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Insensitivity to anorexigenic effects of naltrexone in the VPA rat model of autism

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Abstract

Autism spectrum disorder (ASD) is a complex disorder whose etiology lies in, among others, abnormal neural processing, and improper neural circuitry development. This results in a plethora of aberrant behaviors, including those driven by the brain's reward system and by the endogenous mediators of reward, for example opioids. One of the maladaptive consequences of pathophysiology of the reward system is excessive consumption of palatable foods in people with autism and in ASD model animals, such as valproic acid (VPA)-induced ASD rats. Here, I hypothesized that overconsumption of palatable diets in the VPA ASD rat is caused by abnormal functioning of the opioid circuitry. To investigate this, first I determined sensitivity of sucrose liquid-fed or high-fat high-sugar chow (HFHS)-fed VPA vs non-VPA control rats to the anorexigenic properties of the opioid receptor antagonist, naltrexone (NTX), a drug that reduces eating for palatability, but does not affect feeding for calories. In the follow up immunohistochemical study involving a marker of neuronal activity, c-Fos, I examined differences in brain activation after an injection of the same dose of NTX in VPAs vs non-VPA controls. As expected, NTX did not affect energy-driven consumption of bland chow in non-VPA controls or VPAs. NTX did, however, decrease intake of palatable sucrose water and HFHS chow. Importantly, while 1 mg/kg NTX was sufficient to reduce eating for palatability in non-VPA controls, a 10-mg NTX dose was needed to achieve the same effect in VPAs. c-Fos analysis in the non–VPA controls showed significant differences in neuronal activation in the paraventricular nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC), Dorsomedial hypothalamus (DMH), central nucleus of the amygdala (CeA), and nucleus accumbens shell (NAc – shell). The VPA animals showed a difference only in the CeA. Collectively these data show different responsiveness to NTX at the behavioural (feeding) and brain activation

(c-Fos) level, likely indicative of dysregulation of the opioid signaling and – more broadly – reward processing, in the autistic brain.

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List of abbreviations

ADHD – Attention-deficit/ hyperactivity disorder

AG-RP – Agouti-related peptide

ALC – Adenylyl cyclase

ASD – Autism spectrum disorder

CNV – Copy number variants

DBS – Deep brain stimulation

DMH – Dorsomedial hypothalamus

DMS – Diagnostic and statistical manual of mental disorders

DOR – Delta opioid receptor

GABA – T – γ -Aminobutyric acid – transferase

GABA – γ -Aminobutyric acid

GPCR – G-protein coupled receptors

HAT – Histone acetyltransferase

HDAC – Histone deacetylase enzyme

HFHS – High-fat high-sugar

ID – Intellectual disability

IRK – Inwardly rectifying potassium channels

KOR – Kappa opioid receptor

LHA – Lateral hypothalamus

MC3R – M elanocortin-3 receptor

MC4R – Melanocortin-4 receptor

MOR – Mu opioid receptor

NAc – Nucleus accumbance

NAL – Naloxone

NPY – Neuropeptide Y

NTD – Neural tube defect

NTX – Naltrexone

OXTR – Oxytocin receptor gene

PFC – Prefrontal cortex

POMC – pro-opiomelanocortin

SEED – Study to explore the early development

SNP – Single nucleotide polymorphism

SSD – Semialdehyde dehydrogenase

TSC – Tuberous sclerosis complex

VPA – Valproic acid

VTA – Ventral tegmental area

α-MSH – α-melanocyte-stimulating hormone

1.0 Introduction - Literature review

1.1 Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that manifests through a variety of behavioural and cognitive abnormalities. These include impaired social interaction, difficulties with verbal and nonverbal communication, repetitive/stereotypic behaviours, intellectual disability, and impairments that cannot be better explained by another differential diagnosis. What is known as classical autism today, was first described by Leo Kanner in his 1943 paper that outlined eleven case studies. Donald Triplett, an institutionalized patient in Mississippi, was the first patient diagnosed by Kanner with "early infantile autism". Kanner used detailed notes made

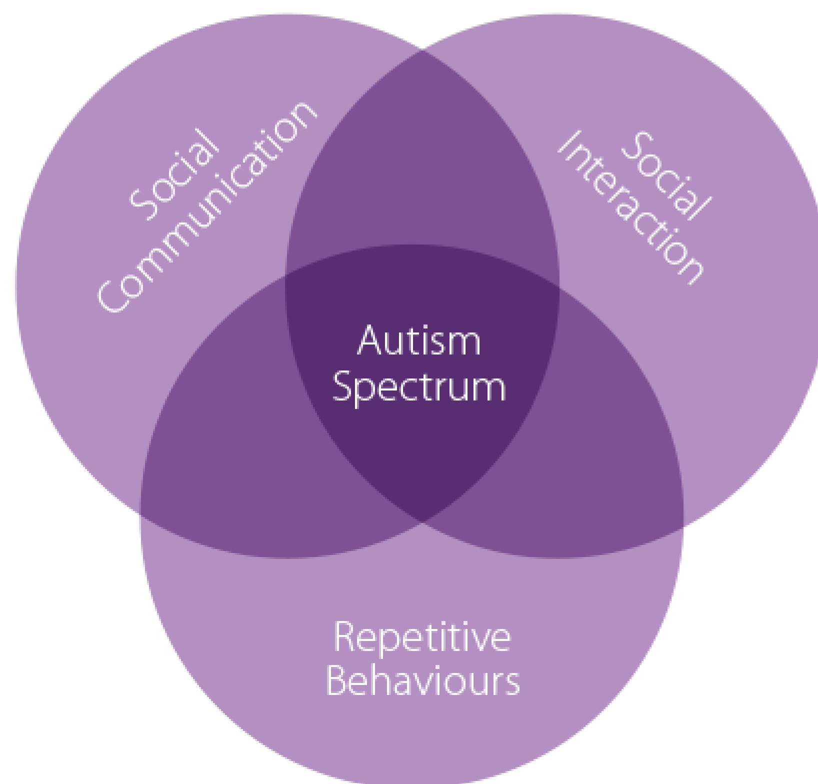

Figure 1.1 – The triad of impairment which was first purposed by Lorna Wing and Judith Gould that describes the three categories of impairment that were seen in Autism before the DSM – V. Image form: https://www.clinical-partners.co.uk/for-adults/autism/symptoms-of-autism

by the patient's parents about Donald's behaviour to develop the diagnostic parameters. The next breakthrough theory in autism was put forward in 1979 with the creation of

what is colloquially known as 'the impairment triad' (*Fig 1.1*) [1]. This work was pioneered by Lorna Wing and Judith Gould and set the foundation for the diagnostic parameters which were used until the release of the Diagnostic and Statistical Manual of Mental Disorders (DSM) – V in 2013. Before the release of the DSM – V, the impairment triad of autism symptoms was used for diagnosis.

However, with the introduction of the DSM – V in 2013, impaired communication and impaired social interactions were combined into a single overarching trait called impaired social communication. This is more aligned with the underlying belief that communication is not just restricted to verbal behaviour. This new term, therefore, encompasses all forms of verbal and non–verbal communication. Verbal and non-verbal communication includes behaviours such as eye contact, body language, mutterings, and speaking. Additionally, the criteria for age of onset is now in the early developmental period rather than a hard cutoff date. In the DSM – III, a diagnosis could not be given to the patient unless the caregivers could present clear evidence that the symptoms began before the age of three. Finally, diagnosis can only be given if symptoms are not explained by delayed global development or a particular intellectual disability. The rationale behind the changes was that clinicians believed the diagnosis was not very clearly defined, and that an overarching diagnosis of ASD was more fitting due to the large overlap and lack of evidence for a differential diagnosis. The generation of ASD as a spectrum leads to the more pragmatic level of impairment assessment which has three levels, where level three is defined as requiring the most support to manage their symptoms (*Tab 1.1*).

Severity level	Social communication	Restricted, repetitive behaviors
Level 3 "Requiring very substantial support"	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.
Level 2 "Requiring substantial support"	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and who has markedly odd nonverbal communication.	Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.
Level 1 "Requiring support"	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful responses to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to-and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

Table 1.1 – The impairment assessment taken from the DSM – V page 52.

ASD today differs from the classical autism model described by Kanner. Previously a diagnosis required all criteria to be met, while today autism is described as a spectrum that encompasses all possible abnormalities in each criterion. An example of this today is that patients do not have to be non–verbal when they present to the clinic, which would have disqualified them under Kanner's diagnostic parameters. This produces a large amount of heterogeneity in the population, as the exact inclusion criteria continue to shift more broadly over time. This heterogeneity makes researching the underlying pathophysiology an arduous task because many of the phenotypes described can have many causes, both neurobiological and psychogenic.

Because of the continued expansion of the inclusion criteria, it is hard to discern if the actual rate of ASD is increasing as data would tend to show. The current worldwide prevalence of ASD is 1 in 100, though this varies widely. The variance globally can be qualitatively influenced by several factors, including the broadening of inclusion criteria, the social awareness differences from region to region, the socioeconomic differences not only within a country but from country to country,

medical infrastructure and access in a country, changes in the method of assessment, and the amount of epidemiological research on ASD being conducted within a country [2]. As a counterpoint, the explosion in the prevalence cannot be solely attributed to these factors alone [3]. In New Zealand, the Ministry of Health puts the prevalence rate at 1 in 100 which is in line with the global average, because a detailed epidemiological study has not been conducted [4]. However, in countries such as the United States and Canada, the prevalence rate is shown to be higher, at 1 in 68 children [5]. The evolution of the inclusion criteria spawned a new colloquial term in ASD known as 'high functioning'. This eludes to the fact that some children diagnosed with ASD can still perform comparably to neuro-typical children in the domains of social interaction and communication. High-functioning ASD has been thought to be the main driver of the increase in prevalence, which has been pushed more by the increase in awareness in western countries [6]. A good example of the upward trend is in Minnesota where the prevalence rate of ASD in 1992 was 0.3 in 100 and in 2002 it was 0.53 in 100 [7]. This is in stark contrast to the current US national average no of 1.47 in 100.

1.2 The heterogenous symptomology of ASD

Intellectual disability (ID) has very high comorbidity with ASD, with roughly 70% of patients also receiving an ID diagnosis [8]. A portion of patients remains minimally verbal or even in some cases non-verbal as they get older. Additionally, many patients have other comorbidities such as attention-deficit/hyperactivity disorder (ADHD), anxiety, depression, impaired coordination, impaired/abnormalities in gait, epilepsy, agitation and aggression above normal, hyperactivity, gastrointestinal distress, altered brain region size, immunological abnormalities, altered neurochemistry, altered brain metabolism and more.

Genetics is being used as a method to approach this heterogeneity of symptoms by elucidating core perturbations in genetics which aid in explaining individual or groups of symptoms to allow for targeted interventions. The underlying understanding of the genetics of ASD has arisen through the research paradigms of twin studies (monozygotic and dizygotic), comparative genetic studies, and the study of highly correlative non-ASD syndromes. Heritability in siblings has been approximated for an ASD diagnosis to be between 12% and 20% under DSM – V classification [9]. But most profoundly, due to twin studies, it has been found that the concordance rate in monozygotic twins is approximately 72%, while in dizygotic it is roughly estimated to be between 20 – 30%. Dizygotic studies showed a larger range whereas some early studies showed no concordance [10-12]. Current work in the field of genetics has moved rapidly from originally using genome-wide association studies which gave a more cursory understanding, to now using technologies such as whole exome sequencing using large databases like the Simons simplex collection to get insights at the level of the nucleotide. This level of resolution allows for the research into gene function and to what effect this mutation produces via in vitro and in vivo methods. Because of this deeper understanding of genetics, it is becoming possible in some particular cases to predict the ASD phenotype, and because of this, genetic testing for the proband and the rest of the known closely related family is recommended if available. At this point when looking into the genetics of ASD, a bifurcation occurs centered around if the ASD is syndromic or non-syndromic. Syndromic is caused by single nucleotide polymorphisms (SNPs), copy number variants (CNVs), or present in the clinic with other known monogenic disorders such as fragile X syndrome or tuberous sclerosis complex (TSC). Non-syndromic ASD, in contrast, is when the underlying genetic causes for the aberration in development are unknown. The reason genetic testing is important is that it can help diagnose monogenic disorders that usually

accompany ASD and some of which may have treatment options like TSC and fragile X syndrome. Both TSC and fragile X have either drugs in development, or studies being done to repurpose other drugs for treatment [13].

The polymorphisms that have been linked to ASD are not random but are all heavily involved in a few fundamental parts of development. These are pathways involved in cell signaling, adhesion, synaptogenesis, dendritic spine formation and growth, and neural migration. In essence, ASD manifests neurobiologically through the disruption of normal connection formation in early development. This disruption occurs through more than one vector because of the interconnectivity of the developmental process. For example, white matter differences have been seen when comparing children with a high risk of developing ASD to neuro-typical children from 6 to 24 months. Since the white matter is indicative of the level of connectivity, it demonstrates the fact that children with ASD have a lower level of connectivity than age-matched neuro-typical children [14]. A particular gene of note which has an immense role in reward and food intake as well as many other ASD phenotypes is the oxytocin receptor gene (OXTR). More than 20 OXTR SNPs have been identified to be correlated with ASD and more are being evaluated for their potential role in the pathophysiology [15]. Though it has been shown that alteration in normal oxytocinergic signaling causes aberrant development of the hypothalamus [16].

Though genetics plays a pivotal role, it is not always the sole driver. When speaking about the environmental role it is important to note that neurodevelopment begins in utero. Aside from maternal environmental conditions, many other factors have been implicated, such as pesticides, pollutants, metals, viruses/ illnesses, pharmaceuticals, and nutrition. For a comprehensive overview of the topic, Karimi et

al 2017 [17].

1.3 Maternal influence

The maternal environment plays a very large role in the early developmental period as the fetus sequesters all of its nutrition from the mother. Before even considering dietary intake, other more general factors such as the health of the mother concerning metabolic health, immune health, and mental health can play a role in development alongside other factors such as age. Studies show that mothers diagnosed with obesity, hypertension, or type two diabetes have an increased risk of having children with ASD [18]. Parental age has come to the fore as one of the most important non-genetic influences of having a child with ASD, though the exact mechanism behind this has not been elucidated [19]. The increase in incidence with maternal and paternal age is thought to be the result of de novo mutations in the germline which accumulate over time [17]. On top of this, paternal ages have been shown to affect many aspects of gene regulation and could also be a contributing factor to maternal age [20]. On the mental health side, during early development, particularly during 21 to 32 weeks of gestation, it has been demonstrated that there may be an association between ASD and maternal mental stressors. These may cause an aberrant gene expression profile which causes a change in neural development. Folic acid intake in pregnant women has also been implicated in ASD via the effects of blunting the formation of neural tube defects (NTD) [21-23]. Children who are diagnosed with ASD are four times more likely to have an NTD compared to the normal NTD rate [24].

1.4 Drugs and environmental toxins

Though the maternal environment is important in the context of her health, wellbeing, and nutrition, another factor that has come to the fore is the role of

environmental toxins and pharmaceuticals as an additive, if not causative mechanism behind the development of ASD. To put this into context, based on the environmental protection agency's most recent chemical data reporting, there were 8,660 chemicals used in the commercial industry in the year 2020 [25]. Many of these chemicals being used have never been studied for their effects on human health. In recent times, companies have had increased due diligence requirements by regulators to show chemicals used in the manufacturing process are not toxic, which has led to the emergency that many chemicals have implications on human health. An important caveat is that the interaction with toxins and the development of a disorder typically requires some underlying genetic susceptibility [26]. Many chemicals commonly used in heavy industry have been linked to neurodevelopmental disorders when children are exposed [27]. These same chemicals have also been shown to decrease life expectancy and increase rates of many other deleterious health issues. A 2012 review paper by Rossignol and Frye examined 190 papers that aimed to investigate toxin exposure and ASD, and out of the 190 papers examined, it was found that 170 showed a correlation [28]. These chemicals included pesticides, biphenyls, heavy metals, and other common pollutants. Though environmental toxins are being shown to have an effect, once again it is thought to come down to a level of genetic susceptibility, with particular SNPs interacting with the chemicals to cause the expression of the phenotype [29].

Pharmaceuticals have also been a contentious issue in relation to a potential mechanism for the development of ASD. One of the most recent examples and potential causes behind some of the increase in ASD rates is opioids. This was found by the US center for disease control and Prevention in their study to explore the early development (SEED) program. Although the study did find a correlation between prescriptions pre-conception and ASD, the study did have many limitations which the authors

acknowledge such as duration of use and dosage [30].

Valproic acid (VPA) is a simple fatty acid that is used clinically as an anti-epileptic and mood-stabilizing medication that has been definitively proven to increase rates of ASD when exposed in utero [31]. In clinical practice, it is primarily used to treat generalized, focal, and absent seizures, but also for bipolar disorder. It has several potential mechanisms for which its clinically used, and the core mechanism is decreasing the rate of γ-Aminobutyric acid (GABA) degradation by inhibition of succinic semialdehyde

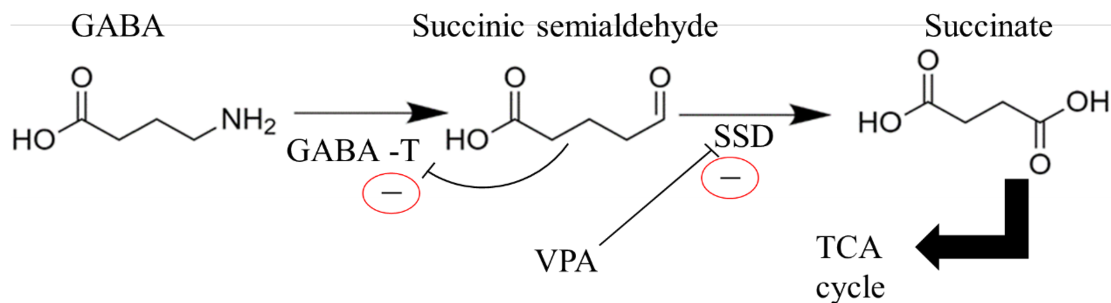

Figure 1.2 – Mechanism by which GABA is processed into Succinate for the TCA cycle and how Valproic acid (VPA) inhibits Succinic semialdehyde dehydrogenase (SSD) to maintain high GABA levels in the brain.

dehydrogenase (SSD). GABA typically over time is converted into succinate which is then used in the TCA cycle. By inhibiting SSD, it causes a building up of the intermediate product succinic semialdehyde which then has a negative feedback loop onto GABA – aminotransferase (GABA – T), thus, raising GABA levels *(Fig 1.3*).

It has other mechanisms which add to GABA modulation such as ERK pathway activation causing BDNF release, IP_3 pathway by inhibiting Inositol monophosphatase, and inhibition of PK C pathways. The last main pathway which is prudent to ASD is the inhibition of the Histone deacetylase enzyme (HDAC).VPA being an HDAC inhibitor would typically not be much of an issue when the brain is close to being fully developed, but during fetal development, there are many genetic changes required to get maximal rates of neurogenesis to support the developing nervous system.

1.5 Animal models of ASD

Continued expansion in the understanding of ASD and its genetic, environmental, and pharmacological causes has led to a revolution in developing models of ASD in animals. Since there is a multitude of different causes behind ASD it leaves many vectors that researchers can exploit to generate animal models of ASD in the lab. Outlined by Belzung et al, there are four main types, genetic models which are either mutations or KNOs, epigenetic models, lesion models, and pharmacological models [32]. To generate the ASD phenotype with minimal intervention only leaves a handful of viable contenders. Mutations in neuropeptides or KNOs require some form of genetic manipulation or extensive breeding which means that there can be a large delay before testing for the phenotype can even begin. However, the main limitation of genetic or neuropeptide models is that only a single variable is changed, which when trying to uncover a particular mechanism or the role of neurochemicals in the phenotype is fantastic. Though this falls short when trying to assess large neural networks which are integrated with one another. Brain lesions have a few issues, that being behaviours are not typically determined by one region, so when assessing behaviours, it can be inconsistent. Lesioning at differing points of development can also alter the phenotype which adds another variable to be accounted for. The Pol-IC method is one that was not mentioned before by Belzung as it is relatively new. It does not face many of the same limitations which were previously mentioned, because it comes under the framework of a developmental model [33]. It perturbs development though trying to reproduce effects seen in epidemiological studies relating to maternal infection and higher incidence of ASD [34]. This means it pharmacologically induces an elevated immune response in the fetus. However, this model is still relatively new and the optimum protocol for generation is still being discussed [35]. Mimicking the same epigenetic environment seen in ASD can be readily done via the administration of VPA in

sufficient doses. This method to produce an ASD phenotype has been shown to align with several related proxies seen clinically in ASD such as oxidative stress, HDAC inhibition, and hyperserotonemia [36-38]. The reason VPA can produce an ASD phenotype is through the disruption of a core developmental process known as neurulation, which refers to the formation of the neural tube in utero.

The process of neurulation has recently been shown to rely heavily on epigenetic changes which cause a dramatic change in the gene expression profile of the cells that form the neural tube and neural crest [39]. The VPA model leverages epigenetics to create a developmental model of ASD. This is achieved by VPA interfering with the histone modification event of deacetylation, as it is an HDAC inhibitor. Acetylation refers to the enzymatic addition of an acetyl group to either Lysine or Arginine residues in the tail region. Histone acetyltransferase (HAT) adds an acetyl group, while HDACs remove them. Mechanistically, acetylation causes the repulsion of the negatively charged DNA molecules from the nucleosome, causing it to become more accessible to molecular machinery. The removal of acetyl groups by HDACs causes the reassociation to the nucleosome thus making the DNA unreadable. Since VPA is an HDAC inhibitor, when it is used during pregnancies, it alters the natural epigenetic changes which are needed for typical neurodevelopment when administered at the correct time. This pharmacologically induced variance in gene expression causes robust downstream genetic, and subsequently morphological differences in the CNS which results in the phenotype that is consistent with ASD. To generate ASD in rodent models, during the beginning of neurulation at postnatal day 12.5, an injection of 500 mg/kg of VPA is administered to the mother. The aforementioned process of neurulation is now perturbed as HDAC1 and HDAC2 are now inhibited causing a cascade of deleterious effects which result in an altered developmental trajectory that will result in the ASD

phenotype [36].

1.6 Aberrant reward processing

One of the key issues surrounding ASD which underlies many of the symptoms is aberrant reward processing. From a neurobiological view, it pertains to key reward structures having altered development or key neuromodulators for reward, such as dopamine, endocannabinoids, and endogenous opioids having noncanonical effects. From a psychological perspective, aberrant reward processing is described by the social motivation hypothesis of autism as "an impaired ability to assign an appropriate reward value to social stimuli" [40]. This dysfunction in the reward system has been shown to permeate many of the symptoms of ASD as an underlying potentially causative force. For many years much of the research on ASD has been around finding treatment options that can ameliorate some of the symptoms such as impaired social interaction and communication issues. One area which has had little attention until recently is food intake.

For neuro-typical children, food intake can already posit a challenge for parents as some children can be picky. However, in ASD food intake can become an issue for caregivers due to many of the symptoms overlapping, such as repetitive behaviours, the rigidity of routine which new meals disrupt, nuances with texture and tastes of particular foods, and generally being picky eaters. These idiosyncrasies seem banal but because of them, they can often lead to strain on the caregivers and to nutrient deficiencies which can further exacerbate altered development or inflame other comorbidities such as GI problems [41]. Due to the heterogeneity of the ASD population, many underlying mechanisms can present with the same phenotype, little is known on a mechanistic level about what drives this pickiness. Food allergies have

been posited as one of the pieces to the puzzle as they are more common in ASD patients with over a twofold increase in occurrence [42]. Because many in ASD have limited to no verbal ability, if an allergy was to be present, it would be hard for caregivers to recognize it if it is not life-threatening, thus, some of the aversion demonstrated to certain foods could be underlined by food allergies. Another important factor that has a neurobiological basis is food aversion due to altered sensory processing. It has been anecdotally and quantitatively shown that alerted sensory processing afflicts those with ASD [43, 44]. Avery et al conducted a study that compared behavioural reactivity tests and fMRIs of participants with ASD to neuro-typical controls [45]. In line with other works, it was found that those with ASD report a much greater reactivity to taste compared to the neuro-typical group. In addition to this, the fMRI showed that this heightened reactivity was based on greater activity in the primary gustatory cortex. This implies a positive correlation between taste reactivity in ASD and brain activation in the corresponding area [45].

The rates of obesity in ASD children are higher than in their neuro-typical counterparts [46]. This increased rate of obesity may highlight that those with ASD may unintentionally choose to consume more highly palatable foods which typically contain a higher caloric content. This then would implicate reward processing and hedonic eating as a core perturbation in those with ASD. The palatability theory nicely intertwines with observations that children with ASD tend to prefer calorically dense, high carbohydrate foods, and dislike vegetables, eating only the average amount compared to neuro-typical children [47, 48]. Looking into the neurobiological differences in their reward system and hedonic eating pathways has been an avenue for research. The reward system encompasses core regions of the mesencephalon such as the mesocotircolimbic system. These regions have been implicated in the development

of many aberrant behavioural patterns ranging from impaired social ability to the reinforcement of drug addictions [49]. Recent fMRI studies have further implicated the reward system as a possible explanation for the aberrant behaviour around food. Cascio et al conducted a study on 19 children with ASD in which after a minimum four-hour fast they were shown images of food while an fMRI was being conducted [50]. The study design had four blocks of images in which one block was of palatable food (ice cream, pizza, etc.) while another block was also of the images but blurred to the point where they were unable to be identifiable to act as a control. The other two groups were unrelated. They found that the reward system in those with ASD compared to neuro-typical children was enhanced when images of palatable foods were shown. This is consistent with other data showing obese children have the same trend in activation, though this paradigm measured pre and post-meal activity by fMRI [51]. These findings in conjunction with the findings of Avery et al have begun to show that in ASD at a neurobiological level there are differences in activity from the same stimuli. This difference in brain activity suggests a shift from homeostatic/ energy-driven consumption to hedonic/ palatability-driven consumption.

The reward system begins with the ventral tegmental area (VTA) which then innervates the nucleus accumbance (NAc). The VTA has a heterogeneous population of neurons, and the composition breakdown is roughly 65% dopaminergic neurons while the remainder 35% is primarily GABAergic or glutamatergic [52]. Projections from the VTA are widespread and permeate much of the surrounding structures such as the prefrontal cortex (PFC), amygdala, hippocampus, lateral hypothalamus (LHA), and more. All of these regions also send projections back, thus creating a large integrative center for information processing. The orchestration of dopamine release from the VTA is a highly regulated affair that requires inputs from all these neuronal populations and

self-regulatory mechanisms. The VTA sends projections that initiate core pathways such as pathways related to reward and motivation via its connections to the PFC and NAc. The NAc can be separated into two primary regions, the core, and the shell, which have differences such as dopamine receptor density and input regions. The NAc within the brain is of particular importance to motivation and reward and acts as one of the main integration and propagation centers. Of particular interest to palatable eating and even drug addiction, the shell of the NAc seems to play more of a significant role than the core. Altered activation of the shell has been implicated in hedonic overconsumption and binge eating disorders as well as been shown to be more overstimulated when exposed to drugs [53, 54]. The LHA and the NAc are heavily integrated and are too both connected to the amygdala which acts as a point of intersection between these three systems. This intersection positions the shell to be highly involved in motivation and reward-based processes. In context, the shell receives inputs from the LHA, VTA, and the amygdala, and because of this emotional-based binge eating disorders can arise as these systems are closely linked. In mice, deep brain stimulation of the NAc shell has been shown to eliminate hypercaloric binge eating [55]. In this system is a level of regulation by endogenous opioids which act via endogenous opioid receptors. There are three endogenous opioids that have effects in this system, these are β-endorphin, dynorphin, and enkephalins. These opioids exert their effects through three G-protein coupled receptors (GCPR), β-endorphin acts through the mu-opioid-receptor (MOR), enkephalin acts through the delta-opioid-receptor (DOR), and dynorphin acts though the kappa-opioid-receptor (KOR). The endogenous opioid system has already been implicated by proxy to have some level of dysregulation in ASD in relation to pain sensitively. Numerous studies have shown an increase in pain sensitivity in children and adults with ASD which has disproven the long held notion that those with ASD have a level of pain insensitivity [56].

The interplay between the endogenous opioid system and food intake with particular emphasis on food palatability has been demonstrated robustly with many studies in humans showing opioid antagonists decrease consumption of palatable food after administration [57, 58]. This is in opposition to acute and chronic morphine administration causing increases in consumption of total calories and palatable foods [59]. MOR and to a lesser extent DOR have been shown to be the main drivers behind imbuing food with hedonic qualities which are driven by β-endorphin and enkephalins. β-Endorphin neuroanatomically is limited in scope as it is mainly produced by POMC neurons and has limited projections to regions for instance, the NAc and VTA. This is juxtaposed by enkephalins which are widely distributed throughout the CNS and in the hypothalamus. This duality at a neuroanatomical level can be explained by how each affects the behaviour of food intake when stimulated.

The research surrounding the importance of β-endorphin in its role to relay information about metabolic state is well established. This role allows the modulation of the hedonic properties of food to be altered in real-time with changes concerning the metabolic state of the organism [60]. This enables a dichotomy to exist between β–endorphin, and enkephalin, as the enkephalin functions in a more distributed fashion, particularly in areas that are hedonic hot spots. This means enkephalins function to endow foods with hedonic qualities which drive food-seeking behaviours via actions through the mesocortical pathway, thus acting as the creator of the motivation behind palatable eating. Simultaneously orexin producing neurons that co-express dynorphin are more active due to the current metabolic state, and thus dynorphin increases the dysphoric effect which can be associated with hunger due to the inhibition of dopamine release. This increase in an anxiolytic state paired with the other physiological responses to hunger drives the organism to consume palatable foods. When the

behaviour results in consumption, there is a delay in the time it takes for the neural systems to alter as it is based on circulating metabolic markers. Once these markers become present at the level of the hypothalamus the ARC, thus β-endorphin can act to alter the hedonic properties of the food which had previously been ascribed by the hedonic pathway [61].

β-Endorphin and enkephalin within the reward system act to lower the electrical threshold for stimulation of the reward system. Dynorphin, when released will actively suppress this action by binding to dopaminergic terminals to limit dopamine release [62]. To put this into context, GABAergic neurons within the VTA contain MOR and DORs which actively repress the firing rate of dopamine into the NAc. β-Endorphin and enkephalin bind to these receptors causing the electrical threshold to fire to increase via inward rectifying potassium channels (IRK) and adenylyl cyclase (ALC) mechanisms. This causes more dopamine to be released. The counter-regulatory mechanism to this is dynorphin, which binds to the mesolimbic dopaminergic neurons and causes the electrical threshold to fire to increase through the same mechanisms (*Fig 1.3*). This in turn decreases dopamine in the NAc shell which generates dysphoria. Though this is a generalization as particular studies which use a variety of different opioid antagonists are not as consistent as this model would predict, which is likely due to biased signaling from different receptors dynamic of different drugs. This biased pathway activation in opioid receptors is still not fully understood, but different receptor dynamics could cause the initiation of other signaling cascades.

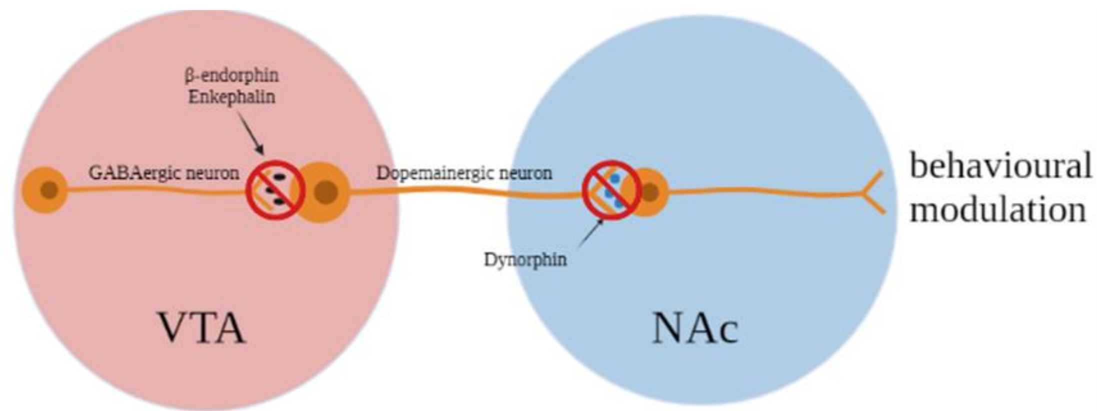

Figure 1.3 – representation of how endogenous opioids regulate the mesolimbic pathway through GABAergic or dopaminergic neuronal modulation. VTA stands for the ventral tegmental area, and NAc stands for nucleus accumbance.

The reward system is modulated by the endogenous opioid system is now a promising avenue of research for the treatment of many reward system-based pathologies such as alcohol abuse, gambling, and weight management. In lab animals, many studies have independently shown that opioid administration, even in sated animals causes a large increase in food-seeking behaviours and food consumption which is dose-dependent [63]. Once opioids were linked to ingestive behaviour, a hypothesis arose that implicated endogenous opioids as a critical part of palatability-driven consumption [64]. Opioids have been shown to not only affect total consumption in terms of calories, but also the intake of specific macronutrients. Acute morphine administration causes an increase in total calories consumed alongside an increase in fat consumption [65]. Though it was initially thought that opioid administration drove mainly an increase in consumption of fats, other works have shown the opposite which is a preference for carbohydrate consumption [66]. At a deeper level, the MOR is shown to be instrumental in this palatability-driven consumption, as rats with diet-induced obesity, through the consumption of HFHS diets, have upregulated MOR mRNA levels in feeding and reward-related circuitry when compared to obesity-resistant rats [67]. Upregulation of MORs is not an isolated event as animals with higher fat intake have

an increase in the amount of proenkephalin mRNA in regions such as the PVN, CeA, and NAc [68]. This is consistent with the notion that the reward system and in particular enkephalins act though imbuing food with its pleasurable qualities and not affecting taste itself [69]. The administration of Naloxone (NAL), an opioid receptor antagonist, has been shown in several studies to consistently affect the intake of palatable foods. Though it is inconsistent with decreasing consumption of "bland" standard laboratory chow unless at much higher doses [59, 66] NAL in operant training of sucrose and water discrimination showed that discrimination of the sweet-tasting sucrose could not solely explain the anorexigenic effects seen [70]. NAL is a similar analog to NTX, though its effects are shorter-lived in comparison. NTX has been shown to have the same ability to modulate palatability-driven food intake in laboratory animals [71]. Laboratory rats administered NTX show a decrease in consumption of highly palatable HFHS chow [72]. In addition to this, chronic exposure to palatable solutions like sucrose increases the activity of the reward system. In these chronically exposed animals, NTX was shown to be more effective compared to rats that had not been exposed to the palatable solutions [73]. In humans, 50 mg of oral NTX has been shown to decrease meal size and meal enjoyment when subjects were given HFHS meal [57]. Though as a monotherapy it was shown to be insufficient causing minimal weight loss because the mechanisms which govern food intake are more complex than one pathway [74, 75].When used in combination with bupropion, a molecule that stimulates α-MSH release from PMC neurons in the ARC, it has been demonstrated to be effective in weight management [76].

1.7 Overarching goals and specific aims

The endogenous opioid system has been shown to play a significant role in the mediation of pleasure/palatability-driven feeding by affecting reward-related processes.

Opioid antagonists successfully reduce intake of palatable foods, whereas their effect on energy needs-induced consumption of bland diets is mostly negligible. Importantly, as mentioned in the earlier sections of the introduction to this thesis, ASD is associated with aberrant reward processing (including the dysregulation of opioid pathways) as well as maladaptive eating behaviour, especially overconsumption of palatable tastants. In the current project I sought to draw upon these two phenomena and assess whether VPA rats (a fundamental laboratory animal model of ASD) display a different sensitivity to anorexigenic properties of a non-selective opioid receptor antagonist, NTX, compared to their non-VPA conspecifics. Furthermore, I mapped expression of an immediate-early gene product, c-Fos, throughout feeding brain circuits to define circuitry specific sites whose altered activity after NTX treatment might have contributed to the changed behavioural response.

2.0 Materials and methods

2.1 Animals

All animals were male Sprague-Dawley rats aged 12 weeks. All were individually housed in transparent polycarbonate cages which contained wood shavings, a wired lid, standard chow *ad lib* (Harlan Teklad, Madison, WI, USA), and a fluid bottle filled with tap water unless specified. All animals were housed in an animal facility that maintained a constant temperature of 22°C and a 12-hour day-night cycle with lights on at 6 a.m. Before and during the experiment all animals had *ad libitum* chow and water availability unless specified. Ethics approval was given by the University of Waikato's animal ethics committee, protocol number 1133.

2.2 Generation of VPA males

Adult female Sprague-Dawley rats of approximately five to six months of age were mated overnight with age-matched Sprague-Dawley males. The following day vaginal smears were used to identify spermatozoa content using a 1% crystal violet stain. Upon successful identification of spermatozoa, the date post conception was noted as E.0.5. At the date E.12.5 the impregnated female Sprague-Dawley underwent a single intraperitoneal injection (I.P) of either a vehicle which was 0.9% saline or 500 mg/kg of sodium valproate (Sigma Chemical Co., St. Louis, MO, USA). All VAP females were healthy and were allowed to raise their litter until weaning at postnatal day 25. 12% of VPA rats developed a crooked tail while a small population developed an acute chromodacryorrhea [77]. All animals used in the experiment were males for homogeneity purposes.

2.3 Confirmation of ASD phenotype

Conformation of ASD phenotype in offspring was performed using a selection of behavioural tests to elucidate traits in line with previously established literature [78-80]. The behavioural tests used were an elevates plus-maze to assess anxiety measures and an open field test for social interactions. The results of these tests confirmed that the animals selected for the experiment exhibited an ASD-like phenotype.

2.4 NTX's effect on energy-driven and palatability-driven consumption in non – VPA and VPA rats

2.4.1 Experiment 1: Effect of I.P NTX on overnight deprivation-induced intake of standard laboratory chow in non – VPA animals. We examined whether NTX affects feeding induced by energy needs, i.e., intake of standard chow after overnight food deprivation in age and weight-matched (320 +/-17 g) non – VPA rats. Chow was removed at 6 p.m. on the day preceding drug administration; water was available. On the next day, pre-weighed chow was returned to cages at 10 a.m. Just before refeeding, rats were given IP saline or NTX at 1 mg/kg, 3 mg/kg, or 10 mg/kg (n = 6 - 7 / group). Food intake was measured after 2 hours.

2.4.2 Experiment 2: Effect of I.P NTX on overnight deprivation-induced intake of standard laboratory chow in control rats. The paradigm was the same as described for Experiment 1 above (n = 6 - 8 / group). However, age-matched VPA rats were used instead (325 +/- 22 g).

2.4.3 Experiment 3: Effect of I.P NTX on HFHS diet intake in non – VPA animals without prior energy deprivation. Rats were preexposed to the HFHS diet to prevent neophobia 7-10 days before the study (2 hours). On the study day, "bland"

chow was removed at 10 a.m. (access to water was unchanged), and 5-10 minutes later the rats were injected I.P with 0.9% saline or NTX at 0.3 mg/kg, 1 mg/kg, or 3 mg/kg body weight (n = 7 – 8 / group). 10 minutes after the drug treatment, they were given the HFHS chow, and the consumption was measured 2 hours later. In this paradigm, the HFHS chow consumption occurs without prior energy deprivation, and thus the motivation to initiate feeding is driven primarily by hedonics. Data from the NTX-injected groups were compared with the saline controls with ANOVA followed by Dunnett's test. Differences were considered significant for $p \leq 0.05$.

2.4.4 Experiment 4: Effect of I.P NTX on HFHS diet intake in VPA rats without prior energy deprivation. The paradigm was the same as described for Experiment 3 above. However, VPA rats were used instead. The animals were injected with saline or NTX (1, 3 or 10 mg/kg) (n = 8 / group).

2.4.5 Experiment 5: Effect of I.P NTX on sucrose solution intake in non – VPA animals without prior energy deprivation. In another paradigm focusing on consumption driven by palatability, rats were preexposed to the sugar solution to prevent neophobia 4 - 8 days before the study (2 hours). On the study day, standard chow and water were removed at 10 a.m., and 5-10 minutes later the rats were injected I.P with 0.9% saline or NTX at 0.3 mg/kg, 1 mg/kg, or 3 mg/kg body weight (n = 7 – 9 / group). 10 minutes after the drug treatment, they were given one bottle containing the sugar solution and the consumption was measured 2 hours later. Data from the NTX-injected groups were compared with the saline controls with ANOVA followed by Dunnett's test. Differences were considered significant for $p \leq 0.05$.

2.4.6 Experiment 6: Effect of I.P NTX on sucrose solution intake in VPA rats without prior energy deprivation. The paradigm was the same as described for Experiment 5 above. However, VPA rats were used instead, and they were injected with saline or NTX (1, 3, or 10 mg/kg) (n = 7 – 8 / group)

2.5 Effect of 1 mg/kg NTX (lowest effective dose in suppressing palatability-driven consumption) on brain activation in on brain activation in non – VPA animals consuming palatable sugar water

Non – VPA animals were separated into four groups Water+Saline, Water+NTX, Sucrose+Saline, and Sucrose+NTX. Every 12 hours for 14 days, from 7 a.m. to 7 p.m. the groups were given the tastant and drug they were assigned. On the experimental day, all animals were injected I.P with the 1 mg/kg of NTX with a 0.9% saline solution to equate volume. 1 hour post-NTX injection the animals were anesthetized using 500 ml of urethane (ethyl carbamate) (35% in isotonic NaCl) I.P. After being anesthetized their body was flushed using 50 ml 0.9% saline followed by 500 ml of 4% paraformaldehyde (in 0.1 M phosphate buffer). The brains were then dissected and placed into 4% paraformaldehyde for 24 hours at 4ºC for post-fixation. After post-fixation, the brains were sliced into 60 μm sections using a vibratome (Leica, Germany; 60 μm coronal sections). For immunohistochemical analysis of c-Fos, a primary antibody of 1:4000 (Synaptic Systems, Australia, stored at 4ºC, made in Rabbit) was used, and then a secondary antibody of 1:400 (Vector Laboratories, Burlingame).

2.6 Effect of 1 mg/kg NTX (lowest effective dose in suppressing palatability-driven consumption) on brain activation in VPA animals consuming palatable sugar water

VPA animals were separated into four groups Water+Saline, Water+NTX,

Sucrose+Saline, and Sucrose+NTX. Every 12 hours for 14 days, from 7 a.m. to 7 p.m. the groups were given the tastant and drug they were assigned. On the experimental day, all animals were injected I.P with the 1 mg/kg of NTX with a 0.9% saline solution to equate volume. 1hour post-NTX injection the animals were anesthetized using 500 ml of urethane (ethyl carbamate) (35% in isotonic NaCl) I.P. After being anesthetized their body was flushed using 50 ml 0.9% saline followed by 500 ml of 4 % paraformaldehyde (in 0.1 M phosphate buffer). The brains were then dissected and placed into 4 % paraformaldehyde for 24 hours at 4ºC for post-fixation. After post-fixation, the brains were sliced into 60 µm sections using a vibratome (Leica, Germany; 60 µm coronal sections). For immunohistochemical analysis of c-Fos, a primary antibody of 1:4000 (Synaptic Systems, Australia, stored at 4ºC, made in Rabbit) was used, and then a secondary antibody of 1:400 (Vector Laboratories, Burlingame).

2.7 Quantification of immunohistochemistry

Imaging was done using a camera attached to a light microscope (Nikon Eclipse 400). All images were taken at 20x magnification bilaterally. Counting c-Fos immunoreactive nuclei were done at the mm^2 level using ImageJ software. Paxinos and Watson Rat brain atlas were used to set site boundaries for distinct brain regions. Means and SEM were calculated, and data were compared with two-way ANOVA with drug and diet set as independent factors.

3.0 Results

NTX was not effective at reducing the intake of standard laboratory chow in energy-deprived rats. This lack of effectiveness of NTX in suppressing energy-driven consumption of the "bland" chow was found in both VPA animals and their control counterparts (*Fig 3.1*).

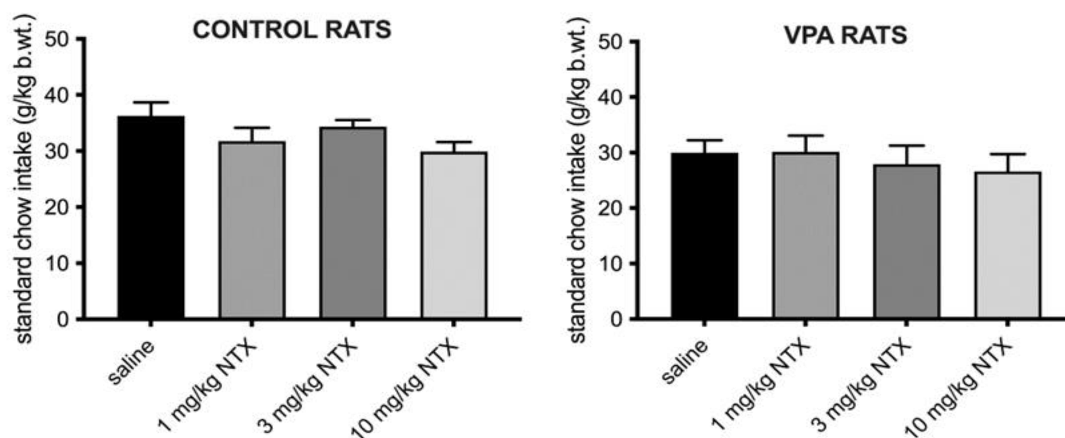

Figure 3.1 - Effect of I.P NTX on overnight deprivation-induced intake of standard laboratory chow in control (right graph) versus VPA rats (left graph). Isotonic saline was used as a vehicle solution. Injections were administered 10 minutes before the meal. Chow intake was measured after 2 hours.

On the other hand, in two paradigms in which rats were eating for palatability, thus in the scenarios where animals were not deprived of energy and received either a palatable and calorie-dense high-fat high-sugar (HFHS) chow for 2 hours (*Fig 3.2*) or a palatable and calorie-dilute sucrose solution for 2 hours (*Fig 3.3*), NTX reduced feeding. Importantly, in control rats, 1 mg/kg NTX and 3 mg/kg NTX suppressed consumption of the HFHS chow ($P=0.036$ and $P<0.001$, respectively) and sugar water ($P = 0.011$ and $P < 0.001$, respectively). Importantly, in VPA animals, only a very high 10-mg/kg dose reduced the amounts of ingested HFHS ($P=0.022$) and sucrose ($P = 0.0011$).

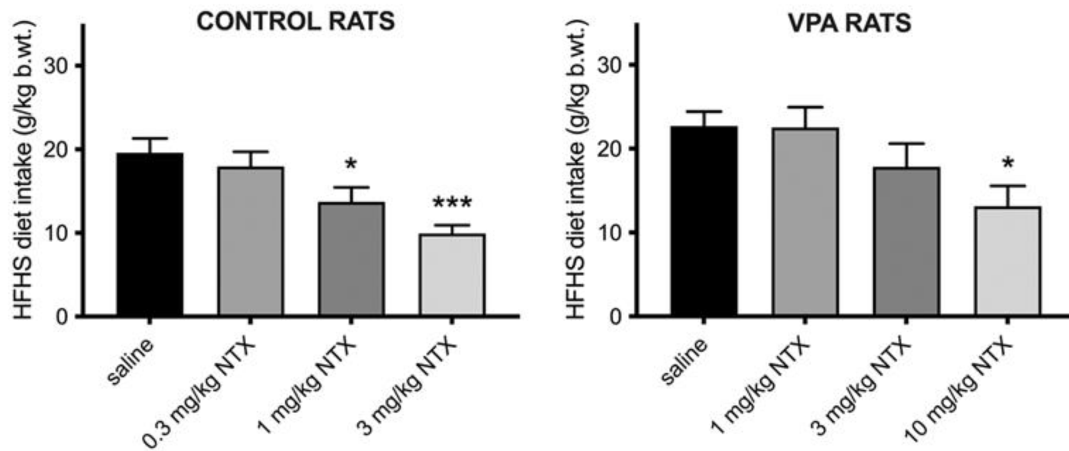

Figure 3.2 - Effect of I.P NTX on HFHS diet intake in control (right graph) versus VPA rats (left graph) without prior energy deprivation. Isotonic saline was used as a vehicle solution. Injections were administered 10 minutes prior to the meal. HFHS diet intake was measured after 2 hours. Standard chow was removed from the cages during the time of the HFHS diet meal.

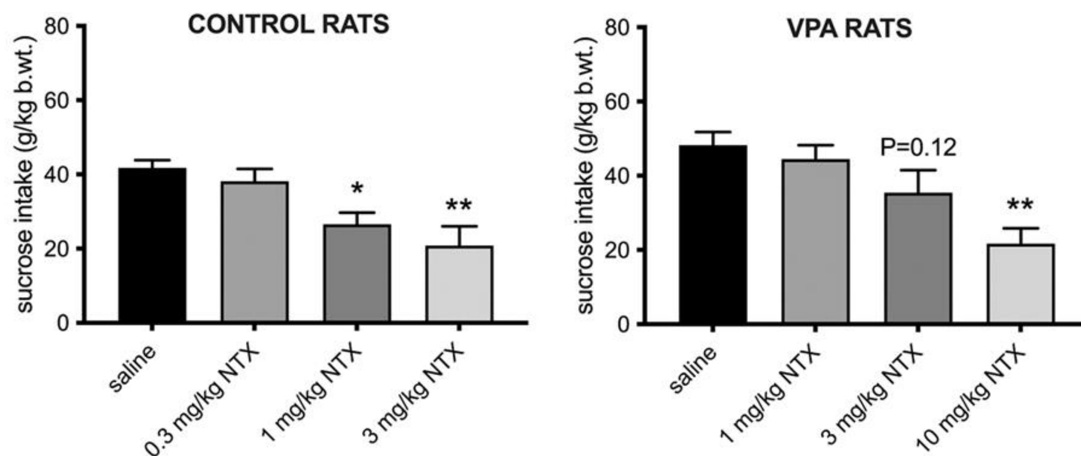

Figure 3.3 - Effect of IP NTX on sucrose solution intake in control (top graph) versus VPA rats (bottom graph) without prior deprivation. Isotonic saline was used as a vehicle solution. Injections were administered 10 minutes prior to the meal. Sucrose solution intake was measured after 2 hours. Standard chow and water were removed from the cages during the time of the sucrose solution presentation.

Immunohistochemical analysis of non-VPA animals showed that in the PVN, SON, ARC, DMH, CeA, and the NAc – shell, NTX caused a significant change in activation levels. In the PVN there was a statistical difference found in the Sucrose+Saline group when compared to the Sucrose+NTX group ($P < 0.0001$). The Water+Saline group versus the Water+NTX was also significant ($P < 0.0001$) (*Fig 3.4)*. Two-way ANOVA showed that both tastant and the drug had an effect ($P = 0.0006$, and $P < 0.0001$,

respectively) and there was an interaction between the two (P = 0.00273) (*Table 3.1*). In the SON for non-VPA animals, there was a similar trend of activation across the groups being compared in controls with the Sucrose+Saline versus the Sucrose+NTX showing high levels of significance (P < 0.0001) (*Fig 3.4*). Two-way ANOVA also

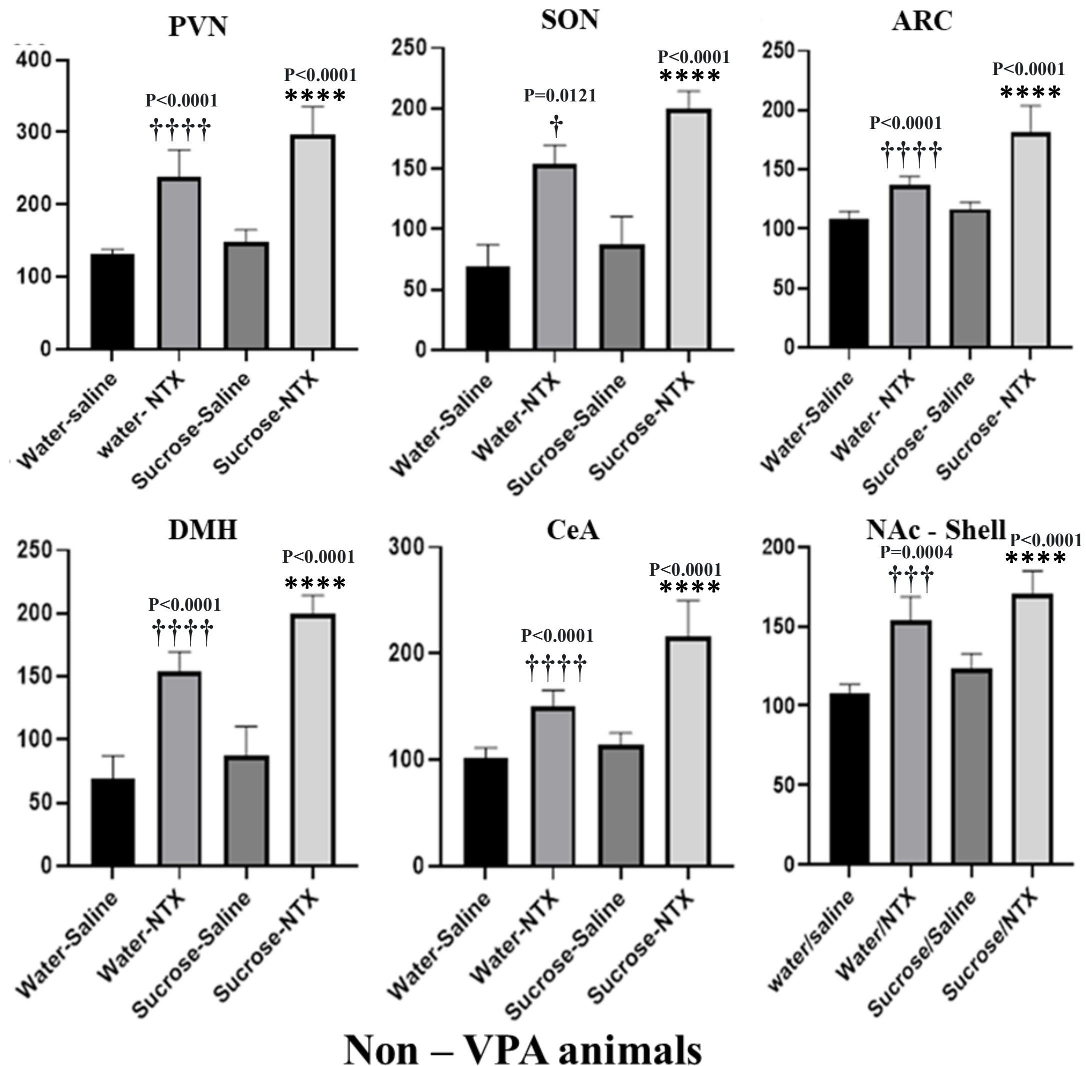

*Figure 3.4 – Effects of I.P NTX on c-Fos expression in non-VPA animals in either Water+Saline, Water+NTX, Sucrose+Saline, or Sucrose+NTX groups. Areas shown in image are PVN (top right), SON (top middle), ARC (top left), DMH (bottom right), CeA (bottom middle), and NAc – shell (bottom left). * Significantly different between Sucrose+Saline vs Sucrose+NTX. † Significantly different Water+Saline vs Water+NTX.*

showed tastant and the drug had considerable effects (both with P < 0.0001). However, the interaction between the two that was detected was not as significant (P = 0.015) (*Tab 3.1*). These results were replicated in the ARC except for the two-way ANOVA

where the P value shifted (P = 0.0023) (*Tab 3.1*). In the DMH of non-VPA animals, Water+Saline versus Water+NTX showed the same level of significance in terms of difference as Sucrose+Saline compared to Sucrose+NTX (both with P < 0.0001). Water+NTX versus Sucrose+NTX showed similar results to those found in the ARC (P = 0.0013) (*Fig 3.4*). This was also seen with the two-way ANOVA as both tastant and drug have P values below 0.0001, and the P value for the drug drink interaction is higher (P = 0.0023) (*Tab 3.1*). In the CeA there was a difference found with the most significant being in the Sucrose+Saline versus the Sucrose+NTX and the Water+NTX versus the Sucrose+NTX groups (P < 0.0001, and P < 0.0001, respectively). The Water+NTX compared with the Sucrose+NTX group also showed significance which is in line with the trend seen in some other regions (P = 0.0004) (*Fig 3.4*). Two-way ANOVA results show that the tastant and the drug have P values below 0.0001, while the interaction between the two only had a P value of 0.0188 (*Tab 3.1*). In the NAc – shell there were observed differences seen in the non-VPA animals. Sucrose+Saline when compared to the Sucrose+NTX group showed a statistically significant difference (P < 0.0001). There was also significance seen when comparing Water+Saline to Water+NTX, and when comparing Water+NTX to Sucrose+NTX (P = 0.0004, and P = 0.0003, respectively) (*Fig 3.4*).

In non-VPA animals the VMH, LHA, BLA, and NAc – core did not show significance with respect to a drug drink interaction after two-way ANOVA (in order, P = 3022, P = 0.9557, P = 0.0544, P = 0.4404). However, individually there were effects detected. In the VMH the tastant and the drug had effects (P = 0.0017, and P = 0.0005). This was also seen in the LHA though to an even greater extent (both with P < 0.0001). In the BLA and the NAc – core there was an effect found with the tastant, only the drug had an effect (P = 0.0007, and P = 0.0080) (*Tab 3.1*).

In VPAs, the only region to show a drug drink interaction was the CeA. In the CeA when the Sucrose+Saline was compared to the Sucrose+NTX group a significant difference was found (P = 0.0003). Water+Saline compared to Water+NTX also showed differences along with Water+NTX versus Sucrose+NTX ((P = 0.428, and P = 0.0195) (*Fig 3.5*). Two-way ANOVA revealed the drug had a large effect (P < 0.0001); this was not seen in the tastant (P = 0.0149) (*Tab 3.5*).

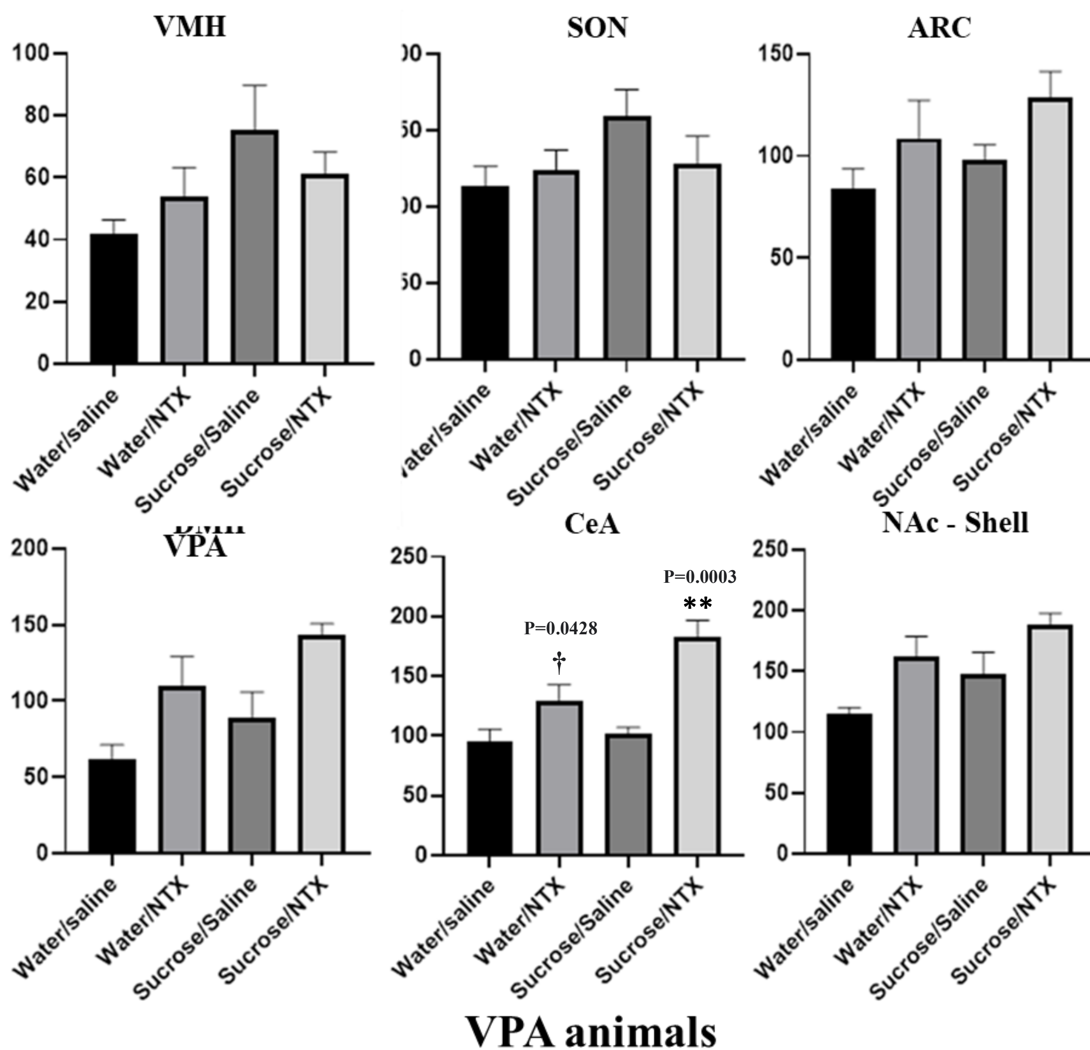

*Figure 3.5 – Effects of I.P NTX on c-Fos expression in VPA animals in either Water+Saline, Water+NTX, Sucrose+Saline, or Sucrose+NTX groups. Areas shown in image are PVN (top right), SON (top middle), ARC (top left), DMH (bottom right), CeA (bottom middle), and NAc – shell (bottom left). * Significantly different between Sucrose+Saline vs Sucrose+NTX. † Significantly different Water+Saline vs Water+NTX.*

In VPAs the PVN, SON, DMH, VMH, LHA, BLA, NAc – core, and NAc – shell showed no significant changes in activation. The NAc – shell showed that the tastant and the drug had some effect after two-way ANOVA (P = 0.0483, and P = 0.0074 respectively). This was also seen in the DMH with a similar trend for the tastant and the drug (P = 0.0425, and P = 0.0015). The PVN did not show an effect of the tastant but did show a significant effect of the drug (P = 0.0009). This was seen though to a lesser extent in the ARC (P = 0.0442) (*Tab 3.2*).

Brain region	Interaction	Drug	Tastant
PVN	P = 0.0273	P < 0.0001	P = 0.0006
SON	P = 0.0150	P < 0.0001	P < 0.0001
ARC	P = 0.0023	P < 0.0001	P < 0.0001
DMH	P = 0.0036	P < 0.0001	P = 0.0001
VMH	P = 0.3022	P = 0.0005	P = 0.0017
LHA	P = 0.9557	P < 0.0001	P < 0.0001
CeA	P = 0.0188	P < 0.0001	P < 0.0001
BLA	P = 0.0544	P = 0.0007	P = 0.0591
NAc - Core	P = 0.4404	P = 0.0080	P = 0.3346
NAc - Shell	P = 0.0411	P < 0.0001	P < 0.0001

Two-way ANOVA – P values non – VPA animals

Table 3.1 – Two-way ANOVA results all brain regions for non – VPA animals in which immunohistochemical analysis was conducted. Mean and SEM from Water+Saline, Water+NTX, Sucrose+Saline, and Sucrose+NTX groups was used.

Brain region	Interaction	Drug	Tastant
PVN	P = 0.9525	P = 0.0009	P = 0.1800
SON	P = 0.2083	P = 0.5309	P = 0.1362
ARC	P = 0.8193	P = 0.0442	P = 0.2031
DMH	P = 0.8249	P = 0.0015	P = 0.0425
VMH	P = 0.1893	P = 0.9157	P = 0.0472
LHA	P = 0.6866	P = 0.1442	P = 0.2281
CeA	P = 0.0445	P < 0.0001	P = 0.0149
BLA	P = 0.7532	P = 0.0691	P = 0.3829
NAc - Core	P = 0.1933	P = 0.7773	P = 0.4740
NAc - Shell	P = 0.8138	P = 0.0074	P = 0.0483

Two-way ANOVA – P values VPA animals

Table 3.2 – Two-way ANOVA results all brain regions for VPA animals in which immunohistochemical analysis was conducted. Mean and SEM from Water+Saline, Water+NTX, Sucrose+Saline, and Sucrose+NTX groups was used.

4.0 Discussion

This initial series of experiments demonstrated that overnight deprivation followed by an injection of NTX did not alter consumption in standard laboratory chow, even at doses up to 10 mg/kg for both non-VPA and VPA rats. This result shows that NTX has little effect on the homeostatic mechanism for food intake after injection. This is in accordance with other work that shows that on a standard laboratory chow i.e low palatability, the NTX groups and controls do not differ [66, 81].

On the other hand, NTX does decrease palatability-driven consumption, which is shown in the two different non-deprived paradigms. When NTX was administered, palatability-driven consumption of HFHS chow was diminished. However, in VPAs it took 3.3x the dose of NTX to elicit a similar response to that seen in their non-VPA conspecifics (3 mg/kg in controls versus 10 mg/kg in VPAs). NTX's ability to decrease consumption of HFHS diets is why it is currently being used in conjunction with other pharmacotherapies to treat obesity [82]. The following experiment validates this, as without deprivation, NTX was able to decrease sucrose consumption in non-VPA and VPA animals. It took 3.3x the amount of NTX to get a similar reduction in consumption of sucrose in VPA models. The ability of NTX to decrease sucrose consumption has historically only been established in non-VPA animals, but not in VPAs [83]. This demonstrates the potential utility of NTX in a clinical setting to acutely dimmish food intake in some models of ASD. This might not be feasible with the standard oral dose being 50 mg, and the maximum dose being 200 mg. The average weight of an adult male in New Zealand is 89kg, meaning the dosage required to match 10 mg/kg would be 890 mg. There has been interest in combining NTX with OTX to curb obesity in neuro-typical patients, but this would be even more beneficial in the ASD population

due to OXT typically being dysregulated. A recent case study in a patient after craniopharyngioma removal showed that low-dose NTX in combination with OXT was able to ameliorate the hyperphagia observed before the intervention [84].

Hughes et al gives an explanation as to why VPA animals need a higher dose of NTX, as they showed that VPA models have differing concentrations of MOR and KOR in and around the area of the hypothalamus [85, 86]. Their work gives credibility to my results as they show that there is a lack of fluctuation when presented with palatable stimuli in VPA animals. Thus, to derive the same level of stimulation, VPA animals require a higher level of consumption. Hughes also found a decrease in enkephalin and dynorphin in the hypothalamus. These both have a robust implication on the motivation to eat via their action through the mesocotircolimbic system. Dynorphin and enkephalin dysregulation in recent years has come to the forefront as being a major contributing factor behind addictive behaviours such as alcoholism, drug addiction, and gambling [87]. The dysregulation in VPAs fits with this notion due to the intertwined nature of hedonic food intake and the reward system [87]. In contrast to the VPA animals, the non-VPA controls showed a good response to the low dosage of NTX with the minimum effective dose being 1 mg/kg. Since the aim of this study was to assess if the endogenous opioid system was a contributing factor to aberrant reward processing, the minimum effective dose of NTX in the non-VPA animals was administered to the VPA animals. This would show a difference in c-Fos expression in feeding and reward-related areas based on the rationale it was producing a different behavioural response.

Immunohistochemical analysis of the hypothalamus and reward centers demonstrated that NTX had little effect in the VPA animals in contrast to their non–VPA conspecifics. The ARC is the brains main connection to the external metabolic

environment and was shown to be affected by NTX in non–VPA animals but not in VPA animals. These results are in line with Hughes et al due to receptor density differences and the fact that the ARC has been demonstrated to be partially regulated by both enkephalin and β – endorphin [88]. Input by endogenous opioids adds an auxiliary layer of regulation, on top of the one which exists due to the antagonistic relationship between pro-opiomelanocortin (POMC) and agouti-related peptide (AG-RP) neuronal populations [89]. A fundamental component in this system of antagonism is neuropeptide Y (NPY), which is synthesized in AG-RP neurons. NPY exerts its effects through receptors Y1, Y2, and Y5. These receptors are found on POMC neurons, and the ARGP neurons deeply innervate this population. When in a fasted state, NPY is released which will cause direct inhibition of the POMC neurons as the NPY acts through its receptors to cause hyperpolarization. This is mainly mediated through the Y1 and Y2 receptors. The Y5 receptor is localized to the POMC synapses which release alpha-melanocyte-stimulating hormone (α-MSH). At the synapse, NPY can stop the release of α–MSH to other structures within the hypothalamus, shutting down anorexigenic signalling. This system of antagonism also has GABA signalling from AGRP neurons to POMC neurons layered in; causing further inhibition to the melanocortin system. However, this system is reciprocally antagonistic, where AGRP neurons contain melanocortin-4 receptor (MC4R) and melanocortin-3 receptor (MC3R) which act to shut down NPY, AGRP, and GABA release. Additionally, β–endorphin acts in an autoregulator manner on MORs which are expressed on POMC neurons, and enkephalin act as an external regulator also by binding to these MORs. This causes a decrease in the release of POMC products, namely α-MSH, stopping anorexigenic signalling. This framework acts in explaining why morphine causes an increase in consumption as it can bind to the MORs. NTX acts in the opposite manner by stopping autoregulation and external regulation by blocking the effects of the opioids, allowing

for the release of α-MSH [90]. This however has never been directly measured in VPA animals, and these results show that 1 mg/kg of NTX is not sufficient in the ARC to stop the effects of autoregulation by β–endorphin or external regulation of enkephalins, which was why there was no difference seen. This is interesting as the 1 mg/kg dose was sufficient in non-VPA animals to cause a statistically significant change in c-Fos expression. This could potentially mean that there is a higher level of autoinhibition in the ARC in VPA animals. If so, the it would be harder for the POMC neuron to overcome this increased level of inhibition and release α-MSH to induce satiety.

The PVN and SON, which both contain magnocellular neuronal populations, were not affected by NTX administration in VPA animals. The PVN contains all types of opioid receptors, is innervated by the ARC, and additionally has efferents from the orexin neuronal populations from the LHA. These receptors have an inverse relationship with magnocellular cells in context to the release of OXT, vasopressin, and CRH. The release of these neuropeptides is controlled by the melanocortin system though α-MSH release [91]. Dynorphin suppresses the release of OXT and other neuropeptides while hungry, due to its co-localization with Orexin. Antagonism of the KOR in the PVN has been shown to increase OXT, vasopressin, and CRH release. MOR agonism at orexin neurons causes a decrease in dynorphin release. This could have a cascading effect on OXT release, due to it not being depressed by KOR agonism on magnocellular cells [92]. OXT has been shown to be anorexigenic in a number of different study designs from ablation of OXT neurons, intraventricular injections, and immunohistochemically where there is increased co-localization between c-Fos and OXT that coincides with meal cessation [93-95]. Peripherally, OXT can modulate many physiological mechanisms, and even IP injections of OXT have been shown to affect food intake, mainly decreasing sucrose intake [96]. The ARC and in particular POMC

neurons have been shown to stimulate the release of OXT via activation of the melanocortin system. Direct administration of α–MSH into the PVN causes robust activation of OXT neurons which project centrally [97]. This is interesting as orexin neurons from the LHA also project into the PVN and work in opposition to OXT by inhibiting glutamatergic signalling [98]. OXT has been implicated to have a wide variety of physiological effects due to it having receptors in many regions. Though in context to reward processing, the NAc and the VTA are of critical importance [99]. As mentioned earlier, mutations in OXT of the OXTR have been implicated as a predictor of ASD. Only in non-VPA animals NTX was able to elicit a change in c-Fos expression in the PVN. In VPA animals, the difference in activity could be the result of NTX, or it could be a downstream effect from the ARC. Because of the increased inhibition at the ARC by an increase in receptor density, this would cause less α-MSH release causing lower levels of stimulation at magnocellular cells in the PVN.

Hughes et al showed that KOR levels were decreased in the hypothalamus but increased in the CeA, giving credibility to these results, as the CeA was one of the main sites of statistical difference in c-Fos expression in both non-VPA and VPA animals. KOR in the CeA has been shown to be critical in anxiety and fear-based conditioning in rats [100]. An increase in KOR density could have implications for ASD. PET scans in neuro-typical adults have shown that there is an inverse relationship between KOR density with social status and sociability [101]. The CeA is one of the few non-hypothalamic regions to affect food intake, and it has recently been shown that the CeA can integrate neuromudulatory chemicals related to feeding. Work around the CeA's involvement in food intake via neuromodulation of hypothalamic regions is under-studied. The CeA is well integrated into the hypothalamus. For example, asthma attacks are thought to be in part modulated by a PVN to CeA connection [102]. One main

connection between the PVN and CeA is that OXT reduced CeA activity through binding to GABAergic neurons [103]. The CeA's role in the context of hedonic eating is through its ability to determine which stimuli are perceived as most rewarding [104]. This is because the CeA has connections to the hippocampus, NAc, VTA, and hypothalamus. A study by Shen et al 2022 has shown that in some cases of ASD, there is an overgrowth of the amygdala as a whole. They also found in fragile X syndrome, there is overgrowth in other areas which pertain to the development of repetitive behaviours [105]. This illustrates that in ASD, the altered neurodevelopmental trajectory can have implications on the CeA. Opioids at the level of the CeA have been shown to be a large regulator of activity [106]. MORs have been shown to tonically inhibit GABAergic signaling in the CeA while KOR has been shown to do the same to glutamatergic signaling [107]. Mahler et al have demonstrated that MOR agonist DAMGO, caused an increase in appetitive behaviours after microinjection into the CeA [104]. They also show that muscimol microinjection had the inverse response and decrease appetitive behaviours. This displays that at the level of the CeA, efferent GABAergic neurons play a role in the regulation of food intake. In addition, muscimol microinjections caused an increase in the prevalence of behaviours associated with fear such as self-grooming. Considering all the factors which can influence the CeA, it is logical to see why there was an observable difference with 1 mg/kg of NTX in VPA animals. This is interesting as the BLA in both non-VPA and VPA animals was shown to not be affected as robustly as the CeA. However, in non-VPA animals, it was trending towards significance ($P = 0.0544$). This can likely be attributed to the fact that the BLA and CeA in non-fear-based settings tend to have independent roles rather than operate as a single unit [108]. However, even though they are independent of one another they both work to regulate the reward processing systems by having a large influence over dopamine release into the NAc (both core and shell) [109]. The BLA contains large

amounts of efferent glutamatergic and GABAergic neurons which project to regions like the NAc which act to stimulate or inhibit them depending on the stimuli. Glutamatergic signaling from the BLA has been demonstrated to facilitate reward-seeking behaviours [110]. Lesioning of the BLA caused an inability for rats to recognize associations with sensory-specific cues surrounding rewarding stimuli [110]. This means that the BLA integrates incoming information from other regions to make sensory associations with the rewarding stimuli. While the CeA on the other hand has a functionally different role which relates to initiating behaviours that lead to consumption [111]. The BLA has been demonstrated to possess one of the highest densities of MORs in the brain, though direct injections of opioids into the BLA have not been done with the intention of measuring palatability-driven consumption [112]. It has been thought due to the location of many of the MORs being on afferent terminals, its role is to allow for the selective inhibitory filtration of weak inputs about external stimuli [112]. Administration of NTX, theoretically, cause asynchronous firing into the BHL that would dimmish its abilities. As mentioned earlier in the discussion, both VPA and non-VPA animals did not show statistically significant c-Fos expression changes across groups. However, based on the limited research on the BHL, it is hard to infer anything about its mechanism in relation to palatability-driven consumption.

Increased activity of the dorsomedial hypothalamus (DMH) has been shown to be associated with decreases in food intake and an increases in physical activity in rats when stimulated [113]. The main mechanism by which the DMH modulates these processes is via leptin, as the DMH contains leptin receptors and additionally receives inputs from the PVNs magnocellular cells. The magnocellular cells in the PVN which secrete CRH innervate the DMH. The dynamics of the ARC when presented with information about metabolic state relay this to the DMH through the melanocortin

system. Additionally, the PVN when stimulated by the melanocortin system releases CRH which works in an additive manner. Though there are direct connections from the ARC to the DMH from the ARC, this allows other neuromudulatory compounds such as β-endorphin and NPY to have effects also [114]. In the DMH it is believed that MORs are present though not in great concentrations when compared to other regions of the hypothalamus [115]. From this, one could hypothesize that changes in the DMH in ASD are due to the regions around it having a perturbed opioid receptor phenotype, which causes the inputs into the DMH to have altered functioning. A way to ameliorate this would be with direct injections of opioids such as morphine or DMAGO to assess behavioural and c-Fos changes, though that is an avenue for potential future research. In relation to weight regulation, neuromodulators like leptin have been demonstrated to increase sympathetic tone and increase brown adipose tissue energy expenditure [116]. Because the DMH has been shown to affect thermogenesis, it is a reasonable hypothesis to think perturbations in the rest of the hypothalamic systems would have cascading effects. Studies have shown that in adults with ASD, their rate of voluntary exercise is lower than those of age-matched neuro-typical participants [117]. By extension, exercise has been demonstrated to have positive effects in children with ASD [118]. The DMH and the endogenous opioid circuitry is an understudied area. Based on the results of the non-VPA animals there are opioid receptors present on the DMH but in the VPA animals the NTX did not induce enough of an effect to change c-Fos expression.

The NAc – core, which is heavily connected to the BLA, was shown in both non-VPA and VPA animals to not be affected by NTX administration by c-Fos expression. One explanation for this is that, like the BLA, its task in context to the reward system is to help respond to environmental cues and initiate the modulation of behaviour towards the rewarding stimuli [119]. The two-way ANOVA showed very minimal drug

drink iteration in the non-VPAs with a P value of 0.4404, which was surprisingly much higher compared to the VPA animals' P value of 0.1933. Although neither of these results meet the threshold for statistical significance it is unexpected. In contrast to the NAc – core, in the NAc – shell, in non-VPA animals there was a statistical difference observed in the c-Fos expressions. Studies have shown that lesioning of the NAc – shell does not affect the recognition of these cues and behaviours. The NAc – shell has many connections to orexin neurons in the LHA. This is how the NAc – shell drives consumption. Injection of MOR agonist DAMGO into the shell has been shown to stimulate the consumption of palatable foods. In fact, not only does the NAc – shell increase consumption, but it also enhances taste reactivity, which was seen by direct microinjections of MORs into rat brains [120]. Deep brain stimulation (DBS) of the NAc – shell has been shown to ameliorate binge eating in animal models when conditioned to do so with HFHS good access for a restricted period [55]. Alongside this, DBS in HFHS diet-induced obesity rat models has been demonstrated to decrease consumption and subsequently body weight [121]. The main mechanism behind this is thought to be lowering the amount of dopamine in the NAc – shell which is caused by the overstimulation in HFHS diet models.

A limitation of this project was that the VTA was not measured immunohistochemically which would have yielded valuable insight into the full overview of the reward system. In conjunction with this, α-MSH antibodies were going to be used to try to illuminate how NTX directly affected the melanocortin system. The stain was tried twice but both times it failed to stain correctly. This could have given useful information about related nuclei such as the PVN, SON, and DMH.

5.0 Conclusion

The findings in this project show that NTX does not affect energy-driven consumption after deprivation. in both non-VPA and VPA animals, it does decrease palatability-driven consumption. The dose required to elicit the same decrease in consumption of both HFHS chow and sucrose solution in VPA animals was 3.3x greater. The c-Fos immunoreactivity analysis revealed that at a minimum effective dose (1 mg/kg) in non-VPA animals the SON, PVN, ARC, DMH, CeA, and NAc – shell had altered neuronal activity. This was not seen in VPA animals with only changes seen in the CeA. These results suggest that in the VPA model of ASD, the endogenous opioid system is dysregulated. This dysregulation appears to be one of the underlying causes behind aberrant feeding reward processing in this model.

6.0 References

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