Associative Learning Deficiencies Underlying Aberrant Feeding in the Valproate Rat Model of Autism

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Abstract

Autism spectrum disorder (ASD) is a heterogenous neurodevelopmental condition prevalent in over 1% of the global population. Aberrant feeding behaviours and feeding dysregulation are a prevalent and understudied difficulty. Individuals with ASD often present with poor health outcomes, including over/under eating, obesity, and restrictive feeding associated with poor dietary habits, however the underlying mechanisms are poorly understood. Here we propose an explanation for some neural mechanisms that may be responsible for feeding dysregulation present in ASD. We demonstrate that valproate rat models of ASD (VPA ASD) have a blunted neural response to LiCl-induced conditioned taste aversion and show transcriptional changes in the arcuate nucleus of the hypothalamus. Findings of this study also show that VPA ASD rats have significantly higher neural activation in the nucleus accumbens and the dorsal vagal complex in response to food, compared to controls. Our research suggests that some of the feeding abnormalities seen in people with ASD may stem from signalling deficiencies in brain areas involved in associative learning responses following ingestion of foods that cause malaise. Extremely restrictive feeding behaviours seen in ASD phenotypes may be caused by difficulties identifying foods that make them sick. These findings contribute to understanding neuromolecular drivers of anomalous feeding behaviours in people with ASD.

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List of Abbreviations

α-MSH – alpha-melanocyte-stimulating hormone

ACC – anterior cingulate cortex

ActB – beta actin gene

ACTH – adrenocorticotropic hormone

ADI-R – autism diagnostic interview revised

ADHD – attention deficit hyperactivity disorder

ADOS-2 – autism diagnostic observation schedule 2nd edition

AgRP – agouti-related protein

AMPAr – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AP – area postrema

ARC – arcuate nucleus of the hypothalamus

ASD – autism spectrum disorder

BBB – blood brain barrier

BDNF – brain-derived neurotrophic factor

CNV – copy number variation

CNS – central nervous system

CRH – corticotropin releasing hormone

CTA – conditioned taste aversion

DA – dopaminergic

DMH – dorsomedial hypothalamic nucleus

DMX – dorsal motor nucleus of the vagus nerve

DVC – dorsal vagal complex

fMRI – functional MRI

GABA – gamma-aminobutyric acid

H^2O^2 – hydrogen peroxide

HFHS – high fat high sugar

HPA – hypothalamic-pituitary-adrenal

i.c.v. – Intracerebroventricular

IP – intraperitoneal

LiCl – lithium chloride

MC3R – melanocortin 3 receptor

MC4R – melanocortin 4 receptor

MQ H_2O – Milli-Q water

NAcc – nucleus accumbens

NMDAR – n-methyl-d-aspartate receptor

NPY – neuropeptide Y

NTS – nucleus of the solitary tract

OFC – orbitofrontal cortex

OT – oxytocin

PBN – parabrachial nucleus

PDD – pervasive developmental disorder

PDD-NOS – pervasive developmental disorder-not otherwise specified

PCR – polymerase chain reaction

PFC – prefrontal cortex

PND – postnatal day

PNOC – prepronociceptin-expressing neuron

POMC – proopiomelanocortin

PPA – propionic acid

PSD-95 – postsynaptic density protein 95

PVN – paraventricular nucleus

qRT-PCR – real-time quantitative reverse transcription PCR

RFU – relative fluorescence units

SNP – single nucleotide polymorphism

TBS – tris-buffered saline

TBP – TATA-box binding protein

US – unconditioned stimulus

VGLUT – vesicular glutamate transporter

VPA – valproic acid

VTA – ventral tegmental area

VMH – ventromedial hypothalamic nucleus

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1. Introduction to Autism

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with broad symptomology, often characterized by cognitive and social impairments. It often, but not always, presents as difficulties with communication, repetitive behaviors, abnormal eating behaviors, sensory abnormalities, altered pain perception, and varying levels of intellectual disability. ASD is a primarily behavioral disorder, characterized by a shift in cognitive and neurological phenotypes and has several known comorbidities, such as depression, irritable bowel syndrome, eating disorders and ADHD (Bougeard et al., 2021; Fletcher-Watson & Happé, 2019; Maenner et al., 2020).

Intellectual delays in people with ASD are common, but not necessary for diagnosis (American Psychiatric Association, 2013). The burden of "lagging behind" can place autistic individuals at higher risk of elevated stress, anxiety, and depression, and often extends to their caregivers (Fletcher-Watson & Happé, 2019).

Autistic individuals often experience difficulties in social situations, interpreting social cues, and understanding nuance and intent in personal interactions. Individuals with ASD commonly report a lack of belonging in social groups and struggles with initiating and maintaining relationships (American Psychiatric

Association, 2013). Several studies implicate impaired amygdala function as an underlying reason for social deficits found in ASD individuals (Afif et al., 2022; Lecavalier, 2006; Sukhodolsky et al., 2008).

Alterations in structural and functional connectivity of the amygdala are common in people with ASD (Phelps & LeDoux, 2005; Wilensky et al., 2006). A study by Schumann et al. shows children with ASD between the ages of 7.5-12.6 years show increased amygdala volume compared to neurotypical peers, but the disparity disappears in groups aged 12.75-18.5 years. Results suggest that while the amygdala initially develops quicker in Autistic children, the volume is the same between both groups when development finishes (Schumann et al., 2009). A post-mortem analysis of 19 individuals by Schumann and Amaral also reported a significant decrease in neuron numbers in the lateral amygdala of people with ASD compared to controls (Schumann & Amaral, 2006). Additionally, Kleinhans et al. conducted an fMRI study where ASD individuals were tasked with matching faces to emotions. The study demonstrated significantly increased activation of the amygdala and left temporal lobe of ASD individuals with high levels of anxiety when shown emotional faces (Kleinhans et al., 2010).

Another core feature of ASD is altered structural brain connectivity, which may affect how people with the condition process stimuli (Irwin et al., 2001; Penzes et

al., 2011). Common impairments people with ASD may suffer from are sensory abnormalities, such as acute sensitivity to sounds, light, touch, et cetera. When presented with triggering stimuli, patients may react in excessive ways, often reporting feelings of pain and physical discomfort when overstimulated (Fletcher-Watson & Happé, 2019). Autistic individuals will often self-soothe with repetitive behaviors (Wolff et al., 2014). Again, the severity and presentation varies, with behaviors ranging from clapping hands, to auditory outbursts and self-harm (Minshawi et al., 2014). Conversely, reduced pain perception is also a symptom of ASD, with research showing that people on the ASD spectrum can have a much higher pain tolerance than neurotypical controls (Ruelle-Le Glaunec et al., 2021). This can sometimes lead to worse health outcomes for those patients, as it may cause them to under-report pain and discomfort in a clinical setting (Nicolardi et al., 2023).

Several fMRI studies and postmortem analyses show anatomical differences in the brains of people with ASD (Amaral et al., 2008; Gotts et al., 2012). Hollander et al. showed a 10% enlargement of the right caudate nucleus in adolescents and young adults with ASD compared to controls, which correlated with higher prevalence of repetitive behaviours (Hollander et al., 2005). Hadjikhani et al. reports cortical thinning of the inferior frontal gyrus, inferior parietal lobe, and superior temporal sulcus in individuals with ASD, and found significant correlation between thinning in

the mirror neuron system and severity of ASD symptoms (Hadjikhani et al., 2006). Conversely, Hardan et al. observed cortical thickness increases in the cerebrum, frontal, parietal, temporal and occipital lobes, but reported no significant differences in total brain volumes of people with ASD compared to controls (Hardan et al., 2006). Additionally, a number of post-mortem studies found reduced Purkinje cell density in patients with ASD (Bailey et al., 1998; Palmen et al., 2004).

ASD is further characterized by hyperconnectivity in local neural circuits, while hypoconnectivity is often observed between larger brain regions (Penzes et al., 2011). A common abnormality found in individuals with ASD is dendritic spine dysregulation. Studies have shown increased spine density on the apical dendrites of pyramidal neurons located in layer 2/3 of the frontal, parietal, and temporal lobes (Hutsler & Zhang, 2010; Irwin et al., 2001). The increased spine density is thought to contribute to local hyperconnectivity, which may disrupt normal synaptic transmission, ultimately leading to the social deficits and cognitive impairments commonly associated with ASD. In typical neurodevelopment, synaptic pruning occurs as part of synaptic maturation, refining neural connections by eliminating excess dendritic spines (Penzes et al., 2011). This pruning process is reduced in people with ASD, leading to an increased number of dendritic spines that continue growing at an excess rate during childhood (Coley & Gao, 2018; Hutsler & Zhang, 2010).

Not all symptoms of ASD are particularly debilitating on their own. Individuals need to meet a number of severity metrics for multiple symptoms to meet ASD diagnosis criteria (Lord, 2012; Rutter et al., 2010). The unique presentation of ASD that a person may suffer can have a massive impact on their quality of life and health outcomes. Consequently, ASD is classed as a disability. Given the variety in severity and presentation, the disorder is broadly referred to as "a spectrum," as an individual with ASD may have a broad combination of symptoms, each presenting with varying magnitude.

1.1. Diagnosis

ASD was originally described as a form of childhood schizophrenia by Eugen Bleuler in 1911. By the end of the 20th century the definition changed to include a set of related symptoms, ultimately being recognized as a spectrum of conditions with varying levels of psychiatric, psychological, and neurodevelopmental challenges (Peralta & Cuesta, 2011). Today ASD is a recognised neurodevelopmental disorder under the DSM-5 (American Psychiatric Association, 2013), and a variety of tools and screening techniques are available to assess children and adults for likelihood of ASD.

ASD was initially classified under the broader category of "pervasive developmental disorders" (PDDs). PDDs encompassed a range of social and communication deficits. ASD was formally distinguished from childhood schizophrenia in 1987 (Spitzer et al., 1987), though the diagnostic criteria remained strict. Efforts were made to refine ASD classifications based on the severity of symptoms in the DSM-4 (Bell, 1994), which introduced subcategories, including autistic disorder, Asperger's syndrome, atypical autism, and pervasive developmental disorder-not otherwise specified (PDD-NOS).

A further revision came in 2013 when the DSM-5 was released. Due to the lack of clear distinctions between different subsets PDDs, the most recent diagnostic systems and the International Classification of Diseases, researchers now use the umbrella term ASD The diagnostic criteria for ASD in the DSM-5 were the subject of some debate due to the inherent subjectivity of observation-based diagnosis, but as of today there is still no alternative method fit for clinical application (Johnson et al., 2013; Singer, 2012). A 2024 review assessed the validity of MRI-based methods for diagnosing ASD and found their performance to be ultimately unreliable, but potentially promising given future advancements (Schielen et al., 2024).

A major change in the DSM-5 was the removal of previous subcategories, including Asperger's syndrome, classic autism, Rett syndrome, etc. Instead, ASD is now

classified by severity, ranging from mild to severe. Severity is determined by the level of support the patients require with social interaction and communication and repetitive or restrictive behaviours. Required support comes in three levels, and ranges from the support needed being "some", substantial, to very substantial (American Psychiatric Association, 2013).

Unfortunately, individuals with ASD are not immune to other neurological conditions by the simple virtue of having autism, and the updated classification enables people to better understand and access the support they need. The revisions that came with DSM-5 allow ASD to be diagnosed alongside other neurodevelopmental conditions such as attention deficit hyperactivity disorder, epilepsy, intellectual disability, and language delay (Lord et al., 2018; Singer, 2012).

1.2. Prevalence and Treatment

While there is a plethora of medications that can ameliorate severity of some symptoms, ASD has no known treatment or cure. Additionally, no *in utero* screening methods exist at present. The best approach to ASD management is early diagnosis, and personalised support therapies (Fletcher-Watson & Happé, 2019). Studies from the past decade estimate that the number of people affected by ASD range from 4 - 60 cases per 10,000 children. The statistics vary significantly

depending on the country, socio-economic status, and awareness. Globally, it is estimated that about 1% of the population has ASD (Bougeard et al., 2021; Maenner et al., 2020).

ASD is often first diagnosed in children, with a 4:1 ratio between men and women cited in most populations, although a recent review suggests a number closer to 3:1 when accounting for diagnostic limitations (Loomes et al., 2017). Studies dating back from 1992 have estimated a rising trend in ASD prevalence globally. An increase in ASD prevalence among children in the UK was observed from 0.27% in 2000 to 1.53% in 2014. In France, prevalence grew from 0.26% in 1997 to 0.41% in 2003 (Bougeard et al., 2021). A recent meta-analysis puts the global prevalence of ASD at 1.18%, just above the 1% estimated by the World Health Organisation. (Talantseva et al., 2023)

Many factors influence epidemiological data, including population diversity, awareness, healthcare infrastructure, socio-economic conditions, and the availability of high-quality, population-based studies. However, it is important to remember the context of data when evaluating prevalence trends. For instance, the observed rise in ASD is likely due to an interplay between expanded diagnostic criteria which allowed for the inclusion of Asperger's and other PDDs into the spectrum in recent years and expanding access to healthcare in developed and

developing countries that lead to better understanding of the condition (American Psychiatric Association, 2013; Fletcher-Watson & Happé, 2019; Hyman et al., 2020). Following this trend, lower-to-middle-income countries with limited healthcare resources tend to report lower instances of ASD (Talantseva et al., 2023).

Global trends indicate a rise of ASD diagnoses world-wide. It is important to keep in mind that the rise of global ASD prevalence should in part be attributed to socio-economic factors that may lead to higher diagnostic rates in certain populations. Additionally, the diagnostic criteria in gold-standard measures may contribute to lower rates of ASD in women. Although diagnostic scores are normalised across sexes, a 2018 study by Ratto et al. found sex to be a significant predictor of likelihood for meeting diagnostic criteria for ASD on the ADI-R, with women being significantly less likely to qualify than men, especially if they had a higher IQ (Lai et al., 2011; Ratto et al., 2018; Rutter et al., 2010). Young girls and women are more likely to be missed for diagnosis than boys of the same age. Females are thought to have more compensatory mechanisms compared to males, such as more developed social skills and higher prevalence of camouflaging (Head et al., 2014; Lai et al., 2016; Loomes et al., 2017).

1.3. Genetics of ASD

ASD does not follow traditional Mendelian inheritance patterns. Hundreds of genes have been proposed as potential contributors to ASD, but their precise roles in the development of the disorder remain largely unclear. ASD does not appear to be linked to any single chromosome, which makes it difficult to pinpoint the causality (Auranen et al., 2002; Cohen et al., 2005; Genetics, 1998, 2001). Additionally, research suggests that genetic mutations may manifest differently in males and females, contributing to the observed differences in ASD prevalence and symptom expression. Sato et al. identified a rare CNV in the SHANK1 gene, a high-confidence ASD-associated gene with male-biased penetrance. The SHANK family of genes is responsible for proper formation of neuronal synapses by encoding scaffolding proteins. Their study found that males with a SHANK1 microdeletion exhibited high-functioning ASD, but female relatives with the same genetic variant did not meet the diagnostic criteria (Sato et al., 2012). Studies using animal models of ASD such as *Shank3*$^{\Delta C/\Delta C}$ mice and prenatally VPA exposed rat and mice also report altered NMDA receptor (NMDAR) functionality, implicating it in the aetiology of the condition (Fremeau et al., 2004; Lee et al., 2015).

A study by He et al. assessed the neurodevelopmental role of VGLUT2 by utilising recombinant Emx1-Cre$^{+/+}$/VGLUT2$^{fx/fx}$ k/o mice. Their results showed that VGLUT2 deficiency during a critical developmental period reduced glutamine transmission in

corticolimbic circuits, and led to reduced dendritic arborization, impaired spatial learning and memory, and increased exploratory activity in adult animals. Some of the behavioural findings mirrored NMDA receptor k/o phenotypes, linking VGLUT2 deficiency to neurodevelopmental disorders (He et al., 2012). However, it is worth noting that although animal models have contributed to our understanding of the link between ASD and NMDAR dysfunction, more research is required to confirm the hypothesis.

Females are hypothesised to require a higher number of genetic mutations to present with ASD due to the X chromosome associated protective factor (Steinberg & Webber, 2013). Current prevalence data support this line of thinking, with males presenting up to four times as often as females. Several ASD-related genes, including FMRP, MECP2, NLGN3, and NLGN4X, are located on the X chromosome, reinforcing its significant role in neural development (Jansen et al., 2017). The MECP2 gene is implicated in Rett syndrome, but is also considered a risk gene for ASD (Gonzales & LaSalle, 2010). Children presenting with Rett syndrome may be diagnosed with ASD due to an overlap in behavioural symptoms such as anxiety and repetitive behaviours. Additionally, the two share a significant overlap in the age of onset (Cohen et al., 2005).

A key genetic factor linked to ASD is duplication of the 15q11-q13 region – associated with severe intellectual disability, language disorders, and seizures (Rineer et al., 1998). Deletion of 22q11 is linked to developmental delay, delayed speech, and higher pain tolerance, which are characteristic of ASD. Additionally, mutations in exons appear to pose a higher risk for ASD than those in introns (Poduri et al., 2013). A 2017 study of large-scale whole-exome sequencing found that postzygotic mutations of pathogenic variants showed elevated expression in the amygdala and striatum, which are involved in social cognition (Lim et al., 2017).

Additionally, there may be epigenetic factors at play, as DNA methylation has been linked to ASD (Kimura et al., 2019; Ladd-Acosta et al., 2014). Wong et al. conducted a study that found common methylation patterns at specific CpG sites that were associated with distinct ASD symptom groups. Additionally, a significant correlation was observed between DNA methylation in the P2RY11 and NRXN1 genes and quantitative autistic trait scores (Wong et al., 2014). These results indicate that epigenetic changes may influence ASD severity and symptom variability, adding another layer of complexity to the disorder.

1.4. Non-genetic Risk Factors

There are other known risk factors that may increase the chances of developing ASD. Environmental factors such as exposure to toxins and certain chemicals may modulate risk genes associated with developing the condition as discussed below.

Valproic acid (VPA) is a branched short chain fatty acid that inhibits depolarising mechanisms of neurons and can prevent aberrant neuronal excitation. After being discovered for its anti-seizure qualities, it was prescribed for treatment of epilepsy and migraines in 1967. VPA has well documented teratogenic effects that were not known when it was first introduced as an anti-epileptic drug (Zarate-Lopez et al., 2024).

Early gestational exposure to VPA increases the risk of neural tube defects in offspring, so much so, that it is now a well-established practice for modelling ASD in animals (Kim et al., 2011; Wagner et al., 2006). VPA also interferes with neurodevelopmental processes, including neuronal precursor proliferation, differentiation, migration, and synaptogenesis, ultimately resulting in altered neuronal connectivity (Favre et al., 2013; Kataoka et al., 2013; Kuemerle et al., 2007). The estimated prevalence of ASD ranges from 1-2.5% in children of mothers who used VPA during early pregnancy (Ornoy, 2009).

Propionic acid (PPA) has also been reported to induce inflammatory neural responses, as well as behavioural changes that mimic ASD. The structure of PPA allows it to penetrate the blood brain barrier (BBB) and induce intracellular acidification in the brain, leading to behavioural changes. Intraventricular infusion of PPA causes hyperactivity and repetitive behaviours, as shown in a 2007 rat study (MacFabe et al., 2007). Another study looking at oral administration of PPA found increased oxidative stress, accompanied by decreased glutathione and catalase activity in PPA-treated rats. Additionally, elevated levels of Interleukin-6, TNFα, and IFNγ further support its pro-neuroinflammatory effects (El-Ansary et al., 2011).

Heavy metal exposure has also been implicated in neurodevelopmental disorders, including ASD. Animal model studies suggest that arsenic, lead, and mercury disrupt calcium homeostasis and impair synaptic function, contributing to neurodevelopmental abnormalities (Grandjean & Landrigan, 2006). Methylmercury in particular affects brain development through oxidative stress and disruptions in calcium and glutamate regulation. Chronic low dose exposure can result in neurobehavioral deficits. In a study conducted by Crump et al. (1998), psychological tests were administered to 237 children aged 6-7 years in New Zealand. The results were then correlated with the mercury concentration in their mothers' hair during pregnancy, revealing an association between prenatal mercury exposure and subtle neurobehavioral deficits in these children (Crump et al., 1998).

Phthalates are commonly used additives in plastic products, such as hygienic tools, water bottles, plastic food containers, et cetera and may be another risk for increasing ASD rates. Research on the neurodevelopmental effects of phthalates has mixed results (Jeddi et al., 2016; Shin et al., 2018). Shin et al. reported inconclusive findings. However, a larger study by Kim et al. measured five phthalate metabolites during mid-term pregnancy and followed 547 mother-child pairs until the children were 4, 6, and 8 years old. Assessing autistic traits using the Social Communication Questionnaire at each stage, they found that prenatal phthalate exposure was associated with increased autistic traits in young children, with a stronger correlation observed in males compared to females (Kim et al., 2017).

2. Eating Behaviours in ASD

Eating disorders in the context of ASD are less researched than behavioral aspects. People on the spectrum often present with atypical feeding behaviors that can range from avoiding novel foods, to exclusively eating a specific food (Mayes & Zickgraf, 2019; Schreck et al., 2004). This can in part be explained by elevated levels of hyponeophagia in people with ASD. Additionally, meal location, presence or absence of stimuli, social cues derived from parents and caregivers, food presentation such as color and dishware, can all affect eating practices (Huxham et al., 2021; Keen, 2008; Schreck & Williams, 2006; Wang et al., 2022). Given the

heterogeneous presentation of ASD, prevalence of abnormal sensory processing, and an aversion to novelty, it is unsurprising that autistic individuals often present with feeding abnormalities.

Pica is a condition characterised by ingestion of non-food materials and is more prevalent in ASD children (Kinnell, 1985; Schreck et al., 2004). Several plausible explanations for pica exist, but the exact cause of this behaviour remains elusive. A 2019 study by Mayes et al. found that >70% of aberrant eating behaviours occurred in children with ASD, compared to just 4.8% in neurotypical children (Mayes & Zickgraf, 2019). Additionally, the study found that out of all subjects, children with ASD were the only ones to have pica.

Feeding behaviour is primarily regulated by mechanisms that respond to hunger, which is the conscious perception of an energy deficit. The termination of feeding often coincides with reaching satiation, even if food is still available. However, feeding is not solely driven by physiological need; it is also influenced by hedonic or "reward-related" mechanisms, which drive food consumption for pleasure rather than necessity (Waterson & Horvath, 2015). The reward system evolved to enhance survival of the individual and therefore species by utilising neural mechanisms that signal a state of well-being. Positive reinforcement promotes behaviours that produce the most favourable outcomes relative to required effort and is generally

associated with essential activities such as eating and mating (Bromberg-Martin et al., 2010; Klein et al., 2019; Steinberg et al., 2014). Recent research indicated this may be a major area of dysregulation in ASD individuals (Cascio et al., 2012; Klockars et al., 2021; Schiavi et al., 2019).

Dopamine is a neurotransmitter credited with signalling pleasure and hedonic reward. The role of dopamine in reinforcement learning and cue-reward associations is likewise well understood (Berridge & Robinson, 1998; Creed et al., 2014; Palmiter, 2008; Salamone, 1994; Schultz, 2010). Dopamine also plays a crucial role in generating motivation and making goals "wanted" by driving actions to attain them. For dopamine to effectively motivate actions that lead to rewards, its release should coincide with rewarding experiences. In line with this, most dopamine neurons exhibit strong activation in response to unexpected primary rewards like food and water, often generating phasic bursts of activity. (Schultz, 1998, 2013). Research indicates neuronal activation of the nucleus accumbens (NAcc) by dopaminergic inputs from the ventral tegmental area (VTA) is sufficient to induce reward responses in rats (Steinberg et al., 2014).

Hypothalamic neurocircuits are directly linked to the mesolimbic reward system, including the VTA and NAcc, which regulate the pleasurable, or "hedonic" aspects of food intake (Morales & Margolis, 2017). The VTA is a brain region involved in

processing reward, motivation, cognition, and aversion (Morales & Margolis, 2017). The VTA is located in the midbrain, and contributes to circuitry involved in learning, reinforcement, and outcome evaluation (Fields & Margolis, 2015; Solié et al., 2022). It is primarily composed of dopaminergic (DA) neurons, which make up about 65% of the population. GABAergic neurons, which produce the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), make up the majority of the non-DA population in the VTA (Nair-Roberts et al., 2008).

The VTA sends DA projections to the NAcc, which is well known for reward anticipation processing (McNamara et al., 2014). VTA projections to the NAcc play a key role in encoding incentive salience, which motivates an individual to seek rewarding stimuli (Berridge & Robinson, 1998). VTA projections to the hippocampus facilitate spatial memory formation (Brown et al., 2012; Steinberg et al., 2014). It also projects to the amygdala, prefrontal cortex (PFC), and the olfactory bulb. Higher cortical areas like the ventromedial prefrontal cortex and orbitofrontal cortex (OFC) integrate homeostatic, hedonic, and sensorimotor components of feeding such as reward to guide future anticipatory behaviours (Grabenhorst & Rolls, 2011).

The arcuate nucleus (ARC) is located within the hypothalamus and contains two distinct populations of neurons involved in feeding regulation: AgRP/NPY and POMC

(Fioramonti et al., 2007). NPY neurons produce neuropeptide Y (NPY), and together with agouti-related peptide (AgRP) producing neurons they stimulate appetite. Pro-opiomelanocortin (POMC) neurons on the other hand produce α-melanocyte-stimulating hormone, which is understood to promote anorexigenic (appetite suppressing) responses (Jais et al., 2020; Vohra et al., 2022). Together, AgRP/NPY and POMC neurons are responsible for regulating food intake via stimulating or suppressing appetite to maintain homeostatic balance (Fenselau et al., 2017). Both NPY and POMC neurons in the ARC have receptors for insulin and leptin, allowing them to receive signals about homeostatic changes using those metabolites (Könner et al., 2009). POMC neurons extend to key hypothalamic regions, including the dorsomedial hypothalamic nucleus, lateral hypothalamus, and ventromedial hypothalamic nucleus (VMH). Importantly, POMC neurons primarily project to second-order neurons within the paraventricular nucleus (PVN). The PVN produces a wide range of neuropeptides involved in sympathetic signalling; and damage to the PVN results in dysregulation of feeding behaviours, as well as obesity (Kannan et al., 1989; Roh & Kim, 2016)

Childhood obesity and higher BMI have been consistently reported in children with ASD, highlighting the complex relationship between dietary preferences and health outcomes in this group (Curtin et al., 2005; Curtin et al., 2014). Conversely, studies

show that people with ASD are also at a higher risk of malnutrition due to increased avoidance of non-preferred foods (Bölte et al., 2002; Sedgewick et al., 2020).

Children with ASD are often described as “picky eaters,” however there is no definitive explanation for ASD associated eating abnormalities. Food selection can be influenced by a multitude of factors, including preference, acceptance, consequences (such as aversion due to food expulsion), motivation, and early nutritional exposure. People with ASD often show great difficulty accepting novel foods or meals presented in an unusual fashion (Keen, 2008). Sensory processing plays a critical role in food selectivity as well, with research demonstrating altered olfactory and gustatory processing in individuals with ASD (Birch, 1999; Shankar et al., 2018). An observational study by Ahearn et al. measured food acceptance and selectivity in children with ASD aged 3 to 14 years. They found that 57% of the study participants showed significant selectivity based on food type and texture. Starch, protein, and fruit had the highest levels of acceptance, but the research notably lacked a control group (Ahearn et al., 2001). A study by Bennetto et al. used salt, sucrose, citric acid, and quinine solutions in measuring olfactory and taste identification in ASD individuals aged 10 to 18 years. Their results show decreased accuracy in sour and bitter taste identification, but no impairments to recognition of sweet and salty flavours in individuals with ASD (Bennetto et al., 2007).

ASD associated food avoidance extends beyond typical taste and texture preferences to include additional factors such as colour, presentation style, and timing. A cross-sectional study by Huxham et al. analysed levels of food acceptance in ASD children aged 3 to 16 years found 67% of the participants were influenced by food presentation, and 51% by the colour. Additionally, the study measured acceptance by food group and found unrefined carbohydrates and vegetables to be the categories with consistently higher or lower acceptance rates respectively (Huxham et al., 2021). Schreck and Williams conducted a study measuring abnormal eating behaviours and food preferences in 4- to 12-year-old children with ASD. They found that 72% of the subjects had restricted food acceptance. Additionally, their study shows that food selectivity in ASD children is related to their family's eating preferences (Schreck & Williams, 2006).

A strong preference for consistency can contribute to restrictive eating habits in autistic individuals. Hyponeophagia, an avoidance of novel foods, and a narrow range of "preferred" foods often leads to a limited diet in children with ASD (Huxham et al., 2021; Wang et al., 2022). ASD patients often display a heightened preference for palatable foods, which together with a limited dietary variety, can increase the risks of obesity and other eating disorders (Healy et al., 2021; Lucarelli et al., 2017).

A number of studies have documented food preferences in autistic children, common examples including: pasta, peanut butter, rice, pizza, French fries et cetera (Healy et al., 2021; Mayes & Zickgraf, 2019; Schreck & Williams, 2006; Wang et al., 2022). Notably, many of these foods can be mass produced, which ensures a consistent flavour, texture, and presentation due to modern automation techniques. Interestingly, not all autistic individuals seem to subscribe to the consistency view, with some studies noting a preference for fruit (Schreck & Williams, 2006; Schreck et al., 2004). Together this paints an interesting dichotomy for causal food preferences in ASD individuals.

Altered neural processing is a core feature of autistic brains which may extend to altered perception of food associated reward. The anterior cingulate cortex (ACC) receives input from prefrontal and parietal brain areas through the corpus callosum, and is responsible for integrating emotional, multimodal sensorimotor interoception, and higher cognitive functions with homeostasis together with the insula (Rolls, 2023). The ACC may exhibit anomalous activity due to the increased workload needed to achieve positive feedback from goal-oriented behaviours, as demonstrated by a 2008 study (Schmitz et al., 2008). Their research demonstrated that a monetary incentive for correct responses in a target experimental paradigm increased activation of the left anterior cingulate gyrus in autistic brains compared to controls. Autistic individuals in the study also showed a significant reduction in

periventricular white matter density in the left frontal lobe. In another study, Autistic individuals demonstrated greater activation in the ACC and insula in fMRI scans when food-deprived participants viewed images of appetizing, high-calorie food (Cascio et al., 2012).

However, behavioural tests without complementary techniques such as fMRI or neuromolecular evaluations are insufficient to fully explain the neurological basis of feeding-related dysfunctions in autistic individuals. While behavioural studies and questionnaire-based methods provide valuable insights, they are not without limitation. Assessment methodologies can vary, impacting the consistency of findings. Many studies are conducted in regions with readily available high-calorie foods, which may influence dietary decisions and contribute to differences in body composition across racial groups (Huxham et al., 2021; Mayes & Zickgraf, 2019; Schreck & Williams, 2006; Schreck et al., 2004). Other factors, such as biases in patient recruitment, and mealtime discipline and expectations can also influence feeding habits, highlighting incomplete understanding of the phenomena.

2.1. Food Toxicity Response

The dorsal vagal complex (DVC) in the brainstem processes gastrointestinal sensory input and plays a key role in coordinating efferent responses influencing caloric intake, gastric and intestinal motility, and pancreatic exocrine secretion. It includes

nucleus of the solitary tract (NTS), area postrema (AP), and the dorsal motor nucleus of the vagus nerve (DMX) (Sobrino Crespo et al., 2014; Troadec et al., 2022).

The area postrema is another component of the DVC and plays a crucial role in initiating the vomiting response. Area postrema receives input from multiple brain regions involved in feeding regulation, including the NTS, parabrachial nucleus (PBN), and the PVN. The AP contributes to initiation of the emetic response and broader feeding regulation by responding to various circulatory neuromodulators like ghrelin, peptide YY, and glucagon-like peptide-1 (Browning & Carson, 2021; Price et al., 2008). As a circumventricular organ, AP allows hypothalamic hormones to enter the bloodstream without compromising the integrity of the BBB. Additionally, AP enables substances that cannot cross the BBB to influence brain function (Ganong, 2000).

The DMX regulates gastrointestinal functions, influencing processes like gastric emptying, intestinal transit, and nutrient absorption. Integrated signals from the DVC, midbrain, and forebrain are relayed to the DMX to generate appropriate physiological responses at the digestive tract level (Troadec et al., 2022; Zhong et al., 2021).

The brainstem provides significant contribution to protective defence mechanisms that prevent further exposure or ingestion of harmful substances following detection of toxic chemicals in the body (Zhong et al., 2021).

Conditioned taste aversion (CTA) is a defence mechanism that protects against voluntary ingestion of toxic foods. CTA forms a strong association between the sensory properties of taste and the experience of illness, even when the food itself is not toxic (Chambers, 2018; Lin et al., 2017). Humans and animals have a tendency to attribute illness and malaise to “something they ate”, even when symptoms appear hours after consumption. Lithium Chloride (LiCl) can be used to induce an aversive response in association with food, when injected before a meal (Bernstein & Goehler, 1983; Meachum & Bernstein, 1990).

CTA is typically induced by administering an intraperitoneal injection of LiCl after the consumption of a highly palatable, novel solution, such as sucrose flavoured water. LiCl acts as an unconditioned stimulus which triggers nausea and vomiting. A key result following CTA establishment involves acquiring a learned association between a recently consumed food, which is often novel, and future avoidance of the perceived toxic edible (Etscorn & Stephens, 1973).

Pal et al. recently found that VPA rats are resistant to CTA acquisition. In their study, healthy controls and VPA rats were given a 3 mEq/kg dose of LiCl to examine the effects of CTA on neuronal activation and transcriptional changes. VPA rats showed no changes in c-Fos IR in the hypothalamus or amygdala but had differential regulation of five genes related to feeding and stress in the amygdala and PVN compared to controls. Notably, even a high dose (6 mEq/kg) of LiCl was insufficient for inducing CTA in VPA rats, who continued consuming saccharin at a similar rate to saline treated controls. (Pal et al., 2025) (Figure 1)

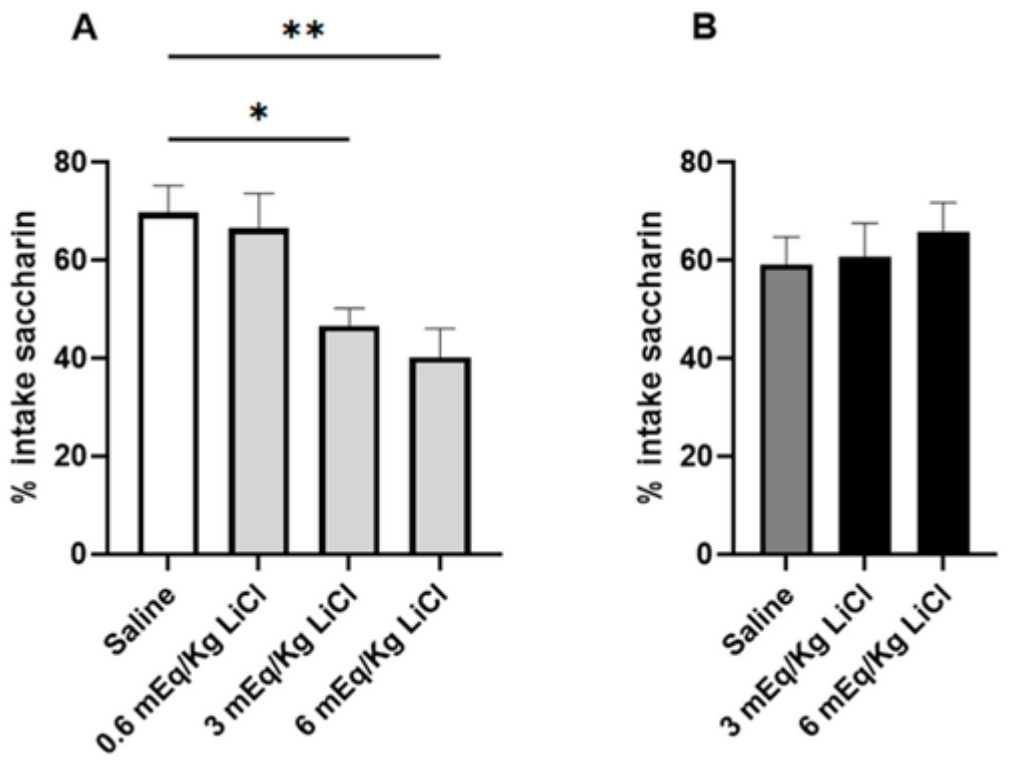

Figure 1

Note. Effect of i.p. saline and LiCl on the acquisition of CTA to 0.1% saccharin. The graphs show the % intake of the saccharin solution during a two-bottle test in which a choice between water and saccharin was given. (A) Intake in control animals (N =

*10/group). (B) Intake in VPA animals (N = 8/group). Data are expressed as mean ± SEM * p < 0.05, ** p < 0.01. From "Neuromolecular Basis of Impaired Conditioned Taste Aversion Acquisition in Valproate-Induced Rat Model of Autism Spectrum Disorder", by Pal et al. Genes 2025, 16(2), 203, Results section, Figure 2. (https://doi.org/10.3390/genes16020203). Included with permission from authors.*

3. Animal Models in ASD Research

3.1. Use of Laboratory Animals

The animal model for autism is idiosyncratic, meaning it is not "true" ASD. The VPA rat model of ASD is a replica that mirrors behavioural and neurological traits seen in people with the condition. While rats lack the cognitive and social complexity of humans, they provide a controlled system for studying mechanisms associated with ASD. Although observational human studies using questionnaires and fMRI scans offer valuable insights into the condition, animals offer the only ethical way to conduct research that involves directly manipulating the brain.

Most models are imperfect but are a valuable tool for researching potential causes and treatments of any ailment, not just ASD. Animal research has a long history and is widely acknowledged as one of the most important *in vivo* tools available to scientists (Robinson et al., 2019). Animal models like the VPA rat allow us to explore neural pathways, test interventions, and develop hypotheses that can later be examined in human studies. There is no comparable alternative to using animals

in the discovery, development, and application of new medicines and treatments that can be used in advancing both human and veterinary medicine at this time.

3.2. Choice of Species

Rodents offer an effective model for investigating biological mechanisms of ASD by balancing behavioural relevance, genetic accessibility, and practical housekeeping needs. There are many reasons justifying this preference compared to other mammals; their ease of handling, fully sequenced genome, short generation time and ability to be trained to name a few. Mice are favoured in demonstrating ASD models reliant on genetic phenotypes, as they are more accessible for modification and have a fully mapped epigenome (Kuemerle et al., 2007; Wagner et al., 2006). Another advantage of choosing rodents for research are their size and maintenance needs compared to other mammals, which make having larger cohorts feasible even in small scale research.

Unlike mice, rats display more complex and explicit social behaviours, making them better suited for studying those aspects of ASD (Kondrakiewicz et al., 2019). Rats' larger brain and body size also provides a practical advantage, as working with bigger tissue samples improves accuracy and lowers the skill barrier for identifying relevant neurological structures. While using mice could have provided us with a

larger cohort of individuals, their limited behavioural complexity and smaller anatomy made the species less attractive. Additionally, because my research centred on learning associated with food intake, it made sense to use the rat, which is understood to be more food driven than its smaller cousin (Smith et al., 2019). Although VPA animals mimic autistic traits found in humans, like behavioral changes, and anatomic differences, there is no “perfect” way to model ASD in mammals. VPA rats are an idiosyncratic phenotype of this complex and multifaceted developmental condition. Accurately measuring cognitive markers in animals is difficult, subject to much debate, and often highly controversial. Researchers must rely on behavioral presentation when assessing animals for a likelihood of phenotype fitting ASD. Additionally, because ASD is typically more pronounced in males, only male animals were used in the study (Loomes et al., 2017; Werling & Geschwind, 2013).

3.3. Measuring Brain Activity with c-Fos

The acquisition of memory and learning processes is complex and multimodal, involving a several brain areas and a variety of neuropeptides, amino acids, monoamines and other messenger molecules that are simply beyond the scope of this thesis (Cohen & Squire, 1980; Graf & Schacter, 1985). However, products of neuronal activity such as the c-Fos protein and expression of certain

neurotransmitters can give us insights into brain activity following an event or intervention. By looking at c-Fos activity in key brain regions implicated in learning, reward, and toxicity, we can quantify differences in response to treatment.

Immunohistochemical staining of the c-Fos protein can be used to visualise neuronal activation post stimulus or drug injection and its use in neuroscientific research has been well documented since the 90s (Bullitt, 1990). The protein of interest is a product of the c-Fos proto-oncogene which is expressed following neuronal depolarization. It can serve as a marker for neuronal activity throughout the nervous system, though the analysis must be done postmortem. The method, like any, is not without limitations. Staining for c-Fos protein can only indicate excitatory responses, as inhibited neurons do not produce the protein, which restricts our ability to study inhibitory pathways. The lifespan of c-Fos Protein is short making this technique suitable for only a limited type of experiments, and expression is more potent following short or fast stimuli (Piechaczyk & Blanchard, 1994). Additionally, it requires the excision of the brain.

Despite the limitations, c-Fos visualisation offers significant advantages that make it a valuable tool in neuroscience. It can be used in a variety of animal models, its versatility allows for analysing any time of stimulus, and it is a well-researched, and effective technique that can be used to effectively quantify neuronal responses.

Activation of c-Fos can be visualised as a dark spot on a brain tissue slice, corresponding to the location of a recently activated neuron (Sakurai, 2024).

3.4. Genetic Markers

I analysed a selection of genetic markers related to homeostatic regulation, appetite, and neuroplasticity to investigate differences in the CTA response between control and VPA-exposed rats. Beta actin (ActB) and TBP genes were chosen for reference and normalisation of results.

The brain secretes a plethora of signalling molecules such as amino acids, neurotransmitters and cytokines that allow it to process and respond to signals received from the periphery. The brain's role in maintaining homeostasis in the body is exemplified through precise synthesis and methodical release of these molecules that enable its regulation of systemic functions. However, not all signalling molecules require constant production, as their expression and secretion are tightly regulated in response to specific physiological demands (Binder & Scharfman, 2004; Cowley et al., 1999; Rolls, 2023; Wilensky et al., 2006; Xiao et al., 2017). Examining gene expression related to residues or receptors associated with signalling chemical production and uptake can provide us with a snapshot of neural activity within a timeframe of interest.

Neuropeptides are short 3 to 100 amino acid residues synthesised by neurons. They are generally considered secondary messengers due to their slow release and ability to act on distal cells. Neuropeptides are the largest and most diverse class of messenger molecules the brain uses for signalling and are often expressed alongside other neurotransmitters (Hökfelt et al., 2000).

3.4.1. NPY

Neuropeptide Y (NPY) is one of the most abundantly expressed peptides in the mammalian brain (de Quidt & Emson, 1986; Tatemoto et al., 1982). NPY is predominantly expressed by GABAergic neurons in the brain after sustained activation (Vezzani et al., 1999). However, NPY may also pass the blood brain barrier and enter the brain through circulation (Kastin & Akerstrom, 1999). Evidence suggests that NPY modulates neuroplasticity, synaptic transmission, and various forms of memory (Gøtzsche & Woldbye, 2016).

In a study involving rats, NPY has been shown to impair cued and conditioned fear responses following an intracerebroventricular (i.c.v.) administration (Méndez-Couz et al., 2021). In another study, NPY suppressed fear expression when administered during the memory extinction phase (Lach & de Lima, 2013). Additionally, i.c.v. administration of 10 µg NPY or bilateral infusion of 10 pmol NPY into the

basolateral amygdala impaired the acquisition of cued fear conditioning while enhancing extinction retention of conditioned fear responses (Gutman et al., 2008).

3.4.2. OXTR

Oxytocin (OT) is a key hormone that regulates food intake and energy balance. OT has substantial evidence linking it to termination of food intake (Klockars et al., 2015). The release of oxytocin coincides with food ingestion and activates hypothalamic OT neurons. Notably, OT is particularly effective in reducing the consumption of carbohydrates and sweet-tasting foods (Olszewski et al., 2022).

While OT is well-known for enhancing satiation, emerging research indicates that its influence on food intake also arises from its ability to diminish the rewarding aspects of ingested foods. OT neurons project to various regions within the brain's reward circuitry, including the mesolimbic reward pathway (Borland et al., 2018; Peris et al., 2017; Xiao et al., 2017). Recent research supports the idea that OT significantly modulates the intake of palatable and sweet foods, reinforcing its dual role in appetite regulation through both satiation and reward modulation. (Head et al., 2021; Herisson et al., 2016; Olszewski et al., 2022; Pal et al., 2022)

3.4.3. BDNF

Brain-Derived Neurotrophic Factor (BDNF) is another key regulator of satiety. Neurons in the VMH primarily receive input from the ARC and project their axons to several brain regions, including back to the ARC, PVN, LH, dorsomedial nucleus (DMN), and NTS. Most VMH neurons express steroidogenic factor 1 alongside BDNF. Studies have shown that selectively deleting BDNF in the VMH leads to excessive eating (hyperphagia) and obesity in mice. Additionally, mutations that disrupt BDNF or its receptor: tropomyosin receptor kinase B, result in severe obesity and hyperphagia in both humans and rodents. (Gray et al., 2006)

BDNF was first purified from mammalian brains by Yves-Alain Barde and Hans Thoenen in 80s (Barde et al., 1982). It was initially identified for promoting sensory neuron growth. Subsequent research revealed its critical roles in learning, memory, synaptic transmission, plasticity, and various neurodegenerative and neuropsychiatric disorders (Binder & Scharfman, 2004). Importantly, BDNF has been recognized for its role in central feeding regulation. Intracerebroventricular infusion of BDNF in rats induced hypophagia and 10–15% weight loss over two weeks. (Pelleymounter et al., 1995). In mouse models of obesity, BDNF retained its anorexigenic effects, significantly reducing food intake in both high-fat diet-fed mice and leptin receptor-deficient (*db/db*) mice (Unger et al., 2007).

3.4.4. CRH

The stress response is initiated by corticotropin releasing hormone (CRH) via the hypothalamus-pituitary-adrenocortical axis (HPA). A functional stress response is a necessity for survival, as it allows the organism to quickly mobilise energy resources in a crisis. The PVN releases CRH when a stressful event occurs. When the anterior pituitary detects CRH, it in turn releases adrenocorticotropic hormone (ACTH), which triggers secretion of cortisol from the adrenal cortex into the circulation. Cortisol in turn acts as negative feedback and inhibits the production of both CRH and ACTH to downregulate the system (Bouarab et al., 2019; Herman et al., 2016).

The HPA stress response is adaptable and highly conserved between mammalian species, including humans and rats. Measuring CRH transcription can give us insight into the stress levels of the animal and provide a basis for future investigation using more direct methods such as blood serum cortisol screening.

3.4.5. MC3R

Orexigenic POMC neurons are found in the ARC of the Hypothalamus. POMC neuronal activity releases a satiety-inducing hormone alpha-melanocyte-stimulating hormone (α-MSH) which acts through melanocortin receptors in the PVH. α-MSH binds to the melanocortin receptors 3 and 4 (MC3R and MC4R) and

activates catabolic pathways that lead to reduced food intake and increased energy expenditure. (Fenselau et al., 2017; Garfield et al., 2015; Krashes et al., 2016; Olszewski et al., 2001; Waterson & Horvath, 2015). MC3R is thought to regulate AgRP neurons in mice, and their dysfunction is linked to food intake and anxiety based anorexia (Sweeney et al., 2021).

3.4.6. PNOC

PNOC neurons are characterised by their expression of prepronociceptin and represent a recently identified regulator of food intake during exposure to a highly palatable diet. They are an orexigenic population of neurons found in the ARC of the Hypothalamus, that form strong inhibitory GABAergic connections to POMC neurons (Joly-Amado et al., 2014). POMC neuronal activity releases a satiety inducing hormone alpha-melanocyte-stimulating hormone (α-MSH) which acts through melanocortin receptors in the PVH (Fenselau et al., 2017; Garfield et al., 2015; Olszewski et al., 2001).

3.4.7. PSD95

Postsynaptic Density Protein 95 (PSD-95) is a scaffolding protein involved in synaptic maturation and plasticity. It is implicated in neurodevelopmental and psychiatric disorders, including ASD and schizophrenia (Coley & Gao, 2018; De Rubeis et al., 2014; Penzes et al., 2011). PSD-95 is essential for the stabilization

and clustering of neurotransmitter receptors at excitatory synapses. Studies have indicated that mutations or dysregulation of PSD-95 can lead to abnormal synaptic signalling, impaired neuroplasticity, and deficits in higher-order cognitive processes, further implicating its role in the aetiology of neurodevelopmental diseases (Ehrlich & Malinow, 2004; Penzes et al., 2011).

3.4.8. VGLUT2

Glutamate is essential for protein synthesis and metabolism in all cells. Neurons utilize specialized mechanisms for its regulated secretion via synaptic transmission Vesicular glutamate transporters (VGLUTs). VGLUTs facilitate the uptake of L-Glutamate (L-Glu) into synaptic vesicles, enabling its role as an excitatory neurotransmitter (Fremeau et al., 2004). Glutamate is produced by the glutaminase enzyme which is present in all types of neurons and glial tissue. L-Glu binds to postsynaptic glutamate receptors AMPA and NMDA, which are hypothesised to produce ASD like symptoms when dysfunctional (He et al., 2012; Lee et al., 2015). Excessive and persistent synaptic L-Glu release can also lead to NMDA receptor-dependent excitotoxicity. There are three known subtypes of VGLUTs (1, 2 and 3), which share structural and functional similarities, but differ in distribution patterns within the central nervous system (CNS) (Blanke & VanDongen, 2009).

The distribution of VGLUTs in the brain is highly conserved between rodents and humans, with VGLUT1 and VGLUT2 as the two predominant isoforms. The isoforms have little overlap in distribution within the brain, hinting at potential functional differences. However, the functional activity between the known isoforms is indistinguishable with present bioenergetic or pharmacological profiles (Vigneault et al., 2015). VGLUT3 is implied to be involved with more subtle local transmission modulation than the two more abundant forms. VGLUT2 mRNA is mainly found in the DVC and thalamus, and its reduced expression is connected to reductions in pyramidal neuron plasticity, spatial learning, and dendritic refinement (Leloup et al., 1994; Pietrancosta et al., 2020; Royo et al., 2022).

3.5. Research Aims

The overarching goal of this thesis is to examine functional response differences in the midbrain and brainstem of VPA rats to conditional taste aversion learning following toxic food ingestion. LiCl is well-researched in simulating a noxious stimulus to induce a food toxicity response. The specific aims formulated to accomplish this are as follows:

Specific Aim One:

Evaluate the food related reward response following an LiCl injection by using c-Fos activation levels in the midbrain of VPA rats. Specifically, changes in c-Fos

expression were looked at in the NAcc shell and core, which are implicated in reward processing and learning behaviours.

Specific Aim Two:

Investigate how LiCl affects c-Fos activation in the dorsal vagal complex of the brainstem of VPA rats. The areas of interest are involved in coordinating efferent responses following toxic food ingestion and include the AP, DMX, and NTS.

Specific Aim Three:

Identify transcriptional changes in the ARC of the hypothalamus for a selection of genes associated with conditional taste aversion, synaptic plasticity, satiety and homeostatic controls in VPA and controls. Differential regulation of transcripts in ASD could be contributing to abnormal feeding behaviours seen in VPA rats.

4. Methods

4.1. Animals

Experiments were carried out on male adult Sprague-Dawley rats. The animals were housed in a temperature-controlled animal facility (22C +-2) inside plexiglass cages with wire tops. The day-light cycle was kept to 12:12 with lights on at 7AM. The animals were kept on a diet of ad lib standard chow (Sharpes New Zealand; energy density: 3.6 kcal/g) with free access to water. The animals were treated with standards set by the National Institute of Health Guide for the Care and Use of Laboratory Animals and National Animal Ethics Advisory Committee. The University of Waikato Animal Ethics Committee approved the procedures described herein (protocol # 1155).

Generation of VPA animals

The animal model of ASD is based around VPA's teratogenicity, which leads to developmental and behavioral abnormalities in offspring if the mother is exposed to the chemical during early stages of embryonic development when the neural tube closure occurs. Exposing a pregnant animal to a high enough dose of VPA can produce a phenotype consistent with the presentation of classical ASD. Although the exact mechanisms underlying pathophysiological effects of VPA are poorly understood, there is a large body of evidence validating the model (Kim et al., 2011; Nicolini & Fahnestock, 2018; Wagner et al., 2006; Zarate-Lopez et al., 2024).

The autistic rat models (hereby VPA) were generated by following well-established procedures described in detail elsewhere (Rinaldi et al., 2008; Schiavi et al., 2019; Schneider & Przewłocki, 2005). Adult female rats were mated overnight with males of a similar age. Vaginal swabs were performed each morning to detect spermatozoa with 1% crystal violet (Sigma). The date of spermatozoa detection was labeled as embryonic day 0.5 (E0.5). On E12.5 the pregnant rats received an intraperitoneal (IP) injection VPA (Sigma) dissolved in saline (0.9%). The injection volume was matched to body weight with each pregnant animal receiving 500 mg/kg. The resulting offspring were kept with the mothers and allowed to nurse until postnatal day (PND) 25, on which they were weaned. Once weaned, the VPA-exposed male offspring were subjected to several behavioral tests to validate the phenotype.

Experimental controls were obtained in the same way as the VPAs, with dames receiving a body weight matched intraperitoneal (IP) injection of 0.9% saline instead of VPA. Some of the VPA animals presented with varying levels of tail deformation in association with VPA toxicity during development (Favre et al., 2013). This is in line with previous research and did not affect my study.

VPA Model Validation

Standard behavioral tests were performed to identify key traits associated with ASD presentation in the VPA rat model. Subsequent analysis of test outcomes was used to confirm that the cohort exhibited aberrant behaviors in line with reports of the animal phenotype for ASD. (Figure 2)

VPA animals have elevated levels of anxiety in line with the ASD phenotype, particularly in novel situations (Olexová et al., 2016; Zarate-Lopez et al., 2024). The elevated plus maze is a behavioral test used to gauge anxiety driven behaviors in rodents (Kondrakiewicz et al., 2019). It consists of a cross shape with two open arms, and two walled ones, with no barriers in the middle. The maze is situated above ground level to provoke a further sense of anxiety in the animals. The subject is then placed in the middle of the maze and time spent in the covered vs open part of the contraption is measured. This test is designed to evoke an anxiolytic response by placing the animal in an unfamiliar environment. Subjects more prone to anxiety will spend less time on average in the uncovered (open) part of the maze and prefer the perceived safety of walled arms.

I followed a modified version of a previously established protocol. The maze was stationary 50 centimeters above ground level. Each rat, aged 35-45 days, was placed into the open field area for 5 minutes, before being placed in the center of

the maze facing the open arm, for a further 5 minutes. The maze was cleaned with a 70% ethanol solution before and after each subject to remove their scent. An entry into the open arm was defined as both front paws being on or beyond the closed arm boundary. The number of entries into the open portion, as well as time spent there was noted alongside time spent in the closed portion. In line with previous research, VPA affected animals had a reduced number of entries into the open part of the maze compared to healthy controls.

VPA animals also display more antisocial behaviors compared to controls (Zarate-Lopez et al., 2024). A social interaction test (Open Field Test) was performed to assess social behavior in rodents by placing them in a familiar environment with a novel individual to stimulate social interaction (Kim et al., 2019). Behaviors such as sniffing, grooming, following, and aggression are observed and quantified, and then compared against control peers to assess the likelihood of ASD-like phenotype.

A modified version of a previously described open field protocol was used, in which a VPA and a control rat were placed in a 44 x 44-centimetre open arena for nine minutes. All sessions were recorded and assessed for key social behaviours, such as sniffing, licking, mounting, crawling over or under, and anogenital inspections. The interactions were scored for each animal individually.

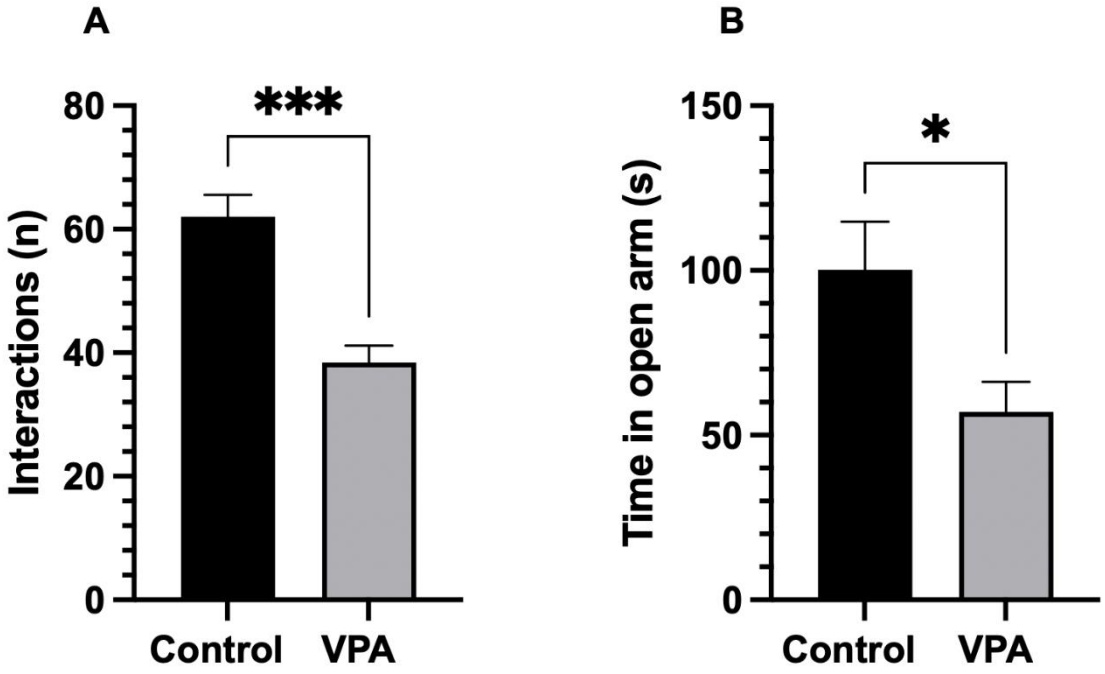

Figure 1

*Phenotypical validation of ASD-like presentation in VPA rats using social interaction tests (graph A) measuring number of social behaviours with novel peers. Graph B shows time spent in the open arms of elevated plus maze. N = 68. Data is expressed as mean± SEM * p < 0.05, ** p < 0.01, p *** < 0.001.*

4.2. Experiments

VPA and control cohorts were randomly assigned to four groups (n=17 per group) with similar body weight averages after the phenotypes were validates. All rats were pre exposed to high fat high sugar chow (HFHS) multiple times prior to the

experiments commencing to rule out aversive behaviours due to hyponeophagia. Animals were food deprived overnight, with the fed groups receiving 10 grams of HFHS chow on the day of the experiment to simulate a normal meal. All groups were non-blinded, and the author was aware of group allocations.

4.2.1. Effect of LiCl treatment on c-Fos IR in VPA and control rats

Adult animals (n=28) were subdivided into two main cohorts: Controls (n=14) and VPAs (n=14). Each cohort was further split into two subgroups each, one receiving food pre injection (Fed), and the other not (Hungry). Therefore, there were a total of four groups with 7 animals each, with the hungry animals of each phenotype acting as controls.

All animals were given an IP injection of LiCl (3 mEq/kg) before being returned to their enclosures for an hour. Animals were deeply anesthetized with 35% urethane in 0.9% saline solution an hour after LiCl was administered. Transcardial perfusions were performed using 50 mL of saline, followed by 500 mL of 4% paraformaldehyde, after absence of palpebral and toe-pinch reflexes was established. The animals were then decapitated, and their brains excised in preparation for microdissection.

Perfusions and Immunohistochemistry

Antibodies were acquired from various sources as stated and were dissolved in a 0.25% porcine gelatine (Sigma) and 0.5% Triton X-100 (Sigma) in TBS solution.

Brains were excised and post-fixed overnight in an aldehyde-based fixative at 4 °C. Coronal sections of 60 µm-thickness were cut with a vibratome (Leica, Germany) and processed as free-floating sections for c-Fos protein immunostaining. Brain sections were rinsed with Tris-buffered saline (TBS, pH 7.4-7.6) before being incubated in 3% hydrogen peroxide (H_2O_2) and 10% methanol in a TBS dilution for ten minutes at room temperature. Another TBS rinse was performed post incubation, after which the tissue was incubated in rabbit-anti-Fos monoclonal primary antibodies for 18 hours at 4 ºC. (diluted 1:12,000; Synaptic Systems, Sydney, Australia). After the first antibody incubation, the sections were washed again with TBS and incubated with biotinylated-secondary goat-anti-rabbit antibody (1:400; Vector Laboratories, Burlingame, CA, USA) for a further hour. Finally, the brain sections were washed with TBS a further four times before being incubated with avidin-biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories) for an hour at room temperature.

Tissue staining was visualized by incubation in 0.05% diaminobenzidine (DAB, Sigma), 0.01% H_2O_2 and 0.3% nickel sulfate, for 15-20 minutes at room temperature. Post staining, the sections were washed in TBS and mounted onto gelatin-coated slides that were air dried and dehydrated using ethanol and xylene Entellan (Merck KGaA, Darmstadt, Germany) to preserve the specimens.

Images of immunohistochemically stained brain sections were obtained using a Nikon Eclipse E400 microscope and an OMAX digital microscope camera attachment. All images were captured using the same photographic settings and resolution and were not subjected to digital processing. Brain region boundaries were defined using Paxinos and Watson brain atlas (Paxinos & Watson, 2013). The number of activated c-Fos nuclei per mm^2 was manually counted using ImageJ (v. 1.54i MacOS, Fiji) for each neuroanatomical region. The measurements were performed to quantify activation density within a mm^2 rather than a raw number of c-Fos nuclei.

Statistical analysis of c-Fos positive nuclei was carried out using GraphPad Prism (v. 10.4.1, California). Density of c-Fos positive nuclear profiles per mm^2 were averaged per individual, and then per group. The data was normalised, and 2-way ANOVA was run on each group. Interactions between factors were assessed to judge significance ($p < 0.05$).

4.2.2. Effect of LiCl treatment on gene expression in the ARC of VPA and control rats

Adult rats (n=40) were obtained, treated, and allocated in the same way as the c-Fos cohort. LiCl was administered to all control and VPA animals, who were then placed back in their enclosures following the procedure. Two hours after LiCl injection, animals were deeply anesthetized with 35% urethane in 0.9% saline solution. After absence of palpebral and toe-pinch reflexes was established, a cervical dislocation was performed, and the brains were excised in preparation for microdissection of the ARC and qPCR.

qPCR Analysis

Primer selection was based on literature review and in silico validation with Primer-BLAST (NCBI), and Multiple Primer Analyzer (Thermofisher, Australia). Both pre-validated primers, as well as custom ones were used to improve binding specificity, which was confirmed using BLAST. Primers were optimised for melting temperatures of around 60 °C, with amplicon sizes ranging from 100-200 bp. (Table 1)

Brain samples were stored in RNAlater (Thermofisher) at -80 °C. Once thawed, they were homogenized in 100µL Trizol (Ambion, Glasgow, UK). Twenty µL chloroform was added before the mixture was centrifuged at 10,000 g for ten minutes at room

temperature. Total RNA was isolated from the clear phase and precipitated in isopropanol for ten minutes in an ice bath. The mixture was centrifuged again at the same settings, in a 4 °C environment. Supernatant was discarded between each wash step, ensuring the pellet was kept intact before washing in 75% ethanol and DEPC-treated water solution, and being centrifuged again at 4 °C. After washing, the supernatant was carefully discarded, and pellets were left to air dry.

After air drying, the pellets were dissolved in 8µL DEPC water and 1µL 10xDNAse buffer (dNature, Gisborne, New Zealand). Each sample was incubated with 1µL DNAse (dNature) at 37 °C. The reaction was stopped after 30 minutes using 1µL stop buffer, and then further incubated at 65 °C for ten minutes.

RNA purity and concentration was quantified (ug/µL) using a spectrophotometer (DeNovix DS-11 FX, Delaware, United States). The RNA was used to synthesize cDNA using iScript Advanced cDNA synthesis kit (BioRad, Hercules, CA, USA). Nanodrop was used to assess obtained cDNA quantity and purity. Obtained cDNA purity and quantity was also assessed with the DeNovix spectrophotometer.

Quantitative real time PCR reactions were carried out using 4µL of 25ng/µL cDNA, forward and reverse primers, 4µL MQ H2O, and iTaw SYBR Green Supermix (BioRad). Housekeeping genes ActB and TBP were selected to normalize findings.

Initial denaturation at 95 °C for 15 minutes. Cycling consisted of 45 repetitions of 95 °C for 15 seconds, followed by 15 seconds at primer specific temperature, and 30 seconds at 72 °C. Thermal profiles of amplified transcripts were visualized using melt peaks in CFX Maestro. Melting temperature analysis of the negative value of the change in relative fluorescence units (RFU) over the change in temperature (°C) was plotted (−dRFU/dT) to determine the specificity of the primers to a given transcript and primer dimer.

qRT-PCR data was analyzed using BioRad CFX Maestro software (v 2.3, BioRad). All results were normalized in relation to housekeeping genes.

Table 1

List of forward and reverse primer oligonucleotides used for amplifying genes of interest and housekeeping genes.

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'
NPY	5'-AGGTAACAAACGAATGGGGCT-3'	5'-TGATGTAGTGTCGCAGAGCG-3'
CRH	5'-TGGATCTCACCTTCCACCTT-3'	5'-TTCATTTCCCGATAATCTCCA-3'
PNOC	5'-CAGGTGAGCCCCCGT-3'	5'-TATGGCAGTGGTGAGCGAAAA-3'
OXTR	5'-GATCACGCTCGCCGTCTA-3'	5'-CCGTCTTGAGTCGCAGATTC-3'
BDNF	5'-TGCAGGGGCATAGACAAAAGG-3'	5'-CTTATGAATCGCCAGCCAATTCTC-3'
VGlut2	5'-CAGCGGATTTGGTTGCGTTA-3'	5'-TGATGAGTCCCCGTTCTGGA-3'
PSD95	5'-CTTCTCAGCCATCGTAGAGG-3'	5'-GAGAGGTCTTCAATGACACG-3'

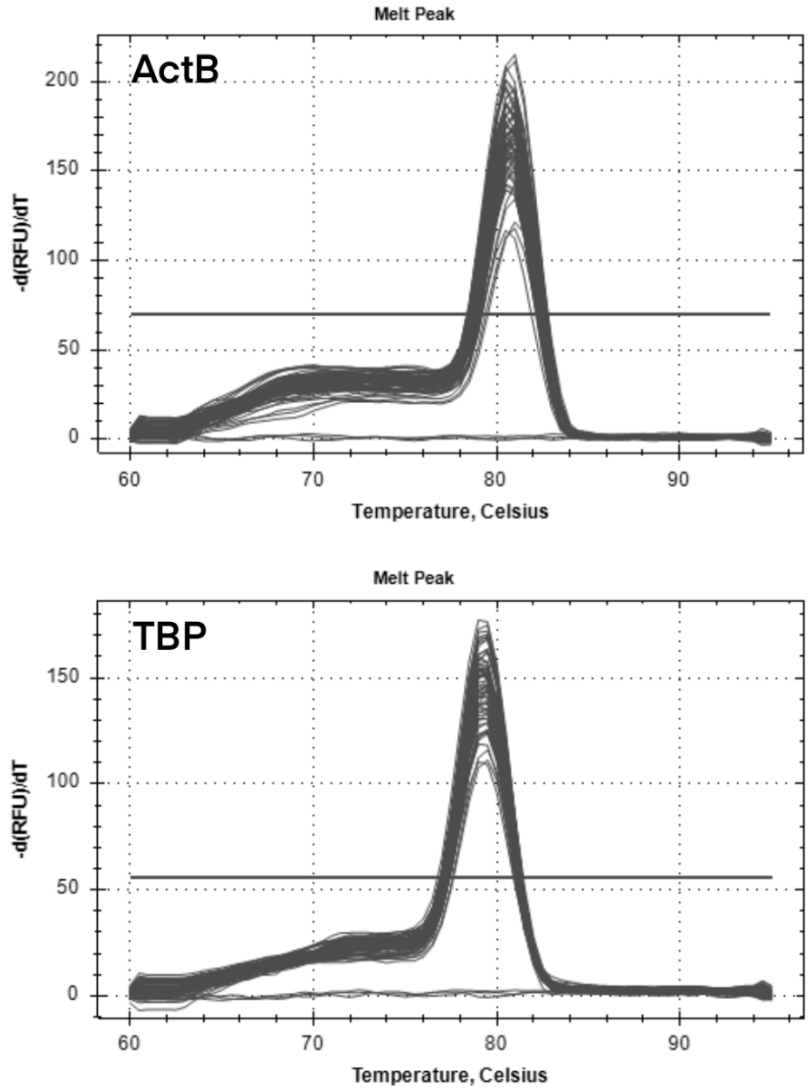

Figure 2

Primer melt curves for housekeeping genes ActB and TBP. Housekeeping genes were extracted from tissue in the ARC and amplified using custom and pre-validated primers.

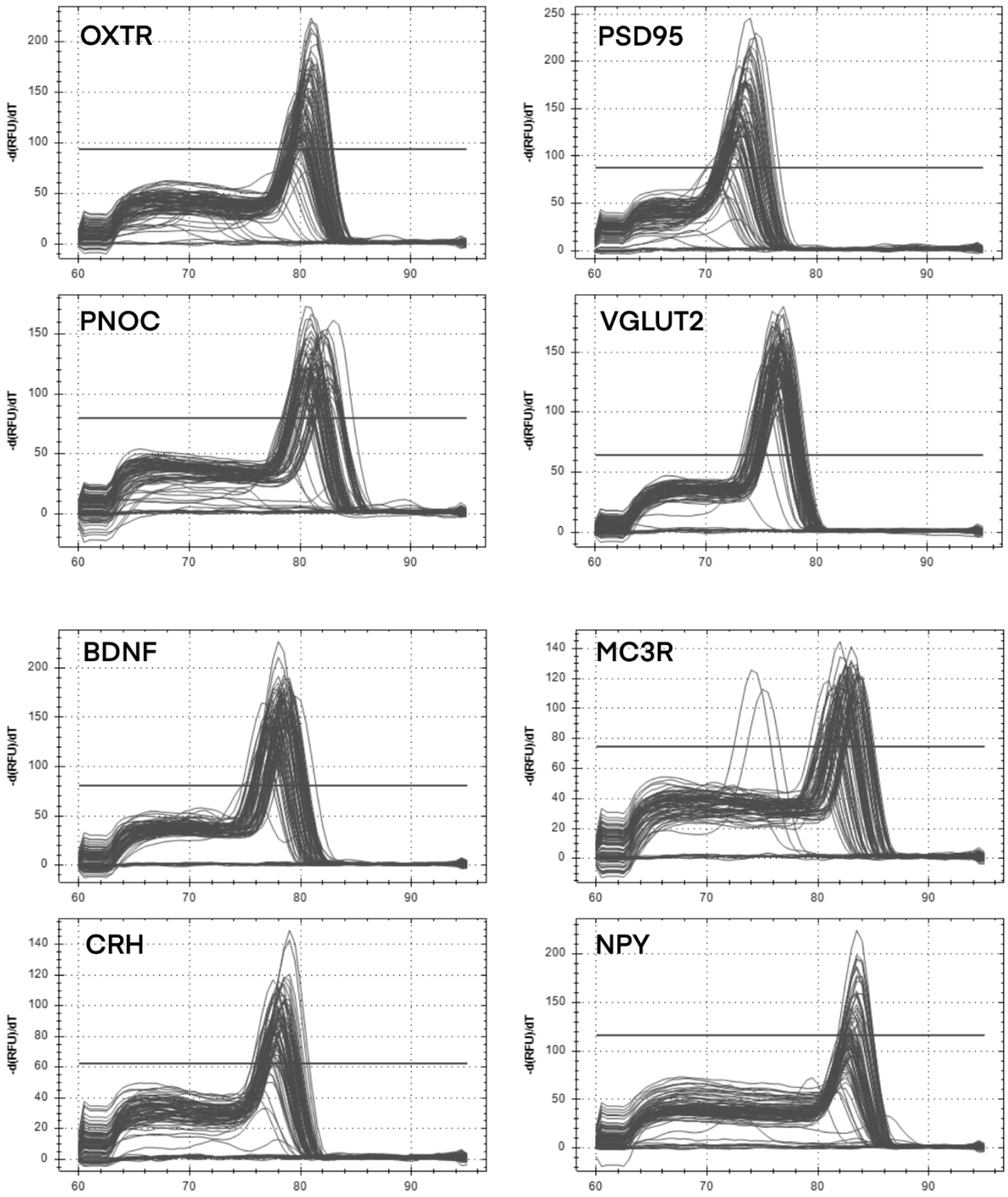

Figure 3

Primer melt curves for genes of interest in the ARC. Genes were amplified using custom and pre-validated primers.

4.3. Data Analysis

c-Fos positive nuclear profiles per mm^2 were averaged per individual (n=27), and then per group. Data between each group (Fed VPA, Hungry VPA, Fed Control, Hungry Control) was compared using GraphPad Prism with a Two-Way ANOVA followed by Sidak's post hoc multiple comparisons test. Shapiro Wilk and the Kolmogorov Smirnov tests were used to verify data normality. Values were considered significantly different for $p \leq 0.05$. One animal was excluded from the study.

Gene expression data obtained from the ARC of the PCR cohort was analysed using GraphPad Prism. Two-way ANOVA was performed, followed by Dunnett's post-hoc test. Normal distribution was validated using the Shapiro-Wilk and the Kolmogorov-Smirnov tests. Values were considered significantly different for $p \leq 0.05$.

rt-qPCR results were normalized with the Actin B and TBP housekeeping genes. Normality of data distribution of ΔCq values was checked using a Shapiro Wilk test. ΔCq values were analysed with a two-way ANOVA assessing the interaction between LiCl treatment and food exposure. Values were considered significantly different when $p \leq 0.05$.

5. Results

5.1. VPA rats show diminished c-Fos activation in response to LiCl.

Immunohistochemical analysis of brain tissue collected from experimental animals revealed a heightened response to i.p. LiCl injection (3mEq/kg) in hungry VPA ASD rats compared to their fed VPA counterparts. Hungry VPA animals show significantly elevated c-Fos immunoreactivity in the NAcc shell ($p = 0.043$) and NAcc core ($p = 0.032$) compared to Fed VPAs and controls. No significant difference was found between the control groups in the NAcc shell ($p = 0.220$) or NAcc core ($p = 0.729$) (Figure 5).

Control animals displayed a heightened activation of c-Fos IR in the dorsal vagal complex (brainstem) compared to VPAs. Fed controls show a significantly higher c-Fos activation in the DMX ($p = 0.006$) and NTS ($p = 0.001$) than controls who were not offered food prior to LiCl administration. Additionally, there is a significant decrease of c-Fos per mm^2 in the NTS ($p = 0.001$) and DMX ($p = 0.001$) in both VPA groups when compared to controls. Fed VPA ASD rats had a significantly lower c-Fos activation in the NTS when compared to hungry VPA ASD rats ($p = 0.020$). Conversely, fed VPA ASD rats had a higher response in the DMX ($p = 0.011$) than hungry VPA ASD counterparts. A significant interaction between model and diet was found in the NTS ($p = 0.006$) (Figure 6).

Differences in c-Fos expression were observed in the AP when comparing VPA ASD rats with controls. VPA ASD rats had a significantly reduced activation of c-Fos ($p = <0.001$) compared to controls, however no significant difference was noted when comparing hungry and fed animals within the models (VPA ASD vs controls) ($p = 0.077$) (Figure 7).

5.2. Fed VPA rats show transcriptional changes in the arcuate nucleus

Follow up analysis of gene expression revealed differential regulation of two genes in the ARC of VPA ASD rats post LiCl injection, and while 3mEq/kg i.p. LiCl does not produce a significant change in controls, however it affects mRNA levels in two of the transcripts in fed VPAs: one related to feeding, and one to synaptic plasticity.

Fed VPA animals show lower PNOC transcription levels in the ARC ($t = 2.300$, $DF = 11$, $p = 0.0338$) compared to their hungry counterparts. Likewise, there is a similar trend concerning PSD95 ($t = 2.280$, $DF = 11$, $p = 0.0431$) receptor expression, where fed VPAs once again show diminished expression. (Figures 8, 9)

5.3. Figures

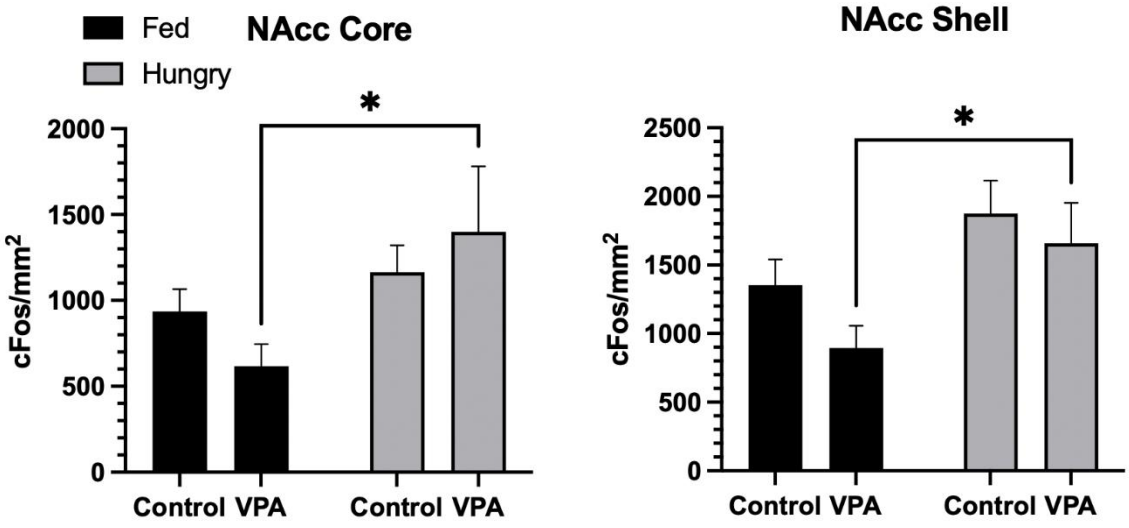

Figure 4

*Effect of i.p. LiCl injection on c-Fos immunoreactivity in the nucleus accumbens. Graph shows c-Fos IR activation per mm² in control and VPA animals that were hungry or fed prior to LiCl administration. The hungry VPA group had one fewer subject (n = 6) than all other groups (n = 7). Data is expressed as mean± SEM * p < 0.05.*

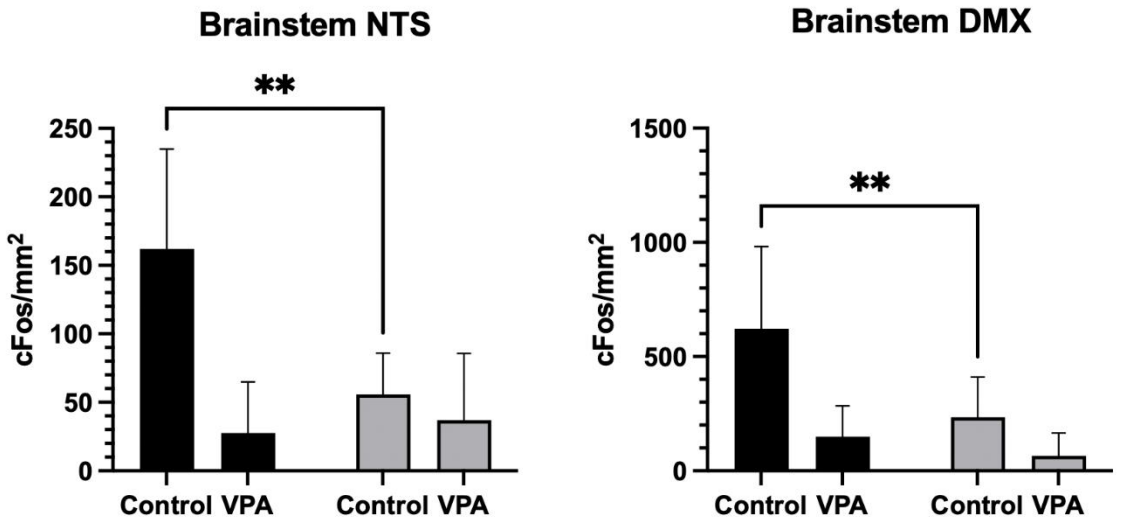

Figure 5

*Effect of i.p. LiCl injection on c-Fos immunoreactivity in the DVC, with NTS shown on the left and DMX on the right. Graphs show c-Fos IR activation per mm² in controls and VPA animals that were either hungry or fed prior to LiCl administration. The hungry VPA group had one fewer subject (n = 6) than all other groups (n = 7). Data is expressed as mean± SEM * p < 0.05, ** p < 0.01.*

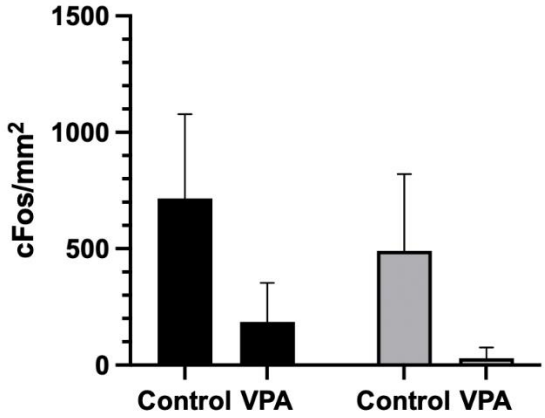

Figure 6

Effect of i.p. LiCl injection on c-Fos immunoreactivity in the AP. Graph shows a significant effect of LiCl c-Fos IR activation in the AP of VPA rats (p = <0.001), however no significant difference between the fed and hungry control animals (p = 0.077). The hungry VPA group had one fewer subject (n = 6) than all other groups (n = 7). Data is expressed as mean± SEM.

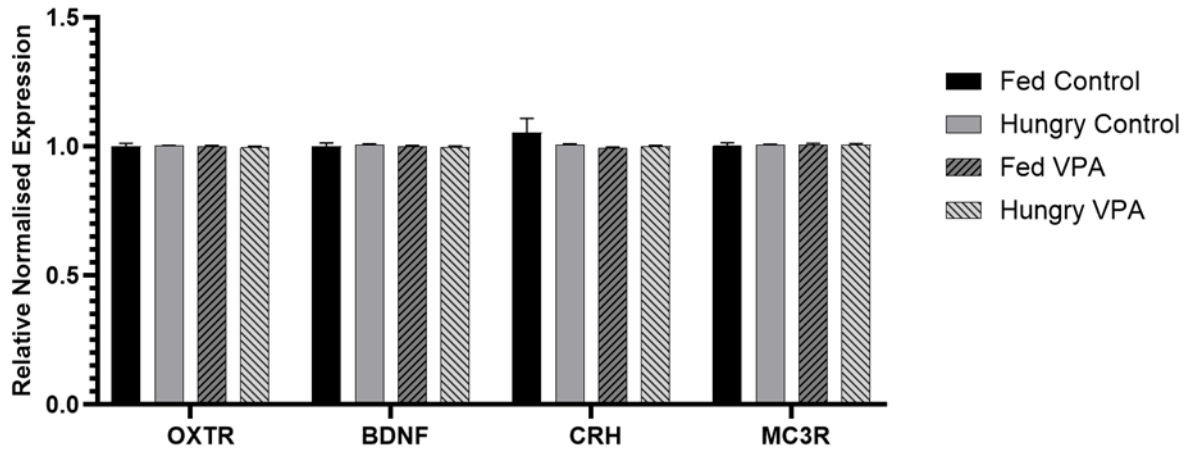

Figure 7

*LiCl-induced mRNA level changes in the ARC of healthy control vs VPA rats. OXTR – oxytocin receptor, BDNF – brain-derived neurotrophic factor, CRH – corticotropin releasing hormone, MC3R – melanocortin 3 receptor. Data is expressed as mean± SEM * $p < 0.05$.*

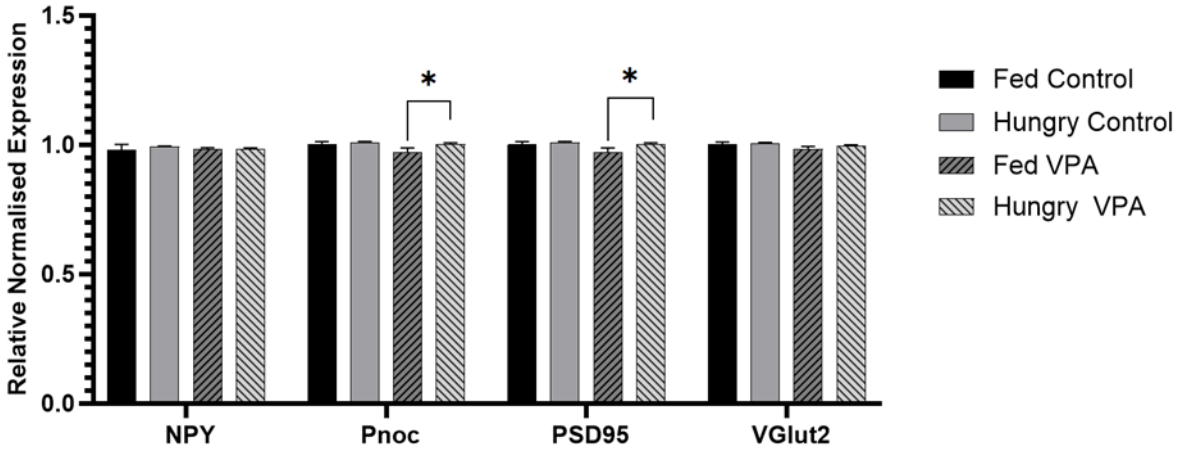

Figure 8

*LiCl-induced mRNA level changes in the ARC of healthy control vs VPA rats. NPY – neuropeptide Y, PNOC – proopiomelanocortin, PSD95 – discs large MAGUK scaffolding protein, VGLUT2 – vesicular glutamate transporter 2. Data is expressed as mean± SEM * $p < 0.05$.*

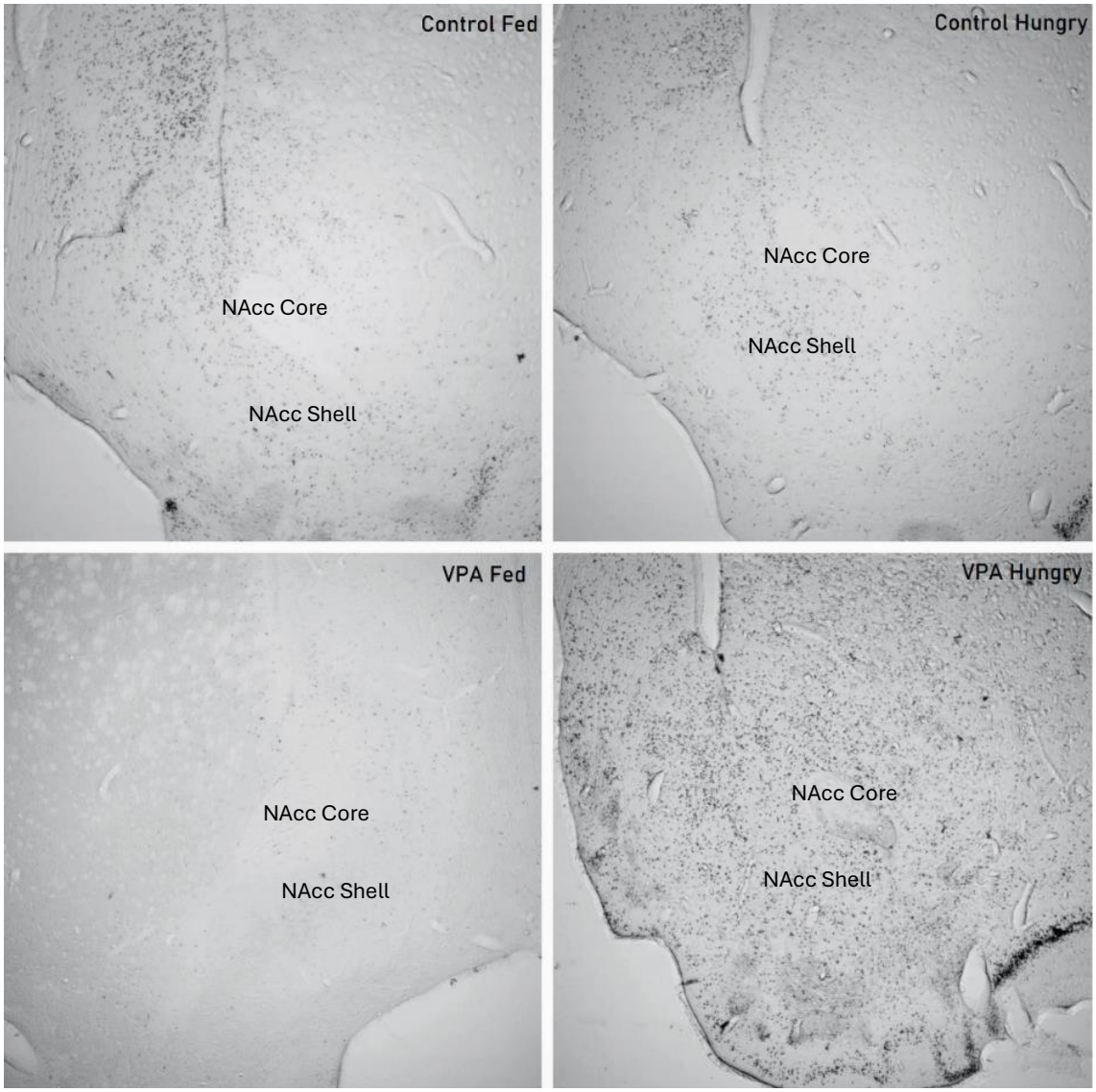

Figure 9

Representative photomicrographs from each cohort. Photos show c-Fos IR levels in the nucleus accumbens shell and core.

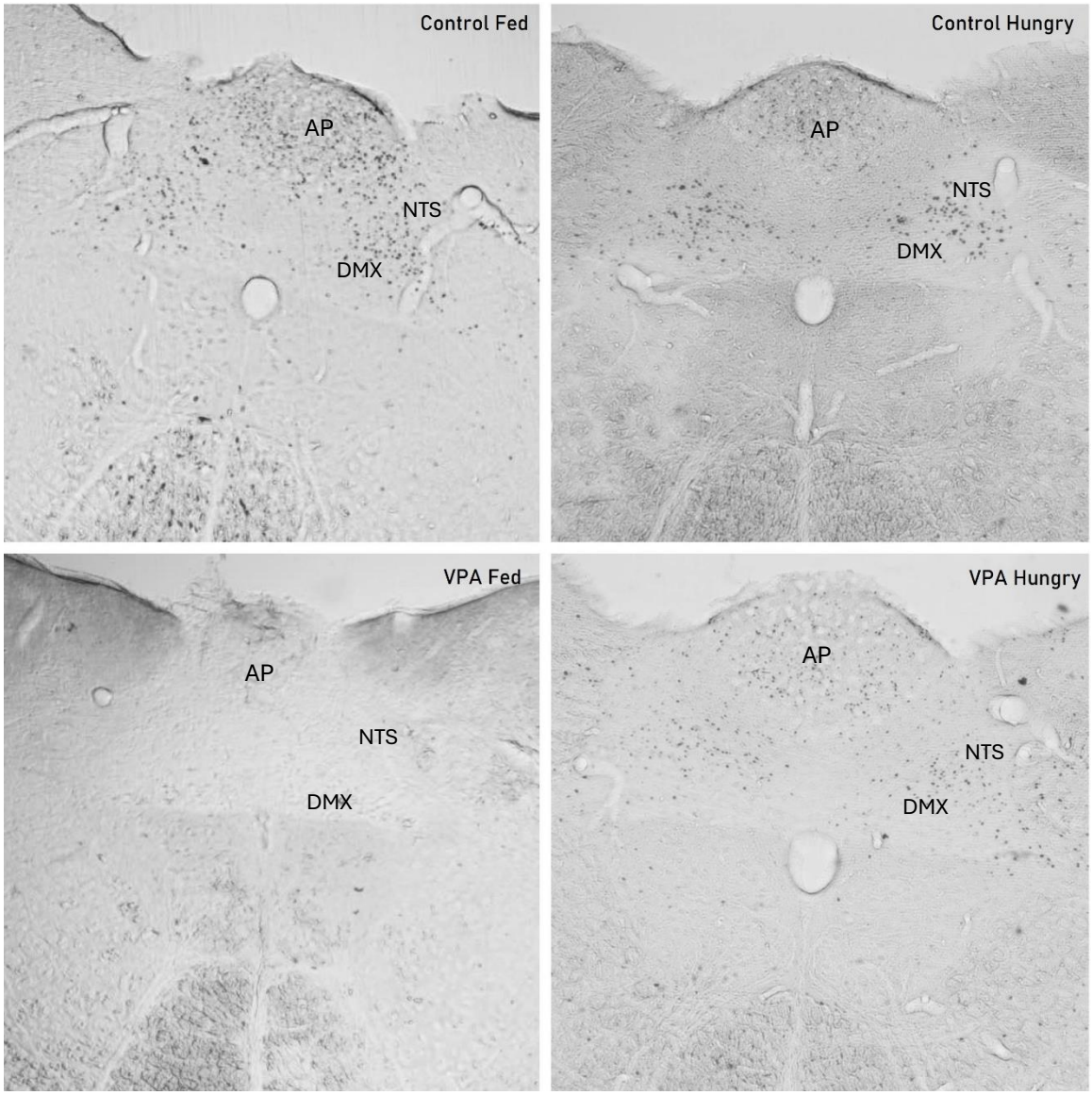

Figure 10

Representative photomicrographs from each cohort. Photos show c-Fos IR levels in the brainstem. DMX – dorsal motor nucleus, NTS – nucleus of the solitary tract, AP – area postrema.

6. Discussion

People with ASD often present with aberrant feeding behaviours, the causes of which are poorly understood. These behaviours have broad impacts on both health and associated care costs for individuals with the condition (Curtin et al., 2014; Healy et al., 2021; Sedgewick et al., 2020). Abnormal feeding behaviours in people with ASD range from extreme dietary restrictions and selectivity, refusal to eat, over- or undereating, and incorporates aspects such as food presentation and timing (Huxham et al., 2021; Lucarelli et al., 2017). A 2019 review estimates the ASD related healthcare costs of caring for a person diagnosed with ASD ranges somewhere between $2.4 million to $3.2 million USD over their lifetime (Rogge & Janssen, 2019).

Autistic individuals have been observed to restrict their diet based on familiarity and perceived “safety” of food (Keen, 2008; Wang et al., 2022). My research demonstrates that ASD feeding habits may be influenced by deficiencies in toxicity signalling mechanisms. I suggest that people with ASD could present with anomalous feeding patterns because they find specific foods rewarding to an extent that overrides their aversive features. Given ASD individuals’ heightened sensitivity towards meals, one might expect increased food selectivity to be associated with previous negative experiences or consequences, such as illness following the consumption of a novel food. Additionally, the prevalence of pica in ASD individuals

who have already experienced adverse effects after consuming inedible substances supports the hypothesis that other underlying factors may influence aberrant eating behaviours in ASD (Mayes & Zickgraf, 2019).

The VPA rat is one of the closest models resembling phenotypical presentation of ASD in humans, and has extensive research backing its use (Favre et al., 2013; Kim et al., 2011; Nicolini & Fahnestock, 2018; Wagner et al., 2006). While prenatal VPA exposure is a reliable way of inducing a phenotype of ASD in mammals, not all resulting offspring will meet behavioural inclusion criteria for ASD studies (Nicolini & Fahnestock, 2018).

This thesis investigated the effects of LiCl-induced CTA on c-Fos IR expression in the brains of VPA and control phenotypes that are hungry or fed, which may elucidate how feeding and reward pathways interact with ASD. For this reason, I chose to perform immunostaining in the NAcc, which part of the dopaminergic reward system, as previous research suggests the reward system may be more active in individuals with ASD (Schiavi et al., 2019). Palatability is also a major factor of hedonic eating; consumption of palatable foods and solutions is governed by both the dopaminergic reward systems in the midbrain such as the NAcc, and those responsible for maintaining homeostasis such as opioids and endocannabinoids.

Klockars et al. reports overconsumption of highly palatable liquid solutions of saccharin, milk, and sugar in VPA rat models of ASD (Klockars et al., 2021).

The current body of research suggests that people with ASD may have an enhanced overall activation of the reward system, which is in line with my findings. My study found elevated levels of c-Fos IR in the NAcc of hungry VPA rats, which may mean that autistic individuals find the anticipation of palatable foods more inherently rewarding than neurotypical controls. Previous research supports this line of thinking, demonstrating that VPA rats have significantly higher activation in areas governing predictive reward and learning (Klockars et al., 2021; Schiavi et al., 2019). Differential processing of reward-based cues in ASD could be the basis for some of the anomalous feeding behaviours seen in individuals with the condition. An fMRI study by Cascio et al. showed that children with ASD had functional reward circuitry and uncovered enhanced responses in the anterior cingulate and insula compared to controls. However, their study showed a reward response comparable to neurotypical controls in the NAcc, amygdala, and orbitofrontal cortex (Cascio et al., 2012). Additionally, Pal et al. recently found that hungry VPA rats had elevated or unchanged c-Fos IR levels in the ARC, amygdala, and NAcc shell compared to fed counterparts. Their study also reported a chronic 15% decrease of non-preferred standard chow consumption in VPA rats compared to controls, resulting in a body weight reduction of approximately 20% (Pal et al., 2022).

Dysregulation of social and reward processing pathways in ASD may be responsible for unexpected changes in my findings. I initially theorised that VPA rats would display heightened c-Fos IR in brain areas associated with aversive learning, in line with restrictive food preferences seen in ASD. Instead, neuronal activation in the DVC of VPA rats was blunted, and the NAcc displayed heightened c-Fos density in hungry VPA animals. Additionally, the fed VPA rats displayed much lower activation in the NAcc than all other groups, suggesting a lower reward response to food. The endogenous opioid system plays a significant role in modulating pleasure and reward associated with eating. The dopaminergic system, striatum, OFC, and amygdala are key brain regions involved in reward processing and the reinforcement of palatable food consumption (Kandel, 2013). Studies also suggest dopamine and therefore DA reward circuitry plays a crucial role in the strengthening of appetitive responses promoting hedonic eating (Berridge et al., 2010; Stice & Yokum, 2016). Given the overlap between social reward processing circuitry, and the controls involved in reward-driven food intake, it is possible to assume that atypical reward perception associated with ASD could influence feeding behaviours in people with the condition.

I used an injection of LiCl to establish CTA in the experimental animals, which is an unconditioned stimulus that acts as a harmful substance in aversive learning acquisition. Although any agent that can impair health or poses a risk to life can be

an unconditioned stimulus, LiCl has long standing research backing its use in CTA paradigms (Etscorn & Stephens, 1973). Consumption of food that elicits a CTA response is often described as a traumatic experience, including discomfort, nausea, sickness, toxicosis, or, more broadly, gastrointestinal malaise (Karniol et al., 1978).

CTA activates areas of the DVC responsible for initiating emesis, gastric emptying, and cessation of eating. Nucleus of the solitary tract serves as a link between the gut and brain and has extensive interconnectivity with the wider CNS, which is why I included it in c-Fos analysis. The NTS projects to and receives input from various midbrain and forebrain nuclei, including the PBN, hypothalamic nuclei, and the VTA (Jean, 1991). NTS neurons express several neuropeptides involved in the regulation of food intake, including glucagon-like peptide-1, NPY and POMC (Sobrino Crespo et al., 2014). Similarly to the ARC, the NTS has a neuroanatomical position relevant to its function. The NTS is located near the area postrema, making it well-suited to receive both neural and hormonal signals from the periphery. Together the AP and NTS contribute to integrating peripheral and central signals related to feeding (Jean, 1991; van der Kooy et al., 1984).

I chose to look at c-Fos activation levels in the DVC because of the significant contributions its components have to CTA acquisition. Given the established link of

novelty-based restrictive food intake in individuals with ASD (Mayes & Zickgraf, 2019; Schreck & Williams, 2006; Wang et al., 2022), I chose to pre-expose the animals to the high fat high sugar chow they received prior to CTA acquisition. By ensuring the food used was palatable, but not novel to the subjects, the consumed amount of chow more accurately simulated the size of a standard meal and avoided novelty-based aversion common to ASD. My findings demonstrate that VPA rats have a blunted c-Fos response in the DVC irrespective of hunger status after a LiCl injection compared to controls.

c-Fos IR showed both VPA groups in my study displayed a significantly attenuated response to LiCl in the NTS and DMX areas compared to controls. Recent findings by Clyburn et al. highlight short-term neuroplasticity at NTS-DMX synapses as a key mechanism in regulating food intake and caloric balance. Their study in rodents showed exposure to a calorically dense, palatable diet initially triggers a brief period of hyperphagia lasting about 24 hours. However, food intake is subsequently reduced after 3-5 days to restore caloric balance. This homeostatic adjustment is associated with increased activation of synaptic NMDA receptors at the NTS-DMX synapse, leading to enhanced excitability of DMX neurons (Clyburn et al., 2018). This heightened neuronal activity may play a crucial role in modulating feeding behaviour during this adaptation period. Aversive learning mechanism deficiencies

in autistic individuals may also contribute to underfeeding seen in previous studies using VPA ASD rat models (Pal et al., 2022).

My results show attenuated levels of c-Fos IR in the AP following CTA acquisition in VPA rats. This may indicate that VPA rats have lower processing in areas governing formation of aversive learning responses following food ingestion after a LiCl-induced toxicity response. Additionally, an attenuated response to LiCl in the DVC may suggest that VPA rats' perception of food safety and dietary habits are primarily influenced by hedonic reward and familiarity rather than avoidance following a negative experience. The AP contributes to initiation of the emetic response following toxin detection. Spencer et al. demonstrated that lesioning the AP reduced c-Fos activation in the region following CTA establishment (Spencer et al., 2012).

As a defence mechanism, CTA needs to act quickly to prevent further exposure to potentially poisonous food. As such, CTA relies on the organism's ability to learn the association between food and malaise after a single exposure. There are numerous examples of one-trial learning in literature supporting this claim (Andrews & Braveman, 1975; Matsuzawa et al., 1983). Autistic individuals may present with aberrant feeding behaviours because of deficiencies in aversive learning mechanisms responsible for identifying foods that cause illness. This is in

line with altered neural connectivity and stimulus processing that characterises ASD brains (Fletcher-Watson & Happé, 2019; Gotts et al., 2012; Hutsler & Zhang, 2010). My findings contribute to the idea that gastrointestinal and homeostatic signals responsible for terminating consummatory behaviour may be insufficient to stop feeding in VPA rats.

Previous research into CTA acquisition supports my findings, showing increased c-Fos IR levels in brain areas associated with aversive learning in control animals in food deprivation paradigms. Parkinson et al. measured neuronal activation and c-Fos expression in C57BL/6 mice that were fasted for 18 hours before a peripheral administration of LiCl or other agents that induce CTA. They found that a peripheral injection of LiCl produced a signalling increase in the VMH of fasted mice but had no significant effect in ARC, PVN, AP, supraoptic nucleus, or the amygdala. Additionally, their study also showed that LiCl treatment produces an increase of c-Fos expression in the PVN, supraoptic nucleus, and central nucleus of the amygdala in food deprived animals (Parkinson et al., 2009). Likewise, A 2013 study by Olszewski et al using C57BL/6J mice found that LiCl treatment produced a significant increase in c-Fos expression in the PVN, supraoptic nucleus, central and basolateral amygdala, NTS, and AP of animals with unrestricted food access (Olszewski et al., 2013). In another study, Olszewski et al. demonstrated higher levels of c-Fos IR following LiCl induced CTA acquisition in the PVN, central nucleus

of the amygdala, NTS and AP of schedule-fed rats that received a LiCl injection compared to saline controls (Olszewski et al., 2010).

By omitting a saline-treated group from this study, I investigated whether the response to LiCl itself is substantially different based on phenotype and energy status. However, the lack of non-aversive controls is also a major drawback of this research. Because all experimental animals were exposed to LiCl, I was unable to look at the effect of the treatment itself. Follow up research should conduct a full-scale experiment that includes a non-aversive saline cohort for multi-level analysis.

This appears to be this is the first study attempting to quantify gene expression in the ARC of animals following CTA establishment, making it a key piece of information in this field. Previous research demonstrated up- or down-regulation of key anorexigenic genes in the brains of rodents that experienced aversive learning. Panguluri et al. investigated gene expression in Sprague-Dawley rats following LiCl-induced CTA on an ad-lib diet. They demonstrated a significant upregulation of OXT mRNA in rats that received LiCl. Notably, they uncovered 30 differentially regulated genes in the amygdala of unconditioned versus LiCl treated animals (Panguluri et al., 2012). Likewise, research by Ma et al. suggests region-specific BDNF regulation may affect CTA learning acquisition in rats. They demonstrated increased levels of BDNF mRNA in the central, but not basolateral amygdala animals

treated with i.p. LiCl following 24-hour water deprivation (Ma et al., 2011). Lindblom et al. found reduced levels of CRH mRNA in the hypothalamus of rats following a 2-day 50% food restriction. Their study also demonstrated an increase in NPY, and a decrease in POMC mRNA levels in the ventral hypothalamus specifically (Lindblom et al., 2005). A study by Pal et al. showed that a high dose of LiCl failed to establish CTA in VPA rats and produced changes in gene expression in the amygdala and PVN compared to controls. LiCl-treated VPA rats in the study had upregulated transcripts in the amygdala for COMTD1, while AgRP, OXT, NPY, DOR, and MC3R were downregulated compared to controls. However, animals in the study showed no significant changes to gene expression in the PVN, despite the site's major involvement in feeding control (Pal et al., 2025). My results indicate differential gene regulation in two transcripts of VPA rats which could contribute to feeding abnormalities.

Hungry VPA rats in my study show an upregulation of PSD-95 in the ARC compared to fed counterparts, which could indicate abnormal regulation of this transcript in ASD phenotypes. My results are in line with previous research showing upregulation of certain transcripts in people with ASD. A whole-exome sequencing study by De Rubies at al. found that over 5% of ASD subjects had higher rates of de novo SNVs that resulted in a loss of gene function. Additionally, the study found enrichment of FMR1 and RBFOX1 genes, and an upregulation of mRNA encoding PSD proteins (De

Rubeis et al., 2014). Previous research links dendritic spine abnormalities often found in people with ASD to the PSD family of genes. (Coley & Gao, 2018; Hutsler & Zhang, 2010; Penzes et al., 2011). Additionally, PSD-95 overexpression enhances AMPA receptor (AMPAr) activity, increasing dendritic spine size and density in hippocampal neurons (El-Husseini et al., 2000). There is also evidence for PSD-95 having a regulatory role in food intake via AMPAr. Rat studies by Hettes et al. demonstrated that intracranial administration of AMPAr agonists and antagonists could either promote or suppress feeding behaviours, depending on the targeted brain regions. AMPAr-mediated transmission is therefore implicated in osmotic changes related to feeding, with AMPA injections to the lateral hypothalamus being found to stimulate feeding (Hettes et al., 2003; Hettes et al., 2010). AMPArs are also known to mediate excitatory glutamatergic synaptic transmission and play a major role in normal brain function (Chang et al., 2012; Royo et al., 2022). Downregulation of PSD-95 transcripts in fed VPA rats could hint at impaired neural plasticity following CTA learning.

Transcripts for PNOC are likewise upregulated in the ARC of hungry VPA rats compared to fed counterparts in my study. PNOC neurons in the ARC are orexigenic and form connections with satiety signalling POMC neurons (Jais et al., 2020; Vohra et al., 2022). A recent study by Sotelo-Hisrich et al. shows that an acute high fat diet can reduce the inhibition of PNOC in the ARC, raising suspicions about its

possible role in appetite regulation (Sotelo-Hitschfeld et al., 2024). This transcriptional change supports the hypothesis that VPA rat models may have abnormal satiety regulation.

Although a number of previous studies show up- or down-regulation of OXT, NPY, MC3R, and BDNF mRNA transcripts in the brains of LiCl-treated rats (Lindblom et al., 2005; Ma et al., 2011; Pal et al., 2025; Panguluri et al., 2012), my investigation found no significant differences in expression of these genes in the ARC. This disparity could be attributed to the fact that my study is limited by the lack of comparative non-aversive treatment groups. The lack of significance could also indicate that VPA and control rats may have similar regulation of OXT, NPY, and BDNF in the ARC. The scope of this thesis allowed the analysis of only a small subset of genes in a single brain region. The regulatory role of the ARC in food intake and energy expenditure made it an attractive choice for looking at transcriptional changes in key regulatory genes related to appetite, satiety, neuroplasticity, fear conditioning and metabolism (Könner et al., 2009; Vohra et al., 2022; Waterson & Horvath, 2015). Prenatal VPA exposure disrupts developmental processes that could contribute to the baseline gene expression differences seen between the VPA and control animals. However, a much broader analysis involving multiple brain areas and genes is required to generate robust understanding of mechanisms responsible for feeding abnormalities in people with ASD.

An interesting factor influencing this research is sex bias. There is a growing understanding that ASD presents differently in men and women (Ratto et al., 2018; Wilson et al., 2016). Despite recent research indicating differential presentation of ASD between the human sexes, I chose to use only male rats for this thesis. While it may seem like an oversight, focusing on the male rat in ASD research has compelling reasons. Disease modelling is a major limitation of research. Animal model reliability depends on their use of diagnostic markers comparable to those found in humans. For a primarily behavioural condition like ASD, the challenge lies in demonstrating whether the observed changes translate to human adults and children. Female rat behaviours are not easily measured compared to their male counterparts (Zarate-Lopez et al., 2024). Additionally, the increased prevalence of camouflaging behaviours seen in autistic women has a risk of translating into the animal phenotype, further lowering confidence of ASD models using female VPA rats (Lai et al., 2016).

In humans, accurate diagnosis of ASD is only possible via observational means due to a lack of other measurable biomarkers (Schielen et al., 2024). When using animals to study human ASD, researchers are limited to simplistic behavioural tests and paradigms for validating the model (Nicolini & Fahnestock, 2018; Smith et al., 2019). Research reports women with ASD are more likely to camouflage autistic

behaviours compared to men, but there is substantial variability in both sexes. Greater camouflaging was also found to be highly co-occurring with depressive symptoms and anxiety, which can further influence diagnostic outcomes concerning validity (Boyd et al., 2011; Lai et al., 2015).

Although the majority of ASD research skewed towards more “severe” presentations of ASD stemming from preferential use of male models, it still generates useful insights for people with more subtle symptomology (Ratto et al., 2018; Werling & Geschwind, 2013). Future research may benefit from including high confidence female ASD rat models to eliminate sex biases. Additionally, because CTA acquisition is age dependent (Chambers, 2018), a study involving cohorts of varying developmental stages would shed further light on adaptive learning differences in ASD.

7. Conclusion

My findings suggest that the associative learning mechanism in VPA rats may be impaired in context-specific situations. VPA ASD animals show a blunted c-Fos response to LiCl induced CTA in brain areas associated with toxicity responses, which indicates a deficiency in learning mechanisms that prevent consumption of harmful substances. Additionally, VPA ASD animals have higher c-Fos activation disparity depending on their satiety levels in brain areas associated with predictive reward following CTA. Although the ARC is well positioned to respond to changes in nutrient and hormonal levels, statistical analysis revealed only two differentially regulated genes in one group of VPA rats post LiCl treatment. The findings of this thesis highlight several neuromolecular bases responsible for adaptive challenges related to aversive learning mechanisms faced by people with ASD.

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