Characterisation of Food Intake and Expression of Feeding-Related Genes in The VPA Rat Model of Autism Spectrum Disorder

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting roughly 1% of the global population. Aberrant food selectivity (AFS) is a common comorbid symptom of ASD which can result in nutritional deficiencies, increased parental stress and reduced quality of life. However, alarmingly little research has been conducted investigating the nature and the underlying neurophysiological mechanisms of AFS in ASD. This study attempts to determine whether the VPA rat model of ASD exhibits AFS when presented with various diet types. These include standard chow and water, palatable sweet tastants (sucrose, saccharin and complex liquid diet) and finally palatable high fat milks. The mRNA expression levels of oxytocin, oxytocin receptor, dynorphin and *kappa*-opioid receptor were then determined. These genes have previously demonstrated to be involved in both feeding and social behaviours. The VPA rats were found to consume less standard chow and water, yet increased intake of the sweet tastants was observed. Additionally, in the VPA rats' oxytocin expression in the hypothalamus was increased, as was dynorphin expression in the hypothalamus and brainstem. Increased expression of the anorexigenic oxytocin may have resulted in the decreased intake of chow and water, and could potentially be a result of increased leptin or melanocortin levels. However, increased dynorphin expression may be responsible for the increased intake of the palatable sweet tastants, via inhibition of proopiomelanocortin or neuropeptide S. The development of effective treatments for AFS in ASD requires an understanding of the underlying neurological mechanisms. This research provides the first evidence of AFS and elevated oxytocin and dynorphin expression in the VPA rat model of ASD, thus paving the way for further research in this area.

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List of Abbreviations

AFS	Aberrant food selectivity
ASD	Autism spectrum disorder
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
Ct	Threshold cycle
DA	Dopamine
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
DYN	Dynorphin
GAPDH	Glyceraldeyde-3-phosphate dehydrogenase
gDNA	Genomic DNA
GI	Gastrointestinal
HT	Hypothalamus
IP	Intraperitoneal
KOR	*Kappa*-opioid receptor
LiCl	Lithium chloride
MC	Melanocortin
MID	Monetary incentive delay
NAc	Nucleus accumbens

NPS	Neuropeptide S
NTS	Nucleus of the solitary tract
OT	Oxytocin
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
qPCR	Quantitative PCR
RNA	Ribonucleic acid
TD	Typically developing
Tm	Melting temperature
ToM	Theory of mind
VTA	Ventral tegmental area
VPA	Valproic acid
WCC	Weak central coherence

1. Introduction

1.1. Autism Spectrum Disorder

1.1.1. Definition and Prevalence

Autism spectrum disorder (ASD) consists of a range of neurodevelopmental disorders, and is estimated to affect close to 1% of the global population[1]. ASD was first described by Kanner[2] in 1943 as a novel syndrome called infantile autism. Over the decades, the name of the syndrome has evolved and the defining characteristics refined and broadened, as it has become apparent that ASD is a heterogeneous disorder with a wide range of comorbid symptoms. Thus, today the syndrome is recognised as a spectrum and now includes a range of previously distinct syndromes including Asperger's disorder and pervasive developmental disorder not otherwise specified. The core symptoms of ASD, as defined in the Diagnostic and Statistical Manual of Mental Disorders 5[3], now consists of defects in social interactions and communication, along with a restricted and repetitive pattern of behaviours and interests. Some common comorbid symptoms are intellectual impairment, language impairment, motor defects, self-injury, depression, anxiety, disruptive behaviour, sensory processing dysfunction, seizures, auditory disorders, gastrointestinal (GI) problems and eating disorders[1,4,5]. Roughly 70% of individuals with ASD also have at least one accompanying comorbid mental disorder[1].

1.1.2. Major Theories of Core ASD Defects

Although the underlying cause of ASD is not yet known, there are several prominent theories, such as theory of mind (ToM) defect, executive dysfunction and weak central coherence (WCC). Each of these theories hypothesise a different core impairment of ASD.

The ToM theory, first put forward in 1985[6], suggests that those with ASD suffer from mind-blindness – or the inability to attribute mental states to others and to understand that others have opinions, beliefs and desires different to their own. Although a popular theory that would explain the social aspects of ASD, it falls short on a few accounts. For example, it fails to explain various defects that occur in ASD individuals before the age ToM typically develops. Additionally, it does not account for some of the non-social symptoms of ASD and ASD performance in ToM tests give variable results[7]. Core non-social symptoms of ASD include restricted and repetitive pattern of behaviours and interests. A decade later, in 1995, the executive dysfunction theory[8] was proposed, followed by the WCC theory[9] in 1996. Executive function is an umbrella term for a range of cognitive functions that allow an individual to maintain problem solving skills for the attainment of future goals[10]. This can include planning, mental flexibility, self-control, impulsivity, working memory, shifting of attention and organised speech. An executive dysfunction would explain many of the non-social aspects of ASD. However, some ASD children have performed particularly well on executive function tasks, which suggests that executive dysfunction, although common in ASD, may not be a core defect of the disorder[11].

The WCC theory suggests that individuals with ASD focus on details and fail to integrate information to see the "big picture". Unlike the aforementioned ToM hypothesis, this theory better explains both the social and non-social aspects of ASD. Rather than suggesting a defect as the other two hypotheses, it states that those with ASD are simply more biased to focus on details rather than the whole. However, results from tests of executive dysfunction in ASD individuals have shown variability similar to that of ToM[7]. Also, although some ASD individuals show greater performance on detail oriented tasks, for example hyperlexia (an enhanced reading

ability coupled with a below-average comprehension of spoken language), this is relatively rare in ASD[12]. Because of this, it has been suggested that WCC is simply a part of ASD but cannot account for all cases and all symptoms[11,13].

As many of these theories do not explain all the symptoms of ASD, and because patients often produce mixed results in tests of these theories, it is likely that ASD may not have a consistent core defect and that ASD patients may have a combination of the defects mentioned.

1.1.3. Social Motivation Hypothesis of Autism Spectrum Disorder

First proposed by Dawson in 2002[14], the social motivation hypothesis has become a strong candidate for the underlying reason for impairments in social attention seen in ASD patients[15-24]. The social motivation hypothesis proposes that a lack of interest in social stimuli from infancy leads to a cascade of disruptions in the reward system of the developing brain. This may lead to impairments later in life such as a lack of social response, face and voice processing, social interactions and social learning. However, to date, there has been relatively little research conducted investigating the neural reward system in ASD, and those that have been conducted predominately consist of fMRI studies on human ASD individuals. More research must be conducted before this hypothesis can be referred to as a major theory of ASD.

Ten[22,25-33] studies investigating the neural response to reward in ASD using fMRI have been conducted, many of which suggest aberrant activation of the nucleus accumbens (NAc), a major component of the reward system. Schmitz *et al.* (2008)[30] conducted the first fMRI study investigating reward system functioning in the ASD brain, using a sustained attention task with monetary reward. Unexpectedly, the results showed hyperactivation in the anterior cingulate cortex of the ASD group during reward achievement. The anterior cingulate cortex modulates emotional responses and motivation[34].

Many studies expanding on this research have used modified versions of the well-established monetary incentive delay (MID) paradigm. Classically, this task monitors reactions to a stimulus presented after an incentive cue to win a reward. Delmonte *et al.* (2012)[25] designed a modified MID task which assessed both social and monetary reward. Results showed no significant difference in response of the typically developing (TD) control group when comparing reward types, however in the ASD group hypoactivation of the dorsal striatum was observed in social reward achievement only. The dorsal striatum is thought to be involved in executive function and reward related circuitry[35].

In 2012 Dichter *et al.* conducted two studies also using modified MID tasks. The first[27] compared responses to either monetary reward or reward in the form of an image of an object previously shown to be salient to the participant (e.g. trains). The second study[26] mimicked the first but replaced object images with social reward. Regarding monetary reward, both studies found hypoactivation of the NAc in the ASD group during reward anticipation, however the initial study also described NAc hypoactivation during monetary reward achievement**.** Additionally, the latter study also found hyperactivation in the frontal lobe during monetary reward achievement. The frontal lobe plays a major role in executive function. Interestingly, no significant differences between ASD and TD controls were seen in the reward circuitry activation in regard to object or social incentives.

Another modified MID was designed by Damiano *et al.* (2015)[32] to assess neural response to social punishment (image of a sad face) and monetary loss. Although hypoactivation of the striatum (including the NAc) was seen in anticipation of both types of punishment, more extensive hypoactivation was seen in anticipation of social punishment. Mikita *et al.* (2016)[33] analysed fMRI data from the large-scale Imagen project. In this study's modified MID task the participants were rewarded

with chocolates. The authors found that participants with high ASD symptoms showed hypoactivation of the prefrontal cortex during reward anticipation and when receiving negative feedback.

Other studies using a variety of different fMRI tasks have also been conducted. Scott-Van Zeeland *et al.* (2010)[22] carried out a study investigating neural responses to both monetary and social reward in an implicit learning task. In contrast with the findings of Dichter *et al.* (2012)[26], this study did find significant hypoactivation of the NAc during both social and monetary reward achievement, however this reduction in activity was more pronounced for social reward. Assaf *et al.* (2013)[29] used a social interactive task – a domino game – where the participant was told they were either playing against a computer or human-opponent. In concordance with the results of Scott-Van Zeeland *et al.* (2010)[22], the ASD group demonstrated hypoactivation of the NAc during reward achievement in the human-opponent runs. There were no significant differences between groups in the computer-opponent runs. Kohls *et al.* (2013)[31] assessed the neural response to both social and monetary reward. Interestingly, NAc hypoactivation was seen during monetary but not social reward processing.

Cascio *et al.* (2012)[28] conducted the only study, to our knowledge, which assesses the neural response in ASD to food. In this study, participants fasted for four hours before viewing images of palatable high caloric food. Unexpectedly, the ASD group showed no differences from controls except for a slight hyperactivation of the anterior cingulate cortex, aligning with the results reported by Schmitz *et al.* (2008)[30] in response to monetary reward. However, it is important to note that the images of foods shown were foods that the parents confirmed the child liked. This may explain the increased sense of wanting that was observed, as ASD children with aberrant food selectivity (AFS) are known to have an intense preference for small range of

particular foods, therefore it would be interesting to observe if the results if the children are shown images of a wide range of foods.

Although inconsistent, these results do point to a dysfunction in the reward circuitry of the brain, in accord with the social motivation hypothesis of ASD. The variation in results may be due to the variation in fMRI task type, age or gender of participants, severity of ASD symptoms, or the typically small number of participants. Additionally, low statistical power has been identified as a major issue in fMRI studies[36] and multiple studies have found that a typical fMRI sample size of <20 is not sufficient to produce reliable results[37-40]. It has been recommended that an ideal participant number of at least 27 is required to ensure sufficient reliability[38], a requirement just one of these studies meet.

fMRI is non-invasive, making it ideal for measuring neural activity in human subjects. Unfortunately, as it identifies only blood flow in the brain - a delayed indicator of activity - it cannot give a more specific view of the precise type and location of that neural activity. Additionally, a recent study found that false positives from fMRI data can occur up to 70% of the time when using the most common fMRI analysis software[41]. Some researchers have even branded fMRI the "new phrenology"[42].

To achieve a more accurate depiction of the specific molecular and genetic differences in the reward system of ASD, factors such as expression of reward related genes in these areas should be analysed. Unfortunately, to do this the brain must be dissected therefore animal models of ASD must be used. The combination of data from more invasive techniques, which are more specific but can only be performed in animal models, and fMRI results, which are not as reliable but can be performed on human patients, complement each other to give a more complete and accurate insight into the neural underpinnings of the reward system in ASD.

1.2. The Rewards System

Oxytocin (OT) is a neuropeptide involved in social bonding and social reward[43-45]. Social reward leads to the activation of similar neural reward pathways as non-social primary reward (e.g. food)[46,47]. OT is primarily synthesised in the hypothalamus (HT) with OT neurons projecting to multiple areas in the brain including the NAc and the brainstem[48,49]. OT receptors (OTR) are expressed in high concentrations in the brainstem (pedunculopontine tegmental nucleus, pons and the NTS) and the ventromedial HT[50,51]. Although classically associated with social reward, OT exerts a much wider range of physiological effects, such as the reduction of both homeostatic and hedonic food intake in both human and animal models[52-57].

Regulation of homeostatic food intake is hypothesised to be mediated by OT action in the HT and the brainstem. The HT is situated close to capillaries so can be easily targeted by circulating hormones such as leptin, which can directly stimulate OT neurons[58,59]. Leptin is primarily produced by adipose tissue and released into the bloodstream. Other researchers have hypothesised that stomach distention resulting from food intake and the satiety hormone cholecystokinin activates the NTS which then projects to the HT to stimulate OT release[57,60].

Melanocortins (MC) also have the ability to decrease food intake, promote social behaviour, and stimulate the release of HT OT. It has recently been hypothesised that aberrant MC expression may be an underlying defect of ASD, and atypical OT expression may be an indicator of this[61].

In contrast, the mechanism by which OT regulates hedonic food intake is thought to be via modulation of the reward system, specifically in the NAc and VTA[62].

Opioids are another class of neuropeptides that have been shown to contribute to the rewarding nature of social stimuli[63] and have also recently been implicated in feeding reward. Dynorphin (DYN) is an endogenous opioid peptide with a high

affinity for the *kappa*-opioid receptor (KOR). KOR is widely expressed in the NAc, HT, ventral tegmental area (VTA; a key component of the reward system) and the brainstem (raphe nuclei, substantia nigra, nucleus of the solitary tract (NTS), medulla and parabrachial nucleus)[64].

In contrast, activation of the DYN/KOR system has been demonstrated to have orexigenic effects[65-67]. Although it is evident that the KOR/DYN exerts orexigenic effects[65-67], the mechanism for this is still unclear. One proposed hypothesis suggests that DYN interacts with anorexigenic proopiomelanocortin (POMC) cells. POMC is a precursor to a range to of neuropeptides, including MC, and POMC expressing cells found in the arcuate nucleus of the HT and the NTS. It has been proposed that DYN inhibits activation of POMC cells, therefore preventing POMC mediated satiety[66,68].

Additionally, activation of the DYN/KOR system has resulted in adverse social motivation and decreased prosocial behaviour in both rats and mice, suggesting the social reward value may be diminished by DYN/KOR activity[69-71]. Although the social aversion may appear maladaptive, it plays an important role in mediating social relationships by identifying aversive social interactions. For example, social aversion allows the individual to identify threatening social cues and respond accordingly, and aids in maintaining sexual relationships by encouraging mate guarding (aggression towards sexual rivals)[71,72].

Therefore, it appears that activation of the DYN/KOR system may decrease the reward value of social stimuli while increasing food intake. This is in stark contrast to OT which causes an increase in the reward value of social stimuli and results in a decrease in food intake. These pathways are summarised in Figure 1.

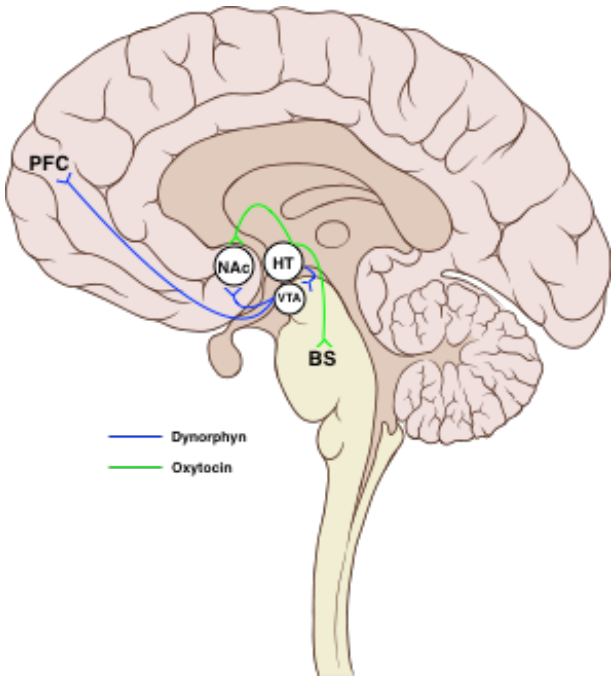

Figure 1. Projections of the neural peptides dynorphin and oxytocin within major reward modulating areas of the brain[49,73,74]. PFC Prefrontal cortex; NAc Nucleus accumbens; HT hypothalamus; Ventral tegmental area; BS brainstem.

1.3. Food Selectivity

1.3.1. Effects and Prevalence

Although a range of ASD associated symptoms can lead to increased parental stress, families with ASD children who exhibit AFS (AFS ASD) have been shown to have increased parental stress and impairment of family life compared to families of ASD children who do not have AFS (NoAFS ASD) and of families with TD children[75-78]. AFS may also lead to nutritional deficiencies, growth retardation and malnutrition. In extreme cases the patient may require invasive procedures such as tube feeding to avoid malnutrition, further increasing stress and reducing quality of life[79,80]. Incidences of scurvy have been reported in multiple AFS ASD children and this is directly caused by lack of vitamin C intake[81]. While this is not a common occurrence

in the AFS ASD population it highlights the fact that serious health issues can arise from severe AFS.

Considering that this is an issue recognised for more than 70 years, along with the heath complications and effects on parental stress AFS ASD has, it is surprising that such little research on the topic has been conducted. A comprehensive meta-analysis by Sharp *et al.* (2013)[79] noted that between 1980 and 2011 just 0.3% of articles published by the *Journal Of Autism And Developmental Disorders* were related to food intake in ASD, and only two articles on the topic were published in any paediatric journals in that same period. These facts in themselves highlight a substantial knowledge gap in the literature. This has resulted in the lack of a standard definition for what qualifies as AFS and therefore estimates of the prevalence of ASD vary greatly, from 46%[82] to 89%[83]. However, the majority of studies investigating prevalence contain major flaws, most notably the lack of control groups. The meta-analysis performed by Sharp *et al.* (2013)[79] included all human AFS ASD studies between 1980 and 2011 that met specific criteria. These criteria included: the presence of a control group, subjects had not been seeking treatment for AFS, candidates were children, and the use of a standardised, replicable evaluation method. This resulted in the exclusion of 661 articles, leaving just seventeen, which combined provided a sample of 881 ASD children. All seventeen studies found that AFS was more prevalent in ASD children and that the overall incidence of AFS in children with ASD was five times that of TD controls. Notably, no significant difference in nutrient intake was seen in fibre, carbohydrates, total fat, vitamins A, C, D or E, iron, zinc or total energy. Additionally, BMI was not significantly different between groups. This contradicts some previous research[84-86], indicating a need for more extensive investigations to reach a definite consensus. There were however, differences seen in calcium and protein intake, with ASD children consuming

considerably less. The long term health effects of ASD patients resulting from nutritional problems has not been well characterised, however there are a small number of recent studies which suggest ASD boys have reduced bone cortical thickness[87] and lower bone density[84] than TD children. Although the relationship between calcium intake and bone heath is well established[88-90], bone abnormalities in ASD have not yet been linked to calcium deficiencies due to AFS. Additionally, some studies have suggested that ASD patients have increased incidences of bone fractures due to these problems, however this has not been confirmed by other research[91,92].

1.3.2. Causes of Food Selectivity

Although the limited research on AFS ASD has not resulted in any widely accepted causes of AFS in ASD, there are several proposed hypothesises. Studies have indicated that GI problems, such as increased intestinal permeability, may play a role. GI symptoms often seen in ASD can include diarrhoea, constipation, nausea, vomiting, bloating or reflux[86,93-95] and AFS ASD children may simply learn to avoid foods that worsen GI symptoms[80]. Some studies take this hypothesis further and suggest that ASD itself is a symptom of abnormally permeable intestines. More specifically, increased intestinal permeability allows peptides formed by casein and gluten digestion to pass into the bloodstream. Some of these peptides such as the β-CM7 are then able to cross the blood brain barrier and exert opioid effects, which can alter behaviour[96-99]. This has been the basis for the GFCF diet, in the hopes that removing these proteins will result in less severe symptoms or even a "cure", yet there have not been any conclusive studies supporting the effectiveness of this diet. In contrast there are a number of studies that have found no link between ASD and GI problems[78,100-102].

Studies that did correlate GI issues with ASD found that GI problems were often not seen in every ASD patient studied. This suggests that even if GI issues contribute

to AFS, there must be other factors influencing AFS. It has been suggested that AFS is simply a manifestation of the core symptoms of ASD. Anxiety, avoidance of novelty, restricted interests, inflexible routines and repetitive behaviours along with sensory processing and motor defects could each contribute to ASD patients having a limited diet. Meal times may simply be stressful to ASD individuals due to the social pressures, the need to use motor skills along with often novel foods which may cause extreme anxiety, therefore reducing appetite[80,103,104].

1.3.3. Food Selectivity and The Reward System

Altered reward processing in ASD patients in response to non-social primary reward, such as food, has received very little attention in the research literature. Food is a very strong stimulator of the reward system, where just images of palatable foods able to stimulate a response[105-108]. Considering that a dysfunctional reward system appears to be common among ASD patients, it could be hypothesised that abnormal reward processing stimulated by food may be a cause of AFS. However, only two studies have investigated the relationship between food the reward system in ASD.

The first study, by Cascio *et al.* (2012)[28], described earlier, found that the food mediated reward system activation is not impaired in ASD children. In fact, the activation was slightly enhanced. The second study by Damiano *et al.* (2014)[109] investigated the sensitivity and hedonic response to the sweet taste of sucrose in ASD and TD adults. They hypothesised that because the hedonic properties of sweet tastes are an indicator of opioid function, and by extension reward processing, ASD individuals would have no change in sensitivity to sweet taste but would have diminished hedonic response and that this would be related to ASD severity. However, as with Cassio's *et al.* (2012)[28] study their hypothesis was incorrect. Both sensitivity and hedonic response were the same as TD controls suggesting the sweet taste mediated activation of the reward system is intact.

These two studies took very different approaches in assessing the relationship between food and the reward system, and have yet to be replicated to confirm results. As these studies only encompass images of palatable foods and sucrose there is also a need to determine reward processing in response to a range of foods such as proteins and non-nutritive sweeteners (non-nutritive sweeteners, such as saccharin, are perceived to taste similar to sucrose yet induce a weaker response from the reward system[110,111]). Other neuronal regions known to be involved in reward processing should be investigated also, such as the HT, NAc and brainstem.

This emphasises the requirement for further studies relating foods to reward processing in ASD, to gain a fuller understanding of the causes of AFS and of ASD itself.

1.4. Animal Models

Research using human subjects is notoriously difficult. With harsh ethics protocols in place, expensive and time consuming recruitment processes, and a limited ability to control the subject's environment it is common practice for researchers to conduct preliminary studies using animal models. There are a range of animal models for ASD, predominantly consisting of rodents that have ASD induced by genetic or environmental manipulations. Several genes have been identified as ASD risk factors in humans, but the genetics contributing to ASD is revealing itself to be complex and the interaction between genetics and the environment is not fully understood. Although it is generally accepted that genetics plays a role in ASD, the importance of this role is up for debate[112]. It is likely that genetics predisposes an individual to ASD, but the environment during development also plays a significant part. Therefore, although genetically modified rodents have been used to produce consistent models

of ASD[113-116] treatment of rodents with teratogens has been shown to be a cheaper, well characterised and arguably better alternative.

In humans multiple teratogens have been proven to increase the incidence of ASD in patients who were prenatally exposed, most notably thalidomide[117], and the antiepileptic drug valproic acid (VPA)[118]. Research in rats however, revealed that although thalidomide induces ASD in primates, the same effect is not seen in rodents. In contrast, just a single VPA exposure in utero is able to induce ASD like symptoms in rodents[116]. Rigorous testing of the VPA rodent model has shown that it exhibits the core symptoms of ASD[119-127], along with many comorbid symptoms such as altered sensory processing, hearing loss, delayed development[121], disrupted sleeping patterns[128], learning difficulties[129], and anxiety[130]. Unfortunately, no studies have been conducted to determine if AFS is present in this ASD model.

Expression of genes associated with ASD in humans have been shown to be reduced in the VPA rodent model[131], and VPA rodents show similar neural biomarkers as ASD humans[132]. It was through these studies that the importance of the VPA dose and time of VPA administration was found. Due to the low bioavailability of VPA in rodents compared to humans[133], a comparatively high dose of 500mg/kg or more to the pregnant dam is often recommended[127] compared to 3–55 mg/kg which is often prescribed to human patients. In rodents, a single exposure to VPA in utero is sufficient in inducing ASD in the pups, however chronic administration seems to be necessary to induce these effects in the human children[127].

The timing of the VPA administration is essential. Embryonic day 12.5 has been identified as the optimal time for VPA administration[121,134]. At this stage of foetal development neural tube closure occurs and motor nuclei of the trigeminal, hypoglossal, and abducens nerves in the brainstem form[135-137]. If the drug is administered after neural tube closure and the formation of these neurons the VPA

will not induce the same effects. Many additional neural dysfunctions similar to that of ASD humans can be seen. These include a reduced number of cerebral Purkinje cells and a smaller interposed nucleus within the cerebellum[134,137-140].

Although both the rat and mouse can be used as ASD models, the rats increased intelligence and more anatomically similar CNS makes it a more accurate model. Additionally, its larger brain makes neuroendocrine examination much easier[141].

1.5. Specific Aims

In this study, we aimed to determine whether the VPA rat model of ASD display aberrant feeding behaviour and to elucidate the molecular changes in the brain that might contribute to abnormalities in eating behaviour. To accomplish this, it was first determined if VPA rats consume different amounts of standard laboratory chow and water. Secondly, it was determined if VPA rats exhibited an increased drive to consume palatable sweet or fatty diets. Finally, the expression of feeding-related genes that are also involved in shaping social behaviours was analysed to determine if expression differed in VPA rats.

2. Materials and Methods

2.1. Animals

Rats were housed in plastic cages with wood chip bedding material at 22 °C under a 12-hr light/12-hr dark cycle with lights on at 07.00 h. Unless stated otherwise, tap water and standard laboratory chow (Sharpes Feed) were available *ad lib.* All procedures described received prior approval from the University of Waikato ethics committee.

2.2. Breeding

Adult female Sprague-Dawley rats were mated in-house, with vaginal smears analysed each morning. The presence of spermatozoa in the vaginal smear was used to determine pregnancy at embryonic day one (E1). Sodium valproate was dissolved in saline to a concentration of 50mg/ml and at E12.5 the females received a single intraparietal (IP) injection of 500mg/kg of the valproate solution. Controls received an equivalent volume of physiological saline. The females were allowed to raise their pups until one month postpartum when the pups were weaned and separated by gender. At ~2 months postpartum males were single housed. Weight was measured regularly. Physical and behavioural abnormalities were used to determine if the offspring were affected by the VPA. In total five VPA treated males and eight control males were used.

2.3. Malformations

The offspring were examined for any physical malformations or conditions, such as tail kinks, previously noted to be common in VPA rats[138].

2.4. Behavioural Analysis

2.4.1. Behavioural Tests

A modified version of the behavioural test "Social behaviour in adulthood" described previously by Schneider & Przewlocki (2005)[121] was performed to confirm that social defects typical of this ASD model were present. In short, a control and VPA treated rat were placed in an arena 44 x 44 cm size for nine minutes. A recording was made, and all social interactions occurring in that period were analysed. Social interactions were considered to be any of the following: sniffing, licking, crawling on, mounting, approaching or following the conspecific. Anogenital inspections were also counted separately.

2.4.2. Statistical Analysis

Data were analysed using Student's *t*-test for independent samples to determine statistical significance. In the case of suspected outliers, Grubbs test was used.

2.5. Food Intake Measurements

2.5.1. Ad Lib. Chow and Water

Chow and water intake was recorded over 48 hours.

2.5.2. Deprivation Induced Chow Intake

Chow intake was then recorded for 24 hours after overnight deprivation.

2.5.3. Sucrose and Saccharin Intake

Water was removed and intake of a 10% sucrose solution was recorded over four hours to expose the rats to the novel diet. This was repeated the following day.

The procedure was then repeated with a 0.1% saccharin solution.

10% sucrose and 0.1% saccharin solutions were given to the rats simultaneously for two hours to expose the rats to the novel combination, and this was repeated the following day.

2.5.4. Complex Liquid Diet Intake

As with the sucrose and saccharin, a complex liquid diet (Protein Fx 100% Whey Chocolate Powder made to packet instructions – 30g added to 200ml tap water) was given to the animals for four hours to expose the rats to the novel diet. This was repeated the following day.

2.5.5. Cow and Goat's Milk

Cow's milk was made according to packet instructions (100g milk powder added to 900ml of tap water). Nutrient composition of the goat and cow's milk is presented in Table 1. Rats were exposed to the milk for 30 minutes and then intake was recorded for two hours the following day.

This process was repeated for goat's milk – also made according to packet instructions (100g milk powder added to 900ml of tap water).

2.5.6. Statistical Analysis

All data were analysed using Student's *t*-test for independent samples to determine statistical significance. In the case of suspected outliers, Grubbs test was used.

Table 1. Nutrient composition of cow and goat's milk.

Fat g/100ml	**Protein** g/100ml	**Lactose** g/100ml	**Ash** g/100ml	**P/E** %	**F/E** %	**C/E** %
0.11	3.04	4.04	0.28	42%	1%	55%

2.6. Quantitative PCR Analysis

2.6.1. Euthanasia and Dissections

All animals were euthanised by decapitation and the brain was rapidly dissected to remove the NAc, HT and brain stem. These were stored at −80°C until analysis.

2.6.2. Primer Design

Primers were needed for two housekeeping genes, glyceraldeyde-3-phosphate dehydrogenase (GAPDH) and beta-tubulin (BTUB), and four genes of interest, OT, OTR, DYN and KOR. Using the online software Primer3 (http://primer3.sourceforge.net/) forward and reverse primers were carefully designed to ensure the best possible amplification of the targeted genes using quantitative PCR (qPCR). The following guidelines were adhered to where possible to optimise primer efficiency and accuracy: the primers had a GC content between 40-60% to ensure stability; an amplicon length of 50 to 150 bases was used to improve amplification efficiency; primers ended with either a C or G residue, to ensure more specific DNA binding; a melting temperature (Tm) of between 54–60°C, with the Tm of the forward and reverse primers being within 1°C of each other; the number of possible nucleotide interactions within and between primers was kept to a minimum to prevent the formation of hair pin loops and primer dimers; to help ensure that the correct target was being amplified, BLAST (Basic Local Alignment Search Tool) was used to compare both the primer and the amplicon sequences against the public database and transcriptome.

2.6.3. RNA Isolation

To homogenise the tissue, each brainstem, NAc and HT sample was added to a 2ml polypropylene RNase/DNase free microcentrifuge tube containing 600µl RNA Lysis Buffer and approximately 50ul of 0.1mm and 0.5mm diameter glass beads. The

tubes were placed into an Alphatech Mini-beadbeater™ and the tissues homogenised at 4800 oscillations per minute for 10 second periods until the tissue was completely broken down. The samples were centrifuged at 13,000xg for one minute to remove foam caused by the homogenisation process.

A Quick-RNA™ MiniPrep kit (Zymo) was then used to isolate total RNA. The supernatant was transferred into a Spin-Away™ Filter (yellow) which was then placed into a collection tube and centrifuged again at ≥10,000xg for one minute. The filter, containing majority of gDNA (genomic DNA), was discarded. One volume of 95% ethanol was added (1:1) to the flow through and transferred to a Zymo-Spin™ IIICG Column (green) in a collection tube, centrifuged at 13,000xg for 30 seconds and the flow through was discarded. To remove any trace gDNA from the column, DNase I Treatment was conducted. To prewash the column, 400µl RNA wash buffer was added and was centrifuged at 13,000xg for 30 seconds and the flow-through was discarded. To prepare a DNase I Reaction Mix, 5µl DNase I DNA and 75µl Digestion Buffer were mixed in a 2ml polypropylene RNase/DNase free microcentrifuge tube. This was then added directly to the column matrix and incubated at room temperature (20-30 ºC) for 15 minutes.

After centrifugation at 13,000xg for 30 seconds, 400µl RNA Prep Buffer was added to the column, before being centrifuged again at 13,000xg for 30 seconds. The flow through was discarded and 700µl RNA Wash Buffer was added to the column, centrifuged at 13,000xg for 30 seconds and the flow-through was again discarded. Another 400µl RNA Wash Buffer was added, and was again centrifuged at 13,000xg but for two minutes. 30µl of DNase/RNase-free water was then added directly to the column matrix and centrifuged 13,000xg for 30 seconds to elute the RNA.

To assess the purity of the RNA, a Nanodrop 2000 (Thermo Scientific) was used to measure the 260/280 absorbance ratio. To minimise degradation, the RNA was immediately used for cDNA synthesis. The samples were then stored at 4°C.

2.6.4. cDNA Synthesis

To synthesise cDNA from the extracted RNA, the HiSenScript™ RH(-) cDNA Synthesis Kit (iNtRON) was used. For each sample, 1µg of total RNA, 10µl of 2X RT Reaction Solution and 1µl of Enzyme Mix Solution were added to a 0.2ml DNase/RNase free PCR tube and made up to a total volume of 20µl with DNase/RNase Free Water. The tubes were vortexed before being spun-down with a centrifuge. The tubes were incubated at 42°C for one hour and then 85°C for five min in a Biorad T100 thermal cycler and then stored at 4°C.

A sample was produced by pooling cDNA from various samples and diluted into 300 µl of DNase/RNase-free water and stored at 4°C until they were subsequently used for measuring the genes expression and primer efficiency in qPCR.

2.6.5. Primer Testing and Efficiencies

Before any qPCR analysis could be performed, the amplification efficiency of the primer pairs must be determined. The pooled cDNA sample underwent a 4-fold serial dilution to give five samples at undiluted, 1:3, 1:6, 1:9 and 1:12, which will be used as the template cDNA in qPCR reactions. A master mix was made containing 0.008µl SYTO (fluorescent DNA dye), 2µl 10x Buffer, 2µl 25mM $MgCl_2$, 0.4µl 10mM DNTP and 9.3µl DNase/RNase-free water, 0.6µl each of the forward and reverse primer and 0.1µl Taq. 15µl of this master mix and 5µl of the template cDNA were added to a 0.2ml RNase/DNase free, thin walled, clear PCR tube (Axygen). Negative controls were made with 15µl and 5µl of DNase/RNase-free. All samples were run in duplicate.

The 20µl mixtures were subjected to the following qPCR programme: Hold at 95°C for 15 minutes; followed by 45 cycles of 95°C for 15 seconds, 56°C for 15 seconds and 72°C for 30 seconds. Fluorescent output was recorded at 80°C and a melt curve for each sample was performed between 60°C and 99°C. A threshold line was set onto the resulting graphs to produce a threshold cycle (Ct) value for each sample. These Ct values were plotted versus the initial amounts of input material on a semi-log10 plot where a line of best fit was applied. From here, the slope of the line and R2 value were calculated, to determine the goodness of fit of the points. Using the equation $E=10^{(-1/s)}-1$, the primer efficiencies were calculated – where E is the primer efficiency and s is the slope of the line.

To confirm just a single product had been amplified, 2% agarose gel electrophoresis was used and the products were visualized by ethidium bromide staining.

2.6.6. Measuring Gene Expression

Using the same master mix and qPCR protocol described in *2.6.5.,* six qPCR analyses were carried out on every sample – one for each of the two housekeeping and four genes of interest. Using the Ct values obtained, Expression Software Tool[142] with the delta delta CT method[143] was used to calculate actual Ct values based on primer efficiencies, and normalise the expression of the genes of interest to the two housekeeping genes. Results were expressed as the fold change in the expression level between ASD and control rats. The expression ratios obtained for the four genes of interest were tested for significance by a Pair Wise Fixed Reallocation Randomisation Test© and were subsequently plotted using standard error estimation using a complex Taylor algorithm. Using the Grubbs' test, outliers were excluded at $p<0.05$.

3. Results

3.1. Weight

No differences were seen between ASD and control groups in the average weights of the rats (Figure 2).

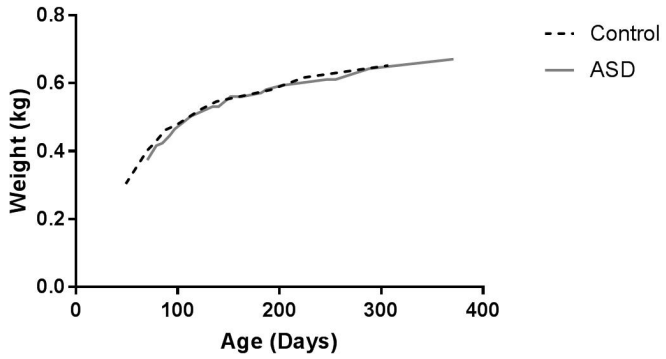

Figure 2. Average weight of ASD and wildtype control rats.

3.2. Malformations

Overall, both the control and ASD groups exhibited good health. However, all five ASD males had some kind of malformation (Figure 3), while no malformations were seen in the control groups. Three of the five ASD males had prominent tail kinks. One had an abnormally short and thick tail. The final ASD male exhibited deficits in one eye, which included a milky discolouration and red crusts forming around it. During brain dissections, it was found that this eye had no optic tract leading to it, therefore the rat was most likely blind in the eye.

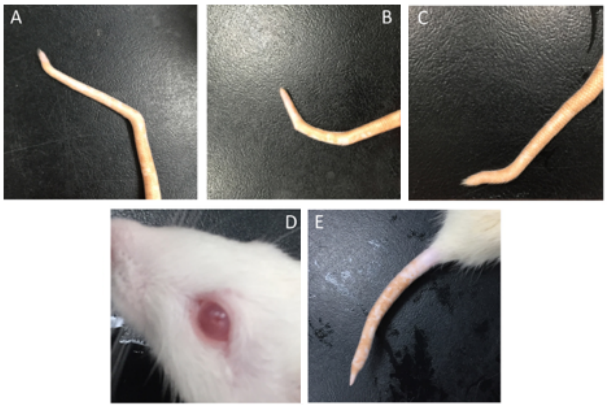

Figure 3. Malformations in VPA rats. Tail kinks are present in three rats (A-C), discolouration was seen in the left eye of one rat (D), and abnormally short and thick tail was seen in one rat (E).

3.3. Behaviour

Statistically significant differences between groups were found for the total number of social interactions, including anogenital investigations ($p=0.0419$) and the latency to anogenital inspections only ($p=0.0348$). There were no significant differences between latency to social interactions including anogenital investigations ($p=0.2154$) or the total number of anogenital inspections only ($p=0.1772$).

3.4. Chow Intake Measurements

3.4.1. Ad Lib. Chow and Water

Significant differences were seen in both chow and water intake, with the ASD group consuming 30% less chow ($p<0.0001$) and 42% less water ($p= 0.0059$) over the 48-hour period compared to the wildtype controls (Figure 4).

3.4.2. Deprivation Induced Chow Intake

During deprivation-induced chow intake, the ASD group consumed 31% less chow than wildtype controls ($p<0.0001$; Figure 5).

3.4.3. Complex Liquid Diet Intake

The ASD group consumed 99% more of the complex liquid diet than wildtype controls ($p=0.0142$; Figure 6).

3.4.4. Sucrose and Saccharin Intake

On average, the ASD group consumed 94% more sucrose solution ($p<0.0001$) and 308% more saccharin solution ($p<0.0001$) compared to wildtype controls when presented separately (Figure 7).

When presented simultaneously, no differences were seen between groups in the consumption of sucrose ($p=0.0845$), saccharin ($p=0.9698$) or total intake of both sucrose or saccharin ($p=0.1236$; Figure 8). However, both groups significantly preferred sucrose over saccharin, with the ASD group consuming 780% more sucrose ($p<0.0001$) and the wildtype controls consuming 971% more ($p<0.0001$).

3.4.5. Cow and Goat's Milk

There were no significant differences between groups for either cow or goat's milk intake when they were presented separately ($p=0.5453$ and 0.5976, respectively; Figure 9A). When both milks were given simultaneously, there was no difference in consumption of cow's milk, goat's milk or total intake of both milks ($p=0.378$, 0.3301 and 0.1745, respectively; Figure 9B).

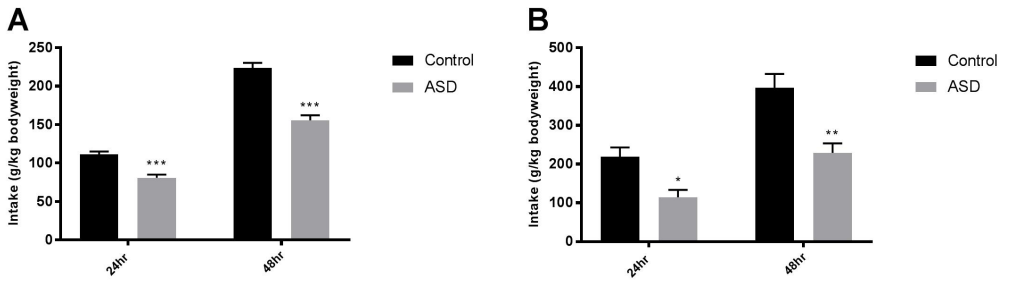

Figure 4. (A) Consumption of *ad lib.* chow. The ASD group consumed 30% less chow compared to controls over 48 hours. (B) Consumption of *ad lib.* water. The ASD group consumed 30% less water than controls over 48 hours. ***P <0.001; **P <0.01; *P<0.05.

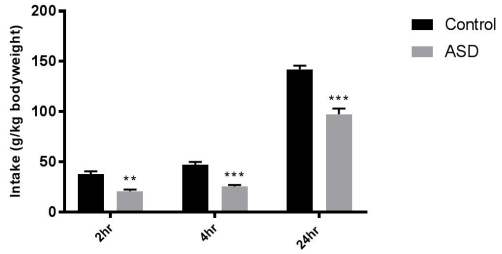

Figure 5. Consumption of chow over 24 hours after overnight deprivation. The ASD males consumed 31% less chow over 24 hours compared to controls. ***P <0.001; **P<0.01.

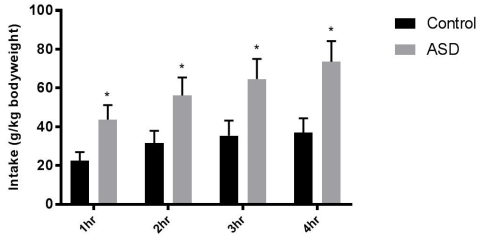

Figure 6. Consumption of complex liquid diet. The ASD group consumed 99% more of the complex liquid diet than controls over four hours. *P <0.05.

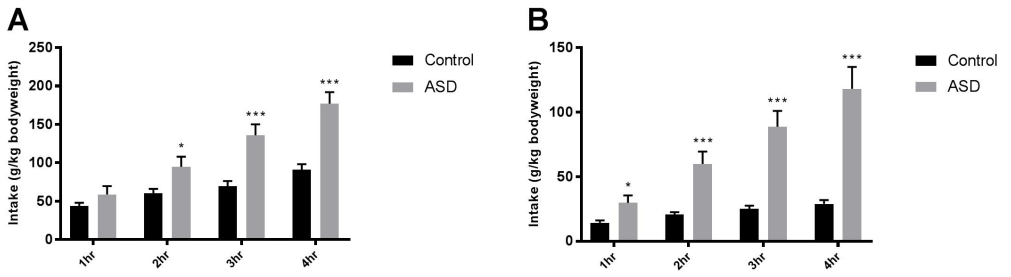

Figure 7. Consumption of 10% sucrose and 0.1% saccharin when presented individually. (A) Consumption of sucrose. The ASD group consumed 94% more sucrose than controls over four hours. (B) Consumption of saccharin. The ASD group consumed 308% more saccharin than controls over four hours. ***P <0.001; *P<0.05.

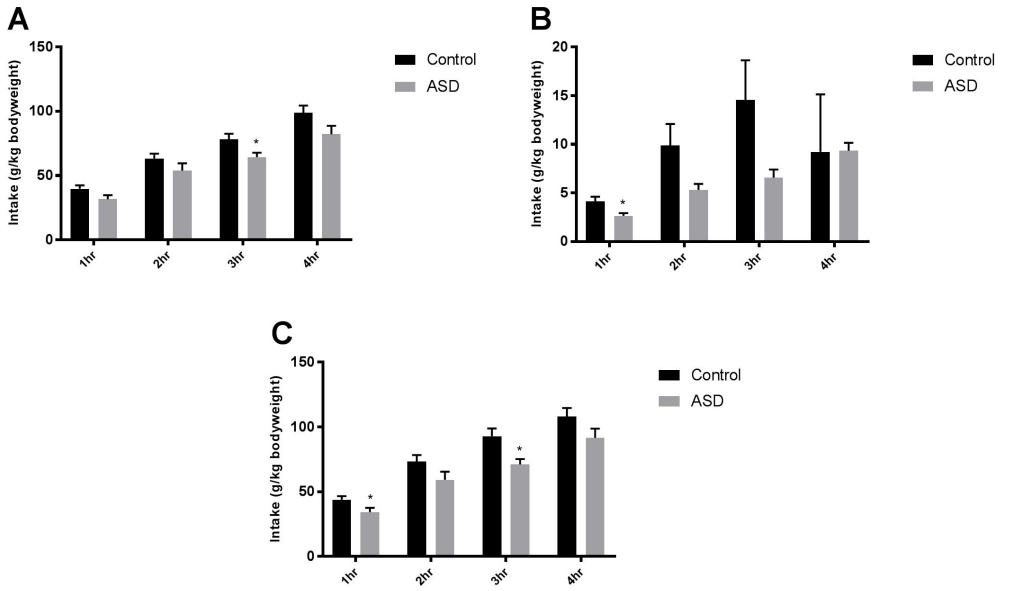

Figure 8. Consumption of 10% sucrose and 0.1% saccharin when presented simultaneously in a two-bottle choice test. (A) Consumption of sucrose. Control rats consumed significantly more sucrose at three hours only. (B) Consumption of saccharin. Control rats consumed significantly more saccharin at one hour only. (C) Total intake of both sucrose and saccharin. Control rats consumed significantly more at one and three hours only. *P<0.05.

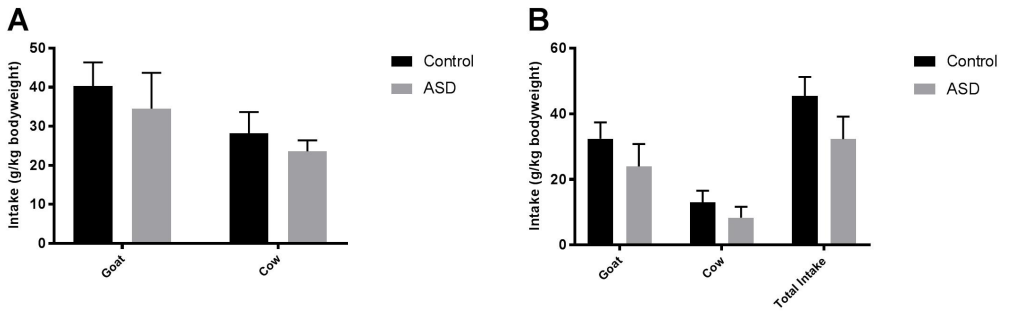

Figure 9. (A): Consumption of goat or cow's milk over two hours when presented separately. No significant differences are observed between groups for either milk type. (B): Consumption of goat or cow's milk over two hours when presented simultaneously in a two- bottle choice test. No significant differences in intake are observed between groups for goat's milk, cow's milk or total intake of both milks.

3.5. Brain Analysis

Expression in the brainstem did not differ in terms of OT, OTR and KOR mRNA ($p=0.7924$ 0.1940 and 0.8377, respectively). However, DYN mRNA expression was 400% higher in the ASD group, on average, then wildtype controls ($p=0.0093$; Figure 10A).

In the HT, an outlier was identified in the DYN data of the ASD, and was subsequently removed from further analysis. The ASD group exhibited, on average, 101% higher expression of OT mRNA than controls ($p=0.0183$) and 349% higher expression of DYN. No significant differences were seen in OTR or KOR mRNA expression in this region ($p=0.8308$, 0.0997 and 0.0770, respectively; Figure 10B).

The expression of OT, OTR, DYN and KOR mRNA did not differ significantly in the NAc ($p=0.3805$, 0.0979, 0.2420 and 0.6079, respectively; Figure 10C).

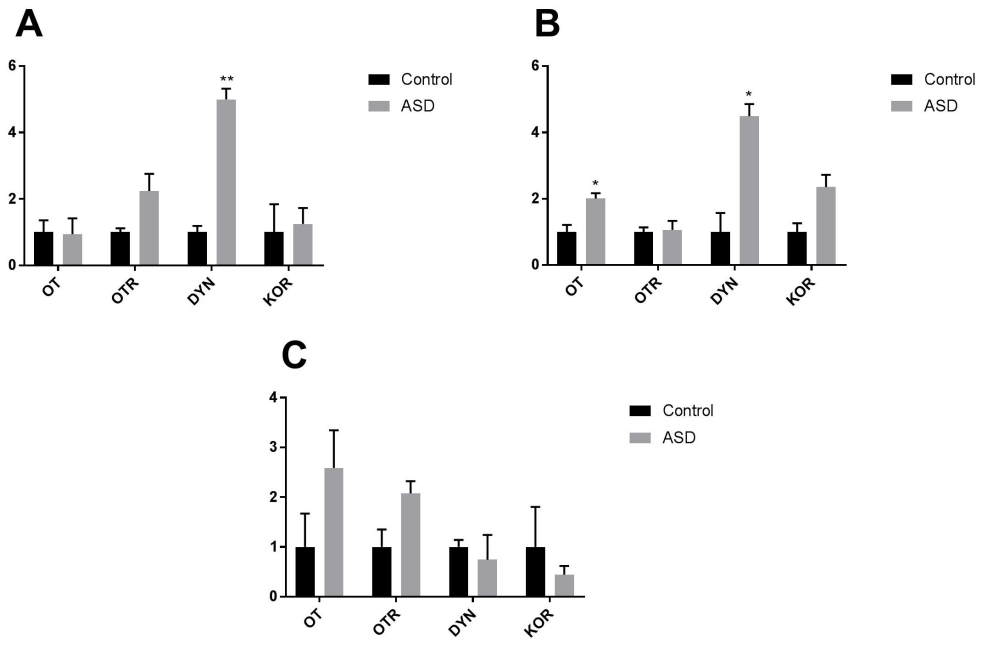

Figure 10. Oxytocin (OT), oxytocin receptor (OTR), dynorphin (DYN) and kappa opioid receptor (KOR) mRNA expression levels. Expression levels are expressed as the fold change between ASD and control rats. (A) Expression in the brainstem. DYN expression was 400% higher in VPA rats compared to controls. No other significant differences are seen. (B) Expression in the hypothalamus. OT expression was 101% higher in VPA rats than wildtype controls. DYN expression was 349% higher in VPA rats than controls. No other significant differences are seen. (C) Expression in the Nucleus accumbens. No significant differences are seen. *P<0.05; **P<0.01.

4. Discussion

4.1. General Discussion

It is obvious that there is a substantial gap in the research literature regarding the nature and underlying neurophysiological mechanisms of AFS in ASD. This study is the first of its kind, elucidating the neural hormonal signalling that may contribute to AFS and characterising food preferences in the VPA rat model of ASD. It is exceedingly evident from these results that this animal model of ASD exhibits aberrant food intake patterns for a range of food types. ASD research over recent years have revealed AFS to be a prevalent and detrimental comorbid symptom of ASD. However, surprisingly little research has been conducted on the nature and neurophysiological mechanisms of this phenomenon. The social motivation hypothesis proposes that a dysfunction in the processing of social reward may be responsible for the social symptoms of ASD. If there is in fact a dysfunctional reward system in the ASD brain, this may affect other reward mediated behaviours, such as food intake. The data produced from this research provides, to our knowledge, the first evidence that AFS and abnormal expression of OT and DYN are seen in the VPA rat model of ASD, and this may provide evidence that a dysfunctional reward system contributes to social abnormalities in ASD and the comorbid symptom AFS.

The presence of physical malformations and abnormal social behaviour of VPA rats confirmed that the VPA treatment in utero had been effective. We then found that these VPA rats indeed exhibited abnormal food intake patterns. VPA rats exhibited anorexigenic behaviour when presented with chow and water. On average, VPA rats consumed 42% less water than controls, 30% less chow and 31% less chow after a previous night's deprivation. However, when it came to highly palatable diets the

ASD group consumed considerably more than controls. Specifically, VPA rats consumed 94% more sucrose, 308% more saccharin and 99% more complex liquid diet, on average, compared with controls. There was however no difference in intake between groups when given a two-bottle choice between sucrose and saccharin. Additionally, there was no difference in goat or cow's milk intake when presented separately or in a two-bottle choice test.

Analysis of mRNA levels of OT and DYN along with their respective receptors, OTR and KOR, suggested differential response of reward system in the VPA rats. Although there were no significant differences seen in the expression of either receptor, OT in the ASD HT was expressed at twice the levels seen in wildtype controls. Additionally, DYN expression in the brainstem was 400% higher compared to controls, and 349% higher in the HT. No differences in expression levels were seen in the NAc.

The elevated levels of the anorexigenic hormone OT in the HT of VPA rats may be responsible for the reduced intake of chow and water in this group. The presence of elevated OT expression in the HT but not the NAc, a central component of the reward system, suggests that OT is not suppressing hedonic mediated reward any more than in controls. Satiety signals induced via gastric motility is thought to reach the HT via the NTS. However, as no differences in brainstem OT were seen this is unlikely to be the case here. Leptin receptors are present on HT OT neurons and due to the close proximity of the HT to capillaries, circulating leptin, a potent satiety hormone, is able to directly activate HT OT neurons[58,144]. Therefore, homeostatic termination of feeding may be mediated by circulating leptins action on the HT, independent of GI input via the brainstem and hedonic NAc signalling. Furthermore, this trend is not seen when palatable sweet tastants are presented and could be due to

the strength of the hedonic response to these palatable foods, which may be explained by the extreme increase in brainstem DYN expression.

Alternatively, an increase in OT expression may suggest increased activation of MC receptors in the HT. Although MC does not directly increase OT levels in the NAc, as was seen in our results, it does potentiate the release of OT in response to social stimuli, which would accelerate and increase the hedonic nature of social reward[61]. Although HT MC expression is associated with general decreased food intake MC expression in the NTS has been demonstrated to specifically reduce palatable food intakes[145]. The fact that elevated OT levels in the VPA rats are seen only in the HT and not the brainstem, aligns with our results where anorexigenic effects were seen in standard chow and water but not the more palatable sweet/fatty tastants.

A proposed mechanism of the orexigenic effects of DYN is by inhibition of the anorexigenic POMC neurons, which are found in the HT and NTS – the two locations where elevated DYN expression was observed in the VPA rats[66]. Intake of palatable foods in rats, including saccharin, has been shown to stimulate POMC expression in these two locations[146,147]. POMC activation may therefore be a mechanism to induce satiety and maintain homeostasis after intake of sweet tastants, which are typically associated with high caloric content.

The fact our data show hypophagia with saccharin, a non-caloric sweet tastant, but not the less palatable caloric chow, supports research suggesting POMC activation is stimulated by hedonic taste-information rather than post-ingestive effects. When hedonic reward influences food intake, satiety is delayed, resulting increased food intake[52].

Additionally, Hill *et al.* (2008)[148] found that in POMC knockout mice there was no difference in the intake of chow compared with controls, suggesting POMC mediated intake of sweet tastants only. Bodyweight was not altered in these

knockouts either, a trend seen in our data supporting the hypothesis that POMC expression is reduced. Conversely, deletion of leptin receptors from POMC cells results in mild obesity but no significant differences in intake of chow[149,150]. Although these studies did not investigate intake of palatable sweet tastants, all three reported no difference in intake of standard chow. This is in concordance with our data that showed no increase in non-sweet diets despite an increase in HT and brainstem DYN expression, and by extension a likely decrease in POMC activation. Although the role of POMC in regulating bodyweight is unclear, our results do imply that PMOC dysregulation does not affect bodyweight.

In the HT, POMC neurons made up just a small proportion of the HT neurons that were activated in response to the non-caloric sweetener sucralose[151]. This suggests that POMC activation in the HT plays just a minor satiety role in response to sweet taste, and there are likely other factors more significantly contributing to this satiety. Increased DYN expression in the HT may further reduce POMC activation, leading to a delay in sweet taste mediated satiety and increased hyperphagia.

The minor effects POMC appears to have in modulating food intake emphasises the role NTS POMC activation may play in the intake of sweet tastants. In support of this, the opioid antagonist naloxone was able to decrease sucrose intake significantly in wildtype mice but these effects were not seen in DYN knockouts, further implicating the role DYN in POMC mediated hedonic food intake[152]. However, this does not explain why no difference between groups was seen when given a two-bottle choice test between sucrose and saccharin.

An alternative explanation for the orexigenic effects of DYN is by inference with neuropeptide S (NPS). NPS has only been identified relatively recently and has been found to reduce food intake and anxiety. When expressed in the HT, food intake of standard diets and palatable sweet tastants is decreased[153-155]. DYN has been found

to directly inhibit NPS expression in the KOR expressing locus coeruleus of the brainstem, and this inhibition may also take place in the HT[156]. Inactivation of NPS neurons contribute to the increased anxiety and aberrant food intake patterns seen in ASD. However, NPS research is still in its infancy and further research will need to be conducted to investigate the locations of NPS expression, its interactions with DYN and its more specific effects on intake of different diet types.

Although there is evidence that *mu*-opioid activation stimulates intake of high fat diets, such as milk, there is no evidence linking DYN/KOR to intake of high fat diets[67,157,158]. Additionally, OT has previously been reported to have no effect on the intake of high-fat diets[159]. The fact that no changes were seen in the intake of milk between ASD and control groups despite differences in OT and DYN expression supports previous research suggesting that OT does not affect intake of fatty diets and suggests that DYN also has no effect.

Due to the fact that OT has been implicated in modulating behaviours and functions that are impaired in ASD, it has long been suspected to play a role in ASD pathophysiology. Specifically, OT is involved in social competence[63,160-163] and repetitive behaviours[163,164], core symptoms of ASD and also some comorbid symptoms such as anxiety[165,166]. However, data investigating the correlation between OT and ASD is conflicting. In ASD children, OT synthesising grey matter in the HT has been found to be diminished[167], and plasma OT levels are reported to be reduced in children[168,169] and adults[170]. However other studies have found no differences between ASD and control groups in adolescent males[171] or children[172]. Conversely, two further studies found that in adults[173] and adolescent girls[171] OT plasma levels were found to be elevated in ASD groups. The inconsistency of these results suggests that OT dysregulation may only exist in a subset of ASD patients.

Interestingly, OT dysregulation was found to be heritable. TD siblings of ASD children were more likely to also have dysregulated OT expression without being afflicted with ASD, and this dysregulation affected social competence regardless of ASD diagnosis[172]. These results suggest that OT may exuberate social impairments in ASD, although it appears not to be essential in the ASD phenotype. Therefore, the differing levels of OT expression seen between the ASD and wildtype control rats in this study may simply be a familial trait, as each group were from a single litter.

Some of these studies also investigated the relationship between plasma OT and social competence, and these results were similarly inconsistent. Modahl *et al.* (1998)[168] reported that while raised plasma OT increased social competence in TD children, it reduced social competence in ASD children. Miller *et al.* (2013)[171] found that elevated plasma OT improved social competence in children while Jansen *et al.* (2006)[173] found no such correlation in adults. The inconsistency of aberrant OT's effects on social competence may be an indicator of OTR dysfunction. OTR polymorphisms are reported to be heritable and increased OT expression may not have the same effects on ASD individuals if its affinity for OTR is impaired. The chromosomal region where the OTR gene is encoded has been identified as a susceptible loci for mutations in ASD[174] and single nucleotide polymorphisms (SNP) in the OTR gene have been correlated with ASD[175]. Therefore, although actual expression levels of the OTR gene may remain unchanged, genetic, and possibly epigenetic, variations may alter the binding ability of OT, or the effects that this binding has on the OTR expressing cells.

It has been suggested the age of subjects may contribute to the effects of OT[176]. This may explain why we observed no difference in intake between ASD and controls when given a two-bottle choice test between sucrose and saccharin. This test was carried out ten months after the initial one-bottle intake tests of sucrose and

saccharin, thus aging may have resulted in anhedonia, reducing hedonic food intake. However, the fact that elevated levels of the orexigenic DYN are seen in VPA rats at the advanced age at which they were euthanised, suggests the dysregulation of another reward mediating system. Furthermore, both groups preferred sucrose to the non-caloric sweet tastant saccharin, suggesting that homeostatic mechanisms are still in place, dictating preference for high calorie diets. Further research investigating both the food intake preferences and DYN expression in both young and aging rats is needed to investigate these claims.

Multiple lines of research have noted POMC, and by extension DYN, dysfunction within subsets of ASD patients[177,178]. Sandman *et al.* (1999)[178] noted that ASD patients that exhibited POMC dysregulation were more likely to exhibit self-injurious behaviour, a comorbid symptom of ASD. Correlations between self-injurious behaviour and eating disorders independent of ASD and have previously been noted, although the mechanisms underlying this relationship are not well understood[179,180].

POMC dysfunction has been hypothesised to be caused by aberrant DYN expression, and this is supported by our data, as discussed earlier. Dysregulation of DYN, and consequently POMC, could therefore account for a number of comorbid ASD symptoms. In addition to AFS and self-injurious behaviour, POMC has been implicated in biological functions which are also impaired in large subsets of ASD patients[68], further supporting the hypothesis that POMC dysfunction plays a role in ASD. These include immune dysfunction[181], sexual dysfunction[182-184] and increased stress response[185,186]. Furthermore, KOR activation has been associated with increased stress which may exuberate some social and non-social symptoms of ASD[187], although it is unclear if this is via POMC activity. This KOR induced stress has been found to be induced specifically by social stress[188] and by KOR activation in the brainstem[189]. Thus, the heightened DYN expression seen in the ASD brainstem

in our results may contribute to increased social stress and therefore the decreased social interactions of these VPA rats that was observed. Additionally, depressive moods, another comorbid symptom, is thought to be caused by KOR activation, potentially in the brainstem[189,190].

The aberrant expression of DYN in the brainstem of the VPA rats, along with the dramatic increase in palatable food intake (sucrose, saccharin and complex liquid diet), supports the hypothesis that inhibition of POMC neurons in the brainstem by DYN is responsible, at least in part, for the orexigenic effects of DYN. The fact that intake of the non-sweet diets did not differ between ASD and wildtype controls may be attributed to DYN, as it appears DYN encourages intake of palatable sweet tastants only. Although the complex liquid diet contained relatively low levels of sugar (0.9%) when made to packet instructions, this appeared to be enough to elicit orexigenic behaviour.

The only fMRI investigation, to our knowledge, of ASD response to foods supports these conclusions, as it was found that ASD children had an enhanced neural response when shown images of palatable foods[28]. These data also support the research conducted by Damiano *et al.* (2014)[109] which claimed that hedonic response to sweet tastants was intact.

Appendix I demonstrated that female rats treated prenatally with VPA also exhibit aberrant food preferences, although these preferences do exhibit some differences to the male VPA rats. While the ASD females also consumed less chow than controls, no other significant differences were seen besides from cow and total milk intake when presented with a two-bottle choice between both cow and goat's milk. No significant differences were seen between groups in the intake of palatable sweet tastants. Unfortunately, the group sizes here were even smaller than the male group, with three VPA rats and seven wildtype controls. This makes it difficult to obtain

significant results that are representative of a larger group, therefore these results do suggest some aberrant food preferences. Additionally, oestrous cycles were not monitored in these females, and stage in the cycle can influence food intake behaviours[191]. Further research will need to be conducted to make more significant claims.

Treatment of ASD with OT administration has been suggested for some time to alleviate social impairments, however it was found to have varied effects in clinical trials[192,193]. However, our results demonstrate that this treatment would not be effective as OT expression levels were either unchanged or elevated in the VPA rats.

Although our data show hedonic responses to food are intact, no differences are seen in expression levels of any of the genes tested in the NAc, therefore suggesting the reward system is not impaired in this ASD animal model. Instead AFS in this model of ASD is most likely due to interactions with satiety mediators in the HT and brainstem. These results do not support the social motivation hypothesis of ASD. This hypothesis proposes that a decrease in social reward value results in reduced motivation to engage in social interactions. An increase in DYN expression in the NAc has previously been demonstrated to reduce social reward value and increase social aggression. Therefore it was hypothesised that an increase of DYN expression in the NAc would be observed, accounting for some of the core social symptoms of ASD.

4.2. Limitations

A major limitation of this study is the small sample size. VPA administration increases the occurrences of foetal reabsorption, with reabsorption rates of 23.1% in Wistar Han rats reported with administration of 500mg/kg, compared to 2.5% in

untreated dams[138]. However, we observed much higher rates of reabsorption, with only one litter born out of dozens of matings. This may be due to that fact Sprague-Dawley rats were used, and reabsorption rates have not been reported for this breed. Due to these limitations in producing viable litters to term, the VPA rats were all from a single litter, so it cannot be ruled out that any differences between groups are simply due to a close genetic relationship, rather than the VPA exposure.

Unfortunately, the perfect animal model of ASD does not exist. Although crucial in preliminary research investigating the specific biochemical properties of a disorder which is difficult to perform in human patients, caution must be taken when translating these results to human patients. The relationship between neurophysiology and behaviour is not always consistent between species, meaning research in human patients would need to be completed before extrapolating these results onto human patients.

4.3. Conclusions and Future Directions

It is clear that the VPA rat model of ASD exhibits aberrant social and food intake behaviours. The elevated levels of OT and DYN likely play a role in these abnormalities. Increased expression of HT OT seen in the VPA rats may be responsible for the decreased intake of standard chow and water. The elevated expression of OT in the VPA rats may indicate increased levels of circulating leptin which has been demonstrated to stimulate release of OT in the HT, leading to decreased food intake. It may also indicate increased expression of the anorexigenic MC, as increased MC expression is thought to potentiate OT release in the NAc in response to social interaction. Investigating OT and MC mRNA expression levels in the NAc immediately following social interaction would provide valuable insight into this hypothesis.

Elevated DYN expression in the HT and brainstem may delay satiety by reducing activity of various anorexigenic neurons, such as POMC or NPS. As POMC is a precursor of MC, elevated levels of DYN would result in decreased POMC expression and subsequently reduced MC and OT expression. However, this is not seen in our data, suggesting that the hypotheses that DYN reduces POMC expression and MC increases OT expression are mutually exclusive, at least in the HT. This discrepancy highlights the fact that there is still much more work to be done in order to fully elucidate the neural mechanisms underlying the aberrant food intake behaviours observed.

Future research should confirm the results obtained here using a larger sample size. Following this, manipulations of the DYN/KOR system, OT and OTR and the subsequent effects on food intake behaviours and social interactions should be investigated. These results will be particularly advantageous if cannula were implanted and aimed at the HT and/or the brainstem – particularly the NTS. This would allow specific antagonism/agonism of DYN and OT. Subsequently, it would be extremely interesting to observe POMC, NPS and leptin levels in the VPA rats, to investigate our claims that DYN interacts with POMC or NPS and that OT is influenced by leptin or MC. This would allow the elucidation of the specific actions of each neuropeptide at specific neural locations.

An understanding of the neurological basis of AFS is essential for the development of effective treatments and cures, therefore improving the quality of life for this subset of ASD patients. This understanding may also provide insight into the social defects of ASD.

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Appendix I: Food Intake Behaviour in a Small Group of VPA Treated Female Rats

Preliminary research on a small group of female VPA treated rats and controls was preformed to determine if AFS is also present in females. Experimental procedures from 2.1. Animals, 2.2. Breeding, 2.5.1. Ad Lib. Chow and Water, 2.5.2. Deprivation Induced Chow Intake, 2.5.3. Sucrose and Saccharin Intake, and 2.5.5. Cow and Goat's Milk were carried out with these females, as described below.

I. Materials and Methods

I.I. Animals

All rats were housed in plastic cages with wood chip bedding material at 22 °C under a 12-hr light/12-hr dark cycle with lights on at 07.00 h. Unless stated otherwise, tap water and standard laboratory chow (Sharpes Feed) were available *ad lib.* All procedures described received prior approval from the University of Waikato ethics committee.

I.II. Breeding

Adult female Sprague-Dawley rats were mated in-house, with vaginal smears analysed each morning. The presence of spermatozoa in the vaginal smear was used to determine pregnancy at embryonic day one (E1). Sodium valproate was dissolved in saline to a concentration of 50mg/ml and at E12.5 the females received a single intraparietal (IP) injection of 500mg/kg of the valproate solution. Controls received an equivalent volume of physiological saline. The females were allowed to raise their

pups until one month postpartum when the pups were weaned and separated by gender. At ~2 months postpartum females were single housed. Weight was measured regularly. Physical and behavioural abnormalities were used to determine if the offspring were affected by the VPA. In total three VPA treated males and seven control females were used.

I.III. Ad lib. Chow and Water

Chow and water intake was recorded over 48 hours.

I.IV Deprivation Induced Chow Intake

Food intake was then recorded for four hours after overnight deprivation.

I.V. Sucrose and Saccharin Intake

Water was removed and intake of a 10% sucrose solution was recorded over four hours to expose the rats to the novel diet. This was repeated the following day.

The procedure was then repeated with a 0.1% saccharin solution.

10% sucrose and 0.1% saccharin solutions were given to the rats simultaneously for two hours to expose the rats to the novel combination, and this was repeated the following day.

I.VI. Cow and Goat's Milk

Cow's milk was made according to packet instructions (100g milk powder added to 900ml of tap water). Rats were exposed to the milk for 30 minutes and then intake was recorded for two hours the following day.

This process was repeated for goat's milk – also made according to packet instructions (100g milk powder added to 900ml of tap water).

II. Results

II.I. Ad Lib Chow and Water

VPA rats consumed 21% less chow than wildtype controls over 48 hours (p=0.0002). No significant differences between groups in water intake was seen, although a trend nearing significance was observed, where the ASD females consumed 17% less water than wildtype controls (p=0.0532; Appendix Figure 1).

II.II. Deprivation Induced Chow Intake

No significant differences were seen in chow intake over 24 hours after overnight deprivation compared to wildtype controls (p=0.7122; Appendix Figure 2).

II.III. Sucrose and Saccharin Intake

No significant differences were seen in sucrose or saccharin intake between ASD and wildtype controls groups when presented separately (p=0.6324 and 0.6328, respectively; Appendix Figure 3).

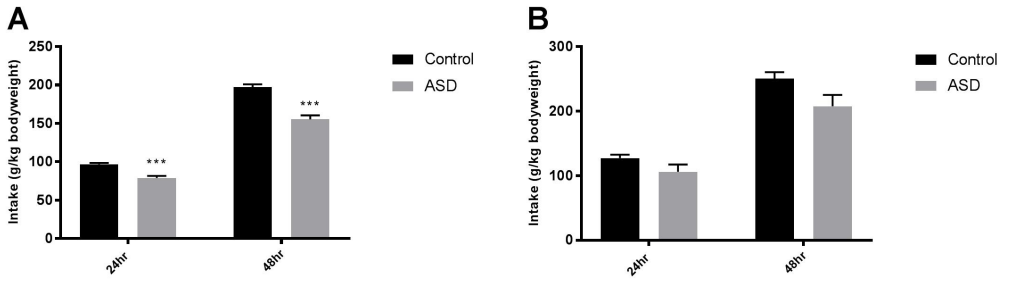
When presented together, intake of sucrose (p=0.0830), saccharin (p=0.2414) and total intake of both sucrose and saccharin together (p=0.0647) did not differ between groups (Appendix Figure 4). However, both the ASD group and wildtype controls preferred sucrose to saccharin, with the ASD group consuming 324% more sucrose compared to saccharin (p=0.0018) and the controls consuming 256% more (p=0.0001).

II.IV. Cow and Goat's Milk

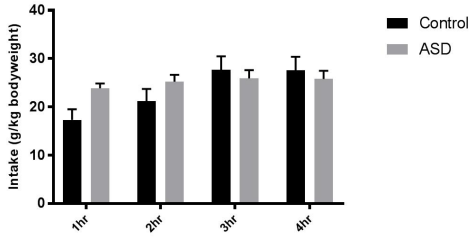
When given goat or cow's milk only, there was no significant differences in intake between ASD and wildtype controls (p=0.2349 and 0.0931, respectively; Appendix Figure 5A).

When both milks were given simultaneously, there was no significant differences in the intake of goat's milk between the ASD and control groups (p=0.2224). However, the control group consumed 64% more cow's milk, and 55% more milk in total

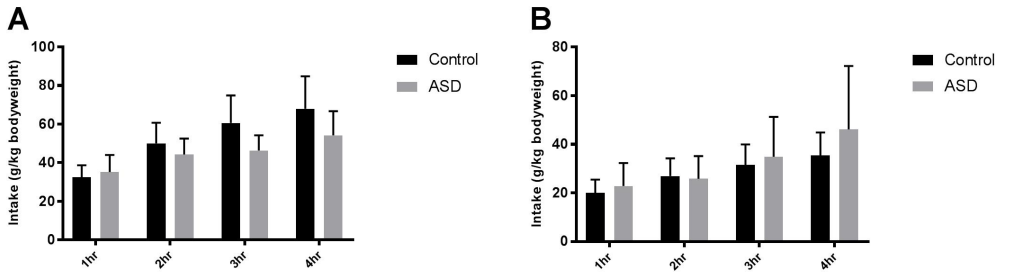
compared to the ASD group (p=0.0213 and 0.0047, respectively; Appendix Figure 5B). While the ASD group had no preference for either milk type (p=0.1148) the wildtype controls preferred cow's milk to goat's milk, consuming 62% more cow's milk compared to goat's milk (p=0.0070).



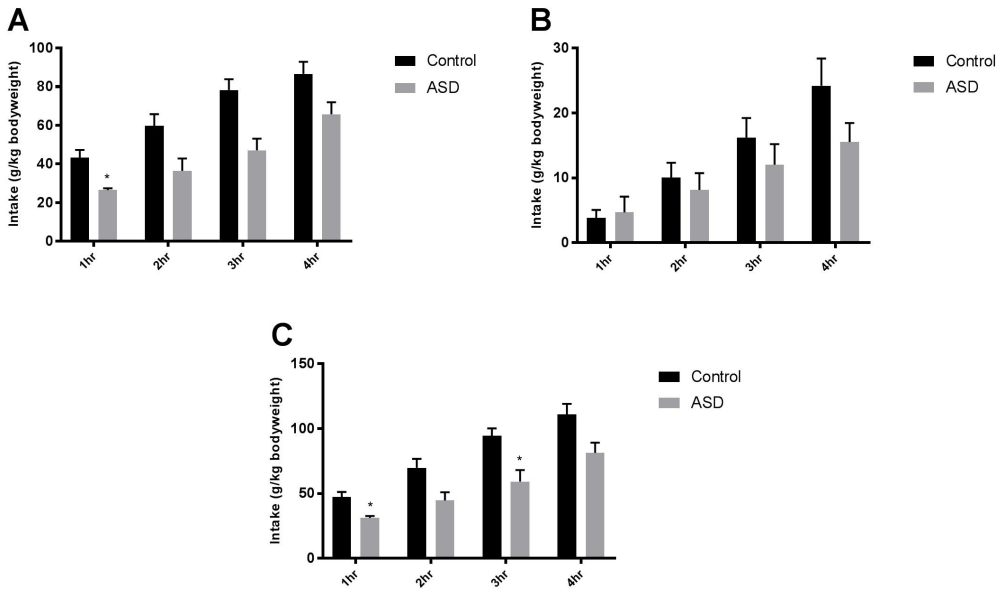
Appendix Figure 1. (A) Consumption of *ad lib.* chow. The ASD group consumed 21% less chow compared to controls over 48 hours. (B) Consumption of *ad lib.* water. No significant differences were seen in water intake. ***P <0.001.



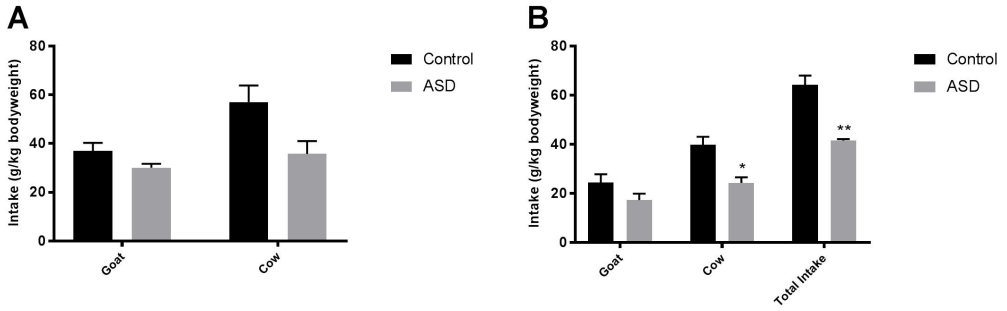
Appendix Figure 2. Consumption of chow over 24 hours after overnight deprivation. No significant differences were seen between groups.



Appendix Figure 3. Consumption of 10% sucrose and 0.1% saccharin when presented individually. (A) Consumption of 10% sucrose. No significant differences were seen between groups. (B) Consumption of 0.1% saccharin. No significant differences were seen between groups.



Appendix Figure 4. Consumption of 10% sucrose and 0.1% saccharin when presented simultaneously in a two-bottle choice test. (A) Consumption of sucrose. Control rats consumed significantly more sucrose at one hours only. (B) Consumption of saccharin. No significant differences were seen between groups. (C) Total intake of both sucrose and saccharin. Control rats consumed significantly more at one and three hours only.



Appendix Figure 5. (A): Consumption of goat or cow's milk over two hours when presented separately. No significant differences are observed between groups for either milk type. (B): Consumption of goat or cow's milk over two hours when presented simultaneously in a two- bottle choice test. No significant differences in intake are observed between groups for goat's milk, cow's milk or total intake of both milks.