. Ad libitum-fed rats served as controls. **p < 0.01.

2.5 Discussion

The fundamental drive that initiates food intake is a feeling of hunger. Hunger increases our motivation to seek high-energy foods and generates an avid feeding behavior upon encountering such ingestants. There are evolutionarily conserved neural and endocrine processes, such as those involving ghrelin or neuropeptide Y (NPY) [31-33], that promote eating for hunger, thereby ensuring that enough energy is obtained in order for the organism to maintain its functioning. However, in the obesogenic environment, where highly palatable and energy-rich diets are readily available, those mechanisms coupled with reward processes appear to be active even in the absence of actual energy needs, producing a feeling of hunger that leads to excessive food intake. Therefore, an important question that arises in the context of peptides that reduce consumption that can be potentially used clinically is whether they are capable of diminishing hunger responsiveness. Several decades of research on anorexigenic properties of OT have indicated that administration of OT supports early cessation of ingestive behavior [for review, see [34]]. Evidence gathered thus far has strongly linked OT with termination of feeding due to enhanced satiation or in response to adverse physiological changes (such as plasma osmolality or stomach distension) that endanger homeostasis. The current set of data shows for the first time that anorexigenic effects of OT do not stem from promoting a reduced feeling of hunger.

Previous experiments have shown beyond reasonable doubt that peripherally injected OT decreases consumption. It seems that OT is particularly effective in reducing intake of energy-dense solid foods regardless of their composition and/or palatability [2; 8; 13; 14]. On the other hand, unlike central OT (particularly, targeting the VTA, AcbC or amygdala) or OT receptor ligands that cross the BBB, peripheral OT is not effective in modifying consumption of calorie-dilute sweet solutions that are ingested primarily for pleasure [3;

5; 10; 35]. Our feeding experiments indicate that i.p. OT decreases calorie-dense palatable food consumption regardless of whether intake is stimulated predominantly by hunger (as in animals deprived for 22 h) or by palatability (as in animals that had the standard chow removed only 2 h prior to episodic access to the HFHS diet). Hence, the relative calorie needs do not impact the ability of OT to produce hypophagia. The effective dose range (0.3–1 mg/kg) is also similar regardless of a feeding paradigm used here and by others.

It should be noted that craving and motivation are not factors that are directly measurable in either our discrimination or consumption procedures. As we and others have reported, simple measures of food consumption do not necessarily relate to motivation to work to obtain food [e.g., [36; 37]]. Assuming there are only two motivating factors driving consumption (reward/pleasure or "free or hedonic" drives) and energy, the "free" or "hedonic" drives would be the key influence mediating consumption under the 2 h deprivation. In our experiments, OT was somewhat more potent in reducing HFHS consumption in subjects that were 2 h-deprived compared to subjects that were 22 h-deprived. OT (0.3 and 1.0 mg) reduced HFHS consumption by approximately 33% in the former group. OT (0.3 mg) did not reduce consumption of the HFHS diet in the subjects food-restricted for 22 h. The reduction in intake under 22-h deprivation was about 25% suggesting that the contribution of the "free or hedonic" food intake may be at least to some extent reduced following the 22-h deprivation. We and others have shown that opioid antagonists are more potent in reducing consumption of sweet food compared to regular chow [for review, see e.g., [38; 39]]. These findings would also support the statement above. Craving is often associated with dependence-like behaviors during times of restriction or deprivation of the item inducing the "cravings." I noted no dependence-like behaviors in subjects in either of our paradigms.

The hunger discrimination protocol that relies on the animals' ability to discriminate between short- (2–3 h) and long-term (e.g., 22 h) food deprivation and "report" it through operant behavior, has defined anorexigenic and orexigenic characteristics of several molecules. As food preloads appropriately change animals' perception of the length of deprivation, the parallel effect of anorexigens given instead of a food preload, indicates that they reduce a feeling of hunger. This approach allowed to implicate CCK and sibutramine as agents that can aid in controlling hunger (and deemed rimonabant ineffective in this process) [27; 40]. Of note is the fact that, in contrast to anorexigens, some orexigens (NPY or ghrelin) make animals perceive the short 2-h deprivation as the state of hunger [28; 41]. The fact that i.p. OT did not reduce operant responding to 22 h of deprivation strongly suggests that OT does not interfere with mechanisms that promote a feeling of hunger. It did not have an effect on hunger discrimination even though it was used at doses that are anorexigenic, and despite the fact that it did decrease bar pressing rate in general. It also produced a trend toward a reduction in chow intake in the 1 h after the completion of the operant test when animals were placed in a transition cage with chow present on the floor. Consequently, it can be inferred that OT induces hypophagia by being part of neuroendocrine processes that facilitate satiation and early cessation of feeding. This is in line with experiments showing a functional relationship between OT and numerous other mediators of satiety [for example, see [42; 43]], including melanocortins, which appear to form a common circuit with OT to support satiation. On the other hand, to our knowledge, there is no evidence that OT might be able to silence activation of, e.g., the NPY system, which could potentially suggest a link with hunger processing.

That i.p. OT differently affects neural processing under deprivation than in satiety is further substantiated by the outcomes of the c-Fos mapping study that revealed distinct

patterns of c-Fos immunoreactivity in response to the OT treatment in hungry vs. sated animals. In individuals subjected to 2 h of deprivation, OT affected c-Fos immunoreactivity in a number of hypothalamic and brain stem sites: an increase in c-Fos staining occurred in the PVN, SON, NTS, and DMV, whereas a decrease was noted in the DMH, ARC, and VMH. The broad change in c-Fos levels in this network of sites that regulate energy intake is consistent with the role of OT as an anorexigen. Outside the hypothalamic-brainstem circuit, elevated c-Fos levels were in the CEA, which might potentially be related to emotional processing related to feeding [44-46] and it aligns well with the ability of OT itself to decrease consumption by acting at this site [5]. Overall, the subset of sites activated by OT is quite similar to what had been reported before in various paradigms unrelated to food intake. For example, Carson et al. found an increase in the PVN, SON and CEA after 2 mg/kg OT in rats subjected to 80-min locomotor testing [47], and Hicks et al. found elevated activation in the PVN, SON, CEA and NTS after 1 mg/kg OT and a brief locomotor test [48]. In another study, Hicks and colleagues reported an increase in the PVN, CEA, and NTS in adolescent animals [49].

In hungry (thus, 22-h deprived) rats, systemic OT induced c-Fos in a smaller subset of areas. In the hypothalamus and brain stem, the PVN, SON, VMH, and—unlike under 2-h deprivation—the AP showed a significant change (it should be noted though that the AP c-Fos levels in hungry animals treated with OT were higher too, though only a trend was observed). No difference was noted in the DMH, ARC, NTS, and DMV. It appears therefore that in the hungry state, OT loses the capacity to engage as broad a network of sites that regulate energy balance as that activated during satiety. rtPCR data showing a decreased expression of the OT receptor gene in the brain stem, a region thought to mediate anorexigenic properties of systemic OT, provide an additional insight into a mechanistic change that might be a key contributor to the changed, hunger/satiety-

dependent receptivity of the CNS to OT. Of note is the fact that the rtPCR analysis revealed global brain stem expression changes and did not include individual areas. Considering that specific brain stem sites (and within these sites, specific neuronal populations) play distinct roles in appetite regulation, future studies will have to refine our understanding of a relationship between energy state, OT receptor, and these particular circuits within the brain stem. Finally, it should be noted that in both 22-h and 2-h deprived rats the amygdala was affected by OT, however in hungry animals, not only the CEA, but also the BLA expressed higher c-Fos immunoreactivity. It allows us to speculate that—considering the role of the amygdala in emotional processing [44-46]—OT affects emotional aspects of feeding regardless of the deprivation level.

We conclude that systemic OT does not diminish a feeling of hunger before the start of a meal. Instead, OT's anorexigenic properties can be manifested once consumption has already begun and this, at least to some extent, is driven by changes in brain responsiveness to OT treatment in the hungry vs. fed state. Therefore, OT's role in feeding control should be viewed as a mediator of early satiation rather than as a molecule that diminishes a perceived need to seek calories.

As I aim to develop a drug combination that targets multiple aspects of feeding behavior, and find that OT predominantly acts on satiety alone, I propose next, to explore the effects of opioid receptor antagonists. While these molecules are known to target another aspect of consumption, in palatability and pleasure, I aim to understand if they might act on the perception of hunger also.

2.6 References

[1] Arletti, R., Benelli, A., & Bertolini, A. (1989). Influence of oxytocin on feeding behavior in the rat. *Peptides, 10*(1), 89-93.

[2] Blevins, J. E., Thompson, B. W., Anekonda, V. T., Ho, J. M., Graham, J. L., Roberts, Z. S., Hwang, B. H., Ogimoto, K., Wolden-Hanson, T., & Nelson, J. (2016). Chronic CNS oxytocin signaling preferentially induces fat loss in high-fat diet-fed rats by enhancing satiety responses and increasing lipid utilization. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 310*(7), R640-R658.

[3] Herisson, F., Waas, J., Fredriksson, R., Schiöth, H. B., Levine, A. S., & Olszewski, P. (2016). Oxytocin acting in the nucleus accumbens core decreases food intake. *Journal of neuroendocrinology, 28*(4).

[4] Ho, J. M., Anekonda, V. T., Thompson, B. W., Zhu, M., Curry, R. W., Hwang, B. H., Morton, G. J., Schwartz, M. W., Baskin, D. G., & Appleyard, S. M. (2014). Hindbrain oxytocin receptors contribute to the effects of circulating oxytocin on food intake in male rats. *Endocrinology, 155*(8), 2845-2857.

[5] Klockars, O. A., Klockars, A., Levine, A. S., & Olszewski, P. K. (2018). Oxytocin administration in the basolateral and central nuclei of amygdala moderately suppresses food intake. *Neuroreport, 29*(6), 504-510.

[6] Klockars, O. A., Waas, J. R., Klockars, A., Levine, A. S., & Olszewski, P. K. (2017). Neural basis of ventromedial hypothalamic oxytocin-driven decrease in appetite. *Neuroscience, 366*, 54-61.

[7] Olszewski, P. K., Allen, K., & Levine, A. S. (2015). Effect of oxytocin receptor blockade on appetite for sugar is modified by social context. *Appetite, 86*, 81-87.

[8] Roberts, Z. S., Wolden-Hanson, T., Matsen, M. E., Ryu, V., Vaughan, C. H., Graham, J. L., Havel, P. J., Chukri, D. W., Schwartz, M. W., & Morton, G. J. (2017). Chronic hindbrain administration of oxytocin is sufficient to elicit weight loss in diet-induced obese rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 313*(4), R357-R371.

[9] Arletti, R., Benelli, A., & Bertolini, A. (1990). Oxytocin inhibits food and fluid intake in rats. *Physiology & behavior, 48*(6), 825-830.

[10] Melis, M. R., Melis, T., Cocco, C., Succu, S., Sanna, F., Pillolla, G., Boi, A., Ferri, G. L., & Argiolas, A. (2007). Oxytocin injected into the ventral tegmental area induces penile erection and increases extracellular dopamine in the nucleus accumbens and paraventricular nucleus of the hypothalamus of male rats. *European Journal of Neuroscience, 26*(4), 1026-1035.

[11] Wu, Z., Xu, Y., Zhu, Y., Sutton, A. K., Zhao, R., Lowell, B. B., Olson, D. P., & Tong, Q. (2012). An obligate role of oxytocin neurons in diet induced energy expenditure. *PloS one, 7*(9), e45167.

[12] Maejima, Y., Iwasaki, Y., Yamahara, Y., Kodaira, M., Sedbazar, U., & Yada, T. (2011). Peripheral oxytocin treatment ameliorates obesity by reducing food intake and visceral fat mass. *Aging (Albany NY), 3*(12), 1169.

[13] Morton, G. J., Thatcher, B. S., Reidelberger, R. D., Ogimoto, K., Wolden-Hanson, T., Baskin, D. G., Schwartz, M. W., & Blevins, J. E. (2012). Peripheral oxytocin suppresses food intake and causes weight loss in diet-induced obese rats. *American Journal of Physiology-Endocrinology and Metabolism, 302*(1), E134-E144.

[14] Klockars, A., Brunton, C., Li, L., Levine, A. S., & Olszewski, P. K. (2017). Intravenous administration of oxytocin in rats acutely decreases deprivation-induced chow intake, but it fails to affect consumption of palatable solutions. *Peptides, 93*, 13-19.

[15] Mitra, A., Gosnell, B. A., Schiöth, H. B., Grace, M. K., Klockars, A., Olszewski, P. K., & Levine, A. S. (2010). Chronic sugar intake dampens feeding-related activity of neurons synthesizing a satiety mediator, oxytocin. *Peptides, 31*(7), 1346-1352.

[16] Kublaoui, B. M., Gemelli, T., Tolson, K. P., Wang, Y., & Zinn, A. R. (2008). Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice. *Molecular endocrinology, 22*(7), 1723-1734.

[17] Verbalis, J. G., McCann, M. J., McHale, C. M., & Stricker, E. M. (1986). Oxytocin secretion in response to cholecystokinin and food: differentiation of nausea from satiety. *Science, 232*(4756), 1417-1419.

[18] Ohlsson, B., Forsling, M. L., Rehfeld, J. F., & Sjölund, K. (2002). Cholecystokinin stimulation leads to increased oxytocin secretion in women. *The European journal of surgery, 168*(2), 114-118.

[19] Renaud, L., Tang, M., McCann, M., Stricker, E., & Verbalis, J. (1987). Cholecystokinin and gastric distension activate oxytocinergic cells in rat hypothalamus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 253*(4), R661-R665.

[20] Bojanowska, E., & Stempniak, B. (2001). tGLP-1 and release of vasopressin and oxytocin from the isolated rat hypothalamo-neurohypophysial system: effects of a tGLP-1 receptor agonist and antagonist. *J Physiol Pharmacol, 52*(4 Pt 2), 781-93.

[21] Ladyman, S., Augustine, R., Scherf, E., Phillipps, H., Brown, C., & Grattan, D. (2016). Attenuated hypothalamic responses to α - melanocyte stimulating hormone during pregnancy in the rat. *The Journal of Physiology, 594*(4), 1087-1101.

[22] Gartner, S. N., Aidney, F., Klockars, A., Prosser, C., Carpenter, E. A., Isgrove, K., Levine, A. S., & Olszewski, P. K. (2018). Intragastric preloads of l-tryptophan reduce ingestive behavior via oxytocinergic neural mechanisms in male mice. *Appetite, 125*, 278-286.

[23] Gartner, S. N., Klockars, A., Prosser, C., Carpenter, E. A., Levine, A. S., & Olszewski, P. K. (2018). Identification of central mechanisms underlying anorexigenic effects of intraperitoneal L-tryptophan. *Neuroreport, 29*(15), 1293-1300.

[24] Lawson, E. A., Marengi, D. A., DeSanti, R. L., Holmes, T. M., Schoenfeld, D. A., & Tolley, C. J. (2015). Oxytocin reduces caloric intake in men. *Obesity, 23*(5), 950-956.

[25] Olson, B. R., Drutarosky, M. D., Chow, M.-S., Hruby, V. J., Stricker, E. M., & Verbalis, J. G. (1991). Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats. *Peptides, 12*(1), 113-118.

[26] Corwin, R. L., Woolverton, W. L., & Schuster, C. R. (1990). Effects of cholecystokinin, d-amphetamine and fenfluramine in rats trained to discriminate 3 from 22 hr of food deprivation. *Journal of Pharmacology and Experimental Therapeutics, 253*(2), 720-728.

[27] Jewett, D. C., Hahn, T. W., Smith, T. R., Fiksdal, B. L., Wiebelhaus, J. M., Dunbar, A. R., Filtz, C. R., Novinska, N. L., & Levine, A. S. (2009). Effects of sibutramine and rimonabant in rats trained to discriminate between 22-and 2-h food deprivation. *Psychopharmacology, 203*(2), 453-459.

[28] Jewett, D. C., Lefever, T. W., Flashinski, D. P., Koffarnus, M. N., Cameron, C. R., Hehli, D. J., Grace, M. K., & Levine, A. S. (2006). Intraparaventricular neuropeptide Y and ghrelin induce learned behaviors that report food deprivation in rats. *Neuroreport, 17*(7), 733-737.

[29] Olszewski, P. K., Klockars, A., Olszewska, A. M., Fredriksson, R., Schioth, H. B., & Levine, A. S. (2010). Molecular, immunohistochemical, and pharmacological evidence of oxytocin's role as inhibitor of carbohydrate but not fat intake. *Endocrinology, 151*(10), 4736-4744.

[30] Fredriksson, R., Hagglund, M., Olszewski, P. K., Stephansson, O., Jacobsson, J. A., Olszewska, A. M., Levine, A. S., Lindblom, J., & Schiöth, H. B. (2008). The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. *Endocrinology, 149*(5), 2062-2071.

[31] Stanley, B. G., Kyrkouli, S. E., Lampert, S., & Leibowitz, S. F. (1986). Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides, 7*(6), 1189-1192.

[32] Bailey, A. R., Giles, M. E., Brown, C. H., Bull, P. M., Macdonald, L. P., Smith, L. C., Smith, R. G., Leng, G., & Dickson, S. L. (1999). Chronic central infusion of growth hormone secretagogues: effects on fos expression and peptide gene expression in the rat arcuate nucleus. *Neuroendocrinology, 70*(2), 83-92.

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

[34] Olszewski, P., Klockars, A., & Levine, A. S. (2016). Oxytocin: a conditional anorexigen whose effects on appetite depend on the physiological, behavioural and social contexts. *Journal of neuroendocrinology, 28*(4).

[35] Herisson, F. M., Brooks, L. L., Waas, J. R., Levine, A. S., & Olszewski, P. K. (2014). Functional relationship between oxytocin and appetite for carbohydrates versus saccharin. *Neuroreport, 25*(12), 909-914.

[36] Jewett, D. C., Cleary, J., Levine, A. S., Schaal, D. W., & Thompson, T. (1992). Effects of neuropeptide Y on food-reinforced behavior in satiated rats. *Pharmacology Biochemistry and Behavior, 42*(2), 207-212.

[37] Jewett, D., Cleary, J., Levine, A., Schaal, D., & Thompson, T. (1995). Effects of neuropeptide Y, insulin, 2-deoxyglucose, and food deprivation on food-motivated behavior. *Psychopharmacology, 120*(3), 267-271.

[38] Olszewski, P. K., Wood, E. L., Klockars, A., & Levine, A. S. (2019). Excessive consumption of sugar: an insatiable drive for reward. *Current Nutrition Reports, 8*(2), 120-128.

[39] Olszewski, P. K., & Levine, A. S. (2007). Central opioids and consumption of sweet tastants: when reward outweighs homeostasis. *Physiology & behavior, 91*(5), 506-512.

[40] Corwin, R. L., Woolverton, W. L., & Schuster, C. R. (1990). Effects of cholecystokinin, d-amphetamine and fenfluramine in rats trained to discriminate 3 from 22 hr food deprivation. *Journal of Pharmacology and Experimental Therapeutics, 253*(2), 720-728.

[41] Davidson, T., Kanoski, S. E., Tracy, A. L., Walls, E. K., Clegg, D., & Benoit, S. C. (2005). The interoceptive cue properties of ghrelin generalize to cues produced by food deprivation. *Peptides, 26*(9), 1602-1610.

[42] OLSON, B. R., DRUTAROSKY, M. D., STRICKER, E. M., & VERBALIS, J. G. (1991). Brain oxytocin receptor antagonism blunts the effects of anorexigenic treatments in rats: evidence for central oxytocin inhibition of food intake. *Endocrinology, 129*(2), 785-791.

[43] Olszewski, P. K., Wirth, M. M., Shaw, T. J., Grace, M. K., Billington, C. J., Giraudo, S. Q., & Levine, A. S. (2001). Role of α-MSH in the regulation of consummatory behavior: immunohistochemical evidence. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 281*(2), R673-R680.

[44] Sun, X., Kroemer, N. B., Veldhuizen, M. G., Babbs, A. E., de Araujo, I. E., Gitelman, D. R., Sherwin, R. S., Sinha, R., & Small, D. M. (2015). Basolateral amygdala response to food cues in the absence of hunger is associated with weight gain susceptibility. *Journal of Neuroscience, 35*(20), 7964-7976.

[45] Beckman, T. R., Shi, Q., Levine, A. S., & Billington, C. J. (2009). Amygdalar opioids modulate hypothalamic melanocortin-induced anorexia. *Physiology & behavior, 96*(4-5), 568-573.

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

[47] Carson, D. S., Hunt, G. E., Guastella, A. J., Barber, L., Cornish, J. L., Arnold, J. C., Boucher, A. A., & McGregor, I. S. (2010). Systemically administered oxytocin decreases methamphetamine activation of the subthalamic nucleus and accumbens core and stimulates oxytocinergic neurons in the hypothalamus. *Addiction biology, 15*(4), 448-463.

[48] Hicks, C., Ramos, L., Dampney, B., Baracz, S. J., McGregor, I. S., & Hunt, G. E. (2016). Regional c-Fos expression induced by peripheral oxytocin administration is prevented by the vasopressin 1A receptor antagonist SR49059. *Brain research bulletin, 127*, 208-218.

[49] Hicks, C., Jorgensen, W., Brown, C., Fardell, J., Koehbach, J., Gruber, C., Kassiou, M., Hunt, G., & McGregor, I. (2012). The nonpeptide oxytocin receptor agonist WAY 267,464: Receptor‐binding profile, prosocial effects and distribution of c‐Fos expression in adolescent rats. *Journal of neuroendocrinology, 24*(7), 1012-1029.

Chapter 3
Effects of opioid receptor ligands on perception of hunger

Abstract

In the previous chapter, I found that while OT inhibits food intake by mediating homeostatic signals of satiety, it does not influence the perception of hunger. In the search for neuropeptides that target different facets of consummatory behaviour, of particular interest are opioid receptor antagonists, known to influence palatability and reward signals, acting to inhibit palatable food intake. What is not known, however, is whether opioid receptor antagonists such as NTX might also influence the third aspect that drives consumption, which is hunger. Some studies suggest a potential involvement in hunger-driven intake, but they suffer from the scarcity of methodologies differentiating between factors that intersect eating for pleasure versus energy. Here, I used a unique hunger discrimination paradigm to test a hypothesis that, since opioids appear to control feeding reward, injection of opioid agonists would not produce effects akin to 22 h of food deprivation. I trained rats to discriminate between 22 h and 2 h food deprivation in a two-lever, operant discrimination procedure. I tested whether opioid agonists at orexigenic doses produce discriminative stimulus effects similar to 22 h deprivation. I injected DAMGO, DSLET, or orphanin FQ in the paraventricular hypothalamic nucleus (PVN), a site regulating hunger/satiety, and butorphanol subcutaneously (to produce maximum consumption). I assessed the ability of the opioid antagonist, NTX, to reduce the discriminative stimulus effects of 22 h deprivation and of the 22 h deprivation-like discriminative stimulus effects of PVN-injected hunger mediator, neuropeptide Y (NPY). In contrast to PVN NPY, centrally or peripherally injected opioid agonists failed to induce

discriminative stimuli similar to those of 22 h deprivation. In line with that, NTX did not reduce the hunger discriminative stimuli induced by either 22 h deprivation or NPY administration in 2 h food-restricted subjects, even though doses used therein were sufficient to decrease deprivation-induced feeding in a non-operant setting in animals familiar with consequences of 2 h and 22 h deprivation. I conclude that opioids do not influence hunger, but promote feeding for reward rather than in order to replenish lacking energy.

3.1 Introduction

Among a host of neuropeptides that affect food intake, some appear to alter consumption associated with energy needs, while others, eating associated with taste and reward [1]. In the previous chapter, I investigated the effects of one such neuropeptide in particular, OT, on whether it induces its anorexigenic effects by altering a feeling of hunger. While OT seems to be associated with modulating aspects of feeding related to energy needs by signaling satiation, I found that it did not reduce a feeling of hunger when examined using an operant procedure, even though it did act to significantly reduce food intake in deprived and non-deprived rats. I therefore aim to investigate here if neuropeptides that modulate feeding associated with palatability and reward might exert their effects by modulating a feeling of hunger, in a similar operant paradigm.

Opioids seem to be involved in the rewarding aspects of eating behavior, particularly related to intake of sugar and fat [2]. In contrast, neuropeptide Y (NPY) primarily modifies feeding driven by energy needs [3]. Food deprivation or restriction increases gene expression of NPY, but decreases gene expression of opioid peptides [4; 5]. As both opioids and NPY are orexigens, one would expect an increase in gene expression of an orexigenic peptide following food restriction if that peptide was indeed involved in energy-related feeding. What adds to the confusion is the fact that opioid receptor ligands,

though particularly effective at modifying intake of palatable diets, at higher doses also affect intake of bland foods in animals motivated to eat by hunger [2; 6]. Interestingly, these effects occur with generalized peripheral or central injections, as well as even when injections are done directly in brain sites typically associated with mediating eating for energy, such as hypothalamic areas [2; 7].

To distinguish whether peptides are involved in eating due to reward or energy needs, most investigators conduct studies with specific macronutrient diets or with diets that are more or less preferred by rats [8; 9]. Such methods do not directly differentiate reward- from energy-induced eating. Rats often prefer a high-fat diet compared to a high-starch (carbohydrate) diet. However, one cannot determine whether preference for fat is due to reward or energy needs since - while fat is preferred and presumably tastes good to a rat - fat is also the most energy-dense macronutrient.

In order to address the issue of feeding for energy versus feeding for pleasure, a discrimination paradigm has been developed in which rats are trained to recognize the difference between discriminative stimuli produced by acute food deprivation (22 h deprivation) compared to discriminative stimuli produced by 2 h food deprivation. Corwin et al. trained rats to discriminate between recent (3 h) and more distant (22 h) food consumption and reported that the satiety peptide cholecystokinin (CCK), or ingestion of sweetened condensed milk, induced effects similar to 3 h food ingestion in chronically deprived rats [10]. This suggests that CCK administration resulted in a state that resembled a satiation (recent food ingestion) rather than deprivation. Using a similar methodology in non-deprived animals, I found that when rats were food-restricted for only 2 h and centrally-injected with NPY or ghrelin, rats responded on the lever associated with a 22 h deprivation [11]. This suggests that internal stimuli associated with NPY or

ghrelin resembled stimuli produced by 22 h of food deprivation. Thus, one might conclude that central injections of NPY or ghrelin result in hunger.

In the current study, I hypothesized that since opioids appear to be involved in the rewarding aspects of eating, injection of opioid agonists would not produce internal stimuli associated with 22 h of food deprivation. To test this hypothesis, I studied the ability of select opioid agonists known to increase food intake to produce discriminative stimulus effects similar to those of 22 h food deprivation. I injected the opioid agonists (DAMGO, DSLET, nociceptin/orphanin FQ) in the paraventricular hypothalamic nucleus (PVN), a brain site that integrates mostly signaling related to energy needs [12]. We also administered butorphanol tartrate, which when injected peripherally, produces the most avid consumption of standard chow [13] Furthermore, I assessed the ability of the opioid antagonist, NTX, at peripheral doses sufficient to decrease deprivation-induced feeding in animals familiar with consequences of 2 and 22 h of food deprivation, to reduce the discriminative stimulus effects of 22 h food deprivation and of the 22 h deprivation-like discriminative stimulus effects of PVN NPY.

3.2 Materials and methods

3.2.1 Animals

In all our studies, I used male adult Sprague-Dawley rats, individually housed in standard polycarbonate cages, under a 12:12 light:dark cycle (lights on at 8:30 AM in all discrimination studies and at 7:00 AM in the remaining experiments) in a temperature-controlled (22 °C) animal facility. Standard laboratory chow (Sharpes Stock Feed, Diet 86; 3.6kcal/g) and water were available ad libitum unless noted otherwise. Animals were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals [14]. The University of Waikato Animal Ethics Committee and the University of Wisconsin-Eau Claire Institutional Animal Care and Use Committee approved all experimental procedures described in this project.

3.2.2 Drugs

Sodium pentobarbital (Sigma Chemical Co., St. Louis, MO) was dissolved in 30 % propylene glycol. Nociceptin/orphanin FQ (Phoenix Pharmaceuticals Inc., Burlingame, CA), NPY, DSLET and DAMGO (all three obtained from Sigma Chemical Co., St. Louis, MO) were dissolved in 0.9 % saline and stored in sealed plastic containers at -20 degrees C. NTX and butorphanol tartrate (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9 % saline and refrigerated at 4 degrees C. All chemicals were slowly warmed to room temperature 20 min prior to administration. In experiments involving central injections, compounds were slowly (60 s) administered via an indwelling cannula directed toward the periventricular nucleus of the hypothalamus (PVN). Injection volumes were 0.5 µl, similar to previously published PVN studies [15; 16]. In peripheral (subcutaneous (s.c.)) injection experiments, butorphanol and NTX were dissolved in 0.9 % saline and administered at a volume of 1 ml/kg.

3.2.3 Operant studies: establishing the effects of drugs on discrimination between 22h and 2h of food deprivation

3.2.3.1 Animals

Experimentally naïve male Sprague Dawley rats (Harlan, Madison, WI) were approximately 12-weeks old at the beginning of the procedures. Food (Harlan Teklad chow, Madison, WI) and tap water were continuously available unless otherwise stated.

3.2.3.2 Operant chambers, reinforcement stimuli and data acquisition

Daily discrimination sessions were conducted in eight standard operant chambers (Med-Associates, St. Albans, VT). Operant chambers were located in ventilated, sound-attenuating cubicles equipped with fans. The chambers were equipped with two response levers. Forty-five mg food pellets (Bio-Serve F#0021, Frenchtown, NJ) reinforced lever pressing and were delivered by a pellet dispenser into a food pellet trough located between the two response levers. A house light located in the back panel of the operant chamber illuminated the operant chambers during experimental sessions. Experimental contingencies and data recording were executed via Med Associates software and a personal computer located in an adjacent room.

3.2.3.3 Training procedure to discriminate between 22 and 2 h of food deprivation

Twenty-four rats were initially food deprived to ~85 % of their free feeding body weight and they were first trained to lever press via the method of successive approximations. Initially, a single lever press was reinforced with a 45 mg food pellet, and response requirements were gradually increased until 15 lever presses (fixed ratio 15; FR 15) were required to produce food. When responding occurred reliably to both levers, rats were given free access to food for 305 days before discrimination training began.

In the discrimination phase of training, rats (having free access to chow except during the deprivation periods) were trained to discriminate between 22 and 2 h acute food

deprivation using multiple cycle training. Under 22 h conditions, food was removed 22 h before the training session. Rats were placed into the operant chamber 5 min before the first training cycle. When the first training cycle began, the house light was illuminated and 15 left lever presses were reinforced with the 45 mg pellet delivery under an FR 15 reinforcement schedule. Incorrect (i.e., right) lever presses were punished with 8 s of darkness under a FR 15 schedule. Training continued until 5 reinforcers were earned or 5 min elapsed. One or more additional training cycles, identical to the first, commenced 30−120 min after the previous training cycle.

Under 2 h conditions, the contingencies were reversed. Right lever presses were reinforced and left lever presses were punished under the FR 15 schedule. Conditions were quasi-randomly assigned with the provision that the same training condition (22 or 2 h of food deprivation) could not be given for more than two consecutive sessions.

Discrimination training continued until the subject emitted 80 % or greater condition-appropriate responses prior to delivery of the first reinforcer and for the entire training session during all training cycles for 8 of 10 consecutive daily sessions.

3.2.3.4 Surgeries

Following acquisition of the deprivation discrimination, animals designated for experiments involving site-specific brain injections, were implanted with a cannula directed toward the PVN. Rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and mounted into a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). A 26-gauge stainless steel guide PVN cannula was positioned according to the coordinates from the Paxinos and Watson rat brain atlas [17]: -1.9 mm anteroposterior, -0.5 mm lateral, and -7.3 mm dorsoventral to bregma. After surgery, rats were maintained on chow and water ad libitum for at least 1 week before discrimination training resumed.

Prior to and after generalization tests, animals were injected in the PVN with NPY (0.5 μg) to ensure that the cannula was functioning properly throughout the experiment: only those animals in whom NPY reliably produced increases in food intake greater than 5 g within 30 min post-injection were retained in the study.

3.2.3.5 Generalization tests I: Effects of opioid agonists in rats deprived for 2h

Following recovery from the surgical procedure and re-establishment of discriminative control, generalization testing began. Initial generalization tests assessed the ability of opioid agonists to induce discriminative stimulus effects similar to those of 22 h food deprivation.

During these tests the rats were food-deprived for 2 h, and during the first response period only right lever presses were reinforced with the 45-mg pellet. If condition-appropriate behavior occurred, rats were slowly injected with saline (0.5 μl, PVN) DAMGO (0.1–3.2 nmol, PVN), DSLET (0.1–3.2 nmol, PVN), orphanin FQ (0.1–3.2 nmol, PVN), or butorphanol (1.0–5.6 mg/kg, s.c.). The small injectant volumes for PVN injections have not been shown to diffuse to other brain areas [16; 18]. After drug administration, rats were placed in stainless steel cages without food or water. During the second response period occurring 1 h after the PVN injections and 2 h after butorphanol injection, responses made on either lever were reinforced under the FR 15 reinforcement schedule.

Generalization tests lasted until the subject earned 5 reinforcers or until 5 min elapsed, whichever occurred first. Appropriate discriminative performance for at least 2 training days (one preceded by 22 h deprivation, one preceded by 2 h deprivation) was required between generalization tests.

3.2.3.6 Generalization tests II: Effects of daily s.c. butorphanol in rats deprived for 2h

Since acute pre-treatment with butorphanol produced significant reductions in lever pressing rates, but did not produce discriminative stimulus effects similar to 22 h food deprivation, I examined the effects of daily butorphanol (3.2 mg/kg, s.c.) administered 2 h before the operant test session. These tests followed the same protocol as described above in section 3.2.3.5.

3.2.3.7 Generalization tests III: Examining the ability of NTX to reduce the discriminative stimulus effects of 22h deprivation and the 22h deprivation-like discriminative stimulus effects of PVN NPY

Additional discrimination tests were designed to assess the potential ability of NTX (injected to either intra-PVN or s.c.) to reduce the discriminative stimulus effects of 22 h food deprivation and the 22 h deprivation-like stimulus effects of PVN NPY. To assess NTX's effects on the discriminative stimulus effects of 22 h deprivation, 22 h deprived rats were given saline (0.5 μl, PVN or 1 ml/kg, s.c.) or NTX (10−100 nmol, PVN or 0.32−10 mg/kg, s.c.) and generalization tests were conducted 30 min after the injection. To assess the effects of NTX on the 22 h deprivation-like discriminative stimulus effects of NPY, 2 h deprived rats were injected with NPY (1.0 μg/0.5 μl). Thirty minutes later, rats were injected with (0.5 μl, PVN or 1 ml/kg, s.c.) or NTX (10−100 nmol, PVN or 0.32−10 mg/kg, s.c.). Generalization tests were conducted 30 min after the second injection. As before, responses toward both levers were reinforced and rats could earn 5 reinforcers during the 5-minute generalization test session.

3.2.4 Home cage feeding studies: Effect of NTX on deprivation-induced chow intake in rats subjected to 2 or 22h of recurrent deprivation

Somewhat surprisingly, even at large doses that typically reduce deprivation-induced food intake [19; 20], NTX failed to affect the discriminative stimulus effects of either 22

h food deprivation or NPY-induced, deprivation-like discriminative stimuli in the aforementioned operant studies. This raised a question of whether NTX retains its ability to reduce consumption in animals subjected to the recurrent 2 h and 22 h deprivation schedule even outside the operant setting, i.e., in their home cage.

Therefore, 12 rats (16 weeks old; b. wt. ~450 g) were given access to food according to a 19-day long familiarization schedule with access to standard chow (Sharpes Stock Feed, Diet 86) after either 2 h or 22 h of recurrent deprivation (see Fig. 3.1 for a flow-chart of study design). Rats were divided into two cohorts (n = 12 per cohort) which received injections prior to re-feeding after 2 or 22 h of food deprivation. NTX (0.1, 0.3, 1.0, and 3.2 mg/kg) or saline (vehicle) were administered s.c. just prior to returning the chow to the hoppers. Food intake measurements were taken 1, 2 and 24 h post injection and corrected for spillage. Each animal received each dose of the drug in a counterbalanced fashion.

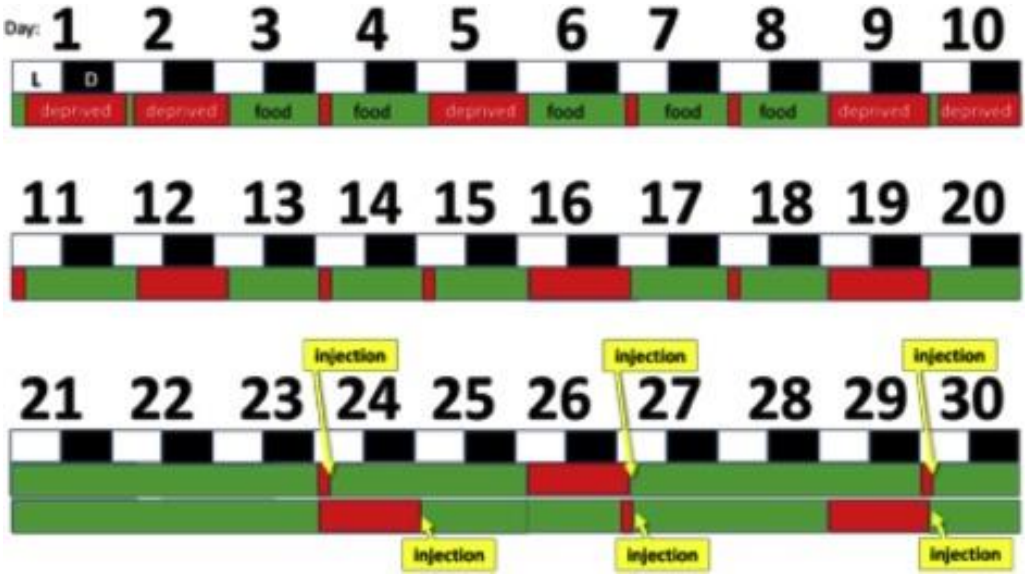


Figure 3.1: Flow-chart delineating drug treatment in rats acquainted with a random, recurrent energy deprivation schedule. In order to assess whether NTX reduces chow consumption in animals subjected to the recurrent 2 h and 22 h deprivation schedule outside the operant setting, i.e., in their home cage, single-housed rats underwent first the feeding schedule familiarization phase followed by the drug treatment phase in which NTX or vehicle (saline) were administered. Familiarization phase (days 1–19): Rats were acquainted with the random chow deprivation schedule (either 22 h or 2 h of food access or food deprivation as indicated by green boxes (access) and red boxes (deprivation)). Three-to-four days of no deprivation were allowed before the beginning of the drug treatment phase (saline, 0.1, 0.3, 1.0, or 3.2 mg/kg NTX were injected subcutaneously). White and black boxes indicate the light (L) and dark (D) phase of the LD cycle.

3.2.5 Data analysis

All descriptive data are presented as means ± the standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to assess the ability of each opioid-agonist to induce the discriminative stimulus effects similar to those of 22 hr food deprivation. One-way, ANOVA was also used to assess NTX's ability to decrease the discriminative stimulus effects of 22 h food deprivation and the 22 h deprivation-like discriminative stimulus effects of neuropeptide Y. Lever pressing rates in all generalization tests were examined using a one-way ANOVA, except for the five day, daily butorphanol tests in which a repeated measures ANOVA was used to analyse response rates. Significant effects were noted if $p < 0.05$. If the results of the ANOVA were statistically significant, Dunnett post-hoc tests were performed to determine conditions that were significantly different than control.

3.3 Results

Rats acquired the 22 h deprivation versus 2 h deprivation discrimination in an average of 77 trials (SEM 12 trials). Generalization tests were conducted under 2 h deprivation and rats were injected with various opioid agonists at doses that have previously been shown to increase food intake in non-deprived rats. Results revealed that opioid agonists failed to induce discriminative stimulus effects similar to 22 h deprivation. PVN-administration of the mu-opioid agonist DAMGO ($F(4,15) = 0.70$. $p = 0.60$, Fig. 3.2a, top panel), the delta-opioid agonist DSLET ($F(4,19) = 0.70$, $p = 0.55$, Fig. 3.2b, top panel), and the NOP receptor agonist orphanin FQ ($F(4,27) = 0.98$, $p = 0.44$, Fig. 3.2c, top panel), did not produce significant increases in 22 h deprivation responding. Response rates were also unaffected by DAMGO ($F(4,21) = 0.22$, $p = 0.92$, Fig. 3.2a, bottom panel), DSLET ($F(4,21) = 0.87$, $p = 0.50$, Fig. 3.2b, bottom panel), and orphanin FQ ($F(4,36) = 0.41$, $p = 0.80$, Fig. 3.2c, bottom panel).

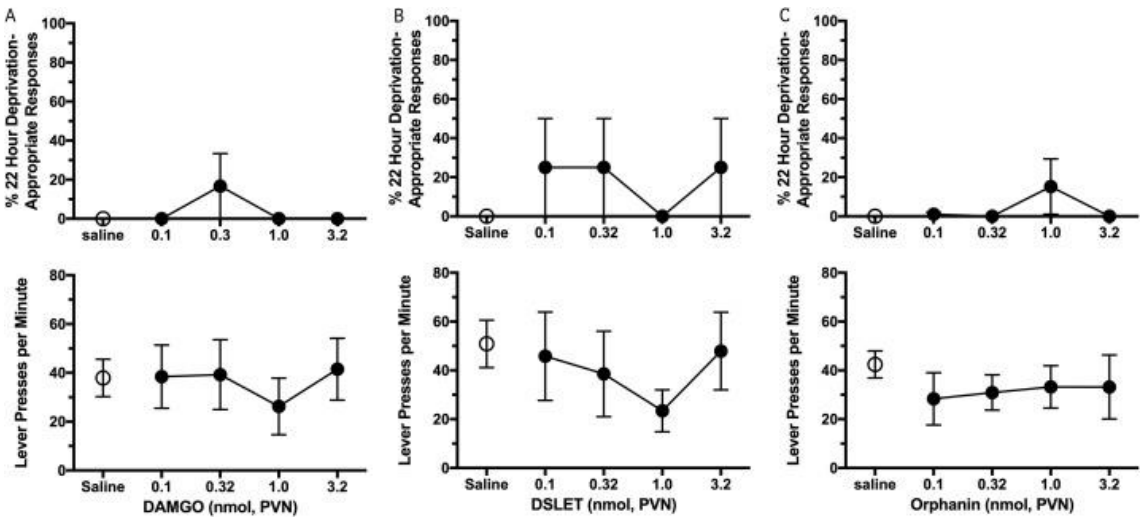


Figure 3.2: Effect of PVN (a) DAMGO, (b) DSLET, and (c) orphanin FQ on hunger discrimination responses and rates of lever pressing. In order to assess whether PVN-administered opioid agonists induce 22 hr deprivation-like discriminative stimuli, rats (n = 16, 3–7 per condition) under 2 h deprivation conditions were injected with saline (open symbols), DAMGO, DSLET, or Orphanin (filled symbols represent data from drug tests) and generalization tests began 60 min later. Generalization test performance is expressed as the average percentage of 22 hr deprivation responses (± SEM, top panel) and response rates (lever pressing expressed as average responses per minute ± SEM, bottom panel). No significant changes in either discriminative performance or response rate were observed following administration of either opioid agonist.

Peripheral administration of butorphanol (1.0–5.6 mg/kg, s.c.) also did not induce increases in 22 h deprivation-appropriate responding, ($F(4,17) = 0.63$, $p = 0.65$, Fig. 3.3, top panel), however lever pressing rates were significantly decreased by butorphanol ($F(4,26) = 6.92$, $p < 0.001$, Fig. 3.3, bottom panel). All doses of butorphanol tested (1.0–5.6 mg/kg, s.c.,) significantly reduced response rates (Dunnett post-hoc test, $p < 0.05$). It is possible that the rate decreasing effects of butorphanol were preventing deprivation-induced discriminative stimuli from being expressed. To test this possibility, six rats trained to discriminate between 22 and 2 h food deprivation were given daily administration of 3.2 mg/kg butorphanol (s.c) for five days. There was an overall effect of butorphanol on response rates, but the rate-decreasing effects of butorphanol were significantly different than response rates following saline administration only following the first day of 3.2 mg/kg butorphanol administration (Dunnett post-hoc test, $p < 0.05$, Fig. 3.4, bottom panel). Although response rates were not significantly different from

saline on days 2–5 of butorphanol administration, daily butorphanol administration did not significantly increase 22 h deprivation-appropriate responses ($F(5,18) = 1.67$, $p = 0.19$, Fig. 3.4, top panel).

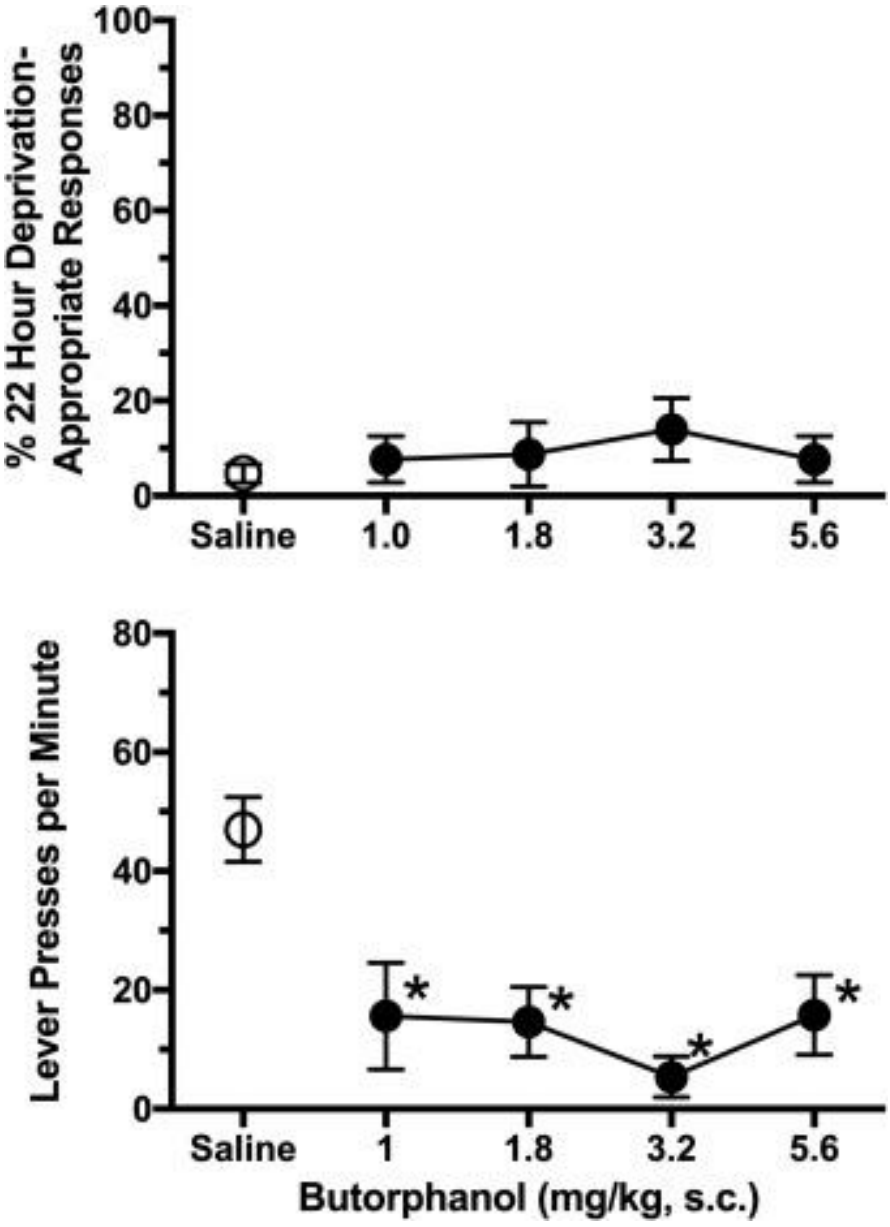


Figure 3.3: Effect of s.c. butorphanol on hunger discrimination responses and rates of lever pressing. In order to assess whether peripherally-administered doses of the opioid orexigenic butorphanol, induced 22 hr deprivation-like discriminative effects, eight rats (4–8 per condition) under 2 h deprivation conditions were injected s.c. with saline (open symbol) or butorphanol (filled symbols). Generalization tests began 120 min after the injection. Generalization test performance is expressed as the average percentage of 22 hr deprivation responses (± SEM, top panel) and response rates (lever pressing expressed as average responses per minute ± SEM, bottom panel). Butorphanol did not produce increases in 22 hr deprivation responding ($F_{(4,17)} = 0.63$, $p = 0.65$) but did significantly reduce response rates ($F_{(4,26)} = 6.92$, $p = 0.0006$). Response rates following 1.0–5.6 mg/kg butorphanol were significantly reduced compared to response rates following saline administration (Dunnett post hoc test, *$p < 0.05$).

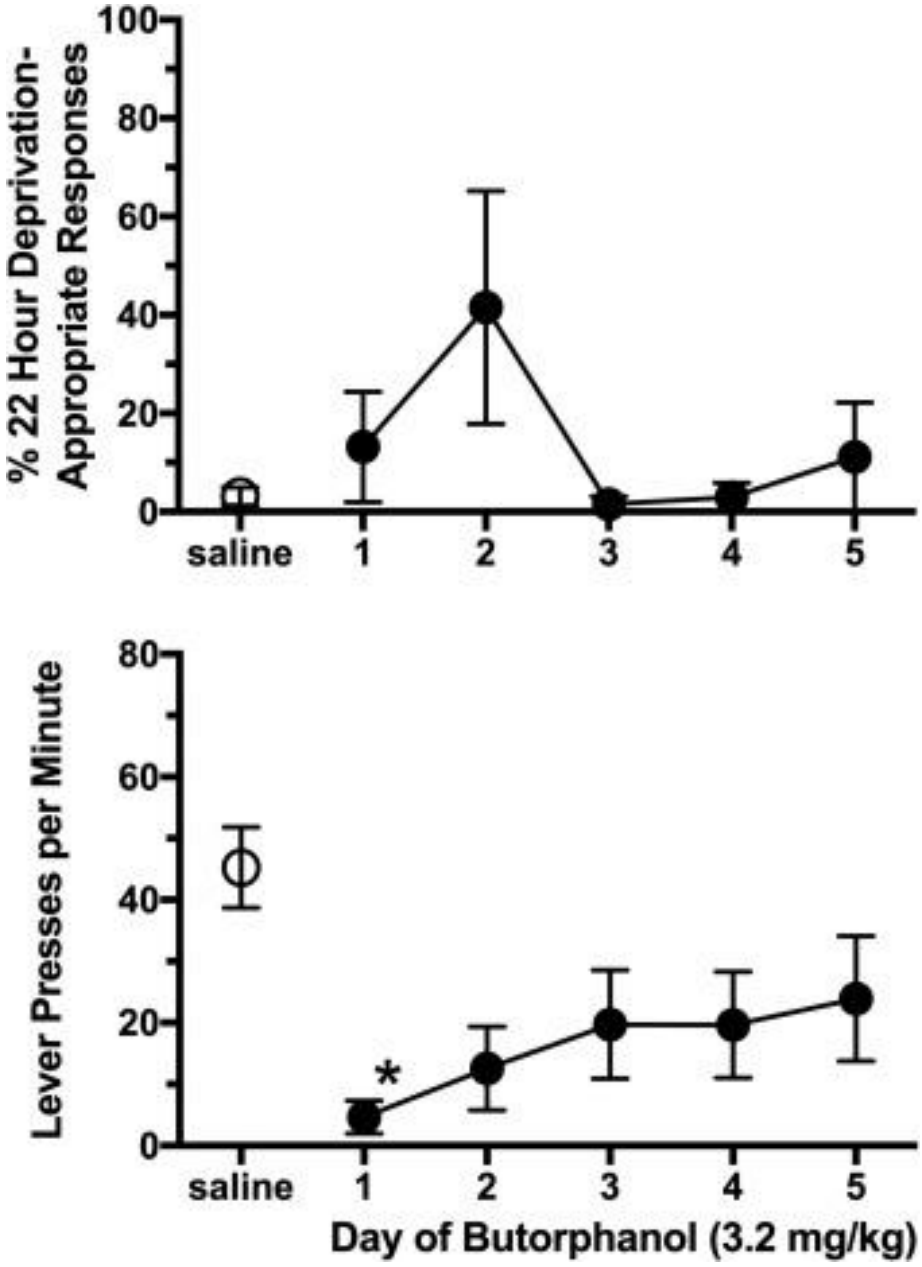


Figure 3.4: Effect of daily s.c. butorphanol administration on discrimination responses. In order to assess whether daily administration of butorphanol induced 22 hr deprivation-like discriminative effects or affected response rates, six rats under 2 h deprivation conditions were injected s.c. with saline (open symbol) or butorphanol (filled symbols). Daily generalization tests began 120 min after the injection. Generalization test performance is expressed as the average percentage of 22 hr deprivation responses (± SEM, top panel) and response rates (lever pressing expressed as average responses per minute ± SEM, bottom panel).When 3.2 mg/kg butorphanol was given to six rats daily for five days, butorphanol did not significantly increase 22 hr deprivation-appropriate responding. ($F(5,18) = 1.67$, $p = 0.65$). Butorphanol did significantly alter response rates ($F(5,25) = 5.91$, $p = 0.03$). Responses rates following butorphanol administration on day 1 were significantly reduced compared to response rates following saline (Dunnett post hoc test, $p < 0.05$). Response rates on days 2 through 5 were not significantly different than control rates following 22 h deprivation (open symbol).

The effects of the opioid antagonist NTX were assessed both in rats under 22 h deprivation conditions and in rats under 2 h deprivation conditions that received NPY (1.0 μg, PVN). NTX administered subcutaneously (0.32–10.0 mg/kg) failed to alter the discriminative stimulus effects of 22 h food deprivation ($F(4,25) = 1.36$, $p = 0.28$, Fig. 3.5) and rates of lever pressing ($F(4,25) = 0.15$, $p = 0.96$, Fig. 3.5). PVN administered NTX also failed to alter either the discriminative stimulus effects of 22 h food deprivation ($F(3,11) = 0.49$, $p = 0.69$, Fig. 3.6) or response rates ($F(3,11) = 0.15$, $p = 0.96$, Fig. 3.6). NPY (1.0 μg) administered into the PVN produced complete generalization to the 22 h discriminative stimulus (Fig. 3.5, Fig. 3.6). The 22 h deprivation-like discriminative-stimulus effects of NPY were not altered by NTX administered subcutaneously ($F(4,19) = 0.52$, $p = 0.72$, Fig. 3.5) or when administered directly into the PVN ($F(3,10) = 0.88$, $p = 0.48$, Fig. 3.6). Response rates following PVN administration of NPY were not altered by NTX administered s.c. ($F(4,19) = 0.29$, $p = 0.82$, Fig. 3.5) or directly into the PVN ($F(3,10) = 0.75$, $p = 0.55$, Fig. 3.6).

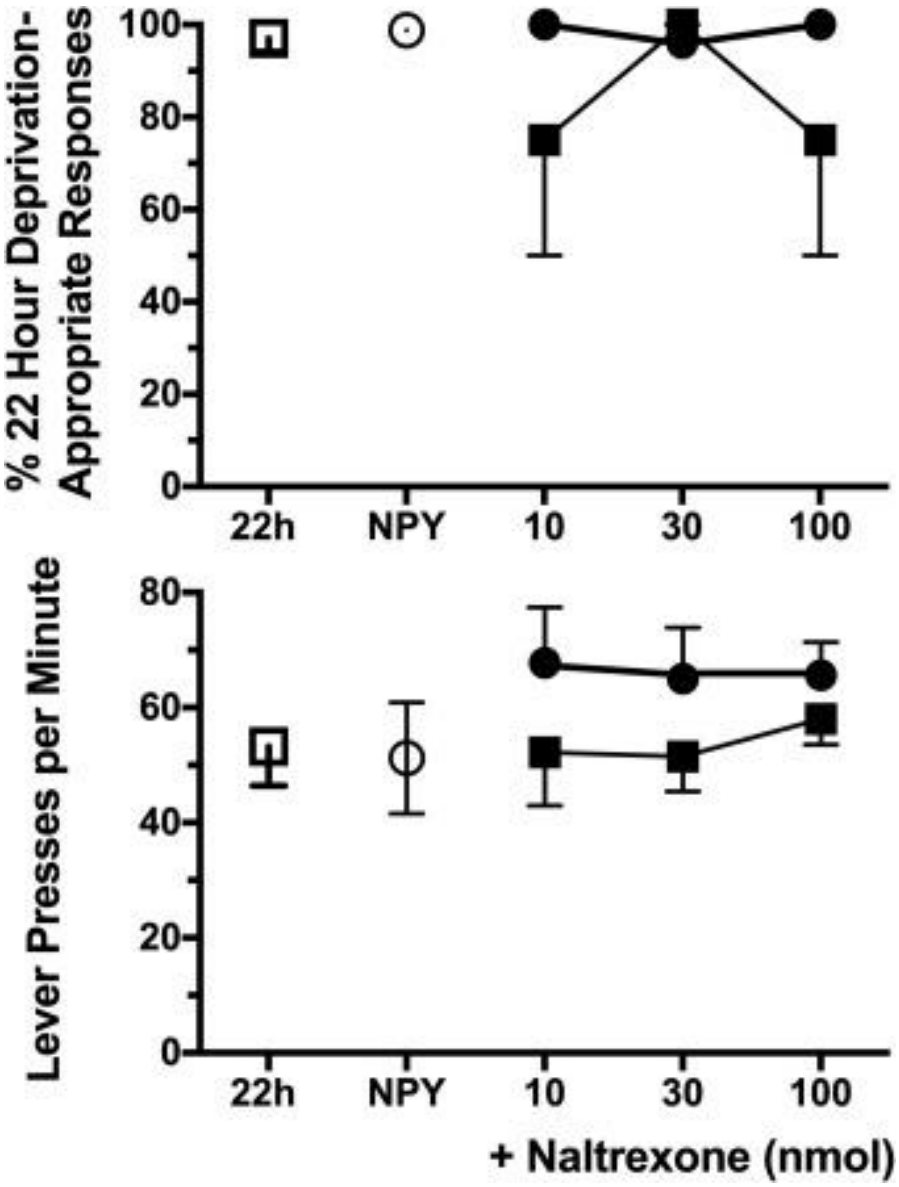


Figure 3.5: Effect of PVN naltrexone on discrimination responses. To examine the possible effects of PVN NTX on the discriminative stimulus effects of 22 h deprivation, four rats were food deprived for 22 h and injected PVN with saline (open squares) or NTX (10−100 nmol, filled squares). During other tests, eight rats (3-6 per condition) were food deprived for 2 h and injected with NPY (1.0 μg, PVN) and injected PVN. with saline (open circles) or NTX (10−100 nmol, filled circles). Generalization tests were conducted 30 min later and the discriminative stimulus effects and rates of lever pressing were assessed. Both 22 h deprivation and NPY administration engendered 22 h-appropriate deprivation responses. NTX did not alter the discriminative stimulus effects of either 22 h food deprivation or NPY administration and NTX did not alter the rates of lever pressing.

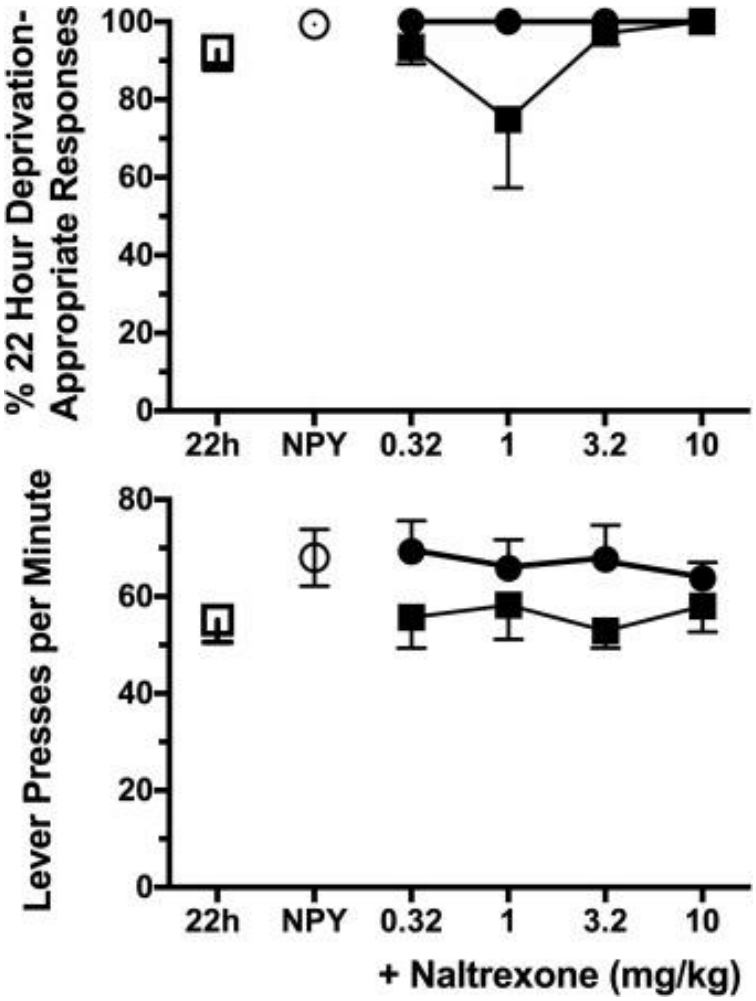


Figure 3.6: Effect of s.c. NTX on PVN NPY and 22 h deprivation discrimination responses. To examine the possible effects of NTX on the discriminative stimulus effects of 22 h deprivation, six rats were food deprived for 22 h and injected s.c. with saline (open squares) or NTX (1.0–10.0 mg/kg, filled squares). During other generalization six rats (3–6 per condition) were food deprived for 2 h and injected with NPY (1.0 μg, PVN) and 15 min later injected s.c. with saline (open circles) or NTX (1.0–10.0 mg/kg, filled circles). Generalization tests were conducted 30 min after the second injection and the discriminative stimulus effects and rates of lever pressing were assessed. Both 22 h deprivation and NPY administration engendered 22 h-appropriate deprivation responses. NTX did not alter the discriminative stimulus effects of either 22 h food deprivation or NPY administration and NTX did not alter the rates of lever pressing.

Administration of NTX at 0.32, 1 and 3.2 mg/kg s.c. prior to re-feeding caused a decrease in chow intake 2 h post-injection ($p < 0.001$) in animals deprived for 22 h (Fig. 3.7). The largest dose of the opioid antagonist (3.2 mg/kg) decreased chow intake already 1 h post-injection ($p < 0.01$). The same doses of NTX failed to affect chow intake in animals deprived for only 2 h, though baseline consumption levels were very low in controls (Fig. 3.7). NTX had no effect on food intake 24 h post-injection in both 2 h and 22 h deprivation scenarios.

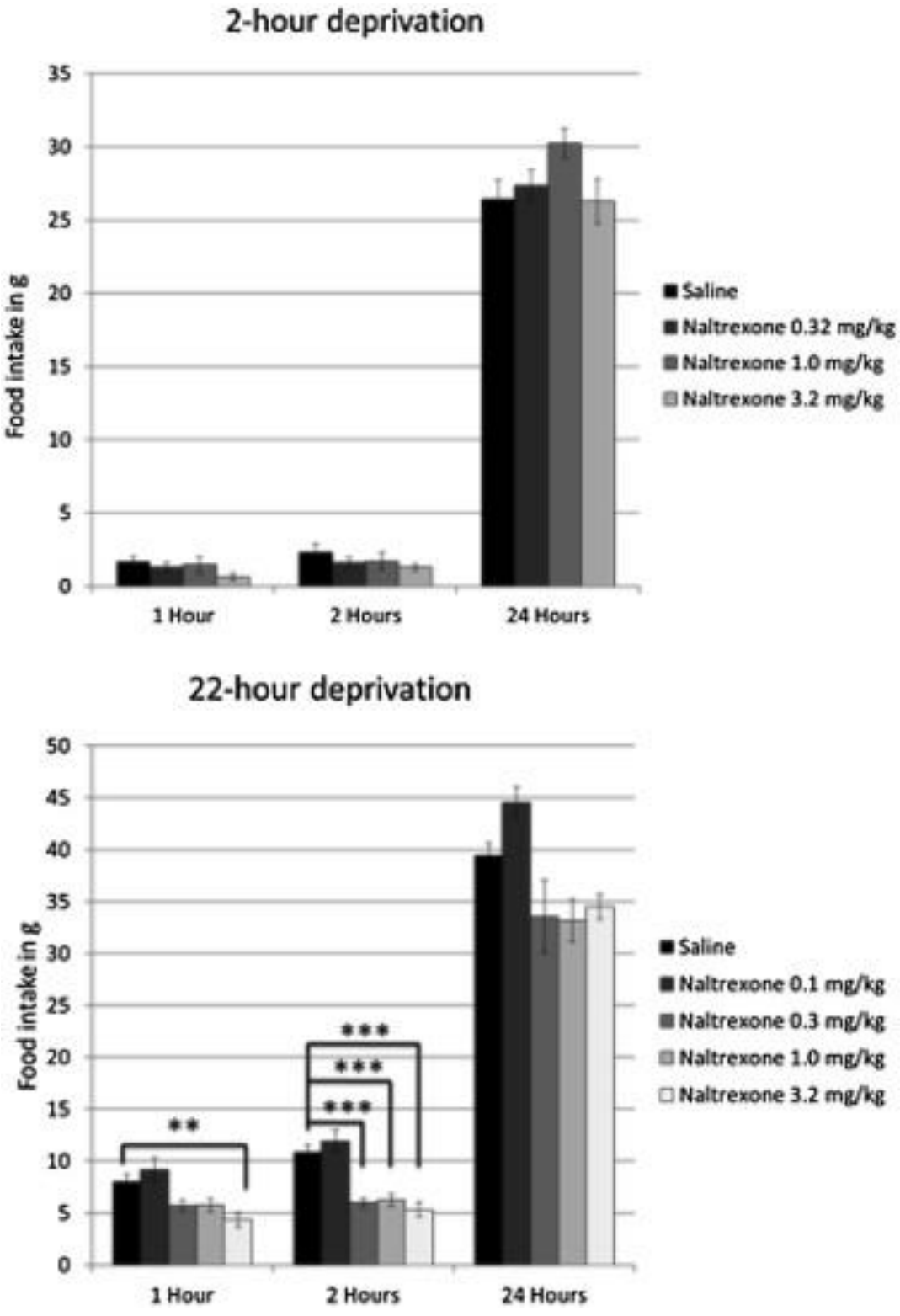


Figure 3.7: Effect of s.c. NTX on chow intake after 2 (top) or 22 h (bottom) of food deprivation. To examine the possible effects of NTX on food intake induced by 2 or 22 h food deprivation, 12 rats were food restricted according to the descriptions in the methods and Fig. 3.1. Following deprivation, animals were injected with either saline or NTX 0.032 mg/kg, 0.1 mg/kg, 0.32 mg/kg, 1 mg/kg and 3.2 mg/kg. Data are presented as means ± SEM. **$p < 0.01$, ***$p < 0.001$.

3.4 Discussion

In 1990, Corwin et al. described a new method to compare the interoceptive stimuli of anorexogenic drugs and satiation in rats [10]. In their experiments, these investigators trained rats to distinguish between 3 h and 22 h food deprivation and press levers in an operant chamber according to recent (3 h) or past (22 h) food access. They found that not only can rats learn to reliably discriminate between 3 h and 22 h food deprivation, but their perception of the length (or severity) of food deprivation can also be influenced by preloads of sweetened condensed milk or administration of CCK. The findings were in line with the hypothesis that satiety factors, including CCK, can produce effects similar to those of a food load or recent food ingestion [10]. The hunger discrimination method has been used in subsequent studies to demonstrate that, for example, sibutramine aids in weight loss by reducing hunger sensations in animals and that intra-PVN administration of NPY and ghrelin increase feeding by simulating hunger [11; 21].

In the present studies, I asked whether opioids are able to regulate hunger-induced feeding. I employed a similar method to train rats to discriminate between stimuli associated with 22 h food deprivation ("hunger") and 2 h food deprivation ("satiation"). After 2 h of food deprivation, opioid receptor agonists DAMGO, DSLET, orphanin FQ (site-specifically) and butorphanol (systemically) all failed to induce discriminative stimuli associated with deprivation. This is in contrast to NPY, which induced 22 h deprivation-like discriminative stimulus effects in animals deprived for 2 h. It should be emphasized that the effect of NPY shown here parallels the effects of another orexigen, ghrelin, in a similar operant experimental scenario [11; 22]. It should be noted that, while it cannot be completely excluded that by using even higher (thus, above 3.2 nmol) doses of intra-PVN opioid receptor ligands, one might be able to affect hunger discrimination, the doses selected for the current set of experiments likely represent a range relevant for

feeding stimulation. For example, 2 nmol PVN DAMGO has been reported to increase chow intake, as little as 3 nmol ICV N/OFQ stimulated chow consumption, and in the case of DSLET, its generalized ICV administration at 10 nmol -- what would be just one ½ log increment higher dose compared to the one used by us site-specifically -- produced hyperphagia (and as little as 100 pmol intra-PVN N/OFQ has been shown to affect water-electrolyte balance) [18; 23-25].

One of the key functional characteristics distinguishing NPY and ghrelin from opioids that may help conceptualize the differential response to opioid receptor ligands in the discrimination context, is the role in eating for hunger versus for palatability. In the vast majority of studies published to date, NPY and ghrelin have produced feeding in response to energy deficit; their release has also been associated with the states of hunger. For example, acute brain injections of NPY increase food intake and repeated administration of NPY into the hypothalamus lead to obesity [26]. PVN perfusions with NPY result in dramatic increases in food intake [27], and PVN NPY-treated rats (unlike DAMGO-injected animals) given a choice between calorie-dense and bland chow versus calorie-dilute and palatable sucrose solution, shift their preference towards chow [28]. Similarly to NPY, central ghrelin increases consumption of "bland" diets, such as a cornstarch mix [1; 29-32]. Plasma ghrelin levels are elevated after a fast and decrease upon glucose stomach infusions [33]. In humans, ghrelin levels mirror a curve of perceived hunger scores between a scheduled lunch and a freely requested dinner [34]. As replenishing lacking calories after a period of deprivation has a component of pleasure regardless of actual palatability of food, NPY and ghrelin have also been shown to affect feeding for reward [35-38], however, that aspect of their function in the central regulation of food intake appears to be much more limited.

Unlike NPY, opioid agonists increase feeding for pleasure and promote maintenance of food intake. This characteristic – to a lesser or greater degree - pertains to virtually all opioid receptor subtypes and, thus, to all of the ligands used here. For example, intra-Acb administration of a mu agonist DAMGO increases the intake of palatable high-fat and high-carbohydrate diets, yet neither nucleus accumbens shell (AcbSh) nor core (AcbC) DAMGO infusions motivate animals to acquire lever pressing for food in an operant chamber; in contrast, food-deprived animals learn this task rapidly [39]. A delta opioid ligand, DSLET, is particularly effective at increasing the intake of a cafeteria diet and enhancing dopaminergic tone in the striatum [40]. Butorphanol tartrate, which acts at both the mu and kappa receptors, promotes particularly vigorous food intake [41]. Finally, orphanin FQ, which acts via kappa-resembling opioid-like receptor 1, is involved in stress-induced binge-like palatable food consumption [42].

That opioid receptor agonists employed in our experiments did not produce 22 h deprivation-like discriminative stimulus effects, further substantiates the notion that peptides belonging to the opioid family do not produce a feeling of hunger. It is more likely that opioid-derived hyperphagia stems from the pleasure-derived component of eating behavior and/or meal prolongation. It should be mentioned that even in the case of opioid-induced consumption observed in the absence of palatable food, orexigenic effects may be related to the fact that calories (and, thus, high-calorie foods regardless of their attractiveness) have a capacity to engage the activity of the reward system [43].

In line with opioid receptor agonists' inability to affect operant responses after 2 h of deprivation, an antagonist of opioid receptors, NTX, failed to diminish 22 h deprivation discriminative stimulus effects. Importantly, NTX also failed to modify NPY-induced responses of animals subjected to 2 h deprivation. The finding of relative insensitivity of

the NPY-elicited operant response to NTX is particularly crucial taking into account the fact that opioid antagonists have been shown to diminish animals' ability to discriminate NPY from vehicle injections [44], as well as affect other NPY-driven parameters, including its anxiolytic effects [45]. It indicates that NPY serves as a stimulus molecule that facilitates hunger-driven eating behavior, whereas opioids may – at best – serve as modifying factors in shaping the magnitude and length of a consummatory response in animals that have already commenced a meal. This hypothesis is supported by earlier reports showing that brainstem-acting NTX weakens but does not completely abolish hyperphagia elicited by PVN NPY injections [46]. Furthermore, that opioid receptor activity is not critical for hunger responses is also reflected by the results of our current experiment in which – in the random schedule of 2 h:22 h food deprivation – lower effective doses of NTX (0.3 and 1 mg/kg) did not change food intake during the first hour post re-feeding, and their effect was visible only during the more advanced portion of a meal, i.e., after 2 h. Only a large 3.2 mg dose of NTX affected food intake during the first hour. Moreover, food intake after 2 h of deprivation, albeit minimal, was unaffected by the opioid receptor antagonist. Thus, for NTX to be effective, a vigorous consummatory activity should already be under way, whereas the feeling of hunger per se is not affected by opioid antagonists.

Our findings are particularly important from the standpoint of addressing a long-standing debate as to why opioid receptor ligands modify intake of foods that are not palatable. It has been shown beyond reasonable doubt that opioid agonists, including DAMGO, DSLET, orphanin FQ and butorphanol also increase consumption of bland diets, whereas antagonists, such as NTX and naloxone, reduce intake of standard laboratory chow [28; 47-54]. It has also been suggested that subpopulations of opioid expressing neurons located throughout a dispersed neuronal network of sites classically viewed as

'homeostatic' (e.g., hypothalamic PVN, arcuate, dorsomedial and ventromedial nuclei) and reward-related (e.g., the nucleus accumbens, ventral tegmental area and amygdala complex) mediate feeding effects of opioids (for review, see [55]). The current set of data strongly suggests that the orexigenic effects of opioids observed in the context of bland foods should not be attributed to a change in hunger levels, but rather be associated with energy balance-unrelated aspects of ingestive behavior, such as consummatory activity-derived reward, which modifies the magnitude of a feeding response. This stipulation is further strengthened by the fact that not only did PVN-injected delta-, mu- and NOP-specific receptor ligands -- thus molecules acting through a small, anatomically isolated population of the opioid receptor expressing neurons -- fail to affect the hunger discrimination (though in the future, it might be potentially useful to test intra-PVN kappa ligands as well), but so did the intra-PVN and systemically administered non-selective, opioid antagonist (NTX) and mixed agonist (butorphanol) of the opioid receptors. Systemic NTX antagonizes effects of mu-, kappa-, and delta-opioid agonists [56-59]. These data serve as yet another example of the complexity of the functional cross-link between intake for calories and intake for pleasure and the dynamic responsiveness of neural networks (sometimes, even seemingly conflicting effects within the same circuit) to energy- and palatability-driven challenges. In this context, in a recent paper, Wei and colleagues found that selective activation of hypothalamic POMC neurons inhibits energy intake in hungry animals, whereas activation of hypothalamic neurons that arise from POMC-expressing progenitors promotes hyperphagia [60].

In sum, I conclude that opioid receptor antagonism using NTX inhibits intake of palatable foods by regulating signals of pleasure and reward, and not by influencing the perception of hunger. Similarly, I found in the previous chapter that OT, while it targets the satiety aspect of feeding behaviour, does not produce its anorexigenic effects by influencing the perception of hunger. While these two peptides do not appear to influence the perception

of hunger, they indeed target distinct facets that drive consummatory behaviour and in doing so, may be able to be combined to address both satiety and reward-processing, and due to the interlinked nature of these two systems, may indeed produce a synergistic effect on reducing consumption when combined.

3.5 References

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

[2] Olszewski, P. K., & Levine, A. S. (2007). Central opioids and consumption of sweet tastants: when reward outweighs homeostasis. *Physiology & behavior, 91*(5), 506-512.

[3] Olszewski, P. K., Shaw, T. J., Grace, M. K., Höglund, C. E., Fredriksson, R., Schiöth, H. B., & Levine, A. S. (2009). Complexity of neural mechanisms underlying overconsumption of sugar in scheduled feeding: involvement of opioids, orexin, oxytocin and NPY. *Peptides, 30*(2), 226-233.

[4] Brady, L. S., Smith, M. A., Gold, P. W., & Herkenham, M. (1990). Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology, 52*(5), 441-447.

[5] Kim, E.-M., Welch, C. C., Grace, M. K., Billington, C. J., & Levine, A. S. (1996). Chronic food restriction and acute food deprivation decrease mRNA levels of opioid peptides in arcuate nucleus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 270*(5), R1019-R1024.

[6] Olszewski, P. K., Alsiö, J., Schiöth, H. B., & Levine, A. S. (2011). Opioids as facilitators of feeding: can any food be rewarding? *Physiology & behavior, 104*(1), 105-110.

[7] Pomonis, J. D., Billington, C. J., & Levine, A. S. (1996). Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. *Neuroreport, 8*(1), 369-371.

[8] Denis, R. G. P., Joly-Amado, A., Webber, E., Langlet, F., Schaeffer, M., Padilla, S. L., Cansell, C., Dehouck, B., Castel, J., Delbès, A. S., Martinez, S., Lacombe, A., Rouch, C., Kassis, N., Fehrentz, J. A., Martinez, J., Verdié, P., Hnasko, T. S., Palmiter, R. D., Krashes, M. J., Güler, A. D., Magnan, C., & Luquet, S. (2015). Palatability can drive feeding independent of AgRP neurons. *Cell Metabolism, 22*(4), 646-657.

[9] Erlanson-Albertsson, C. (2005). How palatable food disrupts appetite regulation. *Basic and Clinical Pharmacology and Toxicology, 97*(2), 61-73.

[10] Corwin, R. L., Woolverton, W. L., & Schuster, C. R. (1990). Effects of cholecystokinin, d-amphetamine and fenfluramine in rats trained to discriminate 3 from 22 hr food deprivation. *Journal of Pharmacology and Experimental Therapeutics, 253*(2), 720-728.

[11] Jewett, D. C., Lefever, T. W., Flashinski, D. P., Koffarnus, M. N., Cameron, C. R., Hehli, D. J., Grace, M. K., & Levine, A. S. (2006). Intraparaventricular neuropeptide Y and ghrelin induce learned behaviors that report food deprivation in rats. *NeuroReport, 17*(7), 733-737.

[12] Woods, S. C., Seeley, R. J., Porte Jr, D., & Schwartz, M. W. (1998). Signals that regulate food intake and energy homeostasis. *Science, 280*(5368), 1378-1383.

[13] Kim, E. M., Shi, Q., Olszewski, P. K., Grace, M. K., O'Hare, E., Billington, C. J., & Levine, A. S. (2001). Identification of central sites involved in butorphanol-induced feeding in rats. *Brain Research, 907*(1-2), 125-129.

[14] Council, N. R. (2010). *Guide for the care and use of laboratory animals*. National Academies Press.

[15] Sweet, D. C., Levine, A. S., Billington, C. J., & Kotz, C. M. (1999). Feeding response to central orexins. *Brain research, 821*(2), 535-538.

[16] Glass, M. J., Billington, C. J., & Levine, A. S. (2000). Naltrexone administered to central nucleus of amygdala or PVN: neural dissociation of diet and energy. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 279*(1), R86-R92.

[17] Paxinos, G., & Watson, C. (2006). *The rat brain in stereotaxic coordinates: hard cover edition*. Elsevier.

[18] Giraudo, S. Q., Billington, C. J., & Levine, A. S. (1998). Effects of the opioid antagonist naltrexone on feeding induced by DAMGO in the central nucleus of the amygdala and in the paraventricular nucleus in the rat. *Brain research, 782*(1-2), 18-23.

[19] Brown, D. R., & Holtzman, S. G. (1979). Suppression of deprivation-induced food and water intake in rats and mice by naloxone. *Pharmacology Biochemistry and Behavior, 11*(5), 567-573.

[20] Bodnar, R. J., Glass, M. J., Ragnauth, A., & Cooper, M. L. (1995). General, μ and κ opioid antagonists in the nucleus accumbens alter food intake under deprivation, glucoprivic and palatable conditions. *Brain research, 700*(1-2), 205-212.

[21] Jewett, D. C., Hahn, T. W., Smith, T. R., Fiksdal, B. L., Wiebelhaus, J. M., Dunbar, A. R., Filtz, C. R., Novinska, N. L., & Levine, A. S. (2009). Effects of sibutramine and rimonabant in rats trained to discriminate between 22-and 2-h food deprivation. *Psychopharmacology, 203*(2), 453-459.

[22] Davidson, T., Kanoski, S. E., Tracy, A. L., Walls, E. K., Clegg, D., & Benoit, S. C. (2005). The interoceptive cue properties of ghrelin generalize to cues produced by food deprivation. *Peptides, 26*(9), 1602-1610.

[23] Krowicki, Z. K., & Kapusta, D. R. (2006). Tonic nociceptinergic inputs to neurons in the hypothalamic paraventricular nucleus contribute to sympathetic vasomotor tone and water and electrolyte homeostasis in conscious rats. *Journal of Pharmacology and Experimental Therapeutics, 317*(1), 446-453.

[24] Olszewski, P. K., Billington, C. J., & Levine, A. S. (2000). Fos expression in feeding-related brain areas following intracerebroventricular administration of orphanin FQ in rats. *Brain research, 855*(1), 171-175.

[25] Levine, A. S., Grace, M., Billington, C. J., & Portoghese, P. S. (1990). Nor-binaltorphimine decreases deprivation and opioid-induced feeding. *Brain research, 534*(1-2), 60-64.

[26] Stanley, B. G., Kyrkouli, S. E., Lampert, S., & Leibowitz, S. F. (1986). Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides, 7*(6), 1189-1192.

[27] Paez, X., & Myers, R. (1991). Insatiable feeding evoked in rats by recurrent perfusion of neuropeptide Y in the hypothalamus. *Peptides, 12*(3), 609-616.

[28] Giraudo, S. Q., Grace, M. K., Billington, C. J., & Levine, A. S. (1999). Differential effects of neuropeptide Y and the μ-agonist DAMGO onpalatability'vs.energy'. *Brain research, 834*(1-2), 160-163.

[29] Wortley, K. E., Anderson, K. D., Garcia, K., Murray, J. D., Malinova, L., Liu, R., Moncrieffe, M., Thabet, K., Cox, H. J., & Yancopoulos, G. D. (2004). Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. *Proceedings of the National Academy of Sciences, 101*(21), 8227-8232.

[30] Bomberg, E. M., Grace, M. K., Wirth, M. M., Levine, A. S., & Olszewski, P. K. (2007). Central ghrelin induces feeding driven by energy needs not by reward. *Neuroreport, 18*(6), 591-595.

[31] Olszewski, P. K., Bomberg, E. M., Martell, A., Grace, M. K., & Levine, A. S. (2007). Intraventricular ghrelin activates oxytocin neurons: implications in feeding behavior. *Neuroreport, 18*(5), 499-503.

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

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

[34] Cummings, D. E., Frayo, R. S., Marmonier, C., Aubert, R., & Chapelot, D. (2004). Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. *American Journal of Physiology-Endocrinology and Metabolism, 287*(2), E297-E304.

[35] Perelló, M., & Zigman, J. M. (2012). The role of ghrelin in reward-based eating. *Biological psychiatry, 72*(5), 347-353.

[36] Naleid, A. M., Grace, M. K., Cummings, D. E., & Levine, A. S. (2005). Ghrelin induces feeding in the mesolimbic reward pathway between the ventral tegmental area and the nucleus accumbens. *Peptides, 26*(11), 2274-2279.

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

[38] Brown, C. M., Coscina, D. V., & Fletcher, P. J. (2000). The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens. *Peptides, 21*(8), 1279-1287.

[39] Hanlon, E. C., Baldo, B. A., Sadeghian, K., & Kelley, A. E. (2004). Increases in food intake or food-seeking behavior induced by GABAergic, opioid, or dopaminergic stimulation of the nucleus accumbens: is it hunger? *Psychopharmacology, 172*(3), 241-247.

[40] Robert, J., Orosco, M., Rough, C., Jacquot, C., & Cohen, Y. (1989). Effects of opiate agonists and an antagonist on food intake and brain neurotransmitters in normophagic and obese “cafeteria” rats. *Pharmacology Biochemistry and Behavior, 34*(3), 577-583.

[41] Olszewski, P. K., Klockars, O. A., Klockars, A., & Levine, A. S. (2016). Central oxytocin receptor stimulation attenuates the orexigenic effects of butorphanol tartrate. *Neuroreport, 27*(14), 1012-1017.

[42] Statnick, M. A., Chen, Y., Ansonoff, M., Witkin, J. M., Rorick-Kehn, L., Suter, T. M., Song, M., Hu, C., Lafuente, C., & Jiménez, A. (2016). A novel nociceptin receptor antagonist LY2940094 inhibits excessive feeding behavior in rodents: a possible mechanism for the treatment of binge eating disorder. *Journal of Pharmacology and Experimental Therapeutics, 356*(2), 493-502.

[43] Stice, E., Borcyk, A., & Menke, K. (Compiler) (2016). *Heritability of hyperresponsivity of brain reward regions to high-calorie food*: American Society for Nutrition.

[44] O'HARE, E., Cleary, J., Weldon, D., Pomonis, J., Billington, C. J., & Levine, A. S. (1998). Intrahypothalamic discriminative stimulus effects of neuropeptide Y. *Pharmacology Biochemistry and Behavior, 59*(2), 375-378.

[45] Britton, K. T., & Southerland, S. (2001). Naloxone blocks 'anxiolytic'effects of neuropeptide Y. *Peptides, 22*(4), 607-612.

[46] Kotz, C. M., Glass, M., Levine, A. S., & Billington, C. J. (2000). Regional effect of naltrexone in the nucleus of the solitary tract in blockade of NPY-induced feeding. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 278*(2), R499-R503.

[47] Gosnell, B. A., Levine, A. S., & Morley, J. E. (1986). The stimulation of food intake by selective agonists of mu, kappa and delta opioid receptors. *Life sciences, 38*(12), 1081-1088.

[48] Olszewski, P. K., Grace, M. K., Sanders, J. B., Billington, C. J., & Levine, A. S. (2002). Effect of nociceptin/orphanin FQ on food intake in rats that differ in diet preference. *Pharmacology Biochemistry and Behavior, 73*(3), 529-535.

[49] Stratford, T. R., Holahan, M. R., & Kelley, A. E. (1997). Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake. *Neuroreport, 8*(2), 423-426.

[50] Rudski, J., Billington, C., & Levine, A. (1994). Butorphanol increases food-reinforced operant responding in satiated rats. *Pharmacology Biochemistry and Behavior, 49*(4), 843-847.

[51] Rudski, J., Billington, C., & Levine, A. (1994). Naloxone's effects on operant responding depend upon level of deprivation. *Pharmacology Biochemistry and Behavior, 49*(2), 377-383.

[52] Morley, J. E., Parker, S., & Levine, A. S. (1985). Effect of butorphanol tartrate on food and water consumption in humans. *The American journal of clinical nutrition, 42*(6), 1175-1178.

[53] Spiegel, T. A., Stunkard, A. J., Shrager, E. E., O'Brien, C. P., Morrison, M. F., & Stellar, E. (1987). Effect of naltrexone on food intake, hunger, and satiety in obese men. *Physiology & behavior, 40*(2), 135-141.

[54] Yeomans, M. R., & Gray, R. W. (1997). Effects of naltrexone on food intake and changes in subjective appetite during eating: evidence for opioid involvement in the appetizer effect. *Physiology & behavior, 62*(1), 15-21.

[55] Bodnar, R. J. (2019). Endogenous opioid modulation of food intake and body weight: Implications for opioid influences upon motivation and addiction. *Peptides, 116*, 42-62.

[56] Valdez, G. R., Platt, D. M., Rowlett, J. K., Rüedi-Bettschen, D., & Spealman, R. D. (2007). κ Agonist-induced reinstatement of cocaine seeking in squirrel monkeys: a role for opioid and stress-related mechanisms. *Journal of Pharmacology and Experimental Therapeutics, 323*(2), 525-533.

[57] Grandison, L., & Guidotti, A. (1977). Stimulation of food intake by muscimol and beta endorphin. *Neuropharmacology, 16*(7-8), 533-536.

[58] Zhang, M., Gosnell, B. A., & Kelley, A. E. (1998). Intake of high-fat food is selectively enhanced by muopioid receptor stimulation within the nucleus accumbens. *Journal of Pharmacology and Experimental Therapeutics, 285*(2), 908-914.

[59] Belcheva, M. M., Barg, J., McHale, R., & Coscia, C. J. (1994). Naltrexone induces down-and upregulation of δ opioid receptors in rat brain regions. *Brain research bulletin, 35*(1), 69-72.

[60] Wei, Q., Krolewski, D. M., Moore, S., Kumar, V., Li, F., Martin, B., Tomer, R., Murphy, G. G., Deisseroth, K., & Watson, S. J. (2018). Uneven balance of power between hypothalamic peptidergic neurons in the control of feeding. *Proceedings of the National Academy of Sciences, 115*(40), E9489-E9498.

Chapter 4

Combined oxytocin and naltrexone at subthreshold doses acutely reduces food intake and induces a unique pattern of neuronal activation in feeding-related brain sites in adolescent rats

4.1 Abstract

In our search for neuroactive agents to curb excessive food consumption, those that affect more than one facet of feeding control are of particular interest, as they target a broader range of appetite regulating processes. In the previous chapters, I have investigated two such peptides and characterised which aspects of feeding control they target.

Interestingly, I found that while both OT and NTX have proven effective at inhibiting food intake in certain situations, they appear to target differing aspects of the motivation to consume, without influencing the feeling of hunger. These disparate systems are indeed intrinsically linked, as the opioidergic reward system can act to modulate the magnitude of a homeostatic feeding response by altering the neuronal sensitivity to satiety signals. I therefore hypothesized that by combining peptides that target these different aspects of feeding behavior, such a treatment might produce potentiating effects that can translate to robust therapeutic effects on curbing excessive consumption and seeking of palatable foods. I indeed found that while OT reduces deprivation-induced chow intake, and NTX reduces palatable food intake, that I could combine these drugs at subthreshold doses that have no effect alone, to produce a synergistic hypophagic effect on acute food intake. Furthermore, using c-Fos immunochemistry, I found robust changes in feeding-related

brain regions within the brain stem-hypothalamic network, as a result of this drug combination. I conclude that combining OT and NTX targets multiple facets of feeding behavior that are intrinsically linked, and so produces a synergistic effect on reducing acute food intake by modulating brain regions associated with both satiety and reward.

4.2 Introduction

Within the last two chapters, I have investigated the effects of various neuropeptides that are effective at modulating different aspects of feeding behavior. In the first chapter, I found that OT, while proven to be effective at reducing food intake in deprived and non-deprived rats, produces its anorexigenic effect without altering a feeling of hunger, as explored by an operant testing paradigm. I show that this peptide influences feeding for energy, and acts as a homeostatic mediator of early satiation. However, feeding behaviour can be influenced by multiple factors, and the drive for caloric intake is only one aspect of this. Pleasure and palatability also act to modify the magnitude of a feeding response, and can alter the sensitivity of an organism to the signals that drive the motivation to consume. The opioidergic neuronal systems are commonly attributed to playing a significant role in altering perception of pleasure and reward, and so, can powerfully influence consummatory behavior. In chapter two, I therefore examined whether opioid ligands perhaps also modulate the intake of palatable foods by altering the animal's perception of hunger, as tested with a similar operant paradigm. While some orexigenic peptides such as neuropeptide Y (NPY) indeed proved to be effective at altering a perception of hunger within this paradigm, opioid receptor ligands failed to modify hunger-associated responses, even at doses sufficient to alter deprivation-induced feeding. Non-selective opioid receptor antagonists, such as NTX, indeed significantly reduce consumption of palatable foods, but did not reduce behavioural responses associated with hunger, and so, I conclude, inhibit feeding for reward rather than in order to replenish lacking energy.

While both OT and NTX have proven effective at inhibiting food intake in certain situations, they appear to target differing aspects of the motivation to consume, without influencing the feeling of hunger. These disparate systems are indeed intrinsically linked,

as the opioidergic reward system can act to modulate the magnitude of a homeostatic feeding response by altering the neuronal sensitivity to satiety signals [32].

We therefore hypothesize that by combining peptides that target these different aspects of feeding behavior, such a treatment might produce potentiating effects that can translate to robust therapeutic effects on curbing excessive consumption and seeking of palatable foods.

The past years have brought a surge in interest in exploring anorexigenic properties of the nonapeptide OT. As shown in a wealth of laboratory animal experiments and supported by human trials, OT administration promotes early cessation of ingestive behavior, a reduction of meal size and, under some circumstances, a decrease in consumption of palatable tastants.

In rodent experiments, hypophagia has been observed after peripheral (intravenous, intraperitoneal (IP), subcutaneous, intranasal (IN)) and central (intracerebroventricular and site-specific) administration of the peptide. While IP OT does not diminish an animal's ability to perceive hunger, OT has been consistently shown to decrease meal duration and lower consumption of standard chow. These outcomes are consistent with the fact that increased activation of hypothalamic OT neurons and neurohypophyseal release of the hormone coincide with satiation and stomach distension. Data on OT and feeding for palatability are promising albeit somewhat less conclusive and they indicate that, even though OT tends to decrease consumption of rewarding tastants, the route and chronicity of OT administration, the macronutrient composition, flavor and energy density of a diet, and even social vs non-social setting of a meal, collectively influence the effectiveness of OT treatment. It should be noted that OT administration evokes changes in activity (assessed through c-Fos analysis) of broad brain circuits, from the hypothalamus to the brain stem to the limbic system, involved in various facets of feeding,

including eating induced by energy needs, homeostasis, reward and emotionality. In line with that, energy deprivation and palatable tastant exposure scenarios affect mRNA levels of OT and its receptor in the CNS.

Translational studies in humans have largely confirmed the basic research findings. A single supraphysiological dose of IN OT in obese and normal-weight subjects decreased energy consumption [1-5]. The link between OT and eating for pleasure has also been suggested, though specific aspects of hedonic eating affected by OT remain unclear with some studies reporting preferential effects in reducing consumption of sweet [3, 4, 6], fatty [1] or salty [4] foods. Functional magnetic resonance analyses (fMRI) have found that OT modifies responsiveness of hypothalamic [7, 8] and limbic [5, 7] sites in response to food images, suggesting that not only homeostatic, but also some reward circuits might indeed be modulated by OT.

In a recent human case study, Hsu and colleagues administered OT peripherally to reduce energy intake and body weight in an adolescent male with hypothalamic obesity and uncontrollable hedonic food-seeking caused by craniopharyngioma resection [2]. Considering a potentially suboptimal effectiveness of OT on eating for reward, the authors co-administered OT with NTX, a non-selective opioid receptor antagonist, thereby capitalizing on the primary effect of opioid receptor blockade on diminishing reward-driven consumption. They found that while OT alone reduced body weight and hyperphagia during the first 10 weeks, subsequent co-administration with NTX was a successful adjunctive therapy leading to further improvements in body weight and satiety parameters.

Taking into account the exciting findings of that case report and the lack of any basic research studies pertaining to effects of OT-NTX combination treatment on food intake, in the current set of experiments, I tested the effectiveness of co-administration of subthreshold doses of IP OT and NTX in adolescent male rats given a meal of (i) energy-

dense, palatable high-fat high-sugar (HFHS) chow, (ii) energy-dense, "bland" chow, or (iii) energy-dilute, palatable sucrose solution. By testing for a conditioned taste aversion (CTA), I assessed whether the anorexigenic effect of the combined drugs is independent from gastrointestinal sickness/malaise. I also employed c-Fos immunohistochemistry to determine whether an effective IP OT-NTX combination produces a unique neuronal activation response in select brain areas associated with feeding.

4.3 Materials and methods

4.3.1 Animals and injectants

Experimentally naïve adolescent male Sprague-Dawley rats (postnatal day 28) (average b. wt. 190 g) were housed individually in standard plastic cages with wire tops in a temperature-controlled (22◦C) animal facility with a 12:12 light:dark cycle (lights on at 07:00). Water and standard laboratory chow (Sharpes Stock Feed, Diet 86; 3.6kcal/g) were available ad libitum unless stated otherwise. Animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The University of Waikato Animal Ethics Committee approved all procedures described herein.

Animals were accustomed to receiving IP injections. NTX (Abcam, Cambridge, UK) and OT (Sigma, St. Louis, MO, USA) were dissolved in isotonic saline just prior to use.

4.3.2 Effect of OT and NTX on consumption

4.3.2.1 Effect of IP NTX and OT on palatable HFHS chow intake

Animals (n = 8–9/group) were injected IP with saline or NTX (1, 3 or 10 mg/kg) prior to presenting with 2h access to palatable high-fat high-sugar (HFHS; Research Diets #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) chow. Rodents avidly consume HFHS diet even in the absence of hunger. Food intake was measured 2h post-injection. Water was available at all times. I aimed to find the highest dose of NTX that

did not produce a significant effect alone on consumption, in order to combine with OT for a synergistic effect.

The above procedure was then repeated with OT. Animals (n = 8–9/group) were injected IP with saline or OT (0.1, 0.3 or 1mg/kg) prior to presenting with 2h access to palatable high-fat high-sugar chow. Food intake was measured 2h post-injection. Water was available at all times. I aimed to find the highest dose of OT that did not produce a significant effect alone on consumption, in order to combine with NTX for a potentiating effect.

Since 0.1mg/kg OT was found to be the optimal dose of OT that did not produce an effect on consumption alone, animals (n = 8–9/group) were injected IP with either saline, OT (0.1mg/kg), or OT (0.1mg/kg) combined with NTX (1, 3 or 10mg/kg). Injection was immediately followed by 2h access to palatable high-fat high-sugar chow. Food intake was measured 2h post-injection. Water was available at all times. I aimed to find the lowest effective combined dosage of NTX with OT, for significant, synergistic effect on reducing consumption.

4.3.2.2 Effect of IP NTX and OT on deprivation-induced regular chow intake

Animals (n = 6/group) were food-deprived overnight and injected IP with saline or NTX (0.3, 1 or 3mg/kg) prior to refeeding (food returned to cages at 10:00). Food intake was measured 2h post-injection. Water was available at all times. I aimed to find the highest dose of NTX that does not produce a significant effect alone on consumption, in order to combine with OT for a potentiating effect.

Since 0.1mg/kg OT was found to be the optimal dose of OT that did not produce an effect on consumption alone, animals (n = 6/group) were food-deprived overnight and injected IP with either saline, OT (0.1mg/kg), or OT (0.1mg/kg) combined with NTX (0.3, 1 or 3mg/kg) prior to refeeding (food returned to cages at 10:00). Food intake was measured

2 h post-injection. Water was available at all times. I aimed to find the lowest effective combined dosage of NTX with OT, for a significant, synergistic effect on consumption.

4.3.2.3 Effect of IP NTX and OT on sucrose intake

Animals (n = 6/group) were injected IP with saline or NTX (0.1, 0.3 & 1mg/kg) prior to presenting with 2h access to 10% sucrose solution. Sucrose solution intake was measured 2h post-injection. I aimed to find the highest dose of NTX that does not produce a significant effect alone on palatable-solution intake, in order to combine with OT for a synergistic effect.

Since 0.1mg/kg OT was found to be the optimal dose of OT that did not produce an effect on food intake alone, animals (n = 9/group) were injected IP with either saline, OT (0.1mg/kg), or OT (0.1mg/kg) combined with NTX (0.1, 0.3 or 1 mg/kg) prior to presenting with 2h access to 10% sucrose solution. Sucrose solution intake was measured 2h post-injection. I aimed to find the lowest effective combined dose of NTX with OT, for a significant, synergistic effect on palatable-solution intake.

4.3.2.4 Conditioned taste aversion test

To investigate whether the reduced consumption effect was due to an aversive effect of the drugs, animals (n=6/group) were initially deprived of water overnight before being given access to a novel 0.1% saccharin solution for 1hr, during which time, regular chow was removed. Immediately following this, animals were injected IP with either lithium chloride (LiCl) as a positive control for CTA (Sigma; 0.56 mEq/kg body weight or 23.9mg/kg; isotonic solution), saline, OT (0.1mg/kg), NTX (3mg/kg), or OT/NTX combined. After 2 days without deprivation, animals were then deprived of water overnight, and on the following day, regular chow was removed and animals were presented with a two-bottle choice test, with 2h access to both water and 0.1% saccharin solution. Bottles of saccharin and water were weighed before and after the two-bottle choice test to determine the amount of liquids consumed, and the percentage of saccharin

intake in the cumulative (saccharin and water) consumption was established. Results were compared to LiCl group for evidence of CTA.

All descriptive data are presented as means ± the standard error of the mean (SEM). One-way analysis of variance (ANOVA) (GraphPad Prism) was used to assess the effects of OT and NTX in reducing food intake and compared against control group. Tukey HSD post hoc tests were performed following significant ANOVA values to determine differences among conditions. Significance was set at $p \leq 0.05$.

4.3.3 Establishing OT-NTX-induced c-Fos immunoreactivity (IR) in feeding-related brain sites in adolescent rats

Age-matched male Sprague Dawley rats were divided into four groups (n = 9 per group) and each received an IP injection of either isotonic saline, 0.1 mg/kg OT, 3mg/kg NTX or 0.1mg/kg OT/ 3mg/kg NTX. An hour after drug administration, animals were deeply anesthetized with urethane (35% dissolved in 0.9% saline, IP), and perfused through the aorta with 50ml of saline followed by 500ml of 4% paraformaldehyde in 0.1 phosphate buffer (pH 7.4). Brains were excised and post-fixed overnight in the same fixative at 4◦C. 60 μm-thick coronal sections were cut with a vibratome (Leica, Germany) and later processed as free-floating sections for standard single antigen immunostaining of c-Fos. Sections were rinsed in 50 nM TBS (pH 7.4–7.6), and then pretreated for 10min in 3% H2O2, 10% methanol (diluted in TBS). After rinsing in TBS they were incubated overnight at 4◦C in the primary rabbit-anti-Fos antibody (diluted 1:3000; Synaptic Systems, Australia) washed in TBS, and subsequently incubated for 1 h at room temperature in the secondary goat-anti-rabbit antibody (1:400; Vector Laboratories). Following four washes in TBS, sections were incubated for 1 h with the avidin–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the tissue was visualized with 0.05% diaminobenzidine (DAB), 0.01%

H2O2 and 0.3% nickel sulfate (12-min incubation). Sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried, dehydrated in ascending concentrations of ethanol, soaked in xylene (Merck KGaA, Germany) and embedded in Entellan (Merck KGaA, Germany). The number of Fos-positive nuclei per 1 mm2 was counted bilaterally for each neuroanatomical region of interest using ImageJ Software, with boundaries defined according to the Paxinos and Watson brain atlas, on 2–4 sections per animal. Images provided by a CCD camera attached to a Nikon Eclipse 400 microscope were analyzed using Nikon NIS Elements image software. The following areas were analysed (in the parentheses, anterior-posterior ranges of bregma levels of sections used to analyze each site are shown): AcbC—nucleus accumbens core (1.28–0.96); AcbS—nucleus accumbens shell (1.28–0.96); AP—area postrema (−13.92 to −14.16); ARC—arcuate nucleus (−2.16 to −2.52); BLA—basolateral amygdala (−2.64 to −2.92); CEA—central nucleus of the amygdala (−2.64 to −2.92); DMH—dorsomedial nucleus of the hypothalamus (−3.00 to −3.24); DMNV—dorsal motor nucleus of the vagus (−13.76 to −14.16); NTS—nucleus of the solitary tract (−13.76 to −14.16); PVN—paraventricular nucleus of the hypothalamus (−1.56 to−1.92); SON—supraoptic nucleus (−0.96 to −1.2); VMH—ventromedial nucleus (−3.00 to −3.24); LH-lateral hypothalamic nucleus (-1.20 to -1.44); BNST-bed nucleus of the stria terminalis (-0.24 to -0.48).

Densities of Fos-positive nuclear profiles (per 1 mm^2) were averaged per individual, and then per group. Group means were compared using a one-way ANOVA (GraphPad Prism) with Tukey's post-hoc test. Values were considered significantly different for $p \leq 0.05$.

4.4 Results

IP NTX at 10 mg/kg was effective at decreasing episodic HFHS chow intake in non-deprived rats, whereas 3 and 1 mg/kg doses did not produce any change (Fig. 4.1 A). Only the highest, 1mg/kg, dose of IP OT reduced the amount of HFHS diet consumed during

the 2-h meal (Fig. 4.1B). When animals were injected with the subthreshold 0.1 mg/kg OT in combination with NTX, both 3 and 10 mg/kg NTX combined with OT generated hypophagia in the HFHS diet meal (Fig. 4.1C).

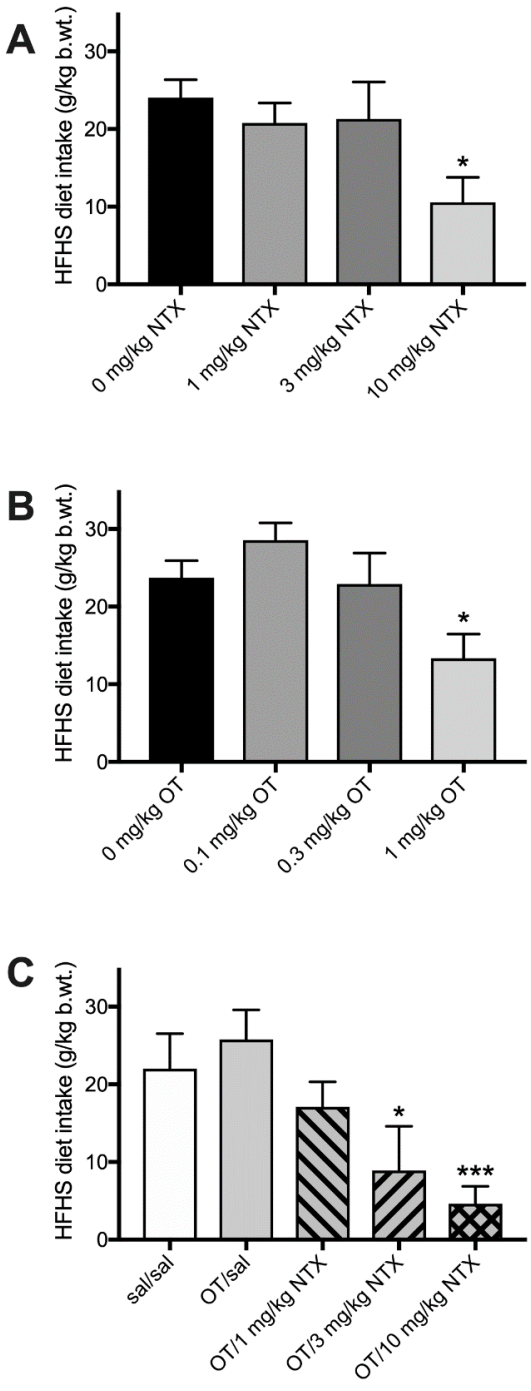


Figure 4.1: Effect of IP NTX (A), OT (B), and OT–NTX combination (C) on 2-h HFHS chow intake in non-deprived rats. Doses are shown in mg/kg body weight. In (C) 0.1mg/kg OT and 1, 3 and 10 mg/kg NTX were administered. Isotonic saline served as the vehicle. *$p < 0.05$; ***$p<0.001$.

The CTA test revealed that, unlike IP LiCl, the anorexigenic 0.1 mg/kg OT – 3 mg/kg NTX drug combination did not produce an aversion to the 0.1% saccharin solution (Fig. 4.2).

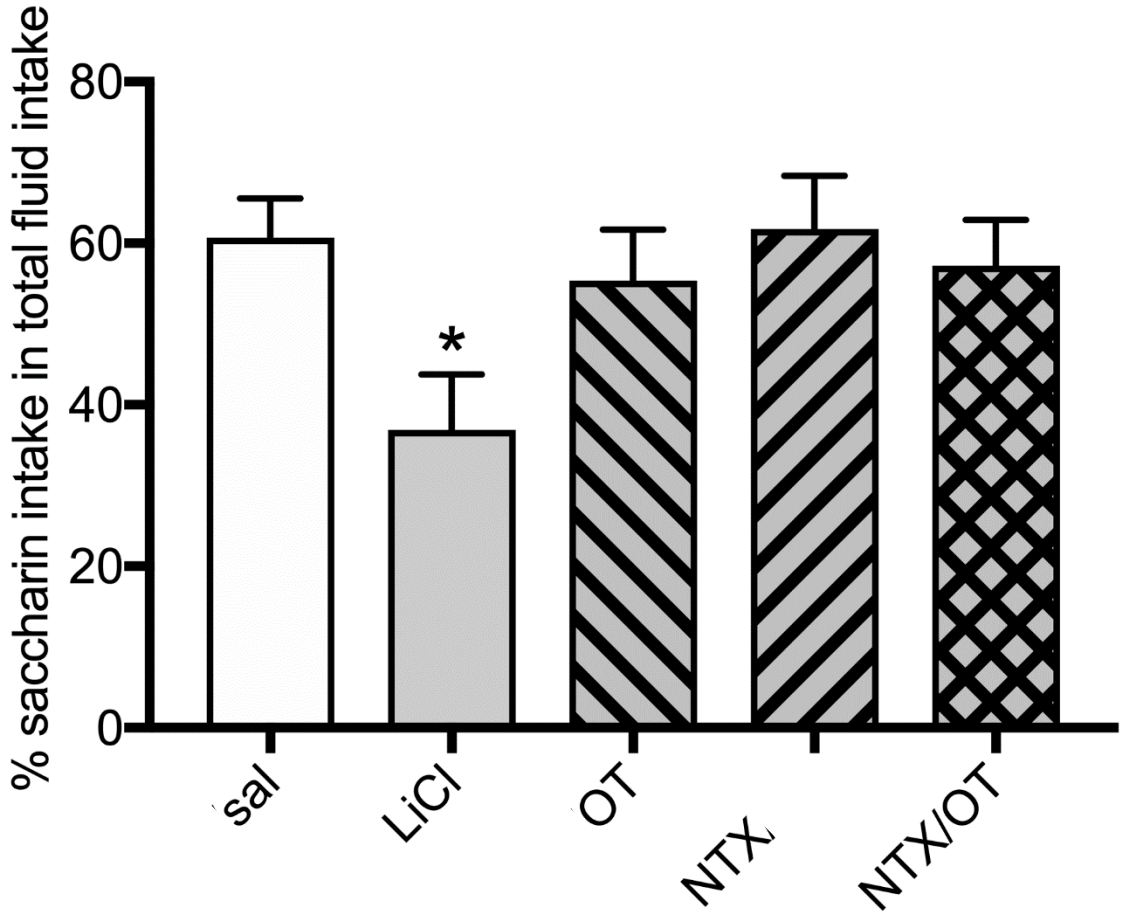


Figure 4.2. Conditioned taste aversion test. Effect of OT (0.1mg/kg), NTX (0.1mg/kg), OT/NTX combination and LiCl (23.9mg/kg) on preference for 0.1% saccharin solution. LiCl following ingestion of 0.1% saccharin solution by rats significantly affected their later preference for the saccharin solution compared with controls. *$p < 0.05$. Administration of OT, NTX or OT/NTX combined did not alter saccharin consumption compared with controls.

IP NTX alone was ineffective at decreasing episodic deprivation-induced intake of standard chow at all doses, up to 3mg/kg (Fig. 4.3A). Similarly, 0.1mg/kg OT did not produce any change in consumption. However, when this subthreshold dose of 0.1mg/kg OT was administered in combination with NTX, 3mg/kg NTX combined with OT

generated hypophagia (Fig. 4.3B). Notably, a trend toward a hypophagic response was observed at lower doses of NTX (0.3 and 1mg/kg) when combined with OT, however did not reach significance (P=0.0681 and P=0.0678 respectively).

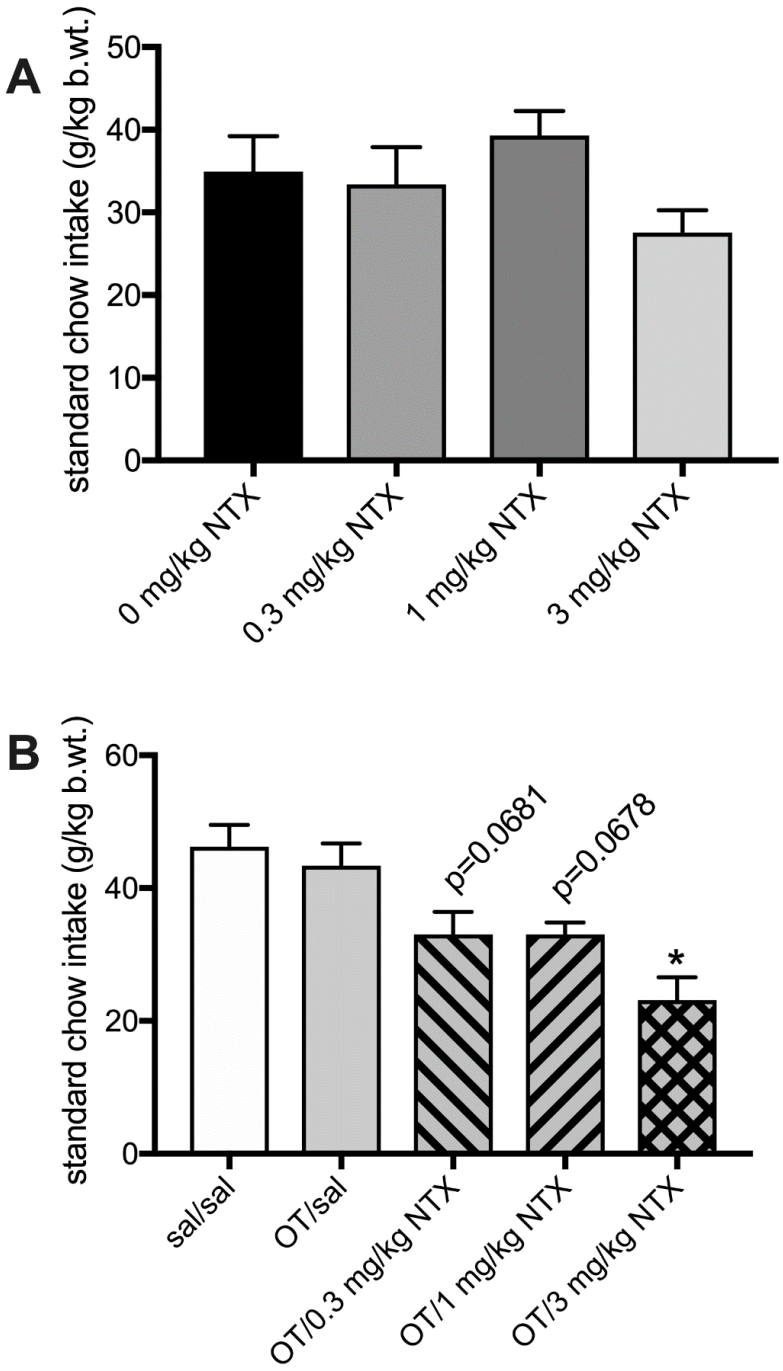


Figure 4.3. Effect of IP NTX (A) and OT/saline and OT-NTX combination (B) on 2-h deprivation-induced chow intake in rats. Doses are shown in mg/kg body weight. In (B) 0.1mg/kg OT, and 0.3,1 and 3 mg/kg NTX were administered. Isotonic saline served as the vehicle. *$p < 0.05$.

NTX at 0.3 and 1mg/kg was effective at decreasing episodic sucrose solution intake in non-deprived rats, whereas 0.1mg/kg did not produce any change (Fig. 4.4A). While 0.1mg/kg OT did not produce any change in sucrose intake, when this subthreshold dose of OT was administered in combination with NTX, doses as low as 0.1mg/kg NTX combined with OT generated hypophagia (Fig. 4.4B).

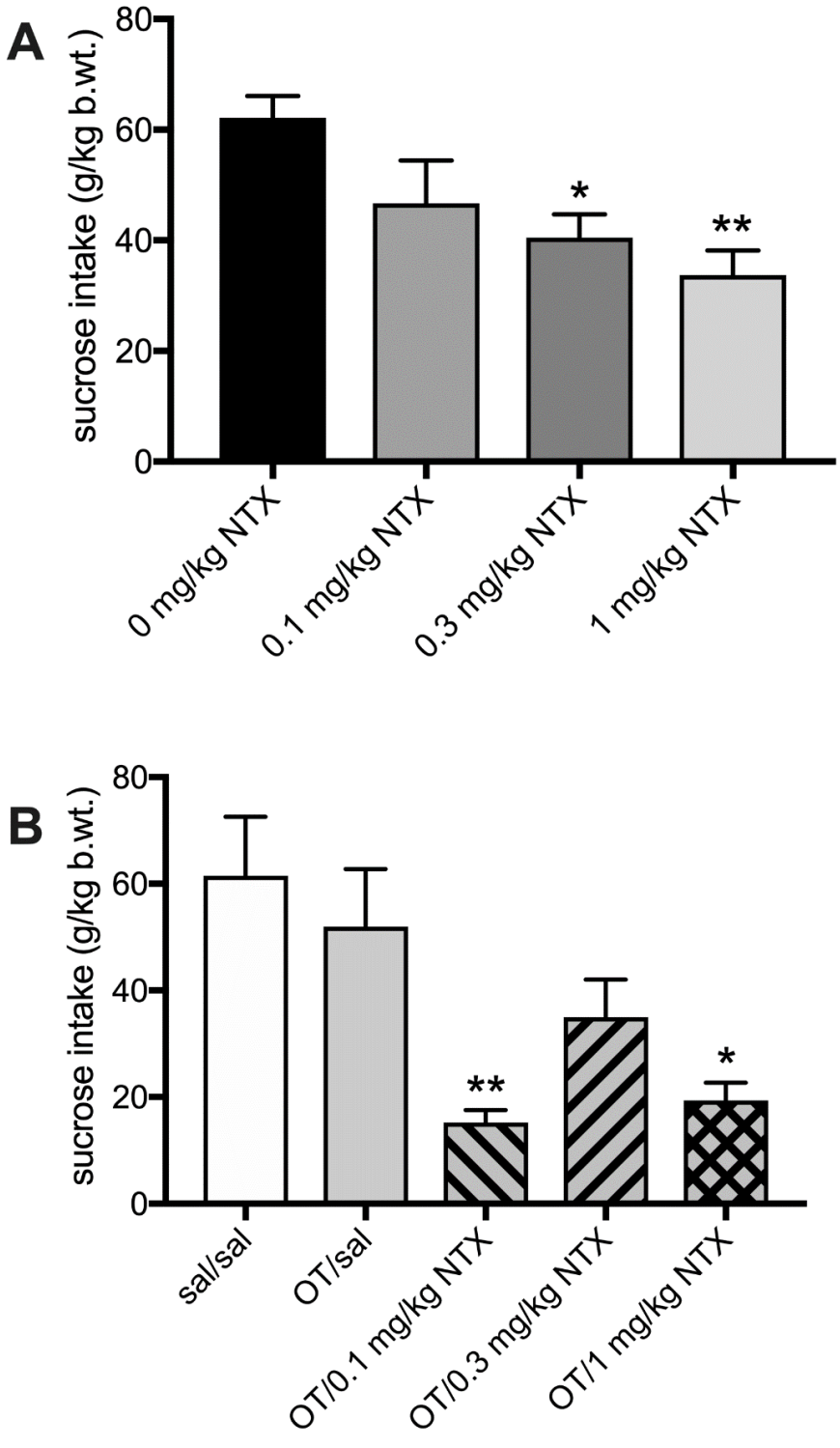


Figure 4.4. Effect of IP NTX (A) and OT/saline and OT-NTX combination (B) on 2-h sucrose solution intake in non-deprived rats. Doses are shown in mg/kg body weight. In (B) 0.1mg/kg OT and 0.1, 0.3 and 1 mg/kg NTX were administered. Isotonic saline served as the vehicle. *$p < 0.05$; **$p<0.01$.

IP combination of OT and NTX at doses found to induce acute hypophagia in all episodic meal scenarios (OT 0.1mg/kg and NTX 3mg/kg) produced robust changes in neuronal activation as measured by cFos IR, particularly in regions associated with satiation. Notably, activation of DMH and CeA was significantly increased by the drug combination, when compared to vehicle and OT or NTX alone (Fig. 4.5). Inversely, activation of ARC was significantly reduced by the drug combination (Fig. 4.5), when compared to vehicle and OT or NTX alone.

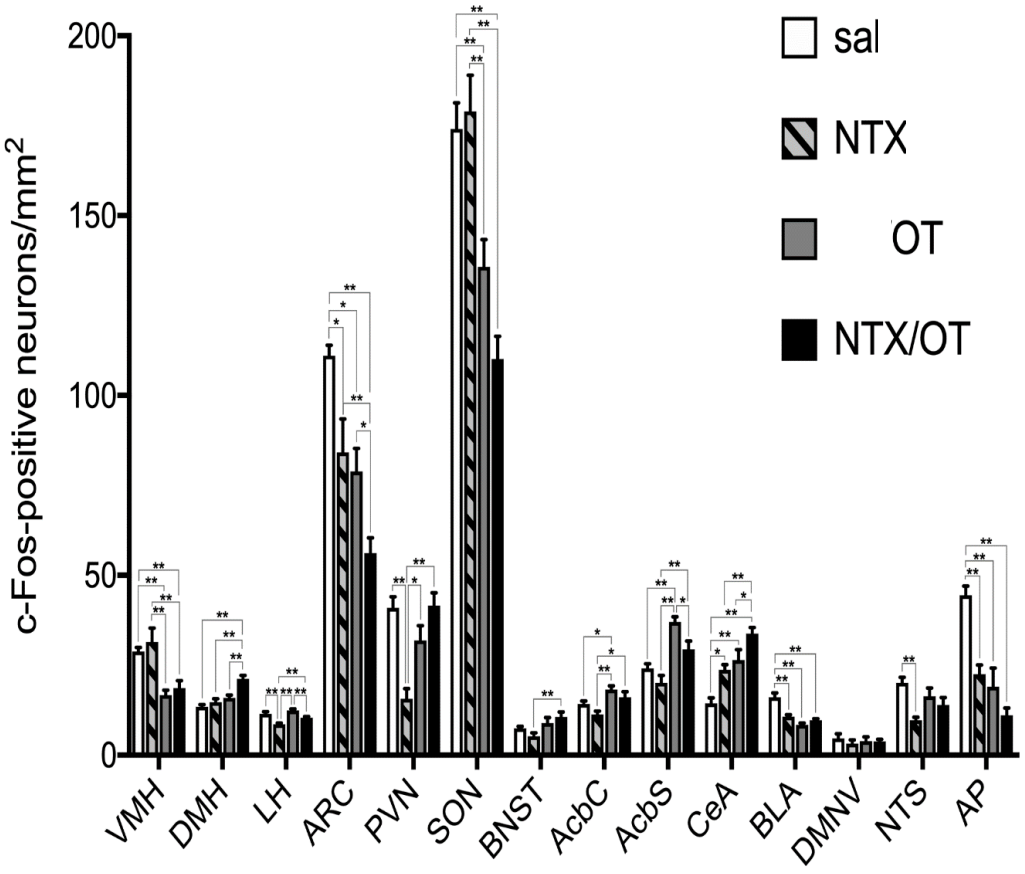


Figure 4.5: Effect of IP OT-NTX combination on cFos IR activation in adolescent rats (A). Doses of 0.1mg/kg OT and 3mg/kg NTX were administered. Isotonic saline served as the vehicle. Panel (B) presents photomicrographs depicting sites that showed a significant difference in c-Fos levels. Densities of Fos-positive nuclear profiles (per 1 mm^2 of a site) were averaged per individual, and then per group. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; AP, area postrema; ARC, arcuate nucleus; BLA, basolateral amygdala; CEA, central nucleus of the amygdala; DMH, dorsomedial nucleus of the hypothalamus; DMNV, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus; VMH, ventromedial nucleus; LH, lateral hypothalamic nucleus; BNST, bed nuclei of the stria terminalis. *$p < 0.05$; **$p<0.01$.

4.5 Discussion

A case-study of an adolescent male suffering from craniopharyngioma described how this condition led to uncontrollable overeating of palatable foods and excessive increases in body-weight [2]. Treatment by co-administration of OT and NTX was trialed, and the authors reported a significant decrease in the seeking and intake of palatable foods, and in turn, in the subject's body weight. That study may outline a synergistic effect of OT and NTX when combined. Therefore, in the current study, I utilized adolescent male rats to investigate the effects of OT and NTX directly administered intraperitoneally, and observed changes in food intake and neuronal activation.

Initial studies showed that the anorexigenic peptide OT regulates gastric motility, responds to stomach distention and to elevated osmolality, and blocks consumption of toxic foods. Most recently, OT has been proposed to act as a mediator of general and carbohydrate-specific satiety and regulator of body weight. It is now understood that OT plays a role as a homeostatic inhibitor of consumption, capable of mitigating multiple aspects of ingestive behavior and energy metabolism [9].

We have showed in a previous chapter that OT does not diminish a feeling of hunger before a start of a meal. Instead, OT's anorexigenic properties are manifested once consumption has already begun, which is—at least to some extent—driven by changes in brain responsiveness to OT treatment in the hungry vs. fed state. OT should therefore be viewed as a mediator of early satiation rather than as a molecule that diminishes perceived hunger [10].

In the present set of experiments, I first investigated the effect of IP NTX administration on acute intake of HFHS palatable chow. The data showed that only the highest of the three doses of NTX (10mg/kg) was effective in reducing HFHS intake 2h after injection. NTX alone has been previously reported to produce only a relatively limited effect on consumption. NTX has been found not to reduce feeding for energy, but only to reduce

consumption of palatable diets, in line with opioid involvement in reward [11]. NTX reduces consumption of novel HFHS diets and sucrose solutions, and inhibits development of preference for high-sucrose diets, yet is ineffective in altering consumption of standard chow when animals are not deprived [12]. This is consistent with the finding shown in the previous chapter that opioid-receptor antagonists were effective at reducing deprivation-induced consumption, while not reducing perceived hunger.

This study was then repeated with the IP administration of OT, which only produced a reduction in HFHS chow intake by the highest dose of OT (1mg/kg). This dose of OT has consistently been reported to produce hypophagia in adult rodent models [10]. Interestingly, when subthreshold doses of OT and NTX were combined, i.e. doses that did not produce an effect alone, this resulted in a significant reduction in HFHS intake. This experiment showed that a dose of 0.1mg/kg OT alone, while insufficient to reduce HFHS intake, when combined with 3mg/kg NTX, also found to be ineffective at reducing HFHS intake alone, produced significant reductions in palatable HFHS intake. This finding is remarkable as neither of these doses were effective at reducing food intake alone, yet when combined produce a synergistic hypophagic effect.

This phenomenon of synergy between OT and NTX was then replicated in other episodic feeding scenarios, firstly with deprivation-induced standard laboratory chow intake. Again, NTX was ineffective at inducing acute decrease in deprivation-induced chow intake at all doses tested. Similarly, OT (0.1mg/kg) alone was ineffective at reducing food intake, however, when combined with a subthreshold dose of NTX (3mg/kg), induced significant acute hypophagia 2h after injection. Notably, in this deprivation-induced standard chow intake paradigm, combinations of OT (0.1mg/kg) with NTX at 0.3 and 1mg/kg also generated a trend toward reduced consumption.

By establishing that these hypophagic doses of OT-NTX did not produce a conditioned taste aversion, we were able to confirm that the hypophagic effect of this drug combination was not the result of malaise or an aversive association. This is important considering that previous reports have shown that opioid receptor blockade with naloxone can potentiate the aversive effects of LiCl treatment, though one should note that in order for this to occur, the doses of naloxone need to high [14]. Similarly, opioid receptor agonism has been found to completely block the acquisition of conditioned taste aversions in some paradigms [15]. Therefore, as the aversive properties of LiCl and other malaise-inducing agents, are mediated by both the opioid system, and OT and vasopressin cells in the PVN and SON [15], the finding that the hypophagic dose of this drug combination did not produce an aversion similar to that of LiCl is encouraging.

Furthermore, using a non-caloric palatable tastant feeding paradigm, such as episodic intake of sucrose solution, similar findings were observed. Both NTX 0.1mg/kg and OT 0.1mg/kg alone were insufficient to induce acute hypophagia, yet when these subthreshold doses were combined, they produced a significant reduction in sucrose intake. Of interest is the observation that in the sucrose solution paradigm, doses of 0.3 and 1mg/kg of NTX alone, were indeed sufficient to significantly reduce sucrose intake. In contrast, these same doses of 0.3 and 1mg/kg NTX did not produce this hypophagic effect toward more calorie-dense tastants, such as HFHS chow in sated animals and standard chow in deprived animals. This outcome is in concert with the notion that NTX, being a non-selective opioid receptor antagonist, specifically influences the reward aspect of feeding, or eating for pleasure, rather than energy. It therefore explains how a lower dose of the opioid receptor antagonist was necessary to influence feeding for reward in the palatable sucrose solution experiment, than was required to induce hypophagia in paradigms involving caloric consumption.

Following the completion of food intake studies, the subsequent experiment focused on identifying neuronal activation patterns induced by (OT 0.1mg/kg and NTX 3mg/kg) treatment. This aimed to elucidate cFos changes in neuronal circuitry relevant to the observed alterations in feeding behavior. I analyzed expression in feeding and reward-related brain regions for significant changes in neuronal activation, when compared to saline vehicle, and each drug alone. The combination of OT and NTX was found to induce some robust changes in neuronal activity, particularly in regions associated with satiety. Of particular interest was a significant increase in cFos IR in the dorsomedial hypothalamic (DMH) nucleus. Activity in this region has previously been associated with satiation, or the lack of hunger. Renner et al. reported that satiation, by refeeding fasted rats, evoked a significant increase in cFos expression in the DMH, and that this activation was elicited by satiation, rather than craving for food; in fact food presentation without consumption did not induce increased Fos activation in the DMH [16].

One of the commonly accepted theories of appetite control involves gut-derived hormones that signal hunger and satiety to brain circuitries regulating homeostatic and hedonic aspects of feeding [17, 18]. These hormones and signals act via anorexigenic and orexigenic pathways originating in the hypothalamic arcuate nucleus (ARC) that hosts the proopiomelanocortin (POMC) and neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons, which in turn relay signaling to the hypothalamic paraventricular nucleus (PVN) [19-21]. The ARC and PVN pathways also converge in the lateral parabrachial nucleus, which sends anorexigenic calcitonin gene-related peptide (CGRP) projections to the central nucleus of the amygdala (CeA) [22]. The CeA, among other forebrain areas, has been shown to integrate homeostatic and motivational aspects of feeding, and to receive sensory input from the brainstem [23-26].

In line with this, after treatment with OT and NTX combined, I observed a significant decrease in cFos activation in the ARC. Interestingly, ARC neurons have been shown to

be activated during fasting, via the inputs stimulated through ghrelin input derived from the empty stomach [27]. Inversely, PYY is released when the stomach is full, and in turn, it inhibits ARC activation. This corresponds with the finding of this study that combination of OT and NTX significantly reduced both consumption and activity of the ARC, previously associated with positive energy-balance states.

Similarly, increases in cFos activation in the CeA have previously been associated with satiety, and shown to correlate with reduced food intake [26]. This parallels the finding from this study that the combination of OT and NTX significantly increases cFos activation of the CeA, and effectively reduces consumption. The presence of both OT and opioid receptors in the DMH and the CeA may contribute to the effect of this drug combination on food intake [28-30].

Taken together, these results imply that OT and NTX combination reduces food intake synergistically, possibly by combining the properties of each drug component to reduce both homeostatic and hedonic pressures for consumption. OT may have promoted satiety through activating the DMH, and reducing of activity of the ARC, whereas NTX affected the reward circuitry component i.e. the CeA, acting to reduce hedonic pressures for consumption. Moreover, since these systems are intrinsically linked, the drug combination not only produced additive effects, but also appear to have acted synergistically to produce a combined effect greater than that of its components, and therefore is effective in reducing consumption and food-seeking by targeting multiple aspects of the motivation to eat.

It should be noted, adolescents may be more responsive to anorexigenic signals than adults. Rigamonti et al. found that obese adolescents produced an increase in endogenous anorexigenic peptides GLP1 and PYY in response to slow-rate feeding, while obese adult subjects did not. However, postprandial responses of insulin and triglycerides were higher in obese adults than in obese adolescents [31]. This suggests that adolescents may be

more responsive to satiety cues than adults, and therefore anorexigenic treatments may be more effective in adolescents.

In conclusion, I find that the combination of OT and NTX administered I.P. in adolescent rats at subthreshold doses produces significant reduction in acute intake of a range of tastants. Changes in neuronal activation found in brain regions associated with satiety and reward processing may underlie the hypophagic effects of this drug combination.

4.6 References

1. Lawson, E.A., et al., *Oxytocin reduces caloric intake in men.* Obesity, 2015. **23**(5): p. 950-956.

2. Hsu, E.A., et al., *Oxytocin and naltrexone successfully treat hypothalamic obesity in a boy post-craniopharyngioma resection.* The Journal of Clinical Endocrinology & Metabolism, 2018. **103**(2): p. 370-375.

3. Thienel, M., et al., *Oxytocin's inhibitory effect on food intake is stronger in obese than normal-weight men.* International journal of obesity, 2016. **40**(11): p. 1707-1714.

4. Burmester, V., S. Higgs, and P. Terry, *Rapid-onset anorectic effects of intranasal oxytocin in young men.* Appetite, 2018. **130**: p. 104-109.

5. Spetter, M.S., et al., *Oxytocin curbs calorie intake via food-specific increases in the activity of brain areas that process reward and establish cognitive control.* Scientific reports, 2018. **8**(1): p. 1-11.

6. Ott, V., et al., *Oxytocin reduces reward-driven food intake in humans.* Diabetes, 2013. **62**(10): p. 3418-3425.

7. Plessow, F., et al., *Effects of intranasal oxytocin on the blood oxygenation level-dependent signal in food motivation and cognitive control pathways in overweight and obese men.* Neuropsychopharmacology, 2018. **43**(3): p. 638-645.

8. van der Klaauw, A.A., et al., *Oxytocin administration suppresses hypothalamic activation in response to visual food cues.* Scientific reports, 2017. **7**(1): p. 1-5.

9. Olszewski, P.K., et al., *Oxytocin as feeding inhibitor: maintaining homeostasis in consummatory behavior.* Pharmacology Biochemistry and Behavior, 2010. **97**(1): p. 47-54.

10. Head, M.A., et al., *Effect of oxytocin on hunger discrimination.* Frontiers in Endocrinology, 2019. **10**: p. 297.

11. Apfelbaum, M. and A. Mandenoff, *Naltrexone suppresses hyperphagia induced in the rat by a highly palatable diet.* Pharmacology Biochemistry and Behavior, 1981. **15**(1): p. 89-91.

12. Levine, A.S., et al., *Naltrexone infusion inhibits the development of preference for a high-sucrose diet.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2002. **283**(5): p. R1149-R1154.

13. Jewett, D.C., et al., *Effects of opioid receptor ligands in rats trained to discriminate 22 from 2 hours of food deprivation suggest a lack of opioid involvement in eating for hunger.* Behavioural Brain Research, 2020. **380**: p. 112369.

14. Flanagan, L.M., J.G. Verbalis, and E.M. Strieker, *Naloxone potentiation of effects of cholecystokinin and lithium chloride on oxytocin secretion, gastric motility and feeding.* Neuroendocrinology, 1988. **48**(6): p. 668-673.

15. Olszewski, P.K., et al., *Opioids affect acquisition of LiCl-induced conditioned taste aversion: Involvement of OT and VP systems.* American Journal of Physiology - Regulatory Integrative and Comparative Physiology, 2000. **279**(4 48-4): p. R1504-R1511.

16. Renner, E., et al., *Activation of neurons in the hypothalamic dorsomedial nucleus via hypothalamic projections of the nucleus of the solitary tract following refeeding of fasted rats.* European Journal of Neuroscience, 2010. **31**(2): p. 302-314.

17. Berthoud, H.-R., *Metabolic and hedonic drives in the neural control of appetite: who is the boss?* Current opinion in neurobiology, 2011. **21**(6): p. 888-896.

18. Murphy, K.G. and S.R. Bloom, *Gut hormones and the regulation of energy homeostasis.* Nature, 2006. **444**(7121): p. 854-859.

19. Cowley, M.A., et al., *Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat.* Neuron, 1999. **24**(1): p. 155-163.

20. Garfield, A.S., et al., *A neural basis for melanocortin-4 receptor–regulated appetite.* Nature neuroscience, 2015. **18**(6): p. 863-871.

21. Shi, Y.-C., et al., *Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase neurons in the PVN.* Cell metabolism, 2013. **17**(2): p. 236-248.

22. Carter, M.E., et al., *Genetic identification of a neural circuit that suppresses appetite.* Nature, 2013. **503**(7474): p. 111-114.

23. Areias, M.F.C. and P.O. Prada, *Mechanisms of insulin resistance in the amygdala: influences on food intake.* Behavioural brain research, 2015. **282**: p. 209-217.

24. Becskei, C., et al., *Lesion of the lateral parabrachial nucleus attenuates the anorectic effect of peripheral amylin and CCK.* Brain research, 2007. **1162**: p. 76-84.

25. Morris, J.S. and R.J. Dolan, *Involvement of human amygdala and orbitofrontal cortex in hunger-enhanced memory for food stimuli.* Journal of Neuroscience, 2001. **21**(14): p. 5304-5310.

26. Breton, J., et al., *Gut commensal E. coli proteins activate host satiety pathways following nutrient-induced bacterial growth.* Cell metabolism, 2016. **23**(2): p. 324-334.

27. Riediger, T., et al., *Peptide YY directly inhibits ghrelin-activated neurons of the arcuate nucleus and reverses fasting-induced c-Fos expression.* Neuroendocrinology, 2004. **79**(6): p. 317-326.

28. Yoshida, M., et al., *Evidence that oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice.* Journal of Neuroscience, 2009. **29**(7): p. 2259-2271.

29. Cassell, M.D., L.J. Freedman, and C. Shi, *The intrinsic organization of the central extended amygdala.* Annals of the New York Academy of Sciences, 1999. **877**(1): p. 217-241.

30. Greenwell, T.N., et al., *Colocalization and shared distribution of endomorphins with substance P, calcitonin gene-related peptide, γ-aminobutyric acid, and the mu opioid receptor.* Journal of Comparative Neurology, 2007. **503**(2): p. 319-333.

31. Rigamonti, A., et al., *Anorexigenic postprandial responses of PYY and GLP1 to slow ice cream consumption: preservation in obese adolescents, but not in obese adults.* Eur J Endocrinol, 2013. **168**(3): p. 429-436.

32. Pecina, S., Cagniard, B., Berridge, K. C., Aldridge, J. W., & Zhuang, X. (2003). Hyperdopaminergic mutant mice have higher “wanting” but not “liking” for sweet rewards. Journal of Neuroscience, 23(28), 9395-9402.

Chapter 5

Determining the chronic effects of a combination of oxytocin and naltrexone at subthreshold doses on food intake and body weight in adult rats, and the corresponding changes in gene expression in feeding-related brain regions

5.1 Abstract

As I have shown in previous chapters, OT and NTX indeed target different facets of feeding control. For this reason, combining these anorexigens holds promise as an effective treatment in reducing excessive consumption by influencing a broad range of appetite regulating processes. While OT influences satiety, and NTX modulates aspects of consumption related to palatability and pleasure, their combination at subthreshold doses has proven to synergistically induce acute reductions of food intake in adolescent animals. In the present study, I therefore aimed to investigate if the OTNTX combination might produce beneficial reductions in total caloric intake and body weight when administered chronically over 24 days. I found here that in adult animals, OTNTX did not appear to produce a significant acute effect on standard chow intake as in adolescents, while I still observed the acute hypophagic effect in palatable feeding scenarios. Futhermore, while the combination indeed had a cumulative effect on reducing palatable food intake with chronic administration, the beneficial effects on caloric intake and body weight were offset by compensatory feeding of non-palatable standard chow in the remainder of the day. The results of the PCR gene expression analysis across multiple brain regions showed that the drug combination produced robust changes in brain stem-hypothalamic networks relating to reward and pleasure, as well as homeostatic signals for feeding. This change in neuromolecular activation aligns well with the behavioural results,

showing decreased cumulative consumption of HFHS palatable food intake presented immediately after OTNTX drug administration. However, this did not result in a significant, beneficial reduction of total caloric intake or body weight over the 24-day period, as acute effects of the drug combination appear to be offset by a compensatory increase in standard chow intake later in the day.

5.2 Introduction

In the previous chapter, I investigated the effect of the OT and NTX drug combination on meal size in adolescent rats, which found robust effects on acute food intake, and associated changes in neuronal activation of homeostatic- and reward-related brain regions. These results align well with the results of the adolescent human case-study, which showed significant reduction in food intake after the drug combination [15].

Translational studies in humans have largely confirmed the basic research findings. A single supraphysiological dose of IN OT in obese and normal-weight subjects decreased energy consumption [15-19]. The link between OT and eating for pleasure has also been suggested, though specific aspects of hedonic eating affected by OT remain unclear with some studies reporting preferential effects in reducing consumption of sweet [17, 18, 20], fatty [16] or salty [18] foods. Functional magnetic resonance analyses (fMRI) have found that OT modifies responsiveness of hypothalamic [21, 8] and limbic [19, 21] sites in response to food images, suggesting that not only homeostatic, but also some reward circuits might indeed be modulated by OT.

After more than a century of search for pharmacological treatments that combat obesity, our choice of FDA-approved pharmaceuticals is currently limited to five. Two of those drugs, Qsymia (phentermine + topiramate) and Contrave (bupropion + NTX), are combination medicines: this fact reflects the need to simultaneously target many neural and neuroendocrine systems in order to increase the likelihood of effectively treating this multifactorial condition.

The combination medicine approach served as the basis of the recent case report in which hypophagic properties of OT, a molecule being subject of several clinical trials related to disordered appetite and obesity, were successfully augmented by co-administration of NTX in a patient with hypothalamic obesity caused by craniopharyngioma resection [15]. In the recent human case study, Hsu and colleagues administered OT peripherally to reduce energy intake and body weight in an adolescent male with hypothalamic obesity and uncontrollable hedonic food-seeking caused by craniopharyngioma resection [15]. Considering a potentially suboptimal effectiveness of OT on eating for reward, the authors co-administered OT with NTX, a non-selective opioid receptor antagonist, thereby capitalizing on the primary effect of opioid receptor blockade on diminishing reward-driven consumption. They found that while OT alone reduced body weight and hyperphagia during the first 10 weeks, subsequent co-administration with NTX was a successful adjunctive therapy leading to further improvements in body weight and satiety parameters.

While the results of the previous chapter are promising, an important drawback of the study was that the adolescent period in rodents is very short (ca. 2 weeks), which did not allow us to examine the longer-term effects of these drugs on food intake and body weight. I therefore sought to evaluate the validity of this therapeutic combination over an extended period. A recent meta-analysis shows that OT alone does not significantly reduce food intake over long-term use, and therefore utilizing a drug combination that aims to target multiple aspects of feeding may alleviate this issue [1].

For this purpose, adult rodents were studied over a 24-day period here, and body weight was monitored. The current study utilized a feeding paradigm in which animals maintained on standard laboratory chow were given a daily meal of high-fat high-sugar (HFHS) palatable chow for 2 hours. During this time, standard chow was removed from the cages. Animals were given daily intravenous (I.V.) injections immediately before

HFHS presentation, and the intake of the HFHS chow, and total calorie consumption over the 24hr period was measured. I used subthreshold doses of both OT and NTX alone, in order to investigate if their combination might produce a significant, potentiating effect on food intake and body weight.

Similarly, while our adolescent study investigated effects in cFos expression, showing the immediate neuronal activation in response to the treatment, in a longer-term project, I wanted to see if this had any effect on expression of genes related to food intake, and if the effect of the drug combination can be associated with changes in related transcript profiles.

5.3 Materials and methods

5.3.1 Animals and injectants

Adult male Sprague-Dawley rats (average b. wt. 555 g) were housed individually in standard plastic cages with wire tops in a temperature-controlled (22°C) animal facility with a 12:12 light:dark cycle (lights on at 07:00). Water and standard laboratory chow (Sharpes Stock Feed, Diet 86; 3.6kcal/g) were available ad libitum unless stated otherwise. Animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The University of Waikato Animal Ethics Committee approved all procedures described here. Animals were accustomed to receiving intravenous (I.V.) injections in the tail vein. NTX (Abcam, Cambridge, UK) and OT (Sigma, St. Louis, MO, USA) were dissolved in isotonic saline just prior to use. Drugs were injected as combination.

5.3.2 Feeding studies

First, by using acute feeding paradigms, we confirmed that the doses of individually administered OT and NTX were too low to reduce consumption for energy (intake of energy-dense standard chow in food-deprived animals) and for palatability (intake of

energy-dilute 10% sucrose solution in sated animals). The effect of the combined OT and NTX was established in these conditions.

5.3.2.1 Acute effect of low doses of IV OT, NTX and OT+NTX on deprivation-induced intake of energy-dense standard chow

Low doses of peripheral NTX and OT were chosen based on previously published studies: in the case of both ligands, we selected doses that were previously reported as ineffective in reducing deprivation-induced intake of standard chow [2]. Standard chow was removed at 16:00 on the day preceding administration (water was available at all times). At 10:00 on the experimental day, animals were injected IV with either isotonic saline (control), OT (0.3μg/kg), NTX (0.1mg/kg) or OT (0.3ug/kg) + NTX (0.1mg/kg) (n=10/group). Immediately after the injection, standard chow was returned, and consumption was measured 2 hours later. Data from drug-injected groups were compared to saline controls with ANOVA followed by Dunnett's post-hoc test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leqslant 0.05$.

5.3.2.2 Acute effect of low doses of IV OT, NTX and OT+NTX on calorie-dilute, palatable 10% sucrose solution intake in non-deprived rats

Low doses of peripheral NTX and OT were chosen based on previously published studies: in the case of both ligands, we selected doses that were previously reported as ineffective in reducing intake of sucrose solutions in non-deprived rodents [2]. Rats maintained on ad libitum standard chow and water were pre-exposed to 10% sugar water to avoid neophobia 5 and 10 days prior to the experiment (for 2 h on each occasion). On the experimental day, standard chow and water were removed from cages at 10:00, and the animals were given an IV injection of either isotonic saline (control), OT (0.3μg/kg), NTX (0.1mg/kg) or OT (0.3ug/kg) + NTX (0.1mg/kg) (n=10/group). Immediately after the injection, the animals were presented with a 10% sucrose solution and the amount of ingested solution was measured by weighing bottles (in grams) 2 hours later. Data from

drug-injected groups were compared to saline controls with ANOVA followed by Dunnett's post-hoc test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leqslant 0.05$.

5.3.2.3 Effect of 24-day daily single injections of low doses of IV OT, NTX, and OT+NTX on HFHS palatable chow consumption, standard chow intake and body weight in non-deprived rats

Low doses of peripheral NTX and OT were chosen based on the outcome of the studies described above as well as on the previously published experiments [2]. Rats were pre-exposed to the calorie-dense, palatable HFHS chow (Research Diets chow #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) on two occasions prior to the study to avoid neophobia. From experimental day 1 until day 24, the rats were given a daily meal of HFHS chow between 10:00 and 12:00. At the onset of the 2-h HFHS meal, standard chow was removed from the cages and returned immediately afterward the meal (water was present at all times). Just before the HFHS chow presentation, animals (n=9/group) were injected IV with either isotonic saline (control), OT (0.3µg/kg), NTX (0.1mg/kg) or OT (0.3ug/kg) + NTX (0.1mg/kg). HFHS food intake was measured 2h post-injection. Intake of standard chow was also measured daily at the time of removing the standard pellets from the cages at 10:00. Body weight was assessed daily between 08:00 and 09:00. Data from cumulative intake were analysed and shown for 3-day cumulative intervals. Data from drug-injected groups were compared to saline controls with ANOVA followed by Dunnett's post-hoc test. A significance level was set at $p \leqslant 0.05$.

5.3.3 Effect of 7-day IV OT and NTX on gene expression in the hypothalamus, brain stem and nucleus accumbens

Considering that the 24-day exposure period resulted in overall changes in food intake, and these changes became more apparent in the second week of treatment, in order to

understand the effect of the drugs alone when administered longer term, we used a different cohort of rats and subjected them to the same injection regimen, understanding that the food intake changes will not yet be a confound of the PCR study. It will therefore reflect mainly the effect and the ongoing changes in the CNS that later result in cumulative changes in food intake.

Animals (n=8/group) were injected IV with either isotonic saline (control), OT (0.3ug/kg), NTX (0.1mg/kg) or OT (0.3ug/kg) + NTX (0.1mg/kg) at 10:00 daily for 7 days, immediately prior to being presented with palatable HFHS chow for 2 h (as described above). HFHS food intake was measured 2 h post-injection. Animals then received 22h daily access to standard chow. Water was available at all times.

On the final day, animals received their injection 120 minutes before being sacrificed by decapitation. The brain stem, nucleus accumbens and hypothalamus were dissected and placed in RNAlater (Ambion, Stockholm, Sweden) for 2 h at room temperature and the samples were then frozen at −80˚C until further preparation.

5.3.3.1 RNA isolation and cDNA synthesis

A standard protocol of sample preparation and rtPCR was used, as established by our lab previously [3]. Samples were homogenized in TRIzol (Ambion); RNA was extracted with chloroform, centrifuged at 10,000 × g at 4 ˚C for 15 min, and RNA precipitated in isopropanol. After centrifuging, the pellet was washed with 75% ethanol, air-dried, and dissolved in the DNase buffer (NEB). The samples were treated with RNase-free DNase I (Merck, Germany) and the absence of genomic DNA was established by PCR of a 5% template. 100 ng/µl genomic DNA served as a positive control, whereas MilliQ H2O as a negative one. RNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). 5 µg RNA samples were diluted with MilliQ H2O. RNA was reverse-transcribed to cDNA in the master mix

(Promega; 20 µl). Samples were incubated for 1 h (37°C), followed by PCR to confirm cDNA synthesis.

The cDNA for the tissue panels was analysed in quantitative real-time PCR with a CFX96 thermal cycler (Bio-Rad Laboratories, Stockholm, Sweden). Each real-time PCR with a total volume of 20 µl contained cDNA synthesized from 25 ng total RNA, 0.25 M each primer, 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 4 mM MgCl2, 0.2 mM dNTP, SYBR Green (1:50,000). Real-time PCR was performed with 0.02 U/liter Taq DNA polymerase (Invitrogen) under the following conditions: initial denaturation for 3 min at 95 ˚C, followed by 50 cycles of 15 sec at 95 ˚C, 15 sec at 54–61 ˚C (optimal annealing temperature), and 30 sec at 72 ˚C. This was followed by 84 cycles of 10 sec at 55 ˚C (increased by 0.5 ˚C per cycle). All real-time PCR experiments were performed in duplicates, and the measurements where the threshold cycle (Ct) values between the duplicates had a difference of over 0.9 were repeated. A negative control for each primer pair was included on each plate. Sample cDNA template (25 ng) was used per primer, and listed below in Figure 5.1. Expression of three housekeeping genes (TBP, H3B and β-tubulin) was used to calculate normalization factors (GeNorm). Melting point curves were included after the thermocycling to confirm that only one product with the expected melting point was formed.

The CFX Maestro software (Bio-Rad) was used to analyse real-time PCR data and derive Ct values, and used delta-Ct to produce relative expression levels with SD. Melting curves were analysed manually for each individual sample to confirm that only one product was amplified and that it was significantly shifted compared with the melting curve for the negative control. Differences in gene expression between groups were analysed using ANOVA followed by Dunnett's post-hoc test on genes significantly up- or down-regulated in the ANOVA. $p < 0.05$ was used as the criterion of statistical significance for

the ANOVA. Statistics were performed using Prism (GraphPad). Values are presented as means ± S.E.M.

Housekeeping genes	forward primer	reverse primer
BTUB	CGGAAGGAGGCGGAGAGC	AGGGTGCCCATGCCAGAGC
H3B	CCTTGTGGGTCTGTTTGA	CAGTTGGATGTCCTTGGG
TBP	AGAACAATCCAGACTAGCAGCA	GGGAACTTCACATCACAGCTC
Functional genes		
AGRP	AGAGTTCCCAGGTCTAAGTCTG	GCGGTTCTGTGGATCTAGCA
DOR	GCTCGTCATGTTTGGCATC	AAGTACTTGGCGCTCTGGAA
DYN	TACCAGCGTCCCAGAGGAAAA	GGCTTCATCATTCATCCGGTC
GHSR	TGGAGATCGCGCAGATCAG	CCGGGAACTCTCATCCTTCAG
KOR	AGACCGCAACCAACATCTACAT	GCACAGAACATCTCCAAAAGG
MC3R	TCCGATGCTGCCTAACCTCT	GGATGTTTTCCATCAGACTGACG
MC4R	CTTATGATGATCCCAACCCG	GTAGCTCCTTGCTTGCATCC
MOR	CGGACTCGGTAGGCTGTAAC	CCTTGCGCTCTTCTCTGG
NOFQ	CAGACAGGGAGGACATGGAT	GGACTGCAAAGTGCAGACCA
NOP	CAGCCAAGGCTACACAGACA	AGACGTGAGGCTCCAACACT
NPY	ATGCTAGGTAACAAGCGAATGG	TGTCGCAGAGCGGAGTAGTAT
OPRL1	GTTCAAGGACTGGGTGTTCAG	CGTGGTACTGTCTCAGAGGTC
ORX1R	CCCTCAACTCCAGTCCTAGC	CAGGGAGGGCCTATAATTGA
ORX2R	CAATGTTGTTGGGGTGCTTA	TCCCCCTCTCATAAACTTGG
OXT	GACGGTGGATCTCGGACTGAA	CGCCCCTAAAGGTATCATCACAAA
OXTR	GATCACGCTCGCCGTCTA	CCGTCTTGAGTCGCAGATTC
PENK	GAGAGCACCAACAATGACGAA	TCTTCTGGTAGTCCATCCACC
POMC	TGGGCGAGCTGATGACCT	GCCGACTGTGAAATCTGAAAGG

Figure 5.1: **Primer sequences used for PCR analysis** Primer base sequences: A, adenine; C, cytosine; G, guanine; T, thymine. Housekeeping genes: BTUB, beta tubulin; H3B, histocompatibility 3b; TBP, TATA-box binding protein. Functional genes: AGRP, agouti-related protein; DOR, delta-opioid receptor; DYN, dynorphin; GHSR, growth hormone secretagogue receptor; KOR, kappa-opioid receptor; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptor; MOR, mu-opioid receptor; NOFQ, nociception/orphanin FQ; NOP, nociception opioid receptor; NPY, neuropeptide Y; OPRL1, opioid receptor-like; ORX1R, orexin 1 receptor; ORX2R, orexin 2 receptor; OXT, oxytocin; OXTR, oxytocin receptor; PENK, proenkephalin; POMC, proopiomelanocortin.

5.4 Results

5.4.1 Feeding Studies

Acutely administered OT (0.3µg/kg), NTX (1mg/kg), or OT (0.3µg/kg)+NTX (1mg/kg) did not decrease deprivation-induced intake of energy-dense and 'bland' standard chow during a 2-hour meal (Fig 5.2). On the other hand, while the doses of OT or NTX alone were not sufficiently high to decrease intake of the palatable yet calorie-dilute sugar

solution, the combination of OT+NTX significantly decreased consumption in non-deprived animals (p=0.0073, Fig 5.3).

Daily co-administration of OT (0.3µg/kg) + NTX (0.1mg/kg) significantly reduced the cumulative intake of HFHS palatable chow over the 24-day period (p=0.0359; Fig 5.4).

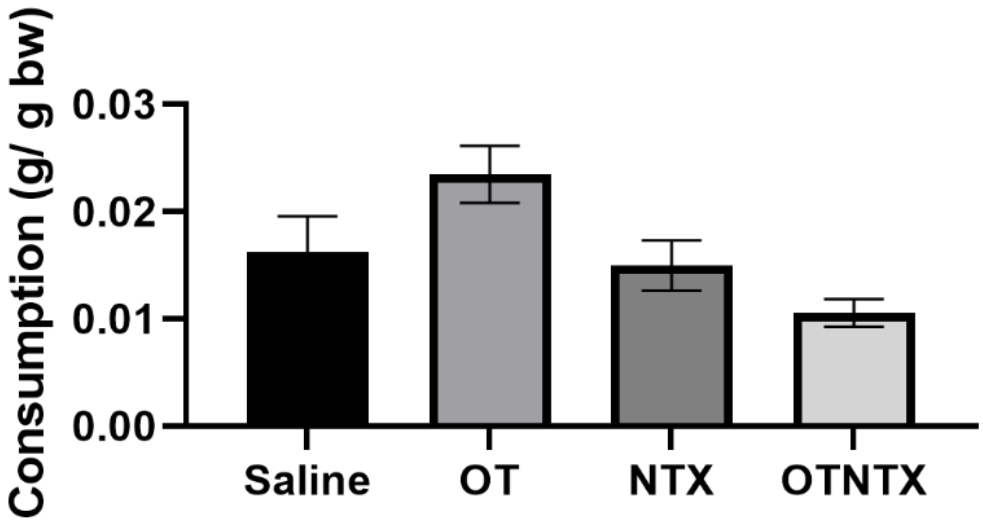


Figure 5.2 Effect of saline (control), OT (0.3µg/kg), NTX (0.1mg/kg) or OT (0.3ug/kg)+NTX (0.1mg/kg) on deprivation-induced intake of standard chow during a 2-hour meal. I.V. administration of either OT (0.3µg/kg), NTX (1mg/kg), or a combination of OT (0.3µg/kg) and NTX (1mg/kg) was ineffective at significantly altering deprivation-induced standard chow intake 2 hours post-injection (Dunnett's post-hoc test).

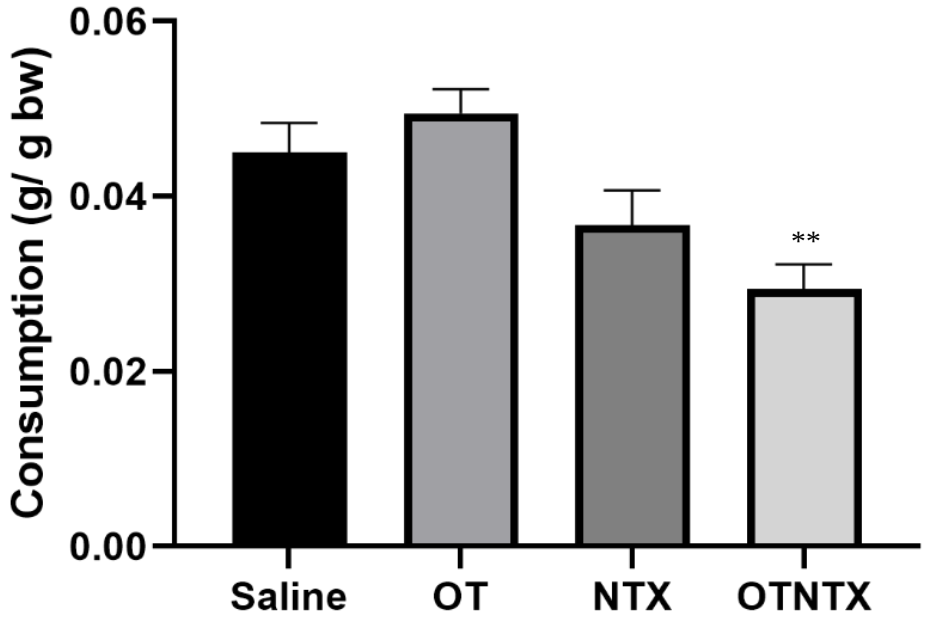


Figure 5.3 Effect of I.V. OT and NTX on sucrose solution intake after 2 hours. I.V. administration of a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) significantly reduced intake of palatable sucrose solution 2 hours post-injection ($p=0.0073$). I.V. administration of OT (0.3µg/kg) or NTX (1mg/kg) alone did not significantly reduce sucrose solution intake. (Dunnett's post hoc test, $^{**}p<0.01$).

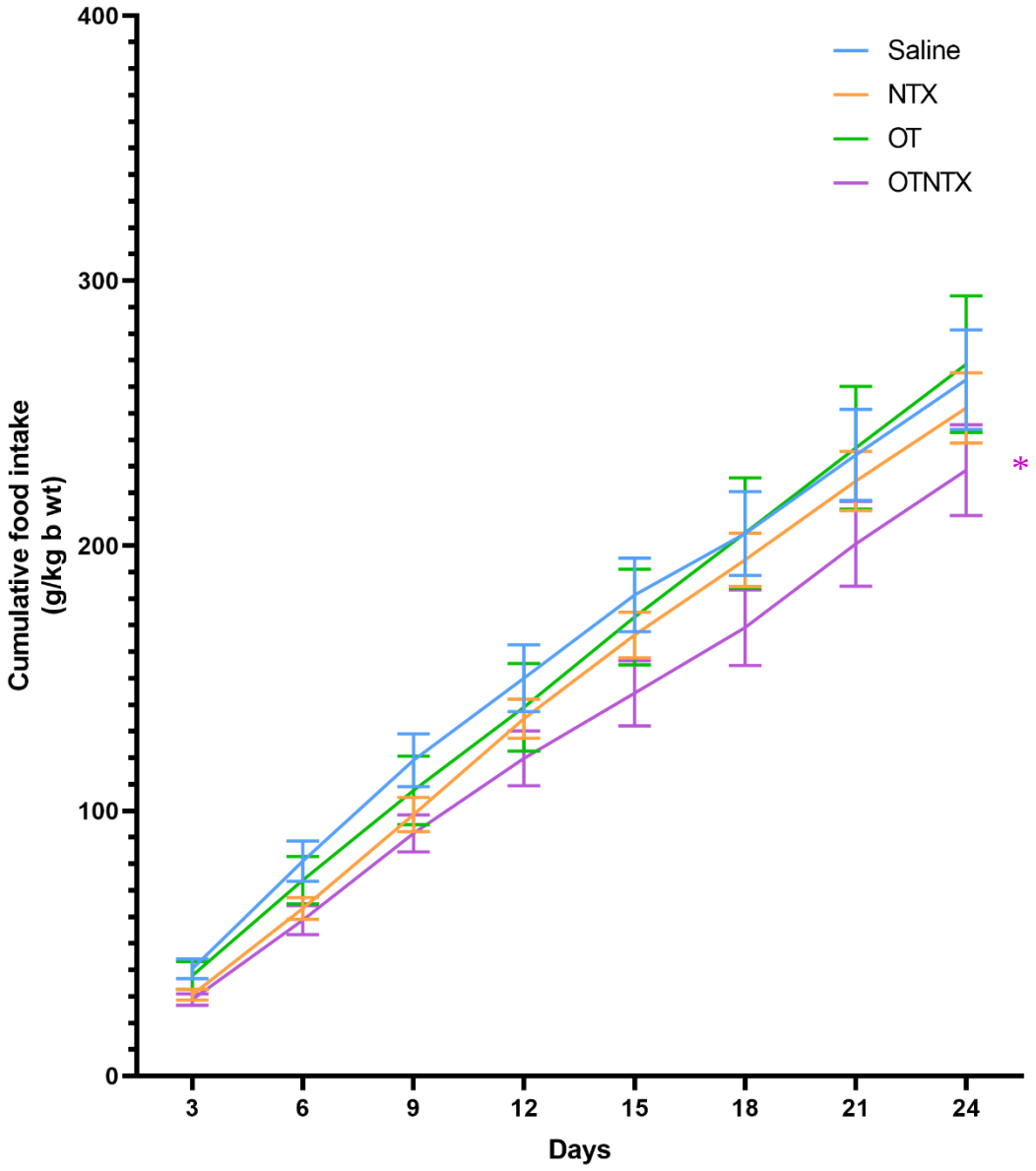


Figure 5.4: Cumulative HFHS intake over 24 days. 2-way ANOVA results show that only the OTNTX-treated group show significantly reduced cumulative consumption of HFHS diet over the 24-day period, when compared to saline control group (Dunnett's post hoc test, *$p<0.05$).

Animals treated with OT (0.3µg/kg) + NTX (0.1mg/kg) consumed more standard chow as established through cumulative intake analysis from day 15 onwards (day 15: p=0.0183, day 18: p=0.002, day 21: p=0.0004, and day 24: p<0.001, Fig 5.5). Administration of OT (0.3µg/kg) or NTX (1mg/kg) alone also produced an increase in cumulative standard chow intake (day 18: OT - p=0.0328 and NTX - p=0.0262; day 21: OT - p=0.0127 and NTX - p=0.0086; day 24: OT - p=0.0037 and NTX - p=0.0028; Fig 5.5). Daily administration of OT (0.3µg/kg), NTX (0.1mg/kg) or OT (0.3µg/kg) + NTX (0.1mg/kg) all significantly increased total calorie intake over the 24-day period (p=0.0107, p=0.0456 and p=0.0412, respectively, Fig. 5.6). Neither of the treatments produced a change in body weight over the 24-day period (Fig. 5.7).

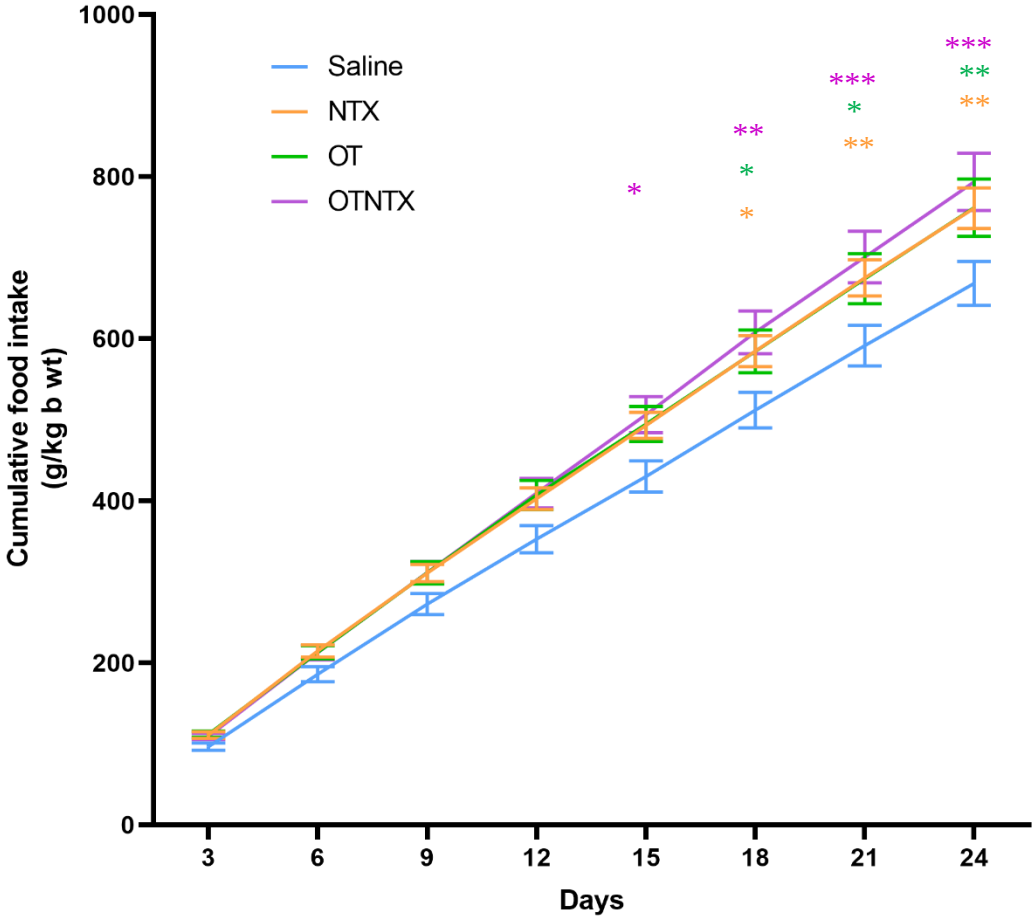


Figure 5.5: Cumulative standard chow intake over 24 days. 2-way ANOVA results show that daily I.V. administration of a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) produced a significant increase in cumulative standard chow intake from day 15 onwards. Similarly, daily I.V. administration of OT (0.3µg/kg) or NTX (1mg/kg) alone, also produced a significant increase in cumulative standard chow intake from day 18 onwards (Dunnett's post hoc test, *p<0.05; **p<0.01; ***p<0.001).

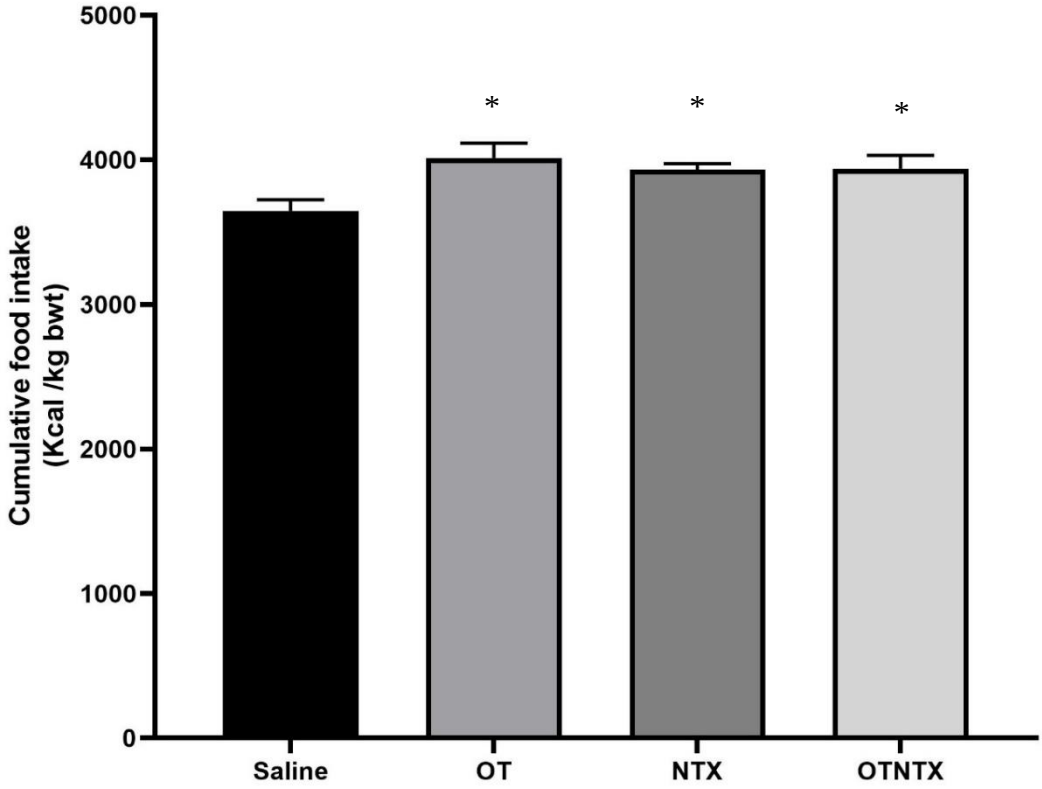


Figure 5.6: Total caloric intake over 24 days. ANOVA results show that daily I.V. administration of OT (0.3µg/kg), NTX (0.1mg/kg) or a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) all significantly increased total caloric intake over the 24-day period, when compared to saline-treated control group (Dunnett's post hoc test, *p<0.05).

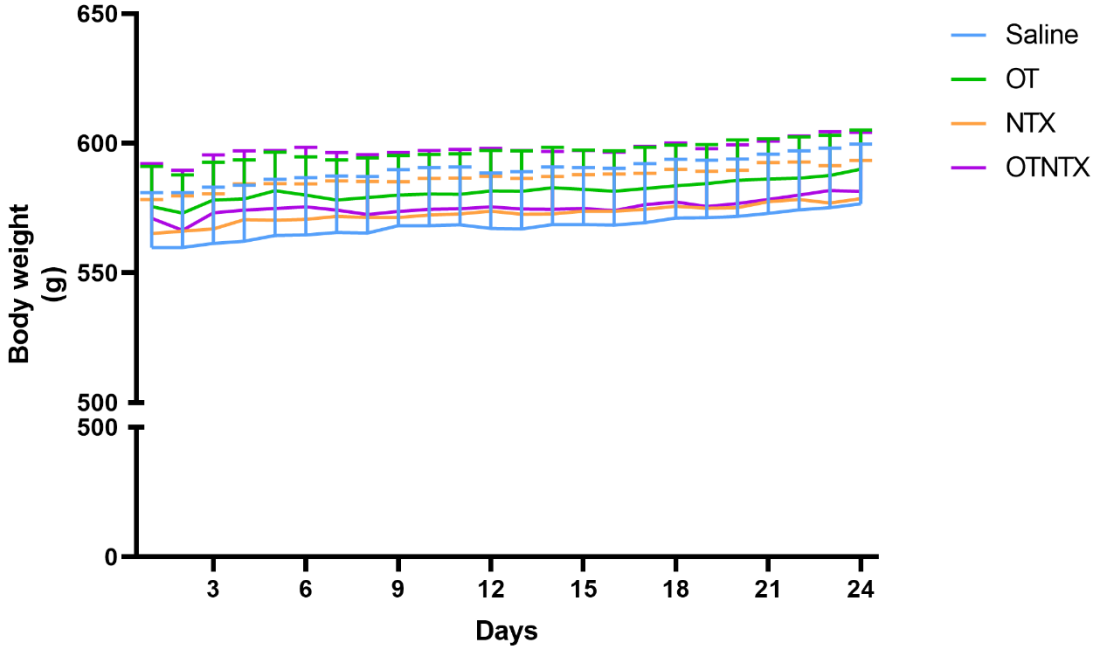


Figure 5.7: Body weight over the 24-day period. 2-way ANOVA with Dunnett's post hoc test results show no significant differences in body weight between treatment groups, over the 24-day period.

5.4.2 Gene expression

5.4.2.1 Hypothalamus

7-days of daily I.V. drug administration resulted in some significant changes in hypothalamus gene expression of homeostatic- and reward-related genes. OT-treated animals (0.3µg/kg) showed a significant increase in expression of KOR ($p=0.0129$, Fig 5.8). NTX-treated animals (0.1mg/kg) showed a significant increase in expression of KOR ($p<0.001$, Fig. 5.8) and POMC ($p<0.001$, Fig. 5.8). Animals treated with a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) showed a significant increase in expression of KOR ($p<0.001$, Fig. 5.8) and POMC ($p<0.001$, Fig. 5.8).

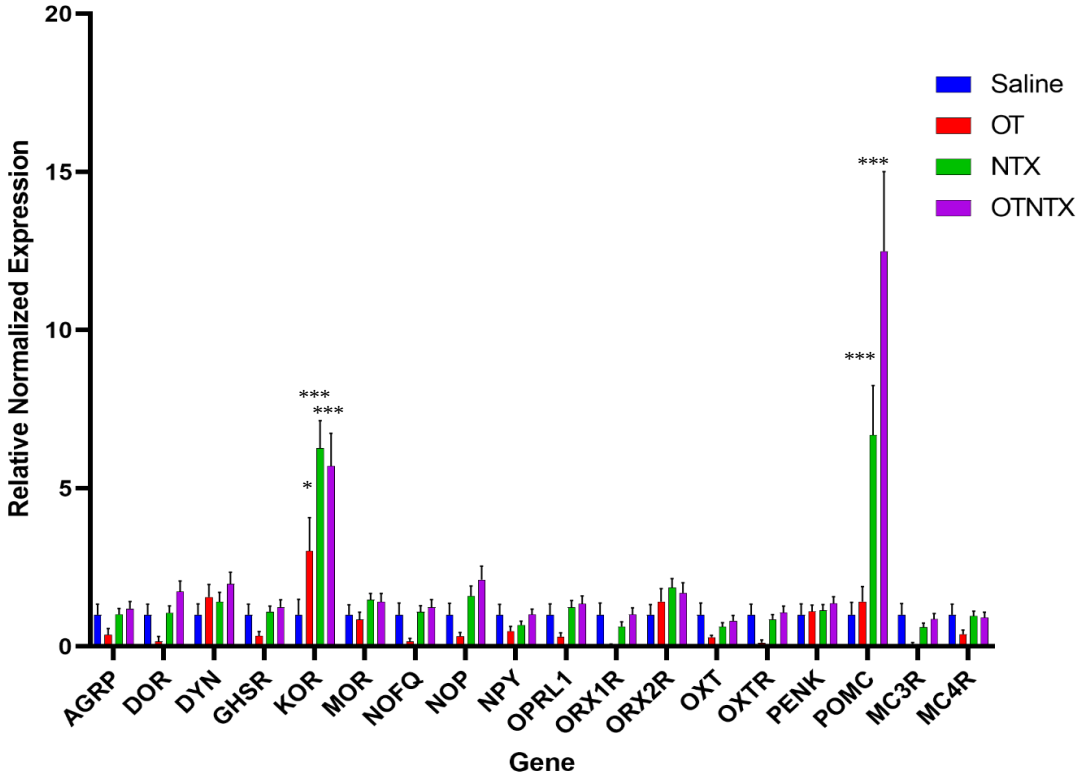


Figure 5.8: Effect of treatment on expression of feeding-related genes established with real-time PCR in the hypothalamus. Hypothalamus gene expression after 7-days I.V. administration of either OT (0.3µg/kg), NTX (1mg/kg), or a combination of OT (0.3µg/kg) and NTX (1mg/kg). AGRP, Agouti-related protein; DOR, delta-opioid receptor; DYN, dynorphin; GHSR, growth hormone secretagogue receptor; KOR, kappa-opioid receptor; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptor; MOR, mu-opioid receptor; NOFQ, nociception/orphanin FQ; NOP, nociception opioid receptor; NPY, neuropeptide Y; OPRL1, opioid receptor-like 1; ORX1R, orexin 1 receptor; ORX2R, orexin 2 receptor; OXT, oxytocin; OXTR, oxytocin receptor; PENK, proenkephalin; POMC, proopiomelanocortin. (Dunnett's post-hoc test, *$p<0.05$; ***$p<0.001$).

5.4.2.2 Brainstem

7-days of daily I.V. drug administration resulted in some significant changes in brainstem gene expression of homeostatic- and reward-related genes. NTX-treated animals showed a significant increase in MOR expression (p=0.0052, Fig. 5.9). Animals treated with a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) showed a significant increase in expression of MOR (p=0.003, Fig. 5.9), ORX2R (p=0.0194, Fig. 5.9) and POMC (p=0.192, Fig 5.9).

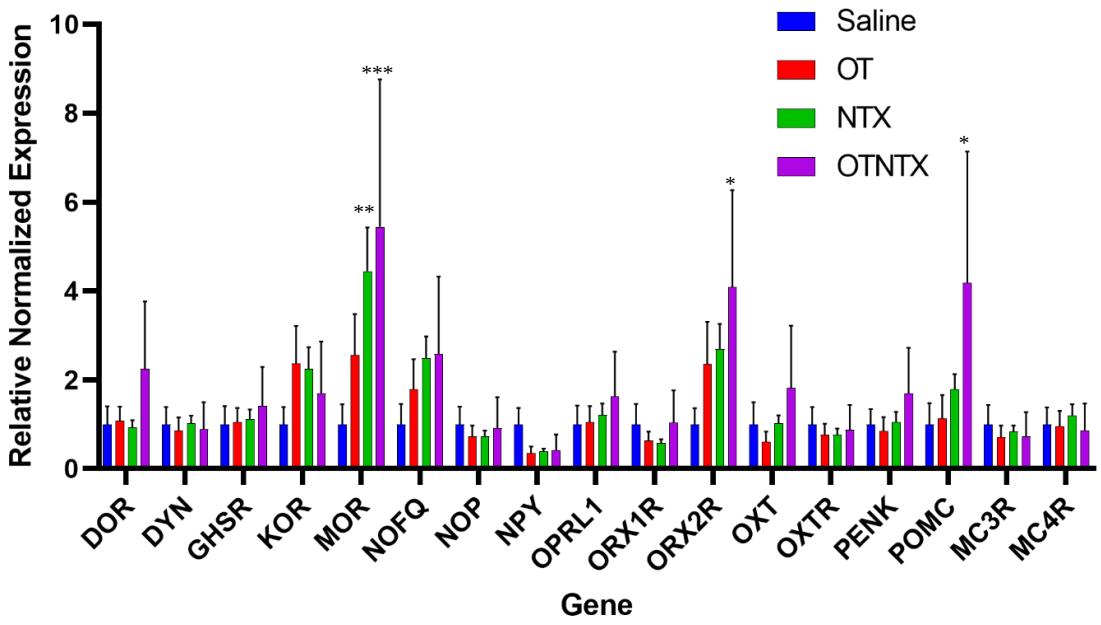


Figure 5.9: Effect of treatment on expression of feeding-related genes established with real-time PCR in the brain stem. Brainstem gene expression after 7-days I.V. administration of either isotonic saline, OT (0.3µg/kg), NTX (1mg/kg), or a combination of OT (0.3µg/kg) and NTX (1mg/kg). DOR, delta-opioid receptor; DYN, dynorphin; GHSR, growth hormone secretagogue receptor; KOR, kappa-opioid receptor; MC3R, melanocortin 3 receptor; MC4R, melanocortin 4 receptor; MOR, mu-opioid receptor; NOFQ, nociception/orphanin FQ; NOP, nociception opioid receptor; NPY, neuropeptide Y; OPRL, opioid receptor-like; ORX1R, orexin 1 receptor; ORX2R, orexin 2 receptor; OXT, oxytocin; OXTR, oxytocin receptor; PENK, proenkephalin; POMC, proopiomelanocortin. (Dunnett's post-hoc test, *p<0.05; **p<0.01; ***p<0.001).

5.4.2.3 Nucleus Accumbens

7-days of daily I.V. drug administration resulted in some significant changes in nucleus accumbens gene expression of homeostatic- and reward-related genes. OT-treated animals (0.3µg/kg) showed a significant increase in expression of PENK ($p<0.001$, Fig. 5.10). Animals treated with a combination of OT (0.3µg/kg) and NTX (0.1mg/kg) also showed a strong trend of increased expression of PENK ($p=0.0508$, Fig. 5.10), although this did not reach criteria for statistical significance.

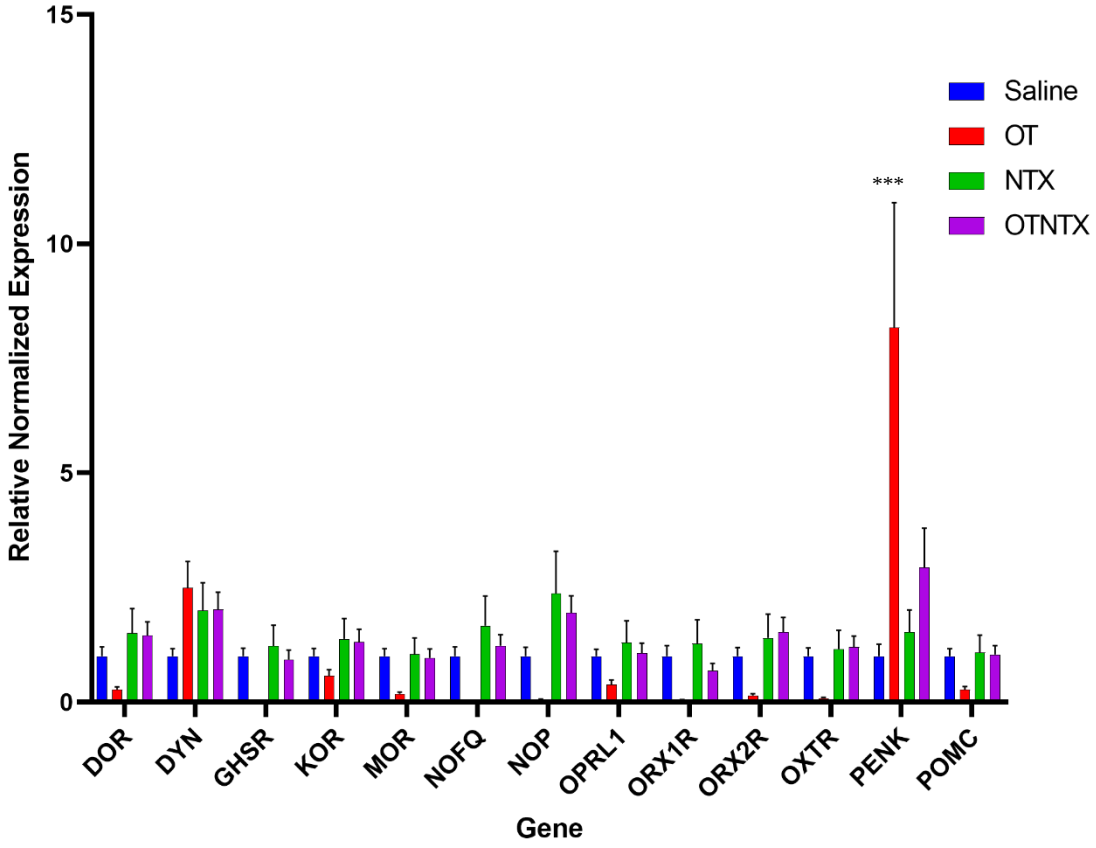


Figure 5.10: Effect of treatment on expression of feeding-related genes established with real-time PCR in the nucleus accumbens. Nucleus accumbens gene expression after 7-days I.V. administration of either isotonic saline, OT (0.3µg/kg), NTX (1mg/kg), or a combination of OT (0.3µg/kg) and NTX (1mg/kg). DOR, delta-opioid receptor; DYN, dynorphin; GHSR, growth hormone secretagogue receptor; KOR, kappa-opioid receptor; MOR, mu-opioid receptor; NOFQ, nociception/orphanin FQ; NOP, nociception opioid receptor; OPRL1, opioid receptor-like 1; ORX1R, orexin 1 receptor; ORX2R, orexin 2 receptor; OXTR, oxytocin receptor; PENK, proenkephalin; POMC, proopiomelanocortin. (Dunnett's post-hoc test, ***$p<0.001$).

5.5 Discussion

As shown in the previous chapter in adolescent animals, administration of OTNTX-combination has a beneficial effect on reducing acute feeding. Here, the data show that in adult animals, the combination does not appear to have this significant acute effect on standard chow intake, as in adolescents. However, in adult animals, I still observe the acute effect on certain palatable feeding scenarios, and the combination has a cumulative effect on reducing palatable food intake with chronic administration, yet the beneficial effects on caloric intake and body weight are offset by compensatory feeding of non-palatable standard chow in the remainder of the day.

A dose of 0.1mg/kg of NTX was selected to be combined with OT, as this was the highest dose that did not produce significantly reduced palatable food intake when administered alone. I then established that I.V. administration of 0.3µg/kg OT alone was insufficient to reduce food intake, and therefore was the optimal subthreshold dose to combine with the 0.1mg/kg subthreshold dose of NTX. Interestingly, while NTX selectively inhibits intake of palatable sucrose, and does not have an effect on deprivation-induced chow intake, I.V. OT administration selectively inhibits deprivation-induced chow intake, but fails to effect consumption of palatable sucrose solutions [2], and so target different aspects of feeding behaviour. Therefore, by combining subthreshold doses of OT and NTX, I aimed to investigate if these drugs would work synergistically to inhibit both the homeostatic- and reward-based drives to consume, and thus produce a significant reduction in food intake, and body weight.

We then verified that this combination of OT (0.3µg/kg) and NTX (0.1mg/kg) indeed acutely produced a significant reduction in intake of palatable sucrose solution 2 hours post-injection, while the individual drugs alone did not produce a significant effect.

After 24-days of chronic I.V. drug administration, I found that cumulative HFHS food intake was significantly reduced by the combination of OT (0.3µg/kg) and NTX

(0.1mg/kg). HFHS food was presented for 2 hours daily, immediately after drug administration, and represents the acute effect of the drug combination on palatable food consumption. Interestingly, this same group that received the combination of OT (0.3µg/kg) and NTX (0.1mg/kg), and which showed reduced HFHS intake, conversely showed a significant increase in standard chow intake over the 24-day period. Animals treated with OT (0.3µg/kg) or NTX (0.1mg/kg) alone, also showed increased consumption of standard chow, although did not show the same significant reduction in palatable HFHS intake. Standard chow was presented for 22-hours daily following the 2-hour HFHS period. This appears to show that while OTNTX combination indeed induced a reduction in palatable chow intake immediately after injection, that animals showed a compensatory increase in regular chow intake. This conclusion is further reinforced by the result of the total caloric intake of the animals, which shows that all three drug-treated groups consumed a significantly greater amount of total calories over the 24-day period, when compared to the control group. Furthermore, the effect of the chronic drug administration on animal body weights shows no significant decrease or change in body weights over the 24-day treatment period. This again shows that while OTNTX treatment indeed produced a significant acute reduction in palatable HFHS food intake, any reduction in HFHS caloric intake was offset by a compensatory increase in consequent standard chow intake, and therefore produced no significant change in body weight over the treatment period.

We had hoped that by blocking the opioid receptors with NTX, acting as a non-selective opioid receptor antagonist to diminish reward-based drives to consume, and with OT stimulating the homeostatic response for early cessation of meal termination, that I might regulate both aspects of feeding motivation, to reduce caloric intake and body weight. This combination treatment has proven to be effective at reducing palatable food intake of the immediate meal, utilizing a dose of NTX that produces no significant

effect on HFHS intake when administered alone, and therefore implying that the combination with OT produced a synergistic, potentiating effect. However, while OT is effective at reducing acute consumption of standard chow [4], OT's anorexigenic effects may have worn off after the 2hr daily HFHS period, and therefore did not combat against a compensatory increase in regular chow intake in the following 22 hours of the day. OT has been shown to be an important signal for early meal termination, however, is primarily effective after the beginning of a meal [4]. For this reason, what might be beneficial is to administer OT directly before each meal. Studies have shown that while OT is effective at reducing food intake acutely, it fails to maintain efficacy with chronic administration, as it is rapidly metabolized within 20 minutes in the CNS [5; 6]. This therefore accounts for the observed effects of the OTNTX drug combination that significantly reduced intake of the immediate meal, but fails to influence a 24-hour feeding period. Indeed, a systematic review and quantitative meta-analysis on the effects of OT on food intake was recently published, which examined over 2000 studies in animals and humans. This review showed that while a single dose of OT was effective at reducing food intake, whether administered centrally or peripherally, chronic administration did not produce a significant anorexigenic effect [1]. It is interesting to note that the drug administration produced a very slight increase in compensatory caloric intake, albeit not enough to significantly increase body weight over the 24-day period.

Furthermore, by utilizing PCR gene expression analysis, we were able to investigate the neuromolecular changes in gene expression that underlie these observed behavioural effects. Within the hypothalamus, a brain region associated with regulating the homeostatic drives for consumption, I observed a significant increase in expression of KOR and POMC mRNA after OTNTX combination treatment. Increases in hypothalamic

proopiomelanocortin (POMC) mRNA have been previously associated with the homeostatic signalling of satiety, and the lack of hunger. POMC mRNA is decreased by food-deprivation and fasting [7]. The POMC gene encodes the alpha-melanocyte-stimulating hormone (α-MSH), which has been shown to stimulate satiety, while inversely, its reduction is associated with obesity [7], and therefore this result implies that the OTNTX treatment significantly increased neuromolecular signals of satiety that act to reduce homeostatic drives for consumption.

Similarly, the observed upregulation of KOR mRNA within the hypothalamus has previously been associated with modulating the orexigenic effect of ghrelin. Ghrelin produces potent orexigenic effects, but recent studies show that the hypothalamic opioid system can modulate these effects, and that hypothalamic KOR blockade can reduce food intake by blocking ghrelin signalling [8]. Our observation here that hypothalamic KOR mRNA has been upregulated by OTNTX treatment may imply that these opioid receptors are being blocked by the drug combination, inhibiting the orexigenic effect of ghrelin release, and thus reducing food intake in this way.

Within the brainstem, administration of the combination of OT (0.3µg/kg) and NTX (0.1mg/kg) produced a significant increase in expression of MOR mRNA, the mu-opioid receptor. NTX is a non-selective opioid receptor antagonist, and it therefore follows that expression of this opioid receptor might be up-regulated as a result of the opioid receptors being blocked, which act to reduce sensitivity to rewarding stimuli, and the drive to consume palatable foods. Glass et al. similarly found that MOR gene expression was significantly increased within the brainstem of rats treated with NTX, and resulted in reduction of food intake [9]. They presented evidence of a distributed functional network of opioid receptor pathways that were responsible for regulating consumption for both reward- and energy-related feeding. They conclude that based on the wide distribution of

CNS opioid receptors, and the presence of other neuropeptides in the vicinity of opioidergic pathways, it seems likely that opioids can indeed affect multiple aspects of feeding [9]. This effect may have been further modulated by the addition of OT, being a prominent neuropeptide in the vicinity of this receptor network, and which also acts on homeostatic aspects of feeding.

We also observed a significant increase in ORX2R mRNA within the brainstem after the OTNTX combination treatment. ORX2R is a G protein-coupled receptor for orexin, a neuropeptide implicated in feeding and circadian sleep/wake cycles. An increase in ORX2R expression has been previously correlated with an inhibition of food intake, as increased orexin peptide levels are shown to increase consumption, and the increased expression of the receptor ORX2R, could imply an upregulation of the receptor due to a decrease in the orexin peptide levels that stimulate feeding and arousal. A study by Sunter et al. found that ORX2R mRNA was expressed in the nucleus of the solitary tract (NTS) of the rat brainstem [10], and showed that this region received orexin immunoreactive fibres originating in the lateral hypothalamus (LH), an area implicated in feeding and circadian control [11]. They proposed that the presence of ORX2R mRNA in the NTS of the brainstem can result in hypophagia, and also influences activity levels [10]. Interestingly, I also observed a significant increase in POMC mRNA expression within the brainstem, which has similarly been implicated with NTS activation. Activation of POMC neurons in the NTS of the brainstem has been shown to immediately produce an inhibition of food intake, while activation of POMC neurons within the hypothalamus, for which I also observed a significant increase in POMC expression, has been implicated with only inhibiting food intake with chronic stimulation [12]. This shows that the administration of OTNTX was capable of increasing POMC mRNA expression that has been demonstrated to reduce food intake acutely, by modulating satiety signals within the brainstem-hypothalamus network.

Within the nucleus accumbens (NAcc), a brain region associated with regulating the reward-based drives for consumption, I found that OT (0.3µg.kg) significantly increased PENK mRNA expression. Increases in NAcc PENK mRNA expression has previously been associated with reducing food intake in rats, specifically the intake of fats and chow [13]. PENK, or proenkephalin has been characterised as an opioid agonist, and may be upregulated due to the blocking of the opioid receptors within the NAcc [14]. This signifies the reduced sensitivity of the animals to reward-related signalling after the drug administration that acts to reduce the consumption of palatable foods. Similarly, I also observed a strong trend of the combination of OT and NTX in increasing the expression of PENK within the NAcc (P=0.0508, Fig. 5.10). While this did not reach statistical significance due to variability in samples, we can see a trend of an increase in this opioid-receptor agonist that may reflect the blocking of the opioid receptors, acting to reduce sensitivity to reward-related signals for consumption, and this reduce palatable food intake.

Taken together, the results of the PCR gene expression analysis across multiple brain regions shows that each of the drugs influences aspects of feeding relating to reward and pleasure of consumption, as well as homeostatic signals for feeding. The OTNTX combination produced the most pronounced change in expression of POMC transcripts relating to satiety. This change in neuromolecular activation is then shown to result in decreased cumulative consumption of HFHS palatable food intake immediately after chronic OTNTX drug administration. However, this did not result in a significant, beneficial reduction of total caloric intake or body weight, as any acute effects of the drug combination appear to be offset by a compensatory increase in standard chow intake later in the day. This may be because OT has shown to be rapidly metabolized in the CNS [1], and therefore OT effects the immediate meal, but does not serve as a long-term tool to curb 24-hour appetite.

While in the human case-study of craniopharyngioma, this single administration schedule produced successful outcomes on body weight, one can imagine that multiple acute drug administrations before each meal, or sustained release of the drug combination may mitigate this effect and prolong the efficacy of the drug combination.

5.6 References

[1] Leslie, M., Silva, P., Paloyelis, Y., Blevins, J., & Treasure, J. (2018). A systematic review and quantitative meta‐analysis of the effects of oxytocin on feeding. *Journal of neuroendocrinology, 30*(8), e12584.

[2] Klockars, A., Brunton, C., Li, L., Levine, A. S., & Olszewski, P. K. (2017). Intravenous administration of oxytocin in rats acutely decreases deprivation-induced chow intake, but it fails to affect consumption of palatable solutions. *Peptides, 93*, 13-19.

[3] Fredriksson, R., Hagglund, M., Olszewski, P. K., Stephansson, O., Jacobsson, J. A., Olszewska, A. M., Levine, A. S., Lindblom, J., & Schiöth, H. B. (2008). The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. *Endocrinology, 149*(5), 2062-2071.

[4] Head, M. A., Jewett, D. C., Gartner, S. N., Klockars, A., Levine, A. S., & Olszewski, P. K. (2019). Effect of oxytocin on hunger discrimination. *Frontiers in Endocrinology, 10*, 297.

[5] Striepens, N., Kendrick, K. M., Hanking, V., Landgraf, R., Wüllner, U., Maier, W., & Hurlemann, R. (2013). Elevated cerebrospinal fluid and blood concentrations of oxytocin following its intranasal administration in humans. *Scientific reports, 3*, 3440.

[6] Mens, W. B., Witter, A., & Greidanus, T. B. V. W. (1983). Penetration of neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): half-times of disappearance of these neuropeptides from CSF. *Brain research, 262*(1), 143-149.

[7] Mizuno, T. M., Kleopoulos, S. P., Bergen, H. T., Roberts, J. L., Priest, C. A., & Mobbs, C. V. (1998). Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes, 47*(2), 294-297.

[8] Romero-Pico, A., Vázquez, M. J., González-Touceda, D., Folgueira, C., Skibicka, K. P., Alvarez-Crespo, M., Van Gestel, M. A., Velásquez, D. A., Schwarzer, C., & Herzog, H. (2013). Hypothalamic κ-opioid receptor modulates the orexigenic effect of ghrelin. *Neuropsychopharmacology, 38*(7), 1296-1307.

[9] Covey, L. S., Glassman, A. H., & Stetner, F. (1999). Naltrexone effects on short-term and long-term smoking cessation. *Journal of Addictive Diseases, 18*(1), 31-40.

[10] Sunter, D., Morgan, I., Edwards, C. M. B., Dakin, C. L., Murphy, K. G., Gardiner, J., Taheri, S., Rayes, E., & Bloom, S. R. (2001). Orexins: effects on behavior and localisation of orexin receptor 2 messenger ribonucleic acid in the rat brainstem. *Brain research, 907*(1-2), 27-34.

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

[12] Zhan, C., Zhou, J., Feng, Q., Zhang, J.-e., Lin, S., Bao, J., Wu, P., & Luo, M. (2013). Acute and long-term suppression of feeding behavior by POMC neurons in the brainstem and hypothalamus, respectively. *Journal of Neuroscience, 33*(8), 3624-3632.

[13] van den Heuvel, J. K., Furman, K., Gumbs, M. C., Eggels, L., Opland, D. M., Land, B. B., Kolk, S. M., Narayanan, N. S., Fliers, E., & Kalsbeek, A. (2015). Neuropeptide Y activity in the nucleus accumbens modulates feeding behavior and neuronal activity. *Biological psychiatry, 77*(7), 633-641.

[14] Vucetic, Z., Kimmel, J., & Reyes, T. M. (2011). Chronic high-fat diet drives postnatal epigenetic regulation of μ-opioid receptor in the brain. *Neuropsychopharmacology, 36*(6), 1199-1206.

[15] Hsu, E.A., et al., Oxytocin and naltrexone successfully treat hypothalamic obesity in a boy post-craniopharyngioma resection. *The Journal of Clinical Endocrinology & Metabolism*, 2018. **103**(2): p. 370-375.

[16] Lawson, E.A., et al., Oxytocin reduces caloric intake in men. *Obesity*, 2015. **23**(5): p. 950-956.

[17] Thienel, M., et al., Oxytocin's inhibitory effect on food intake is stronger in obese than normal-weight men. *International journal of obesity*, 2016. **40**(11): p. 1707-1714.

[18] Burmester, V., S. Higgs, and P. Terry, Rapid-onset anorectic effects of intranasal oxytocin in young men. *Appetite*, 2018. **130**: p. 104-109.

[19] Spetter, M.S., et al., Oxytocin curbs calorie intake via food-specific increases in the activity of brain areas that process reward and establish cognitive control. *Scientific reports*, 2018. **8**(1): p. 1-11.

[20] Ott, V., et al., Oxytocin reduces reward-driven food intake in humans. *Diabetes*, 2013. **62**(10): p. 3418-3425.

[21] Plessow, F., et al., Effects of intranasal oxytocin on the blood oxygenation level-dependent signal in food motivation and cognitive control pathways in overweight and obese men. *Neuropsychopharmacology*, 2018. **43**(3): p. 638-645.

5.7 Supplementary Material

5.7.1.1 Establishing effect of different doses of I.V. NTX on deprivation-induced chow intake

We sought to investigate the effect of different doses of I.V. NTX on reducing deprivation-induced chow intake in adult rats. Regular chow was removed at 16:00 on the day preceding administration. At 10:00 on the experimental day animals were injected intravenously with either isotonic saline or 0.1, 0.3, 1 or 3mg/kg NTX (n=7/group) before regular chow was returned, and consumption was measured 2 hours after administration. Water was available at all times. Data were analysed with a Student's t-test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leqslant 0.05$.

5.7.1.2 Establishing effect of different doses of I.V. NTX on sucrose solution intake

We sought to investigate the effect of different doses of I.V. NTX on reducing palatable sucrose solution intake in adult rats. Rats maintained on ad libitum food and water had standard chow and water removed, before receiving an intravenous injection of either isotonic saline (n=15/group) or 0.003, 0.1, 0.3 or 1mg/kg NTX (n=7/group). Animals were then presented with 10% sucrose solution and consumption was measured 2 hours post-injection. The animals had received previous episodic (2 h per day, 5 and 10 days before the study) exposure to this sucrose solution to avoid neophobia. Data were analysed with a Student's t-test. Values are presented as means ± S.E.M and they were deemed significantly different when $p \leqslant 0.05$.

5.7.2 Establishing effect of different doses of I.V. NTX on deprivation-induced standard chow intake

I.V. administration of NTX was ineffective at significantly reducing deprivation-induced standard chow intake after 2 hours post-injection (Fig 4.2).

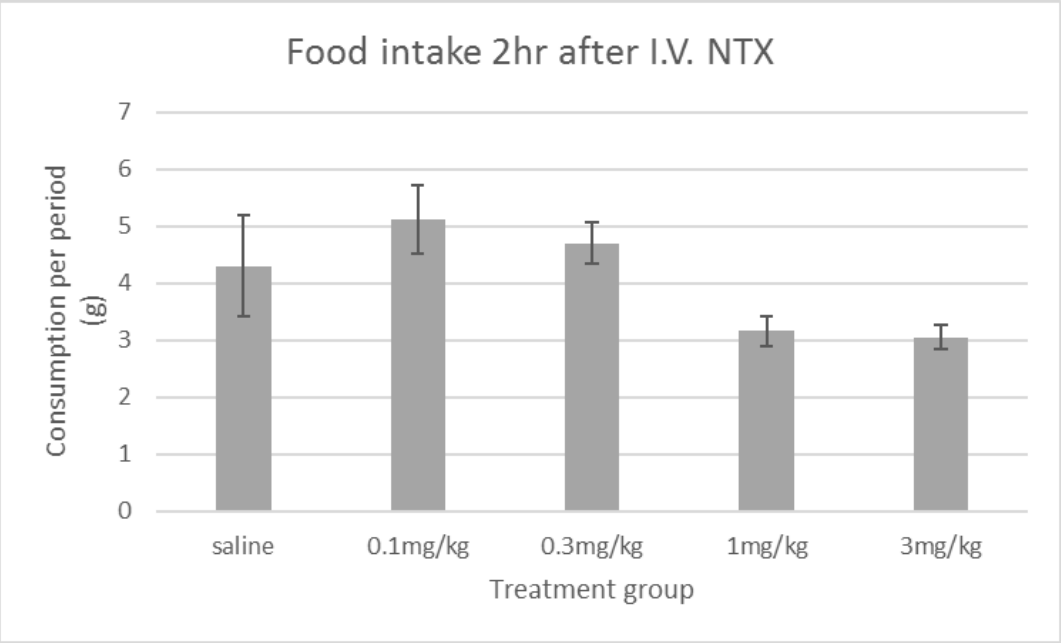


Figure 5.11 Effect of I.V. NTX on deprivation-induced standard chow intake after 2 hours. No significant difference in deprivation-induced standard chow intake 2 hours after I.V. administration of NTX.

5.7.3 Establishing effect of different doses of I.V. NTX on sucrose solution intake

I.V. administration of NTX at 0.3 and 1mg/kg significantly reduced palatable sucrose intake at 2 hours (P=0.0045 and P=0.0016, respectively; Fig 4.5) post-injection. 0.1mg/kg NTX was the highest dose that did not produce a significant effect on sucrose intake alone.

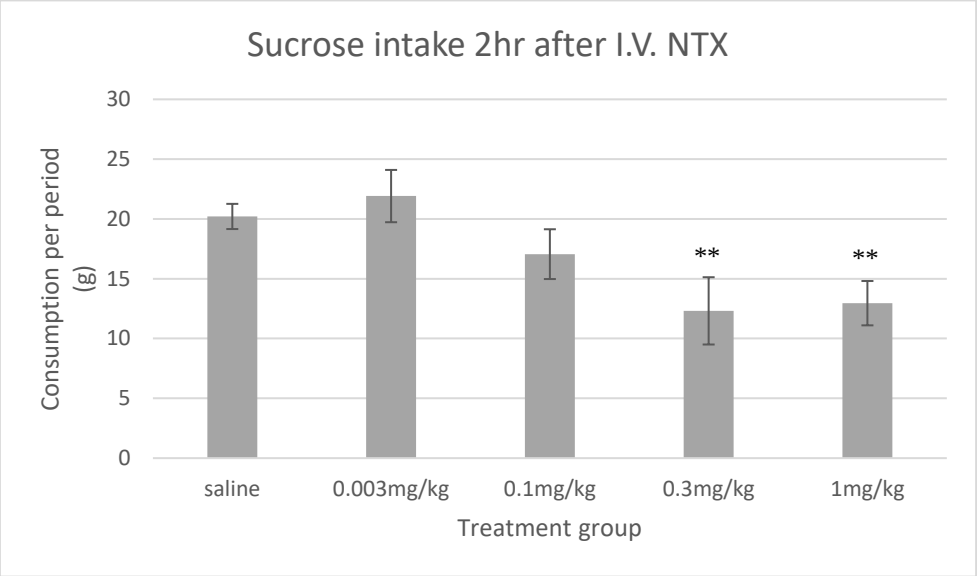


Figure 5.12 Effect of I.V. NTX on sucrose solution intake after 2 hours. **p<0.01.

Chapter 6
Discussion and Perspectives

Overeating, and addiction in general, can develop from a dysregulation of one or more aspects of the complex feedback loops that maintain homeostasis. As repeated stimulation of any one system occurs, biology consistently responds by altering the sensitivity of these systems, until eventually requiring the maintenance of a much higher level of stimulation to reach equilibrium. Over time, this can allow saturated receptor systems to shift the threshold for homeostasis outside regular boundaries, and begin driving behaviour beyond what is healthy, and into dangerous levels.

These factors that drive consumption involve: a feeling of hunger (which determines the motivation to seek calories and food choices), satiation (which underpins the process of termination of ingestive behaviour), and reward processing (which can to a large extent shift the hunger-satiety continuum, adjusting consumption to the 'pleasantness' of food instead of to the actual energy needs of the organism). In our search for neuroactive agents to curb excessive food consumption, those that affect more than one facet of feeding control are of particular interest, as they target a broader range of appetite regulating processes.

One system responsible for regulating these factors is that of OT. While OT has been reliably shown to reduce food intake in deprived and non-deprived rats [1-5], it appears to predominantly act on one of these factors in particular, as a mediator of early satiation, regulating homeostatic drives for consumption. While satiation initiates the early termination of consumption, hunger and reward processing can be powerful in their ability to shift the threshold for satiation signals to occur, and so should also be regulated for any robust treatment aiming to reduce unhealthy consumption. Reward processing is a key factor in this goal, as in this evolutionarily unique time of living in an ‘environment of plenty’, humans in particular

are bombarded with rewarding stimuli, and our physiology responds by constantly shifting the sensitivity of this system to deal with the 'new normal' level of arousal. Aiming to reduce activation of the reward system is one strategy to bring it back into a more balanced state, and so blocking these reward signals would act to allow the system to bring its threshold for equilibrium back to a healthy level. This reward processing system is relatively well understood, and opioid neurotransmitter signals have been consistently implicated as integral in its function [6-8]. Utilizing opioid receptor antagonists has proven effective at reducing the rewarding signals derived from addictive behaviour, ranging from tobacco smoking cessation, consumption of drugs of abuse, overeating palatable foods, and even running in models of anorexia [8-12]. While OT has been shown to communicate homeostatic signals of satiety, opioid antagonists can similarly regulate reward-related aspects of consumption, and so may present a robust therapeutic combination to target multiple facets of the drive to consume.

After more than a century of search for pharmacological treatments that combat obesity, our choice of FDA-approved pharmaceuticals is currently limited to five. Two of those drugs, Qsymia (phentermine + topiramate) and Contrave (bupropion + NTX), are combination medicines: this fact reflects the need to simultaneously target many neural and neuroendocrine systems in order to increase the likelihood of effectively treating this multifactorial condition. The combination medicine approach served as the basis of the recent case report in which hypophagic properties of OT, a molecule being subject of several clinical trials related to disordered appetite and obesity, were successfully augmented by co-administration of NTX in a patient with hypothalamic obesity caused by craniopharyngioma resection [13]. Indeed, the recent case-study describing the therapeutic combination of OT and NTX in treating hypothalamic obesity of an adolescent boy post-craniopharyngioma has shown promise for this strategy [13]. The current set of studies, using a systematic analysis of food intake, body weight and gene expression in laboratory animals, shows for the first time that some beneficial effects

of the OT+NTX combination extend beyond the etiologically unique case described by Hsu et al. I aimed here, to investigate this drug combination and elucidate underlying neuromolecular mechanisms of its action.

To begin with, I note that while this combination seeks to target aspects of feeding related to both satiety, and reward-processing, this strategy has not addressed the important facet that is hunger. This visceral signal alters motivation to seek calories and shapes food choices, while being subtly distinct from satiation, which underlies the termination of ingestive behaviour. Satiation can occur as a response to physiological signals of distress, and is evoked by physical and chemical qualities of ingested food, triggering hormonal responses from the gastrointestinal tract and neural activation within the brain [14]. Signals of stomach distension, where the stomach is becoming full and may burst if feeding continues, or signals of gastric motility, where something toxic has been ingested and its consumption should be terminated, are communicated via OT release [15-17]. One might imagine that drinking a lot of water will eventually fill the stomach, and result in feeling "full" or sated, even though there may remain an energy deficit, and still feel hungry. OT will, in such a case, signal the inhibition of food intake, acting to regulate homeostatic signals for consumption [18]. In this way, satiation is distinct from hunger, or the perception of energy deficit, and so when developing a strategy to target multiple aspects of food intake, I first aimed to investigate if OT or NTX might also influence this third factor driving ingestive behaviour, as they do with satiation and reward-processing respectively.

In the first chapter, I aimed to ask this question of OT, by investigating if, while significantly reducing food intake, OT can alter a feeling of hunger. I utilized a unique hunger discrimination paradigm using operant techniques to allow rats to respond with a lever-press associated with a feeling of hunger. After successful training, rats were able to reliably report on their subjective feeling of hunger, and so we were able to investigate the effects of certain peptides

on this important facet of ingestive behaviour. Importantly, this behavioural paradigm has shown efficacy in parsing satiation from hunger, as I show that neuropeptide Y (NPY) administration indeed produces behavioural responses associated with hunger [19].

We first show that I.P. OT decreases calorie-dense palatable food consumption in both 2h- and 22h-deprived animals, however is more potent in its anorexigenic effect in the 2h-deprived group. Consumption of sweet food after 2h-deprivation would be predominantly stimulated by palatability, whereas after 22h-deprivation, more so by hunger or energy deficit. This begins to suggest that OT's hypophagic effect can influence "free" or "hedonic" intake, but may be less related to hunger, which is then reinforced by the operant behavioural studies. The fact that i.p. OT did not reduce operant responding to 22 h of deprivation strongly suggests that OT does not interfere with mechanisms that promote a feeling of hunger. OT did not have an effect on hunger discrimination even though it was used at doses that are anorexigenic, and despite the fact that it did decrease bar pressing rate in general. It also produced a trend toward a reduction in chow intake in the 1 h after the completion of the operant test when animals were placed in a transition cage with chow present on the floor. Consequently, it can be inferred that OT induces hypophagia by being part of neuroendocrine processes that facilitate satiation and early cessation of feeding, rather than affecting hunger.

Furthermore, that i.p. OT differently affects neural processing under deprivation than in satiety is further substantiated by the outcomes of the c-Fos mapping study that revealed distinct patterns of c-Fos immunoreactivity in response to the OT treatment in hungry vs. sated animals. In animals deprived for only 2h, and thus relatively sated, OT affected c-Fos immunoreactivity in a broad range of hypothalamic and brain stem sites related to energy balance, including the DMH, ARC, NTS and DMV, while this network was unaffected by OT in 22h-deprived, hungry animals. Similarly, our PCR data show a decreased expression of the OT receptor gene in the brain stem of deprived or hungry animals, a region thought to mediate anorexigenic

properties of systemic OT, providing an additional insight into a mechanistic change that might be a key contributor to the changed hunger/satiety-dependent receptivity of the CNS to OT.

We therefore conclude that systemic OT does not diminish a feeling of hunger before the start of a meal. Instead, OT's anorexigenic properties can be manifested once consumption has already begun and this, at least to some extent, is driven by changes in brain responsiveness to OT treatment in the hungry vs. fed state. Therefore, OT's role in feeding control should be viewed as a mediator of early satiation rather than as a molecule that diminishes a perceived need to seek calories.

We find that OT indeed influences one aspect that drives consumption, and that is predominantly satiety alone. In our goal to target multiple facets of food intake, I then hope to explore the effects of NTX, a non-selective opioid receptor antagonist acting primarily on reward-related drivers of consumption, but again must ask if targeting the opioid system perhaps might also influence the third factor, that is hunger. I subsequently utilized our same hunger discrimination paradigm to measure behavioural responses associated with hunger to explore if modulating the opioid receptor network will alter the perception of energy deficit, or hunger. Our behavioural data show that opioid receptor agonists fail to induce operant responses associated with hunger or 22h deprivation, while neuropeptide Y (NPY) administration does significantly induce such hunger-associated responses in sated animals deprived for only 2h. Most importantly, NTX, our candidate molecule to diminish reward-related consumption, correspondingly did not reduce the hunger discriminative stimuli induced by either 22 h deprivation, or NPY administration in 2 h food-restricted subjects. This is interesting given that the doses of NTX used therein were indeed sufficient to decrease deprivation-induced feeding in a non-operant setting in animals familiar with consequences of 2 h and 22 h deprivation. I therefore conclude that the opioid system indeed promotes feeding for reward rather than in order to replenish lacking energy, or the perception of hunger, and

that blocking opioid receptors with NTX, while effective at reducing palatable food intake, does not do so by influencing the perception of hunger.

I find that while both OT and NTX have proven effective at inhibiting food intake in certain situations, they appear to target differing aspects of the motivation to consume, without influencing the feeling of hunger. These disparate systems are indeed intrinsically linked, as the opioidergic reward system can act to modulate the magnitude of a homeostatic feeding response by altering the neuronal sensitivity to satiety signals [29].

I therefore hypothesized that by combining treatments that target these two different aspects of feeding behavior, such a treatment might produce potentiating effects that can translate to robust therapeutic benefits on curbing excessive consumption and seeking of palatable foods. To investigate this, I first aimed to find the subthreshold I.P. doses of both OT and NTX when administered alone, on reducing food intake. Then, using a range of feeding scenarios and different tastants, I found that when subthreshold doses of OT and NTX were combined, i.e. doses that did not produce an effect alone, this resulted in a significant reduction in food intake, including reducing deprivation-induced standard chow intake. This experiment showed that a dose of 0.1mg/kg OT, while insufficient to reduce high-fat high-sugar (HFHS) chow intake alone, when combined with 3mg/kg NTX, also found to be ineffective at reducing HFHS intake alone, produced significant reductions in palatable HFHS intake. This finding is remarkable as neither of these doses were effective at reducing food intake alone, yet when combined, seem to produce a potentiating hypophagic effect.

By subsequently testing these hypophagic doses of OT-NTX in a CTA test, we were able to confirm that the hypophagic effect of this drug combination was not the result of malaise or an aversive association. This is important considering that previous reports have shown that opioid receptor blockade with naloxone can potentiate the aversive effects of LiCl treatment [20]. Similarly, opioid receptor agonism has been found to completely block the acquisition of

conditioned taste aversions in some paradigms [21]. Therefore, as the aversive properties of LiCl and other malaise-inducing agents, are mediated by both the opioid system, and OT and vasopressin cells in the PVN and SON [21], the finding that the hypophagic dose of this drug combination did not produce an aversion similar to that of LiCl is encouraging.

Furthermore, using the combination of OT and NTX at the doses found to induce acute hypophagia in all episodic meal scenarios (OT 0.1mg/kg and NTX 3mg/kg), neuronal activation was investigated with c-Fos immunohistochemistry. This aimed to elucidate changes in neuronal circuitry that underlie the observed alterations in feeding behavior. The findings of this study showed that the OTNTX drug combination resulted in robust changes in brainstem-hypothalamic networks related to satiety and energy, as well as reward-processing regions, thus targeting multiple aspects of feeding behavior.

While this is indeed promising, investigating longer-term effects of the drug combination on total caloric intake and bodyweight is necessary. While here I used adolescent animals to match the human case study, observing chronic effects was not feasible due to the relatively short adolescent period in rats of around 2 weeks [22]. For this reason, further studying the effect of this therapeutic combination on chronic food intake and body weight in adult rats would be advantageous. To do this, I again aimed to find subthreshold doses of the drugs alone on reducing food intake, and combined these for a potentiating effect, before investigating the chronic effect of the drug combination on caloric intake, body weight and gene expression. Interestingly, in adult rats, the OTNTX drug combination, while effective at reducing palatable food intake, did not produce a significant decrease in deprivation-induced chow intake, as it did in adolescent rats. We indeed find that adolescents may be more responsive to anorexigenic signals than adults. Rigamonti et al. found that obese adolescents produced an increase in endogenous anorexigenic peptides GLP1 and PYY in response to slow-rate feeding, while obese adult subjects did not. However, postprandial responses of insulin and triglycerides were

higher in obese adults than in obese adolescents [23]. This outlines that adolescents may be more responsive to satiety cues than their adult counterparts, and therefore anorexigenic treatments may be more effective for adolescents than in adults.

However, in adult animals, I still observed the acute effect on certain palatable feeding scenarios, and found that the combination had a cumulative effect on reducing palatable food intake with chronic administration, yet the beneficial effects on caloric intake and body weight were offset by compensatory feeding of non-palatable standard chow in the remainder of the day. While palatable HFHS chow intake, available for 2h daily after drug administration, showed a significant reduction, total caloric intake over the entire study was not reduced, as animals showed a compensatory increase in standard chow intake in the remaining 22hrs of the day, resulting in no significant decrease in bodyweight over the study period.

We had hypothesised that by blocking the opioid receptors with NTX, acting as a non-selective opioid receptor antagonist to diminish reward-based drives to consume, and with OT stimulating the homeostatic response for early cessation of meal termination, that I might regulate both aspects of feeding motivation, to reduce caloric intake and body weight. This combination treatment has proven to be effective at reducing palatable food intake of the immediate meal, utilizing a dose of NTX that produces no significant effect on HFHS intake when administered alone, and therefore implying that the combination with OT produced a synergistic, potentiating effect. However, while OT is effective at reducing acute consumption of standard chow [1], OT's anorexigenic effects may have worn off after the 2hr daily HFHS period, and therefore did not protect against a compensatory increase in standard chow intake in the following 22 hours of the day. OT has been shown to be an important signal for early meal termination, however, is primarily effective after the beginning of a meal [1]. For this reason, what might be beneficial is to administer OT directly before each meal. Studies have shown that while OT is effective at reducing food intake acutely, it fails to maintain efficacy

with chronic administration, as it is rapidly metabolized within 20 minutes in the CNS [24; 25]. This therefore accounts for the observed effects of the OTNTX drug combination that significantly reduced intake of the immediate meal, but fails to influence a 24-hour feeding period. Indeed, a systematic review and quantitative meta-analysis on the effects of OT on food intake was recently published, which examined over 2000 studies in animals and humans. This review showed that while a single dose of OT was effective at reducing food intake, whether administered centrally or peripherally, chronic administration did not produce a significant anorexigenic effect [26].

By then investigating changes in gene expression, I found robust neuromolecular changes within brain regions associated with energy-intake and reward-processing. I found changes in hypothalamic and brain stem POMC mRNA expression as the most significant changes after anorexigenic drug administration, and evidence supporting POMC activity within the NTS of the brain stem being previously associated with inhibition of food intake [20]. This is interesting as our adolescent study using immunohistochemistry also found changes in brain activation of DMH, ARC and NTS networks associated with POMC neurons that inhibit food intake.

We therefore find that OTNTX combination indeed reduces acute food intake, but did not result in long-term beneficial reductions in body weight, as animals showed a compensatory increase in standard chow consumption that offset the acute reductions in HFHS intake.

In sum, I aimed to target multiple aspects of feeding and found that, while OT predominantly targets satiation, and opioid receptor antagonism by NTX targets eating for palatability, neither of these drugs influence the third aspect driving consumption, which is perception of hunger. Nonetheless, I found by combining these two peptides targeting satiation and reward, that they produce a synergistic effect on reducing acute food intake, as subthreshold doses of these drugs were able to be combined to produce a significant reduction in consumption. This effect was

most pronounced in adolescent animals, which showed acute hypophagia in all feeding scenarios, and may be more responsive to satiety cues than their adult counterparts [23].

We have found that while these peptides target satiety and reward-related drives for consumption, that they do not influence the perception of hunger, and so in future studies, investigation of a third peptide that might also target the final aspect of feeding behaviour, could improve efficacy. I found in the hunger discrimination studies that NPY was indeed capable of inducing hunger-associated behavioural responses, and therefore one might propose that an NPY antagonist or similar may be a candidate for this combination. Cholecystokinin (CCK) is another molecule that has shown merit in influencing hunger in such hunger discrimination paradigms [27], and may be a promising candidate for combination with OT and NTX to further target all aspects driving consummatory behaviour.

In general, I have shown that the strategy of targeting multiple aspects of feeding in order to reduce consumption is a promising approach in developing more advanced treatments for this purpose. This method has proven to produce synergistic hypophagic effects, as the systems of satiety, reward and hunger are indeed intrinsically linked [29]. Therefore, in the long term it might be beneficial to explore further methods of targeting these distinct facets of feeding, especially to further incorporate the elusive role of hunger. This may involve the combination with a hunger-mediating peptide such as CCK, or perhaps stimulation of the vagus nerve, which has recently shown promise in mediating hunger responses without the use of pharmaceuticals [28].

Conclusions

The overarching aim of this doctoral thesis was to investigate the effect of combining OT and NTX at subthreshold doses, on food intake and the corresponding neuromolecular changes. The findings of the study are:

- OT inhibits food intake of the immediate meal by regulating homeostatic signals of satiety, and not by influencing the perception of hunger.
- Opioid receptor antagonism inhibits intake of palatable foods by regulating signals of pleasure and reward, and not by influencing the perception of hunger.
- OT and NTX combination significantly reduces acute food intake by acting synergistically to target both homeostatic- and reward-related brain networks.
- OT and NTX combination reduce acute food intake by targeting multiple aspects of feeding, but does not reduce chronic caloric intake and body weight when administered daily.

References

[1] Head, M. A., Jewett, D. C., Gartner, S. N., Klockars, A., Levine, A. S., & Olszewski, P. K. (2019). Effect of oxytocin on hunger discrimination. *Frontiers in Endocrinology, 10*, 297.

[2] Herisson, F., Waas, J., Fredriksson, R., Schiöth, H. B., Levine, A. S., & Olszewski, P. (2016). Oxytocin acting in the nucleus accumbens core decreases food intake. *Journal of neuroendocrinology, 28*(4).

[3] Klockars, A., Brunton, C., Li, L., Levine, A. S., & Olszewski, P. K. (2017). Intravenous administration of oxytocin in rats acutely decreases deprivation-induced chow intake, but it fails to affect consumption of palatable solutions. *Peptides, 93*, 13-19.

[4] Olszewski, P. K., Klockars, A., Schiöth, H. B., & Levine, A. S. (2010). Oxytocin as feeding inhibitor: Maintaining homeostasis in consummatory behavior. *Pharmacology Biochemistry and Behavior, 97*(1), 47-54.

[5] Olszewski, P. K., Allen, K., & Levine, A. S. (2015). Effect of oxytocin receptor blockade on appetite for sugar is modified by social context. *Appetite, 86*, 81-87.

[6] Gosnell, B., & Levine, A. (2009). Reward systems and food intake: role of opioids. *International journal of obesity, 33*(S2), S54.

[7] Olszewski, P. K., Alsiö, J., Schiöth, H. B., & Levine, A. S. (2011). Opioids as facilitators of feeding: can any food be rewarding? *Physiology & behavior, 104*(1), 105-110.

[8] Olszewski, P. K., & Levine, A. S. (2007). Central opioids and consumption of sweet tastants: when reward outweighs homeostasis. *Physiology & behavior, 91*(5), 506-512.

[9] Covey, L. S., Glassman, A. H., & Stetner, F. (1999). Naltrexone effects on short-term and long-term smoking cessation. *Journal of Addictive Diseases, 18*(1), 31-40.

[10] Volpicelli, J. R., Alterman, A. I., Hayashida, M., & O'Brien, C. P. (1992). Naltrexone in the treatment of alcohol dependence. *Archives of general psychiatry, 49*(11), 876-880.

[11] Apovian, C. M., Aronne, L., Rubino, D., Still, C., Wyatt, H., Burns, C., Kim, D., Dunayevich, E., & Group, C. I. S. (2013). A randomized, phase 3 trial of naltrexone SR/bupropion SR on weight and obesity‐related risk factors (COR‐II). *Obesity, 21*(5), 935-943.

[12] Kanarek, R. B., D'Anci, K. E., Jurdak, N., & Mathes, W. F. (2009). Running and addiction: precipitated withdrawal in a rat model of activity-based anorexia. *Behavioral neuroscience, 123*(4), 905-912.

[13] Hsu, E. A., Miller, J. L., Perez, F. A., & Roth, C. L. (2018). Oxytocin and naltrexone successfully treat hypothalamic obesity in a boy post-craniopharyngioma resection. *The Journal of Clinical Endocrinology & Metabolism, 103*(2), 370-375.

[14] Ritter, R. C. (2004). Gastrointestinal mechanisms of satiation for food. *Physiology & Behavior, 81*(2), 249-273.

[15] Flanagan, L. M., Olson, B. R., Sved, A. F., Verbalis, J. G., & Stricker, E. M. (1992). Gastric motility in consious rats given oxytocin and an oxytocin antagonist centrally. *Brain research, 578*(1-2), 256-260.

[16] Calatayud, S., Quintana, E., Esplugues, J., & Barrachina, M. D. (1999). Role of central oxytocin in the inhibition by endotoxin of distension-stimulated gastric acid secretion. *Naunyn-Schmiedeberg's archives of pharmacology, 360*(6), 676-682.

[17] Nelson, E. E., Alberts, J. R., Tian, Y., & Verbalis, J. G. (1998). Oxytocin is elevated in plasma of 10-day-old rats following gastric distension. *Developmental brain research, 111*(2), 301-303.

[18] Arletti, R., Benelli, A., & Bertolini, A. (1990). Oxytocin inhibits food and fluid intake in rats. *Physiology & behavior, 48*(6), 825-830.

[19] Jewett, D. C., Klockars, A., Smith, T. R., Brunton, C., Head, M. A., Tham, R. L., Kwilasz, A. J., Hahn, T. W., Wiebelhaus, J. M., & Ewan, E. E. (2020). Effects of opioid receptor ligands in rats

trained to discriminate 22 from 2 hours of food deprivation suggest a lack of opioid involvement in eating for hunger. *Behavioural Brain Research, 380*, 112369.

[20] Flanagan, L. M., Verbalis, J. G., & Strieker, E. M. (1988). Naloxone potentiation of effects of cholecystokinin and lithium chloride on oxytocin secretion, gastric motility and feeding. *Neuroendocrinology, 48*(6), 668-673.

[21] Olszewski, P. K., Shi, Q., Billington, C. J., & Levine, A. S. (2000). Opioids affect acquisition of LiCl-induced conditioned taste aversion: Involvement of OT and VP systems. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology, 279*(4 48-4), R1504-R1511.

[22] McCutcheon, J. E., & Marinelli, M. (2009). Age matters. *European Journal of Neuroscience, 29*(5), 997-1014.

[23] Rigamonti, A., Agosti, F., Compri, E., Giunta, M., Marazzi, N., Muller, E., Cella, S., & Sartorio, A. (2013). Anorexigenic postprandial responses of PYY and GLP1 to slow ice cream consumption: preservation in obese adolescents, but not in obese adults. *Eur J Endocrinol, 168*(3), 429-436.

[24] Striepens, N., Kendrick, K. M., Hanking, V., Landgraf, R., Wüllner, U., Maier, W., & Hurlemann, R. (2013). Elevated cerebrospinal fluid and blood concentrations of oxytocin following its intranasal administration in humans. *Scientific reports, 3*, 3440.

[25] Mens, W. B., Witter, A., & Greidanus, T. B. V. W. (1983). Penetration of neurohypophyseal hormones from plasma into cerebrospinal fluid (CSF): half-times of disappearance of these neuropeptides from CSF. *Brain research, 262*(1), 143-149.

[26] Leslie, M., Silva, P., Paloyelis, Y., Blevins, J., & Treasure, J. (2018). A systematic review and quantitative meta‐analysis of the effects of oxytocin on feeding. *Journal of neuroendocrinology, 30*(8), e12584.

[27] Corwin, R. L., Woolverton, W. L., & Schuster, C. R. (1990). Effects of cholecystokinin, d-amphetamine and fenfluramine in rats trained to discriminate 3 from 22 hr food deprivation. *Journal of Pharmacology and Experimental Therapeutics, 253*(2), 720-728.

[28] Bodenlos, J. S., Schneider, K. L., Oleski, J., Gordon, K., Rothschild, A. J., & Pagoto, S. L. (2014). Vagus nerve stimulation and food intake: effect of body mass index. *Journal of diabetes science and technology, 8*(3), 590-595.

[29] Pecina, S., Cagniard, B., Berridge, K. C., Aldridge, J. W., & Zhuang, X. (2003). Hyperdopaminergic mutant mice have higher “wanting” but not “liking” for sweet rewards. Journal of Neuroscience, 23(28), 9395-9402.

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Appendix 1

This thesis is partially based on the following published and peer-reviewed papers:

Title: Effect of oxytocin on hunger discrimination (2019)

Title: Effects of opioid receptor ligands in rats trained to discriminate 22 from 2 hours of food deprivation suggest a lack of opioid involvement in eating for hunger (2020)