Anorexigenic effects of naltrexone in rats

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Abstract

The accessibility of cheap, palatable, and caloric food in the industrialized world has shifted the primary drive of eating behavior from hunger to pleasure; this change has played a significant role in the rise in the prevalence of obesity. Sugary and fatty foods are among the most preferred and, therefore, overconsumed tastants. Opioids are key neuropeptides that propel feeding for reward. Importantly, blocking the opioid receptors in laboratory animals and humans decreases intake of and preference for those diets that are highly palatable. One of those opioid receptor blockers, a non-selective antagonist naltrexone (NTX), has been used in animal and clinical studies related to excessive appetite. Consequently, NTX in combination with another molecule, bupropion, has been approved as a pharmaceutical to treat obesity. This success underscores the need for further characterization of anorexigenic potential of NTX to determine its effects in various eating behavioral contexts/scenarios, in pathophysiological conditions characterized by overeating, as well as in treatment strategies that utilize potential synergy with other molecules that decrease appetite. Therefore, as an **overarching goal** of this thesis I set forth **to further explore anorexigenic potential of NTX as a suppressant of palatability-driven consumption**.

Previous studies indicate that rats cannot discriminate NTX from saline, except at very high doses. However, in those discrimination experiments, NTX-treated animals had not been fed palatable foods, i.e., diets whose consumption affects the endogenous opioid system thereby potentially leading to changes in sensitivity to opioid receptor blockade. Thus, in **Specific Aim 1**, I **investigated the ability of rats to discriminate between NTX and saline subjected to chronic intermittent sucrose consumption**. In the experiments I used the operant setting to determine whether chronic intermittent 25% sucrose solution consumption allows rats to discriminate peripherally injected NTX from saline. Subsequently, I used a feeding regimen that paralleled the discrimination training schedule and examined the consequences of peripheral NTX treatment on c-Fos immunoreactivity (IR) in feeding-related brain regions. I found that rats given intermittent access to a sucrose solution learned to discriminate between NTX doses. None of the rats given only water learned to discriminate between NTX and saline. When access to the

sucrose solution was discontinued for 14 days, the rats lost this ability to discriminate. c-Fos IR analysis revealed a significant drug and diet interaction effect in various brain regions associated with feeding behavior, particularly the amygdala, nucleus accumbens, and hypothalamic sites. I conclude that unlike in animals that are maintained on a "bland" diet (thus those that do not overeat food as a result of hedonic stimulation), in individuals chronically consuming sugar, NTX evokes an interoceptive response that allows them to discriminate the drug. The heightened response of the limbic and hypothalamic areas to NTX suggests that it is associated with a broader involvement of pathways related to both homeostatic and hedonic processes.

Thus far no studies have been conducted on whether NTX reduces palatability-driven feeding in pathophysiological conditions characterized by dysregulated reward processing and excessive consumption of palatable tastants. One such condition is autism spectrum disorder (ASD). Thus, in **Specific Aim 2**, I studied **the effect of NTX on palatable food consumption and feeding-related brain activation in a valproic acid (VPA) rat model of ASD**. I investigated the effect of NTX on episodic and habitual consumption of palatable food (high-fat high-sugar chow (HFHS) and sucrose solution) in VPA ASD rats compared to the control non-VPA rats. Subsequently, I assessed changes in feeding-related brain areas in response to NTX in VPA animals after two weeks of chronic intermittent sugar access using c-Fos IR. I found that, even though NTX significantly suppressed the consumption of the HFHS diet and 10% sucrose solution in VPA rats, the VPAs required a 10-times higher dose of NTX (10 mg/kg) to generate hypophagia compared to controls (1 mg/kg NTX was effective). In order to determine the difference in the neural consequences of 1 mg/kg NTX injection between VPAs and controls that parallels the lack of hypophagia after 1 mg/kg NTX, I found that c-Fos IR response to NTX in several hypothalamic areas was more pronounced in non-VPA control rats, especially those consuming sucrose. On the other hand, there was a significant drug–diet interaction for c-Fos IR in central nucleus of the amygdala (CEA) in both VPAs and non-VPA controls. It suggests that the sensitivity of feeding-related hypothalamic circuit to NTX is particularly affected by the ASD pathophysiology.

Peripherally injected NTX decreases food intake. No animal studies have investigated whether NTX suppresses appetite when administered via a non-invasive intranasal (IN) route and – if so – whether this potential anorexigenic effect can be augmented by IN co-administration of another anorexigen previously shown to act synergistically with

injected NTX. Thus, in **Specific Aim 3,** I investigated **the effect of IN NTX administered either alone or with IN oxytocin (OT; peripheral OT injections decrease appetite) on palatability-driven feeding**. I examined the effect of IN OT, NTX, and OT + NTX on various aspects of consumption in rats: (a) deprivation-induced standard chow intake (b) high-fat high-sugar (HFHS) chow (c) sucrose and Intralipid solutions. In this set of pilot studies, I found that - surprisingly - IN NTX and OT + NTX did not decrease intake of standard chow after deprivation and produced a minimal effect on HFHS diet, sucrose, and Intralipid consumption. When rats were exposed to habitual sucrose consumption for four weeks, neither NTX nor OT + NTX had a significant impact on the sucrose consumption. In contrast, I found that IN OT alone decreases deprivation-induced intake of standard chow as well as of HFHS chow and sucrose in nondeprived rats. In the habitual sugar consumption paradigm, acute IN OT diminishes sucrose solution intake in animals accustomed to the 2-hour/day sucrose meal regimen. Finally, I assessed c-Fos changes in response to the acute IN OT administration in rats subjected to habitual sugar consumption. I found that IN OT alters c-Fos immunoreactivity in brain areas related to energy homeostasis and reward, including the CEA, the hypothalamic paraventricular, and the arcuate nuclei.

In sum, the findings of this thesis further demonstrate that NTX administered via peripheral injections in rats reduces consumption of palatable food, whereas NTX infused IN is a poor feeding suppressant. Chronic habitual consumption of palatable food facilitates the ability to discriminate peripherally injected NTX and this effect is associated with changes in neuronal activation in feeding-related brain sites. Finally, while NTX reduces palatable food consumption in VPA autistic animals, the minimum effective dose is 10 times higher than that in non-VPA controls, and this shift in sensitivity to anorexigenic effects of NTX is reflected by a reduced hypothalamic activity in NTX-treated VPAs.

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Chapter 1

Introduction

Excessive consumption of readily available high-calorie and palatable foods, such as high-sugar and high-fat diets, leads to the accumulation of body fat and weight gain. The brain plays a pivotal role in the regulation of appetite, with the endogenous opioid peptides being key in driving the consumption of palatable (i.e., rewarding) food. It is not surprising therefore that blocking the opioid receptors in laboratory animals and humans decreases intake of and preference for those diets that are highly palatable and preferred. There have been many attempts to use opioid receptor antagonists as pharmacological tools to curb reward-related overeating. Among those opioid receptor blockers, naltrexone (NTX), a non-selective antagonist, has been incorporated into the pharmacological toolbox of drugs that – alone or in combination with other molecules – aid in the treatment of excessive appetite and obesity.

Despite the many years that have passed since the development of NTX, as well as of its closely related counterpart, naloxone, and despite the fact that NTX in combination with bupropion is now used as an FDA-approved drug to treat obesity, there are significant gaps in our knowledge of how NTX affects specific aspects of consumption. For example, it remains to be elucidated whether the action of NTX intersects with hunger-driven feeding and whether this molecule's administration can be interoceptively discerned by an individual. It is unclear whether NTX is at all effective in treating excessive appetite in many of those psychiatric conditions which are accompanied by extreme interest in palatable foods. And it is unknown whether NTX – alone or in combination with molecules that promote satiation (rather than just decreasing a drive to pursue feeding reward) – is effective when administered intranasally (IN), via the route that in recent years has been most avidly explored due to the enhanced likelihood of molecules being able to reach the brain.

In this thesis, I outline the fundamental information pertaining to the regulation of appetite, with particular emphasis on the role of opioids. I will describe the effects of NTX on feeding and on brain mechanisms that shape consumption. Most importantly, I identify the gaps in knowledge of the effects of NTX as a suppressant of food consumption. This critical analysis of the literature leads to the project's specific aims and to basic research studies whose overarching goal is to expand our view of the usability of NTX to reduce palatable food consumption.

1.1 Obesity "Epidemic"

According to the World Obesity Atlas 2022, there are more than 1 billion obese people worldwide with 650 million adults, 340 million adolescents, and 39 million children [1]. It is estimated that 167 million adults and children will be overweight or obese by 2025. The World Health Organization (WHO) predicts that 1 in 5 women and 1 in 7 men will be obese by 2030 [2]. The prevalence of obesity has been increasing rapidly in both developed and developing countries. In high-income countries, obesity rates have been steadily increasing since the 1980s, while in low- and middle-income countries, the rates have been rising rapidly since the 2000s [3, 4].

1.2 Homeostatic regulation of feeding

Feeding is a complex physiological process consisting of three main stages: hunger (necessitating the replenishment of energy), satiation ("fullness", leading to termination of feeding), and satiety (a suppressed drive to seek and ingest calories). They ensue upon dynamic changes in brain signaling, coupled with the endocrine activity of the peripheral tissues, fluctuations in circulating nutrient levels, osmolality, gastrointestinal motility, and others. As the maintenance of internal milieu in the context of calorie intake and energy stores is the ultimate purpose of homeostatic control of feeding behavior, it is not surprising that the hypothalamus and brain stem, two regions reciprocally intertwined with each other and with the periphery, contain discrete sites and synthesize neuropeptides that directly affect hunger and satiety.

1.2.1 Hypothalamic-brainstem circuit components regulating appetite

The PVN was one of the first specific brain sites linked to feeding control. Surgical knife cuts and electrical lesions of the PVN have been found to cause hyperphagia and increased body weight in rats [5, 6]. Similarly, electrolytic lesions within the PVN produce overeating and weight gain in both male and female rats [7, 8]. The PVN neurons produce anorexigenic neuropeptides that terminate feeding and releases neuropeptides that stimulate catabolism, leading to an increased breakdown of fatty acids and lipids [9]. A lack of Sim1 transcription factor which is essential for the development of the PVN, results in hyperphagia and obesity in humans and laboratory animals [10]. The PVN is also involved in regulating water intake and osmotic balance [11]. The PVN is sensitive to various neurotransmitters and neurohormones involved in feeding behavior. Thus, central administration of feeding related neurotransmitters into PVN is used to identify neural pathways in the central regulation of food intake.

The central regulation of food intake is impacted by the lateral hypothalamic area (LHA) through the activity of multiple neurotransmitters that can stimulate or suppress appetite, as well as the receptors and second-order neurons involved in their signaling pathways [12]. Swanson and colleagues revealed that the LHA broadly projects throughout various regions of the forebrain, midbrain, and hindbrain, whereas the absence of a unified output region suggests that the LHA-mediated regulation of food intake is complex and heterogeneous in nature [13]. Many animal studies demonstrated that damage to the LHA results in a significant reduction or complete cessation of food and water consumption, and these animals were unable to survive without intragastric feeding [14]. In contrast, studies using electrical stimulation revealed that activation of the LHA region stimulates food and water intake [15].

The ventromedial nucleus (VMH) has a variety of receptors that respond to signals which impact feeding. Stimulation of the VMH has been reported to induce termination of food intake, while damage to the VMH leads to an increased appetite, excessive thirst, weight gain, and picky eating habits in laboratory animals [8, 16]. The nuclear receptor steroidogenic factor 1 (SF-1) knockout mice, which do not develop the VMH, have demonstrated increased body weight and adiposity [17].

The arcuate nucleus (ARC) has a partially permeable blood-brain barrier, where select hormones and nutrients present in the bloodstream are able to reach the ARC neurons and affect their activity, thereby facilitating the crosstalk between the periphery and CNS in the process of hunger-satiety control [18]. The ARC contains two distinct populations of the orexigenic neurons and anorexigenic neurons that have opposing effects on food intake [19]. A model of the ARC lesions that involves exposing neonatal rodents to monosodium glutamate leads to the development of obesity that persists throughout their lives [20]. Stimulating the neurons in the ARC of mice using optogenetics leads to an increase in their food intake, while decreasing the release of neurotransmitters from these neurons result in a reduction in their body weight [21].

The dorsomedial nucleus (DMH) regulates various physiological processes, including the secretion of hormones, body temperature, and circadian rhythms [22]. It receives input from cells in the ARC and brainstem, and sends projections into the PVN and LHA [23]. Damage to the DMH in rats leads to reduced food intake, decreased physical activity, and a decrease in overall body weight. Restricting the duration of feeding in rats resulted in the synchronization of their daily DMH activity, leading to the highest expression of c-Fos during their scheduled mealtime [24]. In the case of food restriction, rats without DMH lesions exhibited a significant increase in their physical activity, body temperature, and wakefulness before mealtime, but these effects were blocked when their DMH was damaged. DMH-lesioned rats also displayed reduced food anticipatory activity before meals compared to those of sham-operated animals when placed on a restricted feeding schedule [25].

The brain stem nuclei, including the nucleus of solitary tract (NTS), the area postrema (AP), and the dorsal motor nucleus of the vagus (DMNV) form the dorsal vagal complex which senses circulating metabolites and hormones released by peripheral organs [26]. The NTS neurons receive signals mainly from the gastrointestinal tract (via the vagus nerve) and the hypothalamic neurons in the PVN, LHA, and ARC. The NTS sends signals to other regions of the hindbrain [27]. The AP is responsible for detecting toxins in the blood, and damage to the AP reduces the vomiting response [28]. The DMNV controls the muscles involved in swallowing and digesting food, as well as in regulating the rate at which food moves through the digestive tract [29].

1.2.2 Neuroregulators of homeostatic feeding

The intricate circuit of brain sites involved in the homeostatic control of feeding behavior relies on direct neuropeptidergic communication as well as on the signaling conveyed by peripheral hormones that cross the blood-brain barrier or act via the vagus nerve. To illustrate the complexity of mechanisms through which the organism ensures that the consummatory behavior meets the energy needs of the organism, below I present several of these molecules whose action has been tied to maintaining internal balance.

1.2.2.1 Neuropeptide Y

NPY is synthesized by the ARC neurons, and it is one of the most potent orexigens known to date. Upon central administration, it increases intake of highly-caloric tastants and, when injected in animals having a choice between calorie-dense bland food vs low-calorie palatable diets, it is particularly effective at increasing intake of calories despite their relatively low gustatory attractiveness [30]. Laboratory animal studies have found that NPY stimulates appetite by reducing latency to eat, increasing the desire to eat, and delaying a feeling of fullness [31]. When animals were given a selection of different macronutrients, such as carbohydrates, fats, and proteins, injecting NPY resulted in an increased preference for carbohydrate intake, depending on the initial preferences of the animal. The amount of carbohydrate consumed under natural feeding conditions was found to be positively correlated with NPY levels in the ARC and PVN. Rats fed with either a high-carbohydrate or high-fat diet also showed higher NPY gene expression in the ARC when on a high-carbohydrate diet. Studies have shown that blocking the NPY system with NPY antibodies decreases food intake [32]. Inhibiting the expression of NPY in the ARC reduced weight gain and long-term food consumption [33]. However, overexpression or knockout of NPY does not have a profound impact on body weight [34].

1.2.2.2 Agouti-related peptide (AgRP)

AgRP is an endogenous antagonist of the melanocortin receptors that stimulates appetite. AgRP is co-expressed with NPY and these two orexigens produce a synergistic effect on feeding behavior [35]. AgRP neurons show increased activity in response to food deprivation in laboratory animals [36]. Removing AgRP neurons in neonatal mice

resulted in minimal changes in their food consumption and body mass. However, when AgRP neurons were removed from adult mice, it led to their eventual death, as these neurons play a crucial role in stimulating the appetite and motivation to eat. Administration of AgRP directly into the PVN increases food intake, whereas ablation of NPY/AgRP neurons in young mice decreases both their food consumption and body weight [37]. Chemogenetic inhibition of AgRP neurons leads to a notable reduction in food intake, while a complete removal of these neurons leads to starvation in animals [38].

1.2.2.3 Melanocortin peptides

Pro-opiomelanocortin (POMC) is a large polypeptide precursor that is processed to yield a variety of biologically active peptides, including α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH, and adrenocorticotrophic hormone (ACTH). ACTH and α-MSH bind to the melanocortin-4 receptors (MC4R) with similar affinity and regulate food intake. Intracerebroventricular administration of both ACTH and α-MSH suppresses food intake in normal rodents and obesity-prone, genetically modified ob/ob mice. [39]. Further, inhibition of MC4R with specific antagonists and deletion of the MC4R gene in mice is associated with an increased food intake, late-onset weight gain, and hyperinsulinemia[40, 41]. In humans, the presence of autosomal dominant mutations in the MC4R gene has been linked to severe obesity [42].

1.2.2.4 Cocaine- and Amphetamine-Regulated Transcript (CART)

CART is expressed at high levels in select hypothalamic nuclei, such as the PVN, DMH and LHA [43]. Administering recombinant CART peptide through an intracerebroventricular injection has been shown to reduce food intake and weight gain in a dose-dependent manner in both free-feeding and food-restricted conditions. CART administration increases c-Fos expression in brainstem and hypothalamic nuclei that regulate feeding, including the PVN, DMH, parabrachial nucleus and NTS [44]. Administration of CART through an intracerebroventricular or intra-PVN injection effectively reduced food intake in satiated rats that were exposed to NPY-induced overeating [45]. In addition, CART knockout (KO) mice gained weight when they were maintained on a regular chow diet, and when fed a high-fat diet: they consumed significantly more food than the wild-type (WT) controls [46].

1.2.2.5 Oxytocin

Oxytocin (OT) is primarily synthesized in the PVN and supraoptic (SON) nuclei and released throughout the CNS and into the general circulation via the neurohypophysis (for a detailed description of the role of this neuropeptide in feeding control, refer to Section 1.5.1 of this Introduction). OT increases the feeling of fullness after a meal, which leads to a reduction in overall consumption [47]. Central and peripheral administration of OT produces a significant reduction in food intake in rodents. These anorexigenic effects persist in DIO (diet induced obesity) non-human primates and obese humans [48]. OT also stimulates the release of some gut hormones that promote satiety [49].

1.2.2.6 Ghrelin

Ghrelin is secreted from the gastric fundus and the ARC. It is released before a meal and its secretion is influenced by fasting and insulin-induced hypoglycemia [50]. Within the central nervous system, ghrelin receptors are present in neurons that express AgRP/NPY in the ARC [51, 52]. When ghrelin binds to these receptors, it stimulates the release of NPY as well as AgRP, the latter of which inhibits the activity of melanocortin receptor MC4R-expressing neurons in the PVN [53]. This inhibition further increases food intake and counteracts the effects of POMC neurons. Central administration of ghrelin has been found to increase food intake in rodents. Further, administration of ghrelin in humans increases food intake [54, 55].

1.2.2.7 Cholecystokinin (CCK)

CCK is produced mainly in the small intestine and is secreted following the consumption of a meal [56]. CCK receptors are present in various regions of the brain, including the vagal afferent neurons, cerebral cortex, thalamus, hypothalamus, basal ganglia, and dorsal hindbrain [57]. CCK decreases food intake, particularly in the presence of fat, as fat takes longer to digest than other macronutrients, such as carbohydrates and protein [58]. Central and peripheral administration of CCK reduces food intake by affecting meal size in laboratory animals [59]. Intravenous administration of CCK has also been found to reduce meal size and duration of the meal in humans [58, 60]. Rats lacking the receptors for CCK result in increased food intake and obesity.

1.2.2.8 Glucagon-like peptide-1 (GLP-1)

GLP-1, derived from preproglucagon and rapidly inactivated by dipeptidyl peptidase-4 (DPP-4), enhances insulin secretion in response to glucose, suppresses glucagon release, and slows down gastric emptying [61, 62]. It also decreases consumption through GLP-1 receptors located in several regions of the brain, including the ARC, PVN, NTS and AP [63, 64]. In rodents, central and peripheral administration of GLP-1 inhibits food intake, and GLP-1 receptor antagonist, exendin 9-39, increases food intake [65]. Intravenous administration of GLP-1 and DPP-4-resistant GLP-1 analogs suppress food intake [66].

1.2.2.9 Peptide YY

Intestinal L cells synthesize peptide YY (PYY), which belongs to the family of PP-fold proteins [67]. PYY increases c-Fos activation in the ARC and reduces hypothalamic NPY mRNA expression [68, 69]. The amount of PYY that is released is proportional to the calorie content of the meal. PYY decreases food intake in mice, and IV administration of PYY reduces food intake in healthy humans [70]. PYY reduces food consumption in animals lacking melanocortin receptors, indicating that POMC neurons do not play a role in the anorexigenic effect of PYY [71, 72].

1.2.2.10 Leptin

Leptin is secreted from adipose tissue and participates to regulate the long-term energy balance [73]. Leptin and insulin primarily generate adiposity signals, and activate satiety to maintain energy homeostasis through receptors located in the hypothalamus [74]. Leptin triggers the activation of POMC/CART-expressing neurons in the ARC, increasing the concentration of c-Fos expression, whereas leptin inhibits AgRP/NPY neurons by increasing the expression of a suppressor of cytokine signaling-3 [75, 76]. Leptin deficient ob/ob mice, or mice which have mutations in the gene that encodes the leptin receptor (db/db mice), exhibit excessive appetite and become obese [77]. The administration of leptin restores normal body weight and food intake in ob/ob mice[78].

Although homeostatic signals play a significant role in eating behavior, food consumption is greatly modified by hedonic cues (this is especially relevant in the environment in

which foods are plentiful, where an individual can select between various tastants and self-compose the diet). Neuroimaging studies have found that individuals with obesity have an enhanced neural response to high-calorie food cues, and that appetizing food cues encourage reward-driven eating [79]. Thus, in the next section, I will discuss mechanisms underlying non-homeostatic hedonic eating, thus the processes that are critical from the standpoint of managing excessive food consumption in the obesogenic environment.

1.3 Hedonic eating

If our eating behavior were solely dependent on homeostatic control, it is quite likely that the majority of individuals would have a BMI within the healthy range. However, most mammals will consume more calories than their homeostatic requirements dictate if given access to highly palatable food options. Acute and chronic consumption of palatable foods overrides homeostatic appetite regulation by, for example, delaying satiety and enhancing the drive to seek and consume food [80, 81]. Habitual consumption of palatable foods has also been compared to drug dependence, leading to a shift in the homeostatic set point for energy balance [82]. Although homeostatic and hedonic feeding are considered independent mechanisms, they are largely integrated to regulate eating behavior.

1.3.1 Brain reward circuitry: the nucleus accumbens and ventral tegmental area are the key components regulating hedonics of consumption

The brain reward system is a complex circuit involved affecting the perception of pleasant stimuli, motivation to seek them, and reinforcement, as shown by, among others, electrophysiological studies [83] and pharmacological studies involving drug injections into specific brain areas [84, 85]. Among those sites, the nucleus accumbens (NAcc) and the ventral tegmental area (VTA), have a prominent role in regulating reward-driven feeding.

In the VTA, dopaminergic neurons make up roughly 60% of all neurons, whereas GABA is synthesized by approximately 35% of VTA neurons [86]. The dopamine neurons project to several areas of the brain, including the NAcc, prefrontal cortex, and amygdala. These projections are involved in various aspects of reward processing, including motivation, reinforcement learning, and hedonic pleasure [87]. VTA-derived dopamine

is released in the NAcc, which in turn sends GABA signals back to the VTA. This feedback loop is important for synchronizing the VTA-NAcc response upon exposure to rewarding stimuli. VTA lesions significantly reduce the motivation for food consumption. [88]. Lesions of dopaminergic neurons in the VTA result in a reduced consumption of palatable sucrose solutions [89]. Conversely, in hyperdopaminergic mice, there is an increased motivation to consume sucrose-sweetened fluids [90]. Activation of VTA GABAergic neurons using optogenetics in mice promotes an immediate onset of ingestive behavior and leads to a significant increase of palatable food consumption in the sated state [91].

The NAcc is located in the ventral striatum and is composed of two primary subregions, the core, and the shell. The core of the NAcc is primarily involved in processing the hedonic value of rewards, while the shell is involved in the motivational aspects of reward processing [92]. Both regions receive input from a variety of brain regions, including the prefrontal cortex, amygdala, and hippocampus, and project to downstream regions such as the ventral pallidum and the basal ganglia [93, 94]. This connectivity allows the NAcc to integrate information from different brain regions and modulate the reward circuit. Lesion studies provide insight into the functional role of the NAcc in reward processing, such as rodents with lesions in the NAcc shell having difficulty locating reward sources [95, 96].

1.3.2 Neuromodulators of feeding reward

Consumption of highly palatable foods, leading to overeating and potentially contributing to the development of food addiction. Among them, dopamine, opioids, and endocannabinoids play important roles in reward-driven eating behaviors.

1.3.2.1 Dopamine

Dopamine (DA) is synthesized in the in the midbrain and DA neurons project to various regions of the brain, including regions classically defined as involved in feeding control, such as the hypothalamus [97]. Dopaminergic signaling is regulated through DA receptors which are G protein-coupled [98, 99]. There are two major types of DA receptors, DA1 and DA2 receptors [100, 101]. The D1 receptors are generally associated with the excitatory effects of DA signaling and they promote reward seeking behaviors,

whereas DA2 receptors are generally associated with the inhibitory effects of DA signaling [102-104]. In the past few decades, the involvement of DA in reward has been consistently studied through lesioning of DA neurons, and via pharmacological modification of DA transmission [105]. In the regulation of food intake, DA has a significant effect on feeding frequency, and DA neurons in the VTA which project to the NAcc, regulate motivation for reward-driven eating [106]. Drugs of abuse and consumption of palatable foods lead to an increase in DA levels in specific regions such as the VTA and the NAcc, which underscores the common reward-related physiological neural platform for the behavioral phenomena observed upon exposure to addictive drugs and palatable tastants [107]. Animal studies have shown that pleasant tastants trigger the release of DA in the NAcc-shell, NAcc-core, and PFC. However, the response of these regions to DA varies depending on the type of stimulus, the degree of pleasure, taste, or novelty [88]. Microdialysis studies have shown that palatable foods elevate extracellular DA release in the NAcc in rodents [88, 108].

Many human and rodent studies have found that the DA pathway is activated in response to palatable food. Animal studies reported that DA is released in the NAcc upon consumption of palatable foods, and human studies with brain imaging showed that DA release in the NAcc is proportional to the pleasure associated with ingested palatable food [109]. DA-deficient animals, specifically those with an inactivated tyrosine hydroxylase gene in dopaminergic neurons, experience fatal hypophagia [110]. When DA is reintroduced into the caudate/putamen or the NAcc of these animals, they begin to eat again and exhibit a high preference for palatable foods [111]. Moreover, peripheral metabolic signals, such as ghrelin, leptin, and insulin, regulate DA signaling and participate in the regulation of eating for palatability [112, 113]. Leptin decreases DA release in the NAcc, and leptin deficiency in mice inhibits DA signaling [114]. Insulin has also been shown to decrease rewarding sensing, and intravenous administration of insulin decreases sucrose intake in laboratory animals [115].

1.3.2.2 Endocannabinoids

Endocannabinoids, the endogenous ligands for cannabinoid receptors, regulate reward-driven eating behaviors. They are produced in the brain and peripheral tissues, and they bind to two main receptors, cannabinoid receptor 1 (CB1) and 2 (CB2). While CB1 receptors are primarily located in the CNS, CB2 receptors are found in the periphery and

are involved in inflammation and immune responses [116]. Activation of CB1 receptors stimulates the release of DA in the brain, which promotes a feeling of pleasure and reward [117]. After prolonged exposure to palatable foods, CB1R binding increases in the mouse midbrain, while short-term exposure leads to a decrease in CB1R binding in the hypothalamus [118]. Obese mice have elevated levels of endocannabinoids and CB1R binding in the hippocampus [119]. Studies in rodents have demonstrated that the administration of CB1 receptor antagonists, which block the activity of CB1 receptors, reduces food intake and body weight [120]. In humans, the use of CB1 receptor antagonists as a weight-loss treatment has been investigated. The CB1R antagonist, rimonabant, reduces food intake and increases lipolysis and energy expenditure to promote weight loss [121, 122].

1.3.2.3 Opioids

Opioids regulate homeostatic and hedonic feeding processes by modulating hunger and satiety, by promoting preference for foods and by supporting the formation of addiction-like responses to palatable diets. Thus, the endogenous opioids' impact on the hedonic aspects of eating behavior contributes to the development of obesity. In the following section, I will focus on the opioid system and its effects on the consumption of palatable foods. Afterward, I will discuss the potential of an opioid receptor antagonist, naltrexone (NTX), to suppress the consumption of palatable foods, and I will identify the gaps in knowledge regarding the effect of NTX on palatability-driven eating.

1.4 Opioid system

Opioid peptides, naturally occurring small molecules, are produced in the CNS and endocrine glands, including the pituitary and adrenals [123]. The endogenous opioid peptides are divided into three families. These families are made up of proopiomelanocortin, which gives rise to β-endorphin; preproenkephalin, which is a prepro-molecule of leucine (Leu)- and methionine (Met)-enkephalins; and preprodynorphin, which is spliced to dynorphins, such as dynorphins A and B, and neoendorphins. All opioid peptides share a common N-terminal tetrapeptide sequence consisting of Tyr (tyrosine)-Gly (glycine)-Gly-Phe (phenylalanine), which has the affinity to the three types of opioid receptors [124-126].

In 1976, Gilbert and Martin classified opioid receptors into three categories, named after the drugs that were used to identify these receptors. The morphine group was labeled μ, the N-allylnormetazocine group was labeled δ, and the ketocyclazocine group was labeled κ [140]. Thus, the current opioid classification comprises μ (MOR), δ (DOR), and κ (KOR) receptors. The MOR is encoded by the OPRM1 gene, the DOR by the OPRD1 gene, whereas the OPRK1 gene gives rise to the KOR [141]. Opioid receptors are seven-transmembrane G-protein coupled receptors (GPCRs) [144]. Opioid receptor signaling involves a complex interplay between G-proteins, secondary messengers, and effector proteins which initiate cellular changes through Gi/Go transduction cascades [145]. When an opioid ligand binds to an opioid receptor, it causes a conformational change in the receptor that activates a heterotrimeric G-protein. The G-protein then dissociates into its alpha, beta, and gamma subunits, which can activate or inhibit downstream signaling pathways, including the adenylyl cyclase-cyclic AMP (AC-cAMP) pathway [146].

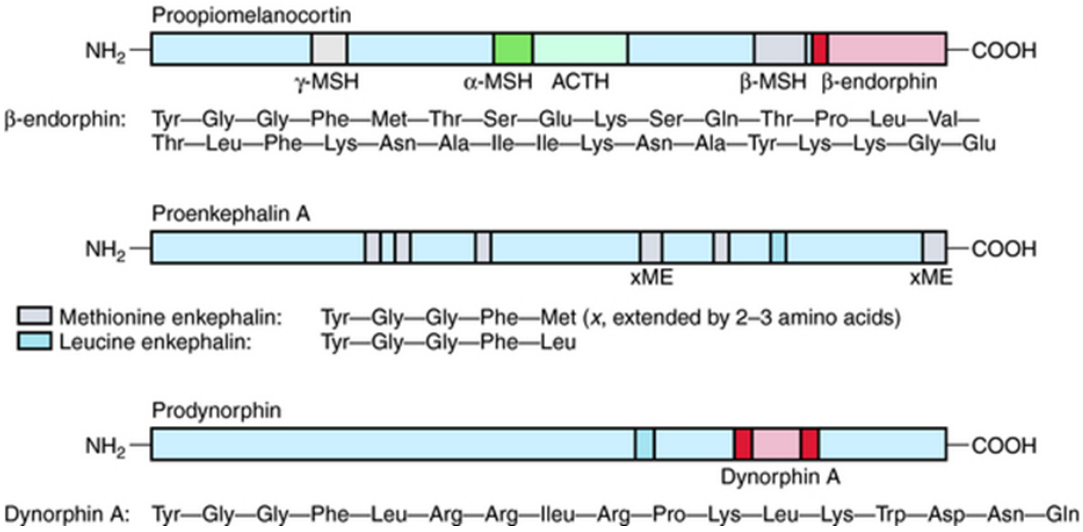

Figure 1.1: Endogenous opioids, their precursors, and main receptors. Adopted from [127]

Endorphins, enkephalins and dynorphins bind their respective receptors with high affinity, although there is some level of cross-reactivity, which can be exploited pharmacologically. β-endorphins primarily bind to the MOR, and to a lesser extent, the

DOR and KOR. Enkephalins activate cellular processes by binding to both MOR and DOR, but have a much greater affinity for the DOR [123]. Enkephalins do not affect KOR though. Dynorphins produce their effects mainly by binding the KOR, but they also bind the MOR and DOR, albeit with a relatively low affinity. [128].

Opioid peptides and receptors are very broadly expressed throughout the CNS, which well reflects their ability to influence reward-related phenomena (from food intake changes to maladaptive behaviors stemming from drug abuse), pain and anxiety (among many functions). Enkephalin-containing neurons are located in the spinal cord, periaqueductal gray region, amygdala and autonomic nuclei in the hypothalamus and brain stem [135]. Enkephalin neurons typically have short axons which suggest that they act locally [136]. Endorphins, including β-endorphin which is oftentimes studied in the context of feeding reward, are present in neurons in the hypothalamus (including the ARC) and in the NTS in the brain stem [137]. Dynorphin is mostly found in hypothalamic neurons. Unlike enkephalin neurons, β-endorphin and dynorphin neurons have long axons that reach far-off the sites where perikarya are localized [138]. The distribution of opioid receptors is broad. In situ hybridization and fluorescent labeling studies in genetically modified mice have demonstrated that binding sites for the opioid receptors are highly present in the limbic system, hypothalamus, cortex, and brain stem [142].

1.4.1 Role of opioids in reward-driven eating behavior

Consumption of palatable foods has been found to trigger the release of endogenous opioids in the hypothalamus, anterior cingulate cortex, and NAcc [129]. In laboratory animals, the administration of opioid agonists has been shown to increase food intake, while the use of opioid antagonists has been found to decrease it [130].

According to the conclusions reached in early studies, the effectiveness of opioid ligands on feeding behavior appeared to be influenced by the micronutrient content of the diet and many studies proposed that these ligands had a greater impact on high-fat diets compared to high-carbohydrate diets [131]. However, many recent findings suggest that the effectiveness of certain opioid antagonists or agonists depends on the individual food preference profile of animals [130]. For example, morphine administration has been linked to increased intake of the macronutrients that align with the pre-existing diet preferences of rats. Gosnell, et al found that rats with a preference for fat consumed a

greater amount of fat after morphine administration, while rats with a preference for carbohydrates consumed a greater amount of carbohydrates after receiving this opioid receptor agonist [132]. Moreover, when rats were given morphine injections in either the NAcc or the ventromedial striatum, they showed hyperphagia and consumed their preferred diets, regardless of the macronutrient content [133]. In addition, administration of another opioid agonist, DAMGO (([D-Ala2, N-MePhe4, Gly-ol]-enkephalin), into the NAcc was also found to stimulate the intake of high-fat diets in rats, but only in those rats that prefer fat [134]. It is noteworthy that the consumption of tastants with no caloric value, such as non-caloric sweeteners, is also increased by opioid agonists, which indicates that the opioid system promotes consumption in dissociation from the calorie consequences that ingesting a given diet will entail [135, 136].

Research suggests that the effectiveness of opioid ligands depends on the palatability of food. For example, the opioid receptor antagonist naloxone is more effective at decreasing the intake of palatable foods compared to less preferred ones [137, 138]. Subcutaneous injection of naloxone (0.01 mg/kg) resulted in a decrease in consumption of a preferred diet, while higher doses up to 3 mg/kg did not have any effect on the intake of a nonpreferred diet [130] . Similarly, Levine and co-workers observed more anorectic effect of naloxone in rats ingesting sweet chow-consuming than in those eating standard chow pellets [139]. Weldon et al reported that naloxone at low doses significantly reduced sucrose intake in ad libitum-fed or food-restricted rats [138]. Further, Kirkham et al revealed that the potential of naloxone to suppress sham feeding was more effective in high sucrose concentration than in less concentrated and less preferred sucrose solutions [140].

Data suggest that opioid receptor ligands affect the size of the meal but are unable to affect the initiation of the meal [131]. Naloxone has been found to decrease the number of pellets ingested by rats that have already eaten enough but it did not affect the initiation of the meal. According to a progressive ratio study by Rudski et al., administration of naloxone to rats that had ad libitum access to food led to a decrease in their breakpoint, indicating a reduction in their motivation to consume food [141]. Further, Rudski et al studied the effect of opioids on the maintenance of feeding[142, 143]. They used operant chamber techniques to study opioid-related feeding using an FR80/FR3 regimen and studied the effect of opioids on the initiation and/or termination of meals. In their

experimental protocol rats were pressing an operant lever 80 times to obtain the first food pellet. This initial stage was followed by an FR3 phase, in which the rats only needed to press the lever three times to receive each subsequent food pellet for the duration of the meal [143]. They investigated how the administration of subcutaneous saline or naloxone (at doses of 0.1, 0.3, 1, 3, and 10 mg/kg) affected feeding induced by NPY at a dose of 5 mg ICV [144]. Consistent with previous findings, the orexigenic effects of NPY significantly increased pellet consumption during a one-hour session, whereas naloxone decreased pellet intake [144]. NPY did not change the time taken for the first response, but it reduced the time taken to complete the FR80 schedule. Administration of naloxone at 3 and 10 mg/kg increased the latency of the first response and prevented NPY-induced completion of the first ratio.

Taste perception in rats and humans remains unchanged when opioid receptors are blocked. In rats, naloxone had no impact on their ability to differentiate sucrose solutions [145]. Arbisi and colleagues reported that blocking opioid receptors had little to no effect on the identification or detection of different tastes, although it did reduce the pleasure experienced from sweet solutions [146].

Interestingly, Pomonis et al found a significant interaction between naloxone and activation the central nucleus of the amygdala (CEA) and lateral division of the periaqueductal gray after long-term sucrose treatment [147]. The rats were given access to a 10% sucrose solution or water for 3 weeks followed by a 10 mg/kg injection of naloxone. Subsequently, limbic and autonomic regions of the forebrain and hindbrain were analyzed for c-Fos immunoreactivity. They revealed that after naloxone administration, c-Fos immunoreactivity. was elevated in the CEA in rats that consumed sucrose but not a standard diet [147]. These findings imply that sucrose consumption increases endogenous opioid tone in the reward circuits.

On the other hand, reward-driven eating behavior has been found to affect opioid circuits, either by promoting opioid release or by altering receptor sensitivity. Jewett et al investigated how sucrose consumption influences the ability to discriminate opiates [148]. Using an operant protocol, they trained rats to discriminate nalbuphine, an opioid antagonist, from saline. Initially, the rats were trained to discriminate buprenorphine at 3.2 mg/kg, and then 0.1-3.2mg/kg doses of nalbuphine were injected to establish a dose-response discrimination curve. Afterward, rats were given 30 minutes of a 30% sucrose

solution or water, but it did not impact their ability to differentiate between nalbuphine and saline. Then, after a waiting period, when the rats were given either sucrose or water for several days, and the ability of rats to discriminate nalbuphine increased by 3-fold.

Palatable food consumption has been found to affect expression of genes encoding opioid peptides in the hypothalamus [149]. Chronic exposure to palatable food can lead to a desensitization of the opioid system: ingestion of the same amount of food later on does not produce the same level of reward. Welch and colleagues examined how the consumption of palatable and bland diets affected gene expression of opioid peptides in the hypothalamus [149]. They conducted experiments in rats, providing them with four types of diets: high-starch diet ad libitum, palatable fat/sucrose diet ad libitum, a restricted fat/sucrose diet that provided the same number of calories as the high-starch diet, and a restricted fat/sucrose diet that provided only 60% of the calories of the unrestricted fat/sucrose diet. As expected, rats avidly consumed the unrestricted fat/sucrose diet and gained more weight than those on the high-starch diet. The rats on the unrestricted fat/sucrose diet also had higher levels of prodynorphin mRNA in the ARC and dynorphin A1-17 peptide in the PVN compared to the rats consuming the high-starch diet. However, there was no significant difference in the expression of proenkephalin or POMC genes in the ARC, nor in the levels of met-enkephalin or beta-endorphin in the PVN. When the fat/sucrose diet was restricted to match the calorie intake of the high-starch diet, mRNA levels of prodynorphin, proenkephalin, and POMC decreased relative to the unrestricted group. These findings suggest that overconsumption of a palatable diet leads to increased hypothalamic dynorphin A1-17 and pro-dynorphin mRNA levels, which may be related to energy consumption rather than taste preference. Notably, when rats were deprived of food, gene expression of opioid peptides in the hypothalamus decreased, indicating that restricting access to palatable food can result in a state of "hedonic deprivation" similar to that seen with food deprivation [150].

1.4.2 Naltrexone and food consumption

Opioid signaling leads to excessive consumption of palatable food, and it stimulates eating in general. Supporting this notion, when the MOR is removed from all cells in the constitutive MOR KO mice, these animals exhibit a lower drive to consume both standard chow and palatable food [151]. Studies on the administration of NTX directly into feeding-related areas of the brain show that NTX reduces intake of both regular and

palatable food, though there are some differences in the precise outcome of NTX treatment. For example, administration of NTX into the amygdala suppresses consumption of preferred food in animals that are not food-deprived, whereas NTX injection into the PVN decreases the intake of high-fat food regardless of initial preference, and of high-sugar food only in animals with a preference for sugar [152, 153]. Additionally, infusions of NTX into the PVN reduce the consumption of standard chow in rats that have been deprived of food. Changes related to the opioid system occur in feeding-related brain areas in response to highly palatable food intake over a period of time, upon both short- and long-term exposure [81]. These palatability-induced changes in opioid signaling can also be demonstrated through responsiveness to NTX treatment. NTX is more effective at reducing consumption of palatable food in rats maintained on high-fat or high-sugar diets compared to rats fed only standard bland chow. In rats, NTX significantly decreased the intake of standard chow and a 32% sucrose solution compared to standard chow alone [154]. Moreover, NTX produced a dose-dependent reduction in food intake in animals that had previously consumed palatable sucrose, Polycose, or saccharin solutions [154].

It has been found that NTX also reduces the positive rewarding effects of sugar in rats, as shown by a taste-reactivity test [155]. Levine et al suggested that NTX inhibits the formation of a preference for sugary foods after abstaining from them for a while [156]. In that study, rats were fed simultaneously a high-sucrose and a high-starch diet for 10 days and it was evident that they preferred sugary food. Afterwards, the rats were given only the starch diet for 10 days, and they were administered either NTX or saline via implanted mini osmotic pumps. The rats that received saline continued to prefer the high-sucrose diet, but those given NTX consumed only 33% of their energy from the palatable food and did not develop a preference for it again. However, when rats were not restricted from accessing the sucrose diet throughout the study, NTX did not affect their preference for sucrose.

In contrast, Sclafani’s laboratory, reported no indication of opioids' involvement in conditioned flavor or place preferences [157-159]. They found that NTX did not have an impact on flavor preference that had been established through the intragastric infusion of 16% sucrose or water. They also noted that the rats developed a preference for the flavor that was paired with the intragastric infusion of sucrose. Although NTX reduced the intake of the solution, it did not prevent the expression or acquisition of the flavor

preference. Bodnar and Sclafani also examined the impact of NTX on the acquisition and expression of a conditioned flavor preference in rats that were sham-fed and given a non-specific flavor paired with either saccharin or sucrose [157]. The rats were tested using a two-bottle preference test to determine the flavor that was paired with the preferred sucrose. Once again, NTX resulted in a decrease in feeding, but it did not have any effect on the acquisition or expression of flavor preference. Several studies showed that rats are generally unable to discriminate NTX from saline, except when given very high doses in operant experiments. It has been found that rats can differentiate between NTX only if they have been consistently treated with an opioid agonist such as morphine. However, the previous studies did not consider the effect of intermittent sucrose consumption on the ability of rats to discriminate NTX.

In humans, NTX reduces the intake of meals, meal size, and eating rate. Yeomans and Gray found that blocking opioid receptors with 50 mg NTX led to a reduction in meal size in male participants [160]. Furthermore, the subjects rated the food as less pleasant, resulting in a decrease in the speed of consumption. A subsequent study confirmed that intake of highly palatable sweet, high-fat, and high-protein foods was indeed reduced by NTX [161]. Correspondingly, Arbisi et al. demonstrated that while NTX did not affect the ability to taste or recognize the taste, it did diminish the pleasantness of sucrose solutions [146].

The fact that NTX selectively suppresses consumption driven by pleasure in animals and in humans, has led to the inclusion of this molecule in pharmacotherapies of obesity. In fact, NTX is now a component of Contrave, an FDA-approved anti-obesity medication that combines NTX and bupropion [162]. Furthermore, based on case studies involving hypothalamic obesity, in which NTX has been used in combination with oxytocin (OT) [163], there is interest in expanding the use of NTX in drug combination therapies, including by co-administering it with OT.

1.4.3 Opioids as part of a broader network that promotes habitual consumption of palatable foods

It is suggested that repeated exposure to palatable food leads to repeated releases of endogenous opioids. Colantuoni and colleagues conducted a study on opioid dependence in rats by providing them with a sweet solution for an extended period of time [164]. The rats were given access to a 25% glucose solution, along with laboratory chow, for 12 hours, and then they were deprived of food for 12 hours. Over a period of 10 days, the rats doubled their intake of the glucose solution and consumed a large amount of it within the first hour of access. After 30 days, the authors measured the binding of DA and opioid receptors in various brain regions of rats that were given access to the glucose solution compared to those given only regular laboratory chow. The group that received the glucose solution showed an increase in DA1 receptor binding in the NAcc shell and core. Further, there was an increase in MOR binding in the hippocampus, locus coeruleus, NAcc-shell, and the cingulate cortex. In another study, Colantuoni et al further reported that the administration of opioid agonists to rats that had been intermittently consuming glucose for a long time caused withdrawal symptoms, such as teeth chattering and anxiety, similar to those seen in food-deprived rats [165]. These withdrawal symptoms were not seen in the control groups that had intermittent access to regular food or ad libitum access to either regular food or sugary food. These findings suggest that intermittent sugar intake causes a dependent-like state.

In addition, different types of emotional and physiological stressors can also increase eating behavior in both humans and animals [166]. Many animal studies reported that stress triggers increased chewing and consumption [167]. For example, Antelman and colleagues found that even a minor tail pinch can cause gnawing, eating, and licking in rats [168]. Further, they found that the administration of antagonists of dopamine receptors decreases tail pinch-induced eating and gnawing. Similarly, Morley and Levine reported that naloxone reduced tail pinch-induced feeding in rats, but did not have any impact on gnawing or licking behavior [169]. This indicates that opioid circuits play a role in stress-related eating. Moreover, the effect of opioids and their receptors on food intake can be influenced by certain types of food. Sweet solutions and other tasty diets affect the plasticity of circuits expressing opioids and opioid receptors. It seems that opioids play a role in a spectrum of food experiences from palatable to aversive and it can be influenced by various environmental factors [81].

The repeated release of opioids in response to palatable food downregulates opioid receptors, reducing the sensitivity of the system and potentially leading to a cycle of overeating and decreased reward sensitivity [81]. This enhanced reward response contributes to a cycle of consumption of palatable foods, as individuals seek out more of these foods to achieve the same level of reward. Over time, this can lead to a reduced ability to feel satisfied with non-palatable foods as well as small quantities of palatable diets, further reinforcing the addiction-like behavior associated with palatable food consumption.

1.4.4 Interactions between the opioid system and other systems governing appetite

Data suggests the functional interplay between the opioid and endocannabinoid systems in hyperphagia, especially that related to hedonics. The cannabinoid receptor type 1 (CB1) and MOR1 co-localize in the same presynaptic nerve terminals and activate a common receptor-mediated G-protein pathway [170, 171]. This co-activation of the CB1 and MOR1 receptors produces an additive or synergistic effect in increasing food intake. Further, chronic opioid exposure has been found to increase endocannabinoid release, subsequent activation of CB1 receptors and, consequently, food consumption [172].

NPY is another orexigenc which seems to be acting in concert with opioids. Administration of NTX into the rostral NTS prior to NPY PVN injection resulted in the decrease in the feeding and thermogenic effects of PVN NPY injections alone [173]. Pomonis and colleagues also found evidence of a functional interaction between opioids and NPY [174]. They used c-Fos, a marker of neuronal activity, to track the effects of NPY injection into the PVN and peripheral injection of naloxone on brain regions related to feeding. The injection of NPY into the PVN increased food intake, which was prevented by peripheral administration of naloxone. PVN NPY also led to increased c-Fos in the PVN, which was independent of food intake. While peripheral naloxone inhibited NPY-induced feeding, it did not affect c-Fos in the PVN. According to Kotz and colleagues [175], both food deprivation and NTX treatment had significant and independent effects on the levels of ARC NPY mRNA and brown fat uncoupling protein mRNA. The increase in ARC NPY mRNA and decrease in brown fat uncoupling protein mRNA levels suggest that there is a complex interplay between hypothalamic NPY and

endogenous opioids in regulating energy balance, possibly reflecting some degree of overlap between eating for pleasure and eating for energy.

Importantly, a functional interplay between opioids and other neuroregulators of feeding is not limited to only orexigens. In fact, many studies revealed an interplay between opioids and the OT system. Opioid receptors are expressed by OT neurons, and opioid fiber terminals have been found in regions where OT cells are localized [176]. Both neuroanatomical and functional analyses suggest the existence of the opioid-OT pathways that have functional relevance for feeding. Studies on taste aversion, for example, have shown that injecting opioid receptor agonists before a malaise inducing toxin, LiCl, prevents the formation of a conditioned taste aversion and it reduces LiCl-induced the percentage of Fos IR OT neurons in the PVN and SON in response to LiCl [177]. Further, opioid receptors have been found in the PVN where OT neurons are clustered.

The subsequent section will provide an overview of the link between OT and food consumption, from homeostatic to reward aspects of ingestive behavior, including the comments on how the functional interaction between OT and opioids can be exploited to reduce a drive to eat palatable foods.

1.5 Oxytocin

OT is a nonapeptide synthesized from a larger precursor protein containing 160-170 amino acid residues which are then cleaved into its active form [178]. In the brain, the OT perikarya are localized in the PVN and SON. The PVN and SON magnocellular OT neurons supply OT into the general circulation, whereas parvocellular OT neurons project and release OT into the CNS [179].

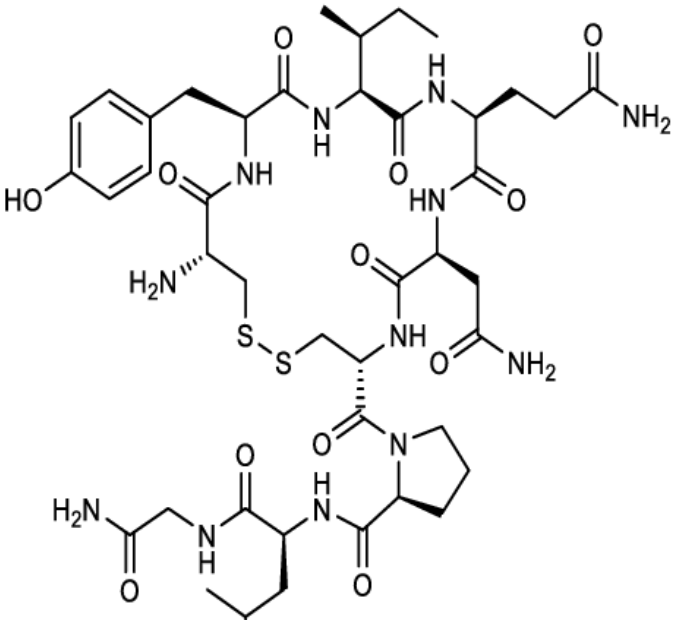

Figure1.2: Molecular structure of oxytocin

The OT receptor a member of the rhodopsin GPCR family. The OT receptor is found in the olfactory system, limbic brain structures, hippocampus, hypothalamus, and brainstem [180].

OT is a pleiotropic peptide which controls a number of processes and behaviors, from parturition to lactation to sociality. It is also involved in regulating food intake. Considering the pleiotropic nature of OT, it is not surprising that dysregulation in OT signaling has been associated with many physio- and psychopathological conditions, including autism, anxiety, anorexia, and obesity [181]. Consequently, in feeding control, OT's action intersects with feeding changes driven by homeostasis and hedonics, as well as by broader psychophysiological factors (such as, for example, those that underlie overconsumption of palatable foods in autism spectrum disorder) [182].

1.5.1 Role of OT on the regulation of homeostatic feeding

A plethora of evidence supports the notion that OT acts as an anorexigen in a "homeostatic" manner as OT facilitates the cessation of eating upon "fullness". Mealtime activation of OT neurons is maximal at the time coinciding with satiety and eating

behavior cessation [183-187]. The levels of OT transcripts in the hypothalamus are greater in rats that are satiated compared to those that are deprived. [188]. Intragastric infusions of nutrients that promote satiety, such as essential amino acids, affect the expression of the OT gene [189]. When the stomach is highly distended, OT neurons are activated and OT released into the peripheral circulation, which underpins the immediate termination of feeding [190].

An increase in food intake and body weight after lesions of the hypothalamic PVN in rodents provided early evidence for the potential role of OT in the regulation of food intake. Arletti and co-workers reported that IP and ICV administration of OT reduced food intake and eating duration in rats and increased latency to the first meal in both normal and food-deprived rats [183]. Since that initial discovery, many studies have demonstrated that central and peripheral administration of OT reduces food intake in laboratory animals [191-193]. This reduction can be prevented by injecting the OT receptor antagonist, which indicates that the OT receptor mediates the effect of OT and the observed anorexigenic action of OT is not dependent on, for example, competitive binding with vasopressin receptors [194].

The location of the OT receptor in the feeding circuit supports its role in homeostatic function. OT neurons in the SON express KATP channels that act as glucose sensors and activate POMC neurons in the ARC [195]. The VMH is also a potential target for dendritic OT, as it has a high density of OT receptors and OT receptor mRNA levels [49]. In addition, OT enhances the firing of VMH neurons and decreases food consumption when injected directly into the VMH [196]. Additionally, OT positive varicosities have been found on the perikarya, dendrites, and axons of DMH neurons that project to the gut [197].

OT receptors are also highly expressed in the hindbrain, specifically in the parabrachial nucleus (PBN), AP, DMHV, and NTS [198-201]. Interaction between OT receptors signaling in the NTS and the gastrointestinal tract during a meal also reduces food intake. The direct synaptic connection between OT neurons and the NTS contributes to the anorexigenic effect of OT as OT fibers originating from the parvocellular PVN region directly innervate the NTS [201]. In rats, administering CCK in the periphery results in the activation of c-Fos in the NTS, which has abundant OT axons. OT has been demonstrated to induce the secretion of CCK from the small intestine, which activates the

vagus nerve and promotes the feeling of fullness [202]. In addition, CCK has been found to stimulate the release of OT from the hypothalamus, which also activates the vagus nerve and contributes to satiation [203].

Several studies have discussed the bidirectional circuit between hypothalamic OT neurons and the dorsal vagal complex in the hindbrain [204]. These pathways connect the central processing of appetite to peripheral mechanisms related to feeding and their activity has been associated with reduced food intake.

OT has been found to act as an anorexigen in both males and females though the effect is more pronounced in males [205]. In female rodents, OT has been observed to reduce food intake across the estrous cycle, except during the proestrus phase [205].

Finally, OT is an important contributor to energy homeostasis dysregulation in developmental and genetic pathophysiologies. Mice with ablation of the SIM1 gene, associated with reduced OT expression, show hyperphagia and increased body weight [188, 206]. Moreover, low-dose intranasal OT reduces appetite drive in individuals with Prader-Willi syndrome (PWS) which is a deficiency in hypothalamic OT neurons as well as a reduction in the peripheral expression of the OT receptor [207].

1.5.2 Role of OT in the regulation of reward-driven eating

OT affects rewarding aspects of feeding: although it has been demonstrated that OT reduces consumption of energy-dense and tasty foods, the effectiveness upon presentation of energy-dilute and single-macronutrient food depends on the composition of these diets [208]. Injections of OT in the VTA and NAcc reduce the intake of sugar solution, whereas blocking the OT receptor in these reward areas increases sugar consumption [209]. ICV administration of OT reduces DA neuron activity in VTA and suppresses the motivation for the consumption of sucrose compared to standard chow consumption in a choice paradigm [210]. Administration of OT into the NAcc has also been shown to reduce the consumption of sucrose and saccharin solutions and reduce methamphetamine-seeking behavior in rats [209]. In contrast, OT administered in the CEA and VMH does not impact eating for pleasure, whereas the consumption of food for energy is reduced by intraparenchymal OT [208].

Many studies have reported that IP, IV, and central injections of OT reduced episodic intake of palatable food consumption while the OT KO, OT deficiency, and OT receptor blockade lead to elevated consumption of palatable foods in animals. IP administration of OT results in a decrease in consumption of HFHS diet after a 2-hour period and decrease responses to sucrose pellets in rats [211]. IP administration of L-368,899, an OT receptor antagonist which can cross the blood-brain barrier, increases consumption of sucrose solution when sucrose is given alone or concurrently with Intralipid in a 2-bottle choice paradigm in rats [193]. OT KO mice tend to consume excessive amounts of sugar and saccharin solutions, as observed in two-bottle choice tests where they show preference for sugar and carbohydrate solutions such as Polycose or cornstarch over water [212]. However, the OT KO mice do not exhibit a heightened preference for Intralipid, either in choice tests or after chronic exposure [196, 209]. The effect of OT KO on food preference is irrespective of the time of the day: OT KO mice during both light and dark phases [213]. In mice consuming sugar, there is a greater meal-end percentage of c-Fos positive OT neurons compared to animals that consume Intralipid [214]. Additionally, a high-sugar diet consumption stimulates OT neurons to a greater degree in rats than low-sugar food does [49].

Furthermore, OT KO mice show a higher frequency of sucrose intake bouts, indicating an elevated preference for sucrose solution [212]. This is in line with IP administration of OT reduces licking behavior for sucrose specifically, but not for NaCl, quinine, or citric acid [215]. Ingestion of sucrose also results in an increase in OT mRNA, and consistently administering an OT antagonist leads to an increase in carbohydrate intake in both choice and no-choice food intake paradigms [216, 217].

The results obtained in animal models were largely replicated in humans. Various studies have shown intranasal (IN) OT to decrease the episodic intake of palatable foods [218-221]. IN OT has also been found to suppress the consumption of palatable food in healthy men and it had a preferential effect on the reduction of hunger-driven food intake in obese men [218]. After a full buffet-style breakfast, when subjects were offered chocolate cookies, rice waffles, and salt crackers as post-meal snack, the intake of these snacks was significantly reduced by administration of OT [217]. The use of IN OT appears to have a significant effect on clinical trials, but there are still some unanswered questions regarding its efficacy following the chronic use of palatable foods.

Interestingly, apart from being linked to homeostatic eating behavior dysregulation in various pathophysiologies (as mentioned earlier in this chapter), altered OT signaling appears to contribute also to aberrant processing of rewarding aspects of consumption. Most notably, autism spectrum disorder (ASD), a neurodevelopmental condition associated with changes in OT circuits, leads to – among other symptoms – an enhanced drive to consume palatable foods. This has been well documented already in the first diagnostic criteria for autism as well as in many recent studies. In animal models of ASD, such as for example, in the valproic acid (VPA) rat model of the disorder, elevated sugar consumption is one of the prominent features of the condition. Peripheral OT partially reduces this drive to overeat in ASD animals [222], though the suppression is not complete likely due to the complexity of neural pathology, including the erroneous processing of pleasant cues due to the reward system dysfunction [223].

Overall, the link between OT and eating for pleasure gives rise to the question as to whether there is a possibility to exploit the potential synergy between opioid receptor blockers (thus, ligands that directly affect receptors that are classically viewed as reward-related) and OT (a peptide that impacts both homeostatic and rewarding facets of consummatory behavior). Early evidence supports the validity of this approach: simultaneous injections of OT and NTX in animals promote hypophagia even when the drugs are at low doses [224, 225]. IN OT and oral NTX may be effective in treating hypothalamic obesity, as evidenced by the recent craniopharyngioma post-resection case study [163]. Unfortunately, no IN NTX-OT co-administration studies have been done to date and neither has NTX been tested in the context of alleviating excessive appetite in neurodevelopmental disorders that incorporate opioid and OT circuit abnormalities, such as autism.

1.6 Overarching Goal and Specific Aims of the Thesis

Palatability-driven eating, which is oftentimes so robust that it results in the consumption of excess energy, can lead to obesity, especially in individuals who habitually overconsume highly caloric, palatable tastants. Among endogenous neuropeptides that increase feeding, opioids are key culprits responsible for driving the overconsumption of palatable food. Pharmacological and genetic blockade of opioid receptors suppresses

intake of preferred diets. It is not surprising, therefore, that over the past decades there have been several attempts to use opioid receptor antagonists in the treatment of obesity.

The recent addition of NTX-bupropion (Contrave) to the repertoire of drugs to treat excessive body weight shows the validity of approaches that target opioid signaling. It draws attention to NTX, a non-selective antagonist of opioid receptors, and the effects it promotes in various eating behavioral contexts/scenarios, in pathophysiological conditions characterized by overeating, as well as in treatment strategies that utilize potential synergy with other molecules that decrease appetite.

The existing gaps in knowledge on the effects of NTX have prompted me to set forth further characterization of the anorexigenic action of NTX as a suppressant of feeding for palatability, as the overarching goal of this thesis. In order to pursue this goal, I conducted a series of laboratory animal studies that centered around the following specific aims:

Specific aim 1: Investigate whether chronic intermittent sucrose consumption facilitates the ability to discriminate NTX from saline in rats

According to the knowledge so far, NTX and naloxone do not affect the discrimination of sucrose in rats trained to discriminate sucrose from water, except under highly excessive doses. However, in these experiments, animals were not given highly palatable foods in an intermittent fashion. We hypothesized that regular and intermittent consumption of sucrose over a long period would enhance opioid activity due to palatability-induced changes in opioid signaling have been reported. Thus, the first chapter of this thesis aimed to study whether chronic intermittent sucrose consumption allows laboratory animals to discriminate NTX from saline. To understand the neural modifications linked to the ability to discriminate NTX in animals given sucrose, I analyzed the concomitant modifications in the expression of c-Fos IR in feeding-related brain regions which mirrored the discrimination training schedule.

Specific aim 2: Investigate the effect of NTX on feeding in VPA-induced autistic rats known for excessive consumption of tasty diets

ASD is a complex neurodevelopmental disorder, and its symptoms include aberrant eating behaviors. Recent human studies suggest that eating disorders in ASD are caused by

irregular mechanisms for regulating food intake, specifically those that control reward-driven eating. Animal models of ASD have provided insights into the link between alterations in reward processing, particularly excessive brain opioid activity, and atypical behaviors of ASD [222, 226]. Recent studies have shown that NTX reduces social behaviors in ASD individuals [227]. To date, no studies have addressed the issue of how NTX affects abnormal food especially palatability-driven consumption in ASD. Thus, as a second aim, I investigated the effect of NTX on episodic and habitual consumption of palatable food in valproic acid-induced ASD rats compared to the control non-VPA rats in the second chapter.

Specific aim 3: Investigate the effect of NTX administered intranasally either alone or concurrently with another anorexigen, OT, on palatability-driven eating in rats

Peripherally injected NTX has been found to decrease food intake in laboratory animals and humans. Thus far, there have been no attempts to use the intranasal (IN) route of NTX delivery in studies focused on appetite control. Therefore, one of the goals of this set of studies was to determine whether IN NTX affects feeding (especially palatability-induced feeding) in rats. As IP and IV NTX has been shown to act synergistically with OT, the ultimate aim of this experimental work was to determine whether IN co-administration of NTX and OT has a more pronounced anorexigenic effect than either of these agents alone. In order to study this potential synergy, I first focused on determining the hypophagic effect of IN OT alone (as well as the relevant brain activation after the treatment), and then I followed with a set of studies in which NTX and OT were administered concurrently, and the feeding effects were investigated.

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Chapter 2

Chronic intermittent sucrose consumption facilitates the ability to discriminate opioid receptor blockade with naltrexone in rats

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2.1 Abstract

The opioid antagonist naltrexone (NTX) decreases the intake of preferred diets in rats at very low doses relative to doses needed to decrease the intake of "bland" laboratory chow. In the absence of an opioid agonist, NTX is not discriminable using operant techniques. In the current study, we found that rats given intermittent access to a 25% sucrose solution learned to discriminate between various naltrexone doses and saline. None of the rats given only water learned to discriminate between naltrexone and saline. When access to the sucrose solution was discontinued for 14 days, the rats lost the ability to discriminate between NTX and saline. We also studied the changes of c-Fos immunoreactivity in selected brain regions in rats treated with saline versus NTX that were drinking water or 25% sucrose. An injection of NTX or saline resulted in a significant drug, diet, and interaction effect in various brain regions associated with feeding behavior, particularly the amygdala, accumbens, and hypothalamic sites. Thus, we found that ingestion of a sucrose solution results in the ability of rats to reliably discriminate naltrexone administration. In addition, sucrose and naltrexone altered c-Fos immunoreactivity in an interactive fashion in brain regions known to be involved in ingestion behavior.

2.2 Introduction

It is well established that opioid circuits are involved in the regulation of food intake [1-3]. In laboratory animals, agonists of opioid receptors increase, and antagonists decrease food intake. Early studies suggested that opioid agonists selectively increased dietary fat consumption [4, 5]. However, Gosnell [6] demonstrated that morphine increased the intake of high-fat diets in rats that preferred such diets, and it produced a similar orexigenic effect in carbohydrate preferers given high-carbohydrate food. Our laboratory published a series of papers that support the latter finding [7-10].

There is a strong interaction between sugars and opioids in the central regulation of feeding and drinking. Naloxone, an opioid antagonist, reduces imbibition of sucrose or saccharin solutions more effectively than intake of water even in sham feeding paradigms [11, 12]. Naloxone can also reduce the intake of sucrose solutions in sham-fed animals, suggesting that postabsorptive signaling is not necessary for a decrease in sucrose intake following naloxone administration [11]. Naloxone does not affect sucrose discrimination in rats trained to discriminate 10% sucrose from water [13]. Humans also continue to discriminate sucrose solutions after opioid receptor blockade [14]. Interest in the longer-acting opioid antagonist naltrexone (NTX) has increased, as this molecule has been clinically tested to decrease addictive behaviors (from alcohol drinking to gambling), and it has been a component of the recently approved anti-obesity medication Contrave (NTX + bupropion) as well as part of combination pharmacotherapies (with oxytocin) in hypothalamic obesity case studies [15-18]. In rats, NTX decreases intake of standard chow and a 32% sucrose solution more effectively than standard chow alone [19]. NTX is effective in decreasing the intake of palatable high-fat, high-sugar chow [20, 21].

However, opioid receptor antagonists decrease the positive hedonic effect of sugar, that is, the pleasantness of sugar in both humans and non-human animals. When given a choice between a high-sugar diet and a high-starch diet, virtually all rats prefer the high-sugar diet. Our laboratory demonstrated that chronic central infusion of naltrexone via a mini-osmotic pump inhibited the re-development of a preference for a high-sugar diet after a period of ingestion of only a less rewarding starch diet [22].

In addition to opioids affecting the ingestion of sweet, palatable diets, sweet foods can affect opioid circuitry. For example, opioid gene expression in the arcuate nucleus is

altered by ingestion of a high-fat, high-sugar diet [23]. Restricted feeding of this preferred diet feeding pair to intake of a bland chow resulted in a decrease in gene expression of opioids, a response similar to that seen with energy deprivation [24]. Some have suggested that sugar ingestion can lead to addictive behaviors similar to those seen with opiates. Colantuoni and colleagues [25] exposed rats to an extended period of intermittent access to sucrose. They then injected these rats with naloxone to evaluate whether chronic sucrose ingestion led to opioid-like withdrawal. They reported withdrawal symptoms, such as teeth chattering, forepaw tremors, and head shakes. Hoebel's laboratory [26, 27] also found that opioid blockade following sugar consumption in rats resulted in a ratio of acetylcholine to dopamine in the CNS, which resembled that seen with morphine withdrawal. Pomonis et al. [28] studied the effect of three weeks of consumption of a 10% sucrose solution on naloxone-induced changes in c-Fos immunoreactivity in the brain of rats. The greatest effect was seen in the central nucleus of the amygdala (CEA). Naloxone injection in rats drinking water instead of sucrose increased c-Fos in the CEA, and sucrose significantly enhanced this effect. Koob [29, 30] noted that the amygdala, especially the CEA, has been implicated in opioid dependence, and negative aspects of opioid withdrawal are mediated largely via this site. Further evidence of sucrose affecting opioid circuits was demonstrated in a study by Jewett et al. They reported that chronic sucrose ingestion enhanced the ability of rats to discriminate doses of the opiate nalbuphine three-fold [31].

A variety of reports indicate that rats cannot discriminate NTX from saline, except at extraordinarily high doses, in operant paradigms [32-34]. Animals can discriminate opioid antagonists when subjects are treated chronically with an opioid agonist [28]. However, in those previous studies, the animals had not been receiving highly palatable, sugary food in an intermittent fashion. Based on the data discussed above, we hypothesized that chronic, intermittent sucrose consumption would increase opioid function. Naltrexone would produce a reduction in this sucrose-induced effect and allow rats to discriminate NTX from saline. In order to better understand neural changes associated with the ability to discriminate NTX in sucrose-given animals, we examined concurrent changes in c-Fos immunoreactivity expression associated with the diet and drug treatment that paralleled the successful discrimination training regimen in a variety of brain sites known to be involved in the regulation of food intake. The c-Fos study was crucial not only from the standpoint of a different opioid antagonist used by us versus Pomonis but also because the animals' exposure to sucrose was intermittent, thus

generating a more robust appetitive response (likely driven by reward processes) than the unrestricted access to sugar utilized in the earlier report.

2.3 Materials and Method

2.3.1 Animals

Male Sprague-Dawley rats aged 12 weeks old (average b. wt. 400 g) were housed individually in standard polycarbonate cages with wire tops under a 12:12 light: dark cycle (lights on at 6.00 a.m in both discrimination studies and c-Fos study) in a temperature-controlled room (22°C). 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. The University of Waikato Animal Ethics Committee approved all experimental manipulations described in this project.

2.3.2 Chemicals

Naltrexone (NTX) (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% saline refrigerated at 4 °C and was slowly warmed to room temperature 20 min prior to administration. Commercially available sucrose was dissolved in tap water.

2.3.3 Operant study: Establishing effects of NTX on discrimination

2.3.3.1 Apparatus

Daily discrimination sessions were conducted in standard operant chambers (Med-Associates, St. Albans, VT) which were equipped with two response levers. The operant chambers were located in ventilated, sound-attenuating cubicles equipped with fans. Forty-five milligrams of food pellets (Bio-Serve F#0021, Frenchtown, NJ) were given to 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 operant chamber illuminated the chambers during experimental sessions. Experimental contingencies and data recording were performed with Med Associates software (St. Albans, VT, USA) and a computer located in an adjacent room.

2.3.3.2 Initial training and intermittent sucrose access

Sixteen 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 the number of responses required to deliver a food pellet gradually increased to 15 lever presses (FR 15). When lever pressing occurred reliably at both levers, rats received unlimited access to standard chow. At the beginning of the dark phase (6.00 pm), rats were divided into three groups, and they received 25 % 0r 32 % sucrose solution or water daily. In the light phase (6.00 am-6.00 pm) all rats received water daily. This alternating sucrose access remained throughout the study unless otherwise indicated.

2.3.3.3 Discrimination training

After fourteen days of intermittent sucrose access, discrimination training was started. At the beginning of the dark phase, rats were given sucrose or water as mentioned earlier. After one hour, rats were injected with naltrexone (NTX) at the dose of 3.2 mg/kg or saline subcutaneously. Rats were placed in the operant chamber and discrimination training was started after 15 minutes of NTX or saline injections. When the first training cycle started, the house light was illuminated, and fifteen correct lever presses (left lever press for NTX injections and right liver press for saline injections) were reinforced with 45 mg food pellet delivery. Rats were punished with 8s darkness for the incorrect liver press (right lever for NTX injections and left lever saline injections). Training continued until 5 reinforcers were earned or 5 min elapsed. After the discrimination training session, rats were placed in the home cage with access to standard chow and sucrose solution. 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.

2.3.3.4 Training procedure to discriminate different doses of NTX

Thirty-two rats were trained to press the levers as mentioned above. Rats were given access to a sucrose solution and discrimination training was performed as described in 3.3.3.3. Rats were divided into three groups and were injected with different doses of NTX as 1.0 mg/kg, 0.32 mg/kg, and 0.1 mg/kg. rats were trained to discriminate between

different NTX doses and saline. The training was continued until discrimination criteria were achieved as described in section 2.3.3.3.

2.3.3.5 Generalization test I: Effect of different doses of NTX on discriminative stimulus effect of NTX

After the rats achieved discrimination ability, the generalization test was started. In the dark cycle, rats received 25 % sucrose solution, and after one-hour rats were injected with different doses of NTX or saline subcutaneously. Rats were placed in the operant chamber and a generalization test was started after 15 minutes of NTX or saline injections. During the test, the house light in the operant chamber was illuminated and 15 lever presses to either lever were reinforced with the 45 mg food pellet delivery. Generalization tests lasted until the subject earned 5 reinforcers or until 5 min elapsed. After the test, rats were placed in the home cage and received access to standard chow and 25 % sucrose solution for the remaining time of the dark phase. Appropriate discriminative performance for at least 2 training days (one preceded by NTX injection, one preceded by saline injection) was required between generalization tests to ensure discriminative ability remains reliable.

2.3.3.6 Generalization test II: Effect of acute water consumption on discriminative stimulus effect of NTX

A subgroup of rats from experiment 2.3.3.3 was selected and given access to water for one of the beginnings of the dark phase. After one-hour rats were injected with different doses of NTX (0.032-3.2 mg/kg) or saline and test sessions were started after fifteen minutes of the injections. A generalization test was performed as described in section 2.3.3.5. After the test, rats were returned to their home cages and received access to standard chow and 25 % sucrose solution for the remaining time of the dark phase (until 6.00 am). Appropriate discriminative performance for at least 2 training days (one preceded by NTX injection, one preceded by saline injection) was required between generalization tests to ensure discriminative ability remain consistent.

2.3.3.7 Effect of chronic water consumption on discriminative stimulus effect of NTX

After rats were given chronic sucrose access, rats from experiment 2.3.3.6 received 24-hour access to water for the next 14 days, and then the discrimination test was started. Rats were given water at the beginning of the dark phase and one hour later they were injected with different doses of NTX and saline. After fifteen minutes of injections, rats were placed in the operant chamber, and discrimination training was started as described above. When test sessions were finished, rats were returned to their home cages and given access to water until 6.00 am. At the beginning of the light phase, water bottles were replaced with freshwater bottles. Discrimination training was continued for 14 days daily and then after discrimination training was suspended. After 14 days of water, rats received 14 days of chronic intermittent sucrose access, and discrimination training was resumed as described in 2.3.3.3.

2.3.4 c-FOS study

Rats were divided into two groups: a sucrose diet-fed group and a control diet-fed group. Rats were fed with standard chow and water ad libitum in the control diet cohort. Rats in the Sucrose fed group were given access to 25 % sucrose solution from 6.00 pm to 6.00 am (dark phase) daily for 10 weeks. In the dark sucrose-fed rats were given 10 g of standard chow to minimize the effect of excessive calorie intake. In the light phase (6.00 am- 6.00 pm), rats were given access to standard chow and water ad libitum.

Rats were given access to a control diet and sucrose diet for 10 weeks in order to mimic the length of the operant chamber studies that developed the ability of rats to discriminate NTX from saline. During this 10-week, the onset of the dark phase, rats were given random daily injections of either saline or NTX (1mg/kg). These injections were given according to the random schedule as half of the injections were saline and half of the injections were NTX. On the final day, both the sucrose-fed group and standard diet-fed groups were subdivided into two and were injected with saline or NTX intraperitoneally. After injections, all foods and water were removed, and rats were euthanized with urethane (35% in isotonic NaCl) after one hour of injections. Then rats were perfused via the aorta with 50 mL saline (room temperature) followed by 400 mL of 4% paraformaldehyde (ice-cold; in 0.1 M phosphate buffer). Brains were excised and post-fixed overnight in 4% paraformaldehyde at 4°C. After 24 hours, 60 μm-thick coronal

sections of the brains were cut with a vibratome (Leica, Germany), and free-floating sections were used for the c-Fos immunohistochemistry study.

Sections were rinsed in 50 nM TBS (pH 7.4–7.6) four times and then treated for 10 min in 3% H2O2, and 10% methanol (diluted in TBS). After four rinsing in TBS, sections were incubated overnight at 4°C the in the polyclonal rabbit antibody against c-Fos (diluted 1:4000; Synaptic Systems, Australia). After overnight, sections were washed in TBS and incubated in the goat-anti-rabbit secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA; room temperature) for one hour at room temperature. Following four washes in TBS, sections were incubated in the avidin–biotin peroxidase complex (ABC; 1:800; Elite Kit, Vector Laboratories, Burlingame, CA, USA) for another hour at room temperature. The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the brain section was visualized with 0.05% diaminobenzidine (DAB), 0.01% H2O2, and 0.3% nickel sulfate in TBS.

The stain 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). Images were taken from the camera attached to a light microscope (Nikon Eclipse 400). The number of Fos-positive nuclei per 1 mm^2 was counted bilaterally for each neuroanatomical region of interest with ImageJ Software. Boundaries of each area were defined according to the Paxinos and Watson brain atlas, on 2–4 sections per animal.

2.3.5 Data analysis

Acquisition data from different NTX training doses were compared using ANOVA followed by Tukey t-tests and statistically significant differences were considered for $p \leq 0.05$. For each training dose, generalization data from different naltrexone doses were compared with ANOVA followed by Dunnett's test. Differences were considered significant for $p \leq 0.05$. Rates of lever pressing following different NTX doses were compared with repeated measures ANOVA followed by Dunnett's test. Differences were considered significant for $p \leq 0.05$. In the c-Fos study, means and SEM were calculated and data were compared with two-way ANOVA with drug and diet set as independent factors.

2.4 Results

After intermittent sucrose access, rats developed the ability to discriminate between NTX (3.2 mg/kg) and saline (Figure 2.1). Different sucrose concentrations did not produce any significant effect on discrimination acquisition. There was no difference in sessions to discrimination criteria between rats were given access to 25 % sucrose solution (M=55, SEM= 15 sessions) and rats were given access to 32 % sucrose solution (M=68, SEM=13 sessions)

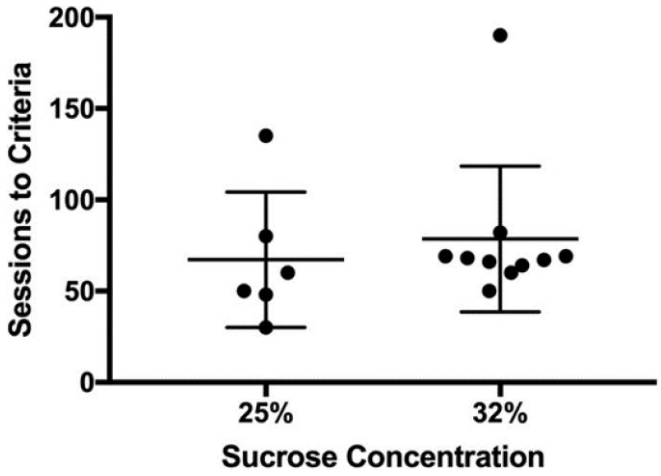


Figure 2.1: Effect of different sucrose concentration on discrimination responses between NTX and saline. There was no significant difference between 25 % and 32% sucrose solution p = 0.58, t (14) = 0.56, Filled circles-individual subject sessions to discrimination criteria; long horizontal lines-mean sessions; short horizontal lines- SEM

Rats were given intermittent access to 25 % sucrose solution and developed the ability to discriminate different doses of NTX (0.32 – 3.2 mg/kg) from saline (Figure 2.2). However, subcutaneous injections of different doses of NTX did not produce any significant difference in sessions to discrimination criteria (0.32 mg/kg- M=119, SEM= 20; 1.0 mg/kg- M=64.5, SEM= 12; 3.2 mg/kg- M=68, SEM= 13) ($F_{(2,14)} = 3.20$, $p = 0.07$). Moreover, 7 of 21 rats were injected with 0.1 mg/kg NTX dose and developed the ability to discriminate NTX from saline, and the training of the other 14 rats was terminated after completing 81 sessions. Rats who were given access to water did not learn to discriminate NTX within 76 training sessions.

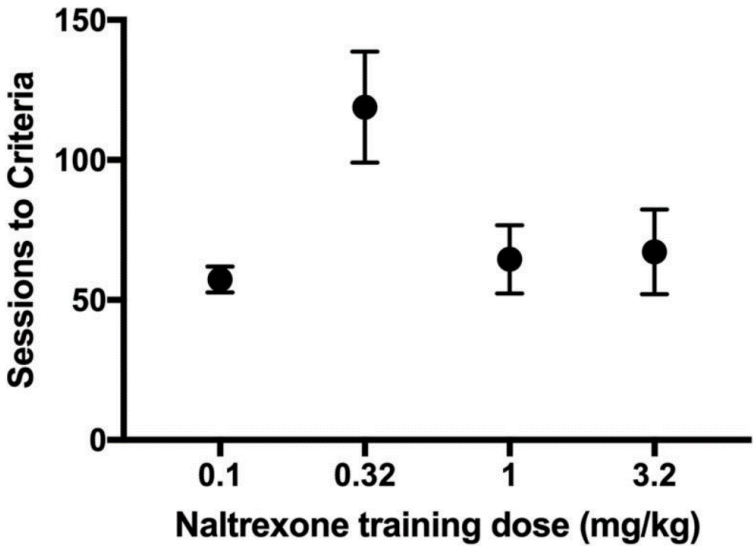


Figure 2.2: Effect of different NTX training doses on discrimination responses. There was no significant difference among NTX training doses (ANOVA followed by Tukey t-tests and statistically significant differences were considered for $p \leq 0.05$).

Naltrexone produced dose-dependent increases in NTX training dose-discriminative stimulus effects (Figure 2.3). Naltrexone's potency to generalize to the training dose was inversely related to the training dose (smaller NTX doses generalized to 0.01 mg/kg, whereas larger NTX doses generalized to 3.2 mg/kg training dose). There was a main effect of naltrexone dose on the percentage of NTX-appropriate responses for each training dose, 0.1 mg/kg NTX $F_{(4,33)} = 20.42$, $p < 0.001$; 0.32 mg/kg NTX $F_{(6,46)} = 6.09$, $p < 0.001$; 1.0 mg/kg NTX $F_{(4,24)} = 20.05$, $p < 0.001$; 3.2 mg/kg NTX $F_{(4,51)} = 11.56$, $p < 0.001$. Response rates were not significantly affected by NTX (Figure 3B).

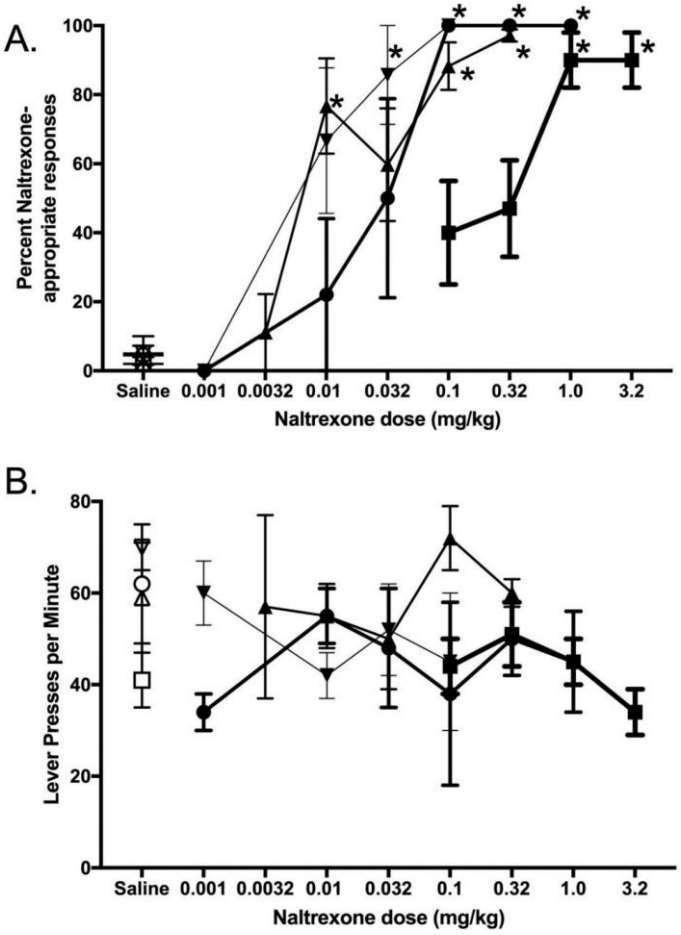


Figure 2.3: Effect of different NTX doses on discrimination responses and rates of lever pressing. NTX dose-dependent effect curves are represented as a function of training dose (inverted triangle illustrates 0.1 mg/kg NTX, triangle illustrates 0.32 mg/kg NTX, circle illustrates 1.0 mg/kg NTX, and square represents 3.2 mg/kg NTX) (A) NTX generalization curves as a function of naltrexone training dose. The percentage of responses associated with the NTX training dose is plotted on the ordinate. (B) Response rate (lever presses per minute) is plotted as a function of NTX dose. Different naltrexone doses were compared with ANOVA followed by Dunnett's test statistically significant differences were considered for $p \leq 0.05$.

Naltrexone's ability to serve as a discriminative stimulus was not altered when water was substituted for 25% sucrose 1 h before the injection of NTX (Figure 2.4). In rats maintained under intermittent sucrose access, 0.32 mg/kg NTX produced a half-maximal effect (Figure 4A), and response rates were not altered by NTX (Figure 4B). When water

was given 1 h before the test session instead of 25% sucrose, the discriminative stimulus effects were not altered (Figure 2.4C). The dose to produce a half-maximal effect was similar (~0.32 NTX), and most doses tested produced similar increases in NTX-appropriate responding. Response rates (Figure 2.4D) were also not significantly affected by NTX when water was substituted for 25% sucrose solution 1 h before the test session.

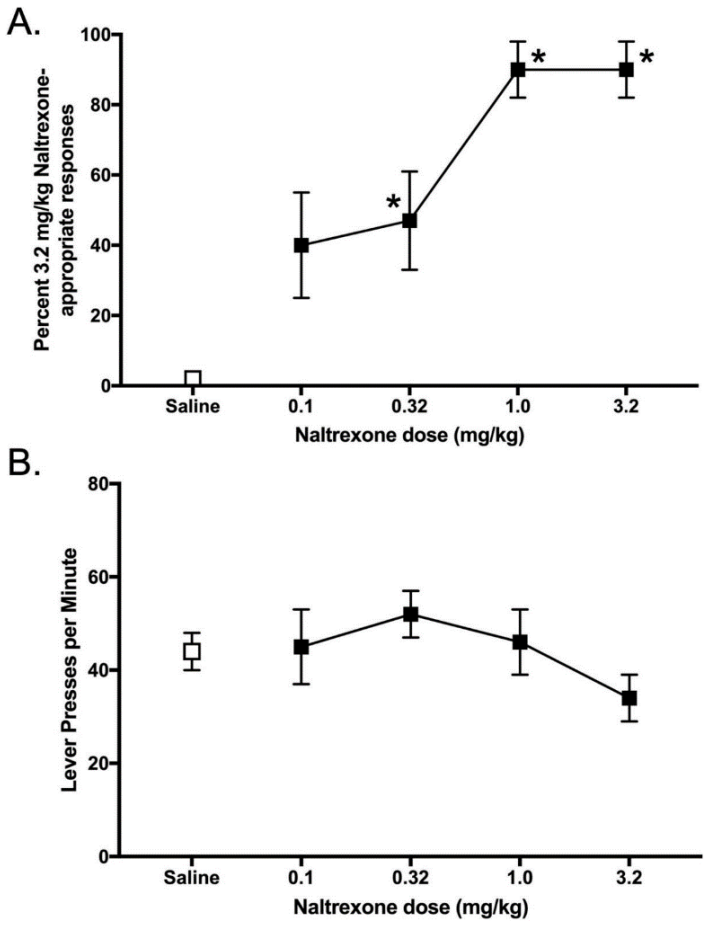


Figure 2.4: Effect of acute water substitution on NTX generalization functions (saline, open squares, and NTX, filled squares) in rats trained to discriminate 3.2 mg/kg NTX from saline. (A) NTX generalization function and (B) response rates in rats with chronic intermittent 25% sucrose solution given 1 h before the test. (C) NTX generalization function and (D) response rates in rats with water given 1 h before the test. * = $p < 0.05$ (significantly different than saline control).

Rats maintained under chronic intermittent sucrose and trained to discriminate 3.2 mg/kg NTX from saline were given 14 days access to water and resumed discrimination training while maintained on water. Under chronic water access, the discriminative stimulus effects of NTX were significantly reduced (Figure 2.5A). $F_{(5,59)} = 2.51$, $p = 0.041$. Under these conditions, performances following saline training sessions did not meet the

discrimination performance criteria. Subjects lost the ability to discriminate between 3.2 mg/kg NTX and saline. Response rates were also significantly higher for NTX training days conducted under the chronic water conditions $F_{(5,59)} = 3.00$, $p = 0.018$ (Figure 5B). When access to 25% sucrose was reinstated for 14 days, rats readily relearned to discriminate between NTX and saline. Figure 6 shows the discrimination reacquisition data for a typical subject. As the training days increased, data tended to be at the top of the graph following NTX administration and at the bottom of the graph following saline administration, indicating the rat was reliably discriminating between 3.2 mg/kg NTX and saline.

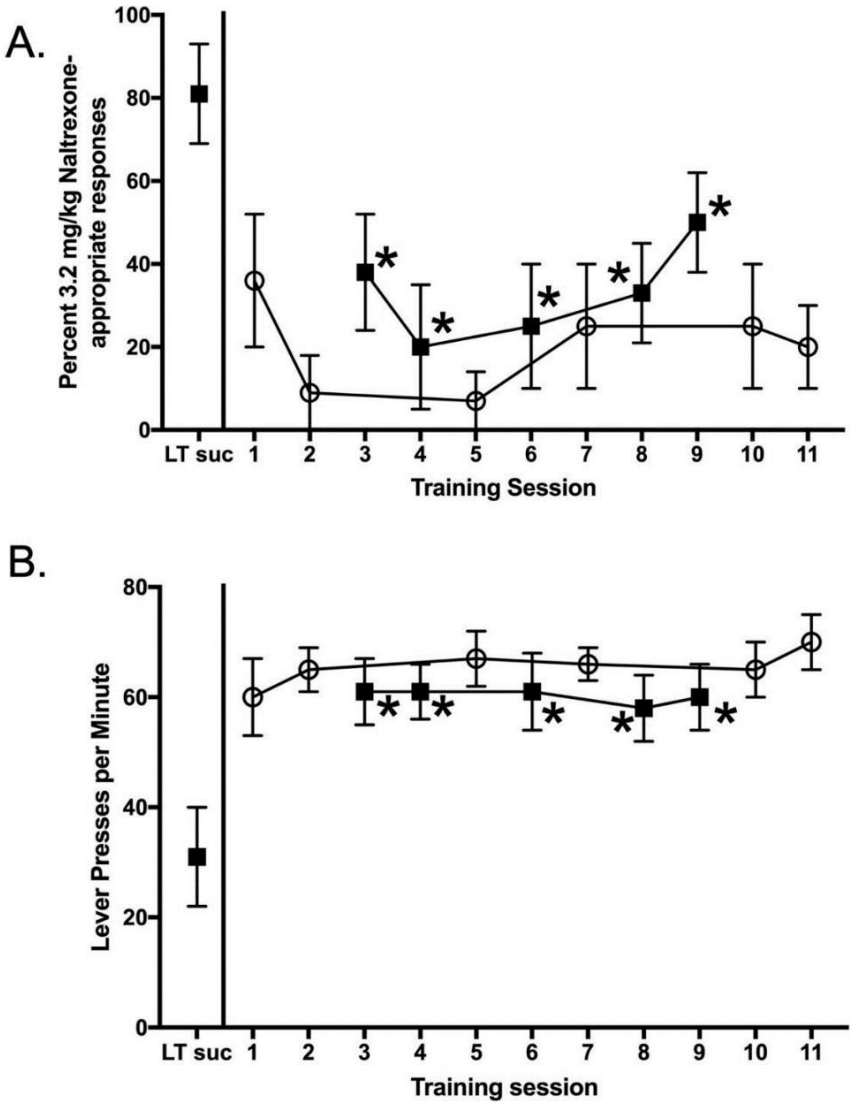


Figure 2.5: Effect of 14-day water access on the ability of NTX to serve as a discriminative stimulus. (A) NTX-appropriate responding and (B) rates of lever pressing for NTX (filled symbols) and saline (open symbols) are plotted as a function of training session. The point above LT suc represents data from the last 3.2 NTX training day before training was suspended, and water access began for 14 days. Training days 1–

11 were conducted under water-access conditions. * = $p < 0.05$ (significantly different than the last NTX training day under chronic, intermittent sucrose conditions).

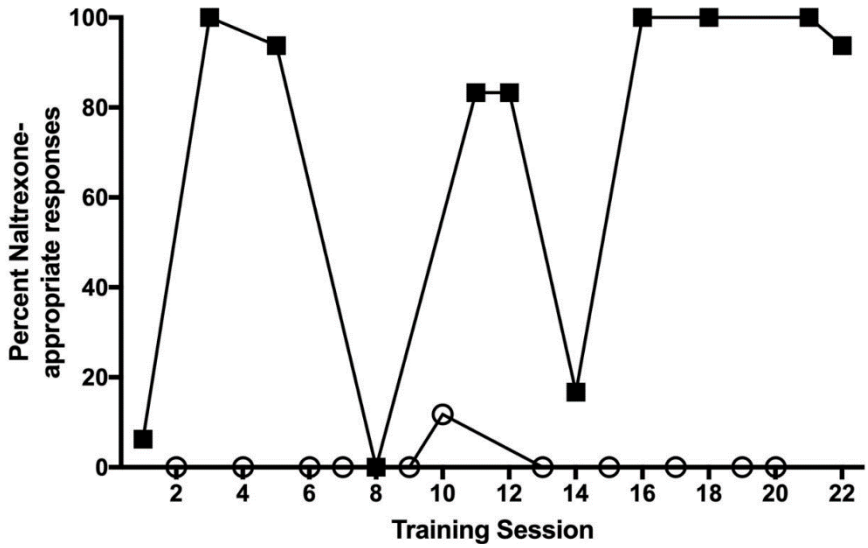
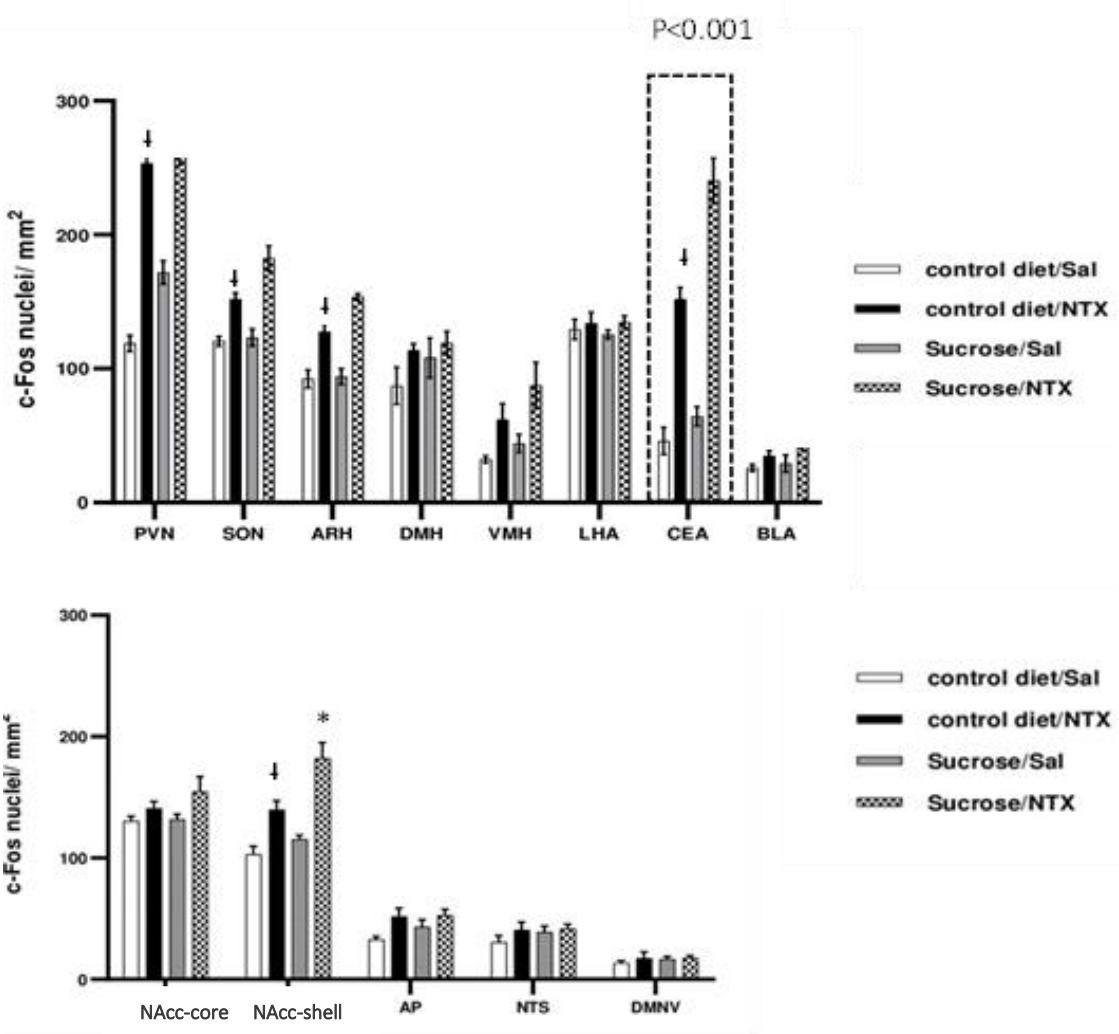


Figure 2.6: Reacquisition of NTX as a discriminative stimulus after 14 days of sucrose access (25%). Data for NTX training sessions (filled squares) and saline training sessions (open circles) from a representative subject are shown. NTX-appropriate responding is plotted as a function of the training session.

In c-Fos studies, a two-way ANOVA analysis showed a significant effect of the drug in the PVN ($p < 0.0001$, $F(1,16) = 168.6$), SON ($p < 0.0001$, $F(1,16) = 50.26$), ARC ($p < 0.0001$, $F(1,16) = 74.05$), DMH ($p = 0.0019$, $F(1,16) = 13.69$), VMH ($p < 0.0001$, $F(1,16) = 53.81$), CEA ($p < 0.0001$, $F(1,16) = 773.9$), BLA ($p < 0.0001$, $F(1,16 = 33.86$), NAcc-shell ($p < 0.000$, $F(1,16) = 36.06$), and NTS ($p = 0.0145$, $F(1,16) = 7.514$).

Per the two-way ANOVA analysis, the effect of diet was significant in the PVN ($p = 0.0021$, $F(1,16) = 13.45$), SON ($p \leq 0.0193$, $F(1,16) = 6.759$), ARC ($p \leq 0.0233$, $F(1,16) = 6.294$), DMH ($p \leq 0.0196$, $F(1,16) = 6.728$), VMH ($p = 0.0017$, $F(1,16) = 14.25$), CEA ($p < 0.0001$, $F(1,16) = 111.7$), BLA ($p = 0.0094$, $F(1,16) = 8.713$), and NAcc-shell ($p < 0.0001$, $F(1,16) = 8.524$). Finally, there was a statistically significant drug–diet interaction in the PVN ($p= 0.0289$, $F(1,16) = 5.760$), SON ($p = 0.0443$, $F(1,16) = 4.762$), ARC ($p = 0.0444$, $F(1,16) = 4.759$), NAcc ($p = 0.0231$, $F(1,16) = 6.308$), and—with the most

statistically significant difference—the CEA ($p < 0.0001$, $F(1,16) = 47.71$) (Figure 7). In addition, a t-test analysis revealed that c-Fos to be more significantly elevated in the control diet/NTX groups in the PVN, SON, ARC, CEA, and NAcc-shell than control diet/saline groups ($p < 0.001$ for all groups). c-Fos IR in the sucrose/NTX groups in the PVN ($p < 0.0001$), SON ($p = 0.0008$), ARC ($p < 0.0001$), CEA ($p < 0.0001$), and NAcc-shell ($p = 0.0008$) was higher than in the sucrose/saline group. Moreover, we found c-Fos to be significantly elevated in the sucrose/NTX groups in the SON ($p = 0.0188$), ARC ($p = 0.0033$), CEA ($p < 0.0001$), and NAcc-shell ($p = 0.0213$) compared to the control diet/NTX group (Figure 7).



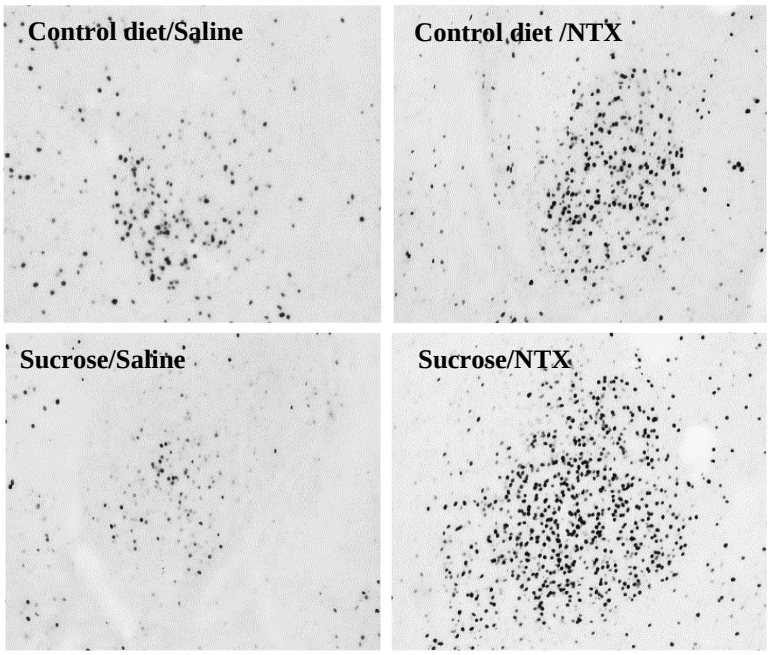


Figure 2.7: The effect of saline vs NTX (1 mg/kg) on c-Fos immunoreactivity in specific brain sites in control diet vs sucrose-consuming rats. Bar graphs depicting c-Fos neuronal activation in various brain regions. The upper panel represents c-Fos expression levels in regions including PVN, SON, ARH, DMH, VMH, LHA, CEA, and BLA. The lower panel illustrates c-Fos activation in NAc-core, NAc-shell, AP, NTS, and DMNV. * Significantly different from the control diet/NTX group. Ł Significantly different from the control diet/saline group. The photomicrographs depict c-Fos immunoreactivity in the central nucleus of the amygdala of rats exposed to standard vs sucrose-enriched diet that received a saline or NTX injection. The CeA was the site where the highest drug x diet interaction level of all regions examined in our study was noted.

c-Fos immunoreactivity was not related to overall daily calorie intake over the course of the study: On average, rats consumed 85.26+/−8.41 g of sucrose solution per night. This feeding regimen produced similar total daily energy intake per day per animal of 90.42+/−6.34 kcal (control diet cohort) and 96.14+/−5.23 kcal (palatable sucrose diet cohort), and average body weight did not differ between the cohorts (609.41+/−16.5 g, control; 621.72+/−26.2 g, palatable sucrose). Since on the experimental day immediately after the saline or NTX injection, all tastants were removed from the cage, the c-Fos data

reflect only the action of the drug without the interference of different amounts of ingested food or liquid.

2.5 Discussion

Sugar-rich diets are readily consumed by humans and laboratory animals independent of actual energy needs of the organism. The idea that sugars can be reinforcing and even addictive has been popularized by the media and some non- and for-profit organizations and has received support from the scientific community [25, 26, 35]. Various neural circuits have been identified that are involved in the reinforcing qualities of sugars. These include the mesolimbic dopamine system as well as endogenous opioid [25, 26, 35]. In the current study, we asked whether sucrose could mimic the effect of morphine on naltrexone discrimination in rats.

Rats naïve to exogenous opioid receptor agonists cannot be trained to discriminate the opioid antagonists naloxone or naltrexone except at extraordinarily large doses [32-34]. Such discrimination trials suggest that the blockade of endogenous opioid binding to opioid receptors does not result in a perceivable interoception. However, if rats are given morphine prior to training for naltrexone, they can be trained to press the appropriate naltrexone-associated lever [32-34]. In the current study, we found that chronic ingestion of a sucrose solution allowed rats to discriminate naltrexone using a standard operant drug-discrimination protocol. This suggests that sucrose can mimic the effects of morphine in such a model perhaps by inducing the release of endogenous opioids or affecting opioid receptors and thereby opioid tone that can be inhibited by NTX. This finding is supported by other research. For example, Jewett et al. [31] found that chronic sucrose ingestion significantly enhanced the ability of rats to discriminate doses of nalbuphine three-fold. Nalbuphine produces discriminative stimulus effects as a low-efficacy, partial agonist at mu-opioid receptors in Sprague–Dawley Rats [36]. The discrimination studies we conducted above expand upon the various opioid-mediated behavioral effects that can be altered by chronic sucrose consumption.

The increase in opioid function allows the opioid-antagonist naltrexone to serve as a discriminative stimulus at doses associated with opioid antagonism. Naltrexone (3.2 mg/kg) represents the largest naltrexone dose in the most relevant literature [37-39]. We examined the ability of different naltrexone doses to serve as a discriminative stimulus.

All subjects learned to discriminate 0.32–3.2 mg/kg naltrexone, and a minority of subjects learned to discriminate 0.1 mg/kg NTX from saline. The subtype(s) of opioid receptors that may contribute to the discriminative stimulus effects of naltrexone in this paradigm is currently unclear. The increase in the opioid function that allows naltrexone to serve as a discriminative stimulus is not dependent on the concentration of sucrose consumed. The acquisition of naltrexone as a discriminative stimulus was not different between subjects drinking 25% sucrose or subjects drinking 32% sucrose. Sucrose intake (g/day) was similar between the two groups (data not shown).

The acquisition and maintenance of the discrimination is related to longer-term changes in opioid function. We determined that acute substitution of water for sucrose (given in the hour before the discrimination session) had no impact on naltrexone's ability to serve as a discriminative stimulus. That indicates that the changes to the opioid system are relatively long-lasting.

We determined that chronic water substitution (2 weeks) results in a decrease or resetting of the opioid system such that naltrexone can no longer serve as a discriminative stimulus. This finding complements the lack of effect of acute water substitution. Reinstating chronic, intermittent sucrose consumption resulted in fairly rapid reacquisition of naltrexone as a discriminative stimulus. As one may predict, the time to reacquire the discriminative stimulus was considerably less than the time to initially learn to discriminate between naltrexone and saline.

In the current study, we also evaluated the effect of naltrexone versus saline injection on c-Fos immunoreactivity in a variety of brain sites involved in feeding behavior in rats drinking a 25% sucrose solution or water in a chronic intermittent exposure paradigm. In order to mimic the discrimination studies, the rats were given not only the sucrose for 10 weeks, but they also received random daily injections of either saline or 1 mg/kg naltrexone throughout this time period (thus, they were familiar with interoceptive effects of both injectants). On the final day, rats were injected with naltrexone or saline. We found that the NTX increases c-Fos immunoreactivity in selected sites, but we found that the magnitude of the change in c-Fos immunoreactivity was higher in the sucrose/NTX group than in the sucrose/saline group in a variety of brain sites. Thus, in line with many reports suggesting that habitual sugar intake elevates opioid tone in the brain [40], in the current experiments, we found that sucrose ingestion enhances the effect of blockade of

opioid receptors with NTX on c-Fos immunoreactivity in a variety of brain nuclei. Among all the sites where a positive drug–diet interaction was found, the CEA was most highly significant (* $p < 0.0001$). These results are similar to those found by Pomonis et al., [28] who studied the effects of three weeks of consumption of a 10% sucrose solution on naloxone-induced changes in c-Fos immunoreactivity in rat brains. They found the greatest effect in the central nucleus of the amygdala and a significant interaction in the periaqueductal gray. In the current study, we found more brain sites that had significant drug–diet interactions reflected in c-Fos immunoreactivity levels; however, it should be noted that in our study, the drug was different (naltrexone rather than naloxone).

Furthermore, it should be emphasized that the sucrose exposure in our experiment was limited to the nighttime. This is a stark contrast with the Pomonis' setup, in which the sugar solution was available at all times, completely abolishing the anticipation of exposure to the palatable diet and making the sweet ingestant into a more "standard" (albeit still palatable and rewarding) food. Nonetheless, while it is intuitive that the CEA or NAcc, areas critical for the emotional processing of food reward, showed a significant change in activation, the scope of changes in hypothalamic regions typically associated with homeostatic control of energy balance is striking. For example, activation of the PVN and SON, areas where oxytocin and vasopressin neurons are amassed, convey termination of feeding, and historically, they have been viewed as critical mediators of "fullness" (understood as a combination of stomach distension and changes in plasma osmolality and circulating nutrient/hormone levels) [41, 42]. Similarly, the ARC where melanocortin, Agouti-related protein, and neuropeptide Y-synthesizing cells are localized is part of the circuit that ensures proper energy balance [42, 43]. Yet, mapping of opioid receptors shows that they are abundantly expressed also throughout the "homeostatic" network of sites [44]. This interrelationship between the palatability of a diet and the pharmacological blockade of opioid receptors serves as yet another indicator of a functional intersection between eating for pleasure and eating for calories. It exemplifies how exposure to rewarding tastants can affect also those brain areas that are involved in the regulation of the hunger-satiation processes, possibly shifting the dynamic balance between the mechanisms that affect energy control [40]. Furthermore, it suggests that drugs, such as naltrexone, and thus molecules that target predominantly eating driven by palatability, produce a profound response in the homeostatic brain circuit, thereby being able to shift reward processing of consumption along with the perceived need for ingesting calories.

The results of the current study reinforce the notion that sucrose/sugars can affect opioid circuits. Both drug discrimination and c-Fos immunoreactivity were impacted by sucrose imbibition. More than a decade ago, Hoebel's laboratory [26, 45-47] found that chronic ingestion of sugars can result in naloxone/naltrexone-induced withdrawal effects similar to those seen after morphine administration. These include central levels of acetylcholine and dopamine sampled by micro-dialysis and behaviors such as teeth chattering and head shakes.

In conclusion, we found that imbibition of sucrose can alter the ability of rats to discriminate naltrexone from saline injections. In addition, both naltrexone and sucrose have significant interactive effects on activation, as reflected by c-Fos immunoreactivity, of brain regions known to be involved in feeding behavior.

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Chapter 3

The effect of NTX on feeding in VPA-induced autistic rats, a model of excessive consumption of palatable diets

3.1 Abstract

Abnormal eating behaviors are commonly observed in individuals with autism (ASD). Altered reward processing and dysregulation of opioid system in the brain are associated with these eating behavioral abnormalities. Opioid receptor ligands have been shown to restore various maladaptive behaviors in ASD, and this includes the ability of naltrexone (NTX) to alleviate social behavioral anomalies in autism. Despite this, the effect of NTX on modifying eating behavior in ASD has not yet been examined. Thus, here I studied whether intraperitoneal NTX acutely affects the consumption of deprivation-induced energy-dense standard chow and palatable high-fat high-sugar diet (HFHS) and sucrose solution intakes in valproic acid-induced ASD (VPA) animals compared to non-VPA animals. Finally, by using c-Fos immunoreactivity, I assessed changes in activity in feeding-related brain areas in response to NTX in VPA animals after two weeks of daily sugar access. I found that NTX had no effect on standard chow intake post-deprivation, but it significantly suppressed the consumption of the HFHS diet and 10% sucrose solution in both non VPA and VPA rats. Importantly, the minimum effective dose of NTX to generate hypophagia was 10 times higher in VPAs (10 mg/kg) than in controls (1 mg/kg). NTX at 1 mg/kg produced changes in c-Fos in a host of brain sites, but the effect was much pronounced in the hypothalamus of control rats than in VPAs. On the other hand, in both control and VPA animals, c-Fos in the central nucleus of the amygdala (CEA) was relatively similar and there was a significant drug–diet interaction. In sum, NTX is effective in reducing elevated consumption of palatable food in VPA animals, but the dose to produce hypophagia in ASD individuals is higher.

3.2 Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder, and its symptoms include aberrant feeding. The atypical eating behaviors in ASD include, among others, an elevated preference for specific foods (especially palatable ones that are rich in sugar and fat), monotonous diet selectivity, and even a particular presentation of food (from its appearance to a location in which it is served) [1].

Recent human studies suggest that eating behavioral abnormalities in ASD stem from aberrant food intake regulatory mechanisms, especially those that govern the pleasure of consumption. For example, in the fMRI study, Cascio et al found that neural responses to food cues were significantly increased in the anterior cingulate cortex (ACC), insula, and nucleus accumbens (NAcc) in mildly food-deprived ASD participants [2]. Similarly, Schmitz and co-workers reported an increase in the activity of the left anterior cingulate gyrus upon reward stimulation [3]. These authors also found a significant reduction in periventricular white matter density of the left frontal lobe in ASD, which links aberrant ASD behavioral processing to not only functional, but also morphological CNS changes. Interestingly, functional connectivity between the NAcc and the ventral tegmental area (VTA), the brain sites that play a major role in food reward processing, is decreased in ASD.

ASD animal models have shed more light on the functional link between changes in reward processing in ASD and abnormal reward-driven behaviors. Buchner et al showed that mice lacking the Cntnap2 (contactin-associated protein-like 2) gene, overconsume palatable food and rapidly gain weight [4]. Mice lacking another autism-associated gene, Mecp2 (methyl-CpG-binding protein 2) display extreme hyperphagia and obesity when given access to highly palatable foods [5]. Furthermore, knockout mice haploinsufficient for the ASD-associated neurobeachin (Nbea), consume more palatable sucrose, glucose, and fructose solutions as well as Intralipid fat emulsion compared to the wild-type controls [6]. Importantly, in recent experiments utilizing a developmental valproic acid (VPA)-induced rat model of autism, Klockars et al found that consumption of palatable sucrose, saccharin, and milk solutions was increased in ASD VPA rats compared to the controls, whereas consumption of a ”bland” cornstarch solution remained unchanged [7]. Data suggest that the dysregulation of opioids signaling underpins a plethora of ASD symptoms, including those related to reward [8]. In line with this hypothesis, an opioid

receptor antagonist, naltrexone (NTX), has been found to reduce self-aggressive behavior, hyperreactivity, and restlessness in children with ASD [9]. NTX has also been reported to normalize opioid levels, such as β endorphin, in autistic children [10]. Importantly, opioids constitute one of the major neuropeptidergic systems that mediates the pleasure of consumption. To date, no studies have addressed the issue of how opioid receptor blockade affects palatability-driven feeding in ASD. Thus, in this chapter I investigated the effect of NTX on episodic intake of energy-dense and palatable high-fat high-sugar (HFHS) chow and palatable and energy-dilute sucrose solution in VPA ASD rats compared to the non-VPA controls.

Since I found that the anorexigenic 1 mg/kg dose of NTX in non-VPA rats was ineffective in VPAs, I investigated which feeding-related brain sites are affected differently by NTX administered just prior to a sugar meal in VPAs compared to controls. Therefore, I used a previously published protocol, in which rats were accustomed to getting a sucrose solution for 2 h per day and, on the experimental day, instead of getting access to sugar water, they were injected with 1 mg/kg NTX or vehicle. I used c-Fos immunoreactivity in saline vs NTX-treated control and VPA animals to define differences in brain activation in response to NTX.

3.3 Materials and methods

3.3.1 Animals and drugs

In feeding studies, we used adult male Sprague-Dawley rats aged 20 weeks old that were single-housed in Plexiglas cages with wire tops and were maintained on a 12:12 light: dark cycle (lights on at 07:00) in a temperature-controlled room (22°C). Adult female Sprague-Dawley rats (approximately five to six months old) mated with age-matched males of the same strain, were used to generate VPA offspring (details below).

Standard laboratory chow (Sharpes Stock Feed, New Zealand; 3.6kcal/g) and water were available ad libitum unless stated otherwise. Animals were maintained 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 experimental procedures described herein. Naltrexone (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% saline and administered intraperitoneally (IP). Commercially available sucrose

and Research Diet high-fat high-sugar chow (HFHS, #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) were used in the palatable food intake experiments.

3.3.2 Generation of the VPA rats and confirmation of the ASD phenotype

A previously established protocol was followed to generate VPA animals [7, 11]. Adult female Sprague-Dawley rats (approximately five to six months old) were mated overnight with Sprague-Dawley males (age-matched). The following day vaginal smears were stained using 1% crystal violet and spermatozoa were detected. After the identification of spermatozoa, the date was designated as the first day of gestation (E0.5). 12.5 days after conception, females received a single IP injection of either 500 mg/kg sodium valproate (Sigma Chemical Co., St. Louis, MO, USA) or isovolumetric 0.9% NaCl (to generate control non-VPA controls). VPA female rats and their offspring were healthy. There was no significant difference in the number of animals per litter between VPA and control animals. Weaning occurred on postnatal day 25. VPA animals had no major morphological differences compared to the non-VPAs, though approximately 12% of male VPA rats developed crooked tails, which is a minor deformity commonly observed as a result of the in utero VPA treatment. Only VPA males were used in the experiments as the autistic phenotype in this ASD model is more prominent in males.

3.3.3 Feeding Studies

3.3.3.1 Effect of NTX on deprivation-induced standard chow in non-VPA control and VPA rats

Animals (body weights: controls 320 ± 17 g and VPAs 325 ± 22) were individually housed. Standard chow was removed at 18:00 on the day preceding drug administration (water was available at all times). At 10:00 on the experimental day, control and VPA animals were injected IP with saline or NTX (1 mg/kg, 3 mg/kg, or 10 mg/kg) (n = 10/group). After the drug or vehicle administration, standard chow was returned to the cages, and consumption was measured after 2 hours.

3.3.3.2 Effect of NTX on episodic intake of the high-fat high-sugar (HFHS) diet in non-deprived control and VPA rats

Individually housed control and VPA rats were pre-exposed to the HFHS chow (Research Diets #D12451) meal to avoid neophobia 3 days prior to the experiment. On the experimental day, standard chow was removed from cages at 10:00 and after 10 min, rats were injected IP with saline or NTX at 0.3 mg/kg, 1 mg/kg, or 3 mg/kg (n=8/group); and VPA rats were injected IP with saline or NTX at 1 mg/kg, 3 mg/kg or 10 mg/kg (n=8/group). 10 min after drug administration, both control and VPA rats were given the HFHS chow, and the consumption was measured after 2 hours.

3.3.3.3 Effect of NTX on episodic intake of sucrose solution in non-deprived control and VPA rats

Finally, we compared the effect of NTX on the intake of palatable 10% sucrose solution in VPA and control rats. All animals were pre-exposed to the 10% sucrose solution for 2 hours to prevent neophobia 3 days prior to the experiment. On the experimental day, standard chow and water were removed from cages at 10:00, and control rats were injected IP with saline or NTX at 0.3 mg/kg, 1 mg/kg, or 3 mg/kg (n=8/group), whereas VPA rats were injected IP with saline or NTX at 1 mg/kg, 3 mg/kg or 10 mg/kg (n=8/group). 10 min after the injection, rats were given a 10% sucrose solution, and consumption was measured after 2 hours.

3.3.3.4 Analysis of feeding data

In the feeding paradigms, the effect of different doses of NTX vs saline on consumption was established separately in control and then in VPA rats. Data in VPA and control animal cohorts were compared with a one-way ANOVA followed by the Dunnett's post-hoc test. Data were represented as means of ± SEM and the significant difference was set at $P \leq 0.05$.

3.3.4 c-Fos study

3.3.4.1 Chronic intermittent sucrose access and NTX/saline injections

Previous studies have shown that opioid receptor blockade prior to sucrose solution presentation in rats anticipating the meal, generates c-Fos immunoreactivity in a number of feeding-related brain areas, including the hypothalamus and amygdala [13]. In order to determine whether the response to NTX is different in VPA rats compared to controls, we employed the sucrose exposure paradigm used in the aforementioned studies and, prior to perfusions, we administered 1 mg/kg NTX, thus a standard dose that decreases palatability-induced feeding in control non-VPA animals. Control and VPA rats were subdivided into two groups: one fed with the sucrose diet and the other fed with the standard diet. In the standard diet groups, control and VPA rats were fed ad libitum with standard chow (access to tap water was unrestricted). Rats on the sucrose diet were given access to a 15% sucrose solution from 18.00 to 06.00 (dark phase) daily for 2 weeks. In the dark cycle, these rats were given only a limited amount (10 g) of standard chow to minimize the impact of excessive calorie intake (as in previously published studies, e.g., [14]) In the light phase (06.00- 18.00), rats had unlimited access to standard chow and water.

On the final day, rats belonging to the sucrose-fed and standard diet-fed groups were injected IP with saline or 1 mg/kg NTX at the time corresponding to sucrose presentation. Immediately after injection, food and water were removed from cages (and sucrose was not given to the animals) to prevent c-Fos induction due to changes in feeding activity. One hour after the treatment, rats were euthanized with urethane (35% in isotonic NaCl). They were perfused via the aorta with 50 mL saline (room temperature) followed by 400 mL of 4% paraformaldehyde (ice-cold; in 0.1 M phosphate buffer). Brains were excised and post-fixed overnight in 4% paraformaldehyde at 4°C. After 24 hours, 60 μm-thick coronal sections of the brains were cut with a vibratome (Leica, Germany) and free-floating sections were processed for c-Fos immunohistochemistry.

3.3.4.2 c-Fos immunohistochemistry

Sections were rinsed in 50 nM TBS (pH 7.4–7.6) four times and then treated for 10 min in 3% H_2O_2, and 10% methanol (diluted in TBS). After rinsing (4x) in TBS, sections were

incubated overnight at 4°C the in the polyclonal rabbit antibody against c-Fos (diluted 1:4000; Synaptic Systems, Australia). Sections were then washed in TBS and incubated in the goat-anti-rabbit secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA; room temperature) for one hour at room temperature. Following four washes in TBS, sections were incubated in the avidin–biotin peroxidase complex (ABC; 1:800; Elite Kit, Vector Laboratories, Burlingame, CA, USA) for another hour at room temperature. The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the sections was visualized with 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, and 0.3% nickel sulfate in TBS. The stained sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated glass slides, air-dried, dehydrated in ascending concentrations of ethanol, soaked in xylene (Merck KGaA, Germany) and embedded in Entellan (Merck KGaA, Germany). Light microscope images were taken with the camera attached to a Nikon Eclipse 400 microscope. The number of c-Fos-positive nuclei per 1 mm^2 of tissue was counted bilaterally for each neuroanatomical region of interest with the ImageJ Software. Boundaries of each area were defined according to the Paxinos and Watson brain atlas, on 2–4 sections per animal [15].Brain sections containing the paraventricular nucleus of hypothalamus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARH), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), lateral hypothalamic area (LHA), the central nucleus of the amygdala (CEA), basolateral amygdala (BLA), nucleus accumbens (NAcc), area postrema (AP), the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus (DMNV) were used in the c-Fos staining analysis.

Densities of Fos-immunoreactive nuclei were compared with a two-way ANOVA with drug and diet set as independent factors. Data were represented as means of ± SEM and the significant difference was set at $P \leq 0.05$.

3.4 Results

We followed earlier ASD VPA rat model reports in the confirmation of the ASD phenotype in the VPA cohort [7, 12] . Behavioral tests to confirm the ASD phenotype included the open field social interaction test and the anxiety-like behavior in the elevated plus maze. In short, in the open field social interaction test, non-VPA and VPA rats were introduced into a 44 x 44 cm open arena for a duration of 9 minutes: social interactions, such as sniffing, licking, crawling over or under, mounting, anogenital inspections were

scored for both cohorts [7, 12]. The social interactions scored in these tests showed a cumulative decrease in these socially driven behaviors in VPA animals. In the elevated plus maze test, the rats were first introduced to the open field arena for five minutes; afterwards they were transferred to the elevated plus maze and observed for five minutes [7, 12]. The maze was positioned 50 cm above the floor and consisted of two open arms and two closed arms, 50 × 10 × 40 cm each, located opposite to each other with an open roof above. The rat was placed at the center of the maze, facing one of the open arms. The duration of time spent in the open arm and the number of entries made into it, as well as the duration of time spent in the closed arms, were recorded. An entry into the open arm was defined as when both of the rat's front paws were placed on or beyond the boundary of the closed arms. When exposed to the elevated plus maze, VPA animals spent a significant amount of time in the closed arm and made fewer entries into and spent less time in the open arm, in comparison to the control group.

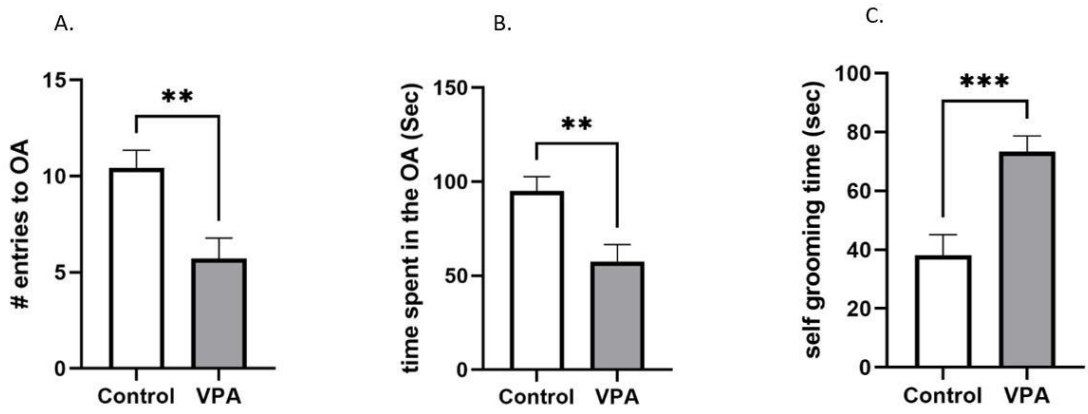

Figure 3.1: Assessment of behavioural patterns in VPA rats displaying traits similar to ASD In the Elevated Plus Maze test, (A) the number of times the rats entered the open arms and the duration spent in the open and closed arms were recorded. Rats showing ASD characteristics tended to stay longer in the closed arms and ventured into the open arms less frequently compared to typical rats. (C) The time spent on self-grooming was similar between the two groups. Data is presented as average ± SEM, with 19 rats in each group. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Acute administration of 1, 3, and 10 mg/kg NTX did not produce a reduction in the consumption of energy-dense standard chow in overnight food-deprived control rats Figure 3.2A). Similarly, NTX was ineffective in VPA rats (Figure 3.2 B).

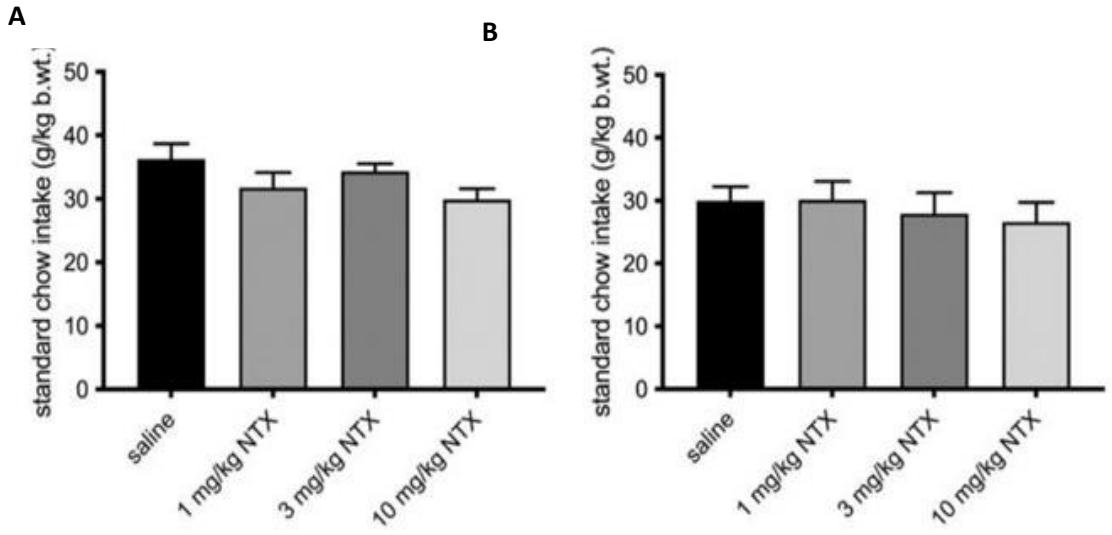

Figure 3.1: Effect of saline or NTX (1, 3, 10 mg/kg) on the consumption of standard chow during a 2-hour meal after overnight food deprivation in control (A) and VPA rats (B)

In the study utilizing the consumption of the HFHS diet, 1 and 3 mg/kg doses of NTX significantly reduced the consumption of the HFHS chow after 2 hours in non-VPA rats (1 mg/kg, p=0.036, 3 mg/kg, p<0.001) (Figure 3.3A). On the other hand, in the VPA rats, only the 10 mg/kg NTX dose produced a significant reduction in HFHS consumption (10 mg/kg, p=0.0222, Figure 3.3B)

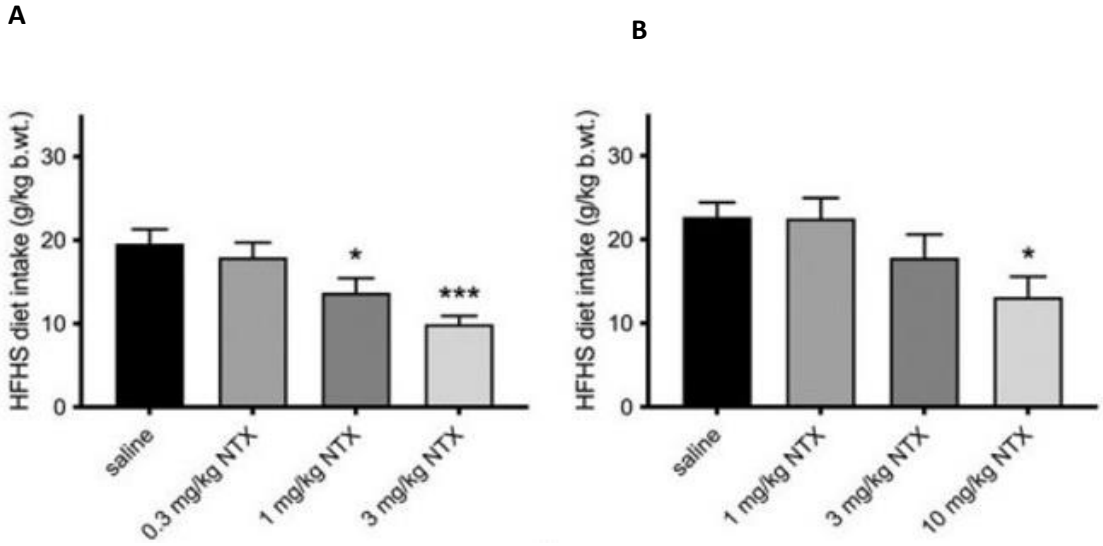

Figure 3.3: Effect of saline or NTX (1, 3, 10 mg/kg) on episodic 2-hour consumption of a palatable high-fat high-sugar chow in non-deprived control (A) and VPA rats (B)

Acutely administered 1 and 3 mg/kg NTX reduced the consumption of a 10% sucrose solution in non-VPA animals (1 mg/kg, p=0.011, 3 mg/kg, p<0.001), whereas 0.3 mg/kg NTX was not effective (Figure 3A). In VPA rats, only 10 mg/kg NTX suppressed sucrose consumption, (10 mg/kg, p=0.001)

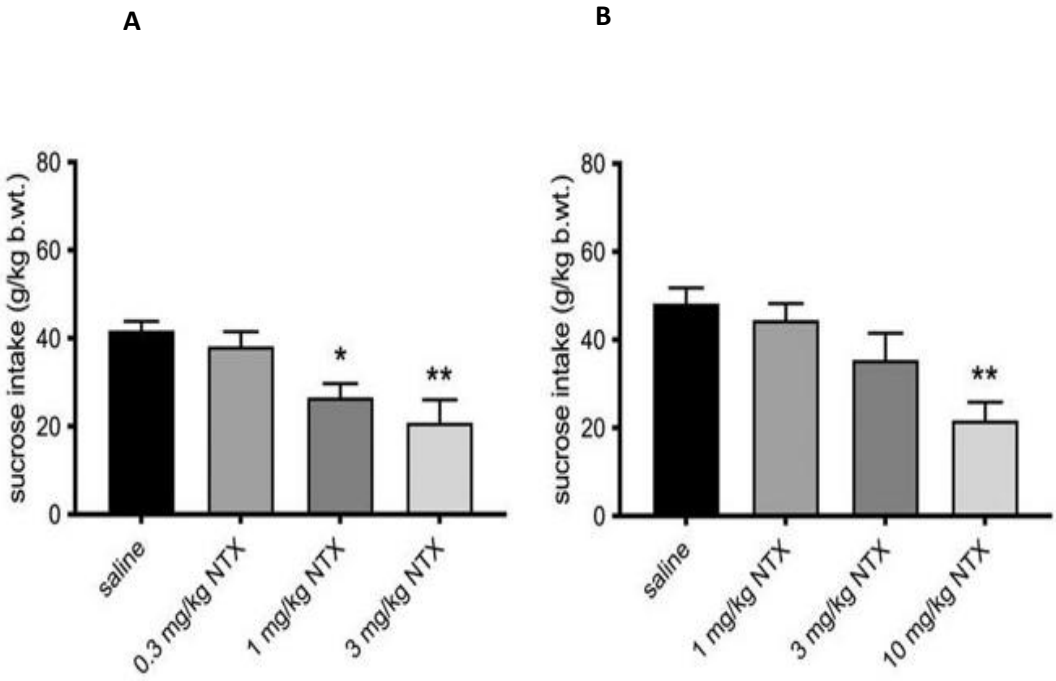

Figure 3.4: Effect of saline or NTX (1, 3, 10 mg/kg) on episodic intake of 10% sucrose solution in control and VPA rats after 2 hours (A) effect of saline or NTX on episodic intake of sucrose solution in control rats (g/kg b.wt.) (B) effect of saline or NTX on episodic intake of sucrose solution in VPA rats (g/kg b.wt.).

In the chronic daily sucrose intake study, a one-way ANOVA revealed that there was no significant difference in daily calorie intake between the control diet-fed group and the sucrose diet-fed group in control animals. However, over a period of 14 days, VPA animals exhibited a tendency towards higher intake of sucrose diet as compared to the control diet intake.

In the c-Fos study in non-VPA rats, a two-way ANOVA showed a statistically significant drug × diet interaction in the PVN ($p=0.0273$, $F(1, 20)=5.668$), SON, ($p=0.0150$, $F(1, 20)=7.08$), ARH ($p=0.0023$, $F(1, 20)=12.24$), DMH ($p=0.0036$, $F(1, 20)=10.90$), CEA ($p=0.0188$, $F(1, 20)=6.536$) and NAcc-shell ($p=0.0411$, $F(1, 20)=4.7$). On the other hand, in VPA rats, only the CEA ($p=0.0411$, $F(1, 20) =4.7$) showed a significant drug × diet interaction.

In non-VPA rats, there was a significant effect of the drug in the PVN ($p<0.0001$, $F(1,20)=166.0$), SON ($p<0.0001$, $F(1, 20)=57.63$), ARH ($p=0.0004$, $F(1, 20)=85.99$), DMH ($p<0.0001$, $F(1, 20)=88.57$), VMH ($p=0.0005$, $F(1, 20)=88.57$), LHA ($p=0.0068$, $F(1, 20)=60.39$), CEA ($p<0.0001$, $F(1, 20)=148.0$), BLA ($p=0.005$, $F(1, 20)=15.79$), NAcc-Core ($p=0.0082$, $F(1, 20)=8.659$), NAcc-shell ($p<0.0001$, $F(1, 20)=70.17$) and NTS ($p=0.0001$, $F(1, 20)=21.92$). Whereas VPA animals showed a significant effect of the drug only in the PVN ($p=0.0009$, $F(1, 16)=16.38$), ARH ($p=0.0442$, $F(1, 20)=4.613$), DMH ($p=0.0015$, $F(1, 20)= 13.43$), CEA ($p<0.0001$, $F(1, 20)=27.24$), NAcc-shell ($p=0.0074$, $F(1, 12)=10.35$).

Furthermore, the diet itself induced a significant change in c-Fos immunoreactivity in non-VPAs in the PVN ($p=0.0006$, $F(1, 20)=16.50$), SON ($p<0.0001$, $F(1, 20)=24.46$), ARH ($p<0.0001$, $F(1, 20)=27.43$), DMH ($p=0.0001$, $F(1, 20)=23.13$), VMH ($p=0.0017$, $F(1, 20)=23.13$), LHA ($p<0.0001$, $F(1, 20)=28.84$), CEA ($p<0.0001$, $F(1, 20)=24.43$), NAcc-shell ($p<0.0001$, $F(1, 20)=27.99$) and NTS ($p=0.0002$, $F(1, 20)=20.37$). On the other hand, in VPA rats there was a significant effect of the diet on the DMH ($p=0.0425$, $F(1, 20)=4.696$), VMH ($p=0.0472$, $F(1, 20)=4.472$), CEA ($p=0.0149$, $F(1, 20)=7.096$) and NAcc-shell ($p=0.0483$, $F(1, 12)=4.832$).

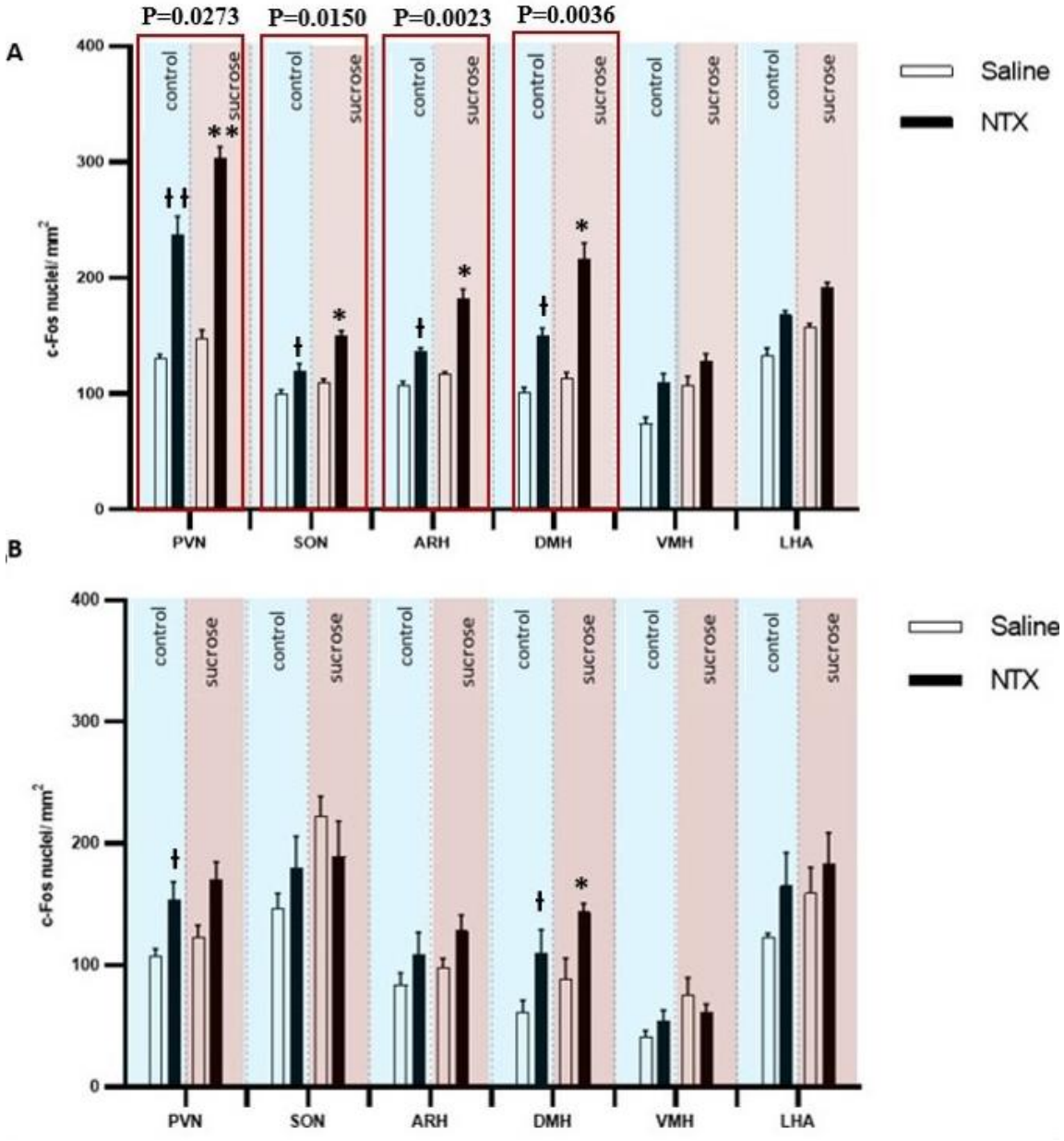

Figure 3.5: The effect of saline vs NTX (1 mg/kg) on c-Fos immunoreactivity in the hypothalamic area in control and VPA animals. (A) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in control rats fed either a control diet or a sucrose diet for 14 days. (B) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in VPA rats fed either a control diet or a sucrose diet for 14 days.

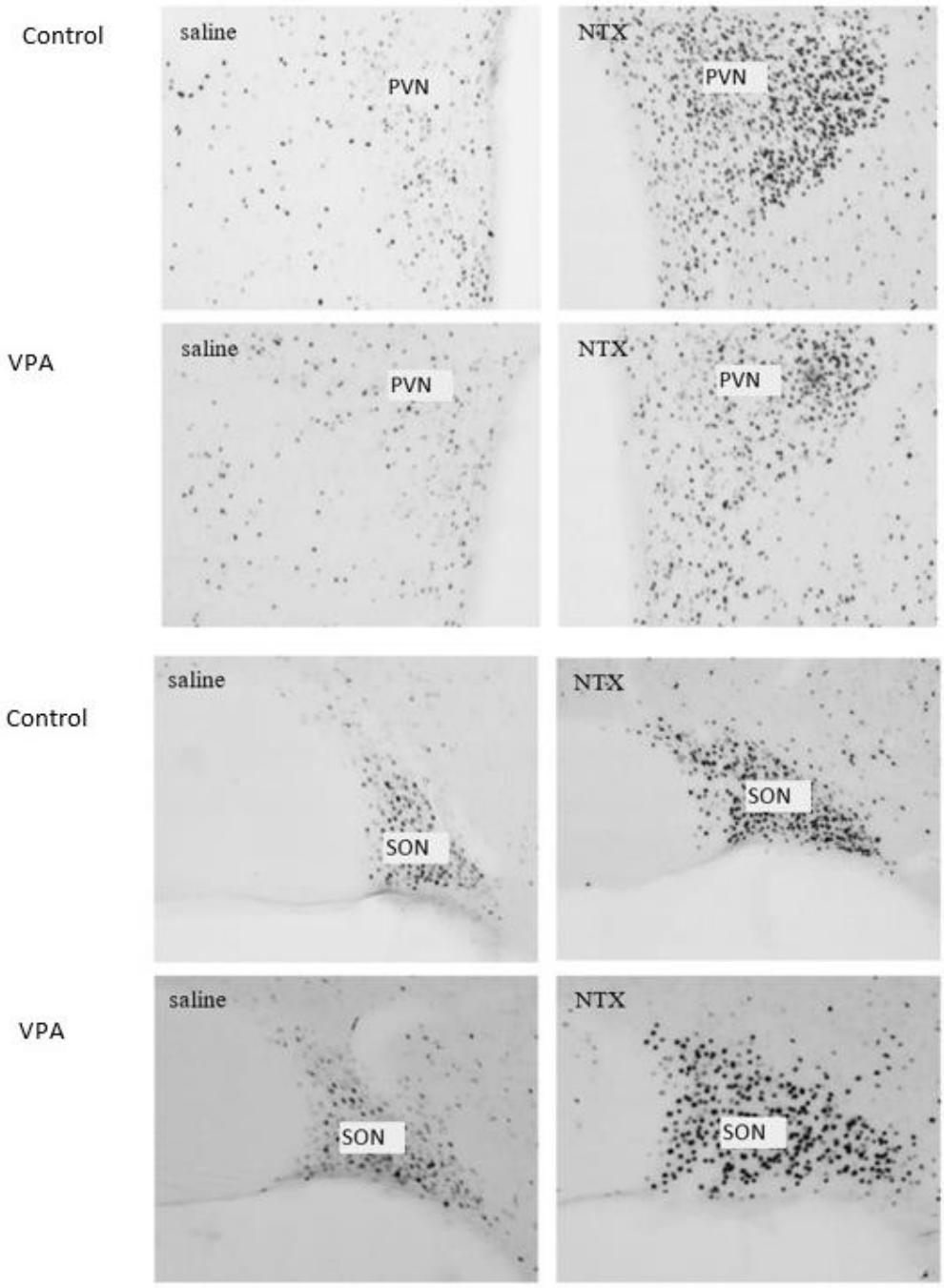

Figure 3.6: The photomicrographs depict c-Fos immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus of the hypothalamus (SON), of control and VPA rats exposed to control diet vs sucrose diet that received a saline or NTX injection.

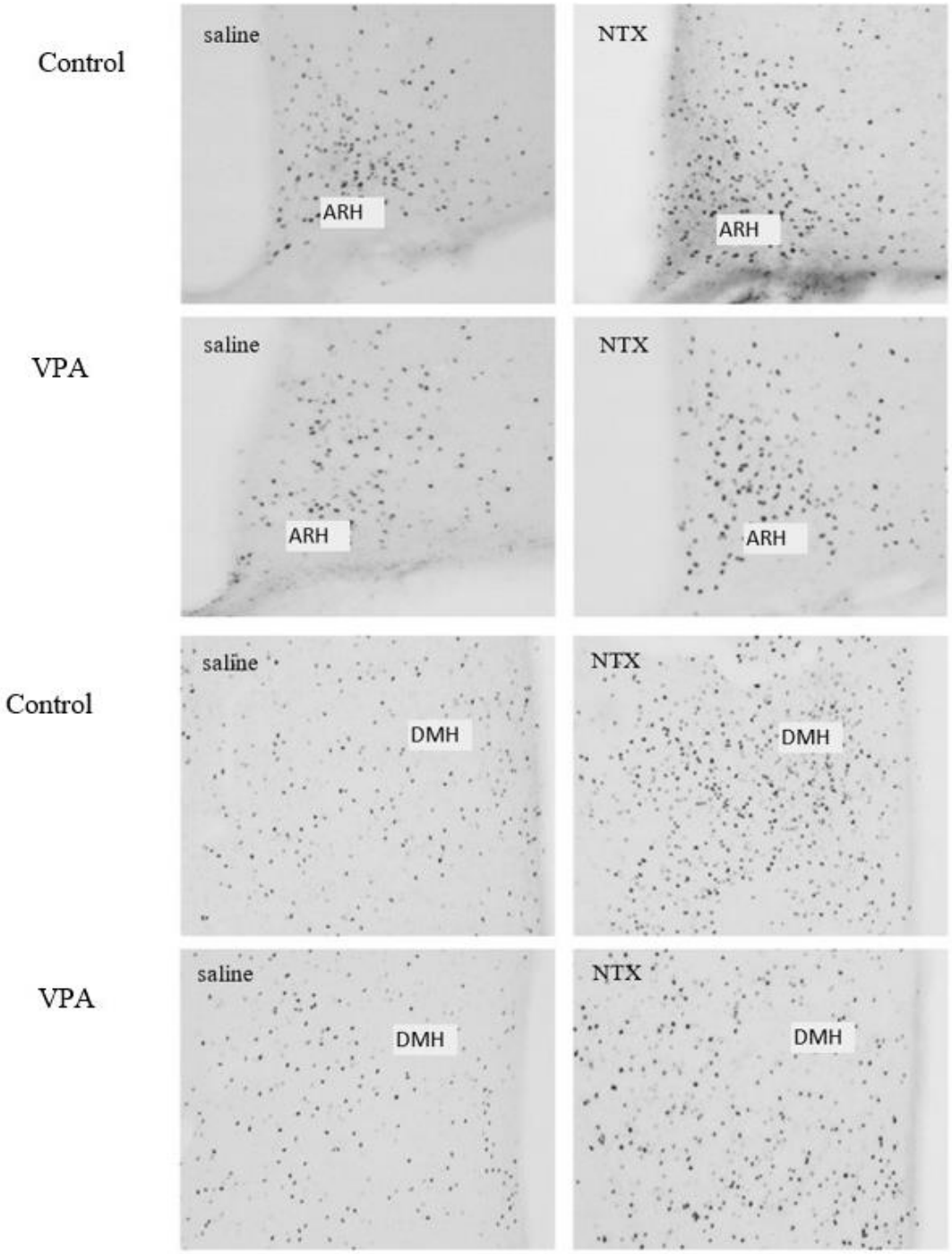

Figure 3.7: The photomicrographs depict c-Fos immunoreactivity in the paraventricular nucleus of the arcuate nucleus of the hypothalamus (ARH), and the dorsomedial hypothalamic nucleus (DMH) of control and VPA rats exposed to control diet vs sucrose diet that received a saline or NTX injection.

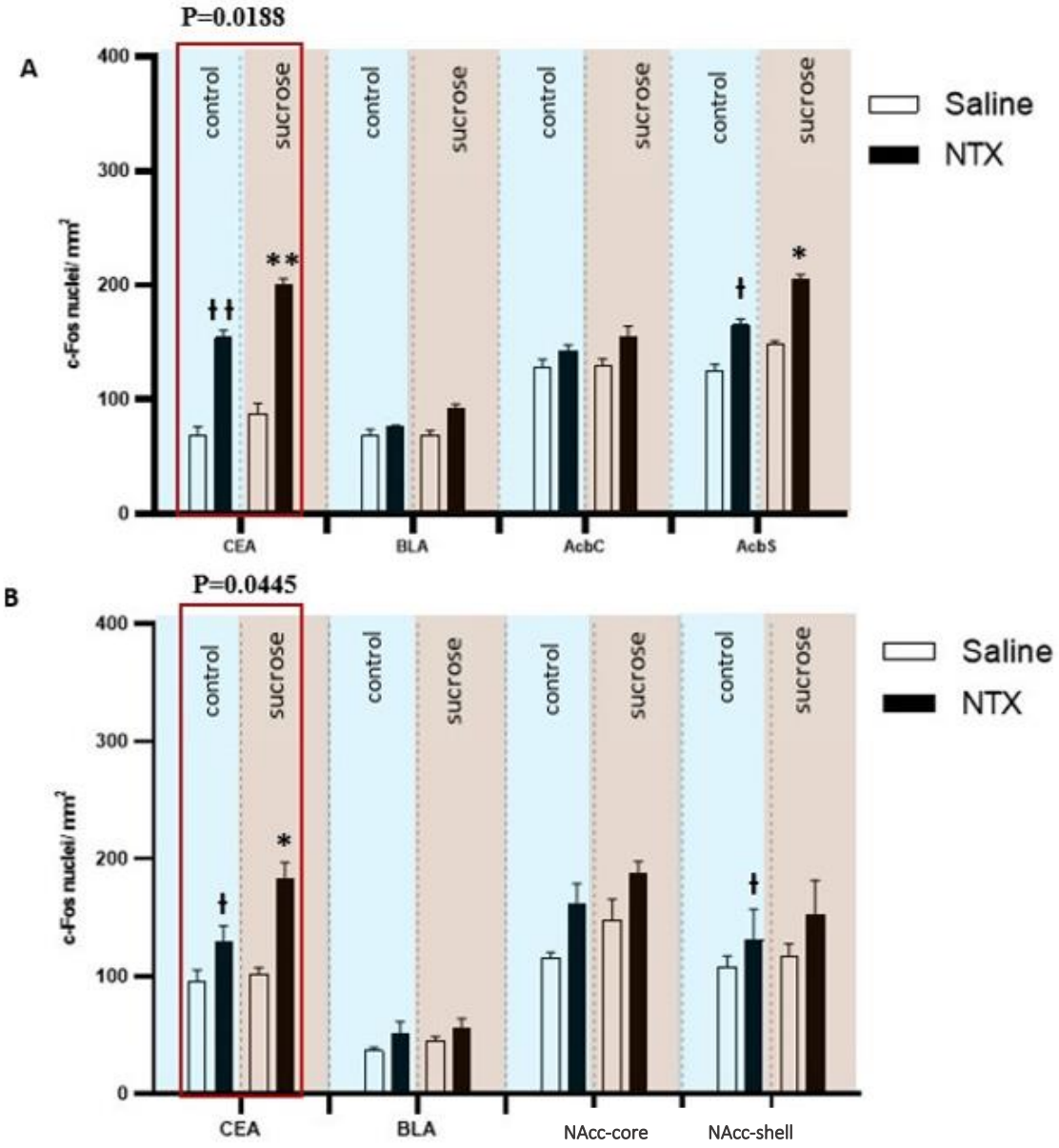

Figure 3.8: The effect of saline vs NTX (1 mg/kg) on c-Fos immunoreactivity in amygdala and nucleus accumbens in control and VPA animals. (A) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in the amygdala and nucleus accumbens in control rats fed either a control diet or a sucrose diet for 14 days. (B) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in the amygdala and nucleus accumbens in VPA rats fed either a control diet or a sucrose diet for 14 days.

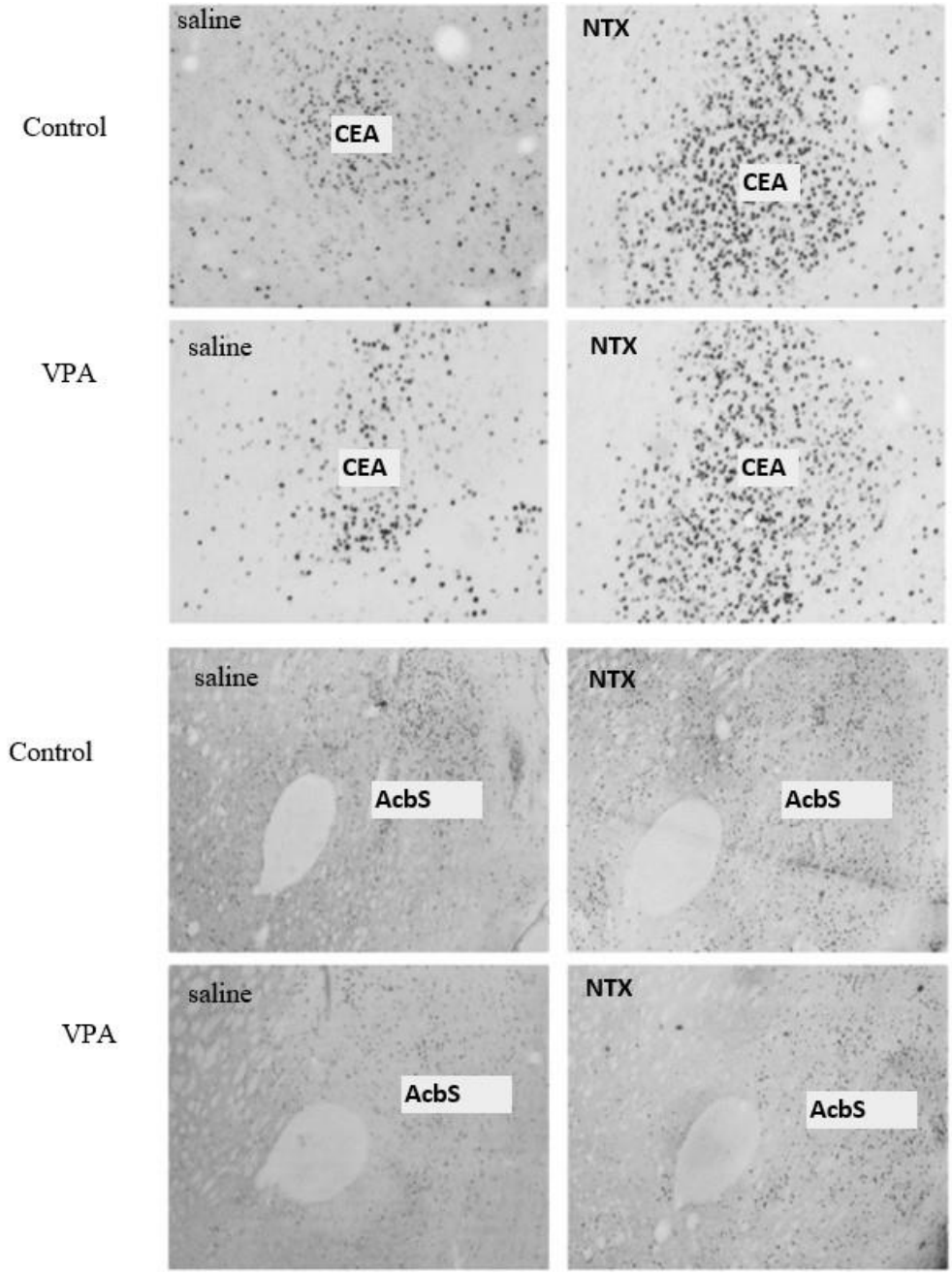

Figure 3.9: The photomicrographs depict c-Fos immunoreactivity in the central nucleus of the amygdala (CEA) and nucleus accumbens shell (NAcc-shell) of control and VPA rats exposed to control diet vs sucrose diet that received a saline or NTX injection. In the control rats, a significant interaction between the drug and diet was observed in the CEA and NAcc-shell, whereas in the VPA animals, a significant drug-diet interaction was only seen in the CEA.

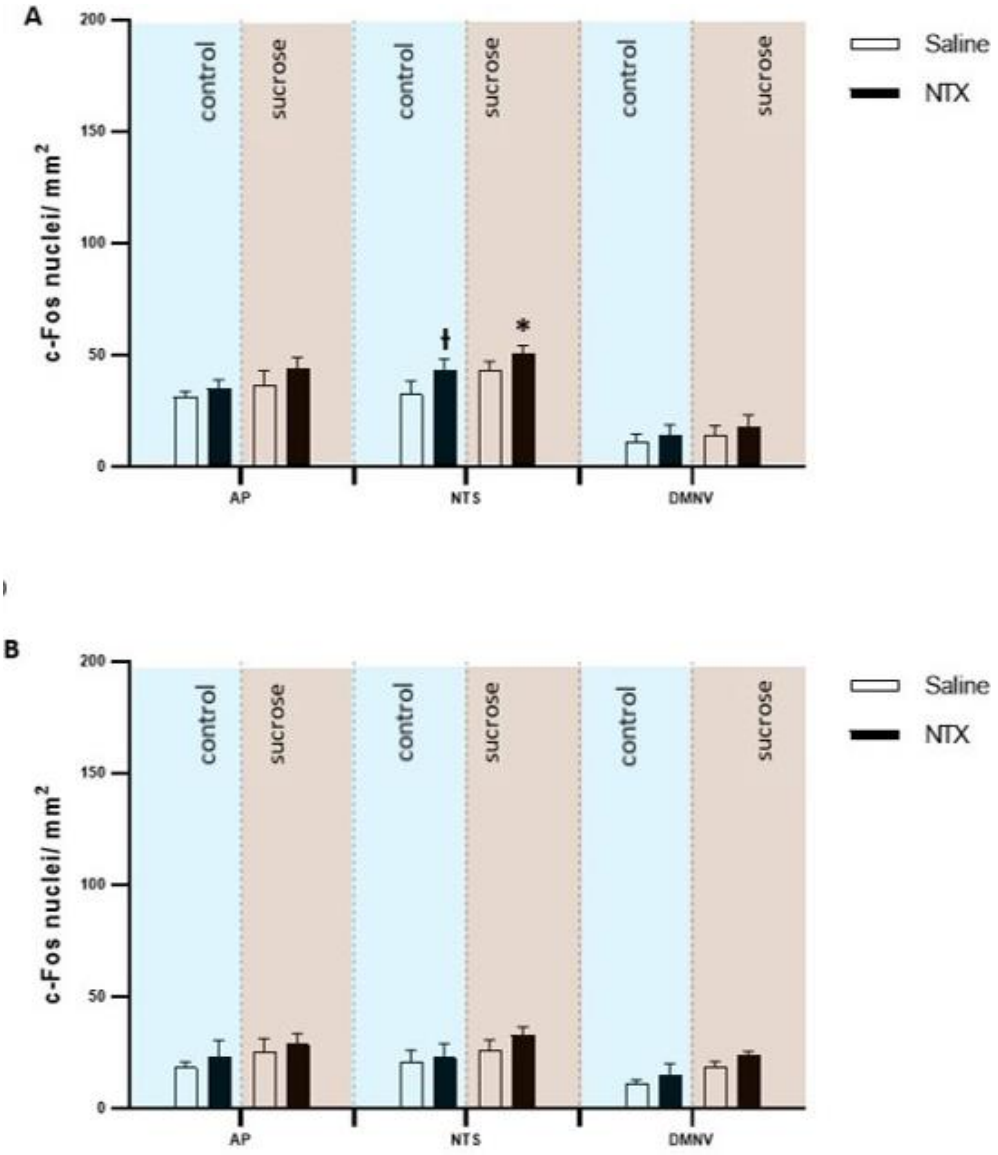

Figure 3.10: The effect of saline vs NTX (1 mg/kg) on c-Fos immunoreactivity in the brain stem in control and VPA animals. (A) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in the brain stem in control rats fed either a control diet or a sucrose diet for 14 days. (B) The effect of saline and NTX (1.0 mg/kg) on c-Fos immunoreactivity in the brain stem in VPA rats fed either a control diet or a sucrose diet for 14 days.

3.5 Discussion

Aberrant eating behavior is one of the well-known facets of autism. Many individuals with ASD exhibit selective (picky) eating habits, which limits their food choices and deters them from trying foods whose texture, color or other physical or sensory characteristics do not fall within a very narrow range of preference. This can ultimately result in long-term consumption of an extremely monotonous diet that poses a risk of nutrient deficiencies, such as previously described for retinol and 25-hydroxycholecalciferol [16]. On the other hand, repetitive food choices and a heightened drive to seek only select foods can lead to another problem, namely, the consumption of high amounts of calorie-dense palatable foods that increase the likelihood of developing obesity. Dysregulation of neurotransmitter and neuropeptide systems essential for feeding control is thought to play a significant role in the abnormal eating patterns of individuals with ASD, with the opioid system proposed as one of the culprits in the peculiar-eating habits of individuals with ASD [17]. Consequently, there has been interest in potentially utilizing opioid receptor ligands in therapies to manage ASD symptoms. One of those molecules is NTX as it has been investigated in the context of its ability to restore social behaviors in ASD. The data presented in the current chapter show for the first time that NTX is an effective suppressant of palatability-induced food intake in VPA animals.

In this study, the acute administration of NTX did not result in a significant decrease in the consumption of energy-dense chow in both controls non VPA and VPA rats. Thus, the effect of NTX (or, rather, the lack thereof) on energy-driven consumption is in line with the long-standing concept of opioids as being generally uninvolved in the regulation of feeding for energy. Previous studies on NTX in non-ASD animal models indicate that bland chow intake is unaffected by NTX. For example, Kanarek and colleagues found that NTX injections have minimal or no effects on the intake of regular chow in laboratory animals [18]. Apfelbaum and Mandenoff reported that NTX have little or no effect on the consumption of laboratory chow in rats [19]. Another opioid receptor antagonist, naloxone, also failed to decrease the intake of the “bland” cornstarch powdered diet in refeeding after food deprivation [20]. Analyzing the data from the standpoint of ASD, it is not surprising either that the effect of NTX on energy-driven feeding was absent: Klockars et al. have reported that VPA and non-VPA animals maintain similar body weight trajectory when they are maintained on the standard laboratory chow, suggesting

no difference in energy balance processing (and, thus, no potential for opioid receptor activation to have a unique calorie consumption-related effect) [7].

The results of my study support evidence that NTX reducing the consumption of palatable foods in general. NTX administration has been shown to decrease intake of standard chow and a 32% sucrose solution more effectively than standard chow alone in rats [18]. Kanarek et al. found that NTX produced a dose-dependent reduction in standard chow intake in rats that had previously consumed palatable solutions [18]. Further, central infusion of NTX inhibited the re-development of a preference for a high-sugar diet after a period of restriction to a less palatable starch diet [21]. In human studies, NTX has been reported to alter the pleasantness of sweet solutions. Yeomans et al. found that following NTX administration, human can taste the sweetness of the sucrose, but the pleasurable or rewarding aspect of the taste is reduced [22].

In the present study, NTX significantly suppressed the consumption of HFHS diet and 10% sucrose solution in both control and VPA rats. It indicates that the ASD rats are sensitive to the hypophagic effect of opioid receptor blockade, similar to what has been described multiple times in the literature in relation to non-ASD animals. It should be emphasized, however, that the minimum effective dose of NTX in VPA animals (10 mg/kg) is 10 times higher than that in control animals (1 mg/kg). The actual nature of this phenomenon remains to be elucidated. One possible explanation might be that VPA animals have a higher sensitivity to the rewarding effects of palatable foods compared to non-VPA animals and, therefore, require a much higher dose of NTX to produce an effect on consumption of palatable foods. This is in concert with evidence showing, for example, heightened activity in the anterior cingulate cortex (ACC), insula, and mesolimbic NAcc-VTA pathway in ASD which coincides with hyper-responsiveness to pleasant stimuli in autism [2]. Hughes and colleagues found that administering valproic acid to animals results in changes in mRNA levels of opioid receptors, indicating that the VPA animal model replicates modifications in the opioid system that occur in humans. [23]. The data are supported by Klockars et al who revealed the consumption of palatable sucrose, saccharin, and milk was significantly elevated in VPA animals compared to control non VPA animals [7].

In the present study, I also assessed how NTX impacts c-Fos immunoreactivity in feeding related brain areas in VPA rats exposed to a 15% sucrose solution for 14 days. With

results of c-Fos analysis, NTX injections showed an elevation in c-Fos immunoreactivity in selected brain areas in both non-VPA and VPA animals. Importantly, the elevation of c-Fos was higher in the sucrose/NTX group than in the water/NTX groups in both control and VPA rats and the magnitude of the changes in c-Fos was greater in control animals than VPA animals. These results support the notion that intermittent sucrose consumption increases opioid tone in the brain and thus it facilitates the hypophagic effect of an opioid antagonist, NTX. Interestingly, paralleling the inability of 1 mg/kg NTX to reduce sucrose intake in VPAs, VPA animals had less c-Fos in the selected brain areas after NTX injection compared to the non-VPA controls.

Interestingly, in both control and VPA animals, c-Fos immunoreactivity in CEA was relatively similar and for both cohorts the drug–diet interaction in the CEA was significant. In VPA animals, among all the sites, the CEA was only the area where a significant drug–diet interaction was found. There is evidence in literature pointing to the amygdala as a crucial site in the context of ASD-reward interaction. Cascio et al. reported similar patterns of increased blood oxygenation level-dependent signal in the bilateral amygdala response to the food images in children with ASD and without ASD [2]. Further, the significant change in activation observed in the CEA following intermittent sucrose consumption and NTX injection is consistent with the results reported by Pomonis et al. and Jewett et al who investigated the impact of naloxone and NTX induced alterations in c-Fos immunoreactivity in rats after chronic intermittent sucrose consumption [13, 14].

Pellissier et al. have discussed μ receptor balance model in ASD which suggests that ASD symptomology might stem from both excessive opioid tone and insufficient opioid tone (i.e., broad dysregulation) [24]. In the present study, we found significant drug interaction in DMH, VMH, CEA and NAcc-shell in VPA animals. These results suggest that NTX has a significant effect in reward-related brain areas. The effect of NTX in ASD individuals has been studied and several studies have found that NTX affects opioid tone in the brain. Leboyer et al. have found that NTX effective in resetting abnormal level of various neurohumoral parameters such as β-endorphin and arginine vasopressin [25]. Bouvard and co-workers also revealed that NTX is effective in treating certain plasma abnormalities in autistic children [10]. Therefore, the results of this study indicate that NTX may be a potential candidate to suppress palatable food consumption in ASD

individuals, though the dose has to be carefully considered since the sensitivity to NTX is different in ASD.

In conclusion, NTX is effective in reducing palatable food consumption in VPA ASD animals and it activates feeding-related brain areas. However, the minimum NTX dose to generate hypophagia in autistic individuals is higher than in non-VPA controls.

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Chapter 4

Intranasal NTX infused alone or in combination with another anorexigenic molecule, oxytocin: Is this administration route the new way to generate hypophagia?

Intranasal (IN) administration of drugs that act via brain receptors has recently become a go-to route in many pharmacotherapies. The effectiveness of IN NTX on opiate-induced responses in humans and laboratory animals has been shown before, but no studies have tested the usability of IN NTX as a suppressant of food intake either as a sole IN drug or as a molecule administered in combination with another IN anorexigenic agent. This set of studies was aimed at closing this gap of knowledge by asking whether IN NTX decreases feeding for energy or for palatability in rats and whether the potential effectiveness of the IN NTX treatment can be enhanced by IN co-administration of oxytocin (OT), i.e., another anorexigen whose co-injection with NTX has been previously shown to enhance hypophagia.

In order to address this issue, the studies were divided into two major projects which are presented in this Chapter as **Part I** and **Part II**:

Considering that thus far anorexigenic effects of IN OT in rodents have only been confirmed in night-time feeding in ad libitum-fed mice maintained on standard chow, in **Part I** of this Chapter I present a thorough analysis of the effects of IN OT on hunger- and palatability-induced food intake in rats, as well as the analysis of c-Fos immunoreactivity changes after IN OT in rats habitually consuming sugar.

In **Part II** of the Chapter I present a pilot study that shows that, unlike OT, IN NTX is a poor suppressant of appetite in rats and that the combination of IN NTX with IN OT does not produce a beneficial (i.e., synergistic) effect.

4.1 Part I: Effect of intranasal oxytocin on palatable food consumption and c-Fos immunoreactivity in relevant brain areas in rats

4.1.1 Abstract

Peripheral and central injections of oxytocin (OT) in laboratory animals decrease feeding for energy and palatability, but the hypophagic response is dependent on the administration route. Human studies rely on intranasal (IN) administration of the peptide, the route underutilized in OT animal feeding studies thus far. Therefore, we examined the effect of IN OT on various aspects of consumption in rats: (a) overnight deprivation-induced standard chow intake, (b) episodic (2-h) consumption of calorie-dense and palatable high-fat high-sugar (HFHS) chow, (c) 2-h episodic intake of palatable and calorie-dilute sucrose and Intralipid solutions, and (d) 2-h sucrose solution intake in rats habituated to ingesting this solution daily for several weeks. Finally, we assessed c-Fos changes in response to the acute IN OT administration in rats subjected to habitual sugar consumption. We found that IN 20ug OT decreased deprivation-induced intake of standard chow and HFHS chow in nondeprived rats without affecting water consumption. IN OT also reduced 2-hour episodic fluid consumption of sucrose, but not Intralipid. In the habitual sugar consumption paradigm, acute IN OT diminished sucrose solution intake in animals accustomed to the 2-hour/day sucrose meal regimen. In rats habitually consuming sucrose, IN OT altered c-Fos immunoreactivity in brain areas related to energy homeostasis and reward, including the central nucleus of the amygdala, the hypothalamic paraventricular and the arcuate nuclei. We conclude that IN OT is an effective appetite suppressant in rats and its effects involve feeding-related brain circuits.

4.1.2 Introduction

Oxytocin (OT), a nine amino-acid neuropeptide primarily synthesized in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, has been implicated in the regulation of food intake. OT neurons are activated, and OT is released, at the time of feeding termination [1]. Food deprivation leads to the downregulation of OT gene expression, whereas overconsumption upregulates OT mRNA in the hypothalamus [2]. Stomach distension and elevated plasma osmolality, i.e., peripheral changes associated with the end of a meal, lead to the release of OT [3, 4].

Reports published since the late 1980's have consistently shown that peripheral and central injections of OT receptor ligands in laboratory animals affect appetite. For example, Arletti et al found intraperitoneal (IP) and intracerebroventricular (ICV) administration of OT reduces standard chow intake at the onset of the dark cycle, the time of the robust feeding activity in ad libitum-fed animals [5]. Standard chow consumption has also been reduced in food-deprived rodents in which OT was injected ICV, IP, subcutaneously (SC), intravenously (IV) and into several specific brain sites that regulate energy balance [6-9].

OT also decreases palatable food intake [10, 11]. Administration of OT in the ventral tegmental area (VTA), nucleus accumbens (NAcc) and amygdala (AMY) – sites involved in reward-related feeding - decreases intake of palatable foods in a dose-dependent manner [12-14]. Conversely, peripheral injections of a blood-brain barrier penetrant OT receptor antagonists, L-366,899, increase palatability driven feeding [15, 16].

It should be emphasized that the route of OT receptor ligand administration and the composition and gustatory characteristics of food impact the effectiveness of the treatment. For example, IP injection of the OT antagonist, L-368,899, robustly increases sugar solution intake, slightly increases saccharin intake, but does not affect intake of fat emulsions [15, 16]. IV OT fails to decrease drinking of sugar water, ventromedial hypothalamic (VMH) and central AMY OT injections do not decrease the amount of ingested sucrose or saccharin, whereas NAcc, VTA and basolateral AMY OT injections suppress sucrose solution intake [6, 9, 12-14]. NAcc OT is more effective at reducing saccharin-sweetened water intake than ingestion of sucrose solutions [13].

Intranasally administered OT to humans decreases total energy intake (see, e.g., [17, 18]). As shown in a recent metanalysis by Leslie and colleagues [19], it is unclear to what extent IN-administered OT diminishes consumption of foods that differ in macronutrient composition, palatability, energy density, and other characteristics (e.g., [1, 20]).

While the effects of IN OT on anxiety and sociality has been extensively studied in animals, the effectiveness of IN OT has only been evaluated in ad libitum chow-fed mice at the onset of the dark cycle [21]. In that study, the 10 µg IN OT decreased chow intake after the first two hours. It remains unknown whether the same dose would be sufficient to reduce post-deprivation chow intake or the consumption of energy-dense, palatable high-fat high-sugar (HFHS) diets. Considering the previously reported discrepancy in the effectiveness of injected OT on episodic sucrose vs fat solution consumption, a similar liquid diet intake assessment in IN-treated animals would be useful.

In the current study we administered OT IN to rats at 10 and 20 µg (doses similar to those shown to decrease night-time chow intake [21] in nondeprived animals within a short, i.e., 2-h timeframe) and examined the IN OT effects on various aspects of consumption. First, we assessed the effect of IN OT on overnight deprivation-induced intake of standard chow. Since that treatment was effective, we subsequently assessed whether IN OT in nondeprived rats suppresses episodic (2-h) consumption of highly caloric and palatable HFHS chow. We also examined whether IN OT affects 2-h episodic intake of palatable and calorie-dilute sucrose and Intralipid solutions. Since only sucrose consumption was suppressed in those episodic intake experiments, we studied whether IN OT acutely suppresses a 2-h sucrose solution meal in rats accustomed to ingesting this solution daily for several weeks. Finally, we assessed c-Fos changes in response to the acute IN OT administration in rats subjected to habitual sugar consumption.

4.1.3 Materials and Methods

4.1.3.1 Animals

Male Sprague-Dawley rats aged 20 weeks old (average b. wt. 500 +/- 15 g) were single-housed in standard polycarbonate cages with wire tops and maintained on a 12:12 LD cycle (lights on at 06:00) in a temperature-controlled room (22°C). Standard laboratory chow (Sharpes Stock Feed, New Zealand; 3.6kcal/g) and tap 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. The University of Waikato Animal Ethics Committee approved all experimental procedures described in this study.

4.1.3.2 Drug preparation and administration

OT (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline at 4°C and slowly warmed to room temperature 20 min prior to administration. The rats were given saline (20µl), 10µg OT (in 20µl saline) or 20µg OT (in 20µl saline) intranasally. Rats were gently restrained and 10 µl of the solution (OT or saline) was infused in each nostril with a pipette. The total administration time was 3 minutes. The doses of IN OT (20µg – effective; 10µg – subthreshold) were chosen based on previously published data [21].

4.1.3.3 Effect of IN OT on deprivation-induced standard chow intake

Animals (n = 15/group) were deprived of standard chow from 15:00 on the day preceding the post-deprivation meal until 09:00 on the experimental day. Water was available at all times. On the experimental day, animals were treated with IN saline, OT (10µg), or OT (20µg). Twenty minutes after drug administration, chow was returned to hoppers and consumption was measured after 2 hours.

4.1.3.4 Effect of IN OT on episodic intake of the high-fat high-sugar (HFHS) chow in nondeprived rats

Rats (n=15/group) were pre-exposed for 2 hours to the HFHS chow (Research Diets #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) to avoid neophobia 3 days prior to the experiment. On the experimental day, standard chow was removed from cages at 07:00 and after 30 minutes rats were treated with IN saline or OT at 10μg or 20μg. Twenty minutes later, rats were given the HFHS chow, and the consumption was measured after 2 hours.

4.1.3.5 Effect of IN OT on episodic intake of 15% sucrose solution in non-deprived rats

Rats (n=15/group) were pre-exposed to the 15% sucrose solution for 2 hours to prevent neophobia 3 days prior to the experiment. On the experimental day, standard chow and water were removed from cages at 07:00 and after 30 minutes rats were treated with IN saline or OT at 10μg or 20μg. Twenty minutes later, rats were given a 15% sucrose solution, and their intake was measured after 2 hours.

4.1.3.6 Effect of OT on episodic intake of intralipid solution in non-deprived rats

Rats (n=15/group) were pre-exposed to the 4.1% Intralipid solution for 2 hours to prevent neophobia 3 days prior to the experiment. On the experimental day, standard chow and water were removed from cages at 07:00 and after 30 minutes rats were treated with IN saline or OT at 10μg or 20μg. Twenty minutes later, rats were given an Intralipid solution, and its intake was measured after 2 hours.

4.1.3.7 Effect of acute IN OT on a sucrose meal intake in rats habitually consuming a sugar solution

Forty-five male rats were given access to a 15% sucrose solution for 2 hours daily for 28 days. The 15% sucrose solution was freshly prepared each morning and it was given daily at 09:00 and taken away at 11:00. Standard chow and water were removed for the time of sucrose presentation and returned to the cages at 11:00. During the rest of the light phase (from 11:00 to 18:00), animals were given standard chow and water ad libitum and consumption were measured at the end of the light cycle (at 18:00). Rats were given

access to standard chow and water ad libitum in the dark phase (18:00 to 06:00) and consumption was measured at 06:00. Animals in the sugar group were consuming daily on average 27 g of sugar water and 29 g of standard chow, whereas the standard diet controls ingested 30 g of chow pellets/day.

Following the 28 days of chronic sucrose access, rats were administered saline or OT at 10 or 20μg/ 20μl 30 min before the daily 15% sucrose solution and the consumption was measured after 2 hours.

4.1.3.8 c-Fos immunoreactivity in the feeding-related brain circuit in response to acute IN OT in rats habitually consuming sugar water

Rats were subdivided into two cohorts based on their diet: the control diet cohort and the sucrose diet cohort. The control diet cohort was maintained on standard chow and water ad libitum. The sucrose diet cohort was given daily 2 hours of access to a 15% sucrose solution from 09:00 to 11:00 (during this time chow and water were removed from the cages) and it had unrestricted access to standard chow and water in the remainder of 22 hours, as described in Section 2.7. This daily feeding schedule was continued for 45 days.

On the final day, the control cohort and the sucrose-fed cohort were each subdivided into two groups that received a different drug treatment: one group was given IN saline and the other IN OT (20 μg) at 09:00 (at the time corresponding to the beginning of the sugar/water presentation in the sucrose-fed cohort). Following IN OT or saline administration, chow and water were removed from the cages of both the control diet cohort and the sugar diet cohort. Sucrose solution was not given to the sugar cohort rats.

Sixty minutes later the rats were deeply anesthetized with urethane (35% in saline) and perfused via the aorta with 50 mL saline (room temperature) followed by 400 mL of 4% paraformaldehyde (ice-cold; in 0.1 M phosphate buffer). Brains were excised and post-fixed overnight in paraformaldehyde at 4°C. After 24 hours of postfixation, 60 μm-thick coronal sections of the brain were cut with a vibratome (Leica, Germany) and free-floating sections were used for c-Fos (single staining) and c-Fos and OT (double staining) immunohistochemistry. Brain sections containing the PVN, SON, arcuate nucleus (ARH), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), lateral hypothalamic area (LHA), central nucleus of amygdala (CEA), basolateral amygdala (BLA), nucleus

accumbens (NAcc), area postrema (AP), nucleus of solitary tract (NTS), dorsal motor nucleus of the vagus (DMNV) were used in the single c-Fos staining, and sections containing the PVN, and SON were stained for c-Fos and OT double staining.

Sections were rinsed in 50 nM TBS (pH 7.4–7.6) four times, and then treated for 10 min in 3% H_2O_2, 10% methanol (diluted in TBS). After four rinsing in TBS, they were incubated overnight at 4°C in the polyclonal rabbit antibody against c-Fos (1:4000; Synaptic Systems, Australia). After overnight, sections were washed in TBS and incubated in the goat-anti-rabbit secondary antibody (1:400; Vector Laboratories, Burlingame, CA, USA) for one hour at room temperature. Following four washes in TBS, they were incubated in the avidin-biotin-peroxidase complex (ABC; 1:800; Elite Kit, Vector Laboratories, Burlingame, CA, USA) for another hour at room temperature. The vehicle for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the brain section was visualized with 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, and 0.3% nickel sulfate in TBS. The stained 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).

Images were taken with the camera attached to a light microscope (Nikon Eclipse 400). The number of Fos-positive nuclei per 1 mm^2 was counted bilaterally for each neuroanatomical region of interest with ImageJ Software. Boundaries of each area were defined according to the Paxinos and Watson brain atlas (Paxinos and Watson, 1986), on 3–4 sections per animal.

4.1.3.9 Statistical analysis

In the feeding paradigms involving the comparison of two doses of IN OT to saline, data were analyzed with one-way ANOVA followed by Dunnett's test. In paradigms where only one dose of IN OT and saline were administered, the comparison was made with Student's t-test. In the c-Fos study, densities of Fos-immunoreactive nuclei were compared with two-way ANOVA with drug and diet set as independent factors. Data are presented as means of ± SEM. The significance threshold was set at $P \leq 0.05$.

4.1.4 Results

In animals motivated to eat by food deprivation, 20µg IN OT significantly decreased intake of standard chow during the 2-hour meal [chow intake (g), p=0.0461, Figure 1A; chow intake (g/g b.wt.), p=0.0296, Figure 1B], whereas the 10µg dose of OT was not effective. Neither 10 nor 20µg OT affected water intake in these animals (Figure 1C-D).

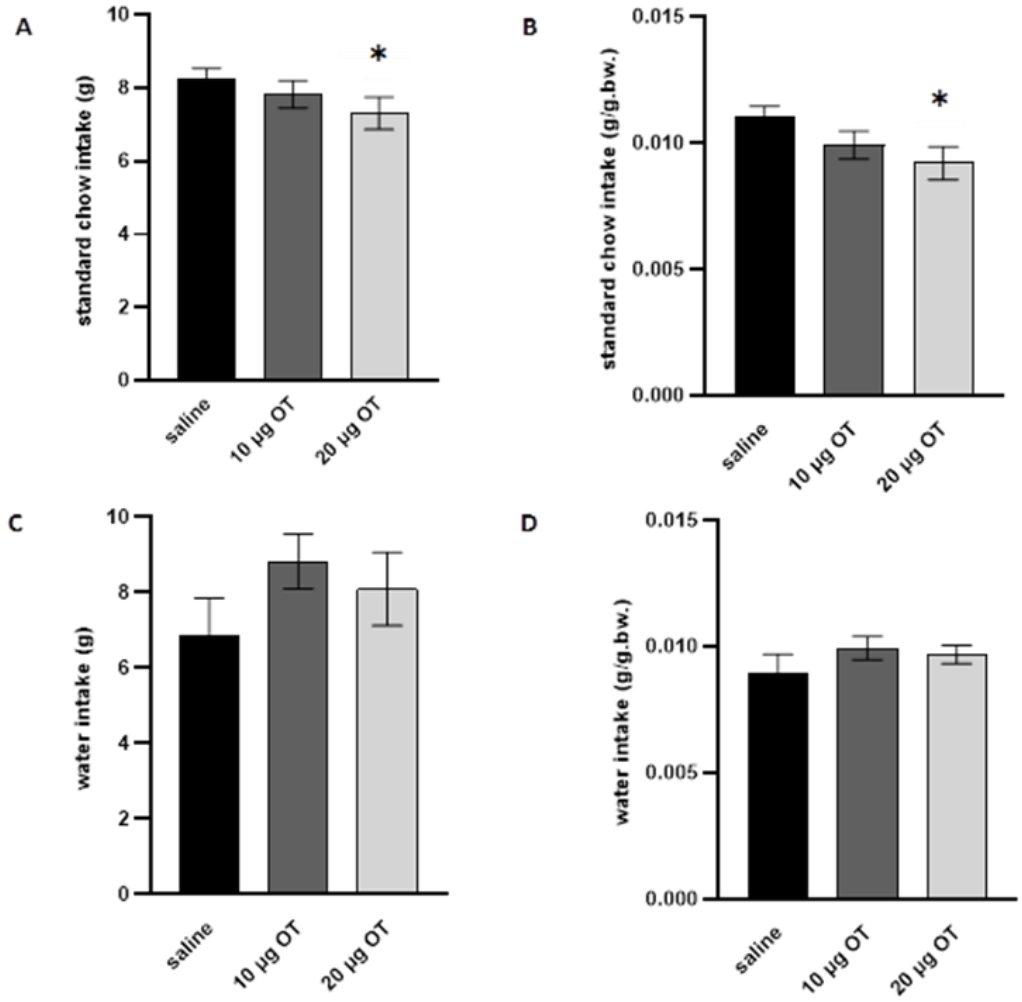

Figure 4.1.1: Effect of IN saline or OT (10µg and 20µg) on standard chow and water consumption during a 2-hour meal after overnight food deprivation. (A) standard chow intake measured in grams; (B) standard chow intake expressed in grams per gram of body weight (C) water intake measured in grams; (D) water intake expressed in grams per gram of body weight. Data are means ± SEM; n=15/group; * - significantly different from saline ($p \leq 0.05$)

In the study involving episodic 2-hour access to the HFHS chow in non-deprived rats, 20µg IN OT significantly reduced HFHS diet intake (HFHS intake in grams, p=0.002, Figure2A; HFHS intake in g/g.b.wt., p=0.0015, Figure2B), whereas 10µg OT did not result in a statistically significant reduction (HFHS intake in grams, p=0.184, 3A; HFHS intake in g/g.b.wt., p=0.102, Figure 2B). Neither of the OT doses affected water intake in animals given the HFHS diet during the 2-hour period (Figure 2C-D).

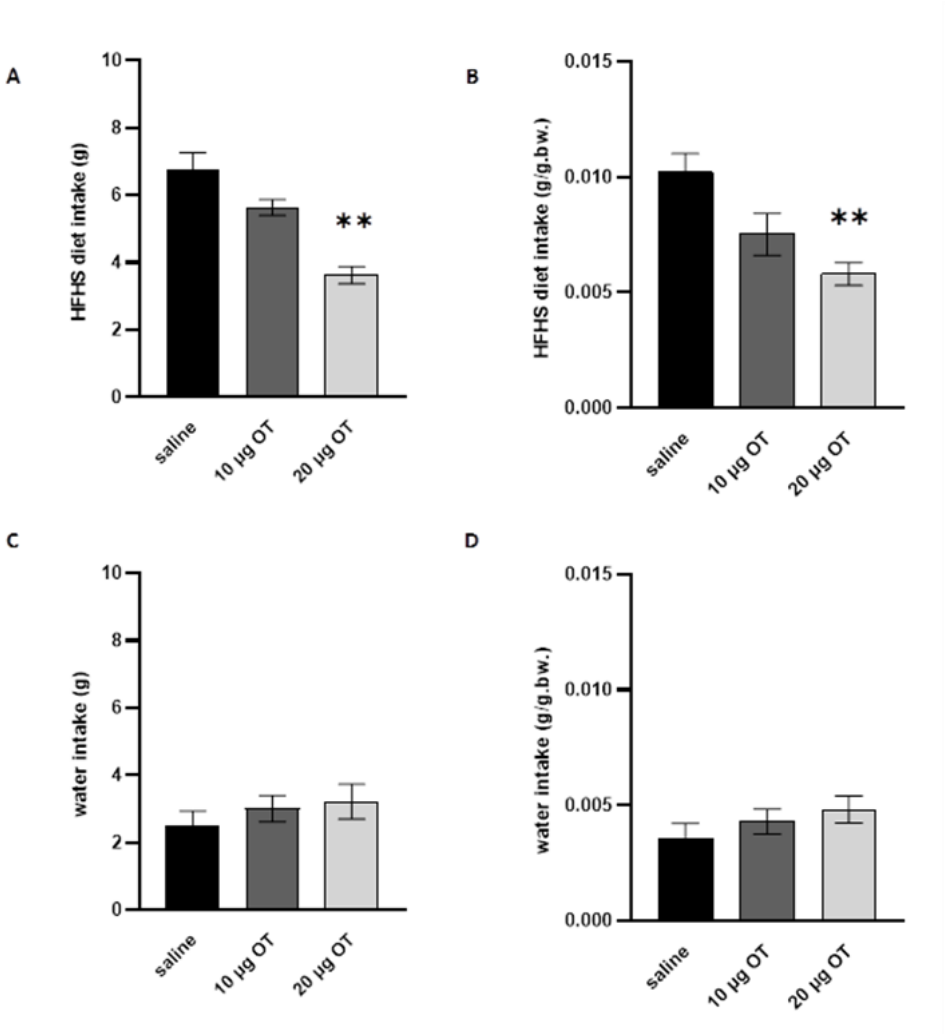

Figure 4.1.2: Effect of IN saline or OT (10µg and 20µg) on the intake of palatable HFHS chow and water during a 2-hour meal in non-deprived rats. (A) HFHS chow intake measured in grams; (B) HFHS chow intake expressed in grams per gram of body weight (C) water intake measured in grams; (D) water intake expressed in grams per gram of body weight. Data are means ± SEM; n=15/group; ** - significantly different from saline ($p \leq 0.01$)

In studies involving consumption of palatable liquids, IN OT was effective in suppressing episodic intake of a palatable sucrose solution in non-deprived animals. While 20µg OT significantly reduced sucrose intake (sucrose intake in grams, $p<0.0001$, Figure 3A; sucrose intake in g/g. b.wt., $p<0.0001$, Figure 3B), 10µg produced a trend toward significance (sucrose intake in grams, $p=0.0758$, Figure 3A; sucrose intake in g/g.b.wt., $p=0.0926$, Figure 3B). OT was not effective in suppressing episodic consumption of Intralipid, though the 20-µg dose generated a change that approached significance (Figure4A: Intralipid intake in grams; 10µg, $p= 0.1629$; 20µg, $p=0.0726$, Figure4B: Intralipid intake in g/g b.wt.; 10µg, $p=0.1294$; 20 µg, $p=0.0692$).

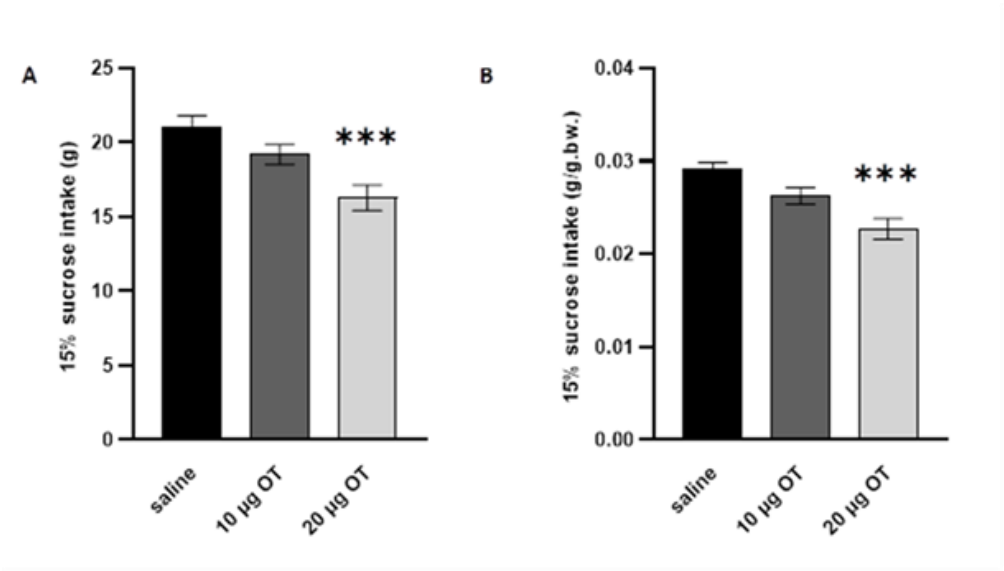

Figure 4.1.3: Effect of IN saline or OT (10µg and 20µg) on the episodic intake of palatable 15% sucrose solution during a 2-hour meal in non-deprived rats. (A) sucrose solution intake measured in grams; (B) sucrose solution intake expressed in grams per gram of body weight. Data are means ± SEM; n=15/group; *** - significantly different from saline ($p \leqslant 0.001$).

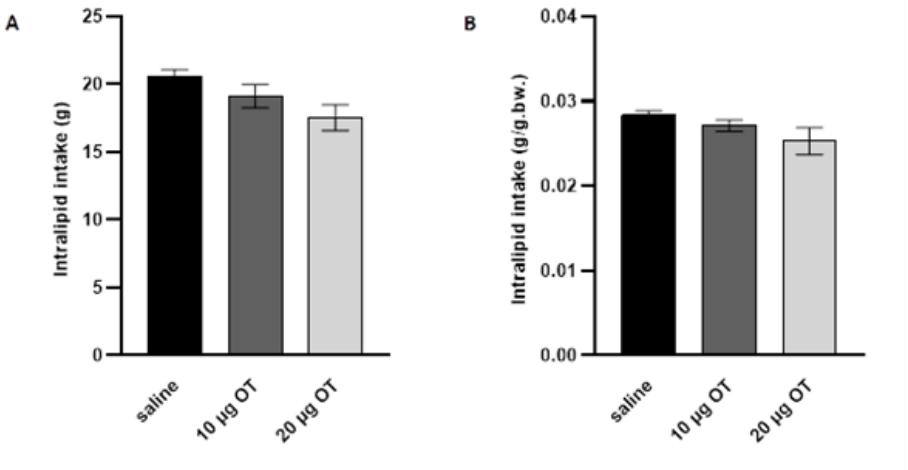

Figure 4.1.4: Effect of IN saline or OT (10µg and 20µg) on the episodic intake of palatable Intralipid solution during a 2-hour meal in non-deprived rats. (A) Intralipid intake measured in grams; (B) Intralipid intake expressed in grams per gram of body weight. Data are means ± SEM; n=15/group

After 28 days of daily habitual sugar consumption, rats received 10 and 20µg of OT. The 20-µg dose was effective at suppressing intake of 15% sucrose (sucrose intake in grams, p=0.0332, Figure 5A; sucrose intake in g/g.b.wt., p=0.0160, Figure 5B), whereas a decrease after 10µg OT did not reach significance (sucrose intake in grams, p=0.0804, Figure 5A; sucrose intake in g/g.b.wt., p=0.0735, Figure 5B).

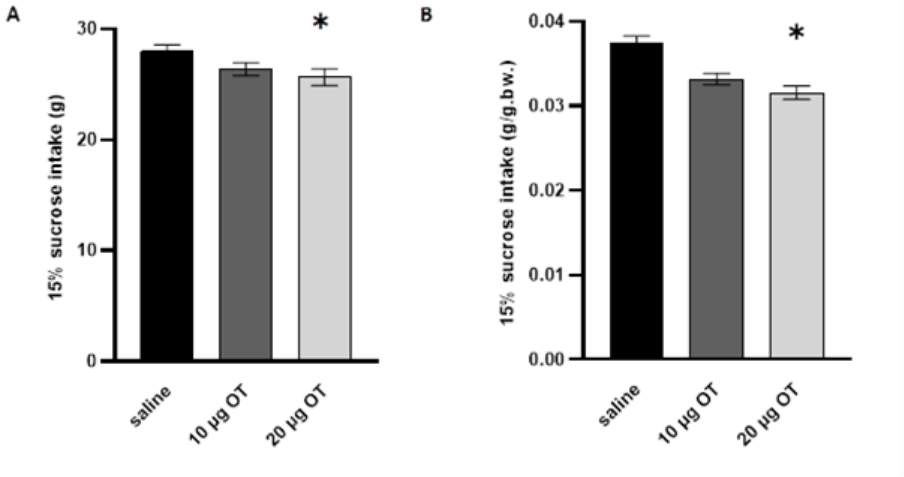

Figure 4.1.5: Effect of IN saline or OT (10µg and 20µg) on the habitual intake of palatable 15% sucrose solution during a 2-hour episodic meal in non-deprived rats accustomed to getting daily 2-hour access to sucrose for 28 days prior to the IN treatment. (A) sucrose solution intake measured in grams; (B) sucrose solution intake expressed in grams per gram of body weight. Data are means ± SEM; n=15/group; * - significantly different from saline ($p \leqslant 0.05$).

A two-way ANOVA in the c-Fos staining analysis showed a significant drug × diet interaction in the PVN ($p=0.0430$, $F(1, 20)=4.668$), ARH ($p=0.0321$, $F(1, 20)=5.305$), and CEA ($p=0.0165$, $F(1, 20)=6.843$). There was a significant effect of the drug on c-Fos IR in the PVN ($p< 0.0001$, $F(1, 20)=286.1$), SON ($p=0.0083$, $F(1, 20)=8.583$), ARH ($p=0.0009$, $F(1, 20)=15.29$), CEA ($p< 0.0001$, $F(1, 20)=144.1$), BLA ($p=0.0009$, $F(1, 20)=15.08$), AP($p=0.0054$, $F(1, 20)=9.716$), NTS ($p=0.0070$, $F(1, 20)=9.027$), and NAcc-shell ($p=0.0128$, $F(1, 20)= 7.473$). The effect of diet was significant in the PVN ($p< 0.0001$, $F(1, 20)=62.50$), DMH ($p=0.0003$, $F(1, 20)=19.08$), VMH ($p=0.0102$, $F(1, 20)=8.042$), LHA ($p=0.0002$, $F(1, 20)=21.52$), CEA ($p< 0.0001$, $F(1, 20)=112.8$), BLA ($p=0.0135$, $F(1, 20)=7.333$), AP ($p< 0.0001$, $F(1, 20)=31.79$), NTC ($p=0.0003$, $F(1, 20)=18.76$), NAcc-core ($p=0.0119$, $F(1, 16)=8.054$), and NAcc-shell ($p=0.0014$, $F(1, 20)=13.84$).

Furthermore, c-Fos levels in the OT-treated animals eating the control diet were higher than in the saline group maintained on the same food in the PVN ($p<0.0001$), CEA ($p<0.0001$), BLA ($p=0.0116$), and AP ($p=0.0278$). c-Fos immunoreactivity was elevated in the OT-treated control diet group compared to saline-infused control diet animals in the PVN ($p< 0.0001$), SON ($p=0.0404$), ARH ($p=0.0045$), CEA ($p< 0.0001$), BLA ($p=0.0366$), NTS ($p=0.0254$), and NAcc-shell ($p=0.0043$). Moreover, c-Fos in the OT-treated rats eating sucrose was higher than in the saline-infused animals on the same diet in the PVN (<0.0001), ARH ($p=0.0011$), DMH ($p=0.0132$), LHA ($p=0.0088$), CEA (<0.0001), AP ($p=0.0258$), NTS ($p=0.0001$), and NAcc-shell ($p=0.0011$) (Figure6).

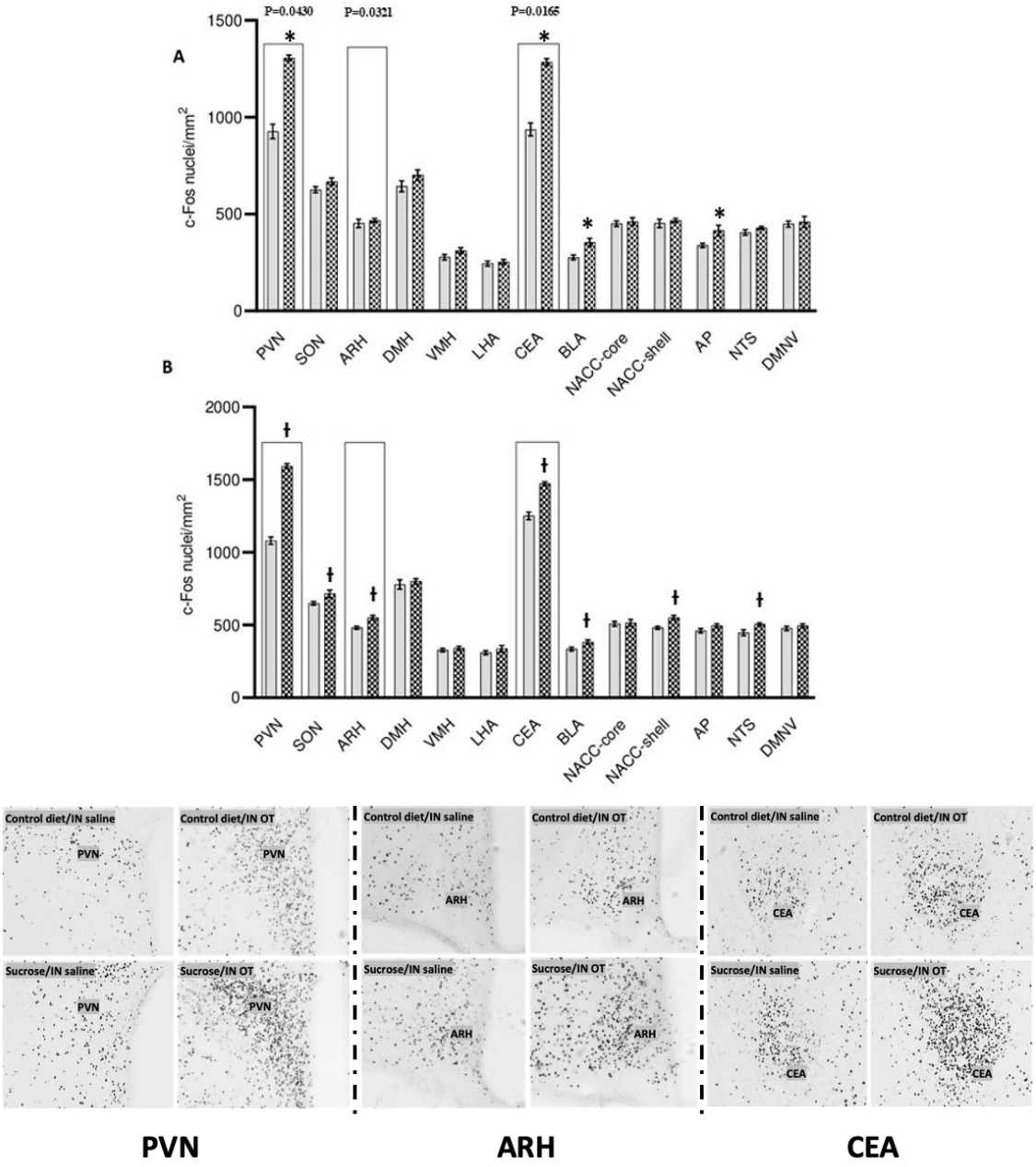

Figure 4.1.6: Effect of IN saline (solid bars) or 20µg OT (patterned bars) on c-Fos immunoreactivity in feeding-related brain sites in rats maintained on the standard chow control diet (A) and in rats accustomed to getting daily 2-hour access to sucrose (B). The IN treatment was administered at the time corresponding to sucrose presentation, however no sucrose solution. Representative photomicrographs depicting the PVN, ARH and CEA are shown in (C). * Significantly different from the control diet/OT group. Ł Significantly different from the control diet/saline group

4.1.5 Discussion

Most animal studies evaluating the effect of OT on food intake have been conducted using central or peripheral injections of OT. In contrast, human trials evaluating the effect OT on food intake and body weight have administered OT intranasally, a well-tolerated route allowing entry of the OT molecule into the brain [22]. Maejima and colleagues demonstrated that IN OT decreased nocturnal feeding in mice. However, no studies have evaluated the effect of IN OT on macronutrient intake and palatability-driven consumption. In the current studies we demonstrate that in rats IN OT decreases food intake driven by energy deprivation as well as by palatability, and we provide evidence that IN OT is an effective tool in curbing not only episodic but also habitual consumption of palatable sucrose solutions.

When Maejima et al [21] administered OT intranasally in mice at the onset of the dark phase, they found that 10 µg OT produced a statistically significant effect within the first two hours post-infusion, whereas the lower 1-µg dose did not change food intake until after 6 hours of the night-time feeding in ad libitum-fed animals. In our study, deprivation-induced feeding during the lights-on period was decreased by IN administration of 20µg OT. Since the 10-µg dose seemed to have a slight effect, it seems that the 10-20 µg OT dose range may be effective at generating hypophagia in rodents offered standard chow diets.

We found a moderate effect of IN OT on post-deprivation feeding. The amount of ingested food in OT-treated rats was approximately 15% lower than in the saline controls. This is a modest decrease compared to the 20-50% range previously reported in free feeding mice given IN OT [21], and in free-feeding or deprived animals injected with OT peripherally [5, 6] or centrally [5, 9, 23]. Also, we have not found an effect of IN OT on water intake, which is in concert with previously published studies involving central, peripheral or intranasal administration, in which water consumption was either not affected or its decrease paralleled a decline in food intake [5, 9, 12, 23].

It is noteworthy that while the effect of IN OT on deprivation-induced intake of standard chow in rats was moderate, the 20-µg dose of IN OT almost halved the consumption of the palatable HFHS chow. This is similar to the effect of 1 mg/kg b. wt. IP OT on HFHS diet intake in rats [24]. Thus, one can presume that in meal paradigms in which highly

caloric food is also palatable, IN OT produces a substantial decrease in consumption. This effect of OT on energy dense and palatable foods is substantiated not only by animal experiments [24-27], but also in human studies (for example, see [28-30]).

OT gene knockout (KO) increases consumption of both sweet and non-sweet and carbohydrate solutions [31]. OT KO mice exhibit an elevated frequency of sucrose intake bouts and more avid consumption of sweet solutions during both light and dark phases of the LD cycle [32]. Pharmacological blockade of the OT receptor in conventional mouse strains also enhances intake of carbohydrate solutions, however, the consumption of those sweetened with sucrose appears to be greater [33]. IP administration of OT reduces operant responsiveness for sucrose pellets in both male and female rats [34]. In contrast, experimental manipulation of OT signaling by either OT knockout, by peripheral injections of a blood-brain barrier penetrant antagonist, L-366,899, or by central administration of OT or OT receptor antagonists, fails to affect consumption of a palatable lipid emulsion, Intralipid [16, 23, 35-37]. The current dataset shows for the first time that IN OT in rats decreases consumption of a sugar solution, but not Intralipid. IN OT reduced episodic (non-habitual) sugar water intake by approximately 25%, which is similar to findings using central injections of OT in an episodic feeding paradigm (for example, [14]). In contrast, IN OT did not change intake of Intralipid.

Habitual consumption of palatable tastants (such as sucrose) produces broad changes in the brain, from reorganization of synaptic connectivity to altered expression of genes encoding appetite-related peptides, receptors and transporters, and generates behavioral changes resembling drug addiction [38-40]. Endocannabinoids, opioids and dopamine have been typically linked with this phenomenon and, not surprisingly, drugs that target those specific systems are particularly effective at modifying long-term consumption of palatable diets. For example, opioid receptor blockade in rats reduces intake of sucrose and palatable chow more potently than of standard reference tastants [41-43] and inhibits the re-development of a preference for high-sugar food [44]. Aside from OT decreasing sugar solution intake in rats offered occasional and episodic access to this tastant, we found that IN OT acutely suppresses sucrose consumption in animals habitually ingesting sugar (i.e., a 2-hour predictable meal given daily for several weeks).

It has been reported that OT gene expression is upregulated in the hypothalamus in rats schedule-fed a high-sugar diet [45]; however, the percentage of activated OT neurons at

the end of 3 weeks of consuming a high-sucrose meal is lower than in standard diet-fed rats [46]. Peripheral daily administration of OT at low doses does not affect high-fat high-sugar chow consumption [25]. The current data show that IN OT acutely suppresses sucrose solution intake in a habitual intake paradigm, albeit in a modest fashion (i.e., within a 15% range). Despite this moderate decrease, we view IN OT as a promising pharmacological tool to target overconsumption of sweet foods. One could envision using OT as a component of drug combination therapies incorporating other molecules that target reward-driven feeding such as opioid receptor antagonists. Further studies need to be conducted to evaluate whether chronic IN OT administration would maintain its effectiveness as chronicity of treatment is a factor affecting anorexigenic potential of the OT molecule [19].

This impact of IN OT on sucrose intake is accompanied by changes in c-Fos immunoreactivity in the CEA, a brain region known to be involved in food and drug reward. Similar to our CEA findings in IN OT-treated animals on the sucrose diet, Jewett et al and Pomonis et al reported naltrexone administration to rats chronically consuming sweet ingestants resulted in a significant diet x drug interaction in c-Fos in the CEA [47, 48]. The combined drug/diet effect at the CEA is well aligned with the proposed role of the CEA in emotional aspects of food consumption, reward processing, as well as with its mediation of hypophagia after direct stimulation of the OT receptor [14].

In the current study, we also found a diet x drug interaction in two hypothalamic sites, the PVN and ARH, which sheds more light on possible mechanisms through which IN OT may affect appetite. For example, ARH neurons co-express, among others, cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC), which promote termination of food intake [49]. ARH POMC-derived α-melanocyte stimulating hormone elicits PVN OT neuronal activation [50] and peripheral administration of OT activates POMC neurons [51]. The PVN itself is a contributor to the energy homeostatic balance. Lesioning of the PVN results in extreme hyperphagia and obesity [52]) as well as reward-driven feeding [53].

We conclude that IN OT decreases standard chow intake driven by energy needs and episodic palatability-driven sugar intake. It is also effective at reducing habitual intake of a palatable sucrose solution and this anorexigenic effect is accompanied by changes in feeding-related brain circuits particularly in the CEA, PVN and ARH.

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4.2 Part II: Effect of intranasal NTX alone or in combination with intranasal OT on food intake in rats

4.2.1 Abstract

As shown in previously published studies and in the data presented in Chapter one of this thesis, injected NTX potently decreases intake of palatable food in laboratory animals. Earlier injection studies have also found that NTX's effect on feeding can be exacerbated by co-administration of anorexigenic oxytocin (OT), a peptide whose IN administration produces hypophagia (as shown in Part I of this Chapter). To date there have been no laboratory animal experiments assessing the effectiveness of intranasally (IN) infused NTX on feeding. There have also been no attempts to co-administer NTX and OT intranasally and determine a potential synergistic effect of the two molecules in termination of feeding. In this set of pilot studies, I found that - surprisingly - IN NTX at a dose that counteracts morphine-induced analgesia - is a poor appetite suppressant. IN NTX did not decrease intake of standard chow after deprivation, palatable high-fat high-sugar chow (episodic), sucrose (both episodic and long-term) or Intralipid (episodic) in rats. The co-administration of IN NTX and IN OT did not produce a hypophagia greater than that generated by administration of OT alone. I conclude that the IN route is not optimal for generating hypophagia with NTX and that the IN co-administration of NTX and OT does not produce synergy similar to that previously reported in injection studies.

4.2.2 Introduction

As shown in previously published studies and in the data presented in chapter two of this thesis, NTX potently decreases intake of palatable food in laboratory animals, whereas its effectiveness on standard chow consumption is limited [54-56]. It should be noted that in the vast majority of animal experiments, NTX has been administered via intraperitoneal (IP) [54, 57, 58] subcutaneous (SC) [56, 59], or intramuscular (IM) [60] injections. This makes translation of the results to humans somewhat challenging as injections do not constitute a preferred administration route in the clinical setting.

Recent years have brought interest in intranasal (IN) drug delivery: this route is particularly advantageous for administration of molecules that target receptors in the brain [61]. As shown in Part 1 of the current Chapter, IN drug treatment is a promising tool in reducing excessive food consumption: IN oxytocin (OT) produced anorexigenic effects similar to those reported for peripherally injected peptide; the effects also paralleled the findings of human trials [21].

Though Contrave (NTX + bupoprion) relies on oral administration, a potential shift to IN-administered opioid antagonists in the treatment of eating behavioral abnormalities has garnered a lot of attention. This is particularly imminent in light of the findings showing, for example, the effectiveness of IN naloxone in individuals who overdosed opiates or the ability of IN NTX in laboratory mice to reduce cognitive impairments and motor alteration after 10 mg/kg morphine and 60 mg/kg oxycodone [62].

Yet despite this interest in the IN administration of opioid receptor ligands, to date there have been no laboratory animal experiments assessing the effectiveness of IN NTX on feeding. Therefore, in this pilot project I sought to investigate whether IN NTX suppresses deprivation-induced intake of standard chow as well as intake for palatability (high-fat high-sugar chow and sucrose solution) without prior deprivation. I also studied whether the combination of IN NTX and IN OT (just as previously reported in peripheral injection experiments [63, 64] produces a synergistic effect, i.e., it generates a more profound decrease in consumption than when either of the molecules is administered alone.

It should be noted that the experiments presented here did not go beyond a very limited, pilot study phase: this is due to the fact that (as shown in the Results section), unlike IN oxytocin, IN NTX was found to be a very poor suppressant of food intake.

4.2.3 Materials and Methods

4.2.3.1 IN administration of NTX, OT and NTX + OT

NTX (Sigma, St. Louis, MO, USA) and OT (Sigma, St. Louis, MO, USA) were dissolved in 0.9% saline and refrigerated at 4°C and slowly warmed to room temperature 20 min prior to administration. The rats were given saline or NTX (3 μg/20μl), OT (20 μg/20μl) or NTX (1.5 μg/10μl) + OT (10 μg/10μl) intranasally 2 hours after the beginning of the light phase. The doses of NTX and OT were based on previously published reports and the minimum effective dose of NTX (3 μg) and OT (20 μg) on food intake was established by pilot experiments.

4.2.3.2 Feeding studies

Deprivation-induced standard chow

Animals (n = 15/group) were individually housed. Standard chow was removed at 15:00 on the day preceding administration (water was available at all times). At 09:00 on the experimental day, animals were infused with saline or NTX (3 μg/20μl), OT (20 μg/20μl) or NTX (1.5 μg/10μl) + OT (10 μg/10μl). After 30 min of administration, standard chow and water were returned and consumption was measured after 2 hours.

Episodic intake of the high-fat high-sugar (HFHS) diet in non-deprived rats

Rats (n=15/group) were individually housed and were pre-exposed to the HFHS chow (Research Diets #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) to avoid neophobia 3 days prior to the experiments. On the experimental day, standard chow was removed from cages at 07:00 and after 30 min, rats were given saline or NTX (3 μg/20μl), OT (20 μg/20μl) or NTX (1.5 μg/10μl) + (10 μg/10μl). After 30 min of administration, rats were given the HFHS chow and water, and the consumption was measured after 2 hours.

Episodic intake of 15% sucrose solution in non-deprived rats

In this paradigm, we studied the effect of NTX, OT and NTX + OT on the intake of palatable 15% sucrose solution non-deprived rats. All experimental rats (n=15/group) were pre-exposed to the 15% sucrose solution for 2 hours to prevent neophobia 3 days prior to the experiment. On the experimental day, standard chow and water were removed from cages at 07:00 and animals were administered saline or NTX (3 μg/20μl), OT (20 μg/20μl) or NTX (1.5 μg/10μl) + OT (10 μg/10μl). After 30 min of drugs and saline administration, rats were given a 15% sucrose solution, and the intake was measured after 2 hours.

Episodic intake of palatable Intralipid solution in non-deprived rats
Long term (28 days) consumption of 15% sucrose solution

Sixty male rats were housed individually and were given access to 15% sucrose solution for 2 hours daily for 28 days. At the beginning of the light phase, animals were given freshly prepared 15% sucrose solution at 09:00 taken away at 11:00 and the consumption was measured at 11:00. Standard chow and water were removed for the time of sucrose presentation and returned to the cages after 2 hours (at 11:00). During the rest of the light phase (from 11:00 to 18:00), animals were given standard chow and water ad libitum and consumption were measured at the end of the light cycle (at 18:00). Rats were given access to standard chow and water ad libitum in the dark phase (18:00 to 06:00) and consumption was measured at 06:00. Following the 28 days of chronic sucrose access, rats were administered saline or NTX (3 μg/20μl), OT (20 μg/20μl) or NTX (1.5 μg/10μl) 30 min before the daily 15% sucrose solution and the consumption was measured after 2 hours.

4.2.4 Results

In deprivation-induced standard chow intake experiments, NTX (3 µg) was ineffective at decreasing chow consumption during a 2-h meal. On the other hand, IN administration OT (20 µg) produced a significant decrease after 2 hours (chow intake (g), p=0.0432, Figure 1A; chow intake (g/g b.wt.), p=0.0148, Figure 1B). The combination of NTX (3 µg) + OT (20 µg) did not produce a significant reduction in food consumption (Figure 1A-B). NTX, OT, or NTX + OT did not affect water intake in food-deprived animals at 2 hours (Figure 1 C-D).

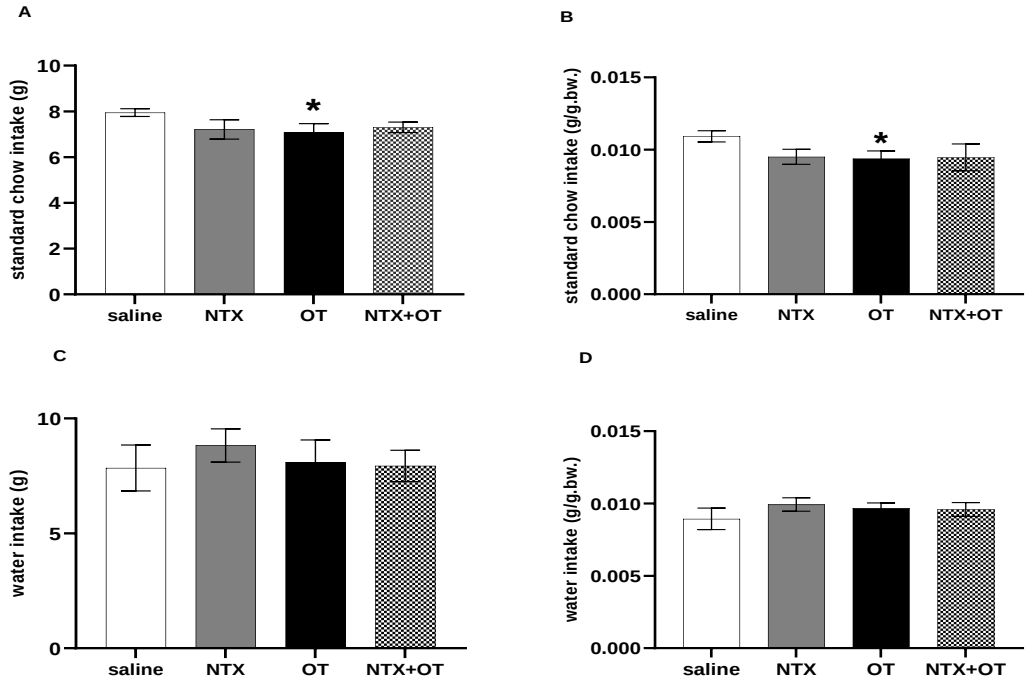

Figure 4.2.1: Effect of saline (control), NTX (3 µg), OT (20 µg), or NTX (1.5 µg) + OT (10 µg) on deprivation-induced intake of standard chow during a 2-hour meal. (A) effect of NTX, OT and NTX +OT on intake of standard chow (g) (B) effect of NTX, OT and NTX + OT on intake of standard chow (g/g b.wt.) (C) effect of NTX, OT and NTX + OT on intake of water (g) (D) effect of NTX, OT and NTX + OT on intake of water (g/g b.wt.). Data are expressed as average intake ± SEM, n = 15/group * Significantly different from the corresponding saline group ($p \leq 0.05$, ANOVA followed by Dunnett's test).

In the HFHS diet consumption experiments, while IN OT (20 µg) significantly reduced the consumption of the HFHS chow after 2 hours in non-deprived rats (HFHS intake (g), p=0.0024, Figure 2A; HFHS intake (g/g.b.wt.), p=0.0036, Figure 2B). However, NTX or OT + NTX did not decrease consumption of this palatable diet. There were no effects on water intake of the treatments during the 2-hour period (water intake (g) Figure 2C, water intake (g/g b.wt.), Figure 2D).

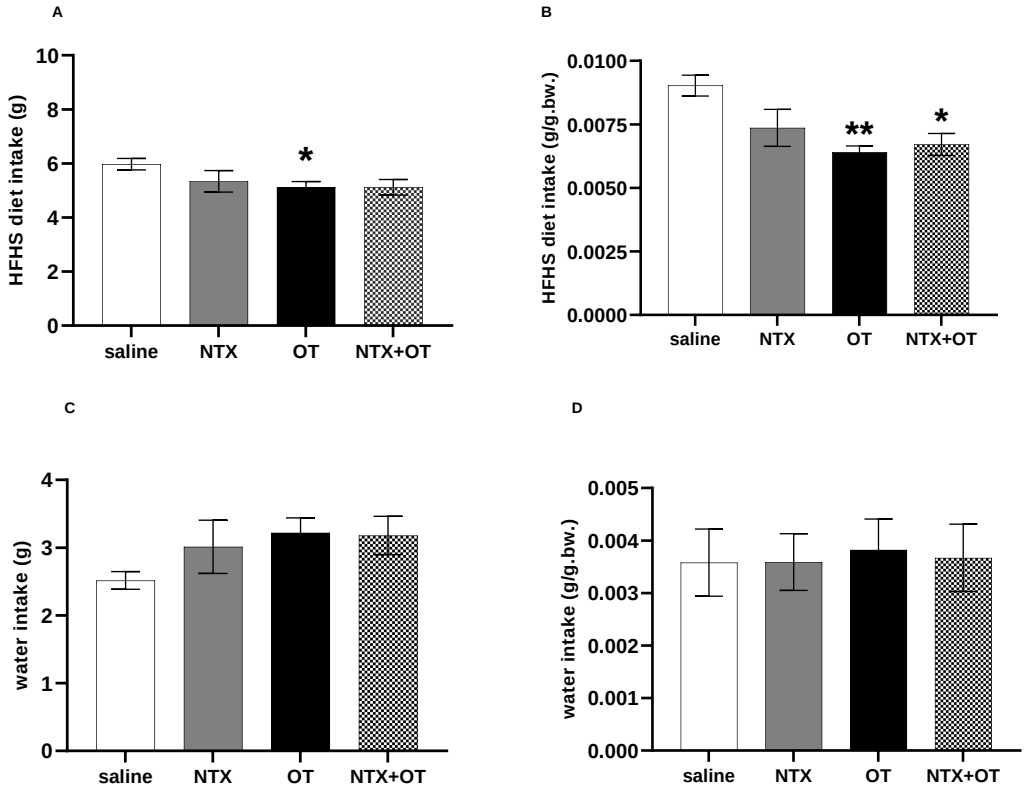

Figure 4.2.2: Effect of saline (control), NTX (3 µg), OT (20 µg), or NTX (1.5 µg) + OT (10 µg) on episodic intake of HFHS diet and water in non-deprived rats. (A) effect of NTX, OT, and NTX + OT on intake of HFHS diet (g) (B) effect of NTX, OT, and NTX + OT on intake of HFHS diet (g/g b.wt.) (C) effect of NTX, OT, and NTX + OT on intake of water (g) (D) effect of NTX, OT, and NTX + OT on intake of water (g/g b.wt.). Data are expressed as average intake ± SEM, n = 15/group * Significantly different from the corresponding saline group ($p \leq 0.05$, ANOVA followed by Dunnett's test).

In rats having episodic (non-chronic) access to the 15% sucrose solution, acutely administered IN NTX, IN OT, or the combined IN NTX + OT produced a significant reduction in episodic intake of 15% sucrose solution after 2 hours (sucrose solution intake (g), NTX p=0.0455, OT p<0.0001, NTX + OT p=0.0362, Figure 3A; sucrose solution intake (g/g.b.wt.), NTX p=0.0652, OT p<0.0001, NTX + OT p=0.0258, Figure 3B).

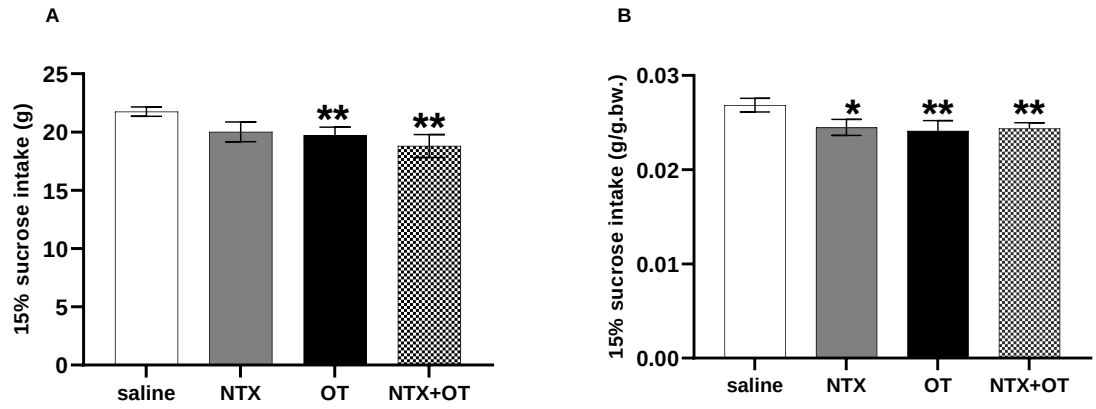

Figure 4.2.3: Effect of saline (control), NTX (3 µg), OT (20 µg), or NTX (1.5 µg) + OT (10 µg) on episodic intake of 15% sucrose solution in non-deprived rats. (A) effect of NTX, OT and NTX + OT on intake of 15% sucrose solution (g) (B) effect of NTX, OT and NTX + OT on intake of 15% sucrose solution (g/g b.wt.). Data are expressed as average intake ± SEM, n = 15/group * Significantly different from the corresponding saline group ($p \leq 0.05$, ANOVA followed by Dunnett's test).

Unlike in the sucrose solution experiment described above, neither of the IN treatments affected episodic intake of a palatable lipid emulsion, Intralipid, during a 2-hour exposure (intralipid solution intake (g) Figure14A; sucrose solution intake (g/g.b.wt) Figure14B).

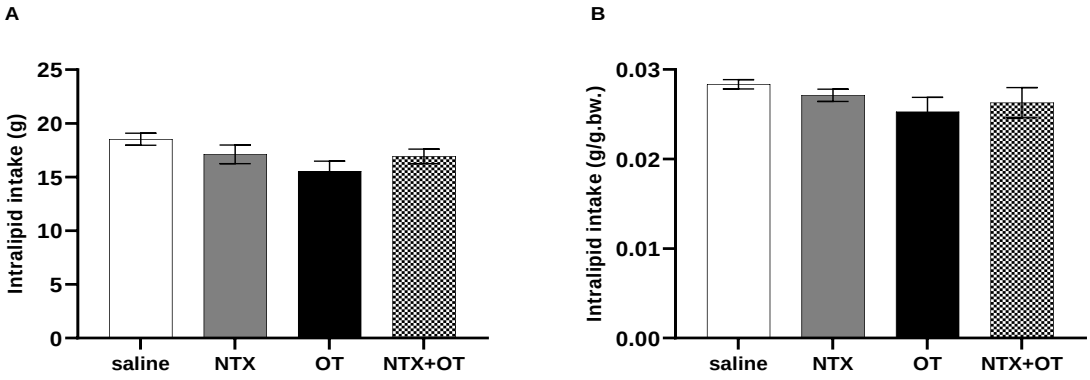

Figure 4.2.4: Effect of saline (control), NTX (3 µg), OT (20 µg), or NTX (1.5 µg) + OT (10 µg) on episodic intake of 4% Intralipid in non-deprived rats. (A) effect of NTX, OT and NTX + OT on intake of Intralipid solution (g) (B) effect of NTX, OT and NTX + OT on intake of Intralipid solution (g/g b.wt.). Data are expressed as average intake ± SEM, n = 15/group * Significantly

Finally, in the study involving long-term (28 days), habitual sucrose solution consumption, IN OT decreased sugar water intake (sucrose solution intake (g), p=0.0332, Figure 5A; sucrose solution intake (g/g.b.wt.), p=0.0160, Figure 5B), whereas all the remaining treatments were ineffective.

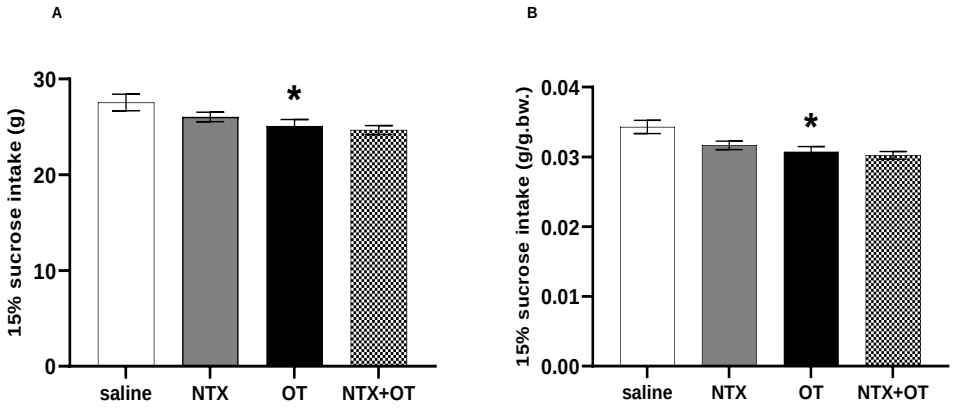

Figure 4.2.5: Effect of saline (control), NTX (3 µg), OT (20 µg), or NTX (1.5 µg) + OT (10 µg) on episodic intake of 15% sucrose solution after habitual daily exposure to the sugar solution. (A) effect of NTX, OT, NTX + OT on intake of 15% sucrose solution (g) (B) effect of NTX, OT, NTX + OT on intake of 15% sucrose solution (g/g b.wt.). Data are expressed as average intake ± SEM, n = 15/group * Significantly different from the corresponding saline group ($p \leq 0.05$, ANOVA followed by Dunnett's test).

4.2.5 Discussion

There is a broad consensus that injections (both peripheral and central) of blood-brain barrier penetrant opioid receptor ligands, including NTX, decrease consumption of palatable diets, while being poor suppressants of "bland" foods [54, 56, 58, 65, 66]. Many studies have demonstrated that the administration of NTX decreases the intake of palatable foods to a greater degree than the intake of less palatable ones, and that in preference experiments, antagonism of opioid receptors leads to a decrease in the consumption of the less preferred tastant [54, 56]. In line with that, injected NTX as well as (in humans) oral NTX, suppresses intake of diets that are sweet, high in fat or high in sugar (or those rich in both fat and sweet carbohydrates). Central administration of NTX also inhibits the re-development of a preference for a high-sugar diet [67].

This pilot study shows for the first time that IN administered NTX does not affect many of those aspects of feeding that have been previously linked with opioid receptor signalling. It could be expected that NTX (regardless of the administration route, including IN) would not reduce hunger-driven consumption of standard laboratory chow. However, it was much more surprising to determine that IN NTX even at a similar dose to the one that had been previously shown to counteract the effects of morphine, does not change feeding for palatability [62]. In fact, IN NTX did not reduce HFHS chow intake (even though peripheral injections of this opioid receptor antagonist have been shown to be effective in the HFHS meal paradigm [64]. It failed to decrease consumption of Intralipid, even though appetite for fat is one of the facets of consummatory behavior most robustly affected by opioids [68, 69]. And the only paradigm in which IN NTX was effective was episodic sucrose consumption (though in the chronic sugar intake scenario, again, it failed to decrease sucrose intake). It leads me to conclude that the IN route is not optimal for generating maximum anorexigenic effects. While it is difficult to speculate on the reasons behind this phenomenon, it should be noted that there has been another IN study (albeit clinical and utilizing naloxone rather than NTX), which also produced somewhat disappointing results. The UK team lead by Dr. Treasure investigated the potential of IN naloxone in the treatment of bulimia nervosa (BN) [70]. Though they observed minimal side effects of the treatment in BN patients, IN naloxone in Phase II trial (OPNT001) failed to meet the primary endpoint of minimizing the number of binging days from baseline to week eight.

Importantly, in this pilot study I was able to replicate the outcomes of the IN administration of OT that have been shown by me in Part 1 of this Chapter. Namely, IN OT decreased intake of standard chow, HFHS diet, episodic sucrose and Intralipid solutions, and chronic sucrose. It indicates that the limited hypophagic response after IN NTX was unlikely related to procedural/methodological errors during the experiment.

It is also notable in this context that the combined IN NTX+OT treatment did not produce an enhanced anorexigenic effect. Despite the previous reports showing synergy in decreasing consumption of palatable foods when NTX and OT were injected at very low doses, the outcome generated by the IN co-administered molecules was akin to that induced by IN OT alone.

Overall, the data allow me to conclude that the IN route is not optimal for generating hypophagia with NTX and that the IN co-administration of NTX and OT does not produce synergy similar to that previously reported in injection studies.

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Chapter 5

General Discussion and Perspectives

Feeding-related homeostasis stems from the capacity of the organism to regulate a drive to ingest food (typically arising from the need to replenish lacking energy) and terminate consumption (due to satiation, i.e., upon ingestion of a sufficient amount of nutrients, or in order to cease consumption of, for example, toxic or highly osmotic tastants). This homeostasis-based view of food intake control is, however, oversimplified. In reality, there are many other factors – both external and innate – that influence eating behavior to the point that at times can jeopardize the internal milieu by dysregulating hunger-feeding termination mechanisms [1]. As evidenced by the obesity "epidemic" in the industrialized world, the high accessibility of cheap, tasty, and energy-dense food, oftentimes dubbed (in combination with reduced energy expenditure) as "obesogenic environment," is an essential external modifier of appetite. On the other hand, stress responsiveness, mood, age, pregnancy, and entrainment, to name a few, serve as innately driven motivators of a change in feeding behavior [1, 2].

Human and laboratory animal research has confirmed beyond reasonable doubt that palatability is one of the major reasons why individuals ingest excessive amounts of food. In fact, considering the fact that in the industrialized world, food is easily accessible and inexpensive, palatability has become the key characteristic of a diet which determines how much of this diet is consumed and – consequently – whether it will be overconsumed (i.e., whether calories obtained through this food will exceed energy expenditure of the organism, in a long-term leading to obesity) [2]. It is clear that reward-motivated overeating is accompanied by recurring patterns of seeking tasty ingestants, craving them, and experiencing withdrawal-like effects upon their unavailability, thus it overlaps with addiction [3]. Just as in any addictive behavior, consumption of palatable foods can be so excessive that it threatens the homeostatic balance and – in fact – it appears to silence mechanisms that maintain internal milieu (especially, in the case of excessive feeding, dampen activity of those processes that ensure timely termination of consummatory behavior) [4].

The addictive nature of palatability-driven food intake arises from the fact that consumption of tasty foods activates reward areas in the brain (the nucleus accumbens, ventral tegmental area, amygdala, and prefrontal cortex) and this increased activity leads to an enhanced release of neuroactive substances that mediate reward, particularly opioids [5]. It is not surprising, therefore, that the search for effective pharmacotherapies against obesity has incorporated blood-brain penetrant blockers of opioid receptors, including the one that constitutes the topic of my thesis, naltrexone (NTX). Thus far, this search has led to the development of NTX-bupropion combination for weight loss [6].

The current thesis expands our understanding of action and usability of NTX to curb excessive appetite. By using laboratory animal models, I found that peripherally injected NTX is a potent suppressant of sugar consumption, and its anorexigenic effects are associated with changes in activation of a host of brain sites that regulate appetite, including the reward areas as well as the regions more typically linked to satiety processing, such as the hypothalamus. The minimum effective dose of NTX is affected by the pathophysiological status of the organism: in the case of studies presented herein, autistic individuals (the VPA model of autism spectrum disorder) require a higher dose of NTX than healthy controls. While individuals maintained on standard ("bland") diet that are administered with NTX cannot distinguish it from vehicle (which is a desirable scenario from the standpoint of adherence to pharmacotherapies), in those that have chronic access to palatable sugar, NTX becomes discernable. Finally, I found that the NTX infused intranasally (IN), thus via a well-tolerated route utilized with other anorexigenic drugs that target central nervous system receptors (including oxytocin tested in my experiments), is a poor suppressant of episodic sucrose solution intake and it does not decrease consumption of other palatable and standard diets. Overall, the data gathered over the course of my doctoral studies offer a substantial insight into anorexigenic properties of NTX and, consequently, possess a translational value.

First, the experiments in conventional as well as ASD VPA rats show that NTX has a limited effect on that portion of feeding driven by energy needs. In experiments in which animals were subjected to food deprivation and, afterwards, offered a meal consisting of standard laboratory chow, NTX did not reduce the amount of ingested food during refeeding (Chapter 1). These are important findings as there is still no clear consensus as to whether modification of the opioid tone results in changes in hunger-derived appetite. After all, an opioid receptor agonist, butorphanol tartrate, acts as one of the most powerful

orexigens known to date that elevates chow intake in rodents as potently as NPY does [7]. Morphine and nociceptin/orphanin FQ injections stimulate standard chow consumption, albeit in a modest manner (certainly not as much as the intake of high-fat and high-sugar tastants) [8]. Site-specific and intraventricular injections of a MOR agonist, Tyr-D-Ala-Gly-(me) Phe-Gly-ol (DAMGO), stimulate ingestive behavior in rats maintained on bland diets [9].

Data on opioid antagonists and energy-driven feeding are somewhat confusing, too. For example, Kanarek and colleagues found that NTX injections have minimal effects on intake of regular chow in laboratory animals [10]. Apfelbaum and Mandenoff reported that 0.5 and 2.5 mg/kg doses of NTX have little or no effect on the consumption of laboratory chow in rats [11]. Curiously, Cooper and Turkish found that subcutaneous administration of NTX (0.05-5.0 mg/kg) could even significantly enhance the consumption of standard laboratory chow, however, one should note that this increase occurred only when the chow was offered against highly palatable chocolate-coated cookies (and consumption of those was simultaneously diminished) [12]. This uncertainty is not limited to just NTX. Another widely used non-selective antagonist, naloxone, has also been found to, for example, decrease "bland" corn starch powdered diet intake during night-time feeding in ad libitum-fed rats, but it failed to reduce the intake of the same diet in refeeding after food deprivation [13].

The results of the current experiments on standard chow intake inspire confidence in that NTX does in fact constitute a treatment unlikely to affect consumption of standard food that is rich in macro- and micronutrients (i.e., being acceptable, yet not highly palatable). This is well aligned with the desired characteristics of anti-obesity molecules that have a translational value for possible human use: the main goal of reducing food intake is not to decrease appetite for basic foods that are a good source of nutrients, but rather to diminish interest in hyper-palatable diets of minimal nutritional quality.

Indeed, in line with the notion that palatable food consumption is regulated by the endogenous opioid system [14, 15], this and many other reports indicate that opioid receptor blockade with NTX [4] is an effective way of decreasing consumption of palatable foods. Animal studies have shown that both peripheral and central administration of NTX can reduce feeding for palatability in laboratory animals that are given access to different palatable diets, i.e., those rich in sugar, fat, or sweetened with

non-caloric sweetener, saccharin [16-18]. In humans, clinical studies have found that NTX reduces body weight in individuals who preferentially incorporate highly palatable foods in their daily meals [19, 20].

Importantly, if NTX were to be used in the context of suppressing consumption of palatable diets, one can easily envision it being administered prior to presentation of tasty foods on a regular basis. Earlier studies indicated that not just short-term episodic consumption of sugar, but also long-term chronic intake of sweet tastants, is diminished by NTX (as well as its close counterpart, naloxone) [21, 22]. This makes the usability of NTX as a "dessert suppressant" even more plausible. However, prior to my conducting this research, there was a major gap in knowledge in terms of our understanding of the impact of NTX on individuals habitually consuming palatable tastants. Namely, considering that chronic and habitual intake of a rewarding food is likely to affect the endogenous opioid tone in the brain, is it possible that NTX administration would become discernable in the treated individuals.

Therefore, in Chapter one of this thesis, I investigated whether rats that are placed long-term on a standard versus highly palatable sucrose diet are able to discriminate NTX in an operant setting (according to the procedure employed previously, as in, e.g., [23]). The data clearly show that, while animals fed a standard diet do not distinguish NTX from vehicle, habitual sucrose consumption underpins the ability of individuals to discriminate the drug. These results indicate that palatable food intake changes the ability to discriminate NTX in a similar fashion that has been previously shown with exposure to drugs of abuse. Gellert and Holtzman who used an animal model of morphine dependency [24] found that rats dependent on morphine through scheduled access to the drug, were able to distinguish between injections of saline and NTX (0.1 mg/kg) in a two-choice discrete-trial avoidance paradigm. Similarly, Miksic and colleagues reported that in rats responding on an FR10 schedule of food reinforcement, a single pretreatment of morphine (40 mg/kg, 8 h) enhanced the potency of a low-dose NTX (1.25 mg/kg) to make it an effective discriminative stimulus compared to saline [25]. Easterling and Holtzman found that when animals were maintained on either 20- or 40 mg/kg/day of morphine via an osmotic pump, NTX produced dose-dependent discrimination [26]. Finally, the discriminative stimulus of opioid receptor blockers extends onto other antagonists: Jewett and coworkers, who studied the ability of rats to discriminate nalbuphine after chronic

sucrose consumption, discovered that chronic sucrose intake increases the ability of rats to discriminate different doses of nalbuphine [27].

The precise molecular mechanism underlying this discrimination remains to be elucidated. One hypothesis is the chronic sucrose intake-triggered transformation of opioid receptors into a constantly active state. When the brain expresses a high level of continuously active opioid receptors, an antagonist could potentially have an intrinsic efficacy [28]. This means that the antagonist may bind to these receptors quickly and produce more intense or different effects leading to an altered interoceptive state. This hypothesis is reflected by the outcome of the brain activation study in animals habitually exposed to sucrose that were treated with NTX. NTX increased c-Fos IR, but the magnitude of the change in c-Fos IR was higher in the sucrose/NTX group than in the sucrose/saline group, suggesting that that habitual sugar intake affects opioid tone in the reward-related areas of the brain. Importantly, in the c-Fos study, I found that among all the sites where a positive drug–diet interaction was found, the CEA was most highly significant ($p < 0.0001$). Pomonis and collagenous also reported that naloxone injection to those rats consuming sucrose significantly increased c-Fos immunoreactivity in the CEA compared to rats that were given water [21]. Considering that the CEA is a component of the opioid circuit, part of the reward network, a neuroanatomical contributor of eating for reward control, and an important site regulating emotional processing of various stimuli, an important follow-up question that emerges from my studies is whether the interoceptive effect of NTX during habitual sucrose consumption has a negative or positive value. In essence, one should evaluate whether the ability to discriminate NTX would (if the effect is negative) lead to the avoidance of the drug (and, from translational point of view, a high likelihood of discontinuation of the treatment).

One avenue toward reducing the impact of NTX discrimination is through the reduction of the NTX dose. In a way, NTX-bupropion (Contrave) addresses one strategy to achieve this reduction: this can be done by using NTX in drug combinations, in which both molecules are administered at doses which are less discernable (as the interoceptive effect is diminished with the lower dose). Contrave is an already approved pharmaceutical [29], however, additional options that rely on molecules having synergistic and complementary effects on feeding are desirable. One such attempt was made by me in this body of work: I sought to investigate whether IN OT and IN NTX may produce hypophagia. Unlike previously reported positive outcomes of studies on peripherally injected OT and NTX

[30, 31], unfortunately, IN combination of the two drugs did not prove beneficial (owing to the very poor effect of IN NTX on food intake). Nonetheless, the result of my IN-administration experiment should not be viewed as detrimental to other approaches in which NTX would be administered via non-IN routes with other molecules.

The ability of NTX to act as a suppressant of reward-driven behaviors may depend on the health status of the individual, particularly, if a given pathophysiology involves changes in the reward circuit (especially in the functioning of the opioid system). This has been the case, for example, with Rett syndrome (RS) and autism (ASD), which have been associated with receptor changes in the opioid circuit [32-34]. Consequently, individuals suffering from these disorders display altered sensitivity to opioid agonists and antagonists.

Importantly, opioid signaling dysfunction affects also appetite. Thus, individuals with RS or ASD show excessive intake of palatable foods. In the case of ASD, it has been shown quite extensively in valproic acid (VPA)-induced autistic rat, which exhibit increased consumption of highly palatable sugar, saccharin, and milk solutions, whereas consumption of a" bland" cornstarch solution remains unchanged in these animals [35]. Similarly, in contrast to their wild-type littermates, mice that lack the Mecp2 (methyl-CpG-binding protein 2) gene (which contributes to both RS and ASD) exhibit extreme hyperphagia and consequent obesity when given a highly palatable high-fat diet [36]. ASD mice lacking the Cntnap2 (contactin-associated protein-like 2) gene show faster weight gain trajectory than controls when maintained on a palatable diet [35]. Mice heterozygous for the Nbea gene, which is involved in targeting neurotransmitter release, consume more palatable sucrose, glucose, and fructose solutions as well as Intralipid fat emulsion compared to the wild-type control animals [37].

Overall, the findings indicating an enhanced drive to consume palatable tastants in ASD models support the opioid hypothesis of autism [38], which suggests that dysregulation of the opioid system could be responsible for a variety of ASD symptoms, including aberrant eating behavior. Consistent with this hypothesis, NTX has been found useful in treating symptoms of these disorders which potentially stem from the opioid system dysfunction. For example, NTX has been reported to reduce self-aggressive behavior, hyperreactivity, and restlessness in individuals with ASD (of note is the fact that NTX also modifies some of the respiratory disturbance in RS) [39, 40].

Surprisingly, until undertaking the current set of studies, it had not been examined whether opioid receptor antagonism (including with NTX) would in fact be an effective pharmacological tool to curb overeating in ASD. Thus, in the second chapter, I focused on studying how NTX affects the consumption of palatable food in VPA ASD rats and the activation of feeding related brain areas after NTX injections and consumption of palatable food over an extended time. I report for the first time that NTX is effective in decreasing palatability-driven feeding in ASD VPA animals. This decrease was apparent with calorie-dense and calorie-dilute diets, i.e., it paralleled the earlier findings in conventional non-ASD rodents [10, 11]. However, it should be emphasized that I found that even though NTX significantly suppressed the consumption of the HFHS diet and 10% sucrose solution in VPA rats, the minimum effective dose in VPAs was higher than that needed to suppress consumption in healthy controls.

It can only be speculated why ASD animals require a higher dose of NTX, but it seems plausible that the dysregulation of the opioid system that causes increased sensitivity to rewards may result in a higher requirement for NTX to effectively block the opioid receptors in VPA rats. This difference in sensitivity to opioid receptor blockade may also stem from the aberrant reward processing in general (not just within the opioid circuit, but rather much more broadly). In fact, several fMRI studies in ASD individuals revealed that neural reward responses to palatable food cues significantly are higher than in healthy people in the anterior cingulate cortex (ACC), insula, and NAcc [41]. These results suggest that individuals with ASD may have an increased tendency towards experiencing cravings for palatable foods than healthy individuals, making it more challenging to suppress pharmacologically by targeting only one receptor type [41]. Supekar et al. further supported the notion of reward hypersensitivity in ASD by reporting a reduced density of the mesolimbic NAcc-VTA tract in individuals with ASD. This decrease in density could contribute to the atypical response pattern of the reward system towards pleasant stimuli [42]. Altogether, these findings provide an explanation as to why VPA animals require a higher dose of NTX to reach the same suppressive effect on the consumption of palatable foods as non-VPA animals. It is noteworthy that peripherally injected oxytocin (OT) has a comparable impact to NTX on decreasing the consumption of palatable food in VPA animals. In fact, Klockars et al have shown that OT has a stronger anorexigenic effect on palatability-driven eating in VPA animals compared to control animals [35]. This suggests that not just the dysregulation in the opioid system,

but also aberrant signaling related to other neuropeptides, contributes to maladaptive feeding responses in individuals affected by ASD.

One of the important cues regarding the nature of the appetite control dysfunction in the context of reward (and sensitivity to NTX) comes from the brain c-Fos analysis. I found that though the level of c-Fos IR in the CEA was relatively similar between control and VPA animals on a standard diet, a significant drug-diet interaction was observed in the CEA of both groups. Furthermore, among all the feeding-related brain areas examined in my study, the CEA was the only one where a significant NTX-sucrose interaction was found in VPA animals. It is a crucial finding as the CEA has been implicated in the pathogenesis of ASD in previous studies, including the amygdala theory of autism proposed by Baron-Cohen and colleagues [43], which suggests that damage or dysfunction of the amygdala could contribute to social difficulties in individuals with ASD. We can now propose that this theory be expanded to include also abnormal palatability-driven appetite in ASD which is affected by the ill-functioning circuit encompassing the amygdala. The significant effect of NTX on the CEA in VPA animals indicates that NTX modifies the activation of the CEA in these rats. This potent effect of NTX on the CEA, along with the amygdala theory of autism, may explain why NTX can reduce self-aggressive behavior, hyperreactivity, and restlessness in children with ASD. Moreover, the ability of NTX to reduce palatable food consumption in VPA animals suggests that it could be a potential treatment for addressing unusual eating habits and improving diet selectivity in individuals with ASD. Possibly this reduced palatable food consumption by NTX in ASD may in turn shift the food selection process in a manner that increases the likelihood of trying a broader range of foods and improve the overall nutritional value of a daily diet.

From the standpoint of usability of NTX in ASD (as well as in other scenarios that involve excessive intake of palatable foods), it is disappointing that the intranasal NTX delivery route has not been found – at least in the animal model examined in my thesis – an effective method of administration that would lead to a decrease in food intake. This is particularly discouraging if one considers the fact that IN OT has been already used in clinical trials to improve social behavioral outcomes in ASD children as well as to curb overeating in ASD-unrelated food intake trials; and that my and Maejima and colleagues' [44] experiments confirmed the viability of the well-tolerated IN route to promote OT-driven hypophagia.

While my experiments clearly show that IN NTX is a very poor suppressant of appetite, it remains to be elucidated why this is the case. Taking into account NTX's ability to penetrate into the brain more readily after IN delivery than after the peripheral injections, in future studies, it might be worth revisiting two issues here: (i) whether peripheral opioid receptors do contribute to hypophagia (i.e., whether feeding reward is entirely a brain-centric process or it involves also the peripheral opioid receptors, and (ii) whether peripheral opioid receptor blockade triggers certain endocrine pathways that enhance the effect of NTX (or naloxone) on eating for pleasure (for review, [45]). The two notions are supported by, for example, the studies showing that an opioid agonist butorphanol tartrate enhances feeding more potently when injected subcutaneously than intracranially [46]. It is nonetheless beyond any reasonable doubt that the brain plays the key role in reward signaling, but there may be a direct or indirect peripheral component of opioid-driven reward that strengthens the effectiveness of opioid receptor targeting ligands. It should also be noted that this poor anorexigenic response to IN NTX may not extend onto feeding-unrelated behaviors and physiological processes. IN NTX has been reported to counteract opioid-induced gastrointestinal and CNS effects in rodents. Micheli et al found the effectiveness of IN NTX in decreasing morphine and oxycodone-induced side effects in rats [47]. Several other studies have also shown that IN administration of NTX can reduce the effects of morphine by blocking the μ receptors in the brain.

While the results of IN NTX administration were disappointing, the outcome of IN OT studies is extremely encouraging. On the one hand, my studies showed that IN OT suppresses feeding after deprivation in rats, whereas Maejima et al reported that IN OT reduces standard chow intake in ad libitum-fed mice during the nighttime (i.e., the time when these animals consume the majority of energy) [44]. It is in line with the notion of OT promoting early satiation and reducing overall amount of ingested food. I also found IN OT to decrease episodic intake of palatable sugar solution and HFHS diet, which shows that IN OT targets also that portion of feeding driven by pleasure. In fact, the suppression of sucrose and HFHS diet intake after IN OT was even greater than in the energy-driven consumption models. Importantly, OT was also effective in reducing sugar intake in animals consuming sucrose chronically for weeks, i.e., IN OT was able to counteract the effects of palatability as well as long-term habit-driven behavior. This puts IN OT in a prominent category of experimental molecules effective in breaking an entrained scheduled reward-based eating behavior pattern that recurs daily. This category

has been typically restricted to opioid, dopamine and endocannabinoid receptor blockers, but considering the ease of IN administration of OT combined with its effectiveness against the drive to consume palatable foods, one can envision OT as being incorporated in treatments that focus on hyper-palatable tastant overeating. This is in line with the outcomes of human studies which have consistently shown that subjects given IN OT reduce intake of also those food items that are highly palatable [48, 49]. As the IN NTX study showed relative ineffectiveness of NTX, IN OT may be a good substitute candidate molecule in this context. This is particularly the case since – as shown in my study, this impact of IN OT on sucrose intake is accompanied by changes in c-Fos immunoreactivity in the CEA, the very same reward-related brain region that Pomonis et al found affected (drug × diet interaction) after injections of naloxone in sucrose consuming animals [21]. Thus, by choosing IN OT in pharmacotherapy of overeating, one might in essence target the key brain region (CEA) that underpins the drive to ingest palatable tastants.

Conclusions

The overarching aim of this doctoral thesis was to investigate the anorexigenic action of NTX as a suppressant of feeding for palatability.

The key findings of the studies are:

1. NTX administered via injections is an effective tool to curb overconsumption of palatable food.

2. Chronic habitual consumption of palatable sucrose facilitates discrimination of NTX from saline.

3. Both NTX and sucrose have significant interactive effects on the activation of brain regions involved in feeding behavior, particularly the emotion processing-associated amygdala.

4. While NTX is effective in reducing palatable food consumption in VPA autistic animals, the minimum effective dose is higher than in non-VPA controls.

5. IN NTX administered alone or in combination with low dose IN OT, poorly reduces feeding.

6. IN OT decreases feeding for energy and for palatability, and its suppressive effects on chronic habitual consumption of palatable sugar are associated with c-Fos changes in brain regions that regulate consumption.

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