A Neural and Behavioural Investigation of Aberrant Feeding in Animal Models of Autism Spectrum Disorder

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Abstract

Autism spectrum disorder (ASD), a neurodevelopmental disorder affecting approximately 1.85% of the population, is underpinned by complex interplay between genetics and environment. Thus far, ASD research has focused predominantly on abnormal social communication, anxiety, and cognitive impairments. It omits, however, dysregulated food intake, which is the symptom present in over 60% of ASD individuals. Data suggest that feeding abnormalities include heightened food selectivity, neophobia and food refusal, as well as pica (consumption of non-food items). The current knowledge of the scope and magnitude of abnormal appetite in ASD as well as neuropathology underlying this phenomenon is extremely limited. Thus, the overarching goal of this thesis was to provide systematic evidence pertaining to aberrant food intake in ASD and identify the relevant mechanisms, by utilizing two key animal models of this disorder (one in which ASD stems from a genetic mutation, and the other, in which ASD is induced pharmacologically).

In Specific Aim 1 of this thesis, I investigated the hypothesis that ASD mutant male mice overconsume palatable foods beyond the level observed in neurotypical controls. In this animal model, ASD stems from the deletion of the contactin-associated protein like 2 (*CNTNAP2*) gene encoding a transmembrane neuronal protein which clusters K^+ channels in the nodes of Ranvier. My studies show for the first time that *Cntnap2*$^{-/-}$ males drink more of sucrose, saccharin and intralipid solutions compared to wild-type (WT) controls. A c-Fos immunoreactivity analysis revealed that at the end of a sucrose solution meal, *Cntnap2*$^{-/-}$ mice have blunted activation in key regions associated with satiety and reward. Specifically in the central amygdala (CEA), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH) and the nucleus accumbens (Acb). Excessive consumption of palatable sucrose was ameliorated in mutants by intraperitoneal administration of oxytocin (OT), a satiety promoting neuropeptide whose dysregulated release is also associated with the broad symptomology of ASD, from anxiety to sociality impairments. In line with the injection experiment, the double staining for c-Fos and OT showed blunted OT neuronal activation in the hypothalamus of the mutants after a meal of sucrose. Overall, the data indicate that ASD *Cntnap2*$^{-/-}$male mice show excessive intake of palatable foods, and it is associated with atypical neural activation (including the OT system) in response to a palatable meal.

In Specific Aim 2, I examined whether the overconsumption of sucrose observed in male *Cntnap2*$^{-/-}$ mice also occurs in females. Research on ASD animal models often focuses on males due to concerns about oestrus driven behavioural variability and the subtler presentation of ASD-related behaviours in females. However, feeding issues in ASD affect both sexes. To address this, I evaluated sucrose intake in female *Cntnap2*$^{-/-}$ mice and compared their consummatory behaviour across different stages of the oestrous cycle. Female *Cntnap2*$^{-/-}$ mice exhibited overconsumption of sucrose, which remained consistent across all oestrous phases. This study suggests that palatability-driven overconsumption of sugar occurs in female *Cntnap2*$^{-/-}$ mice and does not appear to be influenced by oestrus phase.

In Specific Aim 3, I investigated the hypothesis that *Cntnap2*$^{-/-}$ mice exhibit heightened hyponeophagia, a common feeding issue associated with ASD. I assessed hyponeophagia in *Cntnap2*$^{-/-}$ mice both in a novel environment and in their home cage. In both contexts, *Cntnap2*$^{-/-}$ mice displayed a significantly greater latency to approach novel food compared to WTs. Notably, in the home cage more instances of presentation of that food were required for the latency in *Cntnap2*$^{-/-}$ mice to decrease to match that of WTs. The analysis of c-Fos immunoreactivity revealed different densities of activation in key brain regions associated with reward, feeding, and fear processing between *Cntnap2*$^{-/-}$ and WT mice in response to novel food presentation. These regions included the ventral tegmental area (VTA), CEA, and arcuate nucleus (ARC). These findings suggest potential impairments in neural processing of food-related novelty cues, which may contribute to the exaggerated neophobic behaviours observed.

Valproate (VPA), an anti-epileptic drug and known teratogen, induces an ASD phenotype in offspring when administered during the period of neural tube closure. Specific Aim 4 tested whether rats prenatally exposed to VPA exhibit impaired acquisition of the conditioned taste aversion (CTA), a protective learning mechanism against ingestion of foods inducing nausea/malaise. Feeding issues in ASD, such as pica, may reflect disruptions in aversion-based learning. Additionally, OT has been implicated in CTA acquisition, and VPA rats exhibit OT signaling abnormalities. Using a standard CTA paradigm with lithium chloride (LiCl) as a nausea-inducing agent, I found that VPA rats failed to acquire a CTA response. Gene expression analysis via qPCR revealed altered expression of catecholamine metabolism-, reward-, aversion-, and satiety-related genes in the CEA in LiCl-treated VPA rats compared to controls. These findings suggest that

prenatal VPA exposure disrupts neural mechanisms critical for aversion-based learning, with the CEA playing a key role.

In summary, this thesis provides novel insights into the neuromolecular mechanisms underlying feeding behaviours in ASD, highlighting distinct anomalies in palatable food intake, food neophobia, and aversive learning. Using *Cntnap2*$^{-/-}$ mice and VPA-exposed rats, the studies demonstrate significant overconsumption of palatable foods in both sexes, prolonged neophobic responses, and disrupted conditioned taste aversion acquisition, all underpinned by aberrant brain activation and gene expression.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

5-HT	5-hydroxytryptamin
AcB	Nucleus accumbens
AcbC	Nucleus accumbens core
AcbS	Nucleus accumbens shell
ADHD	Attention-deficit hyperactivity disorder
AgRP	Agouti-replated peptide
AP	Area postrema
ARC	Arcuate nucleus
ARFID	Avoidant restrictive food intake disorder
ART	Artificial reproductive technology
ASD	Autism spectrum disorder
AUC	Area under the curve
b.wt	Body weight
BBB	Blood-brain barrier
BLA	Basolateral amygdala
BMI	Body mass index
BNST	Bed nucleus of the stria terminus
BTBR	Black and tan brachyury T^{+}ltpr3^{tf}/J
CA	Cornu ammonis
CDFE	Cortical dysplasia focal epilepsy
CEA	Central nucleus of the amygdala
CEN	Central executive network
CNS	Central nervous system
CNTNAP2	Contactin-like associated protein
CNV	Copy number variant
CTA	Conditioned taste aversion
DEPC	Diethyl pyro carbonate
DG	Dentate gyrus
DMH	Dorsomedial hypothalamus
DMNV	Dorsal motor nucleus of the vagus
DSM	Diagnostic and statistical manual of mental disorders

DVC	Dorsal vagal complex
E/I	Excitatory/inhibitory
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GI	Gastrointestinal
GTS	Gilles de la Tourette's syndrome
GWAS	Genome wide association study
HDAC	Histone deacetylase
HPC	Hippocampus
ID	Intellectual disability
ip	intraperitoneal
LHA	Lateral hypothalamus
LiCl	Lithium chloride
MBP	Myelin basic protein
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
mPFC	Medial prefrontal cortex
NPC	Neural progenitor cell
NSC	Neural stem cell
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OCD	Obsessive compulsive disorder
OL	Oligodendrocyte
OT	Oxytocin
OT-R	Oxytocin receptor
P5P	Pyridoxal 5'-phosphate
PB	Parabrachial nucleus
PBS	Phosphate buffered solution
PDD-NOS	Pervasive developmental disorder – not otherwise specified
pFC	Prefrontal cortex
PND	Postnatal day
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
ROS	Reactive oxygen species

RS	Resting state
rtPCR	Reverse transcriptase polymerase chain reaction
RtPJ	Right-temporo-parietal junction
SEM	Standard error of the mean
SN	Salience network
SON	Supraoptic nucleus
St3gal5	ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 55
TBP	TATA-box binding protein
UsVs	Ultrasonic vocalisations
VMH	Ventromedial hypothalamus
vMPFC	Ventromedial prefrontal cortex
VTA	Ventral tegmental area
VPA	Valproate
WT	Wild-type

Chapter 1

Introduction

Autism spectrum disorder (ASD) is a multifaceted neurodevelopmental disorder characterized by impaired social communication and interaction alongside repetitive/restrictive behaviours [1]

The preliminary identification of ASD was made by Leo Kanner in 1943. He outlined childhood autism as an inborn disorder causing language deficits, preference for social isolation, and repetitive/restrictive behaviours [2]. Presently, ASD is categorised as a neurodevelopmental disorder by the latest edition of the diagnostic and statistical manual of mental disorders (DSM-V) alongside attention deficit/hyperactivity disorder (ADHD) and intellectual disability (ID). However, genetic analysis supports the placement of ASD, ADHD and ID on a neurodevelopmental disorder continuum with schizophrenia and bipolar disorder [3].

Feeding issues are common in ASD. Children with ASD are five times more likely to have problems eating than neurotypical children [4]. Bandini *et al* defined feeding issues as having three measurable domains: food refusal, limited food repertoire and high frequency single food intake [5]. Furthermore, Page *et al* found that feeding challenges in ASD are also linked problems with sensory processing and gastrointestinal issues (GI). Issues in feeding typically converge on disruptive mealtimes which heightens parental stress [6].

Despite the high prevalence of maladaptive feeding in ASD, this area remains understudied. This introduction will review the current knowledge on the epidemiology, diagnosis, aetiology and animal models of ASD before delving into the associated aberrant feeding.

1.1 Epidemiology

The prevalence of ASD has been a subject of debate over the past decades. However, there is an agreement that the number of ASD diagnoses is on the rise, with consistent increases observed globally since 2010. In the USA, ASD affected 1:150 in the year 2000, 1:68 in 2010 and 1:54 in 2016. The growth in number of diagnoses in recent generations has led to claims of an ASD "epidemic" [7]. Current worldwide projections place the prevalence of ASD between 0.38-1.85% of the population [7, 8]. ASD cases reported country by country vary. In Europe, ASD affects between 0.38-1.55% of the population, whilst in the USA this range is between 1.7-1.85%. In New Zealand, the prevalence sits at 1.6% for non-Māori and non-Pacifica children, whereas for Māori and Pacifica, this number is only 1.1% [9]. In non-Western areas there appears to be far fewer ASD diagnoses. In Asia autistic individuals make up only 0.36% of the population, but this number varies geographically ranging from 0.31% in South Asia to 0.51% in East Asia. In the Middle East and North Africa there is an even lower average of 0.13%; however, the range in broad: from 0.1% in Oman to 2.51% in Saudi Arabia [10]. Geographical variations in the frequency of ASD can be explained by differing socioeconomic and sociocultural factors, and the way in which diagnostic data is collected and reported [11] [7]. Although the pervasiveness of ASD in different countries varies, the rise in case numbers is consistent globally.

Increasing numbers of ASD diagnoses have been used to justify the presence of an ASD epidemic. However, it is crucial that confounding variables, such as non-etiologic factors, are ruled out before such a claim is made. The relevant non-etiologic factors include increased social awareness and shifts in the diagnostic criteria of ASD [12].

Increased social awareness of ASD has been linked to the growth in the disorder's prevalence between 2000-2005. Liu *et al* found that undiagnosed autistic children who lived in the proximity of diagnosed autistic children had a higher chance of receiving a diagnosis. Social diffusion of information between parents, such as lived experiences of atypical behaviour associated with ASD, contributed to 16% of the increased ASD prevalence between 2000-2005[13].

Increased ASD diagnosis rates can also be attributed to shifts in the diagnostic criteria. Furthermore, awareness continues to increase amongst clinicians with clinical practice refocusing on subtle expression of autistic traits. Improved diagnostic criteria have allowed clinicians to confidently identify ASD, including those who are high-functioning,

or have co-morbid ASD with other neurodevelopmental disorders. This has subsequently broadened the pool of patients who receive mental health treatment [12, 14].
Etiological factors, such as toxicant exposure during pregnancy, parental age and genetics cannot be dismissed [13, 15]. However, increases in prevalence is multifaceted and includes interplay between etiological and non-etiological factors [13].

Lastly, sex differences in ASD presentation play a role in diagnosis rates, with a 4:1 male: female ratio commonly cited [16-18]. However, Loomes *et al* suggest this ratio may be an overestimate with the true ratio likely sitting between 1-3.5:1 [18]. A contributing factor to the lower diagnostic rate in females is the likelihood of 'female presentation' of ASD-associated behaviours [18, 19]. Autistic females are known to exhibit masking which involves the suppression of autistic behaviours so as to appear neurotypical, which can complicate detection of ASD [19]. Common masking behaviours include mimicking facial expressions, forcing eye contact and restraining oneself from talking about special interests extensively [20]. Masking can be quantitatively measured in proxy by finding the discrepancy between external behaviour and the individual's internal status [21]. Work by Lai *et al* provides a link between the neural self-representation response and quantitatively measured masking in females but not in males [19]. Functional magnetic resonance imaging (fMRI) has implicated the right temporo-parietal junction (RTPJ) and the ventromedial prefrontal cortex (vMPFC) with mentalization and self-representation respectively. Lai *et al* found that when compared to their neurotypical counterparts, autistic males demonstrated hypoactivity in the RTPJ and vMPFC when completing mentalizing or physical judgements about themselves or others. Alternatively, both autistic and neurotypical females showed the same levels of activity in both regions [19].

Increases in global ASD diagnoses can be attributed to greater social awareness and evolving diagnostic criteria. While a 4:1 male-to-female diagnostic ratio is often cited, the lower diagnostic rate in females is likely influenced by differences in the presentation of ASD behaviours.

1.2 ASD diagnosis

The term autism (derived from Greek, autós meaning self) was first used by Swedish psychiatrist Eugen Bleuler in 1908. Initially, it was used to describe a symptom of schizophrenia characterized by withdrawal from reality [22]. The modern understanding of autism began in 1943 when Leo Kanner redefined the term to describe a set of symptoms observed in children. These symptoms included social isolation, communication deficits, and repetitive behaviours, distinct from schizophrenia [2]. Around the same time, in 1944, Hans Asperger described a similar condition in children who exhibited social isolation but without significant communication impairments, which he termed Asperger's syndrome. This was later recognized as an "autistic-like" disorder [22].

Kanner proposed a set of diagnostic criteria in 1944, in his paper 'Early Infantile Autism'. These criteria included extreme autistic aloneness, an intense need for sameness, mutism or language that lacked communicative purpose, and an appearance of intelligence [23]. These criteria were based on his observations of 11 children (8 boys and 3 girls) in his original cohort. Over time, Kanner revised and simplified his diagnostic framework into a more generalized method of characterization [24].

During the 1960s and 1970s, significant progress was made in refining autism diagnostic criteria. This work was notably summarized by Rutter and Bartak in 1971, who outlined criteria that included delayed speech, failure to develop interpersonal relationships, ritualistic and compulsive behaviours, all with an onset prior to 30 months of age [24, 25]. Autism was not formally recognized as a distinct disorder in the DSM until its third edition in 1980 (DSM-III), where it was termed Infantile Autism, with diagnostic criteria largely based on Rutter's work. Alongside this, Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) was also introduced in the DSM-III. PDD-NOS encompassed disorders associated with infantile autism that did not meet the exact diagnostic criteria. This category described individuals with conditions such as Asperger's Disorder (which wasn't introduced until DSM-IV), certain syndromic forms of ASD, or those who exhibited some autistic features but did not fully meet diagnostic criteria. This was revised in the 1987 DSM-III-R, where the name was changed to Autistic Disorder, and the requirement for a specific period of symptom onset was removed.

In the DSM-IV, published in 1994, the diagnostic framework continued to reflect Rutter's contributions while introducing three overarching symptom clusters: social dysfunction, speech delay, and ritualistic/stereotypic behaviours. Given the central role of social dysfunction in autism, the diagnostic criteria required individuals to exhibit more pronounced impairments in the social dysfunction cluster compared to the other two clusters. Additionally, Asperger's disorder was formally included in the DSM-IV as a distinct diagnosis, differentiating individuals with significant social dysfunction but without speech delay [24].

Following publication of the DSM-IV it was questioned whether Autistic Disorder, Asperger's Disorder and PDD-NOS were functionally different from each other due to high levels of symptom overlap. This ambiguity often resulted in confusion and misdiagnosis. To address these issues, the DSM-V eliminated PDD-NOS and Asperger's Disorder, consolidating them under the all-encompassing term ASD. Additionally, speech delay was removed as a diagnostic criterion, leaving social impairment and repetitive/restrictive behaviours and sensory abnormalities as the core symptom clusters. While abnormalities in social-emotional reciprocity during speech are recognized in ASD, these are classified under social impairment rather than being considered a distinct speech delay [24].

Despite the long history of identifying abnormalities associated with ASD and the development of diagnostic criteria to describe them, the diagnosis of this disorder remains ambiguous and relies exclusively on behavioural observation. While significant progress has been made in identifying genetic markers and neurobiological changes linked to ASD, there is currently no established method for diagnosing the condition through genetic or physiological means.

1.3 Aetiology

ASD is both multifactorial and highly pleiotropic. This pleiotropy can be identified at a range of levels including genetics, neurobiology and symptomatology. Due to the highly heterogenic nature of ASD, it manifests differently in each instance. As such, ASD results in individuals 'functioning' at different levels along the spectrum. For some, this can be a crippling disorder, whilst others achieve typical milestones throughout their lives [26].

1.3.1 Symptomatology

Impaired social interaction and communication alongside repetitive/restrictive behaviours are hallmarks of ASD [1]. ASD is typically identified during early childhood and historically the diagnostic criteria stated that onset of symptoms must occur before 3 years of age. However, the ability for parents to identify these behaviours is no longer considered relevant in the diagnosis of a lifelong disorder [27]. Additionally, some individuals (females in particular) with more subtle behaviours may go unnoticed until adolescence or later, when the requirements of social interaction complexities have increased with the individuals age [28].

Early abnormalities include reduced eye contact and facial expressions, motor clumsiness and reduced sensory perception skills [29]. Interestingly, several comorbid issues may also arise early in development including problems such as GI issues, sleep problems and very commonly, food selectivity [29, 30].

As discussed previously, significantly fewer females are diagnosed with ASD compared to males. Many theories have been coined to explain why female diagnostic rates are lower, spanning from the extreme male brain theory of autism [31] to the imprinted-X liability threshold model [32]. However, there is still discussion around the validity of these theories [20]. What is known is that ASD behaviours can differ between sexes [33-36]. Difficulty in social relationships is a key behavioural abnormality in ASD. However, the issues which arise in social relationships differ between autistic males and females. When scored on quality of friendship, autistic females demonstrate similar scores to neurotypical men, although both score lower than neurotypical females. Comparatively, neurotypical females tend to demonstrate a greater baseline ability to form and maintain social relationships and empathise than men. When both ASD and sex are considered, autistic females score similarly to neurotypical males on friendship quotients likely due to a sexually determined higher sociability baseline and ability to successfully mask in social situations [37].

The processing of emotions differs notably between autistic males and females. Females on the autism spectrum are more likely to exhibit emotion internalization, as opposed to the externalization observed more commonly in autistic males. Emotional internalization

in females is associated with an increased risk of co-morbid conditions such as anxiety, depression, eating disorders, and self-injurious behaviours. In contrast, the externalization of emotions in males often manifests as aggression and interpersonal challenges [20].

Alongside masking, some autistic females also engage in compensation, deliberately adopting cognitive strategies to emulate processes they may find challenging, such as theory of mind and intuitive understanding of social norms [20]. In females, the combination of emotional internalization, masking, compensatory behaviours, and a relatively greater inclination toward social relationships compared to autistic males may contribute to difficulty in identifying autistic traits [20, 37].

Lastly, there is a notable relationship between intelligence and the rate of ASD diagnosis in females. The co-occurrence of ASD with intellectual impairment is associated with a higher rate of female diagnoses, reducing the male-to-female diagnostic ratio to approximately 2:1. In contrast, this ratio rises significantly to 11:1 when intellectual impairment is not present [38]. The lower diagnostic rates observed in intellectually able females may be partially attributed to compensatory behaviours. These behaviours, which allow individuals to mask or adapt to social challenges, have been linked to higher IQ and executive function. However, individuals who exhibit high levels of compensation also report increased levels of anxiety [39].

1.3.2 Genetic and environmental risk factors

The highly pleiotropic manifestation of symptoms in ASD reflects complex genetic contributions to the disorder. In the absence of a unifying theory, it is likely that interplay between genetic variants and environmental factors drives ASD development. These genetic variants and environmental risk factors often disrupt shared biological pathways during critical periods of development, resulting in diverse physiological and behavioural phenotypes [40].

ASD was first described as an inborn disorder by Leo Kanner in 1943 [2]. However, no genetic studies of ASD were conducted until 1977, when Folstein and Rutter published the first twin-study [41]. Subsequent twin studies have since provided evidence for a genetic component to ASD [42, 43]. The concordance rate of ASD in monozygotic twins is 88%, compared to 31% in dizygotic twins [44]. Despite high concordance in

monozygotic twins, the absence of a 100% concordance highlights the influence of environmental factors on the manifestation of ASD [45].

Genetic contribution to ASD is further confirmed by the presence of ASD manifestation in certain genetic syndromes. Examples of this include Joubert syndrome, Smith-Lemli-Opitz syndrome, Rett syndrome, Tuberous sclerosis and Fragile X syndrome. Although this corroborates the genetic component of ASD these syndromes are rare and the penetrance of ASD within them is only 50%. Thus, syndromic related cases account for less than 1% of all ASD cases [46]. The evidence within genetic syndromes combined with the results from twin-studies puts forward a strong case for environmental influence in the development of ASD.

Of particular interest for this thesis is the genetic contribution of disruption in the contactin-like associated protein 2 (*CNTNAP2*) gene and the chemical challenge (environmental risk factor) of valproic acid during pregnancy on the development of ASD and its associated behaviours (for details pertaining to this mutation and chemical challenge, please refer to Sections 1.3.2.3 and 1.3.2.5 respectively, in this Introduction).

1.3.2.1 Common genetic variants

Common genetic variants are changes in DNA which occur frequently within the population. These variants contribute to normal variation within cognition and behaviour in neurotypical individuals. However, complex combinations of the very same variants can lead to the development of ASD. [46].

A genome wide association study (GWAS) identified five specific loci associated with ASD, along with seven additional loci which are common to ASD and other conditions such as schizophrenia, major depression and educational attainment. The study also revealed polygenic heterogeneity across ASD subtypes, such as low and high functioning presentations, within common variants. Heritability patterns suggested that common variants contribute more significantly to higher-functioning ASD, while rare *de novo* variants are more strongly associated with intellectual disability in ASD [47].

1.3.2.1.1 Rare de novo variants

Copy number variants (CNVs) are the most prevalent form of rare *de novo* variation in ASD. CNVs are sections of DNA which are either duplicated or deleted, resulting in varying numbers of copies of genes. The nature of CNVs, which can vary in size and encompass multiple genes, adds to the complexity of ASD genetics [40].

Interestingly, ASD simplex cases (a single affected individual within a family) exhibit a higher prevalence of CNVs, often deletions, compared to multiplex cases (multiple affected individuals within a family) or neurotypical individuals. Pathogenic CNVs are observed in 10% of simplex ASD cases, compared to 3% in multiplex cases, and only 1% in neurotypical individuals [48] CNVs are also common throughout other neurodevelopmental disorders including ADHD, depression, intellectual disability, bipolar disorder and schizophrenia [49] [50] [51, 52]. Notably, CNV deletions are common in ASD and developmental delay disorders; CNV duplications have less of an impact in ASD and are associated with schizophrenia [52].

1.3.2.1.2 CNTNAP2 and ASD

CNTNAP2 is a gene whose mutation has been associated with ASD. *CNTNAP2* encodes a transmembrane protein in neurons and is a part of the neurexin family [53]. This protein is responsible for clustering K^+ channels in the nodes of Ranvier and is involved in interactions between neurons and glial cells [53, 54]. *CNTNAP2* is one of the largest genes in the human genome, spanning 2.3Mb on chromosome 7q35 [55].

CNTNAP2 was associated with a syndromic form of ASD in 2006 after a homozygous mutation of the gene was identified in an Amish family. This mutation resulted in cortical dysplasia focal epilepsy (CDFE), language regression, hyperactivity, impulsive and aggressive behaviour as well as ID [54]. Interestingly, other mutations in this gene have been linked also with psychiatric disorders, such as schizophrenia, obsessive compulsive disorder (OCD) and Gilles de la Tourette's syndrome (GTS) [56, 57]. In 2003 before any linkage to ASD was identified, an inversion on chromosome 2 and subsequent translocation into chromosome 7 (inv(2)(p23q22),ins(7;2)(q35–q36;p21p23) was found to cause disruption in the *CNTNAP2* gene, leading to co-morbid presentation of OCD and GTS [56]. A 2008 study by Friedman *et al* also demonstrated that changes in the CNVs in the *CNTNAP2* gene resulted in schizophrenia and epilepsy [57]. Further research found

that CNTNAP2 is a language endophenotype for ASD and that *CNTNAP2* transcripts in foetal human brains were restricted to the frontotemporal-subcortical circuits in the brain. These circuits are a precursor to language acquisition and also play an important role in executive functioning [58]. Following these studies, a wealth of data has been generated linking *CNTNAP2* mutations to ASD with evidence converging on endophenotypes related to language delay and disorders [59].

1.3.2.2 Environmental factors

Several environmental factors have been associated with ASD, with the level of impact dependent on the developmental stage at which exposure occurs. The periconceptional, prenatal, and perinatal periods represent the highest-risk windows for environmental influence [60].

During the periconceptional period, environmental risk factors for ASD include advanced parental age (>35 years), the use of artificial reproductive technologies (ARTs), exposure to environmental chemicals and toxins, maternal nutritional status, and certain maternal medications, such as antidepressants [60]. In the prenatal period, factors such as anti-epileptic drug exposure, substance abuse, air pollution, pesticides, heavy metals, maternal nutritional deficiencies, prenatal infections, and maternal immune activation have all been identified as risk factors. Of particular interest to this thesis is the impact of valproate on the developing brain, as well as its utility in animal models of ASD [60]. During the perinatal period, the most significant risk factors include preterm birth (<36 weeks) and complications during labor and delivery [60].

1.3.2.2.1 Valproic acid and ASD

Valproate (VPA) is a short-chain fatty acid commonly prescribed as an anti-epileptic drug. It is also used to treat other conditions, such as mood disorders [61]. Whilst VPA works as an effective treatment for epileptic seizures, it is a known teratogen which has been linked to ASD in children exposed *in utero*. Women who took VPA during pregnancy often gave birth to children with physical abnormalities and developmental disabilities. VPA was epidemiologically linked to a higher risk of ASD development in the 2000s [62] [63], with further studies incorporating larger cohorts confirming this initial finding [64, 65].

The mechanisms of VPA action in ASD are complex and not yet fully elucidated. However, several pathways are likely involved, including dysregulated epigenetics and chromatin remodelling [66-68], alterations in signalling pathways [69, 70], oxidative stress [71, 72], and immune system responses [73]. These mechanisms contribute to neurological abnormalities such as disrupted neurogenesis, altered neuronal organization and migration, and imbalances in excitatory/inhibitory (E/I) signalling. Collectively, these disruptions result in an idiopathic form of ASD [74].

1.3.2.2.1.1 Disrupted histone acetylation

The higher order organisation of DNA into chromatin and chromosomes is facilitated by histones. Histones are small proteins made up of four subunits (H1, H2, H3 and H4) and are used as bases upon which DNA is wound. Acetylation of histones (subunits H3 and H4) causes them to become less tightly packed, resulting in easier access for transcription factors. Histone deacetylase (HDAC) removes these acetyl groups allowing for tighter compaction of the DNA, preventing undesired gene expression [75].

VPA is a known non-selective HDAC inhibitor in the brain [67, 68, 76] [74]. Within the central nervous system (CNS), class I and class II HDACs which include HDAC1 and HDAC2A, play critical roles in neurodevelopment. HDAC1 is expressed in neural stem cells and glial cells whilst HDAC2 is expressed in postmitotic neuroblasts and neurons but is absent in fully differentiated glial cells [77]. Given their neurodevelopmental significance, any changes to the activity of these enzymes can profoundly impact brain development. VPA inhibits HDAC1 by binding to its catalytic site, while it suppresses HDAC2 through the initiation of proteasomal degradation. Both result in the inhibition of their respective enzymes [67]. Furthermore, postmortem analyses of ASD brains have identified shared histone acetylation patterns in 68% of ASD cases, encompassing both syndromic and idiopathic forms [78].

The interaction of VPA with histones has been studied in embryonic mouse cells, revealing a transient increase in acetylation of the H3 and H4 subunits [68]. By day 14, hyperacetylation of H3K9 (lysine residue 9 on the H3 subunit) is observed [79]. H3K9 is a critical regulatory site involved in histone modification, influencing pluripotency and neural differentiation during the embryonic developmental period [74].

VPA-induced hyperacetylation further influences pluripotency and neural differentiation by increasing the expression of the CDKN1A promoter, which drives hematopoietic differentiation [66]. Additionally, hyperacetylation of H3K56, located at the entry and exit sites of histone bound DNA, can modulate chromatin unfolding. This facilitates the repositioning of developmental genes and suppresses the expression of pluripotency-associated genes. Together, these effects which enhance cellular differentiation and reduce pluripotent gene expression, can lead to an abundance of fully differentiated cells and a diminished reserve of pluripotent stem cells early in development [74].

Inhibition of class I HDACs has been shown to increase synapse formation and accelerate synaptic maturation. Both of which are crucial processes for normal brain development. Early brain overgrowth, a hallmark of ASD, is characterized by excessive neural growth and connectivity. VPA-induced HDAC inhibition during prenatal development provides a plausible mechanistic link to this early neuropathological feature of ASD. VPA contributes to early brain overgrowth by increasing synapse number and promoting premature synaptic maturation, [62].

Neurophysiological abnormalities in the prefrontal cortex (pFC) and temporal association cortices are correlated with ASD. These brain regions develop slowly, with complete maturation occurring only in adulthood. This makes them particularly vulnerable to detrimental neurological changes caused by VPA-induced HDAC inhibition [62].

It is also hypothesized that VPA-induced HDAC inhibition leads to downstream dysregulation of critical signalling pathways, including Wnt/β-catenin, PI3K/AKT, and MAPK/ERK. Among these, the Wnt/β-catenin pathway, which plays a pivotal role in embryonic development, is upregulated due to HDAC inhibition. This dysregulation results in increased expression of the oxidative stress marker 4-HNE in both the pFC and hippocampus (HPC). Additionally, proteins known to be dysfunctional in ASD such as CHD8 and PTEN interact with the Wnt/β-catenin pathway, further implicating its dysregulation in the pathophysiology of ASD [74].

1.3.2.2.1.2 Excitatory/inhibitory ratio in neural circuits

Both hyper- and hypoconnectivity across various brain regions has been associated with ASD [80]. A balanced ratio of excitation to inhibition (E/I) is crucial for maintaining

typical brain function. Consequently, it is unsurprising that specific E/I imbalances have been identified in regions implicated in hyper- and hypoconnectivity in ASD. These regions include the medial prefrontal cortex (mPFC), somatosensory cortex, amygdala and dorsal raphe nucleus [61].

VPA is known to exacerbate E/I imbalance by causing impairments in gamma-aminobutyric acid (GABA) neuronal transmission [81, 82], GABA being a key inhibitory neurotransmitter in the brain [62]. Finally, VPA alters the expression of various gene networks in a non-human primate VPA model of ASD, some of which play critical roles in maintaining E/I balance [83] [61].

1.3.2.2.1.3 Neurogenesis, neuronal organisation and migration.

Abnormal brain growth in ASD is reflected by to enlarged brain volume via early brain overgrowth [84]. Post-mortem studies of young individuals with ASD have revealed increased neuronal populations [85], abnormal cortical lamination [86, 87], and cerebral dysplasia within the cortex [88]. Neurogenesis, the process by which new neurons are generated from neural stem cells (NSCs) and neural progenitor cells (NPCs), is highly regulated and must be carefully coordinated with other stages of brain development [89]. VPA exposure is thought to disrupt this regulation by affecting NSC and NPC populations [90-92], thereby decreasing neuroblast production and altering the fate of these cells [93]. Embryonic exposure to VPA between E10.5 and E12.5 has been shown to affect neuronal distribution and density in key brain regions. These regions include the HPC, cerebellum, brainstem motor nucleus, superior olivary complex, and the cerebellar vermis [94-98]. In the HPC, VPA-induced neurogenesis deficits result in lowered cell density within the dentate gyrus (DG) and cornu ammonis (CA) subregions[99] [74]. When VPA exposure persists throughout the entire embryonic period, cell cycle exit by NPCs is increased, leading to an overproduction of neurons projecting into the superficial neocortical layers [100].

Neuronal organization is also disrupted by VPA exposure. Neuronal organization refers to the process by which neurons develop specialized morphology and biochemistry, enabling the formation of broad neuronal phenotypes and the establishment of complex neural networks [74]. Embryonic exposure to VPA interferes with this process, leading to various structural and functional abnormalities. In the cerebellar nuclei, VPA exposure

results in enlarged neuronal soma [97] and altered dendritic pruning, which together reduce the branching at distal sites of cerebellar Purkinje cells [98]. In regions such as the HPC, amygdala, cerebellum, pFC and somatosensory cortex, there is a marked decrease in dendritic density [99] [74, 93, 101]. Furthermore, abnormal compartmentation of cells in the striatum leads to reduced cortico-striatal synapses and disrupted networks connecting the pFC, granular insula and somatosensory cortex [102]. VPA exposure in a concentration-dependent manner is also associated with the development of ectopic axonal branches and an excess of abnormal branches, further contributing to impaired neuronal organization and connectivity[103] [74].

Prenatal VPA exposure also disrupts the migration of granule cells during cerebellar development, which leads to abnormalities in the Purkinje cell layer and the presence of ectopic Purkinje cells [93]. Additionally, VPA alters the expression of CXCR4, a critical factor in NPC migration during hippocampal neurogenesis. This disruption in CXCR4 expression has been associated with an increased susceptibility to seizures [104] [74].

1.3.2.2.1.4 Oligodendroglia and myelination

Oligodendrocytes (OLs) are glial cells responsible for producing and maintaining the myelin sheath around neuronal axons. These sheaths insulate axons and preventing signal misfiring. OLs also support neuronal function by providing trophic and metabolic factors and facilitating efficient signal transmission [105].

VPA exposure reduces levels of myelin basic protein (MBP) in the basolateral amygdala (BLA) and piriform cortex, leading to increased myelin sheath thickness. This increase in sheath thickness has been linked to impaired social behaviour [106]. In the corpus callosum, VPA exposure during postnatal days (PND) 15–36, a developmental period analogous to the infant and juvenile stages in humans, results in fewer myelinated axons and a reduced number of Ols [107, 108] [74].

1.3.2.2.1.5 Serotonergic system

Hyperserotonaemia has been detected in 25.4% of individuals with ASD, along with increased dystrophic 5-HT axons in various brain regions. However, postmortem studies of ASD brains have revealed significantly reduced binding densities in the posterior cingulate cortex and fusiform gyrus of two 5-HT receptors, 5-HT1A and 5-HT2.

Following *in utero* exposure to VPA, hyperserotonaemia has also been observed in the blood and other tissues. Dysregulation of the E/I balance in serotonergic neurons is associated with a range of ASD-related behaviours, including impaired social interactions, repetitive behaviours, heightened anxiety, abnormal sensory processing, delayed developmental milestones, and memory deficits [61].

1.3.2.2.1.6 Neuroinflammation

Neuroinflammation is another factor contributing to the heterogeneous nature of ASD. Maternal infection during pregnancy is a known risk factor for the development of ASD [60]. VPA exposure emulates the effects of maternal infection by inducing an immune response in the absence of an infectious agent. Prenatal exposure to VPA increases immune activation in the brain, leading to elevated numbers of microglia and astrocytes in the pFC, HPC, and cerebellum [73, 109].

Additionally, VPA increases the permeability of the blood-brain barrier (BBB) as indicated by increased permeation by Evans blue dye, which exacerbates immune activation in the brain [110, 111]. This immune response is further amplified by increased levels of reactive oxygen species (ROS) and reduced antioxidant capacity in the HPC and cerebral cortex [112][63]. Beyond these effects, VPA downregulates TREM2, a transmembrane immune receptor expressed exclusively on microglial cells. TREM2 downregulation promotes a proinflammatory microglial phenotype, which may contribute to dysregulated synaptic pruning, a feature commonly associated with ASD [113] [74].

1.3.3 Neurobiology

1.3.3.1 Early brain overgrowth

Early brain overgrowth in children with ASD aged 0–2 years is one of the most consistently replicated findings in ASD neurobiology [114]. Initial investigations relied on head circumference measurements, though establishing a correlation between total brain volume (TBV) and ASD had historically been challenging due to differences in growth rates occurring before a formal diagnosis could be made. A study by Hazlett *et al* addressed this limitation by predicting ASD diagnoses in high-risk children (with a

familial predisposition) with a 94% accuracy. This facilitated precise analysis of TBV growth rates in children under 2 years of age, as well as detailed investigation of specific brain regions using high-resolution MRI. No differences in TBV are observed between ASD and neurotypical children between 6–12 months of age. However, significant differences emerge during the second year of life. Notably, hyper-expansion of the cortical surface occurs between 6–12 months, with the largest increases observed in the left and right middle occipital gyrus, right cuneus, and right lingual gyrus. This hyper-expansion is significantly correlated with TBV overgrowth at 2 years of age. These findings establish a temporal link between abnormal brain growth and the initial signs of atypical behaviour in ASD. Moreover, periods of overgrowth were found to correlate with greater severity of social affect behaviours in children with ASD [84].

1.3.3.2 Altered amygdala function.

The amygdala has long been implicated as a central structure in the brain dysfunction underlying ASD. The "amygdala theory of autism" was first proposed by Baron-Cohen *et al* in 1999 [115]. The amygdala is a small, yet highly interconnected brain region located within the temporal lobe, primarily associated with fear, reward, and anxiety processing [116-119]. It consists of multiple nuclei, among which the BLA and central amygdala (CEA) are key components. The BLA, and to a lesser extent the CEA, receive sensory inputs from the sensory thalamus and sensory cortices. The BLA has extensive bidirectional connections with cortical regions, including the midline pFC, HPC, and sensory association areas. These connections enable the BLA to integrate sensory information and relay it to these cortical regions, while excitatory projections from these cortical areas feed back into the BLA. Additionally, the BLA has unidirectional projections to subcortical regions such as the striatum, nucleus accumbens (AcB), and the bed nucleus of the stria terminalis (BNST). The CEA mediates sensory information either independently or in conjunction with the BLA, translating sensory input into behavioural outputs through its connections with the striatum and the bed nucleus of the stria terminalis (BNST) [120].

The amygdala has been linked to several abnormal behaviours seen in ASD, such as face perception (recognition of faces and facial emotions) and eye contact, as well as violation of others personal space [121]. Individuals with ASD have been shown to complete face perception and personal space tests in the same manner as individuals with amygdala

lesions. Although ASD individuals perform similarly to those with amygdala lesions, there is still disagreement as to whether the amygdala volume is larger in those with ASD [122-127]. It is likely that other factors such as age and sex play a role in this [128].

Interestingly, after clinical examination of individuals with amygdala lesions, no correlation has been found between amygdala lesions and ASD. This indicates that amygdala lesions alone are not enough to produce ASD. Instead, it is probable that connectivity between the amygdala and other structures is impaired. Network level impairments likely contribute to ASD symptoms rather than the amygdala alone [129].

Abnormal connections from the amygdala to the pFC have been identified in ASD [130]. White matter tracts which connect the amygdala to the right cortex show significantly higher mean diffusivity than neurotypical brains, indicating less microstructural density, this is significantly correlated with the deficits of emotional recognition seen in ASD [128].

When the amygdala is divided into subregions based on their cortical connections, reduced microstructural density is observed in the left amygdala's connections to the temporal lobe, which is associated with impaired attention switching in individuals with ASD [128].

1.3.3.3 Hyper/hypo connectivity

Altered brain connectivity has been identified as a significant correlate to the atypical behaviours associated with ASD [131]. Longitudinal studies on brain connectivity in ASD have revealed dynamic changes with age, highlighting the evolving nature of connectivity abnormalities over the lifespan [80, 132, 133].

Resting state (RS) connectivity within the default mode network (DMN), the central executive network (CEN), and the salience network (SN) has consistently been shown to differ in individuals with ASD compared to neurotypical individuals [80, 132]. These three networks are closely interconnected, with the DMN and CEN performing opposing functions and the SN serving as a “switch” between them, regulating transitions based on environmental demands [134].

Haghighat *et al* conducted an age-stratified analysis of brain connectivity in ASD across three developmental stages: children (<11 years), adolescents (11–18 years), and adults (>18 years). This approach enabled a longitudinal investigation into how connectivity patterns differ between ASD and age-matched neurotypical individuals. Their findings revealed both hyper- and hypoconnectivity in various brain regions, all of which are linked to ASD-related behaviours. These age-dependent connectivity differences underscore the role of network-level abnormalities in ASD pathophysiology and provide insights into how these disruptions may evolve over time [80].

1.3.3.3.1 Within network connectivity

Haghighat *et al* identified within-network connectivity abnormalities in ASD, though these varied across age groups [80]. Hyperconnectivity was predominantly observed in children, while adolescents exhibited more hypoconnectivity within networks. Adults displayed a combination of both hyper- and hypoconnectivity. Notably, across all age groups, the connectivity abnormalities were consistently associated with, or centred around, key nodes within the DMN, CEN, or SN, underscoring the critical role of these networks in ASD.

In children, no instances of network hypoconnectivity were observed. Instead, hyperconnectivity was detected in regions, such as the temporal pole, cerebellum, right anterior superior temporal gyrus, anterior cingulate cortex, anterior insula, middle frontal gyrus, right inferior frontal gyrus, and precentral gyrus [80].

In adolescents, only one instance of hyperconnectivity was identified in the temporal lobe, which was the only region consistently exhibiting hyperconnectivity across all age groups. Hypoconnectivity was noted in the middle frontal gyrus and right inferior frontal gyrus, contrasting with the hyperconnectivity observed in these regions during childhood [80].
In adults, hyperconnectivity persisted in the temporal pole and cerebellum, while hypoconnectivity was observed in the right inferior frontal gyrus and right precentral gyrus. This combination of connectivity abnormalities highlights the complex, age-dependent progression of ASD-related connectivity patterns. Haghighat *et al*'s findings suggest that while hyperconnectivity is prominent in childhood, its prevalence decreases with age. Hypoconnectivity becomes more apparent in adolescents and adults, but the number of affected regions diminishes overall in these groups.

These age-dependent changes in connectivity abnormalities are closely tied to the behavioural manifestations of ASD. Each affected region is associated with behaviours that are disrupted in ASD, such as social interaction, communication, and motor coordination [80].

1.3.3.3.2 Between-network connectivity

Between-network connectivity abnormalities in ASD are only evident when groups are stratified by age; no significant differences are observed when all age groups are combined. In children with ASD, differences in between-network connectivity was identified between the SN and the DMN, particularly involving the precuneus. In adolescents, these connectivity differences shifted, with abnormalities detected between the cerebellum and the DMN. Interestingly, no between-network connectivity differences were observed in adults with ASD, which underscored the dynamic nature of ASD's pathophysiology [80].

Cognitive dysfunction across various neurological disorders, including schizophrenia, depression, anxiety, Alzheimer's, frontotemporal dementia, and ASD, has been unified by the triple network hypothesis. This hypothesis suggests that these disorders share underlying connectivity abnormalities involving the DMN, CEN and SN [135].

The DMN is composed of the inferior frontal gyrus (including posteromedial cortex and the angular gyrus), the anterolateral middle temporal cortex, and the mPFC. These regions, distributed across the parietal, frontal, and temporal cortices, reduce their activity during attention-demanding tasks[136]. The DMN is associated with understanding others' intentions, allocentric spatial reference, conceptual processing, and the sense of self [136] [134]. In children with ASD, DMN overconnectivity has been correlated with the severity of ASD symptoms. Such abnormal connectivity likely disrupts attention, salience orienting, and networks involved in affective, visual, and sensorimotor processing [137]. The CEN, whose activation is inversely correlated with that of the DMN, is most active during periods of cognitive concentration. Its primary functions include task selection, executive functioning, attention control, and working memory. The CEN is comprised of the lateral posterior parietal cortex and the dorsolateral prefrontal cortex [134]. In

individuals with ASD, the negative correlation between the CEN and DMN is weaker compared to neurotypical individuals [137].

The SN includes the anterior insula, dorsolateral cingulate cortex, and subcortical structures such as the amygdala and thalamus. It plays a critical role in identifying and filtering the importance of both internal and external stimuli. The SN also interacts with the DMN to determine whether a stimulus requires attention, effectively acting as a switch between the DMN and CEN [134]. Given the role of the SN in modulating cognitive states, connectivity issues within this network can significantly contribute to behavioural impairments in ASD [137].

The DMN, whose dysfunction has been strongly linked to ASD, is thought to contribute to social impairments. It is associated with theory of mind, understanding others' intentions, and processing a sense of self [80].

1.3.3.4 Synaptic pruning

Synaptic pruning is a process in which the CNS undergoes extensive remodelling by removing excess synapses. This developmental process replaces redundant synaptic connections with strengthened and more precise neural wiring, contributing to optimized brain function. Synaptic pruning occurs extensively during the first two years of life and again during adolescence. Key mechanisms involved in synaptic pruning include activity-dependent pruning and immune-mediated pruning. Rather than functioning independently, these mechanisms are interconnected, with neural activity often guiding which synapses are targeted for removal through immune-mediated responses [138].

1.3.3.4.1 Activity-dependent pruning

Activity-dependent pruning can be categorized into two main types: spontaneous activity and experience-driven activity. These mechanisms play crucial roles in determining which synapses to prune and which to strengthen. Early evidence for the role of activity dependence in pruning came from studies of the retinogeniculate system [139-145]. Before eye opening, an excess of retinal ganglion cells forms synapses with relay neurons. During this period, waves of spontaneous activity in the retina help mediate eye-specific segregation. These waves, when relayed to the brain, are synchronized for each eye but they remain asynchronous between the two eyes [146]. This pattern of activity allows for

the identification of synapses belonging to each eye, enabling pruning of redundant synapses and strengthening of the remaining connections [138].

In contrast, experience-driven pruning relies on external stimuli to shape neural connections. Instead of spontaneous activity guiding the selection of synapses, connections that are used frequently are strengthened, while those that are rarely used are pruned [138]. This process was first demonstrated in the visual cortex through studies of monocular deprivation. When one eye was deprived of visual input, synapses connected to the deprived eye weakened, while synapses linked to the functional eye were strengthened, leading to ocular dominance plasticity [147].

1.3.3.4.2 Immune-mediated pruning

Immune-mediated synaptic pruning is primarily executed by astrocytes and microglial cells, with their roles depending on chemical signals, location, and context. Microglial cells are primarily activated through either fractalkine signalling or the complement cascade [138]. Fractalkine, a G-protein coupled receptor, is cleaved in an activity-dependent manner to induce presynaptic pruning [148].

The complement cascade, a component of the innate immune system, involves a series of protein cleaving and activation steps that promote pruning. One key protein in this cascade is C3, which can bind to pathogens and cellular debris for lysis or phagocytosis [138]. Additionally, C3 may colocalize with another complement protein, C1q, to facilitate pruning of retinogeniculate synapses [149]. In the Acb, complement-dependent phagocytosis also plays a role in the removal of dopamine receptor 1 proteins (D1R)[150] [138].

Astrocytes also contribute to synaptic pruning across various brain regions [148, 151, 152]. Crosstalk between astrocytes and microglia often determines which cell type will perform the pruning in a given context [153]. Astrocytes are thought to play a larger role in retinogeniculate synapse pruning, while microglia are typically involved in localized responses. However, astrocytes will occasionally function in localized roles and microglia may also engage in broader synaptic pruning tasks [138].

1.3.3.4.3 Synaptic pruning in ASD

Interestingly, the first wave of synaptic pruning occurs within the first two years of life, which coincides with the first development of ASD behaviours [154-156]. Altered synaptic pruning in this critical stage of development also underlies schizophrenia [157]. Post-mortem tissue samples from ASD individuals have increased synaptic density in the frontal, temporal and parietal lobes, regions associated with the hyperconnectivity discussed earlier [158, 159]. This suggests under-pruning may be a key mechanism contributing to altered brain function observed in ASD. Complimenting this evidence, transcriptomic studies have identified significant differences in the expression immune-mediated pruning genes [160], and animal studies have identified involvement of *FMR1*, *TSC1*, *TSC2*, *PTEN* and *EIF4E* genes as well [158, 161-164]. Additionally, hypomethylation of genes encoding complement cascade proteins C1QA, C3 and C3R, has been observed, further implicating dysregulated immune-mediated pruning in ASD[165] [138].

Beyond the hypomethylation and abnormal activation of certain genes mentioned above, there is evidence that environmental factors also contribute to disrupted synaptic pruning in ASD. External influences during pregnancy, such as maternal infections, obstetric complications, poor nutrition, and exposure to pollutants, can further impact synaptic pruning processes [138]. The "two-hit" hypothesis has been proposed to explain the interplay between genetic predisposition and environmental triggers in aberrant synaptic pruning found in neurodevelopmental disorders [166]. This hypothesis suggests that while risk genes may predispose an individual to disrupted pruning, environmental factors are often necessary to initiate the process of abnormal synaptic remodeling [138].

As illustrated in Figure 1-1 individuals with ASD exhibit higher synaptic density compared to neurotypical individuals. Although some pruning occurs, the density remains elevated over time contributing to altered neural connectivity and brain function [138].

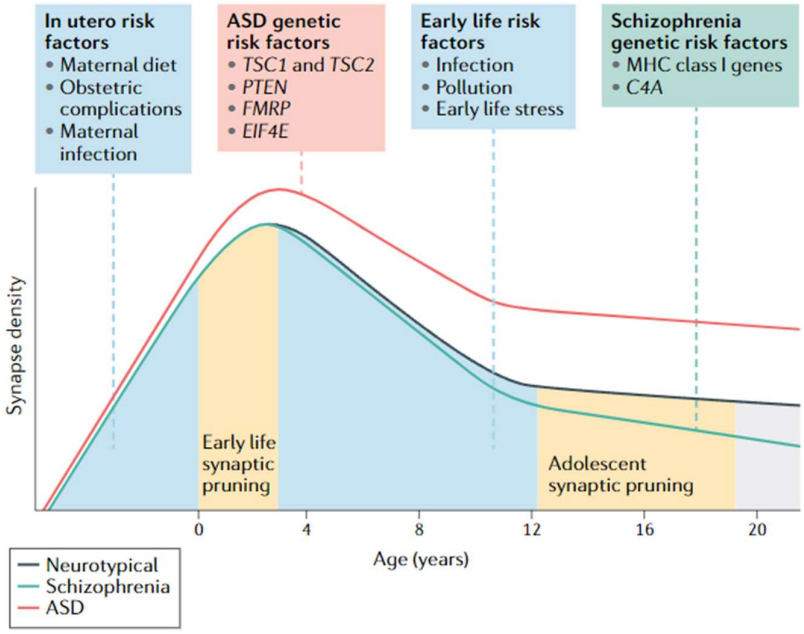

Figure 1-1 Synapse density across neurodevelopmental disorders and age (reproduced with permission from [99].

1.4 ASD animal models

ASD arises from a complex interplay of genetic mutations and environmental challenges during critical developmental periods [40]. Mutations in the *CNTNAP2* gene and *in utero* exposure to VPA are two notable factors implicated in ASD development. The identification of diverse genetic variants and environmental contributors has facilitated the creation of diverse ASD animal models, which fall into three overarching categories: gene inheritance, idiopathic, and environmentally induced [167].

Most genetic variants which that contribute to ASD affect genes involved in synaptic or chromatin structure or remodelling [168]. Also included within this category are syndromic forms of ASD, in which a genetic mutation results in a cluster of symptoms that includes ASD. Examples of animal models which fall into this category include *Nbea*$^{+/-}$, *Mecp2*$^{+/-}$, and *Cntnap2*$^{-/-}$ mice. All three of these animal models display ASD-associated behavioural anomalies, including altered ultrasonic vocalisations (USVs), heightened anxiety, cognitive deficits and social abnormalities [53, 169, 170].

Nbea$^{+/-}$ mice have a neurobeachin haploinsufficiency, whilst *Mecp2*$^{+/-}$, and *Cntnap2*$^{-/-}$ mice demonstrate genetic syndromes in which ASD is one symptom [53, 169, 170]. Neurobeachin (NBEA) is a multi-domain scaffolding protein which is involved in neurotransmitter release and synaptic functioning [171] *Mecp2*$^{+/-}$ mice model of Rett syndrome, which arises from loss of function mutations in the X chromosome linked gene, *MeCP2* which encodes a methyl CpG binding protein [169]. *Cntnap2* (explained in further detail in section 1.4.1) was linked to ASD in humans when a recessive mutation, first identified in an Old Order Amish family, was found to cause cortical dysplasia focal epilepsy (CDFE), language regression, intellectual disability, and ASD [54]. These three animal models demonstrate only a fraction of the many genetic inheritance models available for ASD[167].

In contrast to the wide range of genetic inheritance models, there are only a handful of models which recapitulate idiopathic ASD. The best studied examples are the BTBR+ Itpr3tf/J (BTBR) and BALB/c strains. BTBR mice were originally bred for metabolic studies, while BalB/c mice were established as an inbred albino strain and later extensively genetically characterised [172, 173]. Both of these strains were subsequently identified as displaying significant ASD-like behaviours [172, 174, 175].

Finally, environmentally induced animal models include those generated by prenatal VPA exposure or maternal immune activation (MIA) models. Both antiepileptic drug exposure (VPA) and maternal infection during pregnancy are well characterised environmental risk factors of ASD [60]. Animal models based on these insults recapitulate core behavioural deficits, particularly impairments in social communication and the presence of repetitive and restrictive behaviours [176, 177].

To be effective, these models must demonstrate both construct validity and face validity [53]. Construct validity requires that the model reflects the genetic or mechanistic underpinnings of the human disorder; face validity demands that it exhibits analogous symptoms seen in humans.

While *Cntnap2*$^{-/-}$ and prenatally exposed VPA rats, a specific focus of this thesis, display atypical behaviours associated with ASD [53, 177], achieving perfect face validity in animal models remains a challenge [178]. Furthermore, the absence of definitive biomarkers for ASD complicates both human diagnosis and the evaluation of model

validity, leaving behavioural phenotypes as the primary measure [178]. Although rodents can exhibit ASD-like behaviours, such as heightened anxiety and a preference for non-social stimuli [53, 177], these models are less capable of capturing subtle ASD presentations. Consequently, rodent models are more likely to accurately reflect severe ASD phenotypes rather than high-functioning or mild forms of the disorder.

1.4.1 *Cntnap2*$^{-/-}$ mouse model

A mouse knockout of the *CNTNAP2* gene was first generated in 2003, predating the discovery of its association with ASD by three years [179]. Initially, this mouse model was developed to investigate K+ channels in the juxtaparanodal region of the myelin sheath. The original *Cntnap2*$^{-/-}$ model was created using outbred mice, which are genetically diverse. Such genetic variability is advantageous when studying conditions requiring construct validity, as it reflects the genetic diversity seen in human populations [59]. However, this variability complicates the interpretation of behavioral phenotypes, potentially confounding the effects of background genetics with those of the transgene [180].

To address this, the *Cntnap2*$^{-/-}$ mice were backcrossed with an isogenic (inbred) strain, specifically C57BL/6J mice, to minimize genetic variability and improve the model's face validity [59, 181]. The use of an isogenic background helps ensure that observed behavioral changes are directly attributable to the *Cntnap2* mutation rather than genetic differences unrelated to the knockout.

In wild-type (WT) mice, *Cntnap2* expression in the brain begins around embryonic day 14 (E14). In contrast, the *Cntnap2*$^{-/-}$ mouse model exhibits no expression of this gene [53]. The behavioral phenotype of these *Cntnap2*$^{-/-}$ mice has been extensively characterized and validated by Peñagarikano *et al*, solidifying its utility in studying ASD-related behaviors [53].

1.4.1.1.1 Abnormal neurophysiology in Cntnap2$^{-/-}$ *mice*

Cntnap2$^{-/-}$ mice exhibit spontaneous seizures reminiscent of those experienced by humans with CDFE. In mice, these seizures typically occur after six months of age and are often triggered by mild stressors such as handling. In humans, CDFE seizures are commonly associated with dense hippocampal astroglyosis. Similarly, *Cntnap2*$^{-/-}$ mice display

astroglyosis in the hilus of the HPC, although this does not result in neuronal loss. Additionally, these seizures in *Cntnap2*$^{-/-}$ mice are linked to a reduced number of parvalbumin-positive neurons, further implicating altered inhibitory signalling in the pathology [53].

Beyond seizures, this syndromic form of ASD in humans is also associated with ectopic neurons in the subcortical white matter [53]. Increased white matter density and ectopic neuronal clusters have been identified in the frontal lobes of postmortem ASD brains and are similarly observed in *Cntnap2*$^{-/-}$ mice, with ectopic neurons found in the corpus callosum [53] and cortex [182]. In humans with ASD, neurons in the deep cortical layers frequently exhibit abnormal clustering or migratory patterns. *Cntnap2*$^{-/-}$ mice parallel this with increased numbers of CUX1+ cells in the deep cortical layers [53, 182]. Aberrant cortical cell migration may amplify sensorimotor signalling to the thalamus and motor areas, potentially contributing to repetitive behaviours observed in ASD [182].

The somatosensory cortex in *Cntnap2*$^{-/-}$ mice also exhibits asynchronous firing compared to WT mice. While the firing rate and amplitude of individual neurons remain comparable to WT, the timing of neuronal firing is less synchronized. This suggests that the observed dysfunction arises at the network level rather than from intrinsic abnormalities in individual neurons [53]. Such network-level abnormalities in the somatosensory cortex are similarly implicated in ASD pathophysiology in humans [80, 183].

1.4.1.1.2 Abnormal behaviours demonstrated by Cntnap2$^{-/-}$ *mice*

Cntnap2$^{-/-}$ mice exhibit a range of behaviours reflective of human ASD phenotypes, supporting the validity of this animal model [59]. A comprehensive battery of behavioural tests validating this model is reproduced with permission in **Table 1-1** [53].

Table 1-1 Behavioural results for *Cntnap2*$^{-/-}$ mice. Reproduced with permission [53]

Behavioral domain	Mouse behavioral test	Results in *Cntnap2*$^{-/-}$
General activity	Open field	**Increased activity**
Motor coordination	Rotarod	**Improved performance**
Anxiety	Light-dark box	Normal
Sensory reactivity	Hot plate Startle response Olfaction	**Hypersensitivity** Normal Normal
Sensorimotor integration	Prepulse inhibition	Normal
Learning and memory	Morris water maze (MWM)	Normal
Repetitive behavior, stereotypies, resistance to change	Grooming T maze MWM reverse learning	**Increased** **Deficit** **Deficit**
Communication	UsV in pups	**Decreased**
Social interaction	Juvenile play 3 chamber social interaction	**Deficit** **Deficit**
Housing	Nesting	**Deficit**

Cntnap2$^{-/-}$ mice also show hyper-reactivity to thermal stimuli in the hot-plate test but not to acoustic startle. Interestingly, they outperform WT mice in locating buried food, suggesting heightened olfactory sensitivity [53]. These findings demonstrate abnormal sensory processing, consistent with cortical cell migration abnormalities observed in this model [53, 182].

In cognitive tests, *Cntnap2*$^{-/-}$ mice perform comparably to WT mice in learning the initial location of a hidden platform in the Morris water maze, indicating intact spatial learning. However, they struggle to adapt when the platform is moved, demonstrating behavioural inflexibility. This inflexibility is also observed in the T-maze test, where *Cntnap2*$^{-/-}$mice score lower than WTs in spontaneous alternation, further reflecting difficulties in adapting to new information. These behaviours align with the rigid and repetitive behavioural tendencies characteristic of ASD in humans [53].

Language regression, a hallmark of *CNTNAP2* deletion in humans [54], is mirrored in *Cntnap2*$^{-/-}$ mice, which produce fewer ultrasonic vocalizations (UsVs) as juveniles. UsVs are distress calls typically emitted when pups are separated from their mothers, representing an early form of communication. The reduction in these calls in *Cntnap2*$^{-/-}$ mice is analogous to language regression seen in ASD [53].

Social behaviour is also impaired in *Cntnap2*$^{-/-}$ mice. Nesting behaviour, a social activity involving huddling in communal nests, is reduced by half compared to WTs [53]. Furthermore, in juvenile play tests and adult three-chamber social interaction assays,

Cntnap2$^{-/-}$ mice show less interest in conspecifics. Rather than engaging socially, they exhibit avoidance and engage in repetitive behaviours, such as grooming. *Cntnap2*$^{-/-}$ mice groom three times as often as WT mice, which suggests heightened anxiety and an inclination toward repetition. This preference for repetitive behaviours over social interaction is a core feature of ASD in humans [53].

These results highlight the relevance of *Cntnap2*$^{-/-}$ mice for studying the behavioural and neurobiological underpinnings of ASD, particularly in relation to sensory abnormalities, social impairments, behavioural inflexibility, and communication deficits.

1.4.2 VPA rat model

A teratogenic drug, thalidomide, is associated with physical abnormalities and has been linked to ASD, though not to the same extent as VPA. Thalidomide crosses the placenta, leading to neurobehavioral issues, as well as ear and limb malformations in children [184]. A 1994 study of 100 embryonic thalidomide cases revealed that 30% of children exposed during gestation days 20–24 developed ASD [185]. This exposure period coincides with neural tube closure and the onset of neuronal development in the brainstem motor cranial nerves. Thalidomide exposure during this time resulted in cranial nerve abnormalities, hypoplasia in the brainstem, reduced volume in the posterior cerebellar vermis and hemispheres due to Purkinje cell loss, and injury to the deep cerebellar nuclei [177]. These effects are strikingly similar to the neurotoxic effects of VPA. The link between thalidomide exposure during neural tube closure informed the use of VPA exposure during this critical developmental period (E12.5) in the first behavioural characterization of the VPA animal model [177].

The VPA rodent model demonstrates high construct, face, and predictive validity. Construct validity is confirmed by the model's replication of human neurotoxic mechanisms, including neuroinflammation, E/I imbalance, hyperserotonaemia, and HDAC inhibition, all observed in the human brain exposed to VPA [186]. Face validity is supported by the comprehensive characterization of behaviour, physiology, and development in VPA-exposed animals. Behavioural abnormalities include lowered acoustic prepulse inhibition, stereotypic locomotion, repetitive behaviours, increased latency in social interactions, and a decreased total number of social behaviours.

Physiological and developmental deficits include delayed maturation, lower body weight, impaired motor development, delayed reflex coordination, and abnormal olfactory discrimination. These abnormalities mirror symptoms observed in human ASD [177]. Finally, the predictive validity of this model is high, as pharmacological treatments that produce therapeutic effects in the model show similar efficacy in humans with ASD. This underscores the utility of the VPA rodent model for studying ASD and testing potential interventions [186]. The validity overview of this model is also demonstrated diagrammatically in Figure 1-2.

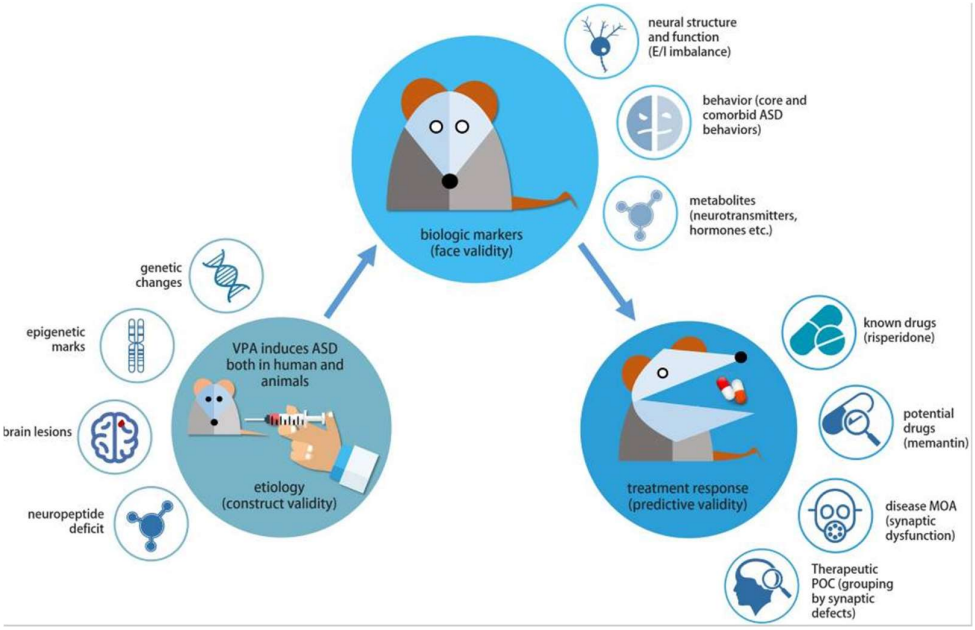

Figure 1-2 Validity of the VPA animal model overview (reproduced from [110] under the terms of creative commons attribution non-commercial license).

1.5 Aberrant feeding in ASD

Children with ASD are five times more likely to develop feeding issues than neurotypical children, with the median of ASD children experiencing these challenges sitting at 62% [6]. Aberrant feeding behaviours affect the majority of individuals with ASD and is one of the first identifiable a-specific symptoms in undiagnosed children alongside sleeping problems, both of which can be identified before two years of age [30].

Extensive research has gone into understanding the relationship between aberrant food intake and ASD [4, 30, 187-194]. It has been identified that core ASD traits such as restrictive/repetitive behaviours, insistence on sameness and impaired social interactions all contribute to the formation of maladaptive feeding [6]. These food-related difficulties are referred to as feeding issues, emphasizing the interactional aspect of feeding compared to the physical act of eating. This distinction is particularly relevant in young children, where obtaining food typically involves parental interaction [194]. Given that ASD impairs social interaction, it is intuitive that the feeding dynamic in children with ASD may differ from that of neurotypical children. Children with ASD may not mimic feeding behaviours modelled by their parents and may appear disengaged during mealtimes [6].

Feeding issues is a broad term encompassing a number of different issues regarding food intake, which are outlined in Table 1-1 [6, 189, 194, 195]. These issues can be broken down into two main categories, food selectivity and eating style [6, 189]. Food selectivity encompasses behaviours regarding selection of food whereas eating style is in regard to the way the child mechanistically eats [6, 189]. Selective intake is the most prevalent feeding issue related to ASD, this is colloquially termed 'fussy eating' [6]. There are other issues which contribute to food selection including sensory issues, neophobia, limited appetite and food refusal [6, 189, 194].

Sensory issues were first described as tactile defensiveness in children that had learning and behavioural issues in 1964 [196]. Sensory abnormalities are one of the central symptoms in ASD and contribute to overstimulation and significantly correlate with restrictive/repetitive behaviour, anxiety, self-harm behaviour and GI issues [197]. These sensory issues are linked with abnormalities in brain connectivity, particularly in the brainstem, cerebellum, and in networks between primary sensory systems to association areas [80, 183]. Sensory abnormalities are considered an overreaction to certain tactile stimuli which do not elicit the same response in neurotypical individuals. It is suggested by both researchers and individuals with ASD that feeding issues may be related to this. Food aversion due to tactile displeasure is commonly observed and can manifest as an aversion to specific food textures or other tactile stimuli associated with eating or related activities, such as brushing teeth [198].

A study examining the dietary variety of children with ASD confirmed that their diets often lack diversity and include fewer food types compared to their neurotypical family

members. The findings showed that many children with ASD frequently consumed highly palatable and rewarding foods such as chicken nuggets, cake, French fries, and ice cream, while very few accepted vegetables. Additionally, their range of fruit intake was limited, although grapes and apples were notably more popular [199]. This dietary pattern reflects a preference for foods high in simple carbohydrates and fats, which are calorie-dense but nutritionally poor, over foods rich in fibre, nutrients, and complex carbohydrates.

Deficiencies in essential nutrients, including pantothenic acid, biotin, folate, vitamin B12, vitamin D, and vitamin E, have been reported among individuals with ASD. Interestingly, some children with ASD exhibit abnormally elevated levels of vitamin B6, suggesting potential dysregulation of pyridoxal kinase, the enzyme that converts pyridoxal and pyridoxine into pyridoxal-5'-phosphate (P5P). During this conversion, vitamin B6 is utilized. P5P is a critical cofactor for numerous enzymatic reactions, including those involved in neurotransmitter synthesis. Impaired activity of pyridoxal kinase may exacerbate abnormal brain functioning by disrupting these key biochemical processes [200].

Eating style poses a separate set of risk factors for feeding in children with ASD. Rather than resulting in a limited diet with anthropometric and dietary impacts, there are safety concerns. Concerning eating styles include eating too fast, too much, the inability to self-feed and pica [189].

Although feeding selectivity and eating style do result in separate problems such as body weight and safety concerns, they both contribute to some of the same issues, which are summarised in Table 1-2. These issues being disruptive behaviour at mealtimes (which leads to stress for parents and other family), and the most significant problem related to feeding issues; a failure to thrive due to lack of proper nutrition, safety concerns and strained mealtimes. Selective intake, sensory issues, neophobia, limited appetite and food refusal all converge on an overall limited diet. This is why it is of utmost importance that aberrant feeding is addressed for children with ASD, and that pharmacological treatments are investigated as an option for these affected children so that they are able to thrive.

Table 1-2 Different types of feeding issues associated with ASD [6, 113, 118, 119, 122]

Feeding Issues	
Food Selectivity	**Eating Style**
• Selective intake • Sensory issues • Avoids foods with certain textures or smells • Neophobia • Limited appetite • Food refusal	• Eats too quickly • Eats too much • Eats non-food items • Lacks ability to self-feed
Leads to	
• Risk of nutrient deficiencies	• Safety concerns such as choking.
Attenuation of disruptive behaviors around meals Lower BMI than typically developing children Stressful mealtimes Failure to thrive	

1.6 Oxytocin, ASD and aberrant feeding

Oxytocin (OT) is a nonapeptide synthesized in the hypothalamus, specifically within the supraoptic nucleus (SON) and paraventricular nucleus (PVN) [201-203]. In these regions, magnocellular neurons produce OT and transport it to the posterior pituitary via neuronal projections. Once in the pituitary gland, OT is secreted into the bloodstream, where it performs various peripheral functions traditionally associated with parturition, lactation, and the milk-ejection reflex [201]. OT exerts its effects through interaction with the oxytocin receptor (OT-R), a G-protein coupled receptor [202].

OT signalling extends beyond peripheral functions; magnocellular neurons also release OT within the brain via somatodendritic projections [204]. In addition to the magnocellular neurons, the PVN contains parvocellular neurons, which do not project to the pituitary gland but instead target various brain regions. These projections extend to the spinal cord, caudal brainstem, amygdala, and substantia nigra, influencing a wide range of neural processes [201].

These parvocellular projections are particularly relevant for OT's role in feeding regulation, as they innervate critical brain regions involved in feeding behaviours. In the brainstem, OT targets the dorsal vagal complex (DVC), which includes the area postrema (AP), nucleus of the solitary tract (NTS), and dorsal motor nucleus of the vagus (DMNV), as well as the parabrachial nucleus (PB). These regions play key roles in modulating feeding behaviour by relaying peripheral signals, such as those for aversion, GI distension, and osmolarity, to the CNS. OT acts as an anorexigenic neuropeptide, contributing to the homeostatic inhibition of feeding through its signalling pathways in these areas [205]

The AP, which is not protected by the BBB, participates in many autonomic functions within the CNS. Its unique location allows it to monitor blood-borne toxins and physiological changes, making it integral to processes such as taste aversion and nausea [206]. The NTS processes signals from the gut via the vagal nerve, serving as a key hub for gut-brain communication [207]. Additionally, the DMNV houses vagal preganglionic neurons, which regulate gastrointestinal functions [208].

The innervation between the PVN and brainstem regions, including the AP, NTS, and DMNV, is reciprocal. This bidirectional communication ensures that information gathered by the brainstem, such as signals from the vagus nerve or detection of blood toxins, is relayed back to the PVN. This communication enables coordinated regulation of autonomic and feeding behaviors. Furthermore, parvocellular neurons also innervate areas of the forebrain such as the ventral tegmental area (VTA), amygdala, ventromedial nucleus of the hypothalamus (VMH), dorsal raphe nucleus (DR), BNST, and Acb [205]. Research demonstrates that OT specifically inhibits reward-driven consumption rather than hunger-driven consumption. In a study by Ott *et al*, human subjects were administered OT and observed during two distinct eating scenarios. OT administration did not reduce consumption during a buffet-style breakfast following fasting and had no significant effect on hunger levels. However, in a postprandial state, when subjects were offered snacks, OT significantly reduced total snack intake, highlighting its role in modulating reward-driven food intake [209].

OT is known to influence a wide range of behaviours, from promoting satiety to facilitating the interpretation of emotions through facial expressions [201, 210]. It plays a critical role in the evolution of complex social cognition and behaviour in mammals

[211]. Given its established role in social cognition, OT has been extensively studied as a potential therapeutic intervention for common behaviours associated with ASD [212-216].

Research has shown that OT administration can reduce repetitive behaviours, improve speech comprehension, increase eye contact, strengthen social interactions, enhance feelings of trust, and heighten attention to social stimuli compared to non-social stimuli [212-216]. However, a study by Sikich *et al* demonstrated that chronic OT administration did not lead to improvements in social functioning in individuals with ASD [217]. This discrepancy arises from OT's mechanism of action: rather than directly correcting social behaviour, OT enhances the salience of social stimuli and supports neural processing, enabling better responses to such stimuli. In the absence of meaningful social interactions and stimuli, as was the case in the study by Sikich *et al*, no improvements were observed because the experimental design relied solely on behavioural questionnaires without providing a social context [218].

Evidence indicating a reduction in palatable food intake and improvements in abnormal social and repetitive/restrictive behaviours in ASD, demonstrate the impact of OT on food intake in individuals with ASD emerges as a key area of interest for this thesis.

1.7 Aims

ASD is highly heterogeneous in both its aetiology and symptomatology, yet feeding issues are often among the earliest atypical behaviours exhibited by children with ASD. In fact, children with ASD are five times more likely to develop feeding issues than neurotypical children [6]. Restrictive and repetitive behaviours, insistence on sameness, and impaired social interactions are likely contributors to these feeding challenges [6, 194]. However, the underlying brain activation patterns and neural pathways related to abnormal food intake in ASD remain poorly understood.

Feeding behaviour is regulated by a complex interplay of intrinsic and extrinsic factors that influence hunger (motivation to seek caloric sources), satiety (feelings of fullness that terminate feeding), and reward processing (determination of food's pleasantness, often skewing preferences towards highly palatable foods over more nutritious ones).

Observational studies of children with ASD reveal a tendency to prefer a narrow range of simple, nutrient-poor foods—such as chicken nuggets, cakes, and ice cream [199]. This suggests a possible dysfunction in signalling within reward pathways, which may override homeostatic satiety mechanisms.

Additionally, many autistic individuals experience significant anxiety around trying new foods, leading to food refusal and challenges during mealtimes for both the individual and their families. Gaining a better understanding of the mechanisms driving food neophobia and strict dietary preferences in ASD could improve nutritional interventions and help ensure that autistic individuals receive adequate nutrition for their overall health and well-being.

The **overarching goal** of this thesis was to explore feeding behaviours and their underlying neuromolecular mechanisms in animal models of ASD, specifically in VPA-exposed rats and *Cntnap2*$^{-/-}$ mice. The following specific aims address this central objective.

The **first specific aim** was to evaluate how *Cntnap2*$^{-/-}$ mice respond to palatable foods, focusing on their feeding behaviours and associated changes in brain activation.

The **second specific aim** explored whether overconsumption of palatable sugar established in *Cntnap2*$^{-/-}$ male mice is evident also in females throughout the oestrus cycle.

Neophobia and food refusal are common challenges for autistic individuals, often resulting in severely limited dietary diversity. The **third specific aim** explored hyponeophagia in *Cntnap2*$^{-/-}$ mice and examined the associated brain activation patterns.

Given the role of OT in the acquisition of conditioned taste aversion (CTA) responses [219] and evidence of underlying OT deficiency in ASD VPA rats [220], the **fourth specific aim** of this thesis investigated the acquisition of a CTA response in VPA rats compared to unaffected controls following administration of lithium chloride (LiCl), a nausea-inducing agent.

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Chapter 2

Palatable solution overconsumption in the male *Cntnap2*$^{-/-}$ murine model of autism: a link with oxytocin

2.1 Abstract

Dysregulated appetite is common in autism spectrum disorder (ASD), and it includes excessive interest in tasty foods. Overconsumption of palatable fluids has been found in the valproate induced ASD rat. Though ASD has a strong genetic component, the link between ASD related genes and appetite for palatable foods remains elusive. This study focused on the *Cntnap2* gene whose deletion in mice recapitulates human ASD symptoms. We investigated whether *Cntnap2*$^{-/-}$ male mice consume greater amounts of palatable 10% sucrose, 0.1% saccharin, and 4.1% intralipid solutions offered in episodic meals either in a no-choice paradigm or a two-bottle choice test. We examined how sucrose intake affects c-Fos immunoreactivity in feeding-related brain areas. Finally, we determined doses at which intraperitoneal oxytocin (OT) decreases sucrose intake in mutants. In the single-bottle tests, *Cntnap2*$^{-/-}$ mice drank more sucrose, saccharin, and intralipid compared to wild-types (WTs). During a two-bottle choice test between sucrose and intralipid, *Cntnap2*$^{-/-}$ mice consumed more sucrose that WTs. While the standard 1 mg/kg OT dose reduced sucrose intake in WTs, a low OT dose (0.1 mg/kg) decreased sucrose intake in *Cntnap2*$^{-/-}$ mice. Sucrose intake induced a more robust c-Fos response in WT than *Cntnap2*$^{-/-}$ mice in the reward and hypothalamic sites and it increased the percentage of Fos-immunoreactivity OT neurons in WTs, but not in mutants. We conclude that *Cntnap2*$^{-/-}$ mice overconsume palatable solutions, especially sucrose, beyond levels seen in WTs. This excessive consumption is associated with blunted c-Fos

immunoreactivity in feeding-related brain sites, and it can be reversed by low-dose oxytocin.

2.2 Introduction

Autism spectrum disorder (ASD) is manifested by changes in social interaction, communication, and anxiety, to name a few. Its aetiology includes *in utero* effects of toxins and drugs as well as a strong genetic component [1]. *CNTNAP2* encoding a transmembrane neuronal protein of the neurexin family, is one of the most investigated genes in ASD [2]. It facilitates K+ channel clustering in the nodes of Ranvier, synapse formation, and neuron–neuron and neuron–glia interactions [2, 3]. In a family with a homozygous mutation in this gene, a link between *CNTNAP2* and ASD was associated with a high prevalence of ASD symptoms, for example, cortical dysplasia focal epilepsy, language regression, hyperactivity, and intellectual delay [3]. Multiple reports have corroborated the early findings and defined CNTNAP2's role in frontotemporal-subcortical circuits governing executive functions and language acquisition [4].

Cntnap2 deletion in animals recapitulates symptoms in humans. *Cntnap2*$^{-/-}$ mice display social maladaptations, abnormal vocalizations, behavioural inflexibility, and stereotypies. The neuropathology in these mice includes changes in neuronal migration, a lower number of interneurons, and desynchrony of neuronal networks [2].

While the core ASD behavioural domains are well documented in *Cntnap2*$^{-/-}$ mice, effects of this gene's deletion on appetite have not been well studied. A limited focus on feeding is understandable considering the immediate impact of social, intellectual, and communication deficits in ASD. However, abnormal appetite merits attention as it affects developmental, neuroendocrine, and behavioural processes. People with ASD are five

times more likely to display eating abnormalities, for example, excessive interest in palatable foods and narrow preferences, leading to changes in body weight and nutrient deficiencies [5, 6]. It has been reported that the valproate (VPA) induced ASD rats overconsume palatable foods and fluids. Also, expression of reward-related genes and opioid system activity differ between VPAs and controls [7]. In VPA rats, elevated intake of sucrose is reduced by injections of oxytocin (OT), a neuropeptide whose dysregulated tone underlies ASD symptomology [8].

It remains to be elucidated whether excessive appetite for palatable solutions extends beyond VPA-induced ASD to animals whose ASD is due to the *Cntnap2* gene. Interestingly, while *Cntnap2*$^{-/-}$ and wild-type (WT) mice fed standard chow do not differ in feeding or body weight, a mild mutation in the *Cntnap2* gene combined with other genomic changes can affect body weight in high-fat diet-fed animals [9].

We investigated whether *Cntnap2*$^{-/-}$ male mice consume greater amounts of palatable 10% sucrose, 0.1% saccharin, and 4.1% intralipid solutions offered in episodic meals (in a no-choice single-bottle test or a two-bottle choice test). As sucrose was then identified as the most avidly overconsumed tastants by the mutants, we investigated how sucrose intake affects c-Fos immunoreactivity in feeding-related brain areas and the percentage of c-Fos positive OT neurons in *Cntnap2*$^{-/-}$ vs. WT mice. Finally, we examined whether intraperitoneal oxytocin at doses ineffective at reducing feeding in WTs, decreases sucrose solution intake in mutants.

2.3 Methods

2.3.1 Animals

Cntnap2$^{-/-}$ mice (JAX #017482) and their WT background strain were bred through homozygous crosses. They were housed individually in Plexiglas cages with wire tops at 22 °C with a 12:12 L:D cycle (lights on at 07:00) and had *ad libitum* access to standard chow (Sharpes, Carterton, New Zealand) and water unless stated otherwise. Animals were weighed weekly and there were no differences in body weight, daily standard chow [0.21 ± 0.02 g/g b.wt. (WT) and 0.20 ± 0.01 g/g b.wt. (*Cntnap2*$^{-/-}$)] or water intake [0.22 ± 0.02 g/g b.wt. (WT) and 0.22 ± 0.04 g/g b.wt. (*Cntnap2*$^{-/-}$)] between the genotypes. Age-matched adult male mice [>PND 79, at 23.8 ± 0.6 g (WT) and 23.8 ± 0.5 g (*Cntnap2*$^{-/-}$) at the beginning of the studies] were included in the experiments. The experiments adhered to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals, and they were approved by the University of Waikato Ethics Committee in protocol 1138.

2.3.2 Episodic consumption of palatable solutions

2.3.2.1 Single bottle paradigm

We assessed the episodic (1-h) consumption of palatable solutions in *Cntnap2*$^{-/-}$ and WT mice not deprived of energy or water prior to the palatable fluid presentation. The paradigm was based on our previously published studies [10]. Mice were accustomed to receiving 10% sucrose, 0.1% saccharin, or 4.1% intralipid for 1 h/day on 3 days prior to the experiment to avoid neophobia. On the experimental day, mice were given access to one of the solutions from 10:00 to 11:00. Chow and water were removed for the 1-h test. Solution intake was measured by weighing bottles. For each palatable tastant, separate cohorts of 16 WT and 16 *Cntnap2*$^{-/-}$ mice were used.

2.3.2.2 Two-bottle preference test

WT and *Cntnap2*$^{-/-}$ mice pre-exposed to sucrose, saccharin, and intralipid were presented with the choice of two tastants from 10:00 to 11:00. We tested the effect of the genotype on the preference for sucrose vs. intralipid, sucrose vs. saccharin, and saccharin vs. intralipid. Each of the two-bottle tests included separate cohorts of 11 *Cntnap2*$^{-/-}$ and 11 WT mice.

2.3.2.3 Effect of oxytocin on sucrose and intralipid intake

Cntnap2$^{-/-}$ and WT mice were given 1-h access to a 10% sucrose (pre-exposure as described above). Water and chow were removed for the duration of the test. Fifteen minutes prior to sucrose presentation, animals were injected intraperitoneally with saline (n = 8–10/ genotype) or OT (Sigma, St. Louis, USA) at 0.1 mg/ kg (n = 8–10/genotype), 0.3 mg/kg (n = 8–9/genotype), or 1.0 mg/kg (n = 8–9/genotype). The same protocol was used to assess the effect of intraperitoneal oxytocin on 4.1% intralipid intake; animals were injected intraperitoneally with saline (n = 8/group) or 1 mg/kg oxytocin (n = 8–9/genotype).

2.3.2.4 Neuronal activation after sucrose intake

WT and *Cntnap2*$^{-/-}$ animals (the same cohort that had been used in the sucrose study in 'Single-bottle paradigm' section; however, a 'washout' period of 2 weeks elapsed between the two experiments) had chow and water taken away just before being given 10% sucrose for 1-h. Mice given water instead of sucrose were controls: they did not drink. One hour following the removal of sucrose, the animals were anesthetized with 35% urethane and perfused with saline followed by 50 ml 4% paraformaldehyde in PBS. The excised brains were postfixed for 48 h in paraformaldehyde. Coronal 60-µm sections were sliced using a vibratome (Leica, Frankfurt, Germany) and processed as free-floating

sections. Sections were incubated in 10% methanol and 3% H_2O_2 (in tris-buffered saline [TBS]; 10 min). Following a TBS rinse, they were incubated in the rabbit-anti-Fos antibody (Synaptic Systems, Brisbane, Australia) in 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS overnight at 4 °C. The sections were rinsed in TBS and incubated for 1 h in the goat-anti-rabbit antibody (Vector, Burlingame, USA). After TBS rinses, they were incubated in the avidin-biotin complex (Vector, USA) for 1 h. The colour reaction was initiated with 0.05% diaminobenzidine, 0.01% H_2O_2, and 10% nickel sulfate in TBS (15 min). Sections were mounted on gelatin-coated slides, air dried, dehydrated in ethanol, soaked in xylene, and cover slipped in Entellan (Sigma, Darmstadt, Germany). To visualize c-Fos and OT, Fos-stained sections underwent the protocol utilizing the primary rabbit-anti-oxytocin antibody (Chemicon, Tamacula, USA). Nickel sulfate was omitted from the DAB step to achieve brown staining. Images were captured under a Nikon H55OS (Nikon Instruments, Melville, USA) microscope equipped with an OMAX camera (Omax, Kent, USA). The regions of interest, outlined using the Allen Brain Atlas (ABA; anterior: posterior bregma ranges in the parentheses), were: AcbC – nucleus accumbens core (1.28 : 0.96); AcbS – Acb shell (as AcbC); ARC – arcuate nucleus (−2.14: −2.52); BLA – basolateral amygdala (−2.64: −2.92); CEA – central nucleus of the amygdala (−2.64: −2.92); DMH – dorsomedial nucleus (−2.80: −3.24); DMNV – dorsal motor nucleus of the vagus (−13.76: −14.16); NTS – nucleus of the solitary tract (−13.70: −14.16); PVN – paraventricular nucleus (−1.56: −1.92); SON – supraoptic nucleus (−0.96: −1.20); VMH – ventromedial nucleus (−2.80: −3.24); and VTA – ventral tegmental area (−6.72: −6.84). Each region was outlined as per ABA and its area measured in mm2 (ImageJ, NIH, Bethesda, USA). Fos immunoreactivity nuclei were counted on three to four sections per region per animal. The numbers were added for each region in each animal and densities of nuclei per mm2 were calculated. In Fos-OT analysis, four PVN sections containing oxytocin cells were used in each animal. We

assessed the number of oxytocin neurons and the number of oxytocin cells colocalizing with Fos. The % of Fos immunoreactivity oxytocin cells was calculated per animal and averaged for each experimental group.

2.3.3 Statistical analysis

Normality of data was confirmed with the Shapiro–Wilk test. Intakes of each individually presented tastant in WT vs. mutants were compared using a t-test. The effect of genotype on tastant preference was studied with two-way ANOVA. Effects of oxytocin at different doses on palatable tastant intake were compared within each genotype with ANOVA followed by the Dunnett's post-hoc test (saline animals were controls). Densities of nuclei per mm2 per region and % of PVN c-Fos-oxytocin cells were compared with a two-way ANOVA. Significance was set at $P \leq 0.05$.

2.4 Results

In the no-choice single-bottle studies, *Cntnap2*$^{-/-}$ mice drank more sucrose ($P = 0.0001$), saccharin ($P = 0.006$), and intralipid ($P = 0.014$) than WTs (Fig. 2-1a–c). When mice were given a choice between two tastants, *Cntnap2*$^{-/-}$ mice had a higher preference for sucrose vs. intralipid (ANOVA $F(1,40) = 19.07$, $P = 0.00009$, genotype × tastant interaction), whereas the sucrose vs. saccharin and intralipid vs. saccharin preference did not differ between the genotypes (Fig. 2-1d–f).

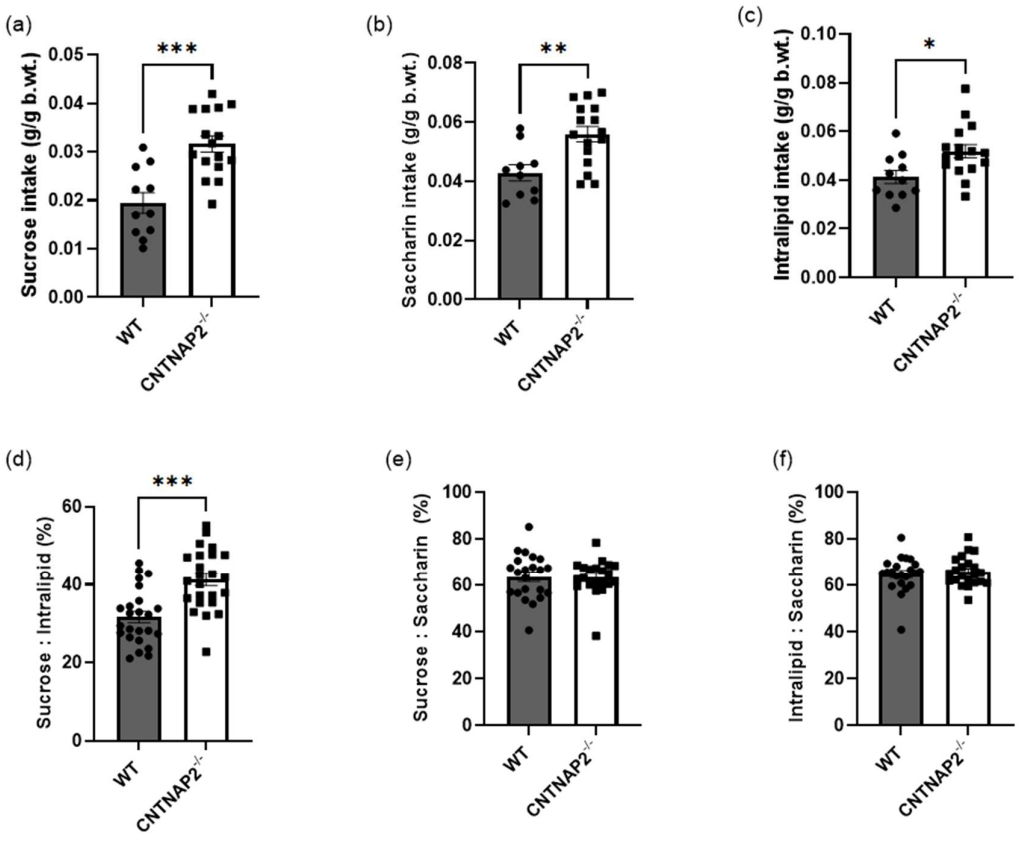


Figure 2-1 Episodic intake of (a) 10% sucrose, (b) 0.1% saccharin, and (c) 4.1% intralipid solutions presented in a no-choice test or as a two-bottle choice test of (d) sucrose vs. intralipid, (e) sucrose vs. saccharin, and (f) intralipid vs. saccharin in WT and *Cntnap2*$^{-/-}$ mice. Animals had not been deprived; chow and water were removed only for the meal-time. (a-c) n = 16/strain, (d-f) n = 11/strain. Data are shown as mean ±SEM. *P < 0.05; **P < 0.01, ***P < 0.001. WT, wild-type

OT at 1 mg/kg reduced sucrose intake in WTs (P = 0.005; F(3,28) = 6.257), whereas 0.1 mg/kg (P = 0.002), 0.3 mg/kg (P = 0.00007), and 1.0 mg/kg (P = 0.00009) decreased sugar consumption in mutants (ANOVA F(3,34) = 12.65, P = 0.00001). Intralipid intake was not affected in either genotype by 1 mg/kg OT (Fig. 2-2).

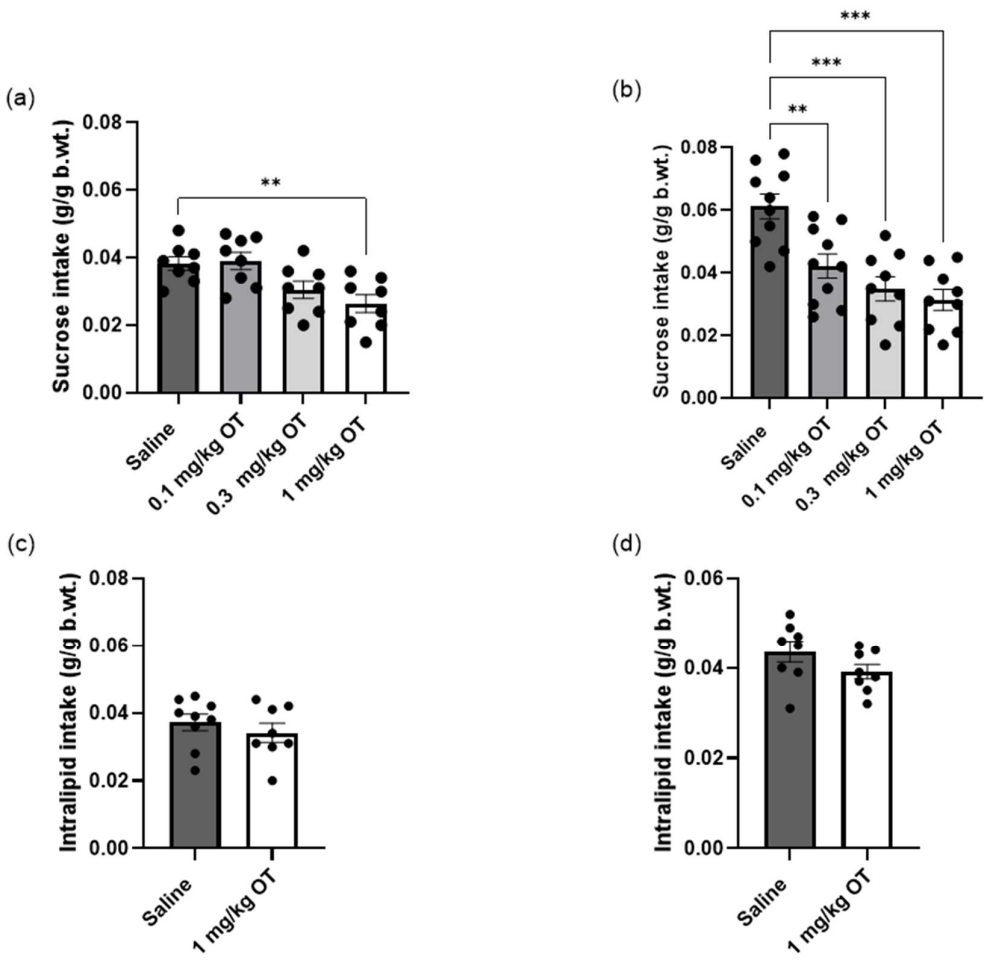
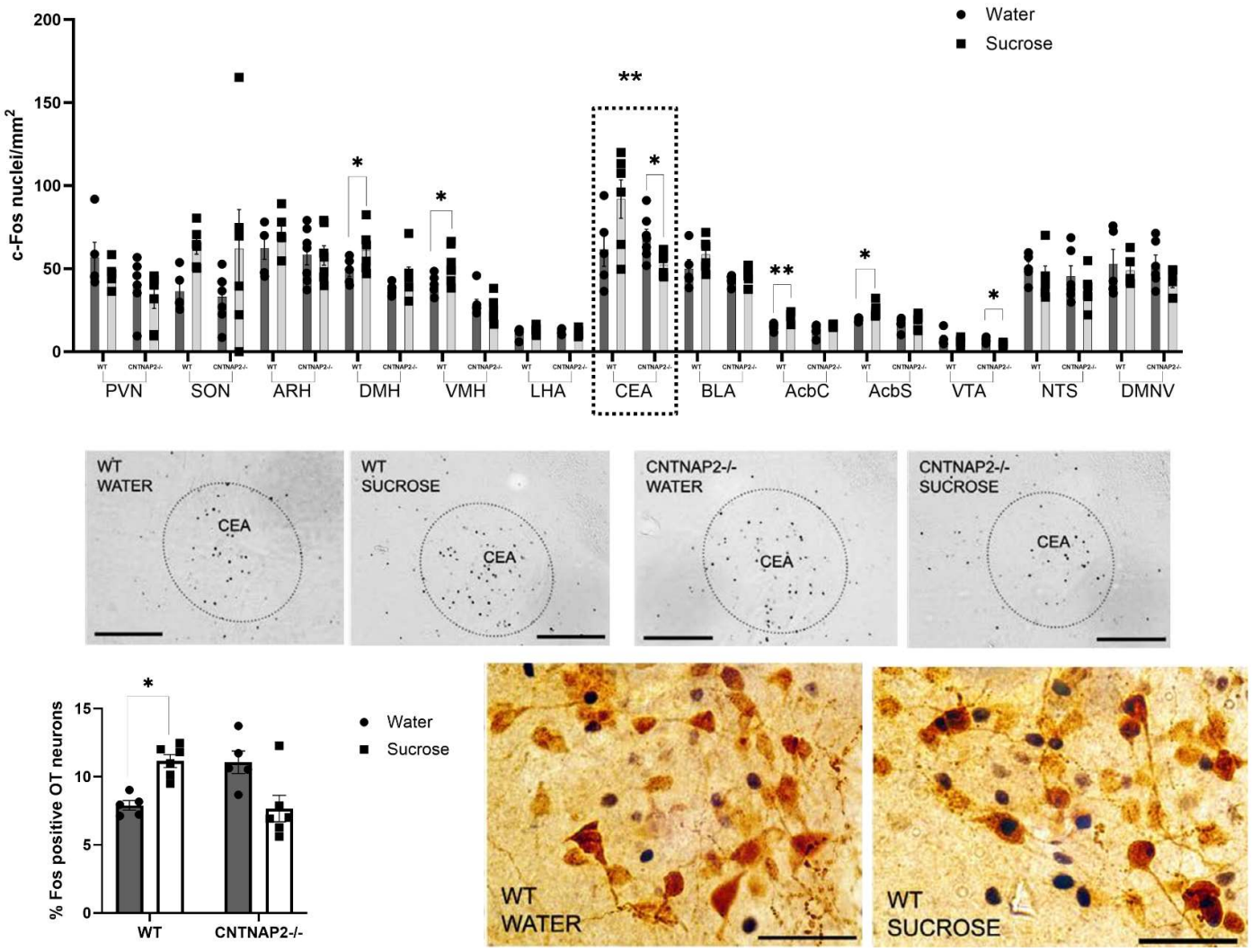


Figure 2-2 Effect of intraperitoneal oxytocin on 10% sucrose and 4.1% intralipid intake in WT (a and c) and *Cntnap2*$^{-/-}$ (b and d) mice. n= 8-10 per dose per strain. Data are shown as mean ±SEM. **P < 0.01; ***P < 0.001. WT, wild-type.

A two-way ANOVA analysis of c-Fos immunoreactivity in WT and *Cntnap2*$^{-/-}$ mice (Fig. 2-3) showed an effect of the treatment (sucrose vs. water control) in the SON (P = 0.034, F(1,6) = 7.446) and AcbC (P = 0.0062, F(1,5) = 20.54), whereas the genotype effect was noted in the DMH (P = 0.029, F(1,6) = 23.38), VMH (P = 0.004, F(1,6) = 19.80), BLA (P = 0.009, F(1,19) = 8.38), AcbC (P = 0.013, F(1.5) = 14.33), and AcbS (P = 0.019, F(1,5) = 11.69). A significant treatment × genotype interaction was found in the CEA = 0.007, F(1,20) = 8.889). In WTs, sucrose consumption increased c-Fos immunoreactivity in the VMH (P = 0.011), DMH (P = 0.048), AcbS (P = 0.033), and AcbC (P = 0.002),

whereas in the mutants, Fos immunoreactivity was lower in the CEA (P = 0.012) and VTA (P = 0.033). There was a significant increase in the percentage of Fos-



immunoreactivity oxytocin neurons in WTs after a sucrose meal (P = 0.017), but there was no effect in *Cntnap2*$^{-/-}$ mice. The treatment × genotype interaction was significant at P = 0.029, F(1,3) = 15.57.

Figure 2-3 Effect of 10% sucrose intake on (a) c-Fos in feeding-related brain areas and (b) the percentage of c-Fos immunoreactivity PVN oxytocin neurons in WT and *Cntnap2*$^{-/-}$ mice. Animals given water were controls. The box around the CEA defines a significant treatment × genotype interaction. n = 5-6 per group. Data are shown as mean ±SEM. *P < 0.05; **P < 0.01; scale bar = 250 μm (a) and 50 μm (b). CEA, central nucleus of the amygdala; PVN, paraventricular nucleus; WT, wild-type.

2.5 Discussion

Surprisingly little research has been done to characterize eating behavioural abnormalities in ASD [5]. Questionnaire studies and anecdotal evidence suggest that abnormal eating behaviours span narrow flavour preferences, insistence on sameness, texture preferences, refusal of non-preferred diets, and neophobia [6, 11]. Caregiver-driven dietary modelling is limited, partially due to social behavioural maladaptations [6, 12]. This limited diet diversity is conducive to weight loss or gain and nutritional deficiencies [12]. Our results support the notion that enhanced responsiveness to palatability contributes to dysregulated eating behaviour in ASD. In human studies, ASD children have been reported to avoid bland foods and overconsume palatable tastants rich in sweet carbohydrates and fat [11, 13]. In animal experiments, rats with VPA-induced ASD consume excessive amounts of milk and sugar water [8]. The current dataset shows that animals in which ASD stems from inactivating the *Cntnap2* gene ingest very high amounts of palatable fluids. In a single-bottle feeding test, *Cntnap2*$^{-/-}$ mice consumed more of each of the palatable solutions compared to WTs. It occurred irrespective of energy density of the liquids (saccharin is noncaloric; 10% sucrose and 4.1% intralipid are isocaloric) or their nutrient composition. It should be noted that mutants and WTs do not differ in chow or water consumption, which indicates that palatable solution intakes in mutants were not driven by thirst or calories. What is striking in the single-bottle test is that *Cntnap2*$^{-/-}$ mice drank about 70% more sucrose than WTs, whereas intralipid and saccharin intakes were about 15%–20% higher than in WTs. The drive to ingest sucrose is also evident in the two-bottle study: *Cntnap2*$^{-/-}$ given a choice of intralipid vs. sucrose drank proportionally about 25% more sugar than WTs. It signifies a functional relationship between *Cntnap2*$^{-/-}$ and appetite for sugar and somewhat resembles the effect of OT receptor blockade [14, 15] or OT knockout [16, 17], which increase intake

of sucrose and preference for sucrose vs. intralipid. Since the OT tone is dysregulated in the *Cntnap2*$^{-/-}$ gene mutation [18] it is not surprising that sucrose intake is particularly affected in *Cntnap2*$^{-/-}$ mice.

PVN OT neuronal activation coincides with sucrose intake termination [10] and this activity is reduced by drugs that promote ingestion of sweet solutions, for example, butorphanol and morphine [10]. In line with the notion of oxytocin's involvement in sucrose overconsumption in *Cntnap2*$^{-/-}$, we found an unchanged percentage of Fos-immunoreactivity OT neurons in mutants after sucrose ingestion. It is plausible that the diminished activity of the OT system permits more robust sugar intake despite stomach distention and plasma osmolality, thus the changes that promote OT-driven feeding cessation. It reflects the dysregulation of the OT system [16] also in the context of feeding. In the injection study, we found that while in WTs 1 mg/ kg oxytocin decreased sucrose solution intake, a 10 times lower dose was effective in *Cntnap2*$^{-/-}$ mice. Thus, even very low oxytocin doses rescue sugar solution overconsumption in the ASD mutant. This result parallels the earlier findings in VPA rats in which low-dose OT prevented palatability-driven overfeeding [8]. Considering the broad palatability-induced overfeeding phenotype in *Cntnap2*$^{-/-}$ mice, the concurrent contribution of disrupted neural systems other than OT is highly probable. While intralipid intake is known to be unaffected by OT (shown also in our injection study in WTs and mutants), and saccharin consumption is poorly modified by oxytocin, opioids, endocannabinoids, and dopamine enhance intake of not just sucrose, but also fat and noncaloric sweeteners [19]. Hence ASD associated dysfunction in one or more of these systems possibly underpins the heightened drive to ingest palatable solutions [20, 21]. c-Fos immunoreactivity differences in reward-related and energy homeostasis-related brain sites between WT and mutants indicate that the ASD phenotype stems from a large-scale dysregulation of neural processing. In WTs,

sucrose consumption led to an increase in c-Fos immunoreactivity in the AcbC and AcbS as well as a trend in the CEA and BLA, areas involved in feeding reward. In line with that, Figlewicz *et al* reported that sucrose self-administration in operant settings in rats increases c-Fos immunoreactivity in the accumbal region [22]. Intragastric sucrose and sucrose sham feeding increase c-Fos in the Acb and amygdala. This change in activation is attributed to elevated dopamine release [23, 24]. Interestingly, in *Cntnap2*$^{-/-}$ mice, sucrose intake did not produce a c-Fos immunoreactivity change in either the AcbC, AcbS, or BLA. In the CEA, mutants had lower c-Fos levels after sugar intake, thus the activity change was opposite to that seen in WTs (a significant treatment × genotype interaction was identified in the CEA). Hypothalamic c-Fos response was more pronounced in WTs (albeit not very high overall, likely due to relatively modest sucrose intake levels in non-deprived animals), with the DMH and VMH showing significant increases in activity.

2.6 Conclusions

We conclude that *Cntnap2*$^{-/-}$ mice, an animal model of ASD, overconsume palatable solutions, especially sucrose, beyond levels seen in WTs. This excessive consumption, associated with blunted activity in the feeding-related brain sites, can be reversed by low-dose intraperitoneal OT.

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Chapter 3

Overconsumption of sucrose in female *Cntnap2*$^{-/-}$ mice and the influence of the oestrus cycle on feeding

3.1 Abstract

Autism spectrum disorder (ASD) disproportionately affects males, with a commonly cited 4:1 male-to-female diagnostic ratio. While the symptoms are similar in both sexes, females show 'masking' traits that , to some extent, diminish detectability of the disorder. While feeding issues are also prevalent in ASD females, whether the enhanced drive to overconsume palatable tastants remains underexplored. While chapter 2 demonstrated sucrose overconsumption in male *Cntnap2*$^{-/-}$ mice, this chapter investigated whether similar behaviour occurs in females and assessed the potential influence of the oestrus cycle. Our findings indicate that female *Cntnap2*$^{-/-}$ mice exhibit significant overconsumption of sucrose compared to WT controls. This behaviour persists across all oestrus phases, with difference 24-h chow or sucrose intake linked to hormonal fluctuations. Additionally, no differences in hunger-driven food intake or refeeding rates were observed between WT and *Cntnap2*$^{-/-}$ mice. Interestingly, *Cntnap2*$^{-/-}$ females consumed less water than WT controls, demonstrating that their sucrose overconsumption is not driven by excessive thirst. These results establish that sucrose overconsumption occurs in female *Cntnap2*$^{-/-}$ mice and remains consistent across all oestrus cycle phases. This study expands our understanding of ASD-associated feeding behaviours in female *Cntnap2*$^{-/-}$ mice, providing a foundation for future research into the neurobiology of feeding difficulties in ASD.

3.2 Introduction

ASD has traditionally been thought to disproportionately affect males, with a commonly cited male-to-female diagnostic ratio of 4:1 [1-3]. However, emerging evidence of a distinct female phenotype suggests this ratio is likely closer to 1-3.5:1 [3, 4]. Notably, the co-occurrence of ASD with intellectual impairment is associated with a higher rate of female diagnoses, narrowing the male-to-female ratio to approximately 2:1. In contrast, when intellectual impairment is absent, this ratio rises significantly to 11:1 [5]. These findings underscore the complexities of ASD diagnosis and highlight the challenges in identifying the condition in females.

Social interaction and communication impairments, core symptoms of ASD, often present differently in autistic males and females. While autistic males typically demonstrate the poorest quality friendships, autistic females form relationships of comparable quality to neurotypical males. Neurotypical females score the highest in friendship quality, which sets a higher baseline for social abilities in females overall [6]. Consequently, the social difficulties experienced by autistic females are often less pronounced compared to males, contributing to lower diagnostic rates.

Females on the autism spectrum are also more likely to engage in masking and compensatory behaviours, further complicating diagnosis. Masking involves mimicking neurotypical behaviours, such as forcing eye contact, copying facial expressions, and refraining from discussing special interests excessively. Compensation refers to the deliberate adoption of cognitive strategies to navigate social norms and emulate processes like theory of mind [7]. While these strategies can help autistic females appear more socially typical, they can obscure key diagnostic behaviours, leading to underdiagnosis or misdiagnosis.

Additionally, autistic females process emotions differently from autistic males, contributing to a higher prevalence of certain comorbidities, including anxiety, depression, and eating disorders [7, 8]. This distinct presentation of social and emotional behaviours in autistic females not only affects diagnosis but also emphasizes the importance of studying ASD in both sexes to better understand its varied manifestations, including feeding behaviours, which remain underexplored.

In the *Cntnap2*$^{-/-}$ model of ASD, a limited number of studies have investigated sexual dimorphisms in behaviour and neurobiology [9-11]. These studies have revealed that female *Cntnap2*$^{-/-}$ mice do not exhibit the same degree of social deficits as males in social behavioural assays [9]. Additionally, the neurobiological differences underlying social behavioural aberrations appear to be less pronounced and less deleterious in females compared to males [9-11].

As discussed in chapter 2, individuals with ASD are five times more likely to experience feeding difficulties than neurotypical individuals [12], with one report suggesting that maladaptive feeding may affect more autistic females than males [13]. Studies characterizing the diets of autistic children with feeding difficulties have found that up to 67% of these children completely omit vegetables from their diets. While certain foods are entirely excluded, a greater variety of foods is consumed within preferred food groups, predominantly starches and proteins [14]. Additionally, selective overconsumption and eating in the absence of hunger are more likely to occur in ASD individuals, with the overconsumed foods typically being highly palatable sweet or salty, bread-based items [15].

Selective overconsumption of palatable tastants has also been observed in male *Cntnap2*$^{-/-}$ mice (see Chapter 2), aligning with these findings in humans. Given that overconsumption of palatable foods has been reported in both humans [15] and male *Cntnap2*$^{-/-}$ mice (chapter 2), it was of interest to determine whether female *Cntnap2*$^{-/-}$ mice overconsume sucrose compared to their WT counterparts.

Furthermore, it was important to assess whether palatable food intake in female WT and *Cntnap2*$^{-/-}$ mice fluctuates across the oestrous cycle. To investigate this, food intake data were collected from freely cycling female mice, enabling the evaluation of intake across oestrous phases.

3.3 Methods

3.3.1 Animals

Female *Cntnap2*$^{-/-}$ mice (JAX #017482) and their WT background strain were bred through homozygous crosses. They were housed individually in Plexiglas cages with wire tops at 22 °C with a 12:12 L:D cycle (lights on at 07:00) and had *ad libitum* access to standard chow (Sharpes, Carterton, New Zealand) and water unless stated otherwise. Age-matched adult mice [>PND 96 WT and *Cntnap2*$^{-/-}$ mice at the beginning of the studies] were included in the experiments. The experiments adhered to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals, and they were approved by the Ethics Committee in protocol 1138.

3.3.2 Standard chow intake

3.3.2.1 24-h standard chow intake

Ad libitum intake of chow and water was measured over 24-h periods (10:00-10:00) for both WT and *Cntnap2*$^{-/-}$ mice (n= 9-11/genotype). Pellets of standard laboratory chow were weighed at the start and end of each period, while water bottles, including any drips caused by inversion, were also weighed before and after. Vaginal smears were collected at the end of each 24-h period, and the oestrous stage was assigned to the preceding day, as phase transitions typically occur mid-light phase [16].

3.3.2.2 Standard chow intake following 16-h food deprivation

To assess refeeding, food was removed from WT and *Cntnap2*$^{-/-}$ mice (n= 9-11/genotype) just before the start of the dark cycle (17:00) and returned 16 hours later during the light cycle (09:00). Food intake was measured at 1-, 6- and 24-h. Animals maintained *ad libitum* access to water during the experiment.

3.3.2.3 Episodic sucrose intake

Non-deprived WT and *Cntnap2*$^{-/-}$ mice were acclimated to 1-h daily access to 10% sucrose solution on three days prior to the experiment to minimize neophobia. On the experimental day, animals were provided access to 10% sucrose for 1-h (10:00–11:00), with chow and water removed during the period of sucrose presentation. Sucrose intake was measured by weighing bottles and correcting for any drips caused by bottle inversion. Vaginal smears were collected immediately after sucrose access ended to prevent handling stress from influencing intake. To collect data across all oestrus phases, the experiment was repeated over multiple sessions, each separated by at least two days to prevent chronic sucrose consumption.

3.3.3 Oestrus cycle determination

Oestrus phase was determined by taking a vaginal smear using sterile physiological saline (0.9% NaCl) in a pipette and aspirating saline into the vaginal opening as reported previously [17]. Smears were air dried on glass microscope slides prior to 0.1% crystal violet staining. The oestrus stage was determined by evaluating the relative ratios of cell types (epithelial cells vs. leukocytes) and cell morphology (nucleated epithelial cells vs. cornified squamous epithelial cells), following the criteria outlined by McLean *et al*. [17]. Proestrus was identified by the exclusive presence of well-defined nucleated epithelial cells, oestrus by a predominance of cornified squamous epithelial cells, metestrus by the presence of both leukocytes and cornified squamous epithelial cells, and diestrus by a higher ratio of leukocytes to cornified squamous epithelial cells.

3.3.4 Statistical analysis

Sucrose intake and 24-h standard chow and water intakes (independent of oestrus phases) were analysed using a t-test. Refeeding differences were evaluated by comparing the area under the curve (AUC). Variations in 24-h food intake and sucrose intake across oestrus phases were assessed using a one-way ANOVA. Statistical significance was defined as $P \leq 0.05$.

3.4 Results

Ad libitum food intake, independent of estrus phase, did not differ between WT and *Cntnap2*$^{-/-}$ mice over a 24-h period ($P = 0.145$), however, during this same period *Cntnap2*$^{-/-}$ mice drank significantly less water ($P = 0.006$) (Fig 3-1 a-b).

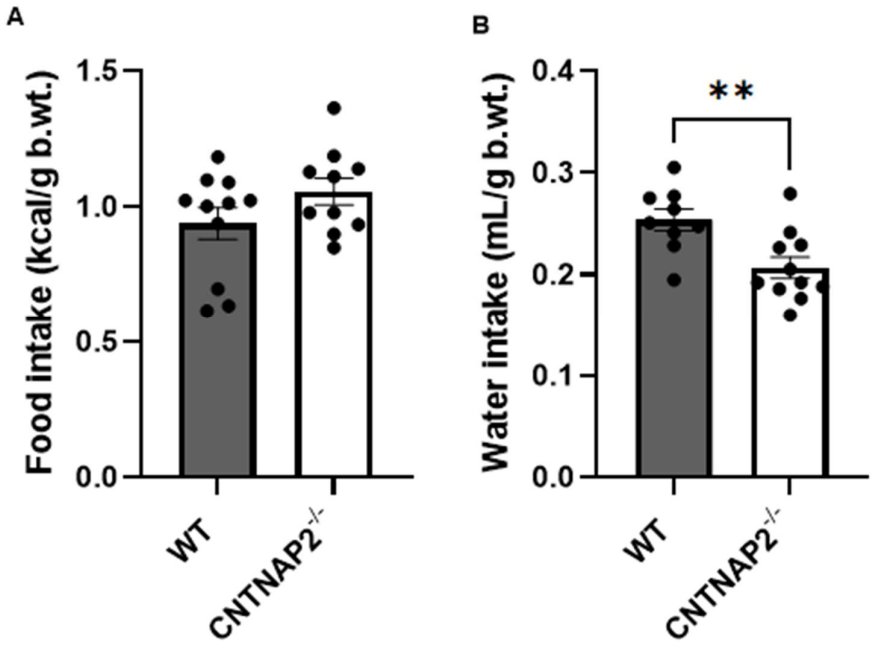

Figure 3-1 *Ad libitum* intake of food and water in WT and *Cntnap2*$^{-/-}$ mice (independent of oestrus cycle). (A) Standard chow intake over 24-h. (B) Water intake over 24-h. n= 9-11/strain. Data are shown as mean ±SEM. **P < 0.01. WT, wild-type;

Furthermore, no differences in refeeding following a 16-h fast were observed (P = 0.1992 AUC comparison) (Fig 3-2)

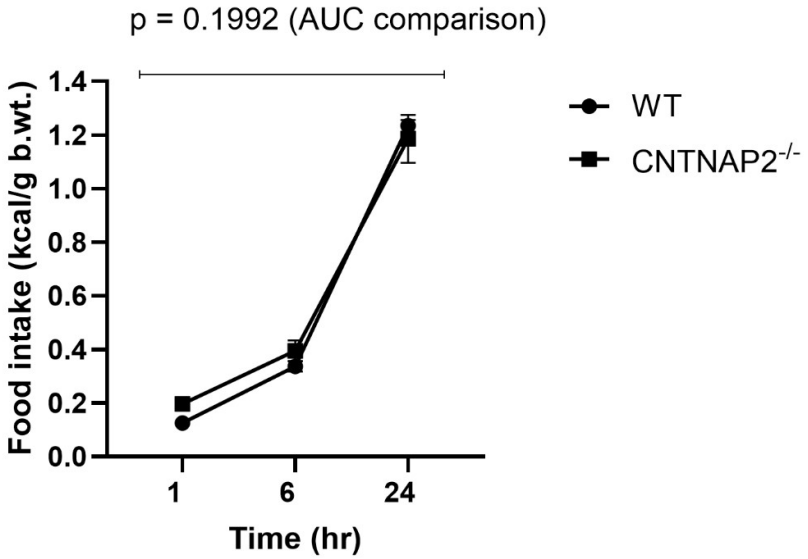

Figure 3-2 Cumulative standard chow intake after a period of 16-h food deprivation in WT and *Cntnap2*$^{-/-}$ mice. n = 9-11/strain. Data are shown as mean ±SEM. WT, wild-type; AUC, area under curve.

Female *Cntnap2*$^{-/-}$ mice, independent of estrus cycle, overconsumed 10% sucrose compared to their WT counterparts (P = 0.006). (Fig 3-3).

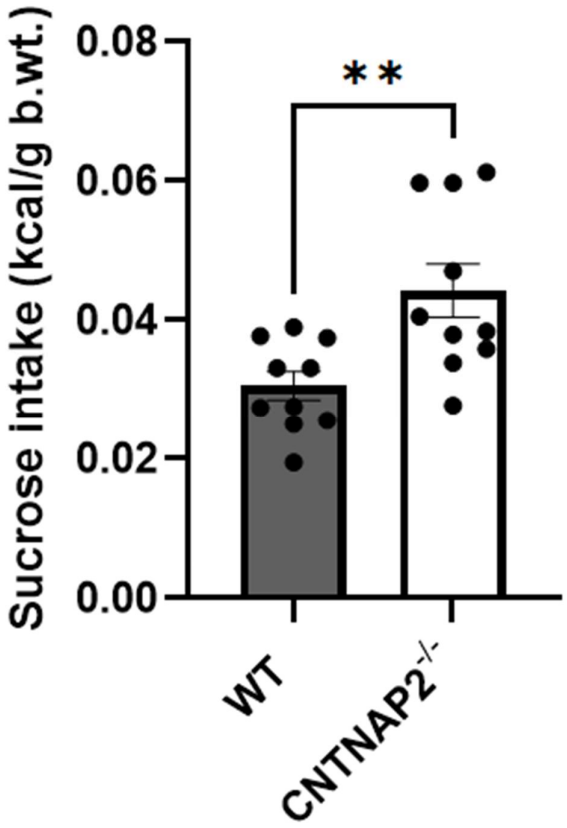

Figure 3-3 10% sucrose consumption in female WT and *Cntnap2*$^{-/-}$ mice. n = 9-11/strain Data are shown as mean ±SEM. **P<0.01. WT, wild-type.

24-h food intake remained constant over the entire estrus phase for both WT ($F(3,31) = 1.104$, $P = 0.3624$) and *Cntnap2*$^{-/-}$ mice ($F(3,26) = 0.3114$), $P = 0.8169$). Although *Cntnap2*$^{-/-}$ mice consumed more sucrose overall, the consumption of sucrose remained constant over each estrus phase for both WT ($F(3,27) = 1.470$, $P = 0.2449$) and *Cntnap2*$^{-/-}$ ($F(3,23) = 0.3844$, $P = 0.7652$) mice (Fig 3-3 a-d).

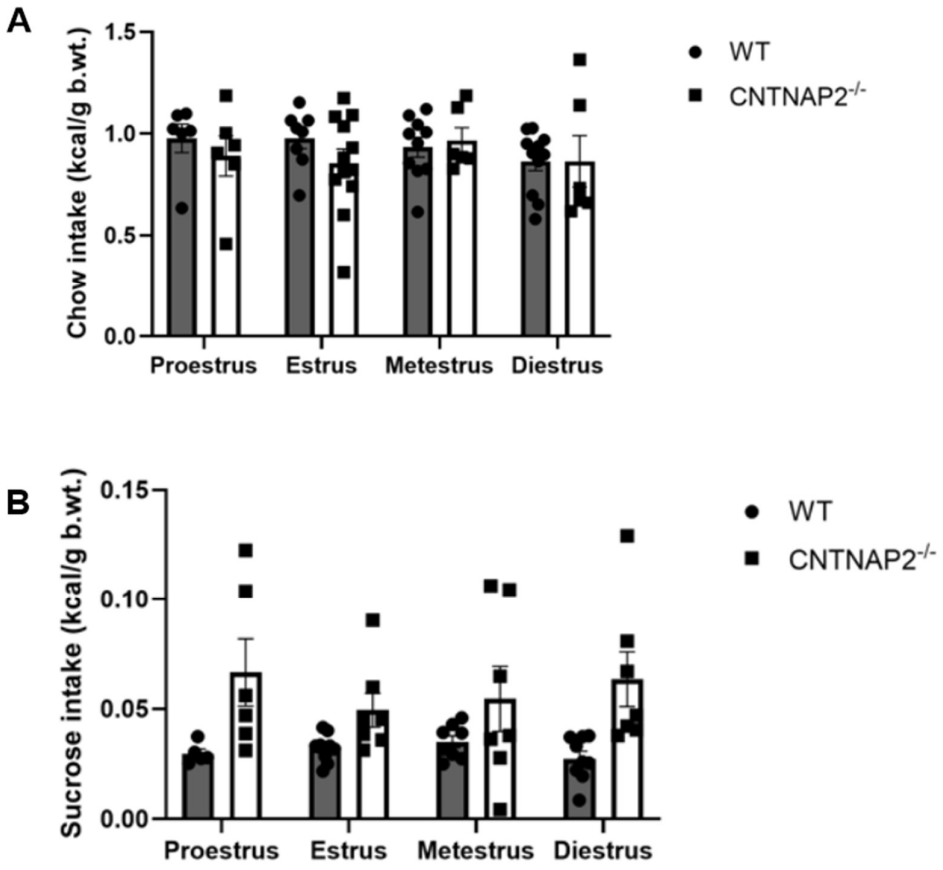

Figure 3-4 Consumption of standard laboratory chow and 10% sucrose for WT and *Cntnap2*$^{-/-}$ mice over each oestrus cycle stage. (A) 24-h laboratory chow intake over the oestrus cycle. (B) 1-h 10% sucrose intake over the oestrus cycle. n = 9-11/strain/estrus phase. Data are shown as mean ±SEM. WT, wild-type.

3.5 Discussion

The prevalence of ASD among females remains highly debated. While a 4:1 male-to-female diagnostic ratio is commonly cited, this is likely an overestimate, with recent studies suggesting the true ratio may be closer to 1–3.5:1 [3]. Although there is limited evidence suggesting that feeding problems may affect autistic females more often than their male counterparts, feeding problems as a whole were compared between sexes rather examining the burden of specific maladaptive feeding behaviours [13]. This means that

although females may be affected by feeding issues more frequently than autistic males, it is unknown whether the specific problems which affect each sex are the same. Therefore, it was of interest to determine whether female *Cntnap2*$^{-/-}$ mice demonstrate different feeding behaviours compared to WTs, as we did with male *Cntnap2*$^{-/-}$ mice in chapter 2. Furthermore, to the best of our knowledge, there are no studies comparing whether food intake over the oestrus cycle differs between autistic and non-autistic females.

Previous studies have shown that female *Cntnap2*$^{-/-}$ mice exhibit fewer social deficits than males in social behavioural assays. These differences are underpinned by sex-specific neurobiological mechanisms, such as decreased spine density of pyramidal pre-synapses mediated by microglial activity, observed only in males [9]. Additional studies have linked maternal immune activation in *Cntnap2*$^{-/-}$ mice to increased expression of the cortical-stimulating hormone receptor in males, which correlates with deficits in social recognition [11]. Furthermore, disruptions in cortical circuitry functional responses are more pronounced in male mutants [10], providing further evidence of sexual dimorphism in the neurobiological underpinnings of this model. However, to date, no studies have specifically investigated potential sexual dimorphisms in food intake in *Cntnap2*$^{-/-}$ mice, leaving an important gap in our understanding of feeding behaviours in this model.

Previous studies examining feeding behaviour in mice across oestrus cycles have yielded conflicting results. Over a full cycle (~4 days), no sex-based differences in feeding behaviour variance were observed [18]. However, studies directly accounting for oestrus phase show inconsistencies: some report decreased food intake during proestrus and oestrus, while others find no differences [16].

Interestingly, in the context of palatable food consumption, WT female mice show resistance to weight gain on a high-fat diet, mediated by oestradiol's regulation of

circadian rhythms. This resistance is lost in ovariectomized mice, which exhibit rapid weight gain driven by increased food intake, a phenomenon reversed with cyclic oestradiol treatment [19]. These findings suggest that oestradiol exerts anorexigenic effects, potentially suppressing food intake during proestrus and oestrus due to reproductive priorities. However, the significance of this anorexigenic effect in normally cycling female mice remains unclear, possibly contributing to the variability across studies.

To date, no studies have investigated the effect of oestrus cycle phases on sucrose consumption or whether feeding over the course of the oestrus cycle differs between autistic and non-autistic individuals. Combined with conflicting evidence on standard food intake in WTs across the oestrus cycle, this knowledge gap prompted the inclusion of these analyses. Our findings indicate no significant variation in standard 24-h food intake or sucrose consumption across oestrus phases.

This result enabled a direct comparison of overall consumption between WT and *Cntnap2*$^{-/-}$ female mice, independent of oestrus cycle influence. Female *Cntnap2*$^{-/-}$ mice mirrored the feeding behaviours observed in their male counterparts as well as in male VPA rats [20], with no significant difference in standard chow intake between the mutants and WTs. The trend in water consumption between female *Cntnap2*$^{-/-}$ and WT mice was different to our findings in males (chapter 2). Females *Cntnap2*$^{-/-}$ drank less than WT mice, whereas we found no different in water intake for male *Cntnap2*$^{-/-}$ and WT mice. This female water consumption data is also in contrast to that presented by Pal *et al* which showed no difference in water intake between male VPA rats and controls [20].

While standard chow intake did not differ between genotypes, female *Cntnap2*$^{-/-}$ mice displayed significant overconsumption of sucrose, even in the absence of hunger. This behaviour was further amplified by reduced water intake in *Cntnap2*$^{-/-}$ females compared to WT controls.

3.6 Conclusion

We, therefore, conclude that over consummatory behavior for sucrose but not standard food occurs in female *Cntnap2*$^{-/-}$ mice. Furthermore, both female WT and *Cntnap2*$^{-/-}$ mice show no variability in both energy-driven and hedonic feeding with estrus cycle phase.

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Chapter 4

Heightened hyponeophagia in *Cntnap2*$^{-/-}$ mice: a neural and behavioural analysis

4.1 Abstract

Feeding abnormalities, including extremely narrow food selectivity, are prevalent symptoms of ASD. Hyponeophagia, the reluctance to ingest novel foods, is driven by a combination of appetite-, anxiety-, and sensory-related processes. Despite its relevance to ASD-related feeding challenges, hyponeophagia remains understudied. This study addresses this knowledge gap by investigating whether *Cntnap2*$^{-/-}$ mice, a model recapitulating core ASD features, exhibit heightened hyponeophagia. We evaluated hyponeophagia in male *Cntnap2*$^{-/-}$ and wild-type (WT) mice by measuring the latency to consume a novel tastant in both familiar and novel environments. To explore the neural correlates of hyponeophagia, we assessed c-Fos immunoreactivity in feeding-related brain regions following novel tastant exposure. *Cntnap2*$^{-/-}$ mice displayed significantly delayed consumption of novel tastants compared to WT controls, regardless of environmental familiarity. These delays persisted across repeated exposures, requiring multiple presentations of the tastant for mutant mice to match the feeding latency of WT mice. Analysis of c-Fos immunoreactivity revealed genotype-dependent differences in the amygdala, hypothalamus, and reward-related brain regions, suggesting disruptions in the neural circuits regulating novelty processing and feeding behaviours. Overall, our findings indicate that *Cntnap2*$^{-/-}$ ASD mice display exacerbated hyponeophagia and hyponeophagia-driven brain activity changes, which likely contribute to the narrow food selectivity in ASD.

4.2 Introduction

Hyponeophagia, an aversion to novel foods, is a pervasive feeding challenge in ASD that contributes to restricted dietary variety and nutritional deficiencies [1]. This behaviour often leads to reliance on specific, nutritionally poor foods, and exacerbates health challenges in individuals with ASD [2]. Core traits of ASD, such as restrictive and repetitive behaviours, insistence on sameness, and impaired social interactions, are thought to play a role in the development of feeding issues [3]. Additionally, the mealtime environment can further exacerbate these challenges. For instance, Provost *et al* found that autistic children had greater difficulty eating in certain settings outside the home, such as non-fast food restaurants and schools [4].

In humans, food neophobia is typically associated with lower body mass index (BMI) in children with subclinical autistic traits. However, when food neophobia co-occurs with elevated autistic traits, it is correlated with higher BMI, suggesting a mitigated risk of being underweight in these children [1], or potentially overconsumption of preferred palatable foods.

A related condition, avoidant/restrictive food intake disorder (ARFID), is characterized by the inability to meet nutritional and caloric needs without specific dietary options, which are often highly palatable but nutritionally poor [5]. ARFID can arise from sensory aversions to food, fear of adverse consequences from eating, or a general lack of interest in food. Its estimated prevalence in the general population is approximately 4.51%, with ASD accounting for 12.5% of ARFID cases [6].

These restrictive feeding behaviours are reflected in animal models of ASD. For example, VPA rats consume less standard laboratory chow [7], and both *Cntnap2*$^{-/-}$ mice and VPA

rats exhibit a heightened preference for palatable sweet and fat liquid diets to which they have been previously habituated (chapter 2) [8].

Hyponeophagia, is a behaviour thought to have evolved as a protective mechanism against ingesting large quantities of novel, i.e., potentially poisonous, food. Its magnitude depends on a complex combination of factors, including anxiety (induced by food novelty alone or by the additional novelty of a new environment), food palatability (and the inherent drive to seek feeding reward), and hunger, to name a few [9, 10].

To date, no studies have assessed whether ASD animals exhibit altered hyponeophagia responsiveness. Therefore, this study aimed to determine whether *Cntnap2*$^{-/-}$ mice display heightened hyponeophagia compared to WT mice. Specifically, we measured the latency to consume a novel tastant in both familiar and unfamiliar environments and investigated whether repeated exposure to the novel tastant reduced this latency. To identify neural correlates of hyponeophagia, we analysed c-Fos immunoreactivity in feeding-related brain regions following exposure to a novel diet.

4.3 Methods

4.3.1 Animals

Cntnap2$^{-/-}$ mice (JAX #017482) and their WT background strain were bred through homozygous crosses. Age matched adult male mice (PND 85 at onset) were housed individually in Plexiglas cages with wire tops at 22 °C with a 12 : 12 L:D cycle (lights on at 07:00) and had *ad libitum* access to standard chow (Sharpes, Carterton, New Zealand) and water unless stated otherwise. The experiments adhered to the guidelines of the NIH

Guide for the Care and Use of Laboratory Animals, and experimental procedures were approved by the University of Waikato Ethics Committee under protocol 1138.

4.3.2 Hyponeophagia to a novel tastant in a novel environment

Cntnap2$^{-/-}$ (n=14) and WT (n=8) animals were placed in a brightly lit, plexiglass arena (43 x 26 x 19 cm, L x W x H). At the center of the arena, a white dish (measuring 4.5 x 4.5 x 1.0 cm, L x W x H) was placed containing 500 µL of saccharin solution (0.1%). Each animal was placed at the edge of the arena at the start of the test and the latency to consume the tastant was determined (s) alongside the number of grooming episodes (n). The test cut off time was 10 minutes. The arena was cleaned with 70% ethanol and allowed to dry before and after each trial.

4.3.3 Hyponeophagia and habituation to a novel tastant in a familiar environment

Cntnap2$^{-/-}$ (n=14) and WT (n=8) animals were presented with a novel tastant (strawberry Kool Aid, sweetened with 0.1% saccharin) in a dish (as in 2.2.1) at one end of their home cage where the food hopper and water bottles are typically placed. Animals were recorded and their latency to try the tastant was measured (s). Standard chow and water were removed just before the presentation of the solution. The pre-determined time for this test was 10 minutes. This protocol was repeated on 3 consecutive days to assess the decrease in latency with repeated tastant exposure.

4.3.4 Neuronal activation following hyponeophagia testing

Cntnap2$^{-/-}$ (*n* =6) and WT (*n* =6) mice (different cohort to previous hyponeophagia tests) were separated into two groups within their respective genotypes. Control WT and *Cntnap2*$^{-/-}$ (*n* =6) groups were pre-exposed to orange Kool Aid sweetened with 0.1%

saccharin each day for 30 minutes a day for 3 days. The tastant was presented in the home cage in a small dish (as in 2.2.1). The remaining groups of WT and *Cntnap2*$^{-/-}$ mice did not receive any pre-exposure to the tastant.

On the experimental day all animals had standard chow taken away 30 minutes prior to receiving 500 µL orange Kool Aid in a small weigh boat at one end of their cage. They were given 10 minutes to ingest the tastant before it was removed. Animals were anesthetized with 35% urethane, 90 minutes after the tastant was removed. Animals underwent transcardial perfusion with saline followed by 50 ml 4% paraformaldehyde in PBS. The excised brains were postfixed for 48 h in paraformaldehyde. Coronal 60-µm sections were sliced using a vibratome (Leica, Frankfurt, Germany) and processed as free-floating sections.

Sections were incubated in 10% methanol and 3% H_2O_2 (in tris-buffered saline [TBS]; 10 min). Following a TBS rinse, they were incubated in the rabbit-anti-Fos antibody (Synaptic Systems, Brisbane, Australia) in 0.25% gelatin and 0.5% Triton X-100 (Sigma, USA) in TBS overnight at 4 °C. The sections were rinsed in TBS and incubated for 1.5 h in the goat-anti-rabbit antibody (Vector, Burlingame, USA). After TBS rinses, they were incubated in the avidin-biotin complex (Vector, USA) for 1.5 h. The color reaction was initiated with 0.05% diaminobenzidine, 0.01% H_2O_2, and 10% nickel sulfate in TBS (15 min). Sections were mounted on gelatin-coated slides, air dried, dehydrated in ethanol, soaked in xylene, and cover slipped in Entellan (Sigma, Darmstadt, Germany).

Images were captured under a Nikon H55OS (Nikon Instruments, Melville, USA) microscope equipped with an OMAX camera (Omax, Kent, USA). The regions of interest, outlined using the Allen Brain Atlas, were: AcbC – nucleus accumbens core; AcbS – Acb

shell (as AcbC);); PVN – paraventricular nucleus; SON – supraoptic nucleus; VMH – ventromedial nucleus; ARC – arcuate nucleus; CEA – central nucleus of the amygdala; BLA – basolateral amygdala; LHA – Lateral hypothalamus ; VTA – ventral tegmental area PVT – Paraventricular thalamus ; NTS – nucleus of the solitary tract; DMX – dorsal motor nucleus of the vagus;

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

4.3.5 Statistical analysis

Each behavioral test compared WT vs *Cntnap2*$^{-/-}$ with a *t-test*. Habituation to the novel tastant was analyzed using an AUC comparison, further comparisons between WT vs *Cntnap2*$^{-/-}$ each day was done using a *t-test*. c-Fos immunoreactivity data was analyzed using a two-way ANOVA with genotype (WT vs *Cntnap2*$^{-/-}$) and novelty (novel vs familiar) as fixed factors along with their interaction (genotype x novelty). Significance was set at $P \leq 0.05$.

4.4 Results

In the novel environment with a novel tastant test, *Cntnap2*$^{-/-}$ mice took longer time to try the novel tastant than their WT counterparts ($P = 0.0038$) (Fig. 1a-b). During this test, *Cntnap2*$^{-/-}$ mice groomed more than WTs ($P = 0.0176$) (fig 4-1 a-b).

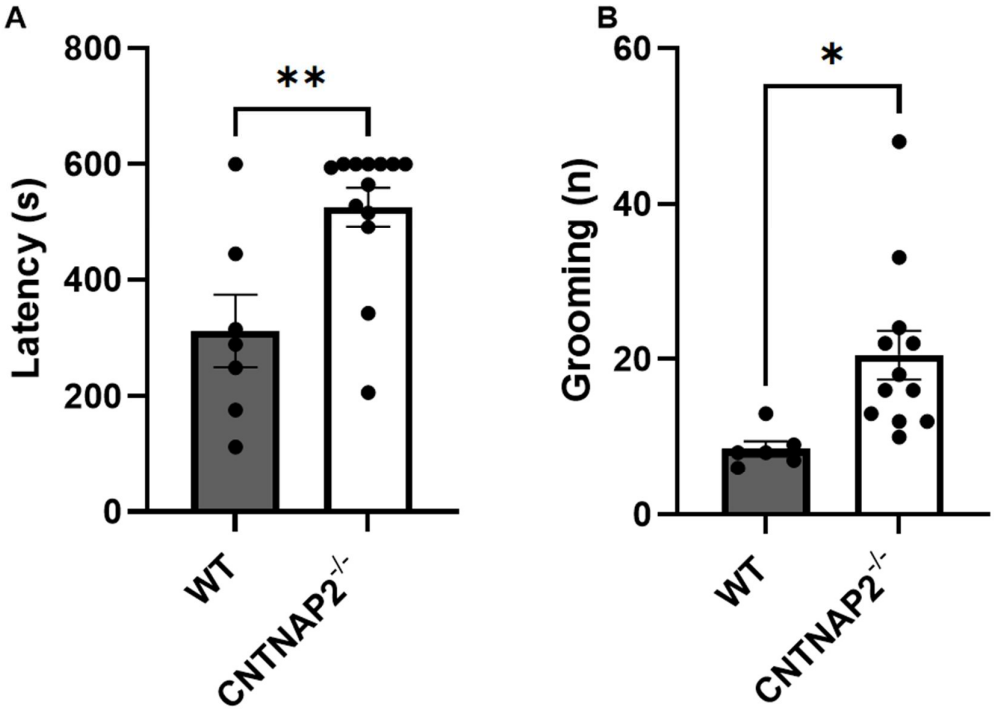

Figure 4-1 Latency to ingest a novel tastant (A) and grooming episodes (B) in WT and *Cntnap2*$^{-/-}$ mice in a novel environment. Data are shown as mean ±SEM *P < 0.05; **P < 0.01. WT, wild-type.

When WT mice were presented with a novel tastant in a familiar environment (home cage) their latency to approach novel food was reduced compared to *Cntnap2*$^{-/-}$ mice ($P < 0.001$). The 3-day habituation test revealed that the latency to ingest the novel tastant remained higher in *Cntnap2*$^{-/-}$ mice than WTs ($P = 0.0495$, AUC comparison of ROC curves), until the third day of exposure when the latency to ingest the tastant was the same between the groups ($P = 0.1839$) (Fig. 4-2).

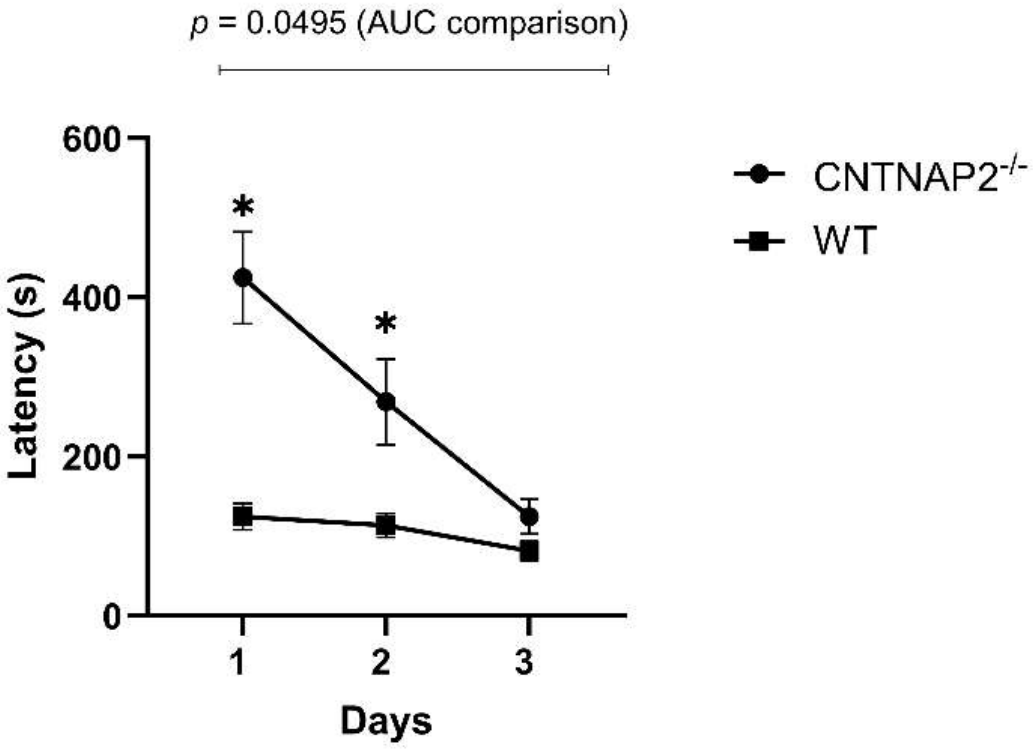

Figure 4-2 Latency to ingest a novel tastant in a familiar environment on the first exposure (day 1) and after repeated exposures on days 2 and 3 in WT and *Cntnap2*$^{-/-}$ mice. Data are shown as mean ±SEM *P < 0.05. WT, wild-type.

A two-way ANOVA analysis of c-Fos immunoreactivity in WT and *Cntnap2*$^{-/-}$ mice (Fig.4-3) showed an effect of genotype in the AcbS (P = 0.0425, F(1,5) = 4.315), AcbC (P = 0.0133, F(1,5) = 14.04), CEA (P = 0.0301, F(1,5) = 8.998), and BLA (P = 0.0423, F(1,5) = 7.344). There are also significant novelty (familiar vs unfamiliar tastant) effects in the AcbS (P = 0.0297, F(1,5) = 9.074), AcbC (P = 0.0002, F(1,5) = 101.8), and CEA (P = 0.0089, F(1,5) = 17.25). A significant genotype x novelty was found in the ARC (P = 0.0367, F(1,2) = 25.79). In WTs, the unfamiliar tastant increased c-Fos immunoreactivity in the ARC (P = 0.01) and CEA (P = 0.0132). In the mutants, c-Fos immunoreactivity was increased in the VTA (P = 0.0379).

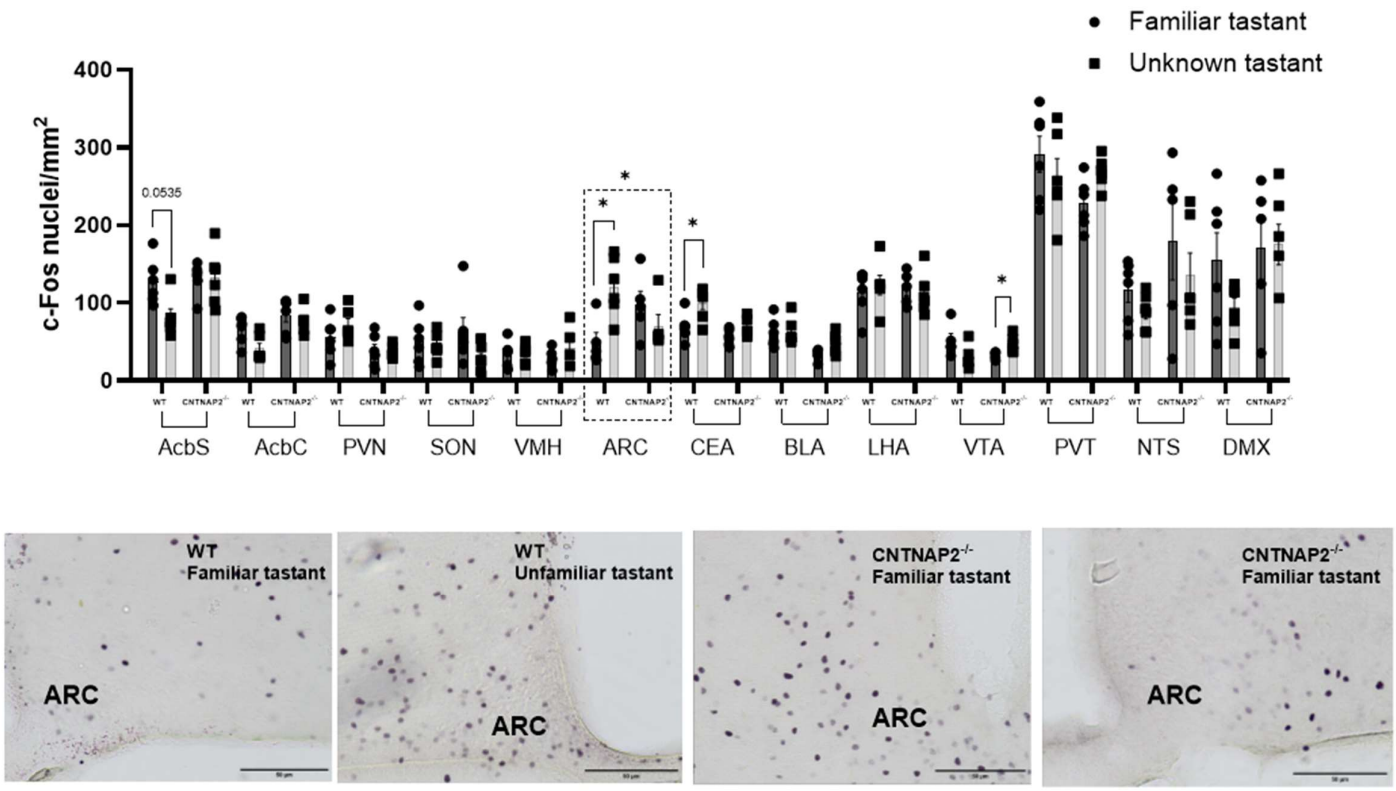

Figure 4-3 Effect of exposure to a novel tastant on c-Fos immunoreactivity in feeding-related brain regions in WT and *Cntnap2*$^{-/-}$ mice. Data are shown as mean ±SEM The box around the ARC defines a significant novelty x genotype interaction. P<0.05. Scale bar = 100 µm. WT, Wild-type, AcbS, nucleus accumbens shell, AcbC, nucleus accumbens core, PVN, paraventricular nucleus, SON, supraoptic nucleus, VMH, ventromedial hypothalamus, ARC, arcuate nucleus, CEA, central amygdala, BLA, basolateral amygdala, LHA, lateral hypothalamus, VTA, ventral tegmental area, PVT, paraventricular thalamus, NTS, nucleus of the solitary tract, DMX, dorsal motor nucleus of the vagus nerve.

4.5 Discussion

One of the core characteristics of ASD is abnormal eating behaviour, with individuals being five times more likely than neurotypical individuals to exhibit issues such as picky eating, narrow food preferences, overconsumption of palatable foods, or insufficient nutrient intake [3, 5, 11, 12]. While limited research has explored these behaviours in animal models, findings thus far suggest that models such as VPA rats and *Cntnap2*$^{-/-}$

mice successfully recapitulate these abnormalities (chapter 2) [7, 13]. Here, we demonstrate for the first time that *Cntnap2*$^{-/-}$ mice exhibit heightened hyponeophagia and that exposure to novel foods elicits distinct neuronal activation patterns in feeding-related brain circuits compared to WTs.

Our previous findings in chapter 2 demonstrated that, similar to VPA rats [8], *Cntnap2*$^{-/-}$ mice overconsume palatable tastants, such as sucrose or saccharin solutions, to which they have been habituated. These mice consume more of those solutions even though they do not exhibit enhanced thirst and do not drink more water. The fact that in the current set of experiments, the mutants had a greater latency to approach and ingest palatable novel solutions, cannot therefore be attributed to a reduced interest in sweet and palatable foods nor can it stem from disturbances in thirst processing. Instead, the delayed consumption of novel palatable liquids parallels abnormally high food selectivity observed in individuals with ASD [14] and it suggests that novelty, rather than taste preference, likely underlies the initial avoidance behavior. This aligns with the clinical presentation of ARFID in ASD, where fear of novel foods often leads to restrictive eating until repeated exposure-based familiarization occurs [15].

Furthermore, it should be emphasized that one of the key components of hyponeophagia is anxiety. This anxiety is induced by the novelty of the food itself, however, in the experimental setting, is oftentimes achieved simply by placing even a familiar food in the novel environment (the combination of the two novelty scenarios, i.e., food and arena, further exacerbates the anxiogenic aspect of the meal) [9, 10]. ASD animals, such as VPA rats or *Cntnap2*$^{-/-}$ mice, show greater susceptibility to some anxiogenic stimuli [7, 16]. Hence, in our two research scenarios, we used settings which differed in their anxiogenic potential by using only novel food or both novel food and environment in which it was

presented. In addition, as evidenced by grooming episode frequency, anxiety is a factor that cannot be overlooked in assessing the motivated behavior of these mutants. We found that *Cntnap2*$^{-/-}$ mice exhibited a more pronounced hyponeophagia regardless of whether the novel palatable solution was given in a familiar or novel arena. Thus, our results demonstrate that novel tastants generate avoidance in *Cntnap2*$^{-/-}$ mice even in the absence of other anxiogenic factors (environment). Furthermore, it took three daily presentations of a novel tastant to *Cntnap2*$^{-/-}$ mice in a familiar environment for their feeding latency to match that of the WT mice. This is striking considering that diet-habituated *Cntnap2*$^{-/-}$ mice overconsume saccharin by 15-20% (chapter 2). This is aligned with previous research showing that ASD children have narrowed preferences for food, which include mainly palatable foods. Autistic children can overconsume preferred foods while at the same time being able to greatly restrict intake of other foods [11, 17].

c-Fos immunoreactivity shows novel food exposure caused activation differences in regions related to reward, food intake and fear processing. In WT's there was an increase in activation in the ARC and CEA in response to a novel food, whereas there was a decrease in activation for the AcbS. There is limited literature investigating c-Fos expression in hyponeophagia; the previous study which delved into this utilised a different hyponeophagia paradigm and investigating different brain regions [18]. However, the changes in c-Fos activation with novelty in WT mice are consistent given the roles of these brain regions.

The Acb is well studied component of the mesolimbic reward pathway [19-23]. Self-administration of sucrose in rats was shown to increase c-Fos expression in the Acb [23]. Furthermore, gustatory reward is known to increase dopamine activity in the Acb [22]. We saw higher c-Fos expression in the AcbS in animals receiving the familiar tastant and

lower activation in the CEA. Thus, novelty suppressed AcbS activation in WT mice. This is consistent with a dampening of reward drive when palatability and anxiety conflict. By contrast, familiar tastants elicited higher AcbS activation, reflecting the unopposed rewarding value once novelty was removed.

The CEA plays various roles in food intake, with involvement in both aversion and voracious feeding [24]. However, it is most well known for its role in fear and anxiety. Previous work illustrated increased c-Fos levels in the CEA following exposure to an open-field arena, a common behavioural test for anxiety. The observed increase in activation within the CEA in the presence of novel food is likely attributable to increased anxiety levels [25].

The ARC is involved in a range of diverse physiological functions from involvement in reproductive, cardiovascular, and anxiety regulation to influencing feeding behaviours and energy expenditure [26-31]. This subregion houses neuronal populations which can either increase or decrease food intake and anxiety. Populations of orexigenic neuropeptide (NPY and AgRP) producing neurons are found alongside anorexigenic hormone (POMC and CART) releasing neurons within the ARC [31]. Interestingly, both NPY and AgRP have been associated with anxiolytic effects [27, 28]. NPY has been found to act similarly to benzodiazepines and barbiturates, which are both used in treatment of anxiety. Investigation using EEG has shown NPY exhibits similar effects as benzodiazepines within cortical regions and the amygdala [28]. AgRP has also demonstrated anxiolytic effects with evidence indicating it decreases anxiety effects during fasting [26].

On the other hand, anorexigenic neuropeptides POMC and CART are also produced by neurons in the ARC, but are instead anxiogenic in nature [29]. Postnatal ablation of POMC neurons results increased anxiety and development of obesity despite decreased food intake [29]. Furthermore, i.c.v administration of CART showed decreased food intake and increased anxiety-like behaviours in an elevated plus maze [30].

Neurons within the ARC are known to have extensive central projections, three regions of interest with these projections are the VTA, CEA, and the Acb [31]. In WT mice we see changes in activation in the ARC, CEA and Acb with novelty. This is attributable to normal processing of a novel food stimulus that had both palatable incentive value and anxiogenic properties.

However, in *Cntnap2*$^{-/-}$ mice the same novel diet resulted only in an increase in c-Fos immunoreactivity for the VTA, other differences seen in the WTs were not produced in *Cntnap2*$^{-/-}$ mice. Amongst other roles, activation of the VTA is known to aid in the acquisition of object recognition. Importantly, the level of salience for a given object will directly impact the formation of object recognition. The more salient an object is, the higher the likelihood is that the object will be remembered [32]. A heightened activation in the VTA in this context may indicate that *Cntnap2*$^{-/-}$ mice find the novel tastant highly salient. However, due to the lack of changes in activation in other brain regions, this may indicate that this salience signal does not get processed in the same manner as WTs, potentially indicating impaired novelty processing or circuit specific dysfunction for *Cntnap2*$^{-/-}$ mice. Furthermore, in the ARC, where activation increases with novelty in WTs, the trend in *Cntnap2*$^{-/-}$ mice is the opposite. As discussed, the ARC is involved directly involved in the regulation of feeding and anxiety behaviours due to the presence of orexigenic NPY/AgRP producing neurons and anorexigenic POMC producing neurons

[33] [26-30]. Moreover, the ARC also monitors corticosterone levels in the blood [34] and is indirectly involved with signalling to and from the Acb in relation to reward processing in the context of food [35]. The observed increase in c-Fos activation in response to novel food presentation in WTs represented normal integration of a simultaneously palatable but anxiogenic food stimuli. In the *Cntnap2*$^{-/-}$ mice the opposite trend accentuates the possibility of impaired novelty processing or circuit specific dysfunction.

4.6 Conclusion

We conclude that *Cntnap2*$^{-/-}$ mice, an animal model of ASD, display heightened hyponeophagia and it is accompanied by different neural processing of novel food exposure in broad circuits that regulate feeding behavior.

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Chapter 5

Impaired conditioned taste aversion acquisition in male rats with sodium valproate-induced autism

5.1 Abstract

Conditioned taste aversion (CTA) is an evolutionarily conserved mechanism which helps organisms avoid foods with which they have previously experienced negative gustatory or nauseating consequences. This mechanism serves to decrease the risk of ingesting toxins which is an inherent risk when consuming food. ASD individuals are often described as "picky eaters" and exhibit restricted dietary preferences and pronounced avoidance of novel foods. This suggests that the perceived safety of specific tastants may be a crucial determinant of dietary acceptance in ASD which helps with avoiding harmful foods. Here, we explored the hypothesis that a CTA, a learned avoidance of foods whose intake promotes sickness, is exacerbated in ASD. To investigate this, we assessed the magnitude of a lithium chloride (LiCl)-induced CTA in the valproic acid (VPA) rat model of autism versus in healthy control rats. We also examined the effect of a standard 3 mEq LiCl dose on gene expression changes in regions key to the development of a CTA response. Surprisingly, we found that while 3 mEq LiCl induced CTA in healthy controls, even the 6 mEq dose was ineffective in generating aversion to a saccharin solution in VPA rats. Gene expression analysis of feeding, stress, reward, and learning-related were differentially regulated in the VPA rats.

5.2 Introduction

Food intake is a critical survival behaviour that inherently carries the risk of ingesting unsafe substances. To mitigate this risk, animals rely on mechanisms to distinguish safe from unsafe foods [1]. One such protective mechanism is nausea, which can be triggered by various factors, including toxin ingestion, motion sickness, and pregnancy. Nausea typically signals impending emesis, although it can occur independently and is rarely observed without nausea [2].

Taste is a primary determinant of food neophobia. Humans and most mammals can distinguish between five basic tastes: sweet, sour, salty, bitter, and umami. Sweet and umami tastes are generally associated with nutrient-rich foods, such as sugars and L-amino acids, and are readily consumed. Conversely, sour and bitter tastes often indicate unripe fruits, spoiled foods, or toxins, eliciting avoidance or smaller consumption volumes. Responses to salty tastes vary based on individual and species-specific nutritional needs [3]. Importantly, these taste-driven behaviours are highly influenced by prior experiences with food.

CTA is a classical conditioning phenomenon in which animals associate the flavour of a novel food with nausea, resulting in subsequent avoidance of that food. The unconditioned stimulus (e.g., toxin), unconditioned response (e.g., nausea), conditioned stimulus (e.g., food flavour), and conditioned response (e.g., avoidance) illustrate how CTA functions as a biological mechanism to identify unsafe foods [1].

In laboratory settings, CTA is typically induced in rodents by pairing a novel tastant with a nauseating agent such as LiCl. LiCl activates vagal and splanchnic afferent nerves, inducing nausea that the animal associates with the novel tastant, forming a long-term

memory of the negative experience. This paradigm has been extensively used to study CTA mechanisms and as a learning and memory model [1].

Rodents lack the brainstem circuitry for emesis and possess anatomical barriers (e.g., diaphragm, stomach, oesophagus) that prevent vomiting [4]. Thus, CTA acquisition is a critical survival mechanism for rodents, enabling them to avoid consuming toxic substances that cannot be expelled.

This study investigates the relationship between ASD and CTA acquisition using the VPA rat model of ASD. VPA is a short-chain fatty acid commonly prescribed for epilepsy and mood disorders [5]. Despite its efficacy in treating epilepsy, prenatal exposure to VPA is teratogenic, leading to physical abnormalities and developmental disabilities, including a higher risk of ASD [6]. VPA-exposed rats display disrupted histone acetylation, excitatory/inhibitory neural circuit imbalances, and altered neuronal organization, along with ASD-like behaviours such as repetitive behaviour, delayed social interactions, and reduced pain sensitivity [7, 8].

While ASD is strongly associated with feeding issues, including heightened food selectivity and pica, little research has explored the neural mechanisms underlying CTA acquisition in ASD models. Prior work has linked abnormal feeding to impaired OT signalling, with exogenous OT administration rescuing feeding deficits in Cntnap2-/- mice (chapter 2). Impaired CTA acquisition has been observed in other ASD models, such as *St3gal5*$^{-/-}$ and BTBR mice [9, 10], but the neurological basis remains poorly understood. Given the prevalence of feeding challenges in ASD, this study explores CTA acquisition in VPA rats, aiming to elucidate potential links between ASD, OT dysfunction, and aversion-based learning.

5.3 Methods

5.3.1 Animals

Sprague-Dawley rats were housed in standard Plexiglass cages with wire tops in a 22ºC temperature controlled room with a 12:12 light: dark cycle (lights on at 07:00). Animals had *ad libitum* access to standard laboratory chow pellets (Sharpes, Carterton, New Zealand; energy density 3.6 kcal/g) and water unless otherwise stated. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all experimental protocols were approved by the University of Waikato Animal Ethics Committee described herein (Protocol #1155).

5.3.2 Sodium valproate exposure

Adult female Sprague-Dawley rats were mated overnight (17:00-07:00) with age-matched Sprague-Dawley males. The following morning, vaginal smears were taken with a pipette containing sterile physiological saline (0.9% NaCl). Smears air-dried prior to 1% crystal violet staining to detect spermatozoa. Upon spermatozoa identification the date was designated as E0.5. Females received a single intraperitoneal (i.p) injection of 500 mg/kg sodium valproate (VPA) or isovolumetric physiological saline on E12.5. Female rats treated with sodium valproate were healthy and there was no significant difference between the litter sizes of VPA and saline treated dams. Females nursed their offspring until PND25. Crooked tails were developed by 50% of males which is higher but still in agreement with previous reports, indicating mild neural tube defects induced by pre-natal VPA challenge [11]. Offspring of dams exposed to saline will be referred to as controls. Due to sex dimorphisms for severity of ASD traits in this model, only males were used [11].

5.3.3 Confirmation of ASD-like phenotype

5.3.3.1 Elevated Plus Maze

In order to confirm the ASD-like phenotype we employed our previously published protocol to assess anxiety in these animals [12]. Animals were each tested for 10 minutes on an elevated plus maze when each rat was PND30-40. The maze was elevated 50 cm above the ground, with two open arms and two closed arms with 50 x 10 x 40 cm dimensions. Arms of the same type (open/closed) were opposite each other and all arms had open roofs. The maze was cleaned with 70% ethanol before and after each rat entered the maze. Number of entries and time spent in open arms were measured. An entry onto an open arm was defined as two paws being on or beyond the boundary of the closed arm. Normality of the data was confirmed using a Shapiro-Wilk test. Independent two-sample t-tests were used, significant difference was defined as $p \leq 0.05$.

5.3.3.2 Open field test for social interactions

A modified behavioural test for social interactions described by [7] was employed. A control and a VPA rat were both placed into an open field arena measuring 44 x 44 cm for 10 minutes. Initiation of social interactions such as following, approaching, sniffing, licking, mounting and anogenital inspections were measured. The initiation of social interactions was scored for both the VPA and the control animal. Normality of the data was confirmed using a Shapiro-Wilk test. Independent two-sample t-tests were used, significant difference was defined as $p \leq 0.05$.

5.3.4 Assessment of LiCl responsiveness for conditioned taste aversion

Age-matched VPA and control rats were separated into groups (n= 8-10 per group).

All animals were deprived of water overnight, prior to a 1-h exposure to 0.1% saccharin. Following exposure, groups were administered an i.p injection of either 0.9% saline,

0.6mEq/kg, 3mEq/kg or 6mEq/kg LiCl. LiCl was made in water to ensure iso osmolarity with saline. Following a 48-h rest period animals were again deprived of water overnight before simultaneous presentation of water and 0.1% saccharin to their cage for 2 hrs. Drips from the bottles were accounted for and consumption was calculated as the percentage intake of saccharin. Data were analysed using a one-way ANOVA to compare LiCl injected groups with the saline group, this was followed up by Dunnett's post hoc test.

5.3.5 Gene expression analyses

5.3.5.1 Microdissection

Control and ASD animals were divided into two groups each (n = 10 animals/group). Animals had food removed and received an i.p injection of either vehicle (0.9% saline) or 3mEq/kg LiCl (dissolved in water). 100 minutes later animals were decapitated, brains were immediately excised and microdissection of the PVN, CEA and ARC regions was carried out. Tissues were immersed immediately in RNAlater (Ambion, Thermo Fisher Scientific, Auckland, New Zealand) at room temperature for 2 hours before freezing at -80^{o}C until further processing.

5.3.5.2 rtPCR Protocol and Data Analysis

Primers were selected based on literature review and in silico validation using Primer-BLAST (NCBI) and Multiple Primer Analyzer (Thermofisher). Previously validated primers were prioritized, and new primers were designed to span exon-exon junctions, with optimal melting temperatures (~60°C) and amplicon sizes (100-200 bp). Primer specificity was confirmed using BLAST.

A standard method of PCR sample preparation was employed, as previously described [13]. Tissues stored in RNAlater were thawed on ice and homogenized in 100 µL Trizol (Ambion). Following this, 20 µL chloroform was added before centrifugation at room temperature for 10 min at 10,000x g. The clear phase containing total RNA was isolated and precipitated using 0.5 mL cold isopropanol and incubated in an ice bath for 10 minutes before another round of centrifugation at 4ºC for 10 minutes at 10,000x g. The supernatant was discarded carefully, keeping the pellet intact before 300 µL of 75% ethanol prepared in DEPC-treated water was added, and the pellet was washed via centrifuging at 4ºC for 10 minutes at 10,000x g. The supernatant was again removed, and the pellets were air-dried.

Dry pellets were dissolved in 8 µL DEPC water and 1 µL10 X DNAse buffer (dNature). Samples were incubated with 1 µL DNAse (dNature) for 30 minutes at 37ºC. The reaction was then halted using 1 µL stop buffer before incubation for a further 10 minutes at 65ºC. Concentrations and purity of RNA were measured (µg/µL) with a spectrophotometer.
cDNA was synthesised from RNA samples using iScript Advanced cDNA synthesis kit (BioRad). Quantification and purity of cDNA were determined using a nanodrop. Quantitative real-time PCR reactions were carried out in duplicate using 4 µL of 25 ng/µL cDNA, 1 µL of forward and reverse primers (5 µM) specific to the transcript (Table 5-1), 10 µL iTaw Universal SYBR Green Supermix (BioRad) and 4 µL MQ H_2O. Expression of housekeeping genes (Actin b, TBP, HPRT1) was used to analyze normalization factors. Nuclease-free water was used as the template for negative controls for each transcript. NCBI-BLAST® was used to check the specificity of primer pairs prior to use in reactions. The amplification protocol used is as follows:

Denaturation at 95 °C for 15 min, followed by 45 cycles of 15 s at 95 °C, 15 s at the primer-specific annealing temperature and 30 s at 72 °C. The final extension was at 72ºC for 30 s.

Thermal profiles of the amplified transcripts were visualized in CFX maestro using melt peaks, where Tm analysis of the negative value of the change in relative fluorescence units (RFU) over the change in temperature (oC) was plotted (-dRFU/dT) to determine the specificity of the primers to a given transcript and primer dimer.

Table 5-1 List of all primers used in qRT-PCR experiments.

Housekeeping Genes		
Gene	**Forward**	**Reverse**
Actin b	5'-AGTGTGACGTTGACATCC GT-3'	5'-TGCTAGGAGCCAGAGCAGTA-3'
TBP	5'-AGAACAATCCAGACTAGCAGA-3'	5'-GGGAACTTCACATCACAGCTC-3'
Genes of Interest		
Gene	**Forward**	**Reverse**
MC3R	5'-AGCAACCGGAGTGGCAGT-3'	5'-GGCCACGATCAAGGAGAG-3'
MC4R	5'-GCACAGTATCGGGCGTTCTT-3'	5'-CCTCAGTTCTTGACTCCGCA-3'
AgRP	5'-CAGAGTTCTCAGGTCTAAGTC-3'	5'-TTGAAGAAGCGGCAGTAGCAC-3'
NPY	5'-AGGTAACAAACGAATGGGGCT-3'	5'-TGATGTAGTGTCGCAGAGCG-3'
COMTD1	5'-TGTGTGCGGAACCTAAACGA-3'	5'-GAAGGTCGCGTGTTCCAGTA-3'
CRH	5'-TGGATCTCACCTTCCACCTT-3'	5'-TTCATTTCCCGATAATCTCCA-3'
MOR	5'-CGGACTCGGTAGGCTGTAAC-3'	5'-CCTGCCGCTCTTCTCTGG-3'
KOR	5'-AGACCGCAACCAACATCTACAT-3'	5'-GCACAGAACATCTCCAAAAGG-3'

DOR	5'-GCRACATTGCGGTCTGCCAC-3'	5'-CGAAGGCGAAGAGGAACACG-3'
PNOC	5'-CAGGTGAGCCCCCGT-3'	5'-TATGGCAGTGGTGAGCGAAAA-3'
OXT	5'-GACGGTGGATCTCGGACTGAA-3'	5'-CGCCCCTAAAGGTATCATCACAAA-3'
OXTR	5'-GATCACGCTCGCCGTCTA-3'	5'-CCGTCTTGAGTCGCAGATTC-3'
BDNF	5'-TGCAGGGGCATAGACAAAAGG-3'	5'-CTTATGAATCGCCAGCCAATTCTC-3'
VGlut2	5'-CAGCGGATTTGGTTGCGTTA-3'	5'-TGATGAGTCCCCGTTCTGGA-3'
CNTNAP2	5'-ACACAGACCAAGACAAGCCAA-3'	5'-CATGTTTGCGAGCACTTCCC-3'
Synapsin1	5'-CACCAGGATGAAGACAAGCA-3'	5'-GTCGTTGTTGAGCAGGAGGT-3'
PSD95	5'-CTTCTCAGCCATCGTAGAGG-3'	5'-GAGAGGTCTTCAATGACACG-3'

5.3.6 Data Analysis

Data from the LiCl dose response were analyzed using GraphPad Prism one-way ANOVA followed by Dunnett's post-hoc test for control and VPA animals after validation of normal distribution using Shapiro Wilk, and Kolmogorov Smirnov test for normality. Values were considered significantly different for $p \leq 0.05$.

rtPCR data was analyzed using BioRad CFX Manager software (BioRad); rtPCR results were normalised with housekeeping genes (*Actin B* and *TBP*). The distribution of ΔCq values was checked using a Shapiro Wilk test for normality. ΔCq values were analysed with a Two-way ANOVA and either a student's t-test or a Mann-Whitney test. Values were considered significantly different when $p \leq 0.05$.

5.4 Results

5.4.1 Verification of ASD-like phenotype

Before the commencement of feeding studies, confirmation of an ASD-like phenotype in VPA rats was carried out. An elevated plus maze and a modified open field test for social interaction were used. VPA rats showed increased anxiety-like behaviour in the elevated plus maze, with significantly less time spent on the open arms ($P = 0.016$). VPA rats also initiated fewer social interactions than controls in the modified open field test ($P < 0.001$) (Figure 5-1 a-b)

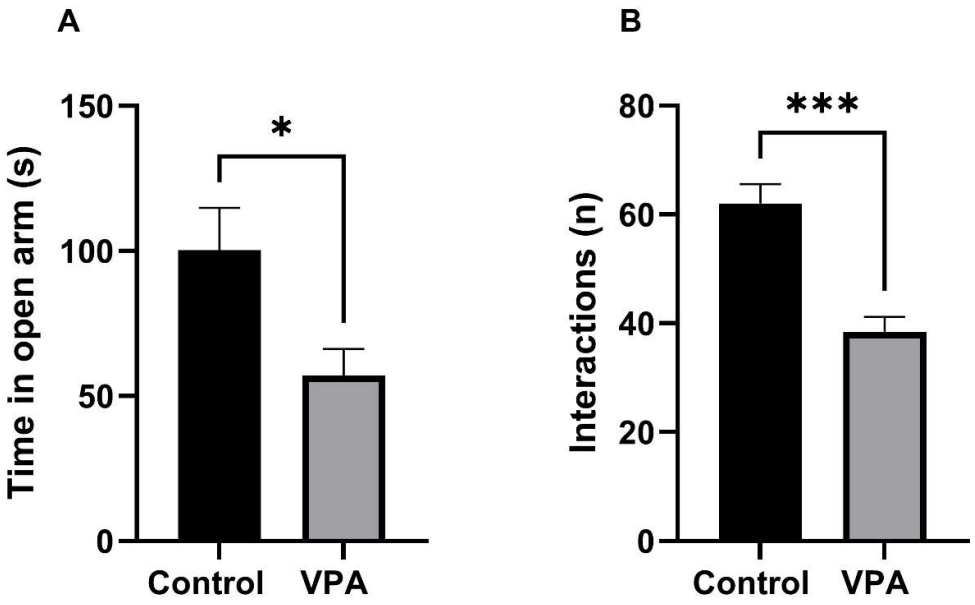

Figure 5-1 ASD-like behaviour phenotype validation using elevated plus maze and open field for social interaction tests. (A) total time spent in the open arm. (B) Total number of social interactions initiated. Data are shown as mean ±SEM, n = 10-13/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4.2 Acquisition of CTA

During the two-bottle test, control animals that received either 3mEq/kg or 6 mEq/kg LiCl consumed significantly less 0.1% saccharin than control animals which received a saline

(F (DFn, DFd): F (3, 33) = 6.592; P=0.001). VPA animals were resistant to CTA acquisition at the same LiCl doeses as evidenced by no change in 0.1% saccharin consumption between the saline treated VPA group and either or the LiCl treated VPA groups (Figure 5-2).

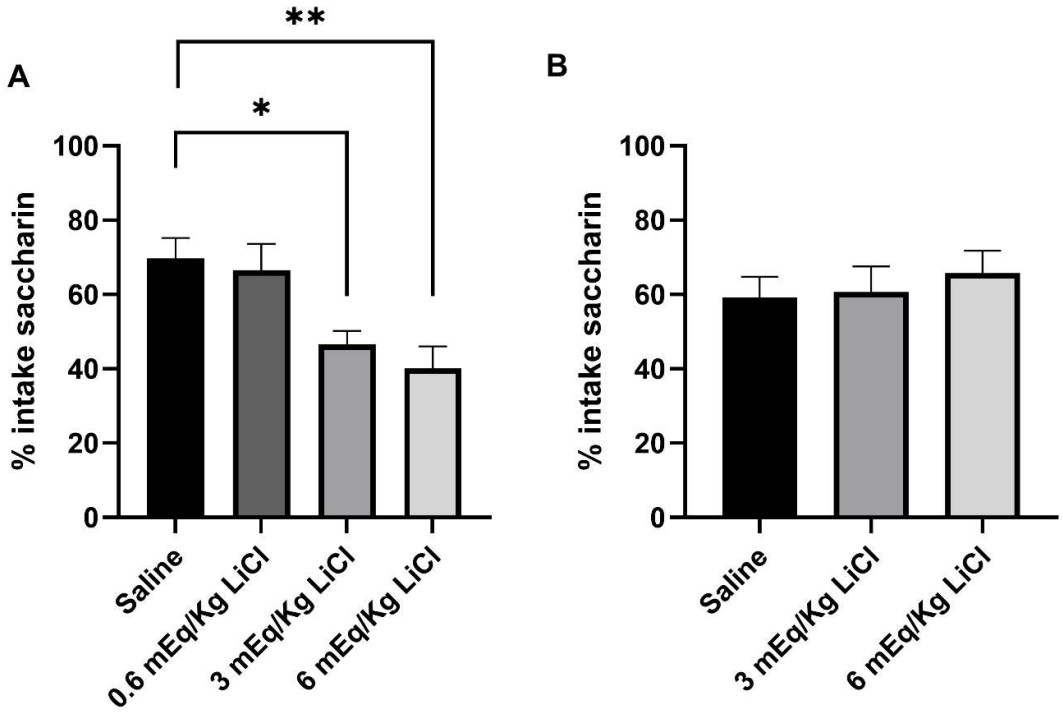

Figure 5-2 Effect of i.p. saline and LiCl on the acquisition of CTA to 0.1% saccharin. Graphs demonstrate % intake of 0.1% saccharin during a saccharin vs water two-bottle test. (A) Intake in control animals (B) Intake in VPA animals. Data are shown as mean ±SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

5.4.3 LiCl induced gene expression changes in the CEA and PVN

In the subsequent gene expression analysis, LiCl treatment altered gene regulation in the CEA of the VPA rats. A two-way ANOVA for the gene expression analysis in the CEA showed a significant VPA exposure X LiCl treatment for COMTD1 (P = 0.004), OXT (P = 0.013), CRH (P = 0.042), AGRP (P = 0.025), NPY (P = 0.003), DOR (P = 0.020) , MC3R (P = 0.012), Pnoc (P = 0.005) and VGlut2 (P = 0.0049) (Table 5-2). Upon LiCl

treatment there was upregulation of COMTD1 (P = 0.004) in the VPA group. However, OXT (P = 0.056), AgRP (P = 0.007), NPY (P<0.001), DOR (P = 0.027), MC3R (P = 0.019) and Pnoc (P = 0.008) were significantly downregulated (Fig4A). Overall, control animals displayed minimal changes in gene expression after LiCl treatment in the CEA apart from DOR which indicated a strong trend towards upregulation (P=0.063) (Fig 5-3 a-b)

Table 5-2 Two-way ANOVA gene expression statistics summary for the VPA exposure X LiCl treatment interaction.

Gene	**F (DFn, DFd)**	**P value**
COMTD1	F (1, 33) = 9.304	P = 0.004
OXT	F (1,31) = 6.904	P = 0.013
CRH	F (1, 33) = 4.496	P = 0.042
AgRP	F (1, 30) = 5.544	P = 0.025
NPY	F (1, 34) = 9.861	P = 0.003
DOR	F (1, 31) = 5.996	P = 0.020
MC3R	F (1, 32) = 7.068	P = 0.012
Pnoc	F (1, 34) = 9.118	P = 0.005
VGlut2	F (1, 34) = 4.183	P = 0.049

Alternatively, in the PVN, there were no significant VPA exposure X LiCl treatment interactions in any genes. However, AgRP (P = 0.008) was significantly downregulated in control animals upon LiCl administration (Figure 5-3)

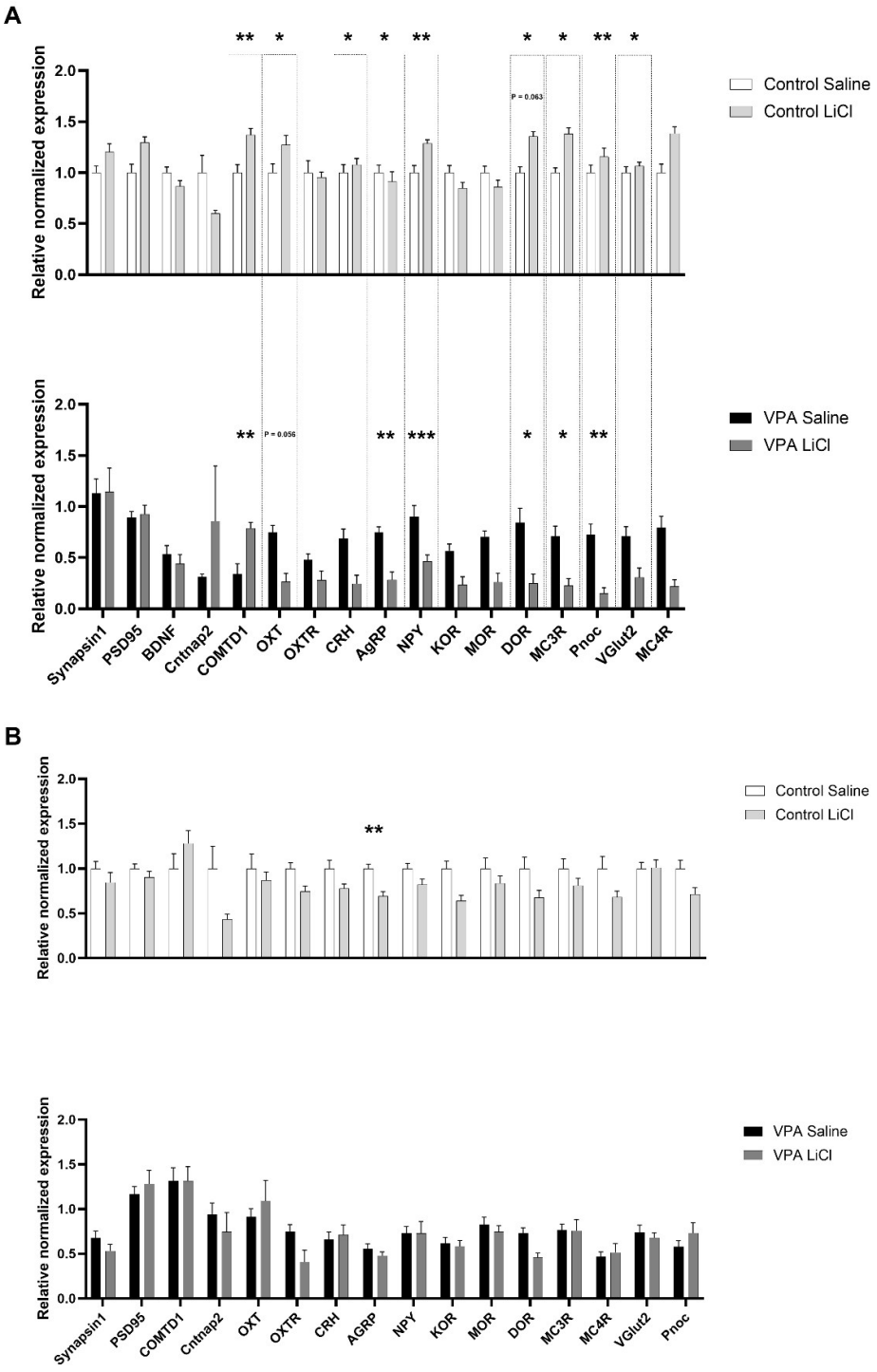

Figure 5-3 Gene expression after LiCl treatment in the (A) CEA (B) PVN. PSD95 - discs large MAGUK scaffold protein 4 (Dlg4), COMTD1 – catechol-O-methyltransferase domain containing 1, CNTNAP2 – contactin associated protein 2, OXT – oxytocin/neurophysin I prepropeptide, OXTR – oxytocin receptor, CRH – corticotropin releasing hormone, AgRP –

agouti related neuropeptide, NPY – neuropeptide Y, KOR – opioid receptor kappa 1 (OPRK1), MOR – opioid receptor mu 1 (OPRM1), DOR – opioid receptor delta 1 (OPRD1), MC3R – melanocortin 3 receptor, MC4R – melanocortin 4 receptor, VGlut2 – solute carrier family 17 member 6 (SLC17A6), Pnoc – Prepronociceptin.

5.5 Discussion

The findings in Chapters 2 and 4 demonstrate that *Cntnap2*$^{-/-}$ mice exhibit heightened hyponeophagia when presented with a novel tastant yet readily overconsume the same palatable tastant once it becomes familiar. This behaviour parallels the heightened food selectivity and refusal commonly observed in individuals with ASD, which are often attributed to behavioural inflexibility and fear of new foods [14, 15]. Additionally, gastrointestinal issues such as constipation, acid reflux, and nausea are frequently associated with feeding abnormalities in ASD, further complicating dietary behaviours [16-18]. Based on this, we hypothesized that VPA rats would exhibit heightened sensitivity to LiCl, leading to the acquisition of a CTA response at lower doses compared to controls.

Contrary to our hypothesis, VPA rats were resistant to developing a CTA response even at doses of LiCl that induced robust aversion in controls. This resistance prompted further investigation into the gene expression of hunger, satiety, reward, and learning-related transcripts in the CEA and PVN, two key regions involved in CTA processing.

Although food refusal in ASD might intuitively suggest a heightened CTA response, previous studies in ASD models (BTBR and *St3gal5*$^{-/-}$ mice) reveal trend of impaired CTA acquisition. These deficits may stem from dysfunctional contingency learning mechanisms [10] or reflect broader behavioural inflexibility [9]. For example, BTBR mice exhibit weakened latent inhibition of CTA [10], while *St3gal5*$^{-/-}$ mice fail to suppress

sucrose preference following LiCl treatment [9]. Our findings in VPA rats align with these observations, further emphasizing that CTA resistance may reflect deficits in aversion formation and contingency learning.

Interestingly, this resistance mirrors feeding challenges in ASD, such as pica, where individuals persist in consuming non-food items despite adverse gustatory or emetic consequences. Pica has been hypothesized to result from diminished aversion learning, further underscoring the role of impaired nausea-based conditioning in ASD [19].

Peripheral administration of LiCl during a CTA paradigm increases c-Fos immunoreactivity in both the CEA and PVN, highlighting their roles in processing aversive stimuli [20]. Notably, the CEA has been strongly implicated in CTA through lesion studies, where electrolytic lesions significantly weaken the aversive response [21-23].

In this study, we observed minimal changes in gene expression within the PVN of VPA rats, suggesting that this region's involvement in CTA processing may not be as significantly altered in this ASD model. However, in the CEA, we identified significant downregulation of genes associated with reward, aversion, and satiety, alongside upregulation of *COMTD1*, a gene responsible for catecholamine metabolism, including dopamine [24]. These changes suggest that the altered gene expression profile in the CEA may play a key role in the impaired CTA acquisition observed in VPA rats, potentially through disruptions in reward processing, aversive learning, or motivational salience.
OT mRNA expression trended toward downregulation in the CEA of VPA rats following LiCl administration, a pattern absent in controls. OT is a well-established satiety signal which also facilitates CTA acquisition [25, 26]. *OT* gene expression deficiencies may

contribute to the failure of VPA rats to acquire CTA. This finding aligns with a study by Dai *et al* which demonstrated that there is a deficiency of central OT in VPA rats [27].

Agouti related protein (AgRP) and neuropeptide Y (NPY), key neuropeptides involved in feeding and stress regulation, also had downregulated transcripts in VPA rats. AgRP promotes food-seeking behaviours and enhances the motivational salience of food-related stimuli [28]. Its downregulation may indicate a diminished ability to assign salience to the tastant, thereby weakening the association between the tastant and the aversive LiCl-induced experience. Similarly, NPY, known for its dual roles in stress adaptation and feeding regulation [29], was significantly downregulated in response to LiCl. This may suggest impaired stress adaptation in VPA rats, further limiting their ability to form aversive associations.

Downregulation was also observed in melanocortin receptor 3 (MC3R) mRNA, which plays a critical role in regulating the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) from AgRP neurons. This function positions MC3R as a key mediator in the control of feeding behaviours and energy homeostasis [30]. Additionally, AgRP neurons in the paraventricular thalamus are innervated by both POMC and AgRP neurons and are activated in response to anorexigenic and aversive stimuli [31]. The observed downregulation of MC3R in the CEA of VPA rats following LiCl administration, combined with decreased AgRP expression, may further impair the integration of aversive signals, contributing to the failure of these animals to acquire a CTA response.

DOR is a key modulator of pain, reward and emotional processing, with roles in reducing stress and anxiety [32], showed lower gene expression in VPA rats following LiCl

treatment. Its transcript downregulation could blunt the aversive and emotional significance of the tastant, further contributing to the failure of CTA acquisition. Notably, in control animals, DOR showed a trend toward upregulation after LiCl administration, aligning with its role in processing aversive stimuli.

Prepronociceptin (Pnoc) encodes the neuropeptide nociceptin, which plays significant roles in learning and memory, hyperphagia, pain perception, and the modulation of stress [33-36]. A previous study reported no significant differences in Pnoc expression in the amygdala between control rats and those exhibiting a CTA response [37]. This observation aligns with the findings in control rats from this study. Interestingly, the observed downregulation of Pnoc mRNA in VPA-treated rats may contribute to their impaired CTA acquisition. Given Pnoc's established roles in pain perception, stress modulation, and cognitive processes, such downregulation could further exacerbate deficits in learning and memory observed in these animals.

COMTD1 participates in the methylation processes, including the metabolism of catecholamines such as dopamine and norepinephrine, through its putative O-methyltransferase activity [24]. Upregulation of COMTD1 mRNA in the CEA of VPA rats following LiCl treatment suggests a potential acceleration in the breakdown of catecholamines. This enzymatic activity could lead to reduced availability of dopamine and norepinephrine, two key neurotransmitters involved in assigning salience and emotional significance to sensory experiences, including novel tastants.

Although corticotropin-releasing hormone (CRH) and vesicular glutamate transporter 2 (VGlut2*)* did not show significant downregulation of gene expression in VPA rats, the VPA exposure X LiCl treatment interaction observed for these genes warrants attention.

CRH plays a pivotal role in activating stress pathways and coordinating behavioural responses to stressors, particularly in the PVN and CEA [38]. Dysregulation of CRH expression could impair the ability of VPA rats to process the aversive properties of LiCl effectively, further contributing to their resistance to CTA. VGLUT2 is essential for glutamate signalling and enables efficient excitatory transmission in brain regions such as the thalamus and brainstem. Loss or reduction of VGLUT2 impairs glutamate loading into vesicles, thereby compromising synaptic efficacy and the strength of excitatory signalling Moechars, 2006 #373}. In this study, the significant interaction between VPA exposure and LiCl treatment suggests that glutamatergic signalling may be contextually disrupted in VPA rats. Specifically, reduced VGLUT2-mediated excitatory drive in key regions like the CEA could impair synaptic plasticity and limit the ability to form aversive associations during CTA.

5.6 Conclusion

In summary, these findings demonstrate that VPA animals, modelling ASD, fail to acquire a CTA response even after administration of 6 mEq/kg LiCl, whereas healthy controls exhibit robust aversive responses at 3 mEq/kg. This lack of CTA acquisition, coupled with significant differences in the expression of key genes related to feeding, stress, reward, and learning, provides valuable insights into the neuromolecular mechanisms underlying this behavioural deficit in VPA animals.

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Chapter 6

Discussions, perspectives and conclusions

Autism spectrum disorder has long been characterized by deficits in social interactions, repetitive/restrictive behaviours, aberrant sensory processing, behavioural inflexibility and feeding issues [1-3] Although ASD was not formally recognized until 1980, its study began over 80 years ago with the pioneering work of Leo Kanner [4, 5]. Since then, extensive research has aimed to understand the breadth of this disorder spanning genetic investigations into its underlying causes, through to pharmacological studies targeting its symptoms. Despite significant progress since 1943, much remains unknown. Diagnosing ASD still relies entirely on behavioural assessments, as no reliable biomarkers have yet been identified. Treatment options remain limited and only address specific symptoms such as depression and aggression.

The most prominent focus of ASD research is on addressing social impairments. This is an understandable priority given the profound impact these have on individuals and caregivers. Impairments in social interaction can, for example, decrease the quality of friendships [6], heighten social anxiety [7], and place a high burden of care on caregivers of autistic individuals [8, 9].

Feeding difficulties, which are among the earliest identifiable symptoms of ASD, affect approximately 62% of autistic individuals [10]. These challenges have been mainly studied in observational and self-report contexts [3, 11-17]. However, the neuromolecular basis underlying problems with feeding in ASD remains relatively understudied.

Common feeding difficulties in autistic individuals include food selectivity, refusal, narrow preferences, sensory sensitivities, neophobia, limited appetite, pica, and

challenges with self-feeding [10, 17-20]. These behaviours are exacerbated by core ASD traits, such as insistence on sameness, sensory abnormalities, and repetitive/restrictive behaviours [10, 17]. These traits often create burdensome mealtimes for both the individual and their family, contributing to heightened anxiety related to food intake. Moreover, individuals with ASD who display narrow food preferences tend to prefer simple highly palatable foods rich in sugar and/or fat, such as French fries, cake and sugary drinks [21]. Predictably, such a diet is associated with adverse health outcomes, including nutrient deficiencies [22] and an increased risk of being underweight or overweight [23, 24].

The purpose of this thesis was to provide systematic evidence of dysregulated feeding in ASD and offer insights into the underlying neuromolecular mechanisms in key animal models of this disorder.

Overconsumption of and preference for sweet palatable foods are symptoms commonly observed in neurotypical individuals but are often exacerbated in those with ASD. Aberrant feeding patterns have been identified across several animal models of ASD. For instance, haploinsufficiency of the ASD- and Rett syndrome-associated gene *Mecp2* in mice results in overconsumption of palatable diets but not standard diets [25]. Similarly, *Nbea* heterozygous mice overconsume palatable sucrose and intralipid solutions, even in the absence of hunger [26]. Prenatally exposed VPA rats exhibit mild hypophagia when consuming standard food but show overconsumption of palatable solutions including sucrose, milk, and saccharin [27, 28].

Adding to this evidence, the data presented in chapters 2 and 3 of this thesis demonstrate that *Cntnap2*$^{-/-}$ mice also overconsume palatable solutions, such as sucrose, saccharin,

and intralipid, while still maintaining normal consumption of standard chow. These findings align closely with the feeding phenotypes observed in *Mecp2+/-* and *Nbea+/-* mice as well as prenatally exposed VPA rats. Pal *et al* demonstrated that VPA rats exhibit dysregulated hunger processing. Food-deprived VPA animals showed similar c-Fos immunoreactivity in key feeding related areas as their fed counterparts, a trend not seen in the controls [27]. Furthermore, VPA animals did not demonstrate the same gene expression patterns as controls, with downregulation of *COMT* only in hungry VPA animals, thus implicating catecholamine metabolism in the hypophagia phenotype [27]. Fukuhara *et al*, linked overconsumption of palatable diets in *Mecp2+/-* mice to disruptions in homeostatic and reward-based feeding circuits, while Olszewski *et al*, found elevated dynorphin expression and heightened sensitivity to naltrexone in *Nbea+/-* mice. In chapter 2, we found blunted neuronal activation in regions involved in homeostatic and hedonic feeding control following a sucrose meal in *Cntnap2*$^{-/-}$ mice. Collectively, these findings highlight a consistent theme of dysregulated feeding across ASD models, which particularly included an overconsumption of palatable foods. These behaviours are consistently underpinned by neuromolecular disruptions in homeostatic and hedonic feeding pathways.

In chapter 2 we also found that consumption of sucrose by *Cntnap2*$^{-/-}$ mice was curbed by doses of OT 10 times lower than what was needed for WT mice. This finding mirrors observations by Klockars *et al*, where sucrose overconsumption in VPA rats was similarly mitigated by OT at doses ten times lower than in controls. Furthermore, we found that activation of OT neurons in the PVN did not increase after a sucrose meal, contrasting with observations in WT animals. OT is typically released during a meal to signal satiety and plays a critical role in feeding regulation [29]. Interestingly, Peñagarikano *et al* found that *Cntnap2*$^{-/-}$ have lower levels of circulating OT and fewer OT producing neurons

compared to WT mice. They demonstrated that social deficits can be rescued by stimulating endogenous OT release using a melanocortin receptor 4 agonist and through an early postnatal treatment of OT [30]. Other ASD models further highlight abnormalities in the OT system. For instance, haploinsufficent reeler mice exhibit lower reduced OT-R expression in the piriform cortex, neocortex, retrosplenial cortex and within regions of the hippocampus [31]. *Fmr1*$^{-/-}$ mice display fewer OT-positive neurons in the PVN [32], while neonatal VPA rats have fewer OT-positive neurons in the SON. Notably, early postnatal treatment of VPA rats with OT also prevents social impairment and reduces repetitive behaviours until adolescence [33].

Taken together, these findings suggest that dysregulation of the OT system may be a common factor in ASD, influencing both feeding and social behaviours. This speculation is further supported by human studies. Many studies have administered OT intranasally to autistic individuals and found increased attentional preferences for faces [34], improved emotion recognition [35], more eye contact [36], strengthened social responses to others and improved the appropriateness of social behaviours [37]. Administration of OT via infusion was found to reduce repetitive behaviours [38]. However, in these studies we again see a strong focus on improving social behaviours with little regard given to the heavily implicated role of OT in feeding regulation.

The data presented in chapters 2 and 3 add to the existing knowledge of abnormal feeding in ASD animal models by implicating overconsumption of palatable solution in both male and female mice. However, further studies could expand upon these data. For example, incorporating further acceptance tests of palatable solutions at varied concentrations would allow identification of the minimum concentration required for palatability and determine whether *Cntnap2*$^{-/-}$ mice differ from WT controls in their acceptance thresholds

In addition, testing solid palatable foods of varying macronutrient compositions would help clarify whether the observed overconsumption reflects a generalised drive for palatable foods or whether nutrient and caloric content modulate that effect.

Chapters 2 and 3 focussed on acute feeding paradigms; expanding to long-term consumption studies would provide valuable insight into sustained feeding behaviour. When given *ad libitum* access to standard lab chow, which is relatively bland, *Cntnap2*$^{-/-}$ mice consume amounts comparable to WT mice and maintain similar body weights. It would therefore be informative to determine long-term access to palatable foods results in persistent overconsumption, or whether intake eventually equilibrates to match energy requirements. Depending on the outcome, additional paradigms such as binge-eating models could be applied. This is particularly relevant as humans with ASD are at an increased risk of eating disorders, including anorexia nervosa, and binge eating; notably, 23% of individuals with an eating disorder also show features of ASD [39]. If *Cntnap2*$^{-/-}$ mice overconsume during acute access to palatable food, they may also be more susceptible to developing binge-like behaviour under limited access paradigms, which tend to involve intermittent or limited access to palatable foods over prolonged periods [40].

Further studies using female *Cntnap2*$^{-/-}$ mice could also address current gaps. For instance, measuring intake of additional palatable solutions such as intralipid or saccharin across the oestrous cycle would mirror the data collected in males and reveal whether cycle stage influences consumption of foods with different macronutrient and caloric properties. Building on this, it would be valuable to test the efficacy of i.p. oxytocin administration in females to assess whether it modulates food intake as it does in males, and whether treatment sensitivity fluctuates across the oestrous cycle. Because oestrogen interacts

with oxytocin [41-43], and because females often present with less severe social deficits than males, it remains an open question whether the feeding abnormalities observed in *Cntnap2*$^{-/-}$ mice arise independently of oxytocin dysregulation.

In chapter 4, we examined hyponeophagia, a feeding difficulty associated with ASD. This behaviour is characterized by the inhibition of feeding in response to novelty, which can lead to restricted dietary variety and nutritional deficiencies [44, 45]. Factors such as food palatability, hunger and anxiety all contribute to the magnitude of feeding inhibition. Hyponeophagia has been linked to the OT system [46]. Olszewski *et al* demonstrated that under conditions of novelty-induced anxiety, an OT-R agonist enhanced food intake, while an OT-R antagonist abolished feeding [46].

To investigate hyponeophagia, we tested WT and *Cntnap2*$^{-/-}$ mice in two environments: a large, well-lit novel arena or in their home cage under normal conditions. In the highly anxiogenic novel environment, *Cntnap2*$^{-/-}$ mice displayed heightened inhibition of food consumption, even when the tastant used was one they typically overconsume once habituated. These findings indicate that hyponeophagia was not due to disinterest in the tastant but likely due to the novelty of the tastant. Additionally, we noted increased grooming behaviour in *Cntnap2*$^{-/-}$ mice, which may reflect elevated anxiety. Although anxiety contributes to hyponeophagia, our results indicate that the heightened feeding inhibition in *Cntnap2*$^{-/-}$ mice persisted regardless of the anxiogenic nature of the environment.

Investigation of the underlying c-Fos immunoreactivity revealed altered neuronal activation patterns in key brain regions, including the ARC, CEA and VTA. Notably, *Cntnap2*$^{-/-}$ mice exposed to a novel food exhibited significantly higher activation in the

VTA, a region involved in reward processing and object recognition. This increased activation may suggest that novel food was perceived as highly salient and rewarding. A functional connectivity study by Uddin et al found that autistic individuals have hyperconnectivity within their salience network [47] which may explain why *Cntnap2*$^{-/-}$ mice found the novel food to be highly salient. However, the lack of observed activation changes in other regions, such as the nucleus accumbens, raises the possibility of dysfunctional signal transmission throughout broader feeding circuits. Human studies have found differences in activity of the ventral striatum during monetary and social reward between autistic and neurotypical individuals [48, 49]. The ventral striatum includes the nucleus accumbens and is a key region in reward processing and may indicate aberrant reward signalling within ASD. It is important to note that while c-Fos immunoreactivity provides insight into neuronal activation, it does not allow for direct analysis of circuit-level dynamics, but trends can be inferred based on relative activation patterns in brain regions which are known to signal one another. Overall, this immunoreactivity data suggests impaired processing within the broader neural circuits that regulate feeding in *Cntnap2*$^{-/-}$ mice. This evidence further supports the hypothesis of dysregulated feeding mechanisms contributing to anomalous food intake behaviours.

The interplay of anxiety and insistence on sameness, both characteristic features of ASD, may exacerbate hyponeophagia. Elevated grooming in *Cntnap2*$^{-/-}$ mice suggests increased anxiety, which could amplify this phenotype. Moreover, it is plausible that hyponeophagia and the overconsumption of palatable foods are interconnected. Given the challenges an autistic individual with food neophobia faces in accepting and incorporating a novel food into their repertoire of 'safe foods,' it is logical that once a food is deemed safe, it may subsequently be overconsumed. Furthermore, any contribution from dysfunctional homeostatic and hedonic regulation of feeding may further exacerbate this behaviour.

Future studies should incorporate pharmacological interventions to investigate the role of the OT system in hyponeophagia in ASD. One approach could involve administering the OT-R agonist WAY267,464 or the OT-R antagonist L-368,899 in *Cntnap2*$^{-/-}$ mice to elucidate the OT system's contribution to this behaviour, as was demonstrated in non-autistic animals by Olszewski *et al* Furthermore, it would be intriguing to determine whether *Cntnap2*$^{-/-}$ mice exhibit altered responses to these drugs at comparable doses, as both *Cntnap2*$^{-/-}$ mice and VPA rats have been shown to respond to lower doses of OT than controls [50]. Exploration with these pharmacological agents could clarify whether the central OT system is involved in the hyponeophagia phenotype observed in *Cntnap2*$^{-/-}$ mice.

Subsequent experiments could explore the direct effects of OT on hyponeophagia using intranasal and/or intraperitoneal administration. While WAY267,464 and L-368,899 are known to penetrate the blood-brain barrier (BBB) [46], OT itself has limited BBB permeability. However, peripheral OT administration has been shown to influence behaviours such as feeding (Chapter 2) and restore social deficits [38, 51]. Interestingly, different routes of peripheral OT administration have produced distinct behavioural effects [52], thus warranting further exploration of how delivery methods impact feeding behaviours.

If peripheral OT administration fails to modulate hyponeophagia, alternative paradigms such as testing social transmission of food preference could be pursued. Chronic early postnatal OT administration has been shown to ameliorate social deficits in *Cntnap2*$^{-/-}$ mice [30]. Investigating whether social transmission of food preference influences hyponeophagia could provide additional insights into the role of OT in regulating feeding

behaviours. These studies would enhance our understanding of hyponeophagia in *Cntnap2*$^{-/-}$ mice, offering a more comprehensive view of how OT dysregulation impacts feeding behaviours in this ASD model.

In chapter 5 we investigated aversion, another feeding behaviour which is pronounced in ASD, by using the pharmacological VPA rat model of ASD. Specifically, we examined the acquisition of a CTA response, a behaviour governed by both the physiological consequences of consuming a toxin and contingency learning mechanisms. Food refusal or aversion in ASD is thought to stem from sensory over-responsivity [20]. In fact, more than 90% of autistic children struggle with sensory abnormalities with these deficits often occurring in the processing of smell, taste and vision [53]. All three of these senses are integral to feeding. Additionally, gastrointestinal issues such as constipation, acid reflux, and nausea are frequently associated with ASD [54-56]. Based on this, we hypothesized that VPA rats would exhibit a heightened sensitivity to LiCl, which would result in CTA acquisition at lower doses than controls.

Surprisingly, our experiments did not support this hypothesis. VPA rats failed to exhibit aversion even after administration of a high dose (6 mEq/kg) of LiCl. In our paradigm, animals were deprived of water overnight before receiving saccharin followed by LiCl injection. The combination of thirst and a 1-h exposure meant that the animals consumed the saccharin despite any potential hyponeophagia. Interestingly, despite experiencing gustatory consequences following the consumption of a novel food, the VPA rats still did not develop and display aversion behaviours.

Two studies have been published showing impaired CTA acquisition in behavioural (BTBR) and genetic (*Stgal5*$^{-/-}$) ASD models [57, 58]. The authors of these studies did not

explore the neuromolecular basis behind this phenomenon, but did conclude that it likely stemmed from dysfunctional contingency learning mechanisms [57] or behavioural inflexibility [58]. This trend of impaired CTA acquisition in ASD models mirrors pica, another anomalous feeding behaviour associated with ASD. Pica is the persistent consumption of non-food items despite the gustatory consequences [59].

Interestingly, the OT system has also been implicated in the acquisition of CTA. Another study by Olszewski *et al* demonstrated that the same OT-R antagonist (L,368,899) which prevented animals from eating novel foods also prevented the acquisition of a CTA response. However, this effect was dependent on timing, as the antagonist blocked CTA acquisition but not retrieval [60]. This suggests that the OT system is involved when the animal associates nausea, the unconditioned response, to the taste of the food, the conditioned stimulus. Dysfunction in the OT system may therefore hinder the development of aversion (the conditioned response). However, it is important to note that CTA acquisition is not solely reliant on the OT system; CTA acquisition involves signalling across multiple brain regions, including the brainstem, hypothalamus, amygdala, and cortex [61-63]. To further investigate, we analysed the expression of genes related to feeding, stress, reward, and learning in the PVN and CEA, two regions previously implicated with the CTA response [64-67].

We observed minimal changes in gene expression within the PVN, suggesting that this region may not play a crucial role in CTA acquisition in this model. However, significant differences were detected in the CEA. Notably, OT expression trended toward downregulation in VPA animals but showed upregulation in controls following LiCl administration. This finding aligns with Olszewski *et al's* report that OT-R blockade prevents CTA acquisition. Interestingly, OT-R expression was not significantly altered in

either region, suggesting that reduced OT expression, rather than OT-R expression, may underlie the observed effects. Additionally, we found evidence of upregulated COMTD1, a gene involved in the metabolism of catecholamines, including dopamine. Since the dopaminergic system is known to regulate aversive learning and memory [68], this upregulation may indicate further dysregulation in reward and learning processes.

This thesis has aimed to weave together data which characterises feeding behaviours in two animal models of ASD. That being said, it is also important to consider the limitations of this research to fully appreciate where it sits within the current literature. When working with animal models, particularly in with such a complex and heterogenous presentation in humans, it is necessary to be address the limited face validity of the models selected. Neither *Cntnap2*$^{-/-}$ mice or VPA rats fully recapitulate the behavioural profile of ASD in humans.

An further limitation is that all experiments were carried out using adult animals. Feeding issues in autism are most commonly observed in children[10], with a previous study indicating that the severity of feeding issues can decrease in adolescence [69]. This may relate to the second round of synaptic pruning which occurs in adolescence [70]. Future work incorporating juvenile animals, and tracking their food consumption across development, would provide a more comprehensive picture.

This thesis also relied on molecular approaches such as immunohistochemistry and gene expression analyses. While these techniques provide valuable insight into protein and mRNA changes in discrete brain regions, they cannot, on their own, establish causal mechanisms or identify circuit-level dysfunction.

Finally, when conducting experiments in VPA rats, multiple offspring from the same litter were included. Although the use of an inbred strain minimised background genetic variability, and the effects observed were large enough that plausible intra-litter correlations would not alter the direction of the conclusions, it is important to highlight this limitation. Because VPA is administered in utero, the dam is technically the experimental unit. Offspring from the same dam share a prenatal and postnatal environment and are therefore, not fully independent data points. In this study, exact litter IDs were unavailable post hoc; however, pups were sampled in modest numbers per litter across multiple dams in both VPA and saline groups, reducing (though not eliminating) concerns about litter clustering.

When considered together, the abnormalities in feeding behaviour explored in this thesis suggest a potential protective mechanism in ASD. Impairment in aversive learning could make a narrowed food repertoire the safest approach to avoid the consumption of toxins. In this context, the novelty of food would reduce consumption until the food is encountered enough times to be deemed safe. Without this cautious hyponeophagic approach, individuals with impaired CTA acquisition might face frequent illness or more severe health consequences depending on what is consumed. This mechanism instead focuses feeding on a limited repertoire of safe, palatable foods, which in conjunction with dysfunctional satiety and reward signalling, means that these foods are overconsumed.
This preference for palatable foods also aligns with the evolutionary advantage of favouring sweet, umami, and salty flavours, which signal the presence of essential nutrients such as sugars, amino acids, and salts, without the presence of toxic elements [71]. Taken together, these findings suggest that hyponeophagia, resulting in a restricted but overconsumed repertoire of foods, may act as a protective strategy in ASD by mitigating the risks associated with impaired aversive learning.

Conclusions

The overarching goal of this doctoral thesis was to investigate feeding behaviours and the underlying neurochemical basis of these behaviours in animal models of syndromic and idiopathic ASD.

The key findings are:

- *Cntnap2*$^{-/-}$ mice show aberrant feeding behaviours
- *Cntnap2*$^{-/-}$ mice overconsume palatable solutions regardless of nutritional or caloric value.
- Lower doses of OT than in WT mice prevent overconsumption of sucrose in *Cntnap2*$^{-/-}$ mice.
- *Cntnap2*$^{-/-}$ mice display heightened hyponeophagia and anxiety-related behaviours in response to novel food.
- *Cntnap2*$^{-/-}$ mice have atypical brain activation in response to a meal of sucrose and presentation of a novel food.
- VPA rats demonstrate impaired CTA acquisition
- VPA rats display anomalous gene expression patterns following LiCl administration, underlying the lack of CTA acquisition.

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