Neuromolecular Basis of Anomalous Feeding Behaviour in the Valproate Rat Model of Autism

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Abstract

Autism spectrum disorder (ASD), which affects circa 1% of the global population, is characterized by impairments in social communication, interaction, language disability, sensory anomalies, repetitive behaviours, and intellectual disability. Aberrant feeding behaviour is a common comorbidity of ASD. ASD individuals often demonstrate extreme food selectivity, refusal, dependence on few very particular food items, neophobia etc. which can lead to nutritional deficiencies affecting development. However, the underlying neurophysiological mechanisms of aberrant food intake in ASD are not well known.

Prenatal exposure to the antiepileptic drug, valproic acid (VPA), leads to autism. The prenatally VPA-exposed rats are model organisms to study ASD and they show ASD-like symptoms of, among others, elevated anxiety and decreased social interaction. Importantly, very little is known about appetite control in these animals. The overall goal of the studies included in this thesis was to identify potential feeding behavioural anomalies and associated neuromolecular changes in the VPA rat model of ASD. We thus hypothesize that ASD is associated with abnormal appetite and reward-driven feeding.

The key factors shaping consumption involve hunger (determines search for calories), satiation (underpins termination of feeding) and reward (consumption for the 'pleasantness' of food, regardless of the energy requirements). Thus, in the first Specific Aim of this thesis, the hunger processing in the VPA animals was investigated. The *ad libitum* intake of ‘bland’ standard laboratory chow was assessed in VPA versus non-VPA controls maintained continuously on this diet. The adult VPA rats ate less ‘bland’ chow than healthy controls did, and it was coupled with a moderately lower body weight than that of the controls. VPA rats also ate less of the standard chow after acute food deprivation. There was an aberrant change in c-Fos immunoreactivity in key brain sites that govern food intake, including the hypothalamic and brain stem areas, in the VPA animals in contrast to the healthy controls upon food deprivation, which indicates differences in neural processing of hunger in autism. This finding is further supported by a lack of change in expression patterns of feeding-related genes

(including oxytocin (OT), Agouti-related protein (AgRP) and mu opioid receptor (MOR)) upon hunger in the hypothalamus in the VPAs as opposed to the controls. Cumulatively, these findings suggest that hunger processing is altered in the VPA rats.

In the second Specific Aim of this project, intake of rewarding diets in the VPA rats was examined to address whether ASD affects pleasure-driven consumption. In episodic meal consumption paradigms, i.e., in scenarios in which energy non-deprived animals were given a brief and non-habitual access to diets that differ in palatability, the VPA rats were found to consume elevated amounts of tasty liquid and solid diets. These data suggest that VPA rats display not only altered energy homeostatis processing (as shown in Specific Aim 1), but also indicate changes in the regulation of eating for palatability.

Considering the abnormal OT signalling in autism and the fact that OT decreases feeding for energy and feeding for palatability, the third Specific Aim addressed whether VPA rats exhibit heightened sensitivity to exogenously administered OT, a peptide known for its anorexigenic properties. Intraperitoneal OT treatment was found to reduce episodic intake of palatable diets as well as post-energy deprivation intake of 'bland' chow; however, the dose needed to generate hypophagia was lower in VPAs than in controls. c-Fos immunohistochemistry revealed that the lower OT dose found to be anorexigenic in VPA rats, was sufficient to affect brain activation in a manner typical for hypophagia, whereas in healthy control rats, it did not induce significant c-Fos changes.

Importantly, OT is involved also in the process of avoidance of toxic foods, i.e., it facilitates the development of a conditioned taste aversion (CTA). Therefore, the goal of the Specific Aim 4, was to assess whether VPA animals display atypical taste aversion acquisition. It was found that VPAs failed to develop aversion to a standard dose of LiCl that induces a CTA in healthy non-ASD controls. In line with the outcome of the behavioural study, the immunohistochemical analysis revealed lower c-Fos-OT colocalization in the PVN in VPAs and a less pronounced broader CTA circuit c-Fos response in ASD animals compared to controls after LiCl treatment.

Taken together, evidence presented in this thesis shows that ASD is associated with abnormal appetite in VPA rats and it offers an insight into the neural basis of this phenomenon. The VPA animals show a slight decrease in intake of standard 'bland' chow, an elevated drive to consume palatable tastants, and resistance to taste aversion. These anomalies in feeding behaviours are underpinned by distinct changes in brain activation and gene expression patterns, as well as altered sensitivity to anorexigenic properties of OT, a neuropeptide known to affect other symptoms of ASD, from social deficits to anxiety.

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5HT – 5-hydroxytryptamin
ACC – Anterior Cingulate Cortex
ADHD - Attention Deficit Hyperactivity Disorder
AgRP - Agouti-related peptide
AHN - Anterior Hypothalamic Nucleus
α-MSH - Alpha-Melanocyte Stimulating Hormone
ARC - Arcuate Nucleus
ASD – Autism Spectrum Disorder
BBB – Blood Brain Barrier
BDNF - Brain-Derived Neurotrophic Factor
BLA – Basolateral Amygdala
BNST – Bed Nucleus of Stria Terminalis
BTBR - Black and Tan Brachyury $T^{+}Itpr3^{tf}/J$
Cacna1C - Calcium channel, voltage-dependent, L type, alpha 1C subunit
CCK – Cholecystokinin
CEA – Central Nucleus of Amygdala
CNTNAP2 - Contactin-associated protein-like 2
CNV – Copy Number Variation
COMT - Catechol-O-methyltransferase
CREB - cAMP response element-binding protein
CTA – Conditioned Taste Aversion
D2R – Dopamine Receptor 2
DAB - 3,3'-Diaminobenzidine
DAG – Diacylglycerol
DCX - Doublecortin
Ddc1 - DOPA decarboxylase 1
DMH – Dorsomedial Nucleus of the Hypothalamus
DMNV – Dorsomedial Nucleus of the Vagus
DSM - Diagnostic and Statistical Manual of Mental Disorders
EMT – Epithelial-to-Mesenchymal Transition
FMRP - Fragile X mental retardation protein

GABA – Gamma Aminobutyric Acid

GABARA1- GABA Receptor A1

GAD - Glutamic acid decarboxylase

GSK3β - Glycogen synthase kinase 3β

HDAC – Histone Deacetylase

i.n. - intranasal

i.p. - intraperitoneal

ICD - International Classification of Diseases

IL - interleukin

IP3 - Inositol trisphosphate

KOR – κ-opioid receptor

LH – Lateral Hypothalamic Area

LiCl – lithium chloride

LNPEP - Leucyl And Cystinyl Aminopeptidase

MC3R - Melanocortin 3 Receptor

MC4R - Melanocortin 4 Receptor

MeCP2 - methyl-CpG binding protein 2

MM – Medial Mammilary Nucleus

MOR - μ-opioid receptor

MPN – Medial Preoptic Area

NAcc (Core) – Nucleus Accumbens (Core)

NAcc (Shell) - Nucleus Accumbens (Shell)

NBEA - Neurobeachin

NLGN3 – Neuroligin 3

NMDA – N-methyl-D-aspartate

NPY – Neuropeptide-Y

NTS – Nucleus Tractus Solitarius

AP – Area Postrema

OFC - Orbitofrontal Cortex

OT - Oxytocin

OTR – Oxytocin receptor

PBN – Parabrachial Nucleus

PDD-NOS - Pervasive Developmental Disorder-Not Otherwise Specified

PFC – Prefrontal Cortex

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PKC – protein kinase C

PND – postnatal day

POMC – proopiomelanocortin

PSD95 - Postsynaptic density protein 95

PVN – Paraventricular Nucleus

RTK – Receptor Tyrosine kinase

rtPCR – real time polymerase chain reaction

SCN – Suprachiasmatic Nucleus

SHANK - SH3 and Multiple Ankyrin Repeat Domains

SIM-1- Single-Minded 1

SNP – Single Nucleotide Polymorphism

SON – Supraoptic Nucleus

St3gal5 - ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 5

TBS – Tris-buffered Saline

VGCC - Voltage Gated Calcium Channel

VGLUT - Vesicular Glutamate Transporter

VGSC - Voltage Gated Sodium Channel

VMH – Ventromedial nucleus of Hypothalamus

VPA – Valproic Acid Sodium Salt

VTA – Ventral tegmental Area

Introduction

Autism spectrum disorder (ASD), is a heterogeneous neurodevelopmental disorder characterized by altered cognitive phenotypes, such as impairments in social communication and interaction, sensory anomalies, repetitive behaviours and varying levels of intellectual disability. The neurological bases of ASD vary with on age [1], and cognitive abilities. In the context of child development, repetitive behaviour, diminished eye contact, imitation of gesture and/or language coupled with a lack of understanding of the context are the signs of social deficits of ASD. While the aforementioned developmental anomalies have been the main target of clinical and basic research as well as of therapeutic strategies, it should be noted that ASD also comprises other well-documented, highly prevalent and - surprisingly understudied - symptoms, including the focal point of this thesis, i.e., abnormal eating behaviour. In the sections below, this Introduction will deal first with the current state of knowledge pertaining to ASD aetiology and then with the evidence published thus far related to aberrant appetite in ASD.

1.1 Epidemiology / prevalence

The issue of prevalence of ASD has been debated for decades. From 1943 to early-2000s, the estimated prevalence of autism was estimated at 4 per 10000 children. However, the demographic studies from past 10 years show figures that vary from 4 to 60 per 10000 children, depending on the country, awareness, socioeconomic status and sampling of the population from which the estimate is derived [2]. A recent review on ASD globally reported the prevalence of 62 in 10,000 children [3]. In a report published by the Centers for Disease Control and Prevention, ASD prevalence of 18.5 per 1000 children aged 8 years (1 in 54) was documented; with a 4.3 times higher prevalence in boys than in girls [4].

Table 1: Prevalence of ASD (adapted from [5] with permission)

Author (15)	Country	Year of estimate	Setting	Method used to estimate prevalence	Age group	Cohort size (N)	Prevalence		M/F ratio
CDC (14)	US	2016	National	Case enumeration and record review	8y	275,419	Total: 1.85% ($CI_{95\%}$ 1.80–1.91%) Male: 2.97% ($CI_{95\%}$ 2.88–3.06%) Female: 0.69% ($CI_{95\%}$ 0.65–0.74%)	1 in 54 children	4.3 $CI_{95\%}$ (4.0–4.6)
Christensen et al. (15)	US	2014	National	Case enumeration and record review	4y	70,887	Total: 1.70% ($CI_{95\%}$ 1.61–1.80%)	1 in 59 children	NR
ASDEU (1)	Europe	2015	International	TNF, SCQ and national registries	7–9y	631,619	1.22%	1 in 89 children	NR
Delobel-Ayoub et al. (16)	France (South-East)	2015	Regional	Regional registries	7–9y	32,342	Total: 0.48% ($CI_{95\%}$ 0.40–0.56%)	1 in 137 children	4.0
	France (South-West)	2015	Regional	Regional registries	7–9y	15,836	Total: 0.73% ($CI_{95\%}$ 0.60–0.87%)	1 in 208 children	5.4
Narzisi et al. (17)	Italy	2016	Regional	Disability certificate census + SCQ & TNF	7–9y	10,138	Total: 0.80% ($CI_{95\%}$ 0.62–0.97%)	1 in 126 children	5.2
Morales-Hidalgo et al. (18)	Spain	NR	Regional	Screening + Clinical assessment	3–5y	2,755	Total: 1.55% ($CI_{95\%}$ 0.89–2.20%) Male: 2.52% ($CI_{95\%}$ 1.34–3.71%) Female: 0.58% ($CI_{95\%}$ 0.01–1.16%)	1 in 65 children	4.3
	Spain	NR	Regional	Screening + Clinical assessment	10–12y	2,827	Total: 1.00% ($CI_{95\%}$ 0.48–1.51%) Male: 1.72% ($CI_{95\%}$ 0.71–2.73%) Female: 0.39% ($CI_{95\%}$ 0.05–0.83%)	1 in 100 children	4.4
NHS Digital (19)	UK	2017	National	Parent report and direct interviews with children	2–4y	1,463	Total: 1.4% ($CI_{95\%}$ 0.70–1.80%) Male: 6.8% Female: 4.2%	1 in 71 children	4.4
	UK	2017	National	Parent report and direct interviews with children	5–19y	7,654	Total: 1.2% ($CI_{95\%}$ 0.90–1.40%) Male: 1.9% Female: 0.4%	1 in 83 children	4.8
	UK	2017	National	Parent report and direct interviews with children	5–10y	3,597	Total: 1.5% Male: 2.5% Female: 0.4%	1 in 67 children	6.5
	UK	2017	National	Parent report and direct interviews with children	11–16y	3,121	Total: 1.2% Male: 1.8% Female: 0.7%	1 in 83 children	2.7
	UK	2017	National	Parent report and direct interviews with children	17–19y	936	Total: 0.5% Male: 1.0% Female: N/A	1 in 200 children	NR
Bachmann et al. (20)	Germany	2012	National	Nationwide health insurance database	0–24y	6,400,000	Total: 0.38% Male: 0.53% Female: 0.20%	1 in 264 children	2.7

*ASDEU, Autism Spectrum Disorder in the European Union; CDC, Centers for Disease Control; CI, Confidence Interval; NHS, National Health Service; NR, Not Reported; SCQ, Social Communication Questionnaire; TNF, Teacher Nomination Form. *As of 2019 when the review was conducted.*

One of the most striking aspects of ASD epidemiological data is the geographic discrepancy in prevalence of the disorder. For example, Bougeard et al reported the prevalence estimates for each country ranging from 0.38% in Germany to 1.85% in the US (Table 1). In the US, ASD prevalence ranged from 1.70% to 1.85% in children. Prevalence estimates of 0.48% and 0.73% were reported for the South-East and South-West region of France respectively. In Italy, Narzisi et al. reported 0.8% prevalence of ASD in children. (reviewed in [5]). ASD affects 1 in 100 New Zealanders. Globally the prevalence of autism shows a clear rising trend.

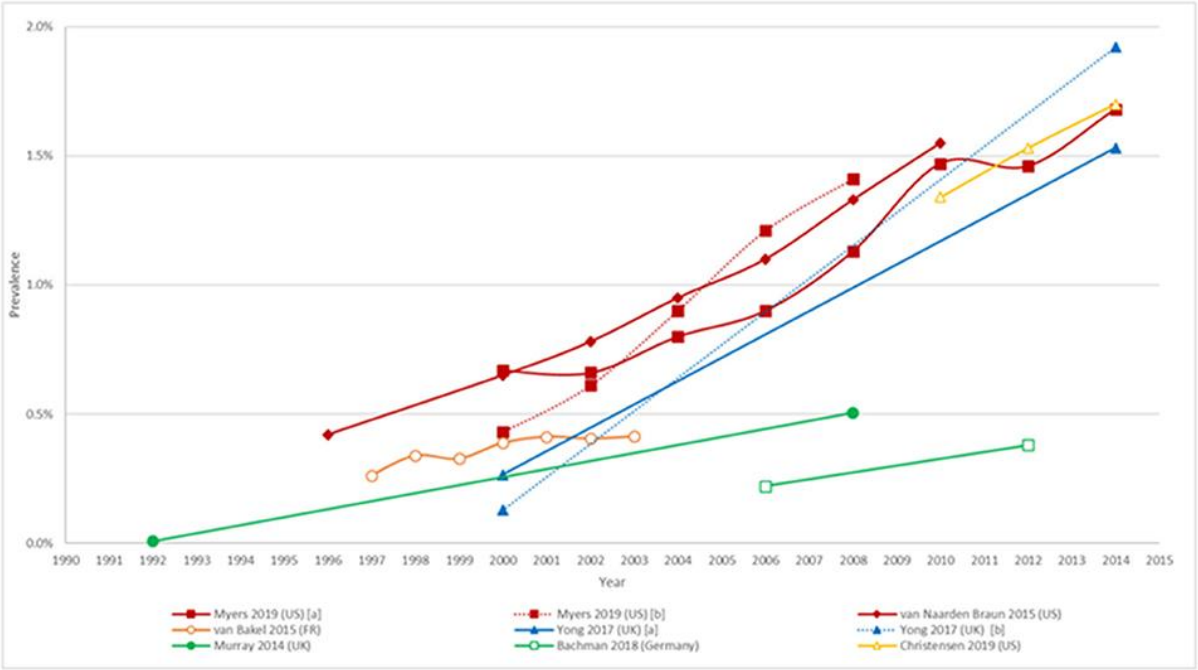


Figure 1-1: Studies with different methodology with differences in age group and geographical scopes estimating the rising trend of ASD prevalence (adapted from [5] with permission)

The Autism and Developmental Disabilities Monitoring (ADDM) network, USA, report ASD prevalence every two year since 2000. Prevalence rates in 4 year-old children were 1.34%, 1.53% and 1.70% in 2010, 2012, and 2014, respectively (yellow). Prevalence in children aged eight years showed a steady increase from 0.43% in 2000 to 1.41% in 2008 (red). Yong et al. reported prevalence to grow from 0.27 to 1.53% in children during 2000 to 2014 in the UK (blue). In France it increased from 0.26% in 1997 to 0.41% in 2003. All these datasets display a consistent upward trend over time (reviewed in [5]) (Figure 1-1).

Importantly, epidemiological data warrant careful interpretation. The rising trend of ASD prevalence in the population is due to the inclusivity of the diagnostic criteria (current definitions include the broader spectrum of neurodevelopmental conditions, such as Asperger's disorder and pervasive developmental disorder) [6], and the genetic variations [7]. Urban areas often show high prevalence due to higher population density and improved access to healthcare [8].Lower-to-middle income countries with less developed healthcare systems tend to show lower estimates of ASD. For example, Hossain et al reported the prevalence of ASD in Indian population

at just 0.09% [9]. The diversity of the population, awareness, healthcare system, the socio economic aspect, availability of high-quality population-based epidemiological studies etc. are some of the determining factors that contribute to the available epidemiological data [10].

Overall, data from the countries shown in Table 1, male: female (M: F) ratio ranged from 2.7 to 6.5. 4:1 ratio is the commonly cited statistic representing a consensus across a wide range of epidemiological studies from different countries over a long period of time, using different iterations of diagnosis. It is postulated that because of the better articulation of speech, females often go undiagnosed of ASD. The underlying mechanism that gives rise to the male preponderance is poorly understood.

It has been hypothesized that females have a higher threshold for presentation of the autistic phenotypes because of 'protective factor' relating to the X chromosomes (gene that 'escapes' the X-inactivation may be protective). Several ASD risk genes are present on the X chromosome (e.g. FMRP, MECP2, NLGN3, NLGN4X) which aligns well with the general role of the X chromosome in neural development. This, in part, explains the apparent increase in prevalence of ASD in Turner syndrome (female partially or completely missing an X chromosome) and Klinfelter syndrome (individuals with an additional X chromosome) [11-13] – both disorders present with intellectual deficits.

ASD inheritance does not appear to follow Mendelian inheritance. Linkage, single nucleotide polymorphism (SNP) and the heritability of the SNPs contribute to the inheritance and presentation of the disorder. Sato et al studied a rare copy number variant (CNV) in ASD and identified an inheritable autosomal variant of –SHANK1, a high confidence ASD gene with male-biased penetrance. Males carrying a microdeletion in SHANK1 showed to be high-functioning autistic individuals, while their female relatives with the same microdeletion presented anxiety, but did not meet the ASD diagnosis criteria [14].

The male-brain hypothesis proposes that 'masculinization' of the cognition - conceptualised as (1) low empathy in males and (2) higher systemizing, the drive to

interact with and understand rule-based systems, predisposes the males to a greater risk of ASD. In sum, increased social motivation and the ability to consciously mask the social impairment may obscure the ASD phenotypes being detected in females. Studies are needed to identify the players that cause sex-specific presentation of the ASD phenotypes.

1.2 Aetiology

1.2.1 ASD: the neurodevelopmental disorder

Considering the breadth of ASD symptomology, it is not surprising that the disorder has been associated with the number of morphological and functional changes in the central nervous system, particularly within those circuits that govern specific facets of behavioural maladaptations that contribute to ASD.

People with ASD often have difficulty understanding the intentions of others, leading to deficits in social interactions and interpersonal relationships [15] [16]. Studies have found this aspect of ASD to be underpinned by altered structural and functional connectivity in the brain, such as those involved in making judgments of cause and effect. For example fMRI scans have shown that people with ASD have wider and more extensive activation of the posterior superior temporal sulcus in the multi-sensory area when processing attribution judgments (i.e., finding causal attribution to an event) [15]. Kana et al. also found that ASD individuals have lower activation of the temporoparietal junction (TPJ) when attributing intentions [17].

Other brain areas important for emotional processing such as the amygdala show altered functionality in ASD. The amygdala is crucial in anxiety and emotional processing [18, 19], and social anxiety is reported in ASD [20]. Several authors have reported impaired amygdala activation as an underlying neural basis of social deficits in ASD [21-25]. In an MRI study with a cohort of ASD and non-ASD children, Zhu et al reported a higher volume of the amygdala in the ASD group [26]. In line with this finding, a meta-analysis by Kovacevic and colleagues indicated an elevated volume of the right amygdala in ASD [27]. Furthermore, a few other authors have reported higher amygdala volume in children with ASD [28, 29]. The trajectory of the volumetric growth

of the amygdala differs between ASD and controls. Schumann et al showed that children with autism of 7.5-12.5 years of age have larger right and left amygdala volumes than the healthy control children, while no differences in amygdala volume were observed between the adolescent groups (12.75-18.5 years). The authors suggested that the amygdala volume increases at a relatively late age (from 7.5 to 18.5 years) in typically developing children, while the amygdala in children with autism is initially larger, but does not continue to grow in size [30]. Additionally, there is evidence of a distinct neurodevelopmental trajectory between ASD with anxiety versus without anxiety: Herrington et al reported that children with ASD and anxiety had a decreased right amygdala volume compared to those having ASD without anxiety and to typically developing children [31].

While diminished amygdala function has been shown to be associated with social deficits in ASD, increased amygdala activity was observed during the perception of emotion and in fear learning [19]. In a face-recognition fMRI task, Herrington et al reported anxiety symptoms to be positively correlated with amygdala activity in adolescent participants with ASD, whereas social deficits were negatively correlated [32]. The reason for the contradicting pattern of activation of the amygdala in elevated anxiety (i.e., increased amygdala activity) that co-occur frequently with poor social functioning (i.e., decreased amygdala activity) in ASD remains elusive. One of the possible explanations could be the context-dependent recruitment of different neuroanatomical microcircuits [33]. In addition, the lateralization of the activation pattern [32] may also bolster the functional specificity of amygdala subregions and/or connections that could be affected in ASD. Another possible explanation could be, the anxious ASD individuals are more sensitive to emotional faces, rendering the interpretation of the emotion and the response to the emotional information from the face more challenging, thus triggering a vicious cycle of avoidance [34].

Atypical connectivity of the amygdala with other higher-order areas can also lead to social deficits (experimentally assessed as a function of face recognition). Hypoactivation of the fusiform face area in different face recognition paradigms (such as person-identity discrimination of expressive faces, person-identity discrimination of neutral faces, categorization task involving expressive and non-expressive faces,

familiar vs. unfamiliar faces, etc.) was reported by several authors [35-38]. While the fusiform gyrus is important for the facial identity perception, the amygdala governs the early-stage processing of facial expression since the amygdala reacts to emotionally potent stimuli. Activation of the amygdala can be induced by images of facial expressions [39]. Bartolotti and colleagues compared ASD children with anxiety vs without anxiety. The authors reported a decreased connectivity between amygdala and dorsal/rostral anterior cingulate (d/rACC) in the ASD-with-anxiety. While decreased amygdala-dorsomedial prefrontal cortex (dmPFC)/rACC connectivity was associated with a more severe social impairment, the disrupted amygdala-striatal connectivity was associated with restricted, repetitive behaviours in ASD [40]. It should be noted that impaired amygdala activation can also be a secondary effect of differential functionality of other higher-order area(s).

Enhanced functional connectivity of mirror neurons compensating for amygdala impairment was reported in ASD [41]. Furthermore, other authors also reported dysfunctional mirror neuron activation in ASD [42-44]. Mirror neurons are present in the precentral gyrus, inferior parietal lobule (IPL), the inferior frontal gyrus (IFG) and the superior temporal sulcus (STS), which are key for language, empathy and imitation. The enhanced functional connectivity of mirror neurons is likely the underlying reason of imitation without understanding a context (echolalia) in ASD.

Anomalous neuronal connectivity of the ASD brain, especially in the limbic region and higher-order cortical areas, has been shown in other MRI studies [45] and in postmortem brains [46, 47] . Differences in activation of the ‘social brain’ - such as the medial prefrontal cortex (mPFC), posterior cingulate gyrus, retrosplenial cortices, anterolateral temporal cortex, precuneus, visual cortex, basal ganglia, hippocampus, amygdala, fusiform gyrus [34, 45, 48-54] have been reported in several fMRI studies conducted in ASD patients compared to their matched healthy controls. Altered motor execution (e.g. primary motor cortex, thalamus, supplementary motor area and cerebellum) [55, 56] and sensory processing [57-59] has been linked to autism as well.

Quantitative analysis of the brain activity in humans is difficult due to inconsistency of the results stemming from the heterogeneity of the disorder, age-related changes in ASD phenotypes, differences in clinical assessment of ASD, and limited structural-functional understanding of the brain. Approximately one-quarter of children with ASD tend to show a regression in language or social skills, generally between 18 and 24 months of age [60, 61]. Several autistic phenotypes are interlinked involving higher cognition: for example, repetitive behaviours may be linked to a desire to compensate for the lack of understanding of a task or gesture with respect to a context. Another example is social camouflaging, i.e., using strategies to mask the autistic characteristics in social situations including the intentional suppression of stimming behaviour in public [62, 63]. Additionally, ASD can co-occur with other conditions including attention-deficit/hyperactivity disorder (ADHD), intellectual disability, language delay, anxiety and epilepsy [64]. Thus, the umbrella term ‘spectrum’ is underpinned by a range of psychiatric, neurological (structural and functional) and metabolic anomalies [65-68] with varying severity.

The changes described above stem from complex interaction of the genetic and chemical factors. Over 140 known genetic polymorphisms have been linked to autism.

(A) Genetic factors

Genetic variations lead to disrupted cell signalling involved in neuronal development, synapse formation, establishment and functioning cumulatively leading to a disruption in synaptic plasticity. The genes involved in ASD and how their mutation underpins the phenotypic outcomes of ASD is under extensive research.

Several loci of chromosome 7q [69], 1p, 3q, 16p, and 15q [70-72] have been identified to be linked to ASD. Although hundreds of different genes have been identified as putative causes of ASD, their precise function in the implication of the disorder is largely not well-understood. For example, children with Rett syndrome may be diagnosed as autistic as many of the behavioural traits fall within the intersection of the two (such as regression, repetitive behaviour, anxiety) and the age when the phenotypes start to be presented overlap quite a bit. The Rett syndrome risk gene

MecP2 is also an autism risk gene. Several genetic syndromes include autism or autistic traits such as Angelman syndrome (a syndrome signified by severe mental retardation, lack of speech and social deficits owing to lack of maternal UBE3A transcription), Down syndrome (trisomy of chromosome 21), Smith–Magenis syndrome (characterized by intellectual disability, delayed speech, sleep disturbances and neurobehavioural issues caused by interstitial microdeletion of 17p11.2) etc. [73].

The type of mutation (such as SNP, CNV etc.) and location (part of the gene where the mutation taken palace such as enhancer, promoter, intron, exon) of the mutation adds to the variability of the ASD-phenotype and the severity of the outcome adding to the complexity of the disorder. Duplication of the 15q11-q13 region leads to clinical phenotypes of autism including severe mental retardation, language disorders and seizures [74]. 22q11 deletion is associated with autistic disorder characterized by developmental delay, delayed speech and higher pain-tolerance. Somatic mosaicism (inheritable postzygotic mutation causing genetically distinct populations of cells within an individual) is being recognized as an important player in various neurodevelopmental diseases including autism [75]. Mutations in the exons poses higher risk than that of the intron regions. With whole exome sequencing of a large cohort of 5,947 ASD-affected families Lim et al showed that the somatic variations in autistic individuals were more likely to be in the critical exons. They also reported postzygotic mutations of pathogenic variants had elevated expression in the amygdala and striatum, areas implicated in social cognition [76].

Altered DNA methylation profile is linked to ASD [77, 78]. Ladd-Acosta and colleagues [79] reported a differential methylation in temporal cortex and cerebellum of post-mortem brains of ASD individuals. Wong et al described an altered methylation in several genes in a study with 50 pairs of monozygotic twins discordant for ASD. Several methylation patterns at some CpG sites were found to be common to symptom groups - for example they reported a correlation between P2RY11 and NRXN1 DNA methylation and quantitative autistic trait scores [80].

The contribution of the vast interactive network of genetic modulators in the aetiology of ASD remains quite obscure. Diverse genetic bases of this disorder underpins the

various phenotypic presentation. Elucidating the interplay between the causative genetic factors and their effect in the disease phenotype would lead to a better understanding of ASD.

(B) Chemical factors

Chemical factors increase the risk of DNA mutagenesis (Figure 1-2). For example, Mitchell et al demonstrated that organic pollutants such as polychlorinated biphenyls and polybrominated diphenylethers can act as developmental neurotoxicants and cause copy number variation of 15q11-q13 and hypomethylation of long interspersed nucleotide element-1 - implicated in autism [81]. , Some of the chemical factors are ubiquitously present in the environment (such as heavy metals, phthalates), while some are pharmacological (such as Valproic acid, misoprostol, thalidomide).

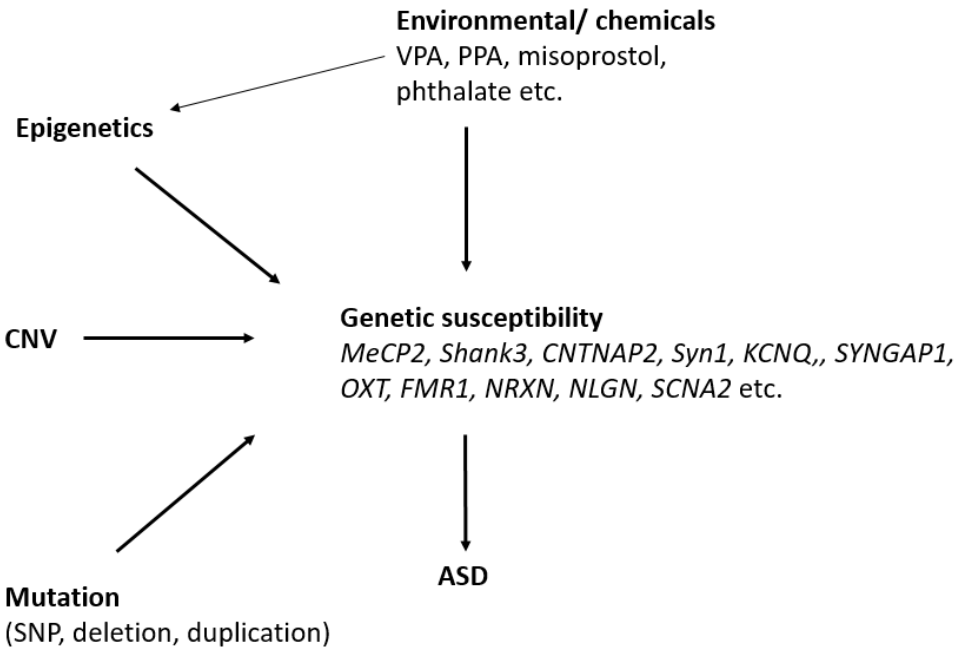


Figure 1-2: Simplified schematic of the multifaceted aetiology of ASD. Both genetic and non-genetic factors modulate the penetrance of risk genes, resulting in a heterogeneous disease phenotype (modified from [82]).

Propionic acid (PPA)

Blood Brain Barrier (BBB)-penetrant PPA, a dietary short chain fatty acid is a common food preservative. It has been reported to induce a number of behavioural changes and neuroinflammatory responses in rats reminiscent of ASD. In the brain, it can cross plasma membranes and induces intracellular acidification, altering neurotransmitter releases and, ultimately, behavioural phenotypes [83].

MacFabe et al showed that intraventricular infusion of PPA can cause repetitive dystonic behaviours, hyperactivity, turning behaviour in adult rats. Biochemical analyses of brain homogenates from the PPA-treated rats indicated increased oxidative stress. Neurohistological examinations of hippocampus and external capsule of the treated rats revealed increased GFAP immunoreactivity and activated microglia suggesting elevated neuroinflammation [84]. El-Ansary and colleagues used oral administration of PPA to young male rats and revealed that PPA-treated rats showed increased oxidative stress, along with decreased glutathione and catalase activity. Elevated IL-6, TNFα, IFNγ further indicates the pro-neuroinflammatory effect of PPA. [83]

Thalidomide

During the late 1950s, thalidomide used to be prescribed to pregnant women to relieve morning sickness. Using thalidomide in early gestation (weeks 3 to 8) leads to birth defects such as ear, cardiac, and gastrointestinal malformations [85, 86]. Ito et al reported that thalidomide induces teratogenicity via inhibiting ubiquitin ligase activity [87]. Using a zebrafish model, they also revealed reduced otic vesicle size, malformed proximal endoskeletal disc of the pectoral fin. Malformed forelimbs were reported in chicks as well.

Misoprostol

Misoprostol is a synthetic prostaglandin medication used to treat stomach and duodenal ulcers and to induce labour. Contradicting opinion persists on the effect of misoprostol in ASD-aetiology. For example in a study autism was found to prevail in both the misoprostol-exposed and misoprostol-unexposed groups [88] while Miller's report indicates otherwise [89].

Phthalates

Phthalates are widely used in personal care products, plastics, children's toys. Rodent studies showed that phthalates cross the placenta to the foetus [90] and also it is found to be excreted in the urine of pregnant females. Using a small number of cases Shin et al found varying and inconclusive results [91]. Kim et al measured five phthalate metabolites during mid-term pregnancy and followed up till 4, 6, and 8 years in 547 mother–child pairs. Using social communication questionnaire they assessed autistic traits of children at each time point and reported exposure during pregnancy is associated with autistic traits in young children and males showed a stronger association as compared to female children. [92, 93].

Heavy metals

Exposure to heavy metals such as mercury, lead, and arsenic has been associated with neurodevelopmental disorders and ASD [94, 95]. The molecular mechanisms revealed using animal models indicate imbalances in calcium homeostasis and impaired synaptic functionality [96, 97].

Methylmercury causes neurodevelopmental disorders via oxidative stress and altered calcium and glutamate homeostasis [98]. Chronic exposure to low doses of these neurotoxins cause neurobehavioural deficits. Crump et al employed psychological tests in 237 children of 6-7 years old from New Zealand and correlated the data with the mercury concentration in their mothers' hair during pregnancy and revealed an association between the mercury-level and subtle neurobehavioral deficits in those children [99].

Pre-pregnancy obesity, maternal diabetes [100-103] and maternal malnutrition of micronutrients [104] have been reported to be associated with ASD-risk. In sum, chemical and environmental factors may affect neuronal development during the critical period indirectly by genetic modifications affecting the brain.

Valproic Acid (VPA)

Women consuming anti-epileptic drugs such as VPA during early gestation tend to have a higher chance of having babies with neural-tube defects (owing to defect in secondary nurulation) and autism [105]. Exposure of a foetus to VPA poses high risk of the baby to autism. The risk is so high and has been established for decades that *in utero* VPA treatment in laboratory animals leads to ASD-like phenotypes and it has become a standard method of generating animal models of ASD.

VPA, a branched short-chain fatty acid, is used to treat epilepsy and migraine [106]. The excitation-inhibition (E/I) imbalance contributes to neuropsychiatric phenotypes, including anxiety, depression [107] and migraine [108]. In epileptic seizures, the mechanisms that inhibit neuronal firing are disrupted leading to aberrant excitation. Thus, anti-epileptic drugs inhibit the mechanisms that depolarize neurons to their firing threshold, or augment the inhibition to aberrant firing.

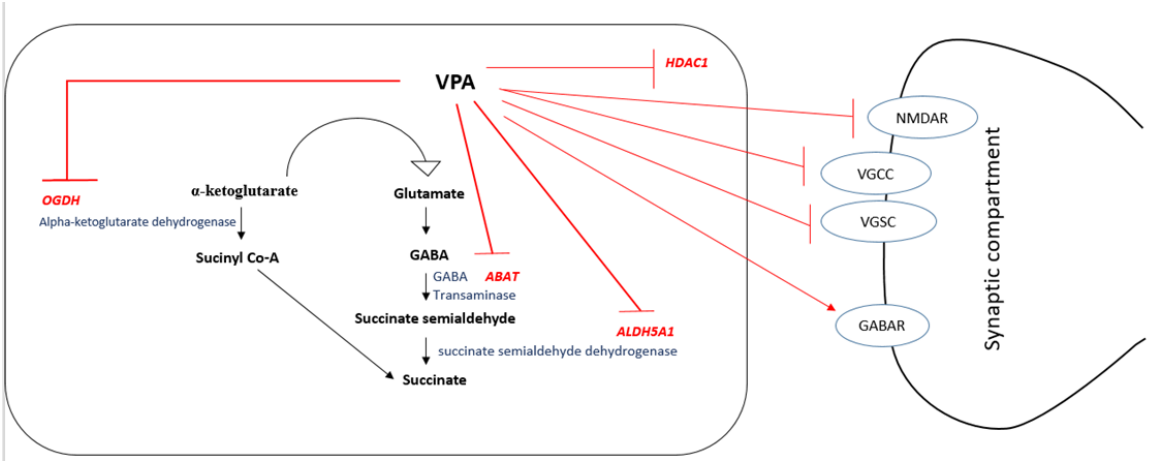


Figure 1-3 : Key mode of action of VPA as an antiepileptic drug. VPA acts as an anti-epileptic drug by (A) acting on enhancing the endogenous GABA levels in the brain by preventing GABA breakdown and increasing the substrate concentration (α-ketoglutarate) to promote production of GABA. GABA is metabolized to succinate semialdehyde by GABA transaminase (gene product of ABAT). Succinate semialdehyde is converted to succinate by succinate semialdehyde dehydrogenase (gene product of ALDH5A1). VPA inhibits ABAT and ALDH5A1 preventing degradation of GABA – the inhibitory neurotransmitter [109]. (B) Interacting with postsynaptic GABA receptors (C) reducing the high frequency burst firing of neurons

by inhibiting Voltage Gated Sodium Channels (VGSC) and Voltage Gated Calcium Channels (VGCC) (D) antagonizing with ionotropic glutamate receptor – NMDAR

VPA increases the activity of glutamate decarboxylase (GAD) that breaks down glutamate to GABA [110] decreasing the excitatory neurotransmitter and increasing the concentration of inhibitory neurotransmitter. It also enhances the response of the GABA receptors [111] (Figure 1-3).

The inhibition of α-ketoglutarate dehydrogenase, leads to lower ATP production in the brain [112] (Figure 1-3). Epileptic seizures are linked to an increase in cerebral energy metabolism. Thus, a decrease in energy production reduces neuronal firing [113]. Also, VPA directly decreases sodium-dependent action potential, thereby inhibits spike generation [114] (Figure 1-3).VPA can directly suppress NMDA-evoked depolarizations, thereby blocking seizures induced by NMDA [115-117]. (Figure 1-3).

Other than these mechanisms of action in the context of epilepsy, VPA is also an inhibitor of Histone Deacetylases (HDACs) [118, 119]. Citraro et al, using WAG/Rij rats showed that early chronic VPA intervention improves depressive-like behaviour [120].

VPA exposure during early gestation disrupts neuronal precursor proliferation, differentiation, migration, synaptogenesis and thereby neuronal connectivity. Teratogenicity, such as neural tube defects and spina bifida following the use of VPA during early gestation are well-established. The prevalence of neural tube defects is approximately 1–2.5% in children of mothers treated with VPA, especially during early pregnancy [121]. Maternal use of valproate during pregnancy is associated with significantly elevated risk of ASD [122-124]. While looking into the molecular pathway involved in anti-epileptic action of VPA, Floriano-Sánchez et al reported VPA may inhibit pro-epileptic gene expression by inhibiting CREB-mediated transcription [125]. CREB is a versatile transcription factor involved in a wide range of contexts - long term potentiation, learning, memory and neuronal development. Perturbation of CREB-mediated transcription by VPA thus may affect neuronal development.

1.3 Diagnosis

Autism was originally described as a form of childhood schizophrenia in 1911 by the German psychiatrist Eugen Bleuler and a result of unkind parenting. Eventually the definition changed as a set of related symptoms, and finally as a spectrum of conditions with varying degrees of psychiatric, psychological and neurodevelopmental impairments. Along with these shifting views, its diagnosis has changed as well. Between 1920-1930s terms such as ‘autistic’, ‘schizophrenic’ and ‘psychotic’ were introduced into the realm of child psychologists. During early 20th century, a growing speculation about the nature of infantile thought processes the cause-effect relation to mental illnesses emerged. Eminent thinkers like Bleuler, Sigmund Freud and Carl Jung proposed childhood traumas being the underpinning reasons of a differential neurodevelopmental trajectory.

Autism was first identified and described in 1943 by Leo Kanner, a child psychiatrist at Johns Hopkins University. He identified a list of cases with similar symptomatology in children and defined it as “extreme autism, obsessiveness, stereotypy, and echolalia”. He describe that his patients were having a “good relation to objects that do not change their appearance and position, that retain their sameness and never threaten to interfere with the child’s aloneness” and he also reported that these children ‘‘... have come into the world with an innate inability to form the usual, biologically provided affective contact with other people’’ [22, 126, 127].

In the mid-20th century the diagnostic criteria of autism were as follows:

1. gross and sustained impairment of emotional relationships with people
2. apparent unawareness of his own personal identity
3. pathological preoccupation with particular objects
4. sustained resistance to change in the environment
5. abnormal perceptual experience
6. acute, excessive and seemingly illogical anxiety
7. speech may have been lost or never acquired
8. distortion in motility patterns
9. a background of serious retardation in which islets of normal, near normal, or exceptional intellectual function or skill may appear (adapted from [128])

Autism was initially identified as a subset of the general diagnostic category of ‘pervasive developmental disorders’ (PDDs) has changed its definition over the years. PDDs are a group of disorders that was used as a terminology to convey the varied range of social communication deficits in Diagnostic and Statistical Manual of Mental Disorders, Third Edition (DSM-3), 1980. DSM-3-R (1987) first differentiated autism from childhood schizophrenia but the diagnostic criteria remained stringent as described earlier. With time, attempts have been taken to sub-divide the phenotypes based on the intensity of features. DSM-4 (1994) specified a series of sub-classifications of autism, such as autistic disorder, Asperger’s syndrome, atypical autism or pervasive developmental disorder – not otherwise specified (PDD-NOS) [129].

Eventually, due to a lack of clear demarcation between different subsets of PDDs the most recent diagnostic systems, DSM-5 (2013) and the 11th edition of the International Classification of Diseases (ICD-11; WHO, 2018) use the umbrella term ‘ASD’, and differentiate individuals using additional clinical specifications [130] . A significant change in the fifth edition is the deletion of the subsets—namely, Asperger's syndrome, classic autism, Rett syndrome, childhood disintegrative disorder and PDD(NOS)—was implemented, with specifiers regarding intensity: mild, moderate, and severe. Both DSM-5 and ICD-11 refer the four separate disorders as a single condition with different levels of severity of symptoms in two core domains 1. Deficits in social and communication behaviours, 2. The presence of restricted and repetitive behaviours. Severity is based on social communication impairments and restricted, repetitive patterns of behaviour, with three levels: Requiring support, requiring substantial support, requiring very substantial support [131].

Additionally, DSM-5 allows autism to be diagnosed in the presence of other conditions – such as ADHD or anxiety – erstwhile co-occurrence would be denied. ICD-11 provides more detail on distinguishing autism with and without intellectual disability [1]. The diagnostic criteria of ASD as described in DSM-5 is tabulated in the appendix section (Appendix 1, Table 5).

Hence, with classical definition of autism, an individual would exhibit repetitive behaviour, social deficit and language impairments while with the evolution of the diagnostic criteria, ASD has become more inclusive. Currently, prenatal diagnosis of ASD is not possible, as it does not present with major morphological deformity.
Taking the variability between people exhibiting different phenotypes owing to similar underlying conditions into account, it is identified as a spectrum.

1.4 Treatment

There is a dearth of pharmacological treatment of ASD owing to the idiosyncratic presentation of the disorder. Medications are used to ameliorate the severity of the symptoms since complete cure is not feasible. Early diagnosis and personalised intervention is key to improve some of the cognitive deficits.

A couple of Food and Drug Administration (FDA)-approved drugs such as aripiprazole and risperidone are commonly used to improve some of the symptoms of ASD. These atypical antipsychotics target the dopaminergic and serotonergic system. These drugs are reported to be safer than other atypical antipsychotics such as clozapine, olanzapine or quetiapine, in terms of weight gain, sedation, prolongation of cardiac QT interval etc. [132].

The activity of aripiprazole depends on the endogenous dopamine concentration and receptor population. It antagonises the D2 receptor under hyperdopaminergic conditions, and is an agonist under hypodopaminergic conditions. Being an agonist to 5-HT1A and antagonist to 5-HT2A aripiprazole improves some of the irritability, aggression and hyperactivity associated with young individuals with ASD. [133] Risperidone inhibits 5-HT2A, D2R, alpha1 and alpha2 adrenergic receptors and is used in the treatment of ASD as well. It has been shown to improve aggression, mood swings, irritability, agitation, social withdrawal and stereotypic behaviour [134, 135] .

However, the neurobiology of the disorder is not limited to dopaminergic and serotonergic systems only. In addition, the long-term impact of aripiprazole and risperidone on brain activity remains elusive. Additionally, long-term usages may cause side-effects. For example, Lian et al showed that risperidone treatment to

juvenile female rats although reduces locomotor activity, on the other hand, leads to an increase in food intake and body weight gain. These effects were supplemented by elevated mRNA expression of hypothalamic orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP), without affecting the hypothalamic proopiomelanocortin (POMC) mRNA expression. Hence, the body weight gain in the juvenile rats was a combined effect of hyperphagia induced by NPY - AgRP pathways and a reduction in locomotor activity [136]. Furthermore, studying the clinical implications of these drugs in the broader spectrum of ASD is required as well.

Targeting neurochemicals, neuropeptides and metabolism-focused treatments are slowly gaining attention (reviewed in [137]). Reports from animal-studies point towards some neuromodulators that show potential as pharmacological substances that can be used in ASD: for example - 5-HT1A agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) has been implicated in improving social deficits in ASD [138]. Oxytocin (OT) is gaining attention as a potential therapeutic candidate in ASD and currently is under clinical trials.

1.5 OT and autism

Diverse underlying genetic causes affect OT functionality. Thus the OT system serves as a common denominator of ASD pathophysiology.

1.5.1. Relevance of OT, oxytocin receptor (OTR) and other related genes in ASD

Although the definition of ASD has changed since 1911, the core symptoms still remain as restricted and repetitive behaviour coupled with a dearth of reciprocal social interactions and communication. Risperidone and Aripiprazole has been approved by the FDA for autism, but it only ameliorates aggression and irritability, not focusing on the sociability and other behavioural shortfalls of ASD.

OT is well-established as a prosocial hormone facilitating pair-bonding. Since Prairie voles develop long-term monogamous relationships, they provide a good model system to look into affiliative behaviour, especially pair bonding [139]. In contrast,

montane voles exhibit polygamy. In a study comparing prairie voles and montane voles, Insel et al found no significant difference in the expression of OT between the species. However, there was a difference in the distribution of OTR in the brain [139]. In prairie voles, they reported OTR expression was more robust in the Nucleus accumbens (NAcc) and the amygdala while the distribution of OTR was higher in the lateral septum and cortical nucleus of amygdala in montane voles. Even though there is a gap in knowledge in terms of how the region-specific distribution of OTR contributes to specificity in partner-preference, this finding indicates the involvement of OT in mediating affiliative behaviour [140]. Furthermore, intracerebroventricular infusion of OT facilitates partner preference in female prairie voles [141] bolstering the role of OT in pair-bonding.

Onset of maternal behaviour in rats includes overcoming natural avoidance towards neonates, which can be induced by novel odour of the pups [142]. Oxytocin is a key regulator of maternal behaviour [143]. Marlin et al showed that oxytocin enables pup retrieval behaviour in female mice by enhancing auditory cortical pup call responses[144]. Endogenous OT promotes associative learning of maternal odour in pups [145]. This suggests that OT is an important neuropeptide for young rats to form associations with their mothers.

Evidences indicate that OT regulates social cognition. OT-knockout (OT-KO) mice show deficits in social recognition memory, with intact spatial learning [146]. Social recognition can be rescued by central administration of OT to the medial nucleus of the amygdala in OT-KO mice [147] suggesting that OT improves social cognition in OT-deficient animals. Adult OTR- KO mice exhibit deficits in social discrimination and aggression than wild type mice [148]. OTR-/- mice show a resistance to change in a learned behaviour projecting to the stereotypic behaviour and restricted interest observed in autism. Increased susceptibility to seizures observed in these animals, interestingly found to be a comorbid symptom of ASD. Intracerebroventricular OT restores social exploration and social recognition, along with aggressive behaviour in OTR-mutant mice [148].

Pair bond formation is also a necessary component of human sexuality: elevated plasma OT was found in response to human sexual responses [149]. OT facilitates mother-infant engagement in humans as well. Nipple stimulation facilitates OT release [150]. Putting this in context with Scatliffe's report of increased maternal OT levels being associated to more affectionate contact-behaviours and engagement of in mothers with infants establishes the role of OT in facilitating attachment [151]. Reports from several authors establish OT's role in social bonding in humans (reviewed in [152])[153].

Reports from human-studies suggest that OT improves memory of facial expressions – a component of social cognition. With intranasal (i.n.) administration improvements were observed in eye gaze number and duration for human faces [154], recognition memory for faces [155] irrespective of the expression of the face (prosocial expression/neutral/angry/ fearful expressions) [156] [157, 158] in human subjects. The amygdala mediates the prosocial and anxiolytic effects of OT. In an fMRI study involving visual matching tasks for different fear-inducing visual stimuli the activity of the amygdala was observed to be reduced post-i.n. OT administration [159]. I.n. OT administration attenuated activity in the lateral and dorsal regions of the anterior amygdala in response to fearful faces, and increased activity in response to happy faces [160].

In sum, all the studies involving animal and human subjects establishes OT-deficiency being linked to social deficits similar to ASD and the potential of OT in improving social deficits. Since OT has been linked to infant-mother attachment, maternal care, pair bond formation, affiliative behaviour, implication of OT is ASD is worth addressing. Aberrant regulation of OT signalling has been shown ASD [161, 162]. Clinical trials for OT's effects on improving social deficits in ASD is gaining attention.

OT-gene loci reside at chromosome 20p13 in humans [163, 164]. Prepro-OT is encoded by OT-Neurophysin-I gene consisting of 3 exons: the first encodes a translocator signal, the hormone, the processing signal, and the first 9 residues of neurophysin; the second encodes the central part of neurophysin; and the third exon encodes the C-terminal region of neurophysin [165]. The OT prepropeptide undergoes

posttranslational processing - leading to mature nonapeptide of OT and its carrier molecule neurophysin-I, remain in the presynaptic compartment and are released upon required neural inputs. OT binds with GPCR-OTR and executes its effect in the target organ in a context-dependent manner - for example uterine contraction [166], milk ejection [167] and it regulates different aspects of social behaviours and food intake.

(A) OT gene mutation in ASD

Multiple SNPs have been reported within or near the OT gene to be linked with ASD. Ebstein and colleagues conducted genome-wide SNP analysis in a large sample size of 170 subjects diagnosed with ASD from 149 families and found associations between ASD and the rs6133010 SNP locus within the OT gene [168]. Haplotype analysis of this cohort also showed significant associations between rs6133010:rs2770378, the later residing near the OT gene. Francis et al further corroborated this finding. They screened 207 ASD-individuals and reported a significant association between OT rs6084258 and ASD [169]. In a subcohort of the individuals included in the study, they measured the association of polymorphisms OT rs6084258, OT rs11697250, and OT rs877172 with plasma OT. With the permutation of allelic substitution, they reported lower plasma OT in presence of the risk alleles since some SNPs may lead to variations in the amino acid sequence. These findings suggest that SNPs on/near OT are associated differential social behaviours, restricted and repetitive behaviours in ASD.

Allen-Brady et al performed a six generations of ASD pedigree scanning of all male cases using genome wide SNP analysis and reported 20q11.21–q13.12 region as an ASD-susceptibility locus, which is near the OT-NPI gene [170]. In this substantially thorough pedigree analysis they reported the 20q11.21–q13.12 region to have a high statistical likelihood to be inherited and predisposes males to ASD. Iourov and colleagues reported a 7-year-old male child with 20q11.21 microdeletion to exhibit mild intellectual disability [171]. The genes within this region do not interact with the OT gene and none of them is implicated in autism, but considering that distant DNA sequences further upstream or downstream to a gene may influence the expression

by means of epigenetic regulation, the possibility of an indirect regulation of OT cannot be completely ruled out.

bHLH single-minded 1 (SIM1) mRNA and protein are expressed during E10.5 in mice when PVN and SON start differentiating and are essential for the development of the magnocellular and parvocellular neurosecretory lineages of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Michaud et al reported that the newborn SIM1 homozygous KO mice pups show drastically reduced immunoreactivity of OT (and other neurosecretory cells as well, such as AVP, CRH, TRH and SS) in the magnocellular PVN as compared to the heterozygous SIM1-deficit mice along with a reduced size of the neurohypophysis [172]. The SIM1 protein binds to ARNT2 forming SIM1/ARNT2 dimer, affecting downstream Brn2 governing the survival and final differentiation of PVN and SON neurons. In heterozygous condition, SIM1-expressing cells either change fate or die around E15.5 in the absence of terminal differentiation. [173, 174]. The role of SIM1 in proliferation of the neuronal precursor cells and in the fate-choice is not known. Postnatal SIM1 deficiency is established to produce hyperphagic obesity coupled with reduced hypothalamic OT mRNA in mice [175]. Several authors have reported SIM1-deficiency leading to a reduction in OT causing hyperphagia [175-179]. In sum, these evidences establish that SIM1 is critical for the development and functionality of central OT signalling. Hovey et al looked into SNPs in SIM1 gene in a population of 1771 children and found significant association between language impairment with SIM1 SNP rs3734354 (Pro352Thr) along with a small association between ASD scores and SNPs in OT gene [180]. Owing to a lower statistical power, their data calls for cautious interpretation but paves to support to the hypothesis that OT and the OT neuron development may have an influence in producing autistic phenotypes [177].

(B) Altered OT-processing in ASD

Alterations in the endocrine OT system due to differential processing of the translated prepro-OT may also underpin ASD. Modahl et al reported lower plasma OT levels in autistic children as compared to normal children in a study with small sample size [181]. Similar data was reported by Green and colleagues [182] suggesting altered OT

processing. They performed radioimmunoassay in plasma samples of 6 to 11 years - old autistic males and matched controls suggesting altered OT processing in autistic children. Their results indicate a reduction in plasma OT levels in autistic individuals along with an elevated level of the immature uncleaved form (C-terminus-extended form). In autistic children, rise in the extended form of OT with age indicates failure in peptide processing, as the level of mature OT peptide tends to remain relatively unaltered with age. Additionally, Munesue's group reported lower plasma OT in ASD patients [183]. Although peripheral measurement of OT levels is insufficient to clearly indicate central processing of the same, these findings may suggest altered activity and/or expression of convertases involved in OT-processing (posttranslational processing, degradation) may also be implicated in autism. When released centrally, OT is degraded within brain tissue by aminopeptidase encoded by LNPEP gene - thus has a half-life of about 20min in the cerebrospinal fluid (CSF). Although there has been no evidence for the involvement of LNPEP in altered human behavioural phenotypes.

(C) OTR mutation in ASD

OTR is a 17kb gene with 3 introns and 4 exons, expressed in various brain regions. In humans, OTR gene is mapped to the locus 3p25-3p26.2 [184].

Wu et al reported the involvement of OTR in the susceptibility to autism in Han Chinese population by analysing the SNPs located within the OTR gene of 195 ASD children. Their results suggest two SNPs at rs2254298 and rs53576 found in over 50% of the participants being associated with the aetiology of ASD [185] suggesting that genetic variability of OTR is a risk factor for autism. Jacob and colleague did not find any association of OTR rs53576 in Caucasian ASD children although they corroborate the finding of Wu et al; rs2254298 was found to be linked with ASD-risk in their cohort as well [186]. This difference in finding is likely due to the racial difference in the cohort and a different M:F ratio. Several other authors link different SNPs of OTR and ASD [186-192]. Furthermore, Kranz et al tested OTR SNPs (rs237889 and rs237897) for association with ASD in German cohorts and found nominal over-transmission for the minor A allele of variant rs237889G>A [190]. One study by Ma et al. reported that the G allele of variant rs35062132C>G was correlated with an increased likelihood of ASD.

Although these SNPs indicate an association with ASD, it does not reveal whether that causes any change to expression levels in central OTR, neither they indicate whether that affects the ligand affinity and/or regional distribution of the OTR in the brain. *In vitro* study showed that rs35062132C>G accelerates OT-induced receptor internalization and recycling [193].

Additionally, whether the SNPs are implicated in epigenetic modification of the OTR is interesting to look into. There is evidence that the 6930G>A allele of rs53576 is linked to increased methylation levels of CpG-islands of the OTR [194, 195] however there is no unified opinion on how increased versus decreased methylation status in different gene-segments would link to the expression-levels of OTR.

DNA methylation status of OTR is associated with the activity of the areas involved in emotional processing such as amygdala, fusiform gyrus, and insula [196]. Dysregulation in methylation of promoter region of the OTR genes is concomitant to ASD [197] (reviewed in [198]). Research focusing on epigenetic modifications and rare variations of the OTR may provide additional evidence for a role of this gene in ASD. In 105 ASD individuals from Japan, Liu et al identified 28 novel variants including a potential functional variant of Arginine150Serine in the intron region [199]. DNA methylation analysis indicated that the hypermethylated promoter of OTR is found to be present in the temporal cortex in autism. In healthy adults, OXTR methylation has been associated with elevated receptor activity in the dorsal ACC and TPJ [196]. This is in line of the findings of Young et al who reported that the sequence variability in the promoter region may account for the difference in regional expression of OTR in different species of voles [200]. Interaction of epigenetic factors during prenatal and perinatal stage also may affect gene expression in the adult. However no such temporal data is available in the context of ASD.

(D) OT synaptic release and ASD

Studies in mice show that the CD38 transmembrane protein implicated in the secretory pathways is important for OT release from the axon terminals and thereby modulating social behaviour [201, 202]. CD38-KO mice show severe social deficits and have been

studied as a rodent model of ASD [203]. CD38 is widely expressed in the human brain as well. Immunohistochemical results indicate expression of this glycoprotein in the human cerebral cortex [204]. The human protein atlas shows expression of CD38 in the hypothalamus [205]. Munesue and colleagues reported highest level of CD38 mRNA expression the human hypothalamus, and substantial expression in the frontal cortex, amygdala, and cerebellum [183]. Immunohistochemical staining of the human brain reported by this group also shows colocalization of OT-immunoreactive (OT-IR) cells with CD38+ cells in the hypothalamus.

SNPs of CD38 (rs3796863 and rs6449197) were reported to be associated with autism in US population [183] [206]. CD38 rs3796863 was also reported in another cohort in Israel [206]. For the rs3796863 SNP, ASD patients carrying the CC were reported to be present with severe repetitive behaviour. They also reported a missense (Arginine140Tryptophan; (rs1800561, 4693C>T)) mutation in 0.6-4.6% of the Japanese population and found association between the SNP and ASD in their sampling cohort [183]. Ceroni and colleagues reported partial deletion of CD38 in a patient with autism and asthma [207].

Various downstream signalling mediators (such as Wnt, GSK3β signalling, ERK pathways etc.) (Figure 1-5) are also implicated in autism that can lead to domino-effect owing to disruption of genes directly involved in OT signalling pathway or could be a combination of multiple independent events. Data obtained from human genetic studies generally calls for cautious interpretation owing to sampling bias, methodological differences and the heterogeneity of the disorder. However, the relevance of OT in ASD in humans is evident.

1.5.2. OT as a potential therapeutic in autism

Harnessing the prosocial properties of OT, modulation of the OT-ergic system is a subject of interest in the field of pharmacology. Evidence from animal models indicate OT to improve some of the phenotypes of autism. It is under clinical trial in the context of improving social deficits in autistic individuals. The findings are controversial and owing to a lack of comprehensive data from large-enough sample size.

Preliminary results using small sample sizes reported the therapeutic potential and safety of administration of i.n. -OT for improving social cognition deficits [208]. Several studies support the fact that OT administration alleviates some of the social deficits in ASD [209-212] (reviewed in [213]). Guastella et al reported an improvement in emotional recognition in a study conducted with 16 ASD male individuals within 12-19 years of age [214]. Improvement in social orientation was also reported by Lin's group [215]. Fragile-X syndrome (FXS), caused by a loss of function of Fragile-X mental retardation protein (FMRP) is one of the most common single-gene defects associated with ASD. FMRP regulates neuroplasticity by negatively regulating translation by affecting ribosomal translocation across transcripts of 842 genes, some of which are also ASD candidate genes [216, 217]. FXS individuals show reduced eye gaze and increased mouth gaze to emotional faces compared to their typically developing counterparts. A randomized double-blind single-dose study by Hall et al demonstrated eye gaze frequency to improve with administration of i.n. OT in males of 13—28 years with a confirmed genetic diagnosis of FXS [218] indicating an improvement of social anxiety in patients with FXS.

However, there are studies reporting no significant difference between i.n.-OT treated group as compared to placebo [219-221]. In a 24-week study involving children and adolescents of 3-17 years receiving i.n. OT and placebo, Sikich et al reported no difference in social behaviours between groups [219]. Dadds and colleagues evaluated parent-child interaction in ASD males (7-16 years old) over a course of 5 days with i.n. placebo or OT once daily. The OT or placebo was administered during parent-child interaction training sessions. They reported no improvement in social interaction skills, repetitive behaviours and emotion recognition [220]. These differences in findings can be due to the methodologies of assessment of social behaviour and parameters of social attributes in question, age group of participants (for example, in Sikich's and Dadd's study), the dosage administered (for instance Sikich's group administered a total daily doses of OT ranging from 8 to 80 IU, based on adverse effects in the participants rather than having a standard dosage depending on age and body weight) etc.

Although many reports support the theory that the OT signalling pathway is related to the pathogenesis and treatment of ASD, they do not explain the implication of this pathway in the heterogeneity of ASD. Some propose that OTR genotype is strongly associated with the social cognition but not ASD diagnosis and finding groups of patients with ASD having OTR sequenced prior to OT-treatment may be beneficial [160]. For instance, Mayer and colleagues recruited ASD male participants analysed for 22 SNPs in the OXTR rs53576 in an fMRI study involving i.n. OT administration. It was expected that carriers of the risk alleles (AA and GA) would show altered activation in amygdala compared to non-carriers post-OT treatment but they observed no effect of OT suggesting heterogeneous effect of OT administration in ASD; the receptor sequence being one of the facets [221].

1.6 ASD and food intake

Anomalous social behaviours tend to be in the limelight of autism research - one of the core yet understudied traits of ASD is atypical feeding behaviour. Being 'picky eaters', is very frequently detected in young children with ASD [222]. There are several components of this peculiar eating practice such as dietary selectivity [223-225], penchant for particular food items and meal location (e.g. definite part of the residence) [223, 226, 227] etc. Varied food exposure, altered sensory processing (e.g. certain noise in the environment like people chewing, getting hands dirty etc.), social cues (such as presence/absence of parent or caregiver, music and/or specific television programme etc.), dislike towards unfamiliar object or event which may be associated with anxiety, texture and taste preferences (e.g. fondness for crunchy [227] and/or salty food), presentation (such as preference for drinking from sippy cups, colour of food) etc. contribute to aberrant food intake of ASD [228]. Because of the idiosyncratic and multi-dimensional nature of this behaviour, it is difficult to pinpoint the root causes of this dysregulation. (Figure 1-4)

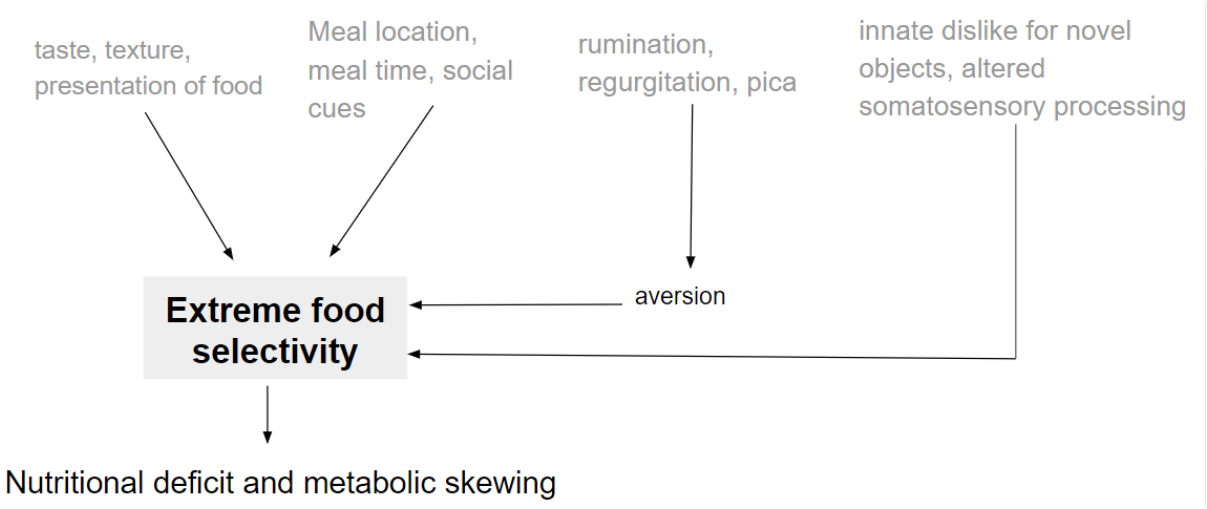


Figure 1-4: components of anomalous food intake in autistic individuals.

1.6.1. Aberrant food intake in ASD

Huxham et al [227] carried out a custom-compiled questionnaire study within a demographically specific region (England) to children with ASD and revealed that difficulty to feed autistic toddlers increases from smooth pureed food items to complex texture of food. Colour, packaging, presentation, smell, temperature, meal composition, sensory perceptions were attributed as contributing factors towards meal acceptance/aversion. Schreck [228] used questionnaires for caregivers of ASD individuals of 5-12 years old children and further corroborated that children with autism had significantly more feeding problems coupled with food-selectivity as compared to children without autism. They also reported the ASD children to prefer specific utensils.

Restrictive eating can be further augmented by certain other factors such as: some families might not want to prepare wide variety of food, parents or caregivers may avoid certain food items they do not like or are allergic to [229].

Food selection is multifaceted e.g. preference (selection of one item over others), liking [230], acceptance, consequences (involving expulsion of food contributing to aversion), and motivation [231], early nutritional exposure (reviewed in [232]) etc. Bennetto showed altered olfactory and gustatory processing in ASD subjects further

strengthening the aspect of atypical sensory processing [233] contributing to food selectivity.

In this context, Pica, the ingestion of non-food materials is important to note [234, 235]. It is hard to point out what exactly causes pica behaviour in autistic individuals - plausible causes may include: altered sensory processing, a proclivity for self-injurious behaviour (which again feeds into altered reward-related processing [236]), abnormal motor activity, altered higher order processing owing to atypical integrity and/or functionality of various brain regions [237].

Keenness for sameness can contribute to restrictive feeding in ASD. Neophobia couple with narrow food preference in autistic children, and at the same time the proclivity to like palatable diet increases the propensity of obesity. While studying food preference in children and adolescents in ASD, Mayes et al reported pasta, pancakes, waffles, peanut butter and jelly sandwiches, rice, bread, crackers, pizza, chicken nuggets, French fries, bananas and apples being preferred in autism [222]. Preference for rice, Ritz crackers and pretzels was also reported [238] . A case-study with a 9 year-old boy with ASD reports that his preference was so restricted that he only consumed corn-crisps, Rich Tea biscuits, French fries and Coca-Cola since age 2 years [239]. Liking for fruit [240] and starch [241] was documented in children with ASD as well. Childhood obesity and higher BMI have been documented in children with ASD [242, 243].

Behavioural studies and questionnaire methods, though elucidate valuable data, have some drawbacks: recruitment of certain parents who are overly concerned with the eating habit of their child, methodologies of assessment. Most of the studies are performed in demographic areas where high calorie food are easily available [222, 227, 228, 240] which also feeds into the fact of racial difference in body composition, degree of discipline for meal-time and expectation set up by the parents or caregivers etc. Behavioural tests unaccompanied by functional Magnetic Resonance Imaging (fMRI) and/or neuromolecular evaluations are insufficient to elaborate the concept of this dysfunctionality in autistic brains.

1.6.2. Functional MRI and anatomical considerations of brains of autistic individuals implicated in the context of food intake regulation

Feeding evolved to initiate in response to hunger (which corresponds to the conscious perception of an energy deficit) and to stop once satiated, even if food remains available thereby regulating the homeostasis. Paraventricular nucleus (PVN), Arcuate nucleus (ARC), lateral hypothalamus (LH), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), Nucleus tractus soletarius (NTS) , Area postrema (AP), Dosomedial nucleus of the Vagus (DMNV) mainly govern internal homeostasis. However, feeding behaviour is also controlled by brain circuits that control hedonic or “reward” – related feeding (consumption for palatability). Midbrain dopaminergic system, striatum, orbitofrontal cortex (OFC) and amygdala are key regulatory areas for reward processing [244, 245]. Endogenous opioid pathway of Ventral Tegmental Area (VTA) also govern the hedonic responses of pleasure [246]. It sends dopaminergic projections to NAcc which is well known for reward anticipation processing. VTA also projects to areas like amygdala, prefrontal cortex (PFC), and olfactory bulb. In addition, Smith described ventral pallidum of basal ganglia as hedonic administrators [247-249]. These two components of feeding interact with each other via different neuropeptides. For example VTA-Dopaminergic-neurons project to the NAcc. The LH receives GABAergic inputs from the NAcc along with melanocortinergic neurons from the ARC of the hypothalamus. Additionally, melanocortin receptors are found on neurons in the VTA and the NAcc. Higher cortical areas like ventromedial prefrontal cortex (vmPFC) and OFC integrate sensory processes with expectation of reward followed by outcome and govern the future anticipatory behaviour [250, 251] and in these higher-centers all the different components (homeostatic, hedonic, sensorimotor) are integrated. Since the neural basis of social-reward processing are not mutually exclusive to that of reward-driven intake, it is logical that the atypical reward-processing that is established in ASD would have an effect in food consumption, at least to some extent.

Kurth and colleagues performed MRI involving autistic children meeting DSM-IV criteria and showed that grey matter volume was significantly lower in supraoptic nucleus (SON) and PVN of the hypothalamus (areas for energy homeostasis) in the

ASD group as compared to age, sex and IQ matched controls [252]. Though this does not reveal anything about neuronal and glial packaging, size and functionality of the neurosecretory cells; yet, it may be a suggestive of possible dysfunctional neural network regulating energy homeostasis.

Altered reward processing is documented in autistic individuals. Cascio et al, delved the neural basis of primary food-reward processing in ASD using fMRI by subjecting mildly food-deprived participants to images of appetizing high calorie palatable food and showed that neural responses to these cues are mostly intact except for the fact that greater response was detected in anterior cingulate cortex (ACC) and insula in autistic individuals [253]. Keeping relevance with this finding, Schmitz and colleagues, using fMRI, demonstrated that monetary incentive upon correct responses to a given target experimental paradigm leads to increased activation of left anterior cingulate gyrus in autistic brains as compared to controls coupled with considerably reduced periventricular white matter density in the left frontal lobe [254]. Insula and ACC acts by integrating physiological homeostasis with emotional, multiomodal sensorimotor interoception and higher cognitive functions. ACC receives input from prefrontal and parietal brain areas via corpus callosum [255] and this anomalous activity of ACC can be empirically explained by greater effort to achieve a 'positive' outcome upon performing goal-oriented behaviour. Taken together, the structural and/or functional anomalies in brain areas relevant to food intake is observed in autism.

1.6.3. Long-term effect of anomalous food intake in ASD individuals

Selective eating affects overall nutrition. A case of scurvy due to vitamin C deficiency has been reported in a 10-year old boy with autism by Kinlin and colleagues [256]. Owing to extreme food-selectivity, medical intervention was required in a case-study [238]. Healy et al reported a 9 year-old boy with low caloric intake for 7 years suffering from malnutrition, mild anaemia, elevated urea and transaminase levels [239]. Furthermore, it is also suggested that patients with ASD are at higher risk of retinol and 25-hydroxycholecalciferol (vitamin D) deficiency [257] due to very limited dietary repertoire. Lower bone mineral density in peripubertal autistic boys was reported by Neumeyer [258]. Retinol (vitamin A) is important in the context of neuronal

development and a dietary deficiency of Vitamin A can lead to impairments in postnatal cognitive functionality [259] [260].

Beside extremely restrictive food intake, differential intestinal permeability leading to malabsorption [261, 262] is also accountable for undesirable effect on nutrition. D'Eufemia and colleagues used sugar intestinal permeability test (IPT) to assess small intestinal mucosal damage in ASD individuals. Urinary excretion of lactulose and mannitol were measured after oral administration as an indication of gut-mucosal permeability. Their data shows that the lactulose/Mannitol recovery ratio in control remained within the normal range. Among the 21 autistic participants, 9 were reported to have a very high lactulose/Mannitol recovery ratio owing to heightened lactulose recovery. This indicates altered intestinal permeability in some ASD individuals [261]. Goodwin and colleagues reported 7 out of 15 ASD-participants to have differential stool and bowel disorders [262]. Even though autism is not always accompanied by gastrointestinal symptoms (70% autistic individuals suffer from GI tract issues such as celiac disease, chronic diarrhoea, changes in bowel movements etc.), the dysregulation of absorption would contribute to the overall deficiency of certain nutrients in ASD.

1.6.4. OT in the context of atypical food intake in autism

(A) OT reduces energy-driven consumption:

Numerous studies and reviews are available confirming the role of OT in meal cessation and the regulation of energy balance ([263-266]). Neuroanatomical studies have shown that most OT neurons are localized in the hypothalamic PVN and SON, and OT-fibers from the PVN project to the brain stem [267-269]. The ablation of the hypothalamic PVN (key brain site for OT-production), as well as the severing of the neuronal connection between the brain stem and the PVN in results in overfeeding and obesity in laboratory animals [270-273]. Release of central OT coincides with feeding in laboratory animals that leads to stomach distension. Increase in plasma OT was observed throughout the course of a meal in rats [274]. Mice and rats allowed to eat until satiation display an elevated number of c-Fos-positive (an immediate early

gene, marker for neuronal activation) cells in the PVN and SON as compared to the time-point of the beginning of a meal. At meal-termination, the percentage of activated OT neurons was reported to remain significantly higher than that of the fasted condition [275, 276]. Apart from stomach distension, elevated plasma osmolality induces OT release in laboratory animals [277-279]. Macronutrient composition also affects the activation of central OT neurons. For example, Hume et al reported high-sugar but not high-fat diet leads to increased activity of SON OT-neurons while plasma osmolality remained unaltered [280].

OT promotes early termination of feeding; exogenous OT as an appetite suppressant. Arletti et al. demonstrated that intracerebroventricular injection of OT causes a significant reduction in deprivation-induced food intake in rats [281, 282]. Upon third ventricular administration of OT, it was showed to be effective in reducing chow intake in rats by increasing latency to begin a meal, decreasing meal duration [283]. Olsen et al showed that intracerebroventricular administration of OT to reduce time-spent in eating and amount of consumed food in rats [284]. Several studies corroborate that not only central, but also peripheral administration of OT causes early meal-cessation with reduced meal-size without affecting the feeling of hunger even after deprivation in laboratory animals [285, 286](reviewed in [287]). Optogenetic manipulation of glutamatergic neurons in the ARC expressing OT receptor was found to induce rapid satiety in mice [288].

A number of neuropeptides that regulate food intake (e.g. alpha melanocyte-stimulating hormone (alpha-MSH) and glucagon-like peptide-1 (GLP-1)) [289, 290], leptin [291, 292], CART [293]) act via OT pathways. On the other hand administration of orexigenic AgRP has been shown to be associated with a decrease in activation of OT neurons in laboratory animals [294].

Developmental abnormality in hypothalamic PVN-OT neurons lead to hyperphagia and obesity as observed in the SIM1-mutation mouse model, and these negative symptoms can be reversed by OT treatment [177]. OT-KO mice also show proclivity for sugar (see below).

I.n. OT decreases food intake and/or body weight in obese humans [295, 296] . OT causes early termination of feeding facilitating early satiation without affecting subjective appetite [300]. (Reviewed in [297]). A reduction in the number of OT neurons has been reported for Prader–Willi syndrome patients characterized by extreme overeating [298].

(B) OT reduces reward-driven consumption

Numerous studies and review articles delineate the role of OT in inducing satiation via curbing reward-related consumption. OT receptor is found to be distributed throughout reward areas NAcc and VTA and PVN OT neurons form synapses with mesolimbic neurons [299-301]. Plethora of evidences from laboratory rodent studies suggest that OT acts directly within the mesolimbic pathway to inhibit food intake [302, 303]. For example, Herrison et al showed that OT acts in the NAcc core to reduce intake of sucrose and saccharin solutions in non-deprived animals without promoting aversion [304]. Mullis and colleagues reported that OT infusion to the VTA decreases deprivation-induced chow intake as well as palatability-driven sucrose consumption in rats. These effects are abolished by OTR antagonist, L-368,899. With L-368,899 administration to the VTA sugar intake was elevated but not chow consumption [303]. Peripheral administration of BBB-penetrant L-368,899 lead to overconsumption of carbohydrate in laboratory animals but not intralipid [275, 305]. These evidences suggest OT receptor blockade to elevate sucrose consumption, indicating a functional relationship between central OT and appetite for carbohydrate. OTR is also present in taste buds [306] which can also contribute to this effect. OT impacts food-motivated behaviours. OT delivered to the VTA reduces motivation to work for palatable sucrose pellets in operant responding task [305]. Chronic and habitual sugar intake shift the endogenous threshold of OT. Mitra et al. showed that daily intake of high-sucrose diets in rats reduces c-Fos expression in OT neurons compared to rats receiving daily low-sugar food [307].

OTR is implicated in non-feeding reward as well. For example, Jarrett and colleagues found that cocaine treatment changes OT receptor binding density in the BNST in female rats [308]. Baracz et al. reported that direct intraparenchymal administration of OT in the NAcc core reduces methamphetamine-seeking behaviour in a dose-dependent manner [309]. Other neuromodulators (leptin, ghrelin, opioid receptor

ligands) contribute to hedonic eating via OT-signalling. In sum, OT acts through brain regions associated with reward and motivation to decrease food intake in rodents and intranasal OT modulates activity in analogous brain regions in response to food cues in humans.

Human research on the effects of OT on eating for reward is warranted. Over the course of the menstrual cycle, food intake (including sugar) is low during ovulation (high plasma OT) and it increases during the luteal phase (low plasma) [310]. Ott and colleagues outlined the effects of i.n. administered OT on feeding behaviour, with special emphasis on rewarding aspects of consumption. OT was found to markedly decrease snack consumption (chocolate cookies, rice waffles, and salt crackers were offered to the subjects) during the postprandial snack test administered shortly after a full buffet-style breakfast [311]. In overweight or obese human males, i.n. OT leads to reduction in Blood Oxygen Level Dependent (BOLD) responses to the reward-areas (VTA, OFC, and amygdala) and enhances activity in cognitive control [312, 313]. Burmester et al. further reported OT to reduce snack intake at in men along with a reduction in the intake of both sweet chocolate cookies and salty crackers without food with lower palatability (oatcake) suggesting that palatability may be the factor in OT's anorexigenic effects [314]. OT is shown to reduce food-cue-induced, CNS-mediated reward anticipation by attenuating functional connectivity between the VTA and brain regions associated with the cognitive, sensory and emotional processing of food images in overweight/obese men in an fMRI study conducted by Kerem et al [315]. (Reviewed in [297]).

Apart from energy homeostasis, OT regulates macronutrient-preferences (carbohydrate) and feeding reward. As stated earlier, OT acts via modulation for rewarding aspects of eating reducing the intake of palatable and or/sweet foods.

Additionally, OT-KO mice demonstrate enhanced intake of both nutritive and non-nutritive carbohydrate and sweet tasting solutions but not lipid [316, 317]. In OT-KO mice Amico and colleagues reported elevated intake of palatable sucrose solutions in the KO mice compared to the WT animals[318] in both dark and light phase [319]. OT-

KO mice show enhanced motivation for sucrose consumption in operant licking paradigm [317] suggesting that OT affects feeding reward.

The OT-KO model findings are largely in agreement with the results of experiments on laboratory animals without genetic modifications in the OT system. Gene expression analysis with real-time PCR showed upregulation of OT mRNA levels in the hypothalamus of rats with scheduled-feeding of high-sugar diet compared to standard food [305] corroborating the finding of Mitra et al [307]. Furthermore upregulated OT transcript was reported in mice given 48-h *ad libitum* access to a 10% sucrose solution compared to animals consuming isocaloric Intralipid during that time[320].

Taken together, evidences support the link between OT and meal cessation, energy balance and reward-driven intake, protection of internal milieu, and metabolic processes. Considering the implication of OT-dysregulation in ASD, it paves the question that OT may play a role in aberrant feeding observed in ASD. OT and OTR-KO animals show ASD-like phenotypes (reviewed in [321]). Pobbe et al reported OTR-KO mice to exhibit decreased reciprocal social interactions, reduced levels of communication, but no changes in repetitive behaviours [322]. Mantella's group reported elevated anxiety-related behaviours of OT-KO female mice [323, 324] and as stated earlier, these animals show overconsumption of sweet tastants. Numerous evidences are available showing exogenous OT to improve social deficits in different knockout-models of ASD. For example exogenous administration of OT to Cntnap2-KO mouse model showed improved social behaviours and OT-immunorectivity in the PVN [325]. Similar observation was documented from other strains of genetic models (reviewed in [321]). This poses the hypothesis that OT might be a pharmacological tool to alleviate aberrant feeding in ASD.

Regulation of food intake is multimodal comprising homeostatic and hedonic components as well as central-peripheral integration. Diffusion Tensor Imaging, MRI, and fMRI, though reveal structural and functional connectomes under a given paradigm, modulation of key regulatory factors like receptor dynamics, status of neuropeptidergic adaptations, synapse dynamics and plasticity, peripheral signals

(stomach distension, leptin, ghrelin, CCK signalling etc.) remain elusive in the context of anomalous food intake in autism. Other brain areas of brainstem (where integration of reward processing, motor coordination such as deglutition), emetic reflexes (which are most likely involved in rumination and/or regurgitation of autism), are unexplored in the context of differential consummatory behaviour in ASD. The role of altered neuronal progenitor cell proliferation, migration, neuronal differentiation, axonal guidance, dendritic arborisation (which can be because of or lead to a vicious cycle of malformed and/or malfunctioning synapses) in aberrant food intake in autism is unknown. Central neural processes are chief governing body of peripheral signalling and behavioural outcomes. At the same time, brain-functionality is a function of behavioural consequences. For example, central reward processing may increase the propensity of sugar intake, which in turn causes obesity, thereby a metabolic shift and changes in brain activity pattern. This, in turn, may impart long-lasting change at the subcellular and molecular level of neuronal functionality thereby, skewing of neural pathways affecting the metabolism and nutrition, triggering a vicious cycle.

Because of the complexity, the neuromolecular bases of atypical food intake in autism is difficult to study in humans. Rodents, with homologous brain areas like humans have relatively simpler neural circuit organization. Therefore, restrictive food intake behaviour as seen in autism was modelled in an established rodent (Sprague Dawley rat) model of autism generated by *in utero* VPA exposure which is an established animal model of ASD.

1.7 *In utero* VPA - induced rat model of autism

1.7.1. Neurodevelopmental basis of the model

At high dose, VPA influences the proliferation and differentiation of neuronal cells by affecting a plethora of signalling cascades involved in cytoskeletal remodelling, cell migration, fate-choice etc. *In vitro* experiments using transformed cell lines like 293T, neuro2A establish the HDAC inhibitory activity of VPA causing hyperacetylation of histones [119] leading to aberrant transcription. This exerts differential effect on the development of nervous system in a context dependent manner - several conflicting

hypotheses tried to explain how VPA might impair brain development by affecting neurogenesis. Laeng et al reported that VPA treatment increased proliferation of neural progenitor cells and neurite outgrowth prepared from embryonic rat cortical or striatal primordial stem cells. They also reported elevated neuronal differentiation in human foetal forebrain stem cell cultures upon VPA exposure [326]. VPA was found to induce neuronal differentiation of multipotent adult hippocampal neural progenitors and to inhibit glial (astrocyte and oligodendrocyte) differentiation [327]. Using primary hippocampal culture from E16.5 rat embryos Yu et al demonstrated that VPA decreases proliferation of embryonic hippocampal neural progenitor cells and induces differentiation. *In vivo* data corroborated VPA treatment to increase neuronal differentiation [328]. These findings are supported by several other studies that described VPA to induce neural differentiation [329-331].

On the contrary, using human cortical organoid culture Cui et al showed neurodevelopmental dysfunction characterized by increased neuron progenitors, inhibited neuronal differentiation and altered forebrain regionalization upon exposure to VPA [332].They also identified aberrant transcription of several genes - some of them are relevant to ASD.

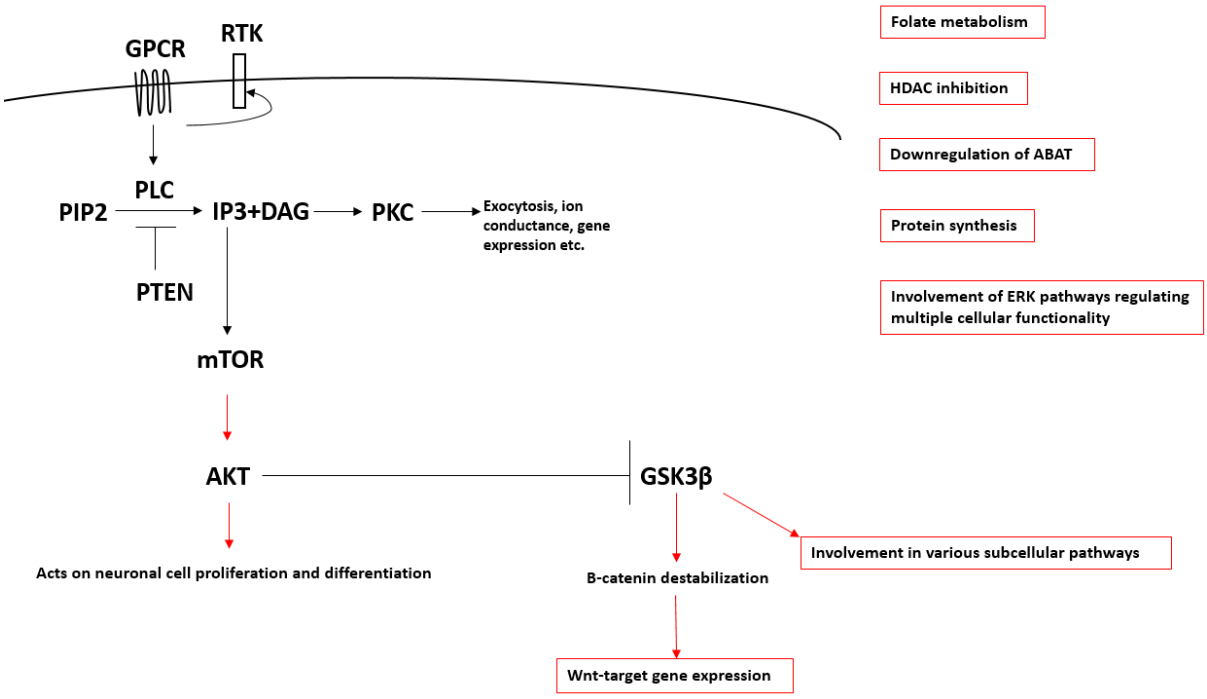


Figure 1-5: Schematic of potential mode of action of VPA in disrupting neuronal development.

Therapeutically VPA is neuroprotective but at a high dose during early gestation, it disrupts major subcellular pathways that govern crucial phases of neuronal development. GSK3β, a multifaceted mediator of many subcellular pathways directly interacts with wnt-βcatenin signalling [333]. VPA interacts with GSK3β and therefore may indirectly affect the wnt-target gene expression. Additionally VPA acts on wnt-target genes is via mTOR/Akt pathway as well [334]. VPA can also directly affect wnt-target gene expression [335]. Other than that, VPA may disrupt subcellular processes involved in neuronal development by affecting folate metabolism [336], protein synthesis [337] and interfering with ERK pathway [338]. Wnt signalling is contextually relevant in autism [339]. (Figure 1-5)

Elevated neuronal differentiation was found to be contributing to γ-amino butyric acid (GABA)+ cells at the expense of astrocytic differentiation [326]. *In utero* VPA exposure in mice was found to affect cortical thickness with no major effect on cytoarchitecture of the cortices. Increased number of excitatory projection neurons via the inhibition of precursor differentiation in embryos lead to elevated number of non-GABAergic projection neurons in the superficial layers of the P21 neocortices upon *in utero* exposure to VPA in mice [340]. This finding is further supported by Kumamaru et al - using cultured cortical neurons from P1 rats, they reported VPA exposure leads to reduced expression of the vesicular GABA transporter (VGAT) whereas other synaptic markers specific for excitatory synaptic transmission, including the vesicular glutamate transporters 1 and 2 (VGLUT1 and 2), remained unaffected [341].

Since disturbance of the excitatory and inhibitory (E–I) balance has been implicated in multiple psychiatric disorders, the disruption in early neuronal development affecting the E-I balance of the neural pathways caused by VPA may contribute to the pathogenesis of VPA-induced neurodevelopmental impairments. When it comes to studying developing brains, the different developmental timeline specific to species, developmental trajectories, molecular pathways specific for certain neuronal types and/or brain areas etc. contribute to the findings. Therefore, it is difficult to pinpoint exactly how and what effect VPA may have on the developing nervous system leading to ASD phenotypes.

VPA, the teratogen that affects neural tube closure, interferes with the migration of the neural crest cells critical for the closure of the neural tube and the neuropores. Using Neural crest cells (NCC) derived from chicken, Fuller et al demonstrated that VPA affects cell migration of the NCC by interfering with cytoskeletal arrangement leading to altered cell morphology while going through epithelial-to-mesenchymal transition (EMT) [342]. Inhibition of cell migration using hESC-derived NCC upon VPA exposure was documented as well [343, 344].

At the embryonic stage, during rapid development of the CNS in the 3-dimensional space, minor differences in timing of VPA exposure can cause major differences at subcellular level. Progenitors tend to have a certain degree of plasticity at the proliferating stage; at later times, when they are committed to attain differentiation, they tend to attain more stable subcellular mechanisms and so are less responsive to perturbation to molecular pathways [345]. Also, the earlier the exposure is, more variables (like gene expression and chromatin remodelling of genes critical for early development) will be affected leading to more widespread and multimodal effect. Other important factors contribute to the differences in findings of the effect of VPA in the developing nervous system are: the model used to study - *in vitro* data although provides important insight to molecular pathways are often hard to replicate owing to culture conditions, variability of the biomolecular detection methods etc. When it comes to animal studies, the type of the animal model used and the route of administration of VPA (which directly affects the bioavailability), timing of administration, dosage are important factors that contribute to the differences in findings while investigating the effect of VPA in the developing nervous system.

1.7.2. Validity of the model

Clinical studies have demonstrated that VPA consumption during early pregnancy is associated with offspring having higher probability of neural tube defects such as spina bifida aperta [346] and autism [347-350] - sometimes referred as foetal valproate syndrome.

Rodents prenatally exposed to VPA show behavioural deficits similar to autism. Rodier et al administered 350 mg/kg of VPA to rat dams on day 11.5, day 12, and day 12.5 of gestation and showed each exposure to reduce the number of motor neurons as compared to their matched controls. They noticed no external malformations [351].

Owing to neuroanatomical, neurophysiological and behavioural anomalies upon *in utero* exposure of VPA in rodents, it is used extensively as a model of human autism for understanding the neurobiological bases underlying phenotypes. Depending on the exposure time point, dosage and rodent species (rat and/or mice), a plethora of behavioural characteristics can be generated in rodents that are a projection of human autism. Prenatal exposure to VPA around E11 to E13 in mice have been shown to generate autistic phenotypes [352-356].

The phenotypic outcome varies with the dosage of VPA, window of exposure, genetic makeup of the rodent, maternal stress, route of administration of VPA etc. Slight seasonal variation of gestational period of laboratory rodents may contribute to the phenotypic outcome as well [357]. Just like the phenotypic characteristics of autism changes with age in humans, so does for the animals' [358]. Thus, it is important to note that the temporal window of behavioural assessment contributes to the findings. The established rat-model of *in utero* VPA-induced autism and their phenotypic characteristics are as follows:

Table 2: Established in utero VPA -exposed rat models of autism

Rat strain	**Dosage and route**	**Temporal window of administration**	**Phenotypes**	**reference**
Wistar rats	600 mg/kg; Single i.p. injection	E12.5	↓ number of social explorations ↓ exploratory activity ↑ latency to social behaviour ↑ repetitive/stereotyped activity ↑ anxiety delayed maturation and motor development	[359]
Wistar rats	500 mg/kg; Single i.p. injection	E12.5	↑ repetitive behaviour ↓ sensitivity to pain ↓prepulse inhibition ↑ anxiety ↑ amygdala-associated fear generalization and fear memory - hyperreactivity and hyperplasticity in the LA due to impairments in the inhibitory system of the amygdala.	[360]
Sprague–Dawley rats	500 mg/kg single i.p. injection	E12.5	Change in locomotor activity over a temporal scale of assessment - PND21 to PND 70	[358]
Sprague–Dawley rats	400 or 500 mg/kg Single i.p.	E12.5	↑ latency to thermal nocipception ↑ exploratory distance ↓ sociability Phenotypes observed in juvenile animals but not in adults	[361]

	injection			
Wistar rats	600 mg/kg i.p. injection	E12.5	VPA rats explored the stranger rat less - indicating lower sociability	[362]
Wistar Han rats	500 mg/kg Single i.p. injection	E12.5	Slightly lower body mass Higher frequency of tail kinks (9%) Presence of Chromodacryorrhea	[363]
Wistar rats	600 mg/kg i.p. injection	E12.5	Impaired learning and memory	[364]
Wistar rats	500 mg/kg, i.p. injection	E12.5	↓ time spent in the open arms of elevated plus maze ↓ number of entries to the open arms	[365]
Sprague–Dawley rats	600 mg/Kg i.p. injection	E12.5	VPA pups showed delayed growth development and social interaction deficits	[366]
Sprague–Dawley rats	400 mg/kg single subcutaneous injection	E12	↓sociability, social preference ↓ electroshock seizure threshold	[367]
Long-	600	E12	faster eyeblink conditioning	[368]

Evans rats	mg/kg single i.p. injection			
Wistar rats	600 mg/kg i.p. injection	E9	↑time in the empty compartment of 3-chamber test ↓ social approaches than controls	[369]
Wistar rats	800 mg/kg oral	E9	Hypermovement in open field test in juvenile male rats	[370]
Wistar rats	800 mg/kg oral	E9	↑ line crossings in the open field ↓ratio of the mean count of locomotor activity during the dark phase to that during the light phase	[371]

(A) Relevance of choosing the time point of E 12.5 for SD rats

Neural tube formation occurs approximately at E10.5–11.5 in rats [372] [373]. Administration of VPA at E12.5 interferes with late-phase of secondary neurulation. (Figure 1-6)

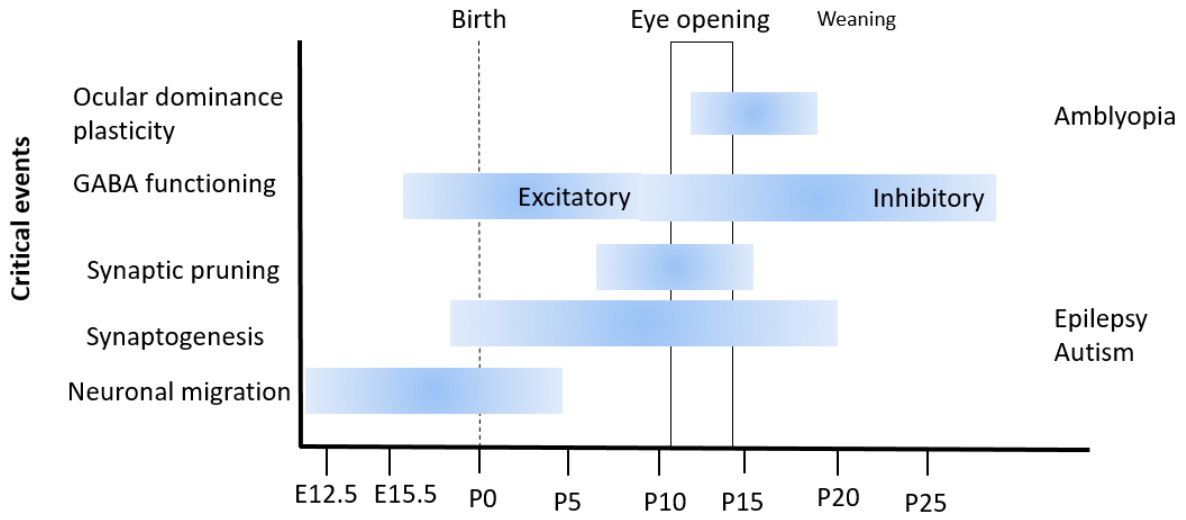


Figure 1-6: Critical events of prenatal and postnatal periods of rat brain development - disruption of which cause an impact on adult brain functionality

Susceptibility to developmental disruption is not restricted to the preclosure period of neural tube only [374]. Induction of neurodevelopmental anomaly can be a result of maternal administration of a chemical insult (in this case, VPA) during early development while rapid growth, division and differentiation of the neuronal precursor cells take place. The temporal window of E9 to E13 in rats is extremely crucial for neuronal development.

Taking the seasonal variation into consideration, the gestational period of Sprague Dawley rats is 21-24 days. This implies a slight difference in neurodevelopmental trajectory. Cell lineage analysis using a detectable marker (usually thymidine analog BrdU or tritiated thymidine) that becomes incorporated into the DNA during S-phase has revealed some interesting findings regarding the exact temporal window of neurodevelopment. These markers are administered intraperitoneally to the pregnant

dams, and they are absorbed into the bloodstream and become available to the embryos. Cells that take up a marker will either go through subsequent S-phases if they continue division, diluting the amount of marker in half with each division. In case they exit the cell cycle (neurons), the level of the marker will attain stability.

By E11, the hypothalamic region (diencephalon) expands ventrally to form the base of the diencephalon and a ventral diencephalic sulcus merges into the optic recess anteriorly. Unlike the cortices, hypothalamus develops as outside-in pattern. By E12–E13 the middle and posterior diencephalic folds develop, the wall of the diencephalon thickens (Figure 1-7). By E14 a mass-like structure appears at the hypothalamic region - the anterior of which develops into the ventromedial region, and the posterior develops into the mammillary region. By E15, the first primitive hypothalamic nuclei becomes identifiable. By E16, stria terminalis and anterior commissure can be identified. By E17, the PVN is defined. By E18 the VMH, ARC and SCN are recognizable [375]. The time points of development of the rat-hypothalamic nuclei are tabulated in Table 3 [373, 376-378].

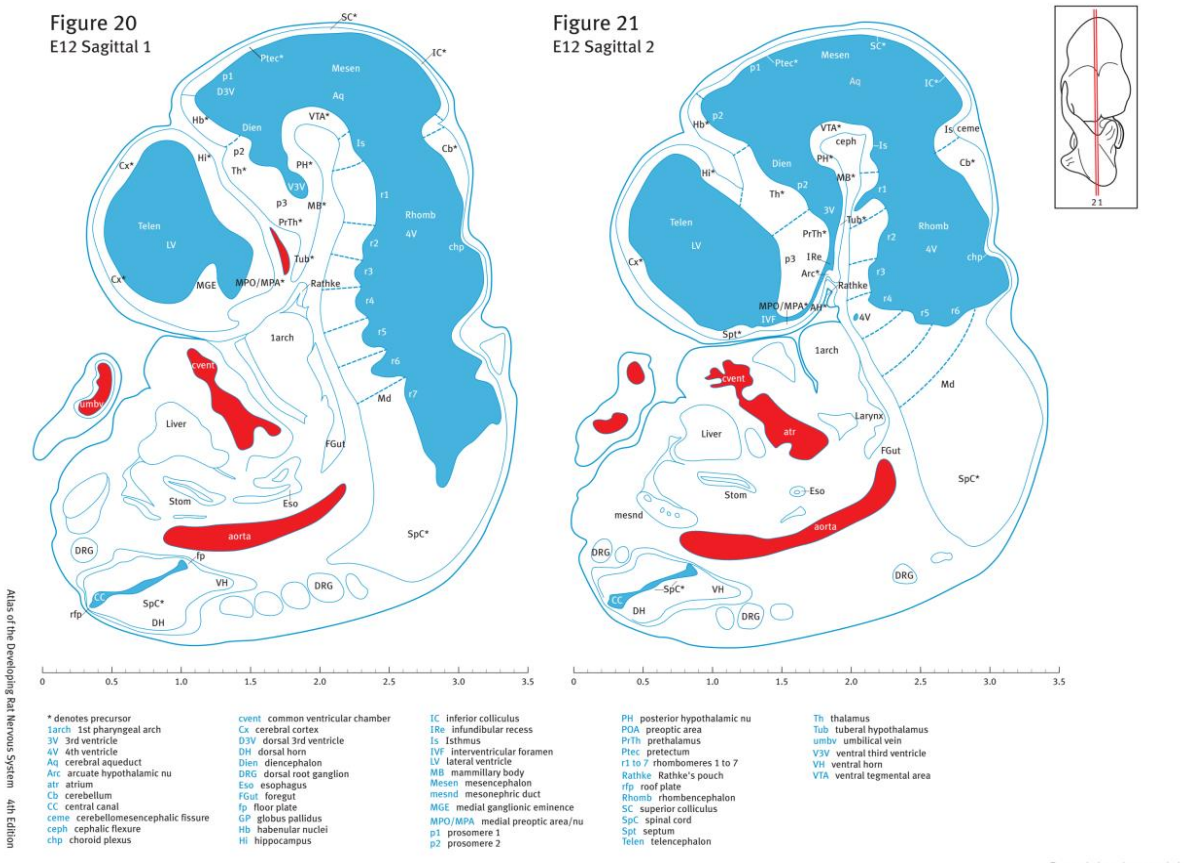
Table 3: time points of development of the rat-hypothalamic nuclei

LPA – Lateral Preoptic Area; MPN - Medial Preoptic Nucleus; LH – Lateral Hypothalamic Area; SON – Supraoptic Nucleus; AHN - Anterior Hypothalamic Nucleus; PVN – Paraventricular Nucleus; SCN - Superchiasmatic Nucleus; VMN – Ventromedial Nucleus; ARC – Arcuate Nucleus; MM – Medial Mammilary Nucleus.

Hypothalamic nuclei	Embryonic day
LPA	E11-E14
MPN (central)	E14-E18
MPN (the area surrounding the center)	E13-E16.
LH	E12-E14
SON	E13-E14
AHN	E12-E15

PVN	E13-E15
SCN	E11-E17
VMN	E13-E16
ARC	E12-E17
posterior nucleus of the hypothalamus	E12-E16
MM	E15-E16
Tuberomammillary	E13-E16

Illustration of spatiotemporal coordinates of the rat embryonic hypothalamus at E12-E13 is as follows [379]



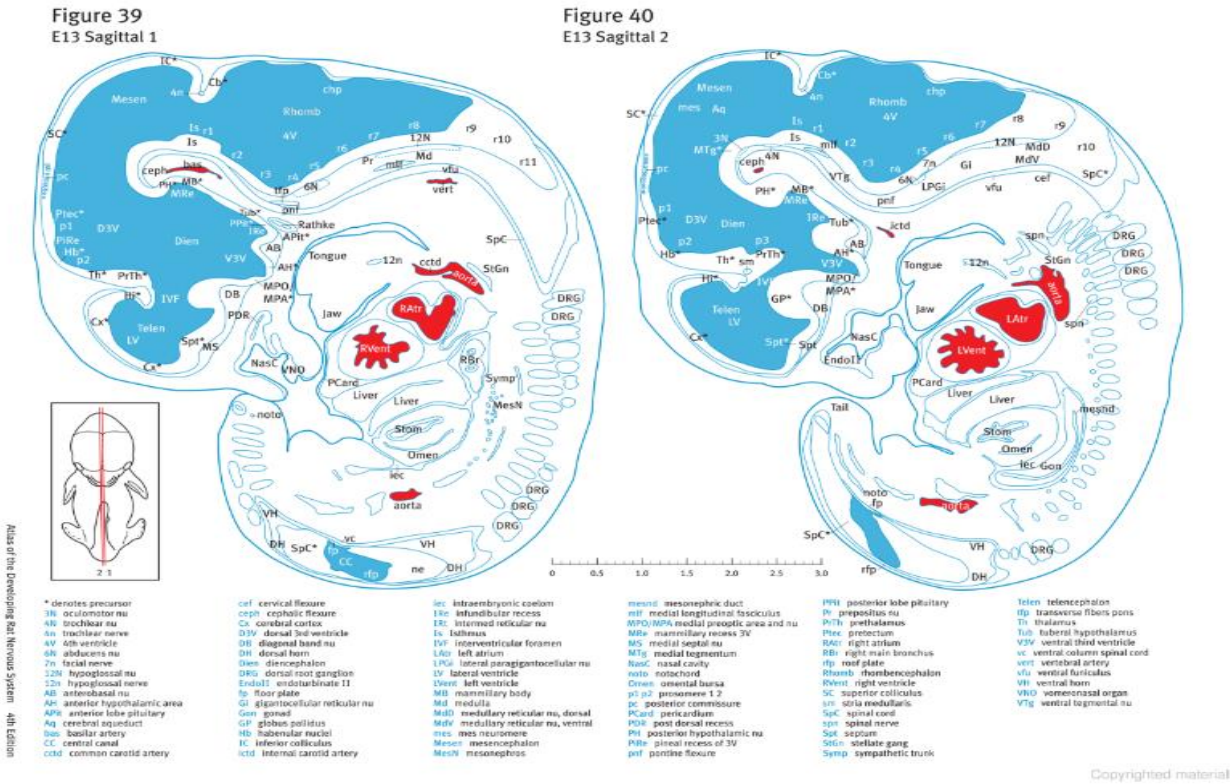


Figure 1-7: sagittal section of E12 and E13 rat embryo showing hypothalamic development (adapted with permission form [379])

The thalamus (integrative area for most motor functionality) develops after the peak proliferative events of the hypothalamus. This is quite interesting in the context of neural tube development - the ventral structures precede the dorsal structures in most cases. By E20-E21 thalamus develops, the mammillothalamic tract extends into the thalamus and the mammillary nucleus becomes distinguishable.

The effect of VPA-induced disruption at E10-E11 would be more global, less specific and more widespread. E12-E13 is the peak of hypothalamus development - the governing-body of energy homeostasis. Administering VPA at E12.5 generates certain phenotypes in rats that can be considered as a projection of human autism. This time point is early enough to cause neurodevelopmental disruption that gives rise to some phenotypic characteristics which are by no means drastic. In later stages, the developing brain regions become less responsive towards chemical insult as the cell lineages commit towards destined fate.

Rat were chosen over mice because they have bigger litter size than mice -
A) VPA causes intrauterine absorption of embryos owing to reproductive toxicity.
B) The ultrasonic vocalisation of *in utero* VPA exposed pups are different than the unexposed counterparts and it is an evolutionary programming of the dams to identify the pups inferior to survival - they undergo cannibalism.
In order to attain an effective sample size without having to use too many animals, rats were chosen, as their litter size is bigger than mice.

(B) Validity of the *in utero* VPA exposure at E12.5 in generating phenotypes that can be projected to human ASD

The speech and language aspect cannot be projected in animal models. However the other autistic traits reported in humans such as atypical nociception [380-382], attention deficit [383, 384] and repetitive behaviour [385] can be modelled in rats (reviewed [386]).

Schneider & Przewłocki reported delay in eye opening (a projection of delay in developmental milestone), increased latency of paw withdrawal upon thermal nociception, lowered tactile threshold, decreased prepulse inhibition, lower exploratory activity, longer latency to social behaviour and decreased the number of social behaviours in prenatal VPA-exposed rats at E12.5 as compared to their matched controls. They also reported a change in these behaviours with age (adolescence and adulthood) [359]. Age related change in exploratory behaviour is reported by Bringas et al as well [358]. Impaired social interactions, repetitive behaviour on a Y-maze, increased latency to thermal nociception, increased anxiety like behaviour in elevated plus maze by reduced amount of time spent in the open arm and abnormal fear conditioning owing to aberrant amygdala activity was reported by Markram et al [360]. Impaired social interaction, elevated self-grooming as a function of anxiety-like behaviour, altered exploratory behaviour upon *in utero* VPA exposure was reported by many other groups as well [366, 387-390].

The general male-bias of ASD is also observed in the VPA-induced animal model. Kim et al used three-chambered sociability test to report a lack of sociability in male rats while that for females remained unaltered. Protein important for synaptic maturation -

GAD 67 expression was found to be decreased in the VPA-exposed male and female offspring and this effect was more pronounced in males [391]. Three-chamber test conducted by Scheggi and colleagues show that male VPA rats showed a reduced sociability index compared to control rats, they had longer latencies initiating social interactions and lower numbers of social interactions. Female VPA rats exhibited milder social deficits in the three-chamber test as compared to the matched controls - there was no significant difference in sociability index and latency to initiate social interaction although they showed lower numbers of social interactions. VPA males but not females showed elevated anxiety like behaviour on Elevated Plus Maze by spending less time in the open arms [389]. Reduced locomotor activity was reported in prenatally VPA exposed male mice while no difference was observed in females. Also, the VPA-males but not VPA-females showed reduced sociability index in three chambered sociability test [392].

(C) OT dysregulation in VPA animals

Epigenomes are sensitive to a variety of external factors, particularly during early development hence are perceived as a critical mechanism underlying changes in behavioural phenotypes owing to environmental disruptions. Epigenetic mechanisms like DNA methylation and histone acetylation influence gene expression levels. There is evidence that DNA methylation and acetylation are linked. DNA methyltransferases of mammals appear to interact with HDACs [393]. Methylation of the promoter region by DNA-methyltransferases (DNMTs) leads to suppression of tissue-specific OT expression [394] while hypermethylation is correlated to ASD (reviewed in section 1.5.).

In vitro data from transformed liver cell line shows, that methylation of the CpG island in the human OTR gene suppresses the transcription [394]. Histone acetylation promotes OTR expression in the mesolimbic reward pathway [395]. Given the fact that VPA mainly inhibits HDAC 1 and HDAC 2 -the precise role of inhibition of HDAC subtypes that regulate OT and OTR expression in the CNS are not yet well-characterized.

OT dysregulation in prenatally VPA-exposed rats have been reported. Administering 600 mg/kg body weight VPA on E12.5 to timed pregnant Wistar rat dams, Dai et al demonstrated a reduced number of OT-IR cells in the SON of the VPA pups than in control pups. They also reported diminished expression of OT mRNA in SON and CSF in VPA-exposed animals as compared to their matched controls [387]. Subchronic exposure to VPA during gestation in rats causes a reduction in OTR density in BNST and CEA depending on the dosage of the subchronic exposure [390]. Zhang et al identified 637 alternatively spliced genes in prenatally VPA-exposed Sprague Dawley rats - 14 of which being implied in OT signalling pathway [366]. On the contrary, Štefánik and colleagues reported an elevated OT mRNA in the SON and PVN nuclei of hypothalamus and elevated OTR expression in piriform cortex, prefrontal cortex and amygdala in *in utero* VPA exposed rat [396]. These contradictory results are likely due to difference in detection methodology, primer sequence of the target transcript, difference in rat strain used, minor variation in temporal window of VPA administration to the rodents, including females in the studies etc. however, an atypical central OT regulation is implicated in the VPA rat model of ASD.

(D) Exogenous OT administration in the context of improving ASD-like phenotypes in *in utero* VPA-exposed rat model of ASD

Interestingly, similar to humans, administration of OT has been reported to ameliorate some of the deficits in VPA rats as well.

Early postnatal administration (from P0 to P7) of i.p. OT showed to have long-term effect on improving ultrasonic vocalisation at early stage and sociability at PND 35-40. Early postnatal intervention of OT has been shown to restore the number of OT-IR cells in the hypothalamus [387]. I.p. and i.n. administration of OT in adulthood (PND 90-97) improves exploratory behaviour and anxiety like behaviour [365]. Administration of intracerebral OT to bilateral CEA was reported to rescue anxiety-like behaviour assessed on elevated plus maze [397]. OT ameliorates reduced exploratory behaviours in the novel-object-recognition task in VPA rats. It also improved oxidative stress parameters [398]. I.n. OT alleviated social interaction deficits in mice prenatally exposed to VPA [399].

In sum, it is hypothesized that OT dysregulation underpins the anomalous social behaviour and atypical consummatory eating behaviour in the *in utero* VPA-induced rodent model of ASD.

1.7.3. Atypical consummatory behaviour in the VPA rat model of ASD

(A) Atypical body weight regulation in VPA-induced rat model of ASD

Body weight and body composition are functions of metabolism and food intake.
Ruhela and colleagues reported body weight of the VPA-exposed group of rats on PND8, 10 and 12 was significantly lower as compared to their matched controls. Milk band observation in early PNDs supported the less food intake in VPA- pups [400]. VPA pups of Sprague Dawley rats show lower body weight at PND 7 and 14 and the trend of lower body weight persisted up to PND 70 [366]. VPA-rats also were reported to have lower body weight by Schneider & Przewłock from PND 23 to PND 180 [359]. This finding is also supported by the results of Tsujino et al where they reported lower body weight of VPA-rats from 4w to 7w of age and then the difference in body weight was not observed at 8w [371]. Ingram et al further corroborated that *in utero* valproate-treated animals were healthy but exhibited a 14.4% reduction in body weight [401]. Kong et al reported a slower increase of body weight in VPA rats [402]. Consistently, lower body weight of VPA rats was reported by Elesawy and colleagues [403] and Di et al [404]. Similar observation of slower growth trajectory was reported in VPA mice as well [405, 406].

The slower growth trajectory in prenatal VPA exposed rat pups poses the question of atypical food intake and the neuromolecular bases of that. The consummatory behaviour of the VPA-model of ASD is not explored.

(B) Atypical consummatory behaviour in VPA rat model of ASD

Consummatory behaviour is not well studied in the animal models of ASD.
Prenatal VPA exposed rats show a tendency of elevated sucrose preference [407-409]. Motivational drive for sugar intake is also elevated in these animals. Chomiak

et a reported that VPA animals tend to over-select for sensory cues associated with reward expectancy - the reward being 30% sucrose [410].

Similarly elevated sucrose preference was seen in prenatal VPA exposed mice [411]. In addition, in transgenic models of ASD, intake for palatability is indicated. Cacna1c heterozygous mice displayed elevated sucrose preference [412]. Tag1 −/− mice demonstrate reduced food intake [413] and this gene is implicated in ASD [414]. OT gene knockout mice tend to have higher propensity of liking sugar [415]. Neurobeachin (NBEA), a gene implicated in ASD, knockdown in mice lead to an elevated palatability driven intake as compared to wild type (WT) mice [416]. Cumulatively, these evidences point towards elevated intake-for-palatability in animal models of ASD.

Aims

Feeding behaviour governed by a variety of intrinsic and extrinsic factors and central-peripheral integration of a wide range of bioactive substances. The key factors shaping consumption involve hunger (determines search for calories), satiation (underpins termination of feeding) and reward (consumption for the 'pleasantness' of food, regardless of the energy requirements). The anomalous brain connectivity feeds into the atypical feeding behaviour in autism, which, as a vicious cycle, skews the brain functionality. For example, not including tryptophan in the diet leads to low production of 5-hydroxytryptamine (serotonin) - leading to depression. Excessive ingestion of sugar topples over the energy-homeostatic machinery and initiates feeding in the absence of energy needs [417]. Dependence on a certain type of diet, especially sugar, skews the metabolism (e.g. obesity) and causes changes in brain functionality.

The neurohistological and neuroendocrine bases of aberrant food intake in autism remain unexplored. Prenatal VPA exposure at gestational day 12.5 in Sprague Dawley rats leads to a projection of autism-like phenotypes such as elevated anxiety and decreased social interaction – provides us with a good model system to study anomalous food intake similar to what is observed in humans. These animals show ASD-like symptomology of, among others, elevated anxiety and decreased social interaction.

The **overarching goal** of this thesis was to investigate whether the ASD VPA rats display abnormal feeding behaviour and to identify maladaptive neural processes that contribute to this phenomenon. The following specific aims were formulated to accomplish this goal.

In the **first Specific Aim** of this thesis, the hunger processing in the VPA animals was evaluated. The *ad libitum* intake of ‘bland’ standard laboratory chow was assessed in VPA versus non-VPA controls maintained continuously on this diet. The adult VPA rats consumed less ‘bland’ chow than healthy controls did even with acute deprivation, and it was coupled with a lower body weight than that of the controls. The VPA animals exhibited a lack of change in c-Fos immunoreactivity in crucial brain regions involved in regulating food intake, such as the hypothalamus and brain stem, when compared

to the healthy controls during acute food deprivation. This indicates differences in how hunger is processed in individuals with autism. Furthermore, the lack of changes in the expression patterns of feeding-related genes (including OT, Agouti-related protein (AgRP), and mu opioid receptor (MOR)) in the hypothalamus of the VPA animals, unlike the controls, further supports this finding. Taken together, these results suggest that the hunger processing is altered in VPA rats.

In the **second Specific Aim** of this project, intake of rewarding diets in the VPA rats was examined to address whether ASD affects pleasure-driven consumption. In episodic meal consumption paradigms, i.e., in scenarios in which energy non-deprived animals were given a brief and non-habitual access to diets that differ in palatability, the VPA rats were found to consume elevated amounts of palatable diets. These data suggest that VPA rats display not only altered energy homeostasis processing (as shown in Specific Aim 1), but also show changes in the regulation of eating for palatability.

Considering the abnormal OT signalling in autism and the fact that OT decreases feeding for energy and feeding for palatability, the **third Specific Aim** addressed whether VPA rats exhibit heightened sensitivity to exogenously administered OT, a peptide known for its anorexigenic properties. Intraperitoneal OT treatment was found to reduce episodic intake of palatable diets as well as post-energy deprivation intake of 'bland' chow; however, the dose needed to generate hypophagia was lower in VPAs than in controls. Analysis of c-Fos immunohistochemistry demonstrated that the lower OT dosage, which resulted in anorexigenic effects in VPA rats, was able to affect brain activation patterns similar to hypophagia. Conversely, in healthy control rats, this dosage did not lead to statistically significant changes in c-Fos expression.

Importantly, OT is involved also in the process of avoidance of toxic foods, i.e., it facilitates the development of a conditioned taste aversion (CTA). Therefore, the goal of the **Specific Aim 4**, was to assess whether VPA animals display atypical taste aversion acquisition. It was found that VPAs failed to develop aversion to a standard dose of LiCl that induces a CTA in healthy non-ASD controls. Consistent with these behavioral findings, the immunohistochemical analysis revealed reduced

colocalization of Fos and OT in the paraventricular nucleus (PVN) in VPAs, as well as a less prominent c-Fos response in the CTA circuit in ASD individuals compared to controls following LiCl treatment.

Taken together, evidence presented in this thesis shows that ASD is associated with abnormal appetite in VPA rats and it offers an insight into the neural basis of this phenomenon. The VPA animals show a slight decrease in intake of standard ‘bland’ chow, an elevated drive to consume palatable tastants, and resistance to taste aversion. These anomalies in feeding behaviours are underpinned by distinct changes in brain activation and gene expression patterns, as well as altered sensitivity to anorexigenic properties of OT, a neuropeptide known to affect other symptoms of ASD, from social deficits to anxiety.

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Chapter 2

Mild Hypophagia and Associated Changes in Feeding-Related Gene Expression and c-Fos Immunoreactivity in Adult Male Rats with Sodium Valproate-Induced Autism

2.1. Abstract

A core yet understudied symptom of autism is aberrant eating behaviour, including extremely narrow food preferences. Autistic individuals often refuse to eat despite hunger unless preferred food is given. We hypothesized that, apart from aberrant preference, underfeeding stems from abnormal hunger processing. Utilising an adult male VPA rat, a model of autism, we examined intake of 'bland' chow in animals maintained on this diet continuously, eating this food after fasting and after both food and water deprivation. We assessed body weight in adulthood to determine whether lower feeding led to slower growth. Since food intake is highly regulated by brain processes, we looked into the activation (c-Fos immunoreactivity) of central sites controlling appetite in animals subjected to food deprivation vs. fed *ad libitum*. Expression of genes involved in food intake in the hypothalamus and brain stem, regions responsible for energy balance, was measured in deprived vs. sated animals. We performed our analyses on VPAs and age-matched healthy controls. We found that VPAs ate less of the 'bland' chow when fed *ad libitum* and after deprivation than controls did. Their body weight increased more slowly than that of controls when maintained on the 'bland' food. While hungry controls had lower c-Fos-IR in key feeding-related areas than their *ad libitum*-fed counterparts, in hungry VPAs c-Fos was unchanged or elevated compared to the fed ones. The lack of changes in expression of feeding-related genes upon deprivation in VPAs was in contrast to several transcripts affected by fasting in healthy controls. We conclude that hunger processing is dysregulated in the VPA rat.

2.2. Introduction

Autism spectrum disorder (ASD) incorporates a cluster of symptoms, including impaired social interactions, communication difficulties, repetitive behaviours and intellectual disability. The umbrella term ‘spectrum’ includes a range of psychiatric and neurological (structural and functional) anomalies with varying severity. The underlying causes are diverse and encompassing genetic polymorphisms, immunological disorders and metabolic abnormalities, environmental factors and fetal chemical insults, all resulting in neurodevelopmental abnormalities with strikingly similar phenotypic symptomology.

Importantly, an increasing body of evidence suggests that dysregulation of neurotransmitters and neuropeptides plays an important role in neurodevelopmental abnormalities in ASD and contributes to behavioural impairments. Previous studies by Zieminska and others examined neurotransmitter changes in a rat model of valproic acid (VPA)-induced autism and found that fetal VPA insult extensively disrupts early brain development and neurotransmitter balance [1-4].

One of the core and yet understudied symptoms of ASD is aberrant eating behaviour. This trait is so common that Leo Kanner, who coined the term ‘autism’, defined it as one of the characteristic ASD anomalies in the first paper describing the disorder [5]. Children with ASD have a five-fold greater risk of developing eating behavioural issues, with picky eating being most common [6]. The peculiar eating practices include dietary selectivity [7-9], rigidity, preference for specific foods and the manner in which they are served [7, 10-12]. Altered sensory processing, social stimuli, food texture and composition, and presentation all affect the willingness of individuals with ASD to eat [10, 12]. To add to the complexity of the problem, reward processing is altered in ASD. Lower density of the mesolimbic pathways has been described and—thus far—functionally linked with maladaptive responsiveness of the reward system to pleasant social cues in ASD [13]. In their fMRI study examining the neural basis of primary food-reward processing in ASD, Cascio and colleagues presented mildly hungry subjects with imagery of palatable food and found the anterior cingulate cortical (ACC) and insular activation to be higher in response to these cues [14]. ASD individuals receiving a monetary incentive upon correct responses to a given target paradigm display elevated activity in the left anterior cingulate gyrus [15].

Altered inflammatory responses have been observed in individuals with ASD [16, 17] and this observation has been further corroborated in animal models of ASD [18-21], along with elevated oxidative stress responses.

Valproic acid (VPA) at a therapeutic level has neuroprotective properties, as demonstrated by Chuang and colleagues in a series of studies *in vitro* [22] and in animal models [23-25]. However, prenatal exposure to VPA at a high dose can lead to an imbalance in redox status and elevated pro-inflammatory cytokines [26].
Under pro-inflammatory conditions and/or under oxidative stress, the regulation of neurotransmitter and neuropeptide release may become altered and, in turn, may lead to aberrant changes in their modulation in response to environmental variation (in this case, food deprivation). One significant player in the regulation of inflammatory responses in the brain is IL6, which is reported to be elevated in the VPA animal model of ASD [27]. Interestingly, IL6 in the hypothalamus also regulates energy homeostasis and protects against obesity [28, 29].

Feeding abnormalities have also been reported in the very scarce feeding-related studies utilising animal models of ASD. For example, Buchner et al. [30] found that the body weight consequences of a mutation of the axonal differentiation and guidance protein, Cntnap2, in mouse were greatly modified by the availability of a palatable diet. Fukuhara et al. reported that mice with a Mecp2-null mutation, a protein essential for normal neuronal functioning, exposed to a tasty diet exhibited extreme hyperphagia, associated with changes in dopamine system gene expression in the reward circuit [31]. Mice heterozygous for the postsynaptic neurotransmitter receptor targeting protein, NBEA, gene overconsumed sucrose and fat and were hyper-sensitive to a hypophagic action of naltrexone [32]. Finally, overconsumption of palatable sugar, saccharin and milk solutions occurred in rats whose autism was induced by *in utero* exposure to valproic acid (VPA) [33].

One of the most puzzling aspects of the extreme food selectivity in ASD is that it occurs despite leading to immediate and long-term problems, from gastrointestinal symptoms (including constipation, diarrhoea and abdominal discomfort) to severe micronutrient deficiencies and malnutrition. Importantly, this extreme selectivity—as reported by parents of children with the syndrome—involves refusal of acceptance of any other

but the preferred food even after a period of energy deprivation, effectively becoming self-imposed food restriction. In fact, initial observation suggested that VPA ASD rats maintained on 'bland' chow eat amounts of this chow below what their healthy counterparts ingest [33].

This striking ability of ASD individuals to not eat despite fasting (unless preferred food is given) led us to hypothesize that, apart from aberrant reward mechanisms, this maladaptive behaviour is underpinned by abnormal hunger processing. Therefore, in the current set of studies utilising a common model of ASD, i.e., an adult male VPA rat, we investigated in detail the intake of 'bland' standard laboratory chow diet in animals maintained on this diet continuously, eating this food after a period of energy deprivation as well as after energy and water deprivation. We also assessed water intake, and we studied changes in body weight increases in adulthood to examine whether this lower food intake came with a cost of a slower growth trajectory. Since food intake is highly regulated by brain processes, we looked into activation (defined through c-Fos immunoreactivity) of key central sites responsible for feeding control in animals subjected to food deprivation vs. *ad libitum*-fed rats. Finally, we measured expression of select genes involved in food intake in the hypothalamus and brain stem (thus, two main regions responsible for energy intake) in deprived vs. sated animals. We performed our analyses in VPA animals as well as their age-matched healthy controls.

2.3. Materials and Methods

2.3.1. Animals

The experiments were conducted on Sprague-Dawley rats. The animals were housed in Plexiglas cages with wire tops in a temperature-controlled (22 °C) animal room with a 12:12 light:dark cycle (lights on at 07:00). Standard laboratory chow pellets (Sharpes, New Zealand; energy density: 3.6 kcal/g) and tap water were available *ad libitum* unless stated otherwise. Animals were treated in accordance with 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# 1088).

2.3.2. Sodium Valproate Exposure

A well-established procedure (including the choice of VPA dose) was followed to generate VPA offspring [34-37]. Adult female Sprague-Dawley rats (16 week old) were mated overnight with age-matched Sprague-Dawley males (approximately 5–6 months old males). Vaginal smears were stained with 1% crystal violet to detect spermatozoa and upon identification of spermatozoa, the date was designated as E0.5. Females received a single intraperitoneal (i.p.) injection of either 500 mg/kg sodium valproate (Sigma) or isovolumetric physiological saline (0.9% NaCl) i.p. on E12.5. Female rats treated with sodium valproate were healthy—similar to previous reports—and no significant difference between the litter sizes of sodium valproate-treated animals and those of controls was found. Females were allowed to nurse and raise their offspring until weaning on postnatal day (PND) 25. Sodium valproate-exposed offspring (from here on referred to as VPA animals) had no major morphological anomaly. However, in line with previous reports on VPA rats, approximately 12% of males developed crooked tails showing mild neural tube defects induced by prenatal VPA exposure [38]. Some VPA animals developed transient chromodacryorrhea, which was of no major concern [35]. Because autism is more pronounced in males [39, 40], we used only males in the experiments.

2.3.3. Confirmation of ASD-like Traits in VPA Rats

In order to ensure that the prenatal VPA treatment indeed produced an ASD-like phenotype in the offspring, we performed select behavioural tests to identify relevant traits consistent with earlier reports on the VPA model of ASD. These tests were selected from the standard battery of behavioural approaches to identify autistic traits in VPA animals (for reference, see [34, 41]). The tests used in our strategy to confirm the ASD phenotype included open field tests for social interactions and the elevated plus maze test for assessment of anxiety-like behaviour. The collective outcome of

these ASD trait analyses confirmed that our VPA cohort exhibited a set of abnormal behaviours in concert with the predicted phenotype.

2.3.3.1. Open Field Test for Social Interactions

A modified version of the behavioural test described previously by [34] was used, in which a control and a VPA rat (PND 35) were placed in an open arena of 44 × 44 cm dimensions for nine minutes and all sessions were recorded. Social interactions such as sniffing, licking, crawling over or under, mounting, anogenital inspections and approaching or following a healthy conspecific were scored for every animal participating in the experiments.

2.3.3.2. Elevated-Plus Maze Test

In order to assess exploration of a novel environment, a modified version of a previously published protocol was followed [40], in which each rat (PND 30–35) was initially placed into the open field arena for 5 min before being placed in the elevated plus maze for another 5 min. The maze was elevated 50 cm above the ground and had two open arms and two closed arms of 50 × 10 × 40 cm dimensions. The arms were opposite each other and there was an open roof arrangement. The rat was always placed at the centre of the plus maze facing an open arm. The maze was cleaned with 70% ethanol before and after each rat was placed inside. The time spent and number of entries into the open arm was noted, along with the time spent in the closed arms for every rat. An entry into the open arm was defined as both paws being on or beyond the boundary of the closed arms. To assess whether the observation was merely a collateral effect of anxiety owing to a novel environment, self-grooming time was also evaluated as a parameter of anxiety-like behaviour [41].

2.3.4. Feeding Studies

2.3.4.1. *Ad Libitum* Standard Chow and Water Intake in VPA Rats versus Healthy Controls

Animals (n = 19/group, PND86, male, BW: ctrl 415.8 ± 8.6 g and VPA 398.7 ± 8.4) were individually housed and were allowed unrestricted access to standard laboratory chow and tap water. Chow and water were changed daily. During the time of the change, the amount of consumed water and food in the previous 24 h was recorded (chow and water bottles were weighed and the amounts subtracted from the values recorded 24 h earlier).

2.3.4.2. Energy Deprivation-Induced Feeding

Individually housed animals (the same cohort as described in Section 2.3.4.1) were deprived of chow overnight (water was available at all times). On the next day at 10:00, chow was returned to the cages and chow and water intakes were measured after two hours of re-feeding.

2.3.4.3. Feeding after Overnight Food and Water Deprivation

As a follow up to Experiment 2.3.4.2. (after a period of one week without any treatment), we wished to investigate whether concurrent deprivation of food and water affected feeding or drinking in VPA rats. Thus, individually housed rats had no access to food or water at night. At 10:00 on the next day, both tap water and standard chow were returned to cages and consumption was measured after 2 h.

2.3.4.4. Body Weight Trajectory

Animals (n = 19/group) were maintained from weaning until PND225 on an *ad libitum* standard chow and water diet and their body weights were assessed weekly throughout that period.

2.3.5. Neuronal Activation in Hungry vs. Fed Animals

2.3.5.1. Deprivation and Tissue Dissection

In the feeding studies described above we found that while VPA and control rats drank similar amounts of water, VPAs ate less of the standard laboratory chow after deprivation. In this study, we therefore wished to investigate changes in neuronal activation in feeding-related brain sites between hungry and *ad libitum*-fed rats. We investigated c-Fos immunoreactivity (IR; a marker of neuronal activation) in healthy

control rats and in the VPA cohort, either having been deprived of food overnight or having unrestricted access to chow during this time.

An independent cohort of animals (n = 10/group) was deprived of chow for 16 h with *ad libitum* access to water (referred to as 'deprived'). Animals with *ad libitum* access to chow and water were used as reference (referred to as '*ad libitum*'; n = 9–10/group). Animals were anaesthetised with single i.p. administration of 35% urethane dissolved in 0.9% saline. Toe-pinch and palpebral reflexes were checked and transcardial perfusion was performed with 50 mL of saline followed by 500 mL of 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Brains were excised and post-fixed overnight in the same aldehyde-based fixative at 4 °C. 60 µm-thick coronal sections were cut with a vibratome (Leica, Germany) and processed as free-floating sections for immunostaining of the protein in question (c-Fos). Immunohistochemistry for simultaneous detection of OT and c-Fos was also performed from the same set of sections.

2.3.5.2. Immunohistochemistry

Immunohistochemistry was performed following the protocol previously published with some modifications [42].

Sections were rinsed in 50 nM tris-buffered saline (TBS, pH 7.4–7.6), and then pre-treated for 10 min in 3% H2O2, 10% methanol (diluted in TBS) at room temperature. After rinsing in TBS, they were preincubated for 1 h at room temperature followed by incubation at 4 °C for 48 h in primary rabbit-anti-Fos antibody (diluted 1:1500; Synaptic Systems, Australia). Sections were washed in TBS and incubated for 1 h at room temperature in biotinylated-secondary goat-anti-rabbit antibody (1:400; Vector Laboratories). Following four washes in TBS, sections were incubated for 1 h with avidin–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). The medium for all incubations was a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The peroxidase in the tissues was visualised with 0.05% diaminobenzidine (DAB, Sigma), 0.01% H2O2 and 0.3% nickel sulfate (15–20-min incubation). Sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried

overnight, dehydrated in ascending concentrations of ethanol followed by xylene, and embedded in Entellan (Merck KGaA, Darmstadt, Germany).

Bright field images of immunohistochemically stained brain sections were acquired using an OMAX digital microscope camera attached to a Nikon Eclipse 400 microscope. Images were not processed digitally before or after the analysis; however, care was taken that the brightness, contrast and resolution of photographs used in software-based Fos-positive nuclear profile counting (Nikon NIS Elements image software) were similar. The number of Fos-positive nuclei per 1 mm2 was counted bilaterally for each neuroanatomical region of interest using ImageJ (Fiji), with boundaries defined according to the Paxinos and Watson brain atlas. Since the analysis of c-Fos immunoreactivity was conducted per mm2 of each site, the actual comparison of the activation levels reflected the density rather than the raw number of Fos-positive neurons. Therefore, any potential morphological changes (not evaluated here as c-Fos immunohistochemistry is not an appropriate method for combination with morphological analyses) did not impact the overall values reported here.
Image acquisition process, resolution and aspect ratio were kept constant using the same setup as described above.

The following areas were analysed (in parentheses, anterior–posterior ranges of bregma levels of sections used to analyse each site are shown): Nacc Core—nucleus accumbens core (1.28–0.96); Nacc Shell—nucleus accumbens shell (1.28–0.96); AP—area postrema (−13.92 to −14.16); ARC—arcuate nucleus (−2.16 to −2.52); BLA—basolateral amygdala (−2.64 to −2.92); CEA—central nucleus of the amygdala (−2.64 to −2.92); DMH—dorsomedial nucleus of the hypothalamus (−3.00 to −3.24); DMNV—dorsal motor nucleus of the vagus (−13.76 to −14.16); NTS—nucleus of the solitary tract (−13.76 to −14.16); PVN—paraventricular nucleus of the hypothalamus (−1.56 to −1.92); SON—supraoptic nucleus (−0.96 to −1.2); VMH—ventromedial nucleus (−3.00 to −3.24); VTA—ventral tegmental area (−6.72 to −6.84).

2.3.6. Gene Expression in Hungry vs. Fed Animals

2.3.6.1. Deprivation and Dissection of Hypothalamus and Brainstem

This study was designed to investigate expression of feeding-related genes in the hypothalamus and brain stem of VPA and control rats that were either hungry or had unrestricted access to food. Experimentally naive control and ASD animals were divided into two groups each. One group (n = 10–12/group) had *ad libitum* access to standard chow and water, whereas the other was deprived of food overnight prior to being sacrificed by decapitation (n = 9–12/group) at 10:00 the next morning. Hypothalami and brainstems were dissected and immersed in RNAlater (Ambion, Thermo Fisher Scientific, Auckland, New Zealand) for 2 h at room temperature and the samples were then frozen at −80 °C until further processing.

2.3.6.2. rtPCR Protocol and Data Analysis

A standard protocol for sample preparation and rtPCR was used, following a protocol previously described [43]. Tissues kept in RNAlater were homogenised in 400 µL Trizol (Ambion), after which 80 µL chloroform was added and samples were centrifuged at room temperature for 10 min at 10,000× g. The clear phase containing the total RNA was isolated and precipitated using 0.5 mL isopropanol in an ice bath for 10 min followed by centrifugation at 4 °C for 20 min at 10,000× g. The aqueous phase was carefully removed, keeping the pellets intact. The pellets were washed in 300 µL ethanol by centrifuging at 4 °C for 10 min at 10,000× g. The supernatant was removed carefully and the pellets were air dried.

Pellets were dissolved in 8 µL DEPC water and 1 µL DNAse buffer (dNature). Samples were then incubated with 1 µL DNAse (dNature) per reaction at 37 °C for 30 min followed by inactivation of DNAse with ‘stop buffer’ (dNature), incubating at 67 °C for 10 min. Removal of DNA was confirmed via PCR using HOT FIREPol Blend Master Mix (dNature), followed with agarose gel electrophoresis. Concentrations of RNA were measured (µg/µL) with a nanodrop.

cDNA was synthesised from RNA samples with iScript Advanced cDNA synthesis kit (BioRad). Quantification and purity of cDNA was determined using a Qubit 4

fluorometer (Invitrogen) and nanodrop. Quantitative real-time PCR reactions were performed in duplicate. An amount of 4 µL of 25 ng/µL cDNA template was used per transcript along with 1 µL of forward and reverse primers (5 µM) specific to that transcript (Table 1), 10 µL iTaq Universal SYBR Green Supermix (BioRad) and 4 µL MilliQ water. Expression of housekeeping genes (Histone H3.3, TBP, Tubulin β3) was used to analyse normalization factors. MilliQ water was used as a template for negative controls for each transcript. NCBI-BLAST® was used to check the specificity of the primer pairs *in silico* prior to setting up the reactions.

Housekeeping Genes		
Gene	**Forward**	**Reverse**
TBP	5'-AGAACAATCCAGATACAGCA-3'	5'-GGGAACTTCACATCACAGCTC-3'
Tubulin β-III	5'-TGCTGGCCATTCAGAGTAAGA-3'	5'-ACTCAGACACCAGGTCGTTCA-3'
Actin b	5'-AGTGTGACGTTGACATCCGT-3	5'-TGCTAGGAGCCAGAGCAGTA-3'
Genes of interest		
Gene	**Forward**	**Reverse**
AGRP	5'- CAGAGTTCTCAGGTCTAAGTC-3'	5'-TTGAAGAAGCGGCAGTAGCAC-3'
BDNF	5'-TGCAGGGGCATAGCAAAAGG-3'	5'-CTTATGAATCGCCAGCCAATTCTC-3'
Catalase	5'-GGCTCTCACATAGCTGCCAA-3'	5'-TTACTGGTGAGGCTTGTGCC-3'
COMT	5'-TGTGTGCGGAACCTAAACGA-3'	5'-GAAGGTCGCGTGTTCCAGTA-3'
D2R	5'-ACCTGTCCTGGTACGATGATG-3'	5'-GCATGGCATAGTAGTTGTAGTGG-3'
DCX	5'-TCGTAGTTTTGATGCGTTGC-3'	5'-GCTTTCCCCTTCTTCCAGTT-3'
Ddc1	5'-ATGTGGCGTCATGTGTGTCT-3'	5'-CACGGCCACACAAAGAACAG-3'
GABARA1	5'-GGCTTGGGAGAGCGTGTAAC-3'	5'-CGTGACCCATCTTCTGCCAC-3'
GAD67	5'-GTGGCGTAGCCCATGGATG-3'	5'-ACTGGTGTGGGTGGTGGAAG-3'
IL6	5'- CTGCAAGAGACTTCCATCCAG-3'	5'- AGTGGTATAGACAGGTCTGTTGG-3'
KOR	5'-AGACCGCAACAACATCTACAT-3'	5'-GCACAGAACATCTCCAAAAGG-3'
MC3R	5'-TCCGATGCTGCCTAACCTCT-3'	5'-GGATGTTTTCCATCAGACTGACG-3'
MC4R	5'-CTTATGATGATCCCAACCCG-3'	5'-GTAGCTCCTTGCTTGCATCC-3'
MOR	5'-CGGACTCGGTAGGCTGTAAC-3'	5'-CCTGCCGCTCTTCTCTGG-3'
OT	5'-GACGGTGGATCTCGGACTGAA-3'	5'-CGCCCCTAAAGGTATCATCACAAA-3'
OTR	5'-GATCACGCTCGCCGTCTA-3'	5'-CCGTCTTGAGTCGCAGATTC-3'

POMC	5'-CAGGACCTCACCACGGAAAG-3'	5'-GTTCATCTCCGTTGCCTGGA-3'
PSD95	5'-CTTCTCAGCCATCGTAGAGG-3'	5'-GAGAGGTCTTCAATGACACG-3'
Shank3	5'-TACAGCACTTGGAGCACCTG-3'	5'-GTAATTGCGGACGTCCTTGT-3'
Synapsin1	5'-CACCAGGATGAAGACAAGCA-3'	5'-GTCGTTGTTGAGCAGGAGGT-3'
VGlut2	5'-CGTGAAGAATGGCAGTATGTCTTC-3'	5'-TGAGGCAAATAGTGCATAAAATATGACT-3'

Table 4: List of all primers used in rtPCR experiments

The amplification protocol was as follows: denaturation at 95 °C for 15 min, followed by 45 cycles of 15 s at 95 °C, 15 s at the primer-specific annealing temperature and 30 s at 72 °C. Thermal profiles of the amplified transcripts were visualised using melt peaks where Tm 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 specificity of the primers in accordance with a given transcript and primer dimer.

2.3.7. Data Analysis

Data from the food intake experiments were analysed via GraphPad Prism using independent two-sample Student's t-tests considering homoscedastic distribution, as outliers were excluded from analysis using Grubb's test of extreme studentised deviate. Significance level was set at $p \leq 0.05$.

Body weight was measured to normalise the food intake. Body weight between groups was analysed using independent two-sample Student's t-tests considering homoscedastic distribution. Area under the curve was calculated and an independent two-sample Student's t-test was used to analyse the data.

Densities of Fos-positive nuclear profiles (per mm^2) were averaged per individual, and then per group. Data between the two groups (control ad lib vs. control deprived and ASD ad lib vs. ASD deprived) in each cohort were compared using independent two-sample Student's t-tests considering homoscedastic distribution. Values were considered significantly different for $p \leq 0.05$.

Analyses of rtPCR data utilised BioRad CFX Manager software (BioRad); rtPCR results were normalised with housekeeping genes and the ΔCq values were analysed with a Student's t-test. Values were considered significantly different when $p \leq 0.05$.

2.4. Results

Prior to undertaking the feeding studies, we conducted select behavioural tests in order to confirm that the VPA rats generated by the *in utero* pharmacological challenge displayed a set of traits characteristic of autism. In those feeding-unrelated behavioural tests, VPA rats showed less interest in social interactions with conspecifics, which is in line with reduced sociality in autism spectrum disorder. The social interactions scored in our pre-test included sniffing, licking, crawling over or under, mounting, and approaching or following the conspecific, which showed a cumulative decrease in these socially driven behaviours ($F = 1.411$, $df = 36$, $p = 0.0357$). Separately evaluated anogenital inspections revealed no differences between VPA and control animals (Figure 2-1A). Subjected to the elevated plus maze (EPM), VPA animals spent a significantly larger amount of time in the closed arm ($F = 1.53576$, $df = 36$, $p = 0.0013$) with fewer entries into ($F = 1.35718$, $df = 36$, $p < 0.0001$), and less time spent in the open arm ($F = 1.53576$, $df = 36$, $p = 0.0013$), compared to controls (Figure 2-1B–D).

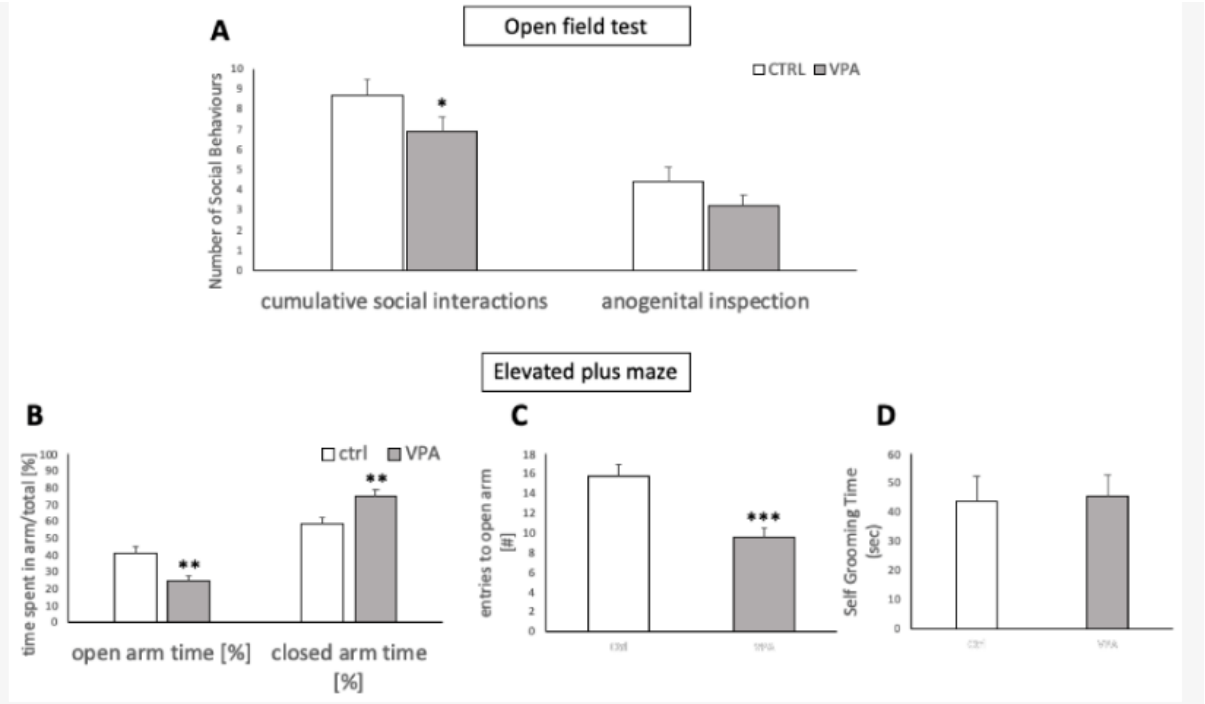

Figure 2-1: Behavioural tests in order to confirm that the VPA rats generated by the *in utero* pharmacological challenge displaying ASD-like phenotypes

(A) Cumulative social interactions including sniffing, licking, crawling over or under, mounting, approaching or following the conspecific were assessed over a temporal window of nine minutes. ASD animals showed a significantly lower number of interactions. Anogenital inspections, evaluated separately, showed no significant difference. (B) Elevated Plus Maze. Time spent in the open arm, closed arm and (C) number of entries to the open arms were recorded. ASD rats spent a greater percentage of time in the closed arms and entered the open arms less often than controls. (D) No significant difference was detected in self-grooming duration. Data are expressed as mean ± SEM, n = 19/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

rtPCR analysis of gene expression profiles showed that IL6 was upregulated ($F = 5.162$, $df = 17$, $p = 0.0303$) in hungry control animals compared to fed controls, while we did not see any change in the gene expression of IL6 in the VPA animals upon fasting. A similar pattern was observed with VGLUT2 expression ($F = 1.451$, $df = 17$, $p = 0.0071$), and Ddc1 expression was increased in hungry control rats ($F = 1.09$, $df = 17$, $p = 0.0035$), but decreased in VPA animals ($F = 2.48115$, $df = 21$, $p = 0.0062$) Finally, while there was no change in COMT expression between fed and hungry

control animals, it was mildly down-regulated in unfed VPA animals compared to their fed counterparts (Figure 2-2).

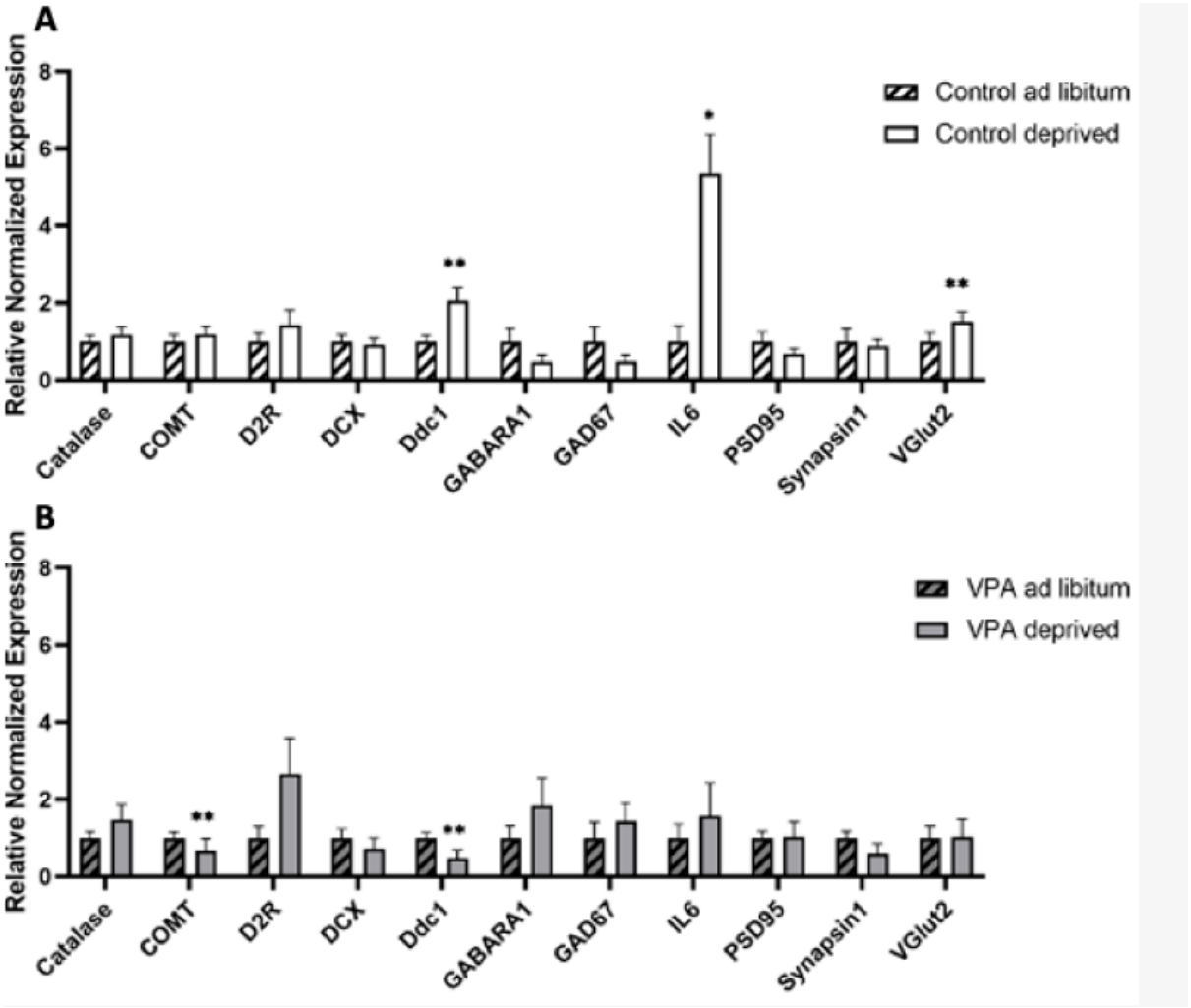

Figure 2-2: Gene expression changes in neurotransmitter receptors, oxidative stress and inflammatory markers

(A) fed and unfed control animals and (B) fed and unfed VPA animals. COMT—Catechol-O-Methyltransferase, D2R—Dopamine Receptor D2, DCX—Doublecortin, Ddc1—DOPA decarboxylase, GABARA1—γ-aminobutyric acid type A receptor, GAD67—glutamate decarboxylase 1, IL6—Interleukin 6, PSD95—discs large MAGUK scaffold protein 4 (Dlg4), VGLUT2—vesicular glutamate transporter 2. Data are expressed as mean ± SEM, n = 9–10/group. * $p < 0.05$, ** $p < 0.01$.

Body weight analysis in adult VPA rats from PND 60 to PND 225 revealed a reduced area under the curve (AUC) (70,118.94 ± 858.85 for healthy animals versus 66,928.55

± 803.65 in VPA animals; p = 0.015). A follow-up time point analysis showed differences in the rate of body weight increase in adult VPA rats compared to controls: while between PND60 and PND170 this did not reach significance, from PND170 onwards, VPA animals weighed less than controls (Figure 2-3).

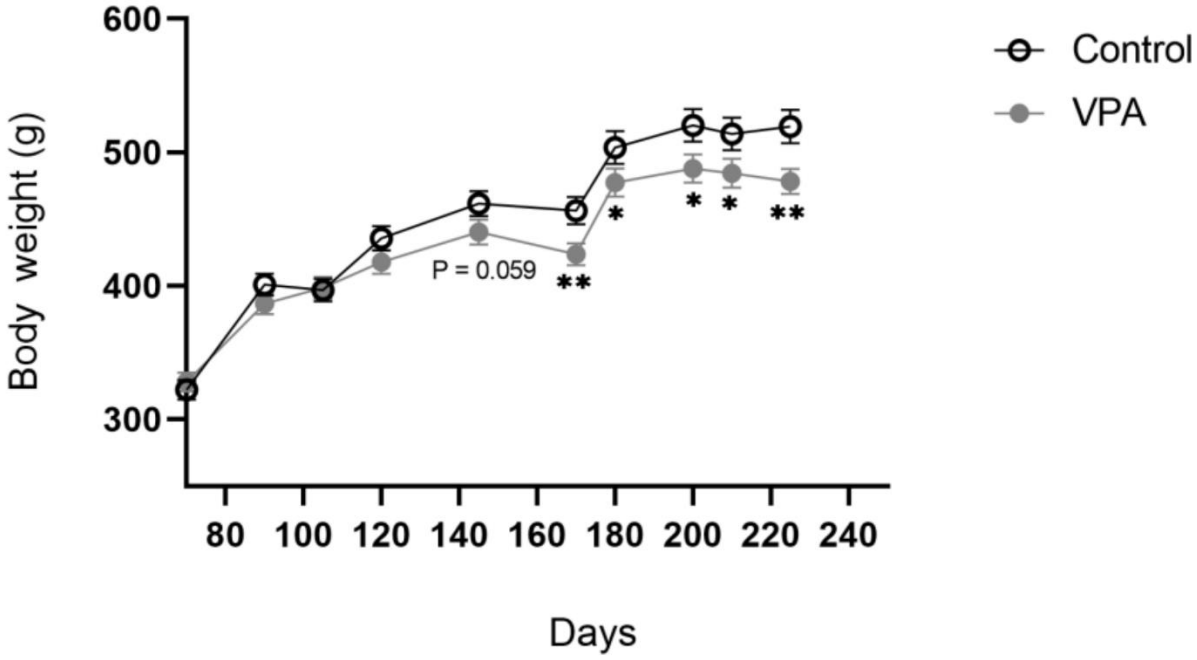

Figure 2-3: Body weight of adult ASD animals was significantly lower than that of healthy controls.

Data are expressed as mean ± SEM, n = 19/group. * p < 0.05, ** p < 0.01.

Consistent with the body weight trajectory, *ad libitum* chow intake in VPA animals was significantly lower than in controls (F = 1.2, df = 36, p = 0.00087), as was deprivation-induced chow consumption regardless of whether only chow (F = 1.4, df = 36, p = 0.0145) or both chow and water (F = 1.48, df = 36, p = 3.71558 × 10−5) were withheld from the animals during the overnight fast. In all of these scenarios, water intake was similar between the VPAs and controls. It should be noted that chow intake experiments were conducted between PND68 and PND86; thus, during the timeframe when body weight differences were not yet significant (Figure 2-4).

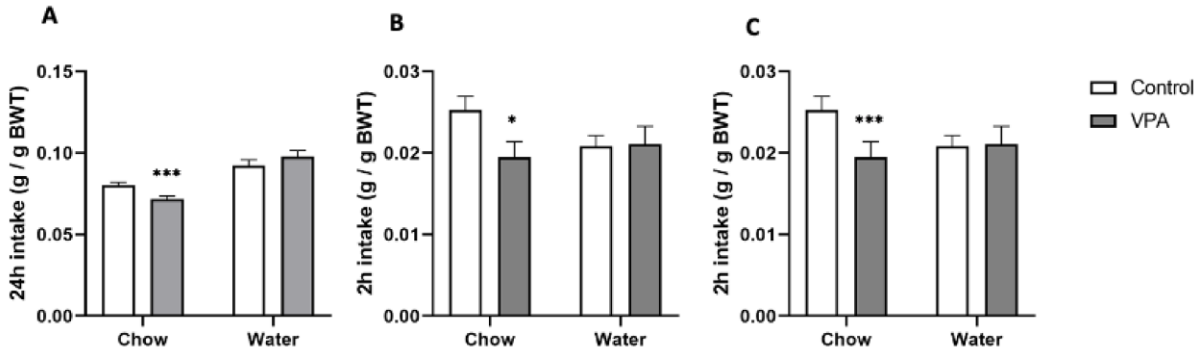

Figure 2-4: Measurement of standard laboratory-chow intake

(A) 24-h chow intake of *ad libitum*-fed animals was significantly reduced in ASD rats. (B) Within 2 h of re-feeding after overnight chow deprivation, ASD animals exhibited significantly lower chow intake with no significant difference in water consumption. (C) Chow intake during 2 h of re-feeding after overnight chow and water deprivation was significantly reduced in VPA animals compared to healthy controls, while water intake was unaffected. Data are expressed as average intake ± SEM, n = 19/group. * $p < 0.05$, $p < 0.01$, *** $p < 0.001$.

The analysis of c-Fos immunoreactivity (IR) in overnight-deprived versus *ad libitum*-fed rats in the healthy control cohort showed that compared to *ad libitum*-fed rats, the fasted animals had lower c-Fos IR levels in the PVN ($F = 1.282$, $df = 15$, $p = 0.0038$) and SON ($F = 141.695$, $df = 15$, $p = 0.0085$) as well as the CeA ($F = 6.584$, $df = 14$, $p = 0.0011$), and a strong trend towards reduced c-Fos IR in the Nacc Shell ($F = 1.757$, $df = 15$, $p = 0.052$). We also observed higher c-Fos IR in the BLA ($F = 2.755$, $df = 16$, $p = 0.0051$; Figure 2-5, Figure 2-6, Figure 2-7, Figure 2-8 and Figure 2-9).

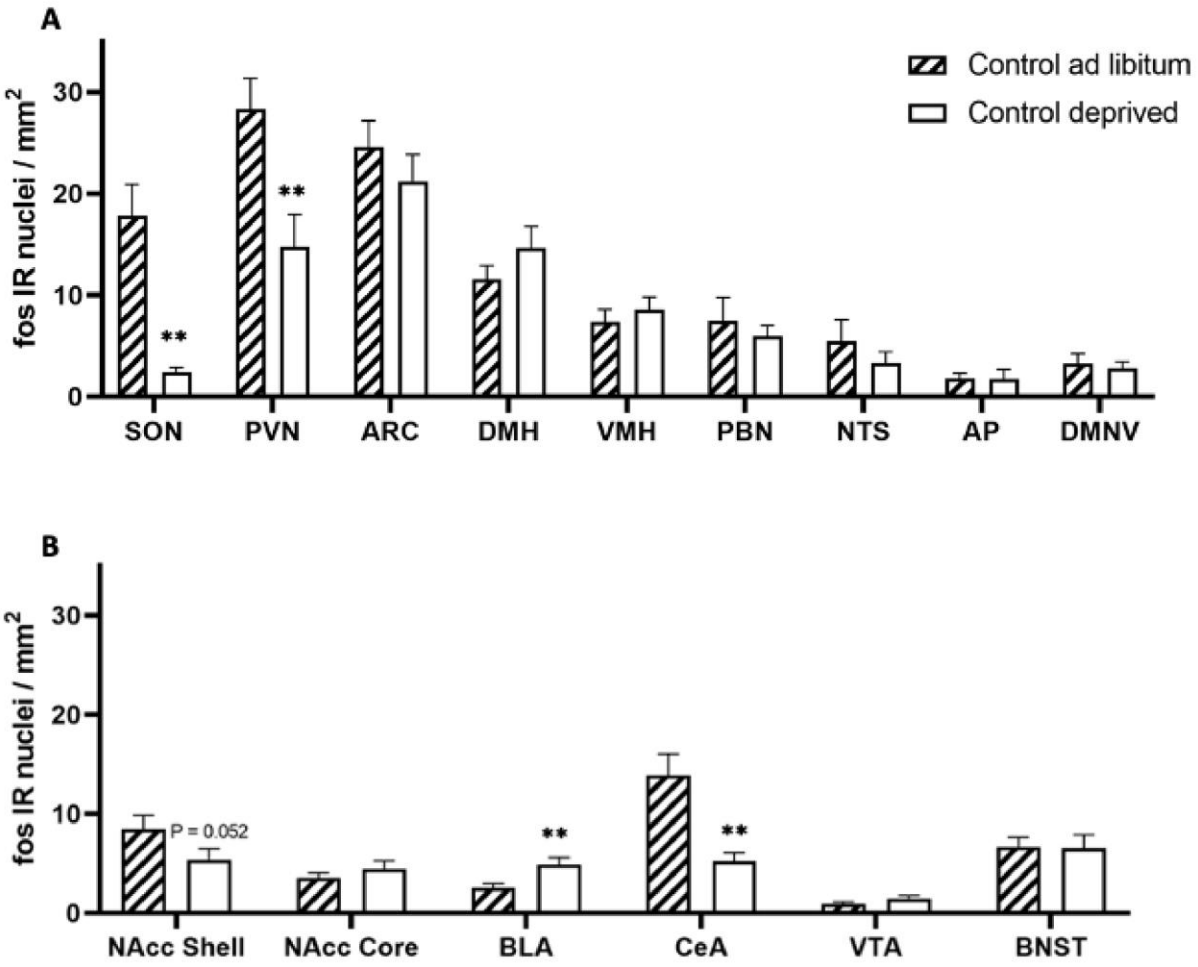

Figure 2-5: c-Fos immunoreactivity in *ad libitum*-fed and food-deprived healthy control animals. (A) PVN—paraventricular nucleus of the hypothalamus, SON—supraoptic nucleus, ARC—arcuate nucleus, LHA—lateral hypothalamic area, DMH—dorsomedial hypothalamus, VMH—ventromedial hypothalamus, PBN—parabrachial nucleus, NTS—nucleus of the solitary tract, AP—area postrema, DMNV—dorsal motor nucleus of the vagus, (B) Nacc Shell—Nucleus accumbens shell, Nacc Core—Nucleus accumbens core, BLA—basolateral amygdala, CeA—Central nucleus of the amygdala, VTA—ventral tegmental area, BNST—bed nucleus of the stria terminalis. Data are expressed as mean ± SEM, n = 9–10/group. $p < 0.05$, ** $p < 0.01$.

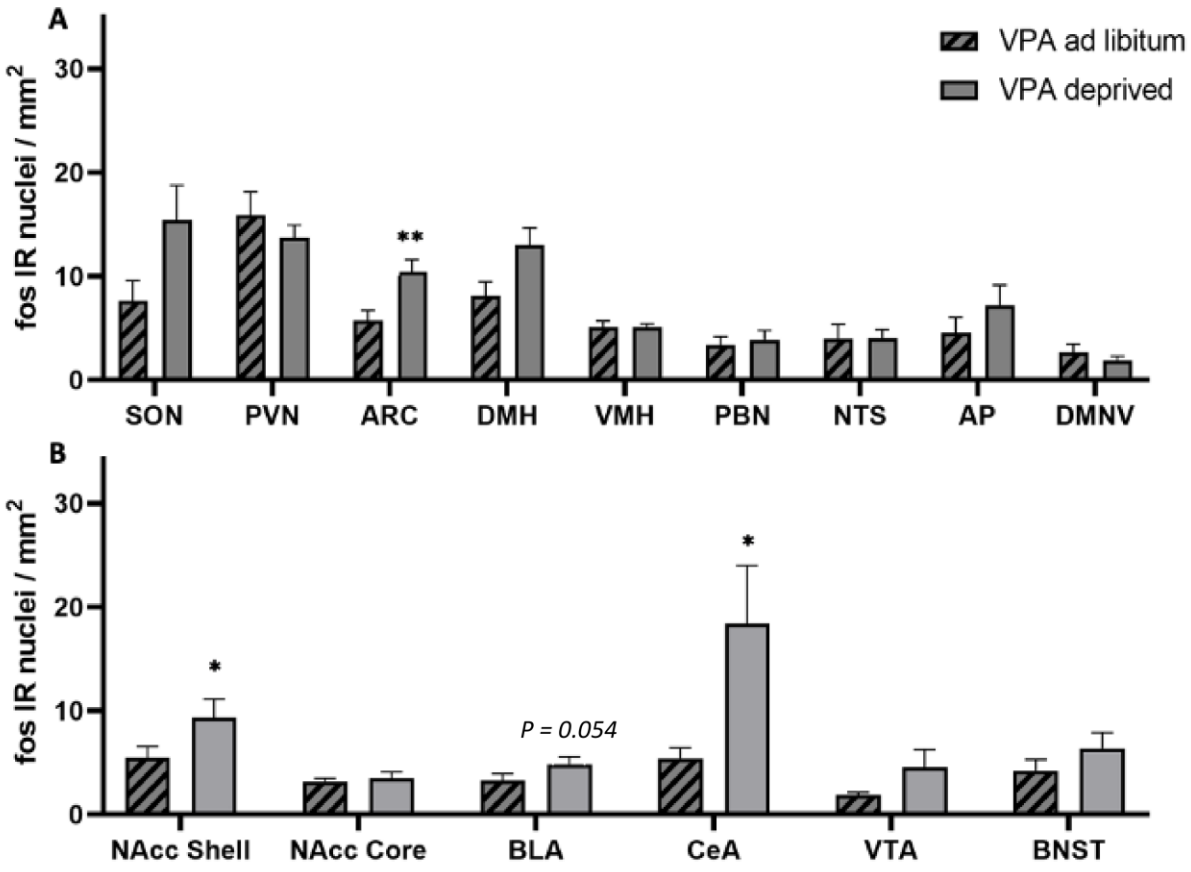

Figure 2-6: c-Fos immunoreactivity in *ad libitum*-fed and food deprived VPA animals. (A) PVN—paraventricular nucleus of the hypothalamus, SON—supraoptic nucleus, ARC—arcuate nucleus, LHA—lateral hypothalamic area, DMH—dorsomedial hypothalamus, VMH—ventromedial hypothalamus, PBN—parabrachial nucleus, NTS—nucleus of the solitary tract, AP—area postrema, DMNV—dorsal motor nucleus of the vagus, (B) Nacc Shell—Nucleus accumbens shell, Nacc Core—Nucleus accumbens core, BLA—basolateral amygdala, CeA—Central nucleus of the amygdala, VTA—ventral tegmental area, BNST—bed nucleus of the stria terminalis. Data are expressed as mean ± SEM, n = 9–10/group. * $p < 0.05$, ** $p < 0.01$.

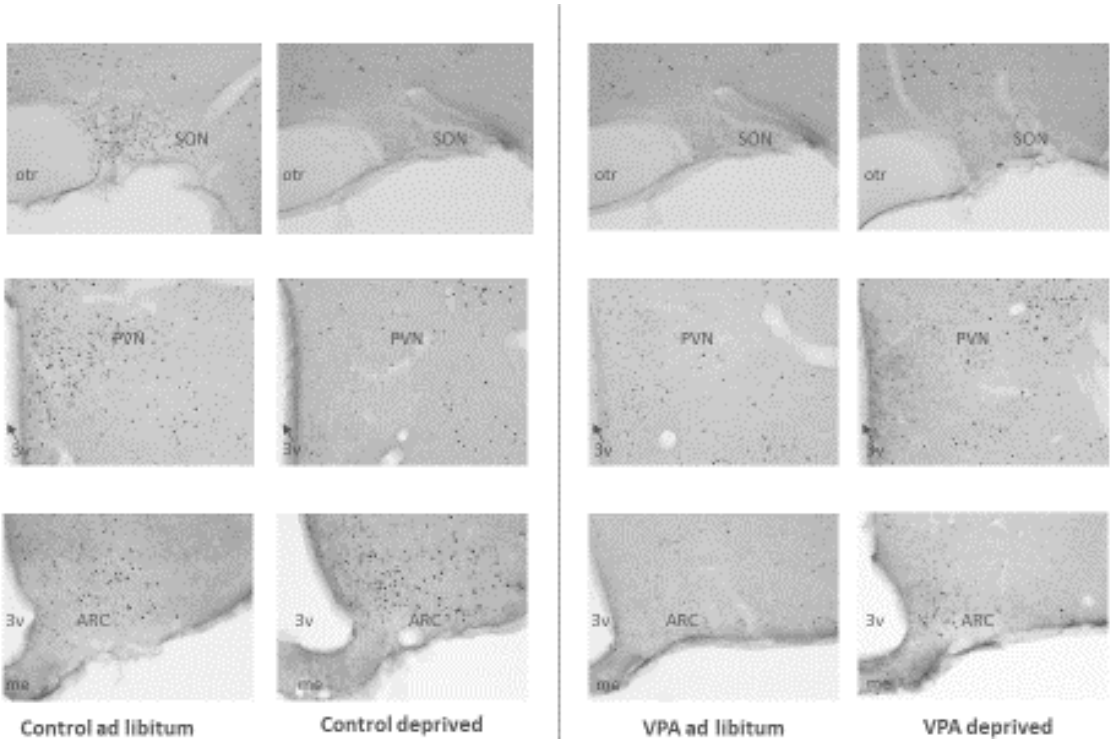

Figure 2-7: Photomicrographs depicting hypothalamic c-Fos IR. PVN—paraventricular nucleus of the hypothalamus, SON—supraoptic nucleus, ARC—arcuate nucleus, 3v—3rd ventricle, otr—optic tract.

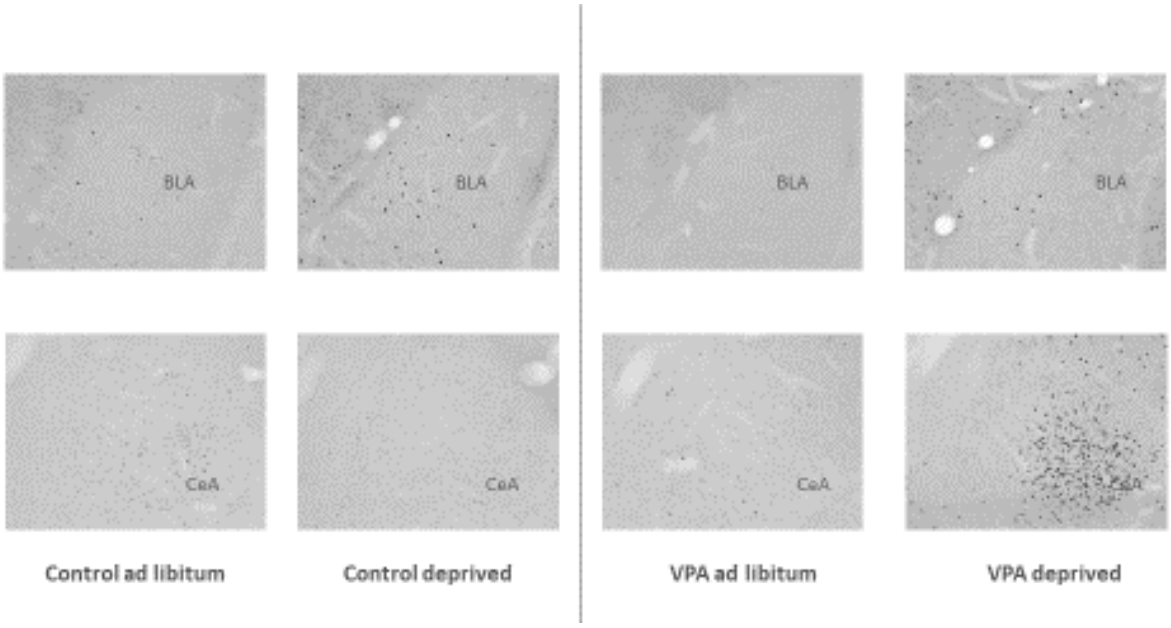

Figure 2-8: Photomicrographs depicting c-Fos IR in the amygdala. BLA—basolateral amygdala, CeA—Central nucleus of the amygdala.

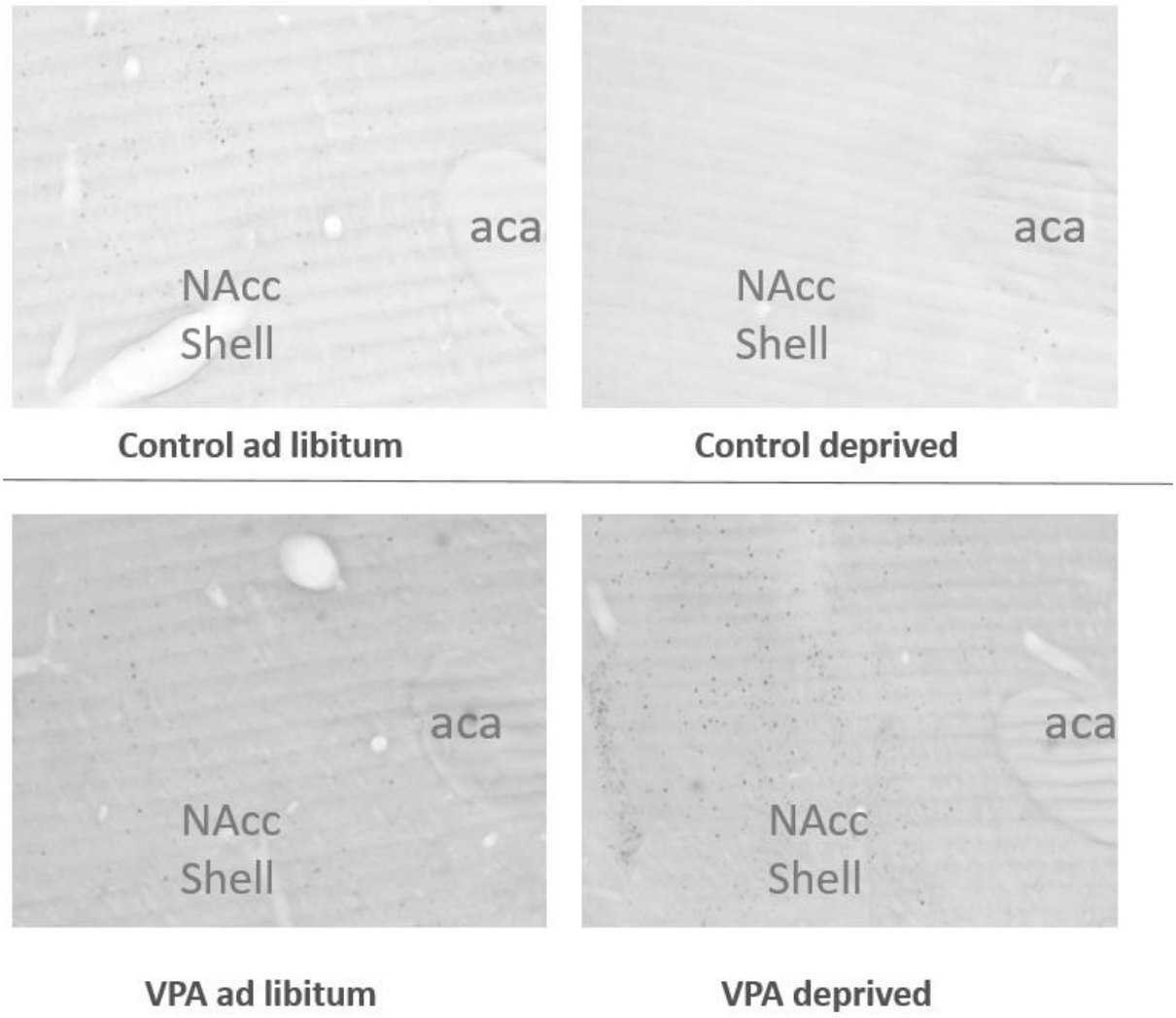

Figure 2-9: Photomicrographs depicting hypothalamic c-Fos IR in the Nacc Shell

Surprisingly, unlike in healthy controls, in VPA fasted rats compared to *ad libitum*-fed VPAs, we did not see changes between the fed and deprived state in the PVN or SON. On the other hand, ARC c-Fos IR increased in deprived VPA rats ($F = 1.317$, $df = 18$, $p = 0.0032$). Furthermore, we noted an increase instead of a decrease in the Nacc Shell and CeA c-Fos IR ($F = 2.453$, $df = 17$, $p = 0.035$ and $F = 30.994$, $df = 17$, $p = 0.022$, respectively; Figure 2-6, Figure 2-7, Figure 2-8 and Figure 2-9).

RtPCR analysis of gene expression profiles in the hypothalamus and brain stem in healthy controls revealed that healthy food-deprived rats had upregulated hypothalamic AgRP and MOR expression compared to fed controls ($F = 1.157$, $df =$

17, p = 0.000287 and F = 1.498, df = 15, p = 0.000295, respectively), while hypothalamic OT mRNA expression was decreased in deprived animals (F = 1.316, df = 15, p = 0.0397). Brainstem gene expression analysis revealed an increase in OTR mRNA levels in unfed animals (F = 1.422, df = 15, p = 0.025).

Importantly, none of these genes' expression levels differed between fed and unfed VPA animals and, instead, only brainstem MC3R expression in unfed VPA animals showed a trend toward statistical significance (F = 1.606, df = 18, p = 0.057; Figure 2-10).

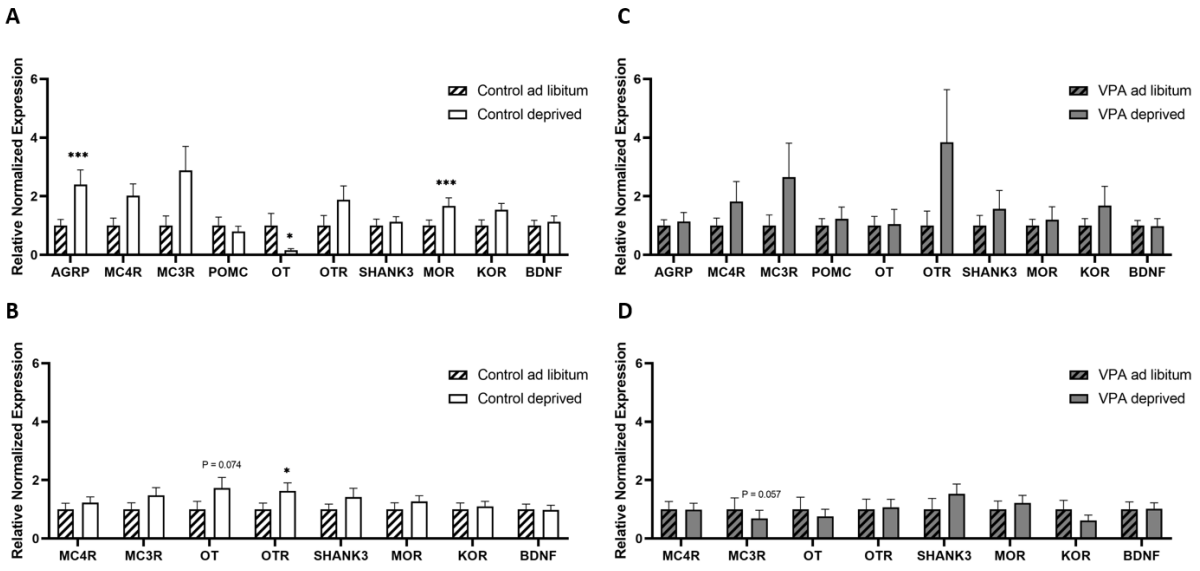

Figure 2-10:Differential gene expression patterns (A) in the hypothalamus of *ad libitum*-fed and food-deprived healthy control animals, (B) in the brainstem of *ad libitum*-fed and food-deprived healthy control animals, (C) in the hypothalamus of *ad libitum*-fed and food-deprived VPA animals and (D) in the brainstem of *ad libitum*-fed and food-deprived VPA animals. AgRP—agouti related protein, MC4R—melanocortin 4 receptor, MC3R—melanocortin 3 receptor, POMC—pro-opiomelanocortin, OT—oxytocin, OTR—oxytocin receptor, Shank3—SH3 And Multiple Ankyrin Repeat Domains 3, MOR—mu-opioid receptor, KOR—kappa-opioid receptor, BDNF—brain-derived neurotrophic factor. Data are expressed as mean ± SEM, n = 9–10/group. * p < 0.05, p < 0.01, *** p < 0.001.

2.5. Discussion

One of the greatest daily challenges for caregivers of individuals with ASD relates to providing a balanced diet that is accepted by a person under their assistance [44, 45]. Acceptance of varied diets is very low in individuals with ASD [44, 46-48]. To some extent, this is driven by sensory dysregulation. Indeed, high-functioning adults with ASD have been reported to avoid foods due to hypersensitivities to certain dietary item characteristics, such as taste, texture, smell or temperature [49]. Consequently, ASD individuals often receive a very limited diet consisting of a narrow range of their preferred foods. Intuitively, in the obesogenic environment in which highly palatable and caloric yet nutritionally substandard food is readily available, this often leads to obesity. Interestingly, data show that many individuals with ASD are also underweight: a persistent avoidance of non-preferred food items in strategies that attempt to incorporate varied diets contributes to this phenomenon [50, 51]. In fact, as shown in case reports, calorie self-restriction in ASD can at times be so severe that it requires medical intervention [52]. Our results indicate that the VPA rats responded with a less avid consummatory behaviour response when challenged with energy deprivation is of importance for understanding the pathophysiology of appetite dysregulation in ASD. Owing to a lower baseline consumption of the bland chow, their body weight trajectory was shown to be rising slower as compared to their matched controls.

By using the VPA animal model of autism, Klockars et al. have reported that VPA rats overconsume sugar, saccharin and milk solutions [33], liquid diets that are highly preferred by rodents (see, e.g., [43, 53]), whereas intake of a less palatable cornstarch solution is unaffected. On the other hand, the current set of results showed that consumption of nutritious yet ‘bland’ standard laboratory chow was significantly lower in VPA rats than in their healthy counterparts, thereby exemplifying reduced energy intake in autistic individuals upon presentation of non-preferred food. Importantly, this reduction was evident regardless of paradigm. In *ad libitum*-fed animals, chow consumption was approximately 15% lower than in controls. This reduction was chronic and persisted despite the fact that it translated into a reduced rate of body weight increase in these animals compared to their non-ASD conspecifics. Furthermore, VPA rats ate approximately 20% less standard chow than controls after an overnight period of energy deprivation (irrespective of whether water was or was

not present during the period of having no access to chow). Thus, the VPA animals consumed less both over the course of a meal when feeding was induced by a strong stimulus of energy deficit, and over 24 h periods of unrestricted access to chow.

On the other hand, we did not see any difference in water intake between VPA and control rats. This was regardless of whether water consumption was measured in the *ad libitum* setting or in the meal scenarios prior to which water had been removed along with food or only food had been removed. Since drinking behaviour is typically affected by food consumption, one would expect water intake to be somewhat lower in VPAs as well. However, it should be noted that human studies have identified autism as a condition in which excessive water consumption occurs quite often in children and adults [54]. Thus, the lack of decline in water drinking in VPA rats, even though they were eating less food, was consistent with the findings in humans.

Changes in the energy state of the organism (i.e., hunger versus satiety) are associated with changes in both brain activation and expression of genes, the latter especially within the hypothalamus and brain stem, the two regions that are key for the regulation of energy homeostasis. Here, we showed for the first time that VPA animals did not show a typical set of neuronal activation and gene expression patterns between the hungry and *ad libitum*-fed (sated) states.

Our gene expression analysis of inflammatory markers revealed that IL6 expression increased in healthy animals upon food deprivation, while we did not observe this change in VPA animals. Interestingly, IL6 has also been shown to improve glucose homeostasis and reduce body weight in obese animals [29, 55], which points to its active involvement in food intake regulation [56] and may potentially amount to an orchestrated interplay of neuronal and glial cells in the context of neuroprotection [57], which is not observed in VPA animals and possibly feeds into the general pro-inflammatory environment of autistic brains. Furthermore, while no changes in COMT (that degrades catecholamines such as dopamine to its inactive metabolite) expression were observed between fed and hungry control animals, we found a decrease in the food-deprived VPA group. Importantly, Hersrud and Stoltenberg identified a correlation between COMT/dopamine dysregulation and maladaptive eating behaviour in undergraduate college women [58].

Earlier reports utilizing c-Fos immunohistochemistry showed that in hungry animals that are not anticipating a meal, the level of neuronal activation is significantly lower in many hypothalamic and extrahypothalamic sites, including the PVN and SON, than in refed or *ad libitum*-fed counterparts [59, 60]. In the healthy control cohort of rats, we found that fasting indeed led to lower c-Fos expression in the PVN, SON, NAcc Shell (trend at $p = 0.052$) and CeA. The only site where c-Fos was elevated was the BLA, which possibly represented a higher motivational drive to search for food [61], other emotional aspects of fasting or the interplay between motivational and homeostatic feeding mechanisms [62, 63]. On the other hand, in the VPA cohort, we did not see any hunger-associated decrease in any of the sites included in our study. In fact, we saw higher c-Fos IR in the ARC, CeA, BLA (trend at $p = 0.054$) and NAcc Shell, although—interestingly—average densities of Fos-positive nuclei in *ad libitum*-fed VPAs were similarly low to the densities in the hungry control cohort. The elevated number of Fos positive nuclei in the CeA of the hungry VPA animals likely indicates elevated anxiety upon food deprivation that could be an underlying mechanism of a reduced motivation to feeding despite hunger [64]. This hypothesis is further strengthened by the lack of change of AGRP gene expression upon hunger in the VPA rats, as AGRP promotes feeding by reducing the anxiety that is associated with negative energy balance [65-67].

While the c-Fos immunoreactivity alone is not sufficient to explain why the VPA animals consumed less than their healthy controls, it is clear that the autistic animals' feeding circuit was differently affected by the energy status of the animal. The notion of aberrant processing of the energy status in VPAs is further supported by the analysis of select feeding-related genes in the hypothalamus and brain stem. This is particularly relevant considering the fact that healthy controls showed significant changes in mRNA levels of genes associated with orexigenic (such as AgRP, mu-opioid receptor) and anorexigenic (oxytocin and oxytocin receptor) processes [68], whereas in VPA animals, only the melanocortin3 receptor transcript approached but did not reach ($p = 0.057$) significance.

'Bland' foods such as standard chow can indeed become rewarding after fasting and trigger dopamine release in similar amounts to a high-caloric palatable food. The neural circuits controlling homeostatic feeding and hedonic feeding are not completely

dissociable. Destruction of dopaminergic neurons (DA) causes profound deficits in feeding, movement, motivation, and learning. In addition to its established role in reward, dopamine signalling is also a critical component of voluntary feeding and motivated behaviours in general [73]. Zhou et al demonstrated that selective disruption of dopamine production causes hypoactivity, adipsia and aphagia in mice [74]. In line of this finding, optogenetic activation of VTA GABAergic neurons leading to reduced dopamine release, showed to inhibit licking for sucrose promoting aversion [75]. Direct synaptic connections between the ventricular AgRP neurons and the VTA DA neurons are relatively sparse, and the mechanism of this circuit is quite unclear in terms of food intake regulation. Dietrich et al showed that impairments in AgRP circuit leads to enhanced spike timing–dependent long-term potentiation in VTA DA neurons and elevated DA in the basal forebrain in mice [76]. This likely suggests that AgRP neurons regulate the set point of the reward circuitry. Additionally, ARC receives projections DA neurons. Optogenetic stimulation of the ARC^{Drd1} neurons showed to induce voracious food consumption in mice [77]. These indicate functional interdependence of AgRP and DA circuits.

ICV delivery of ddc (DOPA decarboxylase; that breaks down L-Dihydroxyphenylalanine to DA) inhibitor showed to reduce chronic alcohol drinking in rats [78] indicating the role of ddc in reward-driven consumption. Furthermore, acute fasting was shown to cause an increase in somatodendritic dopamine release in the VTA [79]. Fasted animals not anticipating food thus would perceive ‘bland’ standard chow rewarding especially when they were food deprived during the dark phase [80]. Our data showing the elevated expression of AgRP and ddc1 in the fasted control animals indicates motivation to eat after fasting which is absent in the VPA rats.

One should note that the current study focuses entirely on central processing of food intake and brain mechanisms that underpin hunger and satiety. However, ASD is characterized by abnormalities in not only the central nervous system, but also in—among others—the gastrointestinal system. In fact, ASD individuals are likely to have digestive issues, from constipation to diarrhoea [69-71], and the symptomology has been linked to both functional gut changes and microbiome alterations [70, 72]. Thus, when considering brain data related to feeding, it should not be neglected that the brain integrates information derived from the gut directly (e.g., via vagal input) or

indirectly (via endocrine communication). Therefore, the observed brain changes might not be the primary, but rather the secondary consequence of broader anomalies associated with ASD.

2.6. Conclusions

In sum, we conclude that adult male VPA animals ate less of the ‘bland’ chow when fed *ad libitum* as well as after deprivation. Their body weight increased slower than controls when maintained on this standard ‘bland’ food. While food deprived control rats had lower c-Fos IR in key feeding-related areas than their *ad libitum*-fed counterparts, in hungry VPAs c-Fos was unchanged or elevated compared to the fed counterparts. The lack of changes in expression of feeding-related genes upon deprivation in VPAs was in contrast to several transcripts affected by fasting in healthy controls. This relative ‘unresponsiveness’ of the central circuits to changes in the energy status of the VPA organism may be an underlying factor in aberrant processing of hunger/satiation upon presentation of foods that do not belong to the preferred category.

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Chapter 3
Anomalous Feeding in Adult Male Rats with Sodium Valproate-Induced Autism

3.1. Abstract

Aside the self-imposed underfeeding of bland diet, autistic individuals often show a propensity to like palatable food, often rich in sugar. Selective consumption for palatability coupled with the keenness for sameness in autism predisposes ASD individuals to obesity. Therefore, we attempted to assess whether ASD-related dysregulation of food intake affects the consumption of palatable foods. Here we used *in utero* VPA-exposed rats, an animal model of ASD to assess the aberrant feeding. We found that these animals show elevated consumption for palatability. While selective eating may be considered a secondary consequence of stereotypy in ASD, there is growing evidence that aberrant food intake regulatory mechanisms — especially those related to reward processing — may be an additional culprit.

3.2. Introduction

Some authors suggest the repetitive food choices in ASD might be the drive for sameness and predictability, whereas others have proposed addictive-like eating behaviour as the underlying cause [1]. This, coupled with the anomalous food-reward processing in ASD [2] suggest an increased food craving for palatability in ASD. Supekar et al reported a lower density of the mesolimbic NAcc-VTA (ventral tegmental area) tract in ASD (already associated with social deficits) contributes to aberrant responsiveness to pleasant stimuli [3]. In line of this finding, high prevalence of pica in ASD can be explained by lower levels of CSF beta-endorphin and met-enkephalin, opioids involved in the context of stress and analgesia – pointing towards a manifestation of reward system's abnormal processing of self-injurious behaviour [4]. The altered functional and structural neuronal connectivity projects to atypical integration of homeostatic state with reward-processing, emotional, multimodal

sensorimotor interoception, and higher cognitive functions leading to an aberrant food intake in autism.

A tendency to prefer sweet solutions has been reported in several psychopathological conditions characterized by dysfunctional reward processing [5]. This, coupled with the dysfunctional neurophysiology poses a question pertaining to the fact that central reward processes differ in ASD is whether it predisposes individuals with ASD to overconsumption of palatable foods. This is an extremely timely issue considering that excessive consumption of palatable foods (including those rich in sugar) is the main contributor to the elevated propensity to obesity in ASD [6].

Reports on palatability-driven appetite in ASD in humans have been scarce. Tavassoli and colleagues reported lower sweet taste-identification-accuracy scores in adult autistic individuals. They also reported highest accuracy in identifying sweet tastant in both ASD and controls [7]. Damiano et al found no difference between ASD individuals and controls in sweet taste sensitivity, although the severity of symptoms within the ASD cohort was negatively correlated with sweet taste sensitivity but not in hedonic response to sweet taste [8].

Data derived from experimental work utilizing animal models of ASD are somewhat more informative and point towards an enhanced drive to consume palatable tastants. Cntnap2 – a high confidence autism-gene is implicated in the context of energy balance. Buchner et al found that the body weight consequences of the mutation in this gene were highly dependent on the general genetic background of the animal as well as on the availability of a palatable diet whose pleasant taste promotes overeating[9]. Fukuhara and colleagues reported that upon knockdown of the autism-associated methylation gene, Mecp2, mice displayed normal food intake and body weight when exposed to a standard chow, but, upon exposure to palatable high fat diet (HFD), these animals showed extreme hyperphagia resulting in obesity compared to their wild-type littermates. Their results show altered levels of genes relevant to regulating food intake in the hypothalamus in Mecp2 +/- animals – elevated orexogenic AGRP level with downregulated anorexigenic POMC. This, along with changes in gene expression in VTA-Dopaminergic system suggest that HFD feeding causes dysregulation of energy balance and affects dopamine reward-circuitry, causing

extreme obesity in Mecp2+/- mice [10]. Mild overexpression of Shank3, a high-confidence ASD-gene leads to elevated body weight and food intake in mice [11]. Here, we conducted brief sets of experiments using prenatally VPA-exposed male rats, to assess the consumption of palatable diets.

3.3. Materials and methods

3.3.1. Generation of VPA Animals and Confirmation of the Autistic Phenotype

All procedures were approved by the University of Waikato Ethics Committee (protocol 1088). Female Sprague–Dawley rats aged 8–12 weeks, housed in a temperature-controlled (22 °C) environment with a 12:12 light:dark cycle (lights on at 07:00), were mated overnight with age-matched Sprague–Dawley males. The following morning, vaginal smears were stained with 1% crystal violet to check for spermatozoa, and a positive test was designated as E0.5. Females received a single intraperitoneal injection of 500 mg/kg VPA (Sigma) on E12.5. Control females were injected with 0.9% NaCl. Animals in the experiments were single housed males, aged 24–27 weeks, with *ad libitum* access to standard chow (Diet 86, Sharpes Stock Feed, New Zealand) and water unless specified otherwise.

The autistic traits of VPA vs. control cohorts were confirmed through standard behavioral tests as described in the previous chapter. Briefly, using an elevated plus maze test was conducted exploration of a novel environment was assessed where each rat (PND 30–35) was initially placed in the open field arena for 5 min before being placed in the elevated plus maze for another 5 min. The rat was always placed at the center of the plus maze facing an open arm. Between animals, the maze was cleaned with 70% ethanol. The time spent (Ctrl 49% vs. VPA 26%, $p < 0.0001$) and number of entries (Ctrl 13 vs. VPA 6, $p < 0.01$) to the open arm were noted, along with the time spent in the closed arms (Ctrl 51% vs. VPA 74%, $p < 0.0001$) – data not shown.

Open field test was used to evaluate social interactions described previously by [12] was used. In brief, a control and a VPA rat (PND 35) were placed in an open arena of 44 × 44 cm for 9 min, and the session was recorded. Social interactions such as

sniffing (Ctrl 22 vs. VPA 19, $p < 0.05$), crawling over or under, and mounting (Ctrl 6 vs. VPA 3, $p < 0.05$) of the conspecific were evaluated.

3.3.2. Episodic Intake of Palatable Sucrose, Saccharin, Glucose and Milk in VPA vs. Control Rats

The paradigm described here was based on the protocol used in our earlier work (e.g., [13, 14]). The palatable solution tests were done in separate crossover tests using different rats. Three cohorts of VPA and control rats were accustomed to receiving palatable 15% sucrose, 0.1% saccharin, or milk (whole milk; Dairy Goat Cooperative [DGC], Hamilton, New Zealand) solutions for 2 h/day for 2 days (10:00–12:00) prior to the experiment to avoid neophobia. On the experimental day, standard chow and water were taken away at 10:00, and the rats were immediately given a bottle containing the palatable fluid. After 2 h, intake of sucrose, saccharin, or milk was measured by weighing bottles, and it was expressed per gram of body weight.

3.3.3. Episodic Intake of High fat high sugar (HFHS) diet in VPA vs. Control Rats

Ad libitum-fed animals were preexposed to HFHS diet (HFHS; Research Diets #D12451; 4.73kcal/g; 35% calories from sugar and 45% from fat) prior experiment. On the experimental day, standard chow and water were taken away at 10:00, and the rats were immediately given a weighed amount of HFHS diet. After 2h, intake was measured and data was expressed per gram of body weight.

3.3.4. Episodic Intake of a “Bland” Cornstarch Solution in VPA vs. Control Rats

In order to stimulate intake of the “bland” carbohydrate, rats were food-deprived overnight, and then, they received access for 2 h (10:00–12:00) to a bottle containing a 10% cornstarch suspension (as cornstarch is insoluble in water, 0.3% xanthan gum was added to the liquids in this experiment, as described previously in [15, 16]. Both standard chow and water were not present in the cage during the 2-h cornstarch meal.

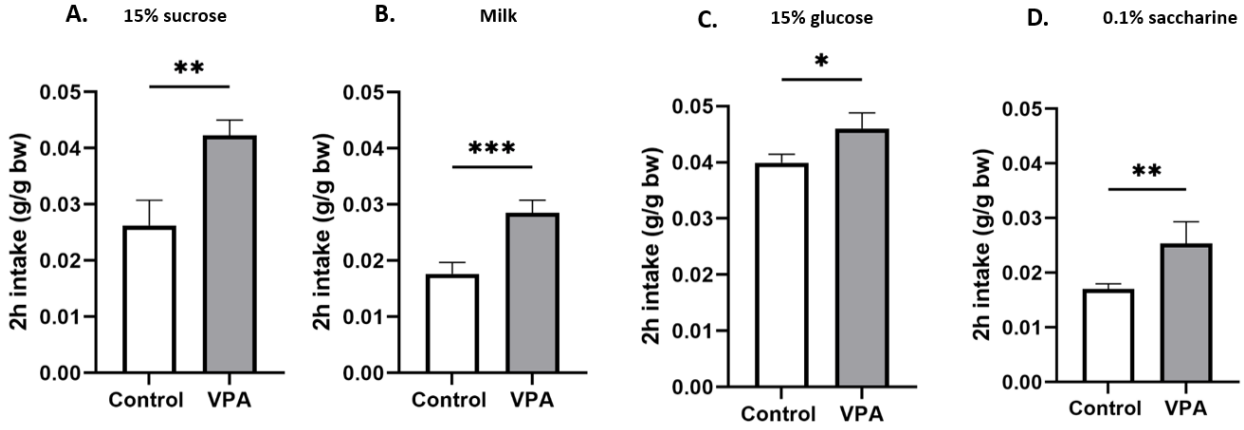
Bottles were weighed; the amount of the solution consumed was reported in g/g of body weight.

3.3.5. Data analysis

The amount of consumed food and fluid (in grams) was divided per gram of body weight. In this and all other feeding experiments involving two-group comparisons (control vs. VPA – 19/ group), the data were analysed with independent sample Student's t-test.

3.4. Results

VPA animals consume significantly more 15% sucrose (t=3.07109, df1=18, df2=18, P=0.002022), 0.1%saccharin (t=2.62089,df1=23, df2=12, P=0.006444), milk (t=3.66548, df1=18, df2=18, P=.000395) and 15% glucose (t=1.90255,df1=17, df2=18, P=0.032676). They show a strong trend of overconsumption of HFHS diet (t=1.50378, df1=18, df2=18, P=0.07068). VPA animals exhibit no change in intake of bland cornstarch.



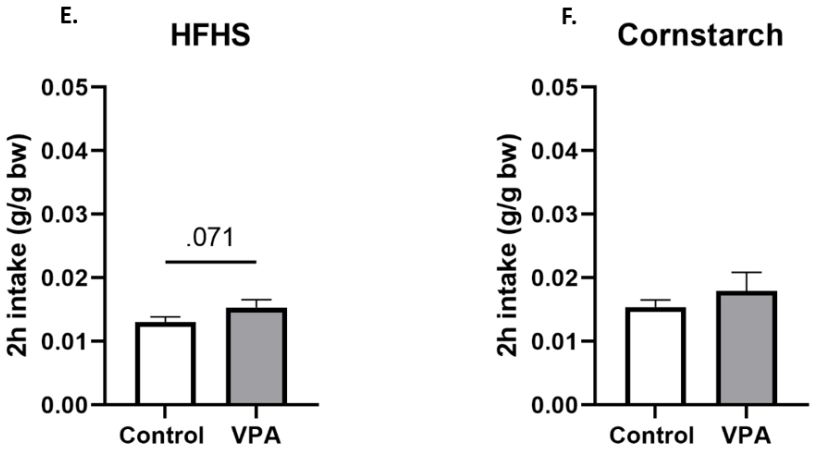


Figure 3-1: Consummatory behavior in VPA (rodent model of autism) versus control rats. Intake of palatable 15% sucrose solution (A), milk (B), 15% glucose solution (C), 0.1% saccharin (D), HFHS diet (E) and non-palatable 10% cornstarch in non-deprived animals

3.5. Discussion

Episodic 2-h intake of palatable sucrose, saccharin, and milk was elevated in VPA animals compared to their control counterparts. This increase in palatability-driven consumption was therefore evident regardless of the tastant. It was independent from fluid intake in general, though, as no effect was seen in the “bland” cornstarch-solution consumption. These data from VPA animal strongly suggest that the ASD phenotype is associated with overconsumption of palatable diets. The diets excessively ingested by ASD animals include, but are not limited to, those that contain sugar. These behavioural data potentially point towards neuropeptidergic dysregulation.

A plethora of evidence implicates impaired OT signalling in the development of key ASD symptoms [17]. Animals with OT-deficit tend to have elevated propensity to consume sugar such as that observed in OT-KO mice [18]. OT is implicated in decreasing palatability-driven eating behaviour, which is consistent with the distribution of the OT receptor throughout the reward system and the presence of OT neuronal terminals in the VTA, NAcc, amygdala, and PFC [19, 20]. Thus, impaired OT signalling could be a direct underpinning to the magnitude of this overconsumption.

3.6. Conclusion

The current literature analysis and pilot data presented here suggest that aberrant neural processing at the reward system level is responsible for overconsumption of palatable tastants, including sugar. Since intake of palatable diets seems to be broadly elevated in individuals with autism, a strategy to limit sugar overconsumption might utilize presentation of alternative palatable food choices that are more adequate nutritionally than sucrose.

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Chapter 4

Acute Intraperitoneal Oxytocin Administration Reduces Food Intake with c-Fos Induction in Feeding-related Brain Areas in Adult Male Rats with Sodium Valproate-Induced Autism

4.1. Abstract

The aberrant feeding behaviour of autism involves extremely narrow food preferences and the propensity to like sugar predisposes autistic individuals to obesity. Oxytocin (OT), a prosocial neuropeptide, widely implicated in ameliorating social deficits in ASD is also an anorexigenic substance. A dysfunctional OT system is established in ASD in both human and animal studies. Utilising prenatally VPA-exposed adult male rats (VPA animals), a model of autism, we examined the baseline expression levels of OT and oxytocin receptor (OTR) as compared to their matched controls. Our results showed that VPA rats exhibited a lower level of OT and OTR mRNA in the hypothalamus and brainstems as compared to the controls. They also had fewer OT-IR cells in the supraoptic nucleus (SON) of the hypothalamus than their matched control animals. Intraperitoneal (i.p.) OT treatment was found to reduce episodic intake of palatable diets as well as post-energy deprivation intake of the standard chow; however, the dose needed to generate hypophagia was lower in VPAs than in controls. c-Fos immunohistochemistry showed that i.p. administration of the lowest effective dosage of OT activates feeding-related brain sites in a manner typical for hypophagia in the VPAs while no significant difference was observed in the controls. We also observed the elevated activity of hypothalamic paraventricular OT-neurons upon peripheral OT administration only in the VPA animals. We thus conclude that exogenous OT employs central sites to diminish food intake in these animals. OT treatment thus has implications in improving some aspect of the aberrant food intake behaviour in VPA rats.

4.2. Introduction

Autism spectrum disorder (ASD), is a heterogeneous neurodevelopmental disorder presented with phenotypes such as impaired in social communication and interaction, sensory anomalies, repetitive behaviours and varying levels of intellectual disability. Such core symptoms are currently untreatable with any medication at a significant level. The nonapeptide oxytocin (OT) is involved in governing various social behaviours in mammals, including social attachment, pair bonding etc. [1]. Harnessing the prosocial effects of OT, the effectiveness of exogenous OT in ameliorating some of the social deficits of ASD makes it a potential pharmacological intervention in autism. OT administration has been shown to ameliorate anxiety-like behaviour, aberrant fear memory and inducing prosocial behaviour in prenatal valproate (VPA)-induced rodent models of ASD. [2] [3] [4]. Intranasal OT has been associated with improving some of the social and emotional deficits in humans [5] [6].

Dysfunctional central and peripheral OT system is established in ASD. Single nucleotide polymorphism of OT gene is often implicated in autism [7]. Modahl et al reported lower plasma OT levels in autistic children as compared to normal children [8]. Green and colleagues performed radioimmunoassay in plasma samples of 6 to 11 years old autistic males and matched controls suggesting altered oxytocin processing in autistic children. Their results indicate a reduction in plasma OT levels in autistic individuals and an elevation in the level of the immature uncleaved form (C-terminus-extended form). In autistic children, the age-related rise in extended form of OT indicates failure of developmental progression in peptide processing with age [9]. Additionally, Munesue's group reported lower plasma OT in 29 ASD patients [10]. Studies also suggest a link between SNPs of oxytocin receptor (OTR) gene and ASD [11] [12] [13] [14] [15] [16] further establishing an involvement of impaired OT functionality in autism.

OT has been demonstrated to reduce food-cue-induced, CNS-mediated reward anticipation by attenuating functional connectivity between the VTA and brain regions associated with the cognitive, sensory and emotional processing of food images in overweight/obese men in an fMRI study conducted by Kerem et al. [17]. Intranasal OT decreases food intake in obese humans [18], attenuates reward-driven consumption

[19] and regulates energy homeostasis and metabolism in humans (reviewed in [20]). In humans, OT exerts its anorexigenic effects in both fasted and sated states which implies that these effects are mediated through both homeostatic (hypothalamus) and hedonic (VTA, PFC, insula etc.) components governing eating behaviour coupled with higher cognitive functionality - thus associated with curbing aberrant food intake and thereby obesity [21] [22] [23]. This anorexigenic effects of OT is also widely studied in laboratory animals [24-26].

Atypical hunger processing was reported in the prenatal VPA-exposed rodent model of autism where animals were seen to have a self-inflicted restriction of consuming bland food [27] while being avid eaters of palatable diets [28]. We hypothesized the central dysregulation of OT that is widely explored to have implication in the context of social deficits of ASD, may be implicated in the context of aberrant consummatory behaviour of ASD as well. We employed neurohistological and neuromolecular analyses on prenatally VPA-exposed rat model of autism (hereby designated as VPA animals) to assess whether there is a baseline difference in central OT signalling. Then we looked into the effectiveness of intraperitoneal (i.p.) OT in terms of diminishing food intake in the VPA animals. In order to understand the mode of action of i.p. OT in reducing food intake, we conducted an analysis of c-Fos immunoreactivity in the brain areas implicated in governing consummatory behaviour in animals.

4.3. Materials and methods

4.3.1. Animals

Sprague-Dawley rats housed in standard cages with wire tops in a temperature-controlled (22 °C) facility with a 12:12 light:dark cycle were provided with *ad libitum* standard laboratory chow pellets (Sharpes, New Zealand; energy density: 3.6 kcal/g) and water unless otherwise specified. Animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The University of Waikato Animal Ethics Committee approved the procedures described herein (Protocol#1118).

4.3.1.1. Sodium Valproate Exposure

The process described in our previous works was used to generate the *in utero* VPA-exposed offspring. Vaginal smears from adult female Sprague-Dawley rats mated overnight were stained with 1% crystal violet. Upon identification of spermatozoa the day was assigned as E0.5. On E12.5, the females received a single i.p. injection of either 500 mg/kg sodium valproate (Sigma) dissolved at a concentration of 50mg/ml or isovolumetric standard physiological saline (0.9% NaCl – referred to as controls). Females were allowed to nurse and raise their offspring until weaning on postnatal day (PND) 25. Sodium valproate-exposed offspring (from here on referred to as VPA animals) were healthy and had no major morphological anomaly - approximately 12% of males developed crooked tails and some VPA animals developed transient chromodacryorrhea. These morphological traits were documented in VPA animals in earlier reports [29]. Only male animals were used in the experiments.

4.3.1.2. Establishing dose-response for i.p. OT

Adult VPA and their matched control animals were assigned to groups (N=10 per group). VPA animals weighed slightly less than the controls [27]. Using episodic feeding paradigms, we confirmed the effective dose of OT to reduce consumption for energy (intake of standard chow upon overnight food deprivation) and for palatability (intake of energy-dilute 15% sucrose solution and milk intake in sated animals).

To assess the effective dosage of i.p. OT (Syntocinon) that reduces consumption for hunger, chow was removed at 16:00 on the day preceding administration. At 10:00 on the experimental day, animals were injected i.p. with saline and OT (0.3 mg/kg, 1 mg/kg and 3 mg/kg). Immediately after the injection, standard chow was returned, and consumption was measured 2h and 4h later and consumption is reported as g/g of body weight. Data from drug-injected groups were compared with saline using one-way ANOVA followed by Dunnett's post-hoc test for control and VPA animals. Values are presented as means ± SEM and were considered significantly different when $p \leq 0.05$. 3 mg/kg dosage was not administered in the VPA animals as their lowest effective dosage was determined to be 1mg/kg.

To assess the effective dosage of i.p. OT that reduces consumption for palatability, animals with *ad libitum* access to chow and water were injected i.p. with saline and OT (0.3 mg/kg and 1 mg/kg). Both standard chow and water were not present in the cage during the duration of presentation of the palatable liquids. Bottles were weighed, drip was calculated; the amount of the solution consumed was reported in g/g of body weight.

4.3.2. Immunohistochemistry

Immunohistochemistry was performed following the protocol previously published with some modifications [27]. Experimentally naïve adult control and VPA animals were injected i.p. with either saline or 0.3mg/kg OT (N = 7 per group), 60 min after the administration, animals were anaesthetised with single i.p. administration of 35% urethane dissolved in 0.9% saline. Toe-pinch and palpebral reflexes were checked for deep anaesthesia. Then animals were transcardially perfused with 50 mL of saline followed by 500 mL of 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Brains were dissected out and 60 µm coronal sections were cut with a vibratome (Leica, Germany) and processed as free-floating sections for immunostaining.

For the c-Fos staining, sections were rinsed in 50 nM tris-buffered saline (TBS, pH 7.4–7.6), and then incubated for 10 min in 3% H2O2, 10% methanol (diluted in TBS) at room temperature. After rinsing in TBS, they were incubated at 4 °C for 48 h in rabbit-anti-Fos polyclonal primary antibody (diluted 1:1500; Synaptic Systems, Australia). Sections were washed in TBS and incubated for 1 h at room temperature in biotinylated-secondary goat-anti-rabbit antibody (1:400; Vector Laboratories). Following washes in TBS, sections were incubated for 1 h with avidin–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). All antibodies were dissolved in a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The tissues were visualised with 0.05% diaminobenzidine (DAB, Sigma), 0.01% H2O2 and 0.3% nickel sulfate (15–20-min incubation). Sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried overnight, dehydrated in ascending concentrations of ethanol followed by xylene, and embedded in Entellan (Merck KGaA, Darmstadt, Germany).

For the OT staining, sections were same immunostaining protocol was followed except the incubation in rabbit-anti-oxytocin primary antibody (Merck) was conducted in a dilution of 1: 8000 for 16h at 4 °C and the tissues were visualised with 0.05% diaminobenzidine (DAB, Sigma) and 0.01% H2O2.

For assessing active oxytocin cells, sections were co-stained with the anti-Fos and anti-oxytocin antibodies, followed by visualisation with DAB, then mounted onto gelatin-coated slides, air-dried overnight, dehydrated in ascending concentrations of ethanol followed by xylene, and embedded in Entellan.

Bright field images of immunohistochemically stained brain sections were acquired using an OMAX digital microscope camera attached to a Nikon Eclipse 400 microscope. Images were not processed digitally before or after the analysis; images across all groups were captured with the same brightness, contrast, resolution and aspect ratio. The number of c-Fos-positive nuclei per mm^2 was counted bilaterally for each neuroanatomical region of interest to reflect the density rather of c-Fos-positive neurons using ImageJ (Fiji). Therefore, any potential distortion caused during tissue processing and/or any possible neuroanatomical defect owing to VPA exposure would not affect the overall values reported. The number of total oxytocin-positive cells, cells with co-localised oxytocin and c-Fos and c-Fos per mm^2 were counted using ImageJ followed by the calculation of percentage of active OT-IR cells.

The neuroanatomical regions were identified with boundaries defined according to the Paxinos and Watson brain atlas in order. The following areas were analysed (in parentheses, anterior–posterior ranges of bregma levels of sections used to analyse each site are shown): Nacc(Core)—nucleus accumbens core (1.28–0.96); Nacc(Shell)—nucleus accumbens shell (1.28–0.96); AP—area postrema (−13.92 to −14.16); ARC—arcuate nucleus (−2.16 to −2.52); BLA—basolateral amygdala (−2.64 to −2.92); CEA—central nucleus of the amygdala (−2.64 to −2.92); DMH—dorsomedial nucleus of the hypothalamus (−3.00 to −3.24); NTS—nucleus of the solitary tract (−13.76 to −14.16); PVN—paraventricular nucleus of the hypothalamus (−1.56 to −1.92); SON—supraoptic nucleus (−0.96 to −1.2); VMH—ventromedial nucleus (−3.00 to −3.24);

4.3.3. Gene Expression analysis

4.3.3.1. Dissection of Hypothalamus and Brainstem

To assess baseline OT and OTR gene expression in the hypothalamus and brainstem of VPA and control rats, hypothalamus and brainstems were dissected from experimentally naive control (N=10) and VPA (N=12) animals. After dissection, the tissues were immersed in RNAlater (Ambion, Thermo Fisher Scientific, Auckland, New Zealand) for 2 h at room temperature and the samples were then frozen at −80 °C until further processing.

4.3.3.2. rtPCR Protocol and Data Analysis

A protocol previously described was followed. Tissues kept in RNAlater were homogenised in 400 µL Trizol (Ambion), 80µL chloroform was added after that and the samples were centrifuged at room temperature for 10 min at 10,000× g. The clear phase containing the total RNA was carefully taken out and precipitated using 0.5 mL isopropanol in an ice bath for 10 min followed by centrifugation at 4 °C for 20 min at 10,000× g. The aqueous phase was removed, keeping the pellets intact. The pellets were washed in 300 µL ethanol by centrifuging at 4 °C for 10 min at 10,000× g. The supernatant was removed carefully and the pellets were air dried.

Pellets were dissolved in 8 µL DEPC water and 1 µL DNAse buffer (dNature) followed by an incubation with 1 µL DNAse (dNature) per reaction at 37 °C for 30 min followed by inactivation of DNAse with ‘stop buffer’ (dNature), incubating at 67 °C for 10 min. Removal of genomic DNA was confirmed via PCR using HOT FIREPol Blend Master Mix (dNature), followed by agarose gel electrophoresis. Concentrations of RNA were measured (µg/µL) with a nanodrop.

cDNA was synthesised from RNA samples with iScript Advanced cDNA synthesis kit (BioRad). Quantification and purity of cDNA was determined using a Qubit 4 fluorometer (Invitrogen) and nanodrop. Assessment was performed using two technical replicates. An amount of 4 µL of 25 ng/µL cDNA template was used per transcript along with 5 µM primer specific to ot and otr transcripts, 10 µL iTaq Universal

SYBR Green Supermix (BioRad) and 4 µL MilliQ water. The gene expression were normalized with housekeeping genes – Actin B, TBP, Tubulin β3 that showed consistent and stable expression in both the cohorts. MilliQ water was used as a template for negative controls for each transcript. NCBI-BLAST® was used to check the specificity of the primer pairs *in silico* and the optimum annealing temperature was assessed by a separate reaction subjecting to a gradient of temperature prior to setting up the reactions for quantitation.

4.3.3.3. List of primers:

Housekeeping Genes		
Gene	F	R
TBP	5′-AGAACAATCCAGATACAGCA-3′	5′-GGGAACTTCACATCACAGCTC-3′
Tubulin beta 3	5′-TGCTGGCCATTCAGAGTAAGA-3′	5′-ACTCAGACACCAGGTCGTTCA-3′
Actin B	5’-AGTGTGACGTTGACATCCGT-3’	5’-TGCTAGGAGCCAGAGCAGTA-3’
Gene of interest		
ot	5′-GACGGTGGATCTCGGACTGAA-3′	5′-CGCCCCTAAAGGTATCATCACAAA-3′
otr	5′-GATCACGCTCGCCGTCTA-3′	5′-CCGTCTTGAGTCGCAGATTC-3′

4.3.4. Data Analysis

Data from the food intake experiments were analysed using GraphPad Prism: one-way ANOVA followed by Dunnett's post-hoc test (each dosage of i.p. OT being

compared to saline) for control and VPA animals after validation of normal distribution using Shapiro Wilk, and Kolmogorov Smirnov test for normality. Outliers were excluded from analysis using Grubb's test of extreme studentised deviate. Values were considered significantly different for $p \leq 0.05$.

Densities of c-Fos-positive nuclear profiles (per mm2) were averaged per individual, and then per group. Data between the two groups (Control saline vs. 0.3mg/kg OT and VPA saline vs. 0.3mg/kg OT) in each cohort were compared using independent two-sample Student's t-tests considering homoscedastic distribution. Outliers were excluded from analysis using Grubb's test of extreme studentized deviate. Values were considered significantly different for $p \leq 0.05$.

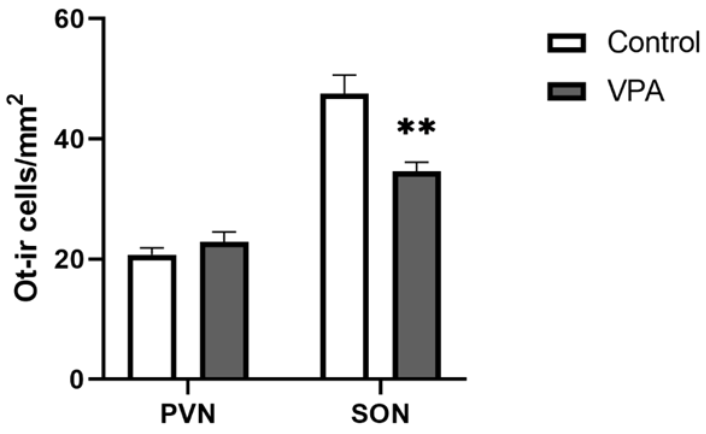
Analyses of rtPCR data utilised BioRad CFX Manager software (BioRad); rtPCR results were normalised with housekeeping genes and the ΔCq values followed by ΔΔCq and plotted as 2^-ΔΔCq. Data were analysed with a Student's t-test. Values were considered significantly different when $p \leq 0.05$.

4.4. Results

4.4.1. Determination of baseline OT expression:

VPA animals showed significantly reduced number of OT-IR neurons in the SON but not in the PVN as compared to their matched controls (t=3.512, df=11, P=0.002) (Fig 4-1.)

A.



B.

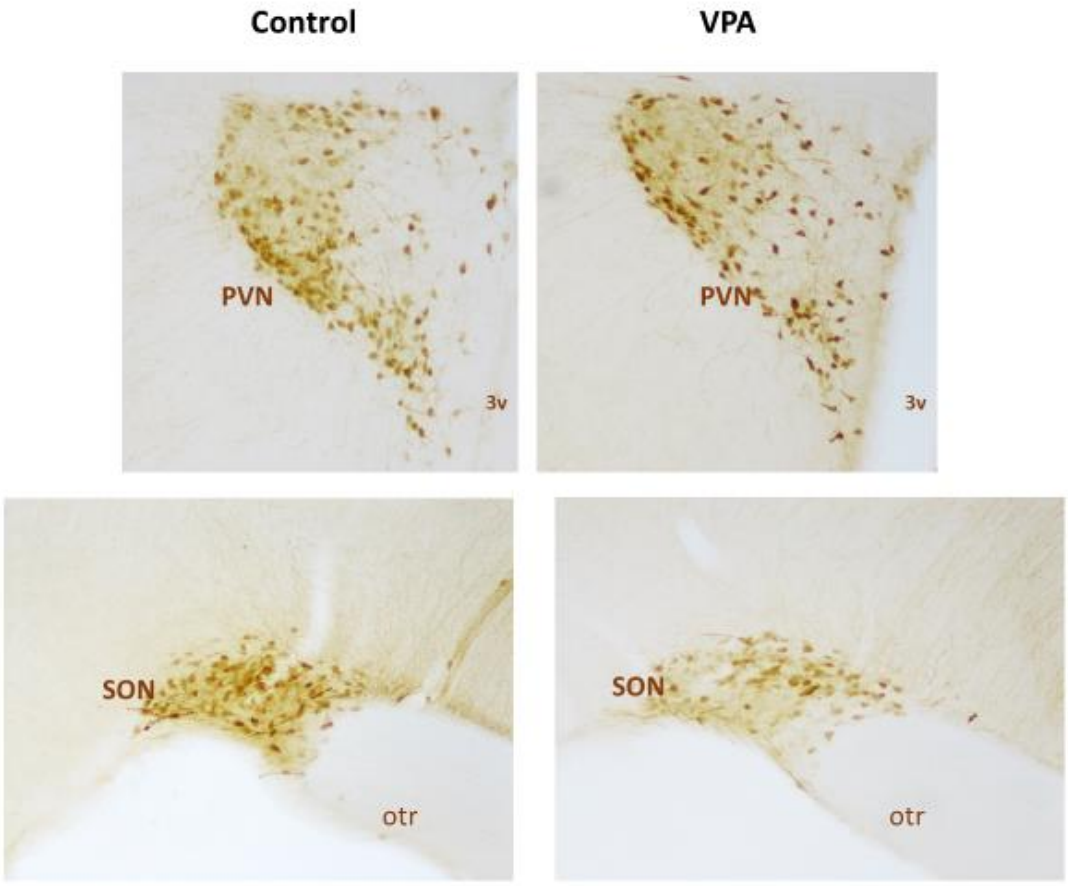


Figure 4-1: VPA animals show central OT deficiency. (A) Panel (B) Photomicrograph representing VPA animals with reduced number of OT-IR cell sin the SON

Data are expressed as mean ± SEM, N = 7/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

qRTPCR analysis indicated a trend of lower OT gene expression in the hypothalamus of the VPA animals (t=1.467, df=16, P= 0.081) while expression pattern of OT (t=2.169, df=16, P=0.023) and OTR (t=2.236, df=19, P=0.019) in the brainstem of the VPA animals were significantly reduced (Fig 4-2.).

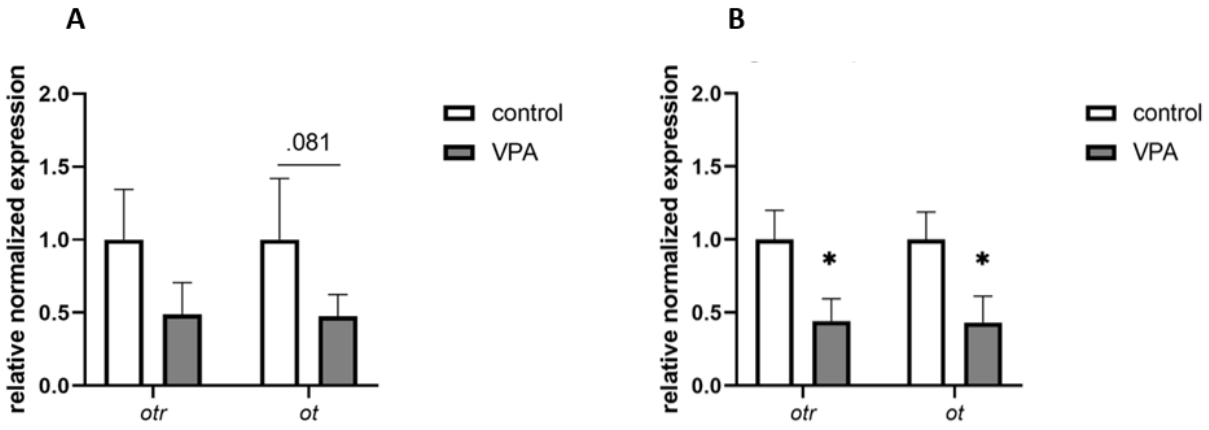


Figure 4-2: Differential expression of pattern OTR and OT in the VPA animals (A) in the hypothalamus (B) in the brainstem

Data are expressed as mean ± SEM, N = 10-12/group. * $p < 0.05$

OT - oxytocin, OTR- oxytocin receptor

4.4.2. Determination of lowest effective dosage of i.p. OT on deprivation-induced intake of standard chow

Acutely administered i.p. OT resulted in a decrease in deprivation-induced intake of standard chow. 3 mg/kg was the lowest effective dose that reduced deprivation-induced intake of standard chow in the control animals (F (DFn, DFd): F (3, 35) = 5.314; adjusted P = 0.033) (Fig 4-3A).

While 1 mg/kg OT was not sufficient to decrease deprivation-induced standard chow intake in the controls, for VPA animals a significant decrease was observed at 1 mg/kg OT (F (DFn, DFd): F (2, 27) = 5.292, adjusted P = 0.006) (Fig 4-3B). After 4h, this effect was abolished in both control and VPA animals

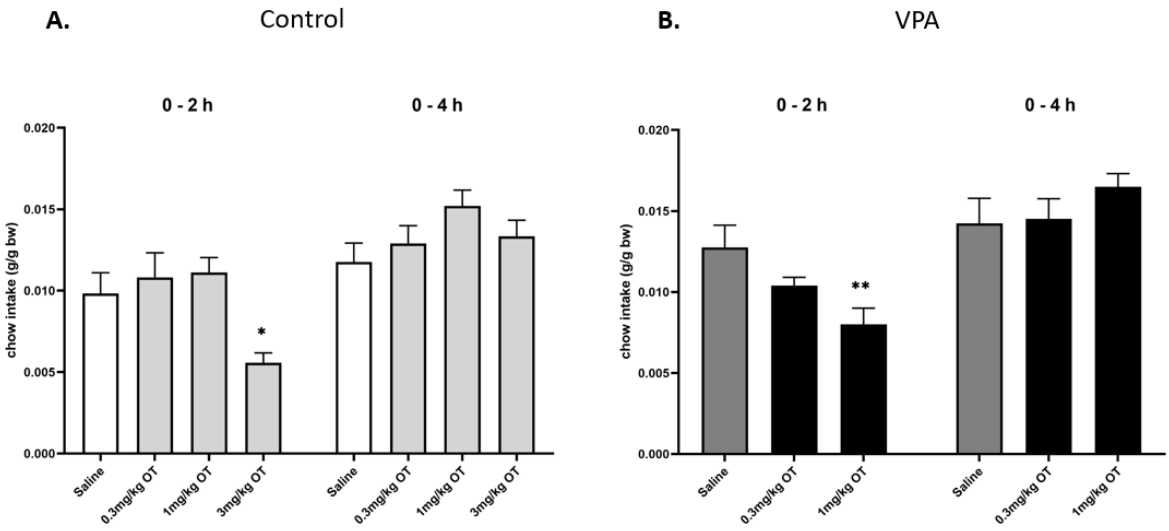


Figure 4-3: Effect of i.p. saline and OT on the intake of standard chow after overnight deprivation-induced intake of standard chow measured after 2h and 4h after administration. (A) Intake of standard chow after overnight deprivation in control animals (B) Intake of standard chow after overnight deprivation in VPA animals. Data are expressed as mean ± SEM, N = 10/group. * $p < 0.05$, ** $p < 0.01$.

4.4.3. Determination of lowest effective dosage of i.p. OT on palatability-driven intake

Acutely administered OT resulted to a decrease in intake of 15% sucrose with the lowest effective dosage of 1 mg/kg over a temporal window of 2h in the control animals (F (DFn, DFd): F (2, 25) = 4.822, adjusted P=0.009). This effect persisted even after 4h of administration (F (DFn, DFd): F (2, 26) = 8.155; adjusted P<.001). (Fig 4-4A).

For VPA animals, a significant decrease was observed at 0.3 mg/kg OT over a temporal window of 2h (F (DFn, DFd): F (2, 27) = 22.87, adjusted $P_{0.3mg/kg\ OT}$<.001, adjusted $P_{1mg/kg\ OT}$<.001). VPA animals continued with a decreased consumption after

4h of administration (F (DFn, DFd): F (2, 27) = 20.45, adjusted P $_{0.3mg/kg\ OT}$<.001, adjusted P $_{1mg/kg\ OT}$<.001). (Fig 4-4B)

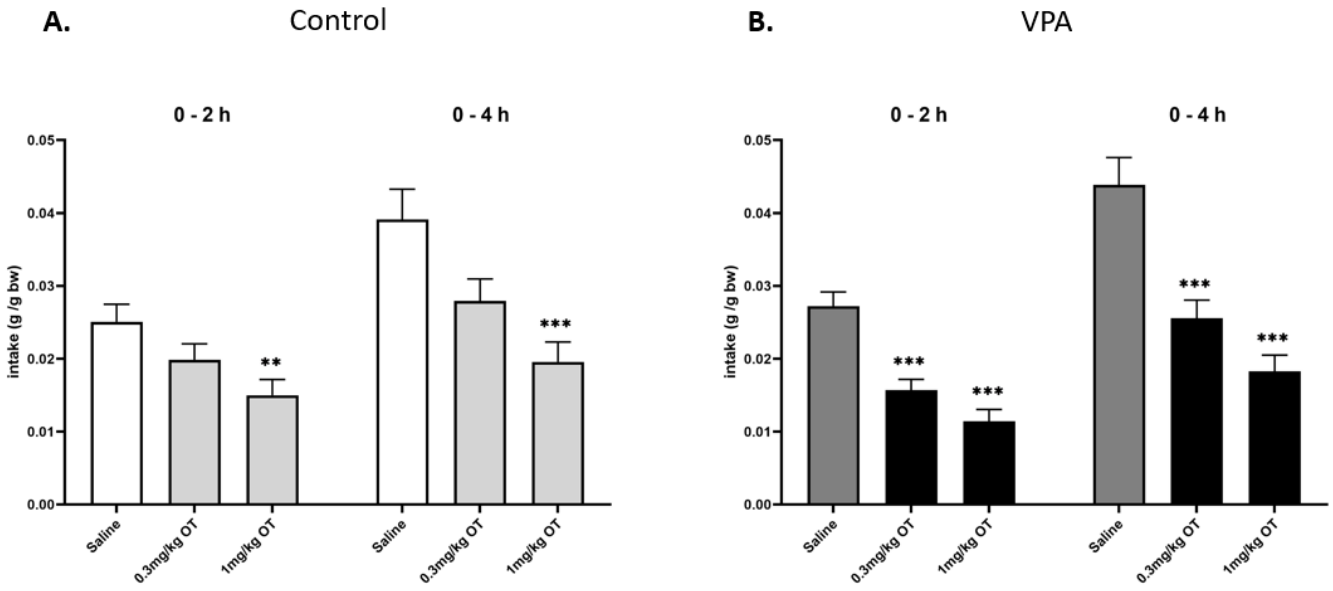


Figure 4-4: Effect of i.p. saline and OT on episodic intake of 15% sucrose while the animals were not energy deprived. (A) intake of 15% sucrose in the control animals (B) intake of 15% sucrose in the VPA animals. Data are expressed as mean ± SEM, N = 10/group. * p < 0.05, ** p < 0.01, ***p<0.001.

Acutely administered OT resulted in a decrease in intake of milk. 1 mg/kg tended towards a reduction in milk intake in the control animals (F (DFn, DFd):F (2, 22) = 1.969, adjusted P=0.114). This effect was abolished after 4h of administration. (Fig 4-5A)

For VPA animals, a decrease was observed upon OT administration over a temporal window of 2h (F (DFn, DFd): F (2, 19) = 6.298; adjusted P $_{0.3mg/kg\ OT}$ = 0.005, adjusted P $_{1mg/kg\ OT}$ = 0.085). They continued to consume reduced amount of milk even after 4h of admninistration (F (DFn, DFd): F (2, 19) = 8.598, adjusted P $_{0.3mg/kg\ OT}$ = 0.002, adjusted P $_{1\ mg/kg\ OT}$ = 0.053 (Fig 4-5B)

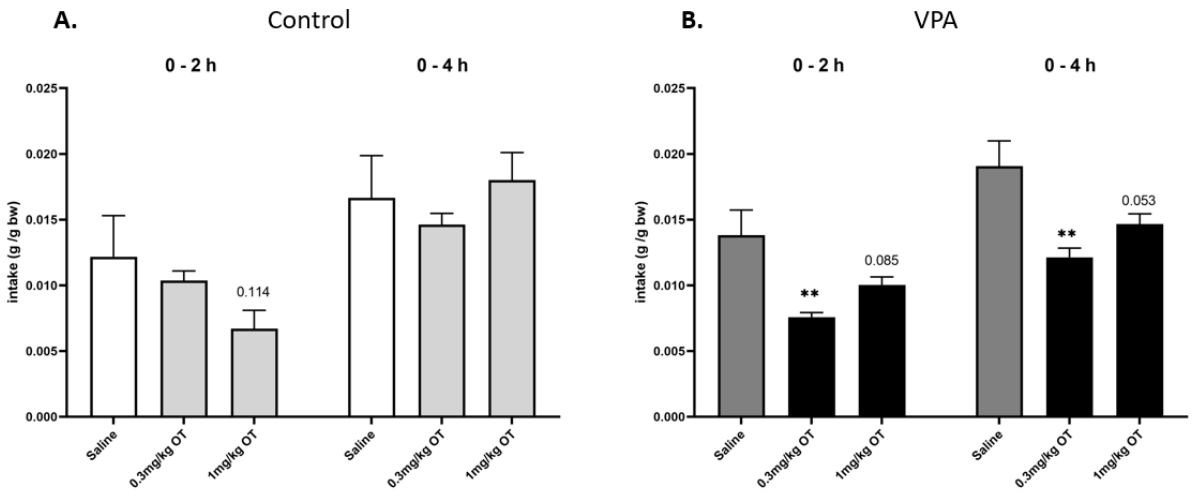
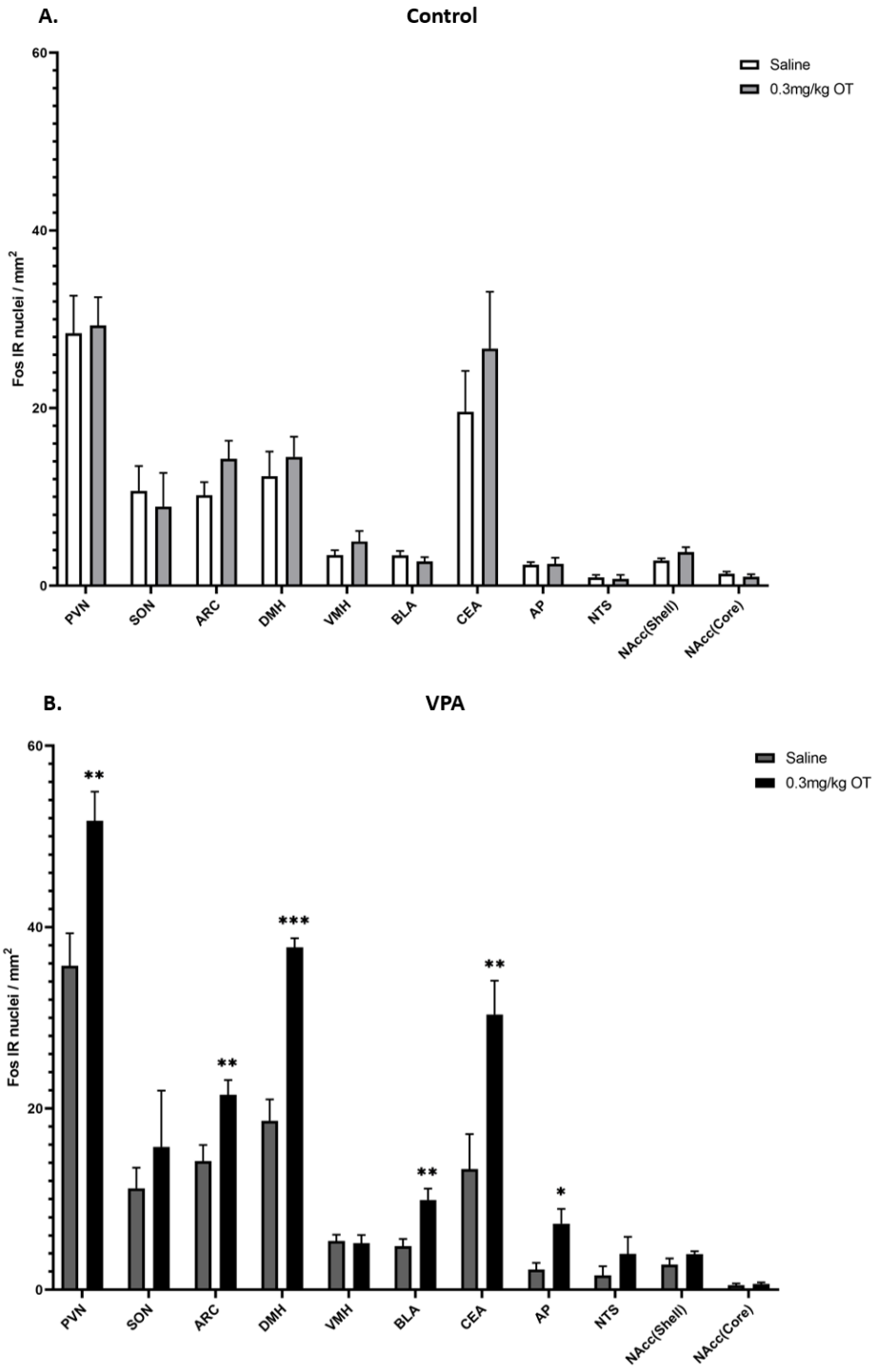
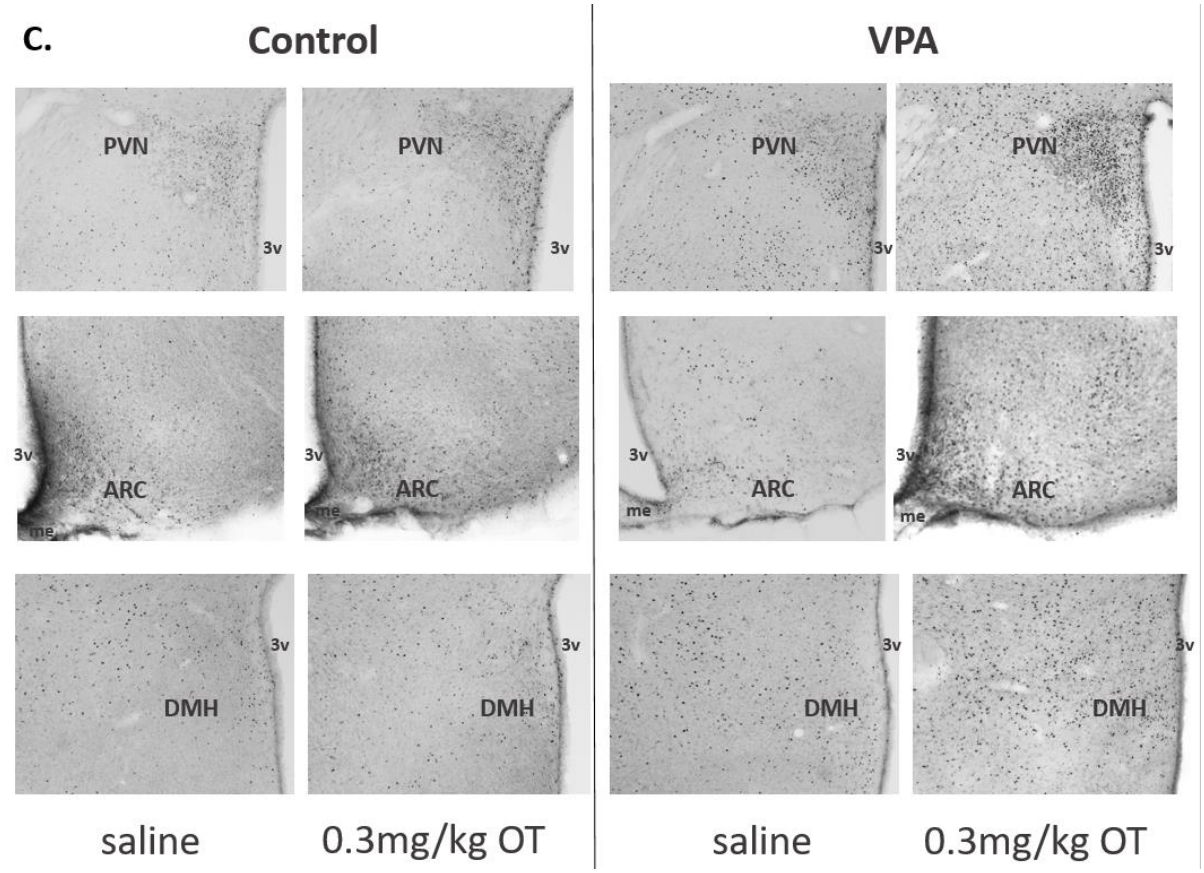


Figure 4-5: Effect of i.p. saline and OT on episodic intake of milk while the animals were not energy deprived. (A) intake of milk in the control animals (B) intake of milk in the VPA animals. Data are expressed as mean ± SEM, N = 10/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

4.4.4. Effect of 0.3 mg/kg i.p. OT administration on the number of c-Fos-IR neurons

Control animals injected i.p. with saline and 0.3 mg/kg OT show no significant difference in c-Fos immunoreactivity in brain regions relevant for food intake (Fig 4-6A). VPA animals showed elevated c-Fos immunoreactivity in PVN ($t=3.273$, $df=11$, $P=0.004$), ARC ($t=2.930$, $df=12$, $P= 0.006$), DMH ($t=6.664$, $df=12$, $P<.001$), BLA ($t=3.533$, $df=13$, $P= 0.002$), CEA($t=3.189$, $df=12$, $P= 0.004$) and AP($t=2.679$, $df=11$, $P=0.011$) upon administration of 0.3 mg/kg OT (Fig 4-6B)


A.
Control
Saline
0.3mg/kg OT
Fos IR nuclei / mm2
60
40
20
0
PVN
SON
ARC
DMH
VMH
BLA
CEA
AP
NTS
NAcc(Shell)
NAcc(Core)
B.
VPA
Saline
0.3mg/kg OT
Fos IR nuclei / mm2
60
40
20
0
**
**
***
**
**
*
PVN
SON
ARC
DMH
VMH
BLA
CEA
AP
NTS
NAcc(Shell)
NAcc(Core)


C.
Control
VPA
PVN
3v
ARC
me
DMH
saline
0.3mg/kg OT
saline
0.3mg/kg OT

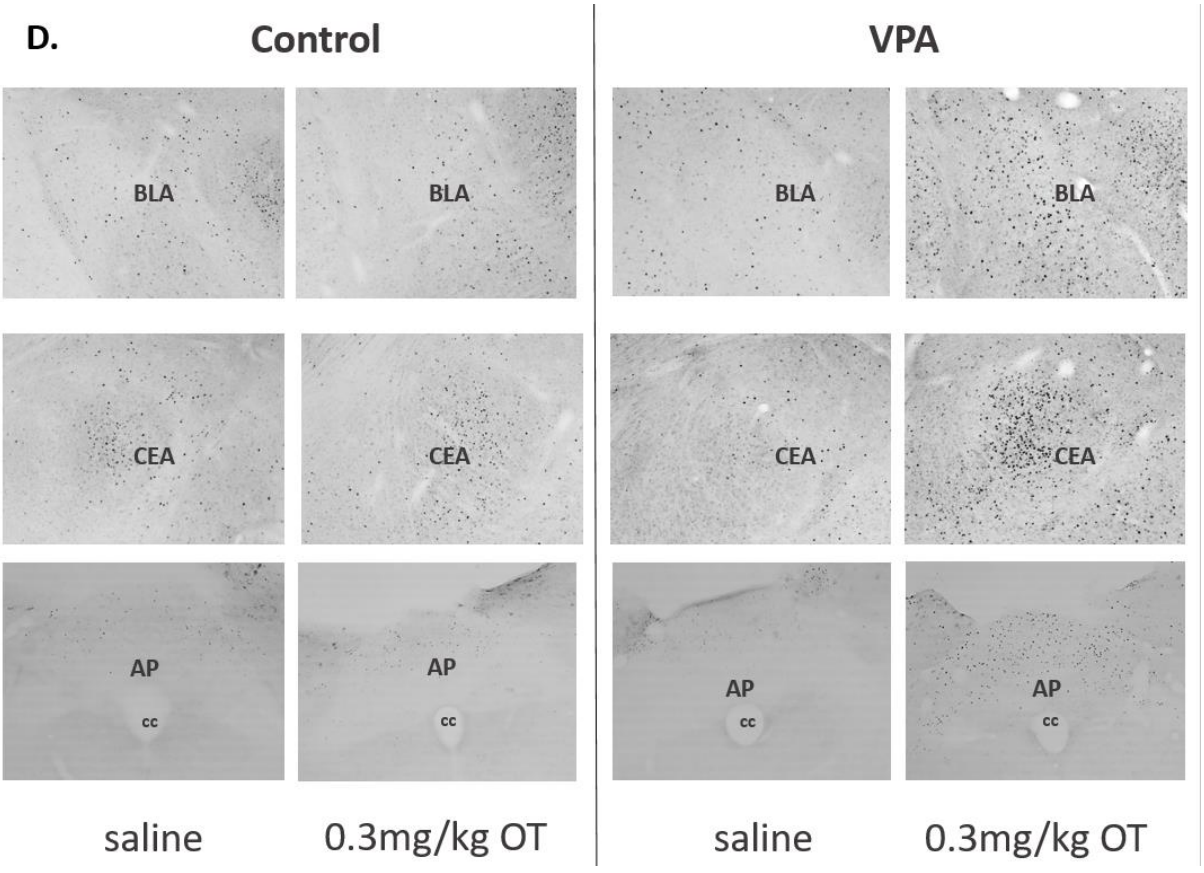
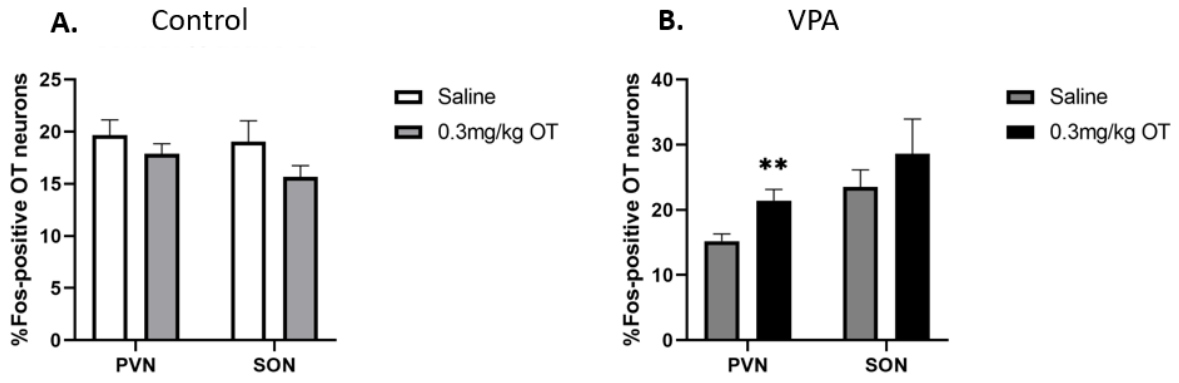

D.
Control
VPA
BLA
CEA
AP
cc
saline
0.3mg/kg OT
saline
0.3mg/kg OT

Figure 4-6: c-Fos immunoreactivity in animals administered i.p. with saline and 0.3 mg/kg OT

(A) Control animals show no significant difference in c-Fos immunoreactivity
(B) VPA animals show elevated c-Fos immunoreactivity in PVN, ARC, DMH, BLA, CEA and AP.
(C) Representative photomicrographs depicting sites that showed a significant difference in c-Fos levels in hypothalamus
(D) Representative photomicrographs depicting sites that showed a significant difference in c-Fos levels in BLA, CEA and AP
PVN—paraventricular nucleus of the hypothalamus, SON—supraoptic nucleus, ARC—arcuate nucleus, DMH—dorsomedial hypothalamus, VMH—ventromedial hypothalamus, NTS—nucleus tractus solitarius, AP—area postrema, Nacc(Shell)—Nucleus accumbens shell, Nacc(Core)—Nucleus accumbens core, BLA—basolateral amygdala, CEA—Central nucleus of the amygdala
Data are expressed as mean ± SEM, N = 7/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

Effect of 0.3 mg/kg i.p. OT administration on the percentage of active OT neurons was assessed. Control animals injected i.p. with saline and 0.3 mg/kg OT showed no significant difference in the percentage of active OT cells in PVN and SON. VPA animals injected i.p. with saline and 0.3 mg/kg OT showed significantly higher percentage of active OT neurons in the PVN of hypothalamus ($t=3.056$, $df=12$, $P = 0.005$) (Fig 4-7)



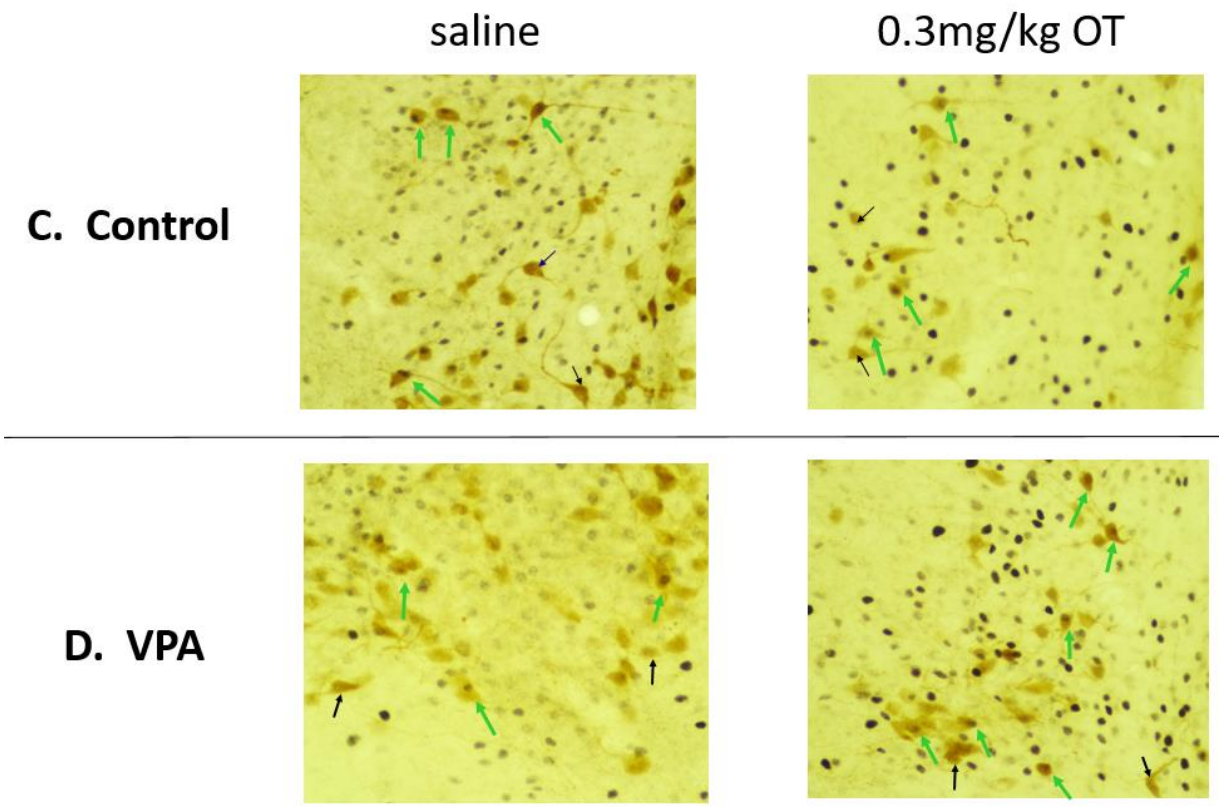


Figure 4-7: Percentage of active OT neurons in animals administered i.p. with saline and 0.3 mg/kg OT.(A) Control animals showed no significant difference in the percentage of active OT cells in PVN and SON. (B) Control animals showed a significantly higher percentage of active OT neurons in the PVN of hypothalamus. (C) Photomicrographs representing c-Fos-positive OT neurons in the control animals (D) Photomicrographs representing c-Fos-positive OT neurons in the VPA animals. Green arrowheads represent the active OT neurons; black arrowheads represent inactive OT-IR neurons.

Data are expressed as mean ± SEM, N = 7/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

4.5. Discussion

Because of the close functional implication of OT in food intake and a dysfunctional OT system established in ASD, we explored for the first time the role of OT in the context of aberrant food intake in the VPA-induced rat model of autism. Here, we report the impairment of the central OT system in the VPA rats. The number of OT-IR cells were lower in the SON of the hypothalamus of the VPA rats compared to their matched

controls and OT gene expression in the hypothalamus and the brainstem was downregulated in the VPA animals. We also observed a shift in dose-response to food intake with acute administration of i.p. OT – there was an elevated responsiveness to lower doses of OT in the VPA animals, while the controls remained unresponsive to the anorexigenic effect of OT at the same dosage showing no decrease in food intake during both hunger-driven intake and palatability-driven intake. We then assessed the effect of acute i.p. administration of the lowest effective dose of OT (0.3mg/kg) on the brain areas responsible to govern consummatory behaviour in rodents. The results reveal an elevated c-Fos-IR in PVN, ARC, DMH, BLA, CEA and AP in the VPA animals while the controls show a lack of significant difference in c-Fos immunoreactivity at the same dosage. Additionally, to assess whether systemic administration of OT causes any change in the activity of OT neurons of the main OT producing brain areas, PVN and SON of the hypothalamus, we found, at the lowest effective dose, the percentage of the active OT cells in the PVN increased significantly in the VPA animals while the controls remained unresponsive.

The fact that the VPA animals have a lower number of OT-IR cells in the SON but not in the PVN corroborates an earlier finding of Dai et al who reported diminished number of OT-IR cells in the SON but not in the PVN in *in utero* VPA-exposed rat pups compared to control pups at infancy [30]. The lower number of OT neurons may be a result of the chemical insult by the teratogenic HDAC-inhibitor VPA [31] at embryonic day 12.5 – the time when the hypothalamus development reaches its peak. VPA interferes with wnt signalling [32, 33] crucial for neuronal differentiation, maturation, migration and establishment of neural network. Previous studies have reported that *in utero* VPA-exposed rats had fewer neurons in several brain regions (such as prefrontal and somatosensory cortices [34] and some motor nuclei [35]). This, in part explains the lower number of OT-IR cells in the hypothalamus of the VPA animals as compared to the controls.

This finding, coupled with the trend of lower expression of OT mRNA in the hypothalamus [30] and downregulated OT and OTR mRNA in the brainstem of the VPA animals point towards a dysregulated OT system. Although hypothalamic PVN and SON are the chief OT-producing sites, OT immunoreactivity was detected in brainstem areas such as mesencephalic trigeminal nucleus, fibres of the pyramidal

tract, trapezoid body, spinal trigeminal nucleus, pontine nucleus and external cuneate nucleus in adult rat brain. [36] OTR is expressed in the brainstem vestibular nuclei, motor and sensory cranial nerve nuclei and autonomic centres-including the NTS [37]. Reduced OTR mRNA expression was reported in postmortem brains of patients with schizophrenia in brain areas implicated in integrative, higher cognitive functionality and motor execution - Brodmann area 10 (BA10), BA21, nucleus caudatus and vermis [38]. Lower plasma OT levels and reduced OTR protein expression were reported in patients with borderline personality disorder [39]. These results support the possible contribution of downregulated OTR in the dysfunctional OT signalling relevant in the context of compromised social cognition in patients with neuropsychological disorders pertinent to ASD as well. Intuitively, lower OT expression would lead to elevated expression of OTR. However, whether the decreased ligand and receptor gene expression are two independent events or whether one downregulation feeds into the downregulation of the other and/or whether the mRNA turnover rate in VPA animals affects the gene expression remain elusive. In sum, our findings support impaired OT system in the VPA rats.

In general, i.p. OT causes an increase in OT concentration in the brain extracellular fluid (ECF) and plasma during the first 30 min after treatment and a subsequent return towards baseline [40]. Even though the blood-brain barrier (BBB) permeability, for i.p. OT is low - the concentration of OT in the blood has to be substantially high (more than 200 times than that of the brain ECF) to initiate the crossing through the BBB [41], this could be a plausible pathway for i.p. OT to act on the brain.

The elevated responsiveness to i.p. OT and a shift in the lowest effective dose in the VPA group may be due to the dysfunctional OT system. Smith et al demonstrated plasma OT levels to remain significantly elevated for longer in OT-KO mice after acute i.p. administration. In OT-KO mice, plasma OT levels return to baseline after more than 120 min, while plasma OT levels in controls return to baseline after only 60 min - indicating a difference in pharmacokinetics [41]. This could be the case in the VPA animals as well. The elevated levels of OT in plasma for longer period would lead to a sustained elevated levels of intracerebral OT [40] [41] [42] owing to slow clearance. Thus, the combined effect of differential pharmacokinetics and slower

clearance of OT from the brain may result to an elevated responsiveness of VPA animals to 0.3mg/kg i.p. OT.

OT reduces the time spent eating and increases the latency to first meal in schedule-fed rats fasted for 21h [43]. OT causes early termination of feeding, thus acts as a mediator of early satiation rather than diminishing the subjective perception of hunger [44]. The lowest effective dose of OT (3mg/kg OT and 1mg/kg OT for the controls and the VPA animals respectively) caused early termination of the meal (deprivation-induced intake of standard chow) - reducing the intake over the window of 2h. Owing to a short half-life of OT in the plasma, the effect was abolished by 4h with a compensatory increase in intake. VPA animals, showing early satiation at a lower dosage, also showed a compensatory increase in feeding by 4h. This supports the previous findings that reports the effectivity of OT to decrease hunger-driven food intake [45, 46] and the VPA rats show an elevated responsiveness to i.p. OT.

Evidence shows that the anorexigenic effect of OT is exerted in the context of reward-driven eating [45]. OT is implicated in reward-motivated behaviours in animals - activation of the OTR in the NAcc core decreases methamphetamine-seeking behaviour [47]. Reward-driven appetite for palatable carbohydrates is affected by intracerebral administration of OT. For example, consumption of 10% sucrose in non-deprived animals was reduced after OT administration in the amygdala while administration of the OTR antagonist L-369,899 produced the opposite effect [48]. The elevated responsiveness to OT in VPA animals in reducing the consumption of palatable sugar solution is an outcome of multiple combinatorial factors - the elevated plasma OT for a prolonged period coupled with the feed-forward release of hypothalamic OT upon peripheral administration of OT [49] enhancing the effect.

Animals with OT deficiency show elevated propensity for palatability-driven consumption and this effect is more prominent in sucrose intake [50] but not lipid [51]. Mice with i.p. administration of BBB penetrant L-368,899 demonstrated elevated consumption of carbohydrate but not lipid solutions [52] implying OT inhibits appetite for carbohydrate [53]. OT mRNA levels in the hypothalamus of rats eating a scheduled, high-sugar diet was upregulated compared to OT gene expression in rats exposed to standard chow [54], and this increase in OT gene expression following the intake of a

high-sugar diet was consistent in short-term exposure studies as well [53] [52], [28] OT-induced reduction of palatable diet intake is more pronounced in sugar and rather blunted in milk owing to the lipid content and the whey content of milk influencing the activity of OT neurons in the PVN [55].

0.3 mg/kg i.p. OT for rats is quite low to induce c-Fos immunoreactivity in the brain areas relevant for food intake and to affect food intake behaviour [25, 28] [24]. Arletti et al reported a dose-dependent decrease in consumption in freely feeding Sprague Dawley rat with the lowest effective dose being 375 µg/kg [43]. Other behavioural data suggest that intraperitoneal 0.3 mg/kg i.p. OT does not cause any change in locomotor activity in male Sprague Dawley rats [56]. The elevated c-Fos expression in some brain areas in response to the same dosage of OT in the VPA animals implies the neurohistological underpinning of elevated responsiveness to exogenous OT demonstrated by reduced food intake upon relatively lower dosage of OT as compared to their matched controls.

The increase in density of c-Fos positive neurons in the PVN may indicate the anorexic action of i.p. OT by inducing release of endogenous OT by feed-forward manner at the PVN which recognized as an integrative centre for feeding and energy metabolism [57]. The neuroanatomical positioning of ARC enables it to sense various nutrients and hormones and convey neural information to other brain areas such as the PVN, where peripheral and central information is integrated [58]. PVN sends OT projection to the brainstem - thus activation of PVN may lead to activation of AP [59]. OT neurons from the PVN also project to the CEA and modulate food consumption by affecting reward seeking aspect of consummatory behaviour through positive-valence mechanisms [60]. Centrally administered OT to the amygdala acts upon OTR and reduces sugar consumption [48]. The elevated c-Fos level in BLA and CEA may possibly be implicated in reward-seeking behaviour, an orchestration between motivational and homeostatic feeding mechanisms and an effect of OT on the emotional aspects of feeding.

Elevated number of c-Fos-positive neurons were found in the AP in the VPA rats upon OT administration. The AP has a leaky BBB - thus the elevated plasma OT may directly affect the neurons in the AP by binding to OTRs. The neurons in the AP project

to the NTS neurons, which in turn project to the ARC and PVN [61]. This can be a mode of action of peripheral OT in suppressing food intake. Previous studies reported i.p. OT administration to bring out elevated c-Fos expression in the NTS [24, 62].Our analysis does not show a significant increase in c-Fos immunoreactivity in the NTS probably because at this dose the neural signal transduction through the AP-to-NTS projection is relatively muted to induce this immediate early gene.

Our finding of the elevated activation status of the OT-IR cells of the PVN implicate the involvement of intracerebral OT signalling in the VPA animals to reduce food intake. The OT-producing parvocellular neurosecretory cells and some collaterals of the magnocellular neurosecretory cells of the PVN are projected to brainstem, NAcc, hippocampus, cortices and amygdala thereby affecting both homeostatic (via ARC, DMH [63]) and hedonic (via BLA, CEA) components of consumption.

Vagal afferent neurons express the OTR mRNA and protein [66] and peripherally administered OT exerts its anorexigenic effects via the vagus nerve [67-69]. We did not observe any difference in c-Fos expression in the DMNV; neither in control nor in the VPA group (data not shown). This is not surprising for the control animals since 0.3 mg/kg i.p. OT for rats is quite low to induce c-Fos immunoreactivity in the brain areas relevant for food intake. Although, the VPA animals show elevated responsiveness to exogenous OT, we did not observe any difference in c-Fos immunoreactivity in the DMNV. It is reported by several authors that ASD individuals, to have low vagal tone [70-74]. Our observation of no significant difference in c-Fos immunoreactivity in the VPA rats likely indicates that 0.3mg/kg i.p. OT is insufficient to cause any effect via the vagus nerve in these animals.

As shown in the previous chapters, VPA animals display atypical hunger processing and they exhibit overconsumption of palatable tastants, including sugar. This is observed in humans as well - ASD individuals are prone to obesity [64] [65] possibly because of a skewed dietary preference – usually sugar. OT- intranasal application of which is already known to ameliorate some of the social deficits in humans has the potential to be an effective pharmacological tool to curb overconsumption of sugar and

other palatable diets with carbohydrate as the major compositional macronutrient in the context of autism.

4.6. Conclusion

In this study, we observed an elevated responsiveness of *in utero* VPA-exposed rats to low doses of OT. 0.3 mg/kg OT administered i.p. were sufficient to reduce food intake in these animals while healthy controls remained unresponsive to the same dosage. This effect was more pronounced when the animals were exposed to sucrose. Combined with a differential brain activity pattern in the VPA animals, these results indicate a higher sensitivity to the anorexogenic effects of peripherally administered OT.

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Chapter 5
Impaired Conditioned Taste Aversion in Adult Male Rats with Sodium Valproate-Induced Autism

5.1. Abstract

In the Conditioned Taste Aversion (CTA) paradigm, animals learn to avoid tastants whose intake has previously been followed by malaise or gastrointestinal discomfort, thereby reducing the dangers of consuming toxic substances. This protective mechanism allows animals to avoid harmful substances for survival. A wide array of memory-, gustatory- and stress-related responses coupled with an orchestrated interplay of central-peripheral signals govern the acquisition of a CTA. As shown in the previous chapters of this thesis, the VPA animals show atypical neural processes governing hunger, satiety and reward-driven intake. Therefore we hypothesize that the acquisition of CTA may be different in the VPA animals because of aberrant neural processes along with a dysfunctional OT system. In this set of experiments we used lithium chloride (LiCl)-induced aversion and showed that the dosage of LiCl that produces neural responses along with activation of the OT neurons in the control animals failed to exert any effect on the VPA animals. This suggests that the responsiveness of the VPA animals to CTA is lower which is indicated by the lack of change in response of the neural system to aversive stimulus of LiCl.

5.2. Introduction

Consummatory behaviour, while fulfilling organism's metabolic and nutritional needs, poses a risk of ingesting plant/animal material harbouring toxins. Thus, there are mechanisms that facilitate avoidance of (potentially) harmful substances. For example, bitter tastes that oftentimes are associated with tainted foods, are generally avoided [1, 2] and novel foods whose postingestive effects have not yet been

experienced by an individual, are consumed with an increased latency and in smaller quantities[3]. Importantly, if the intake of that novel food results in malaise of GI discomfort, animals learn to avoid it in the future through a process of Conditioned Taste Aversion (CTA). Subsequent presentations of an aversive food lead to hypophagia, suppression of hunger, and decreased preference for this tastant, thus induce a complex set of gustatory, stress and memory-based processes [4-8].

CTA is of particular importance to rodents since they do not exhibit an emetic reflex [4], and thus have to rely on a CTA as a crucial mechanism to minimise risks of food-borne toxicity.

In the laboratory setting, a CTA is typically induced by presenting a novel tastant (the unconditioned stimulus) followed by an injection of a noxious substance (the conditioned stimulus), such as lithium chloride (LiCl), to cause short-lived malaise. The animal then associates the sickness with the novel food and will avoid it upon subsequent exposures resulting in a conditioned response [5].

OT regulates CTA. Neurohypophyseal secretion of OT has been observed following administration of aversion-inducing toxins (LiCl, CuSO4) [9]. Furthermore, OTR-antagonist L-368,899 leads to blunted responsiveness of the Central Nucleus of Amygdala (CEA) to peripherally injected LiCl in mice [10]. In addition, agents that reduce activation of OT neurons tend to reduce taste aversion [11]. Panguluri et al showed upregulated OT mRNA expression in CTA acquisition in adult male Sprague-Dawley rats [12]. This reflects the stress associated with the CTA procedure because OT can dampen stress responses involving the amygdala [13]. Additionally, central administration of OT has been shown to influence memory-consolidation in a passive-avoidance learning paradigm [14]. Considering the fact that the malfunctioning OT system in ASD underpins atypical feeding as shown in the previous chapters, one could pose a question whether this affects taste aversion as well.

When presented with highly palatable foods, upon repeated exposures, animals consume large quantities [15, 16]. The dysfunctional OT system observed in ASD predisposes autistic individuals to even greater overconsumption of palatable diets which, in theory, should disrupt the internal milieu triggering sickness. Furthermore,

pica often co-occurs with ASD, where individuals continue to consume inedible substances in spite of having pathophysiological consequences. Previously, a couple of studies with animal models of ASD reported aberrant CTA response but the neural bases is unknown [17, 18].

Here we used a prenatally VPA-exposed rat-model of autism (hereby designated as VPA animals) to assess LiCl-induced CTA responses. We then employed neurohistological analyses with c-Fos immunoreactivity to examine brain activity patterns following the malaise-inducing LiCl exposure in these animals.

5.3. Materials and methods:

5.3.1. Animals

Sprague-Dawley rats housed in standard Plexiglas cages with wire tops in a temperature-controlled (22 °C) animal room with a 12:12 light:dark cycle were provided with *ad libitum* standard laboratory chow pellets (Sharpes, New Zealand; energy density: 3.6 kcal/g) and water unless otherwise specified. The University of Waikato Animal Ethics Committee approved the procedures described herein (Protocol #1155).

5.3.2. Sodium Valproate Exposure

The process described previously [19] was used to generate the *in utero* VPA-exposed offspring. Adult female Sprague-Dawley rats were mated overnight with age-matched Sprague-Dawley males. Early the next day, the vaginal smears were stained with 1% crystal violet to detect spermatozoa and upon identification of spermatozoa, the date was assigned as E0.5. Females received a single intraperitoneal (i.p.) injection of either 500 mg/kg sodium valproate (Sigma) or isovolumetric physiological saline (0.9% NaCl) i.p. on E12.5. Female rats treated with sodium valproate were healthy and no significant difference between the litter sizes of sodium valproate-treated animals and those of controls was observed. Females were allowed to nurse and raise their offspring until weaning on postnatal day (PND) 25. Sodium valproate-exposed offspring (from here on referred to as VPA animals) were healthy and had no major

morphological anomaly - approximately 12% of males developed crooked tails and some VPA animals developed transient chromodacryorrhea [20]. Animals prenatally exposed to physiological saline will be referred to as controls. Only male animals were used in the experiments.

5.3.3. Confirmation of ASD-like phenotypes (Elevated plus Maze test)

In order to assess exploration of a novel environment, each rat (PND 30–35) was initially placed into the open field arena for 5 min before being placed in the elevated plus maze for another 5 min. The maze was elevated 50 cm above the ground and had two open arms and two closed arms of 50 × 10 × 40 cm dimensions. The arms were opposite each other and there was an open roof arrangement. The rat was always placed at the centre of the plus maze facing an open arm. The maze was cleaned with 70% ethanol before and after each rat was placed inside. The time spent and number of entries into the open arm was noted, along with the time spent in the closed arms for every rat. An entry into the open arm was defined as both paws being on or beyond the boundary of the closed arms. To assess whether the observation was merely a collateral effect of anxiety owing to a novel environment, self-grooming time was also evaluated as a parameter of anxiety-like behaviour. Data was analyzed by independent two-sample Student's t-tests considering homoscedastic distribution. Values were considered significantly different for $p \leq 0.05$.

5.3.4. Establishing responsiveness to i.p. LiCl

Adult VPA and their matched control animals were assigned to groups (N=8-10 per group) with no difference in body weight between groups. VPA animals weighed slightly less than the controls as we observed previously as well.

Experimentally novel adult control animals deprived of water overnight were exposed to 0.1% saccharin. After 1h Saline, 0.6mEq/kg, 3mEq/kg and 6mEq/kg LiCl (N=8/group) were administered i.p. 48h after that animals were deprived of water overnight (16h). Water and 0.1% saccharin were presented simultaneously to the cage

and 2h intake was measured. Drips from the bottles were calculated; the amount of the solution consumed was reported in percentage intake of saccharin.

Data from drug-injected groups were compared with saline using one-way ANOVA followed by Dunnett's post-hoc test for control and VPA animals. Values are presented as means ± SEM and were considered significantly different when $p \leq 0.05$.

5.3.5. Immunohistochemistry

Immunohistochemistry was performed following the protocol previously described with some modifications.

Adult control and VPA animals were injected i.p. with either saline or 3mEq/kg LiCl (N = 8-10 per group), 60 min after the administration, animals were anaesthetised with 35% urethane dissolved in 0.9% saline i.p. Once toe-pinch and palpebral reflexes were absent, transcardial perfusion was performed with 50 mL of saline followed by 500 mL of 4% paraformaldehyde in phosphate buffered saline (pH 7.4). Brains were excised and 60 µm coronal sections were cut with a vibratome (Leica, Germany) and processed as free-floating sections for immunostaining.

For the c-Fos staining, sections were rinsed in 50 nM tris-buffered saline (TBS, pH 7.4–7.6), and then incubated for 10 min in 3% H_2O_2, 10% methanol (diluted in TBS) at room temperature. After rinsing in TBS, they were incubated at 4 °C for 16 h in rabbit-anti-Fos monoclonal primary antibody (diluted 1:12000; Synaptic Systems, Australia). Sections were washed in TBS and incubated for 1 h at room temperature in biotinylated-secondary goat-anti-rabbit antibody (1:400; Vector Laboratories). Following four washes in TBS, sections were incubated for 1 h with avidin–biotin peroxidase complex (1:800; Elite Kit, Vector Laboratories). All antibodies were dissolved in a solution of 0.25% gelatin and 0.5% Triton X-100 in TBS. The tissues were visualised with 0.05% diaminobenzidine (DAB, Sigma), 0.01% H_2O_2 and 0.3% nickel sulfate (15–20-min incubation). Sections were washed four times in TBS to stop the reaction, mounted onto gelatin-coated slides, air-dried overnight, dehydrated in ascending concentrations of ethanol followed by xylene, and embedded in Entellan (Merck KGaA, Darmstadt, Germany).

For assessing active oxytocin cells, sections were co-stained with the anti-Fos and anti-oxytocin antibodies (1:5000, Merck), followed by visualisation with DAB. Then, sections were mounted onto gelatin-coated slides, air-dried overnight, dehydrated in ascending concentrations of ethanol followed by xylene, and embedded in Entellan.

Bright field images of immunohistochemically stained brain sections were acquired using an OMAX digital microscope camera attached to a Nikon Eclipse 400 microscope. Images were not processed digitally before or after the analysis; images across all groups were captured with the same brightness, contrast, resolution and aspect ratio. The number of c-Fos-positive nuclei per mm2 was counted bilaterally for each neuroanatomical region of interest using ImageJ (Fiji), with boundaries defined according to the Paxinos and Watson brain atlas. Thus the comparison of the activation levels reflects the density rather than the raw number of Fos-positive neurons. Therefore, any potential morphological changes caused during tissue processing and/or a probable effect of the VPA exposure did not impact the overall values reported here.

The number of total oxytocin-positive cells, cells with co-localised oxytocin and c-Fos and c-Fos per mm2 were counted using ImageJ. The percentage of active oxytocin cells was calculated as well.

The following areas were analysed (in parentheses, anterior–posterior ranges of bregma levels of sections used to analyse each site are shown): Nacc Core—nucleus accumbens core (1.28–0.96); Nacc Shell—nucleus accumbens shell (1.28–0.96); AP—area postrema (−13.92 to −14.16); ARC—arcuate nucleus (−2.16 to −2.52); BLA—basolateral amygdala (−2.64 to −2.92); CEA—central nucleus of the amygdala (−2.64 to −2.92); DMH—dorsomedial nucleus of the hypothalamus (−3.00 to −3.24); NTS—nucleus of the solitary tract (−13.76 to −14.16); PVN—paraventricular nucleus of the hypothalamus (−1.56 to −1.92); SON—supraoptic nucleus (−0.96 to −1.2); VMH—ventromedial nucleus (−3.00 to −3.24);

5.3.6. Data Analysis

Data from the food intake experiments were analysed using GraphPad Prism one-way ANOVA followed by Dunnett's post-hoc test for control and VPA animals after validation of normal distribution using Shapiro Wilk, and Kolmogorov Smirnov test for normality. Outliers were excluded from analysis using Grubb's test of extreme studentized deviate. Values were considered significantly different for $p \leq 0.05$. Two way ANOVA was used to assess VPA X treatment interaction.

Densities of c-Fos-positive nuclear profiles (per mm^2) were averaged per individual, and then per group. Data between the two groups (control saline vs. LiCl and VPA saline vs. LiCl) in each cohort were compared using independent two-sample Student's t-tests considering homoscedastic distribution. Values were considered significantly different for $p \leq 0.05$.

5.4. Results

Prior to undertaking the feeding studies, we conducted selected behavioural tests in order to confirm that the VPA rats generated by the *in utero* pharmacological challenge displayed a set of traits characteristic of autism. In those feeding-unrelated behavioural tests, VPA rats showed elevated anxiety-like behaviour (assessed by increased self-grooming ($t(24)= -4.03903$; $P=0.000477$) in a novel spatial-exploration paradigm (Elevated Plus maze). The reduced number of entries to the open arms ($t(24)=3.27469$; $P=0.003204$) coupled with lesser amount of time spent in the open arms ($t(24)=3.12196$, $P=0.004636$) further bolster the anxiety-like behaviour in these animals (Figure 5-1)

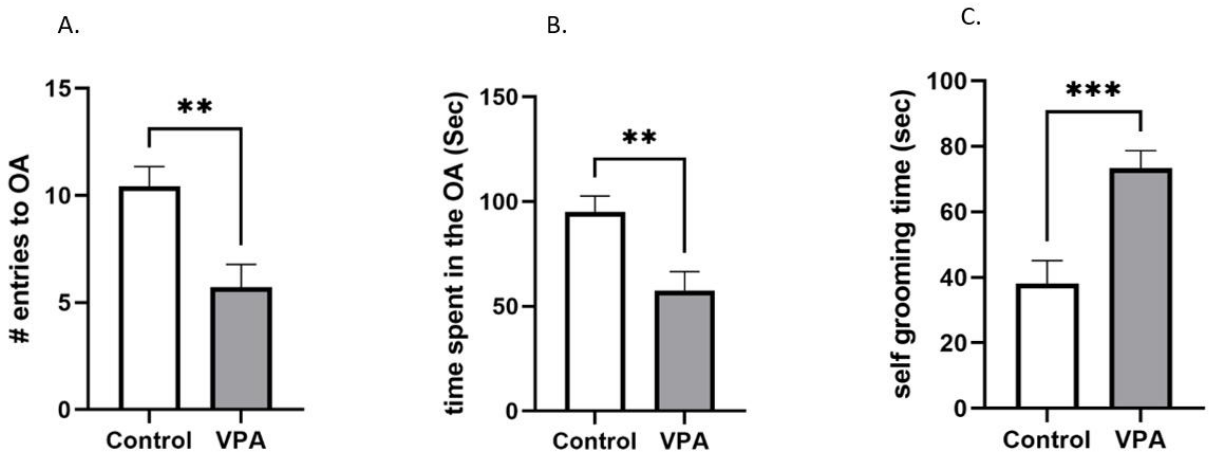


Figure 5- 1: Behavioural tests in order to confirm that the VPA rats generated by the *in utero* pharmacological challenge displaying ASD-like phenotypes in Elevated Plus Maze. (A) Number of entries to the open arms (B) time spent in the open arm were recorded. ASD rats spent a greater percentage of time in the closed arms and entered the open arms less often than controls. (C) Elevated self-grooming was observed in these animals. Data are expressed as mean ± SEM, n = 12-14/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Body weights of the in-utero VPA-exposed animals were lower compared to their healthy controls (control 584.625 ±9.377511741; VPA 461.5 ± 20.11315214) ($t(32)=6.349$;$P< 0.00001$).

During the two-bottle test, non-VPA animals that had been treated with 3mEq/kg and 6 mEq/kg LiCl ingested significantly less of the 0.1% saccharin solution than their saline-treated counterparts (F (DFn, DFd): F (3, 33) = 6.592 adjusted $P_{3mEq/kg}$ LiCl = 0.025; adjusted $P_{6mEq/kg}$ LiCl = 0.002). On the other hand, VPA animals treated with LiCl even at a dose as high as 6mEq/kg, did not develop a CTA. (Figure 5-2)

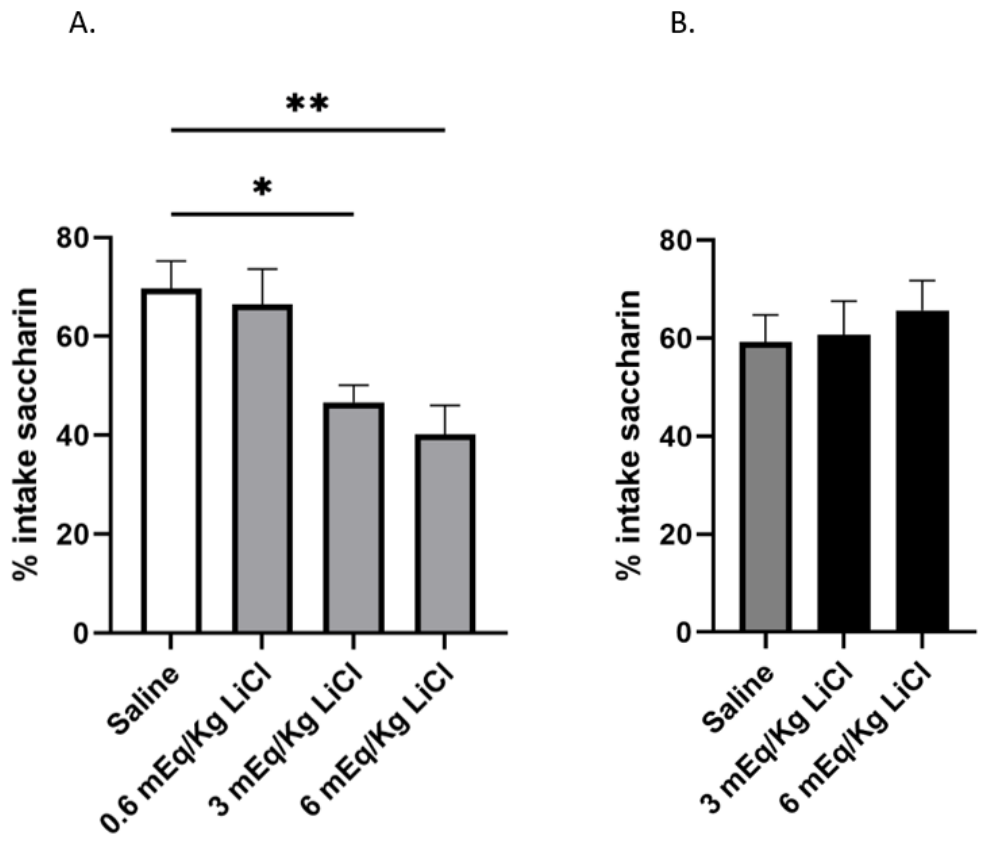
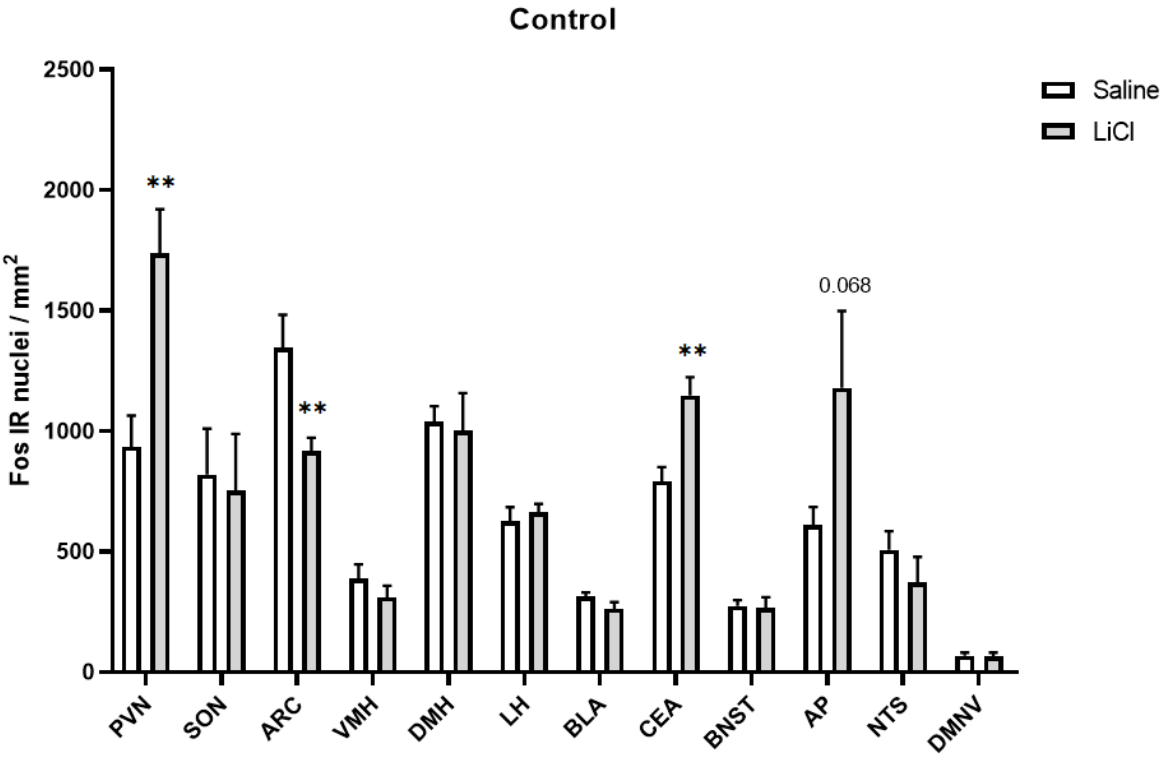


Figure 5- 2 : Effect of i.p. saline and LiCl on the acquisition of CTA to 0.1% saccharin. The graphs show the % intake of the saccharin solution during a two-bottle test in which a choice between water and saccharin was given. (A) Intake in control animals (N=10/group) (B) Intake in VPA animals (N=8/group). Data are expressed as mean ± SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

In the follow-up immunohistochemical analysis, we found different neuronal activation levels after saline or 3 mEq/kg LiCl in the VPA versus control rats. Control animals injected i.p. with saline vs 3mEq/kg LiCl show significantly elevated c-Fos IR in the PVN ($t=3.492$, $df=13$, $P= 0.002$), CEA ($t=3.742$, $df=11$, $P=0.002$) and a strong trend in the AP ($t=1.612$, $df=11$, $P= 0.068$), whereas c-Fos IR was significantly lower in the ARC ($t=3.076$, $df=11$, $P=0.005$). (Fig 3A). In contrast, 3 mEq/kg LiCl did not induce changes in c-Fos IR in those regions in VPA animals (Fig 3B). Two-way ANOVA shows strongest VPA X treatment interaction in the CEA ($P=0.008$) and a strong trend of interaction in the PVN ($P=0.061$) and in the AP ($P=0.051$) (Supplementary Fig. 1)


Control
Saline
LiCl
Fos IR nuclei / mm2
2500
2000
1500
1000
500
0
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PVN
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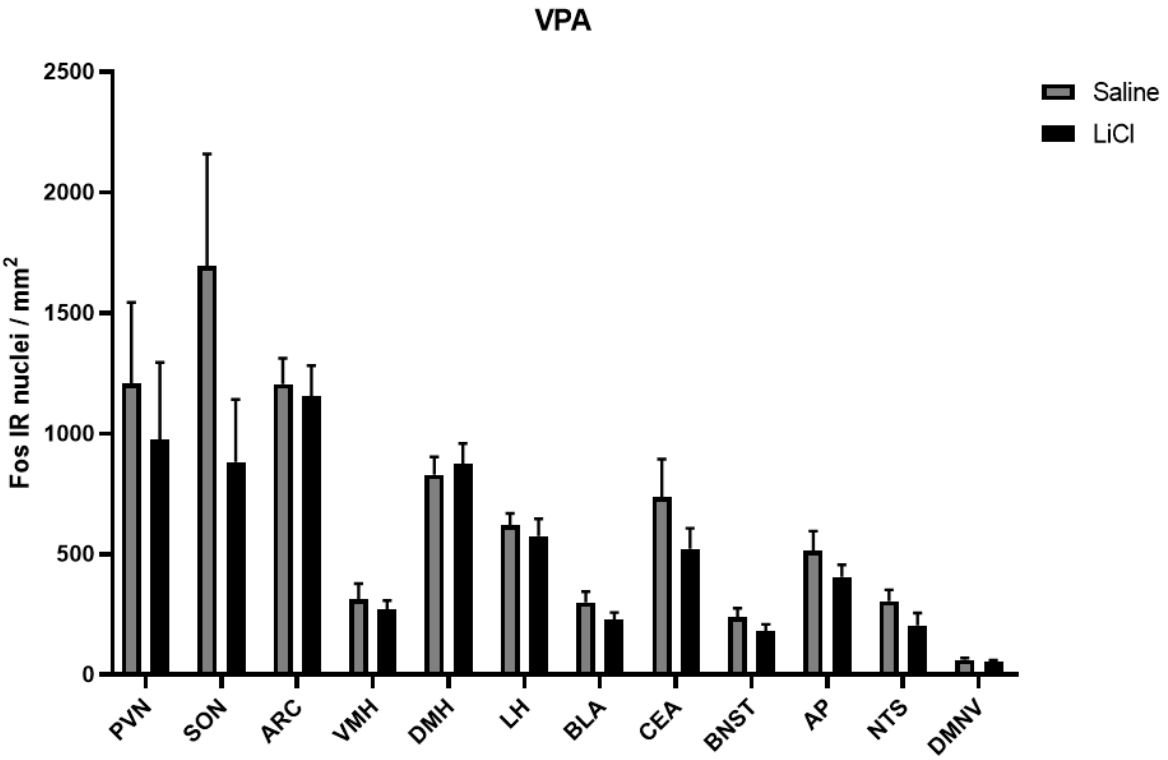
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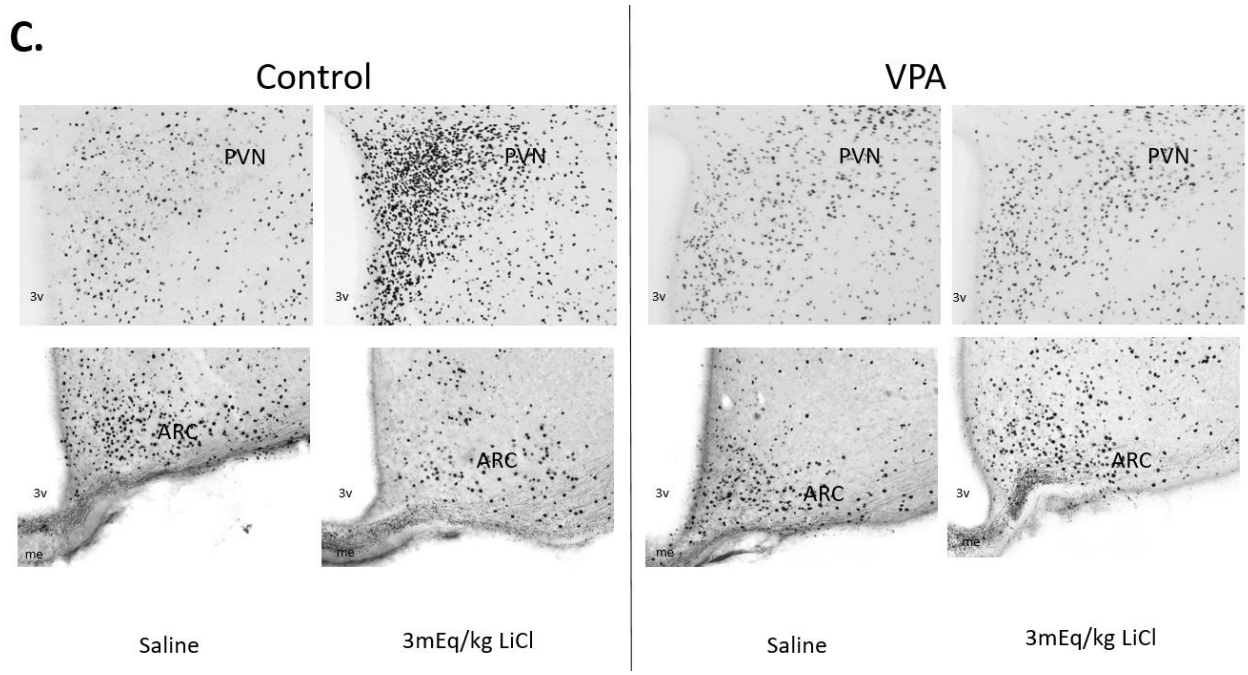
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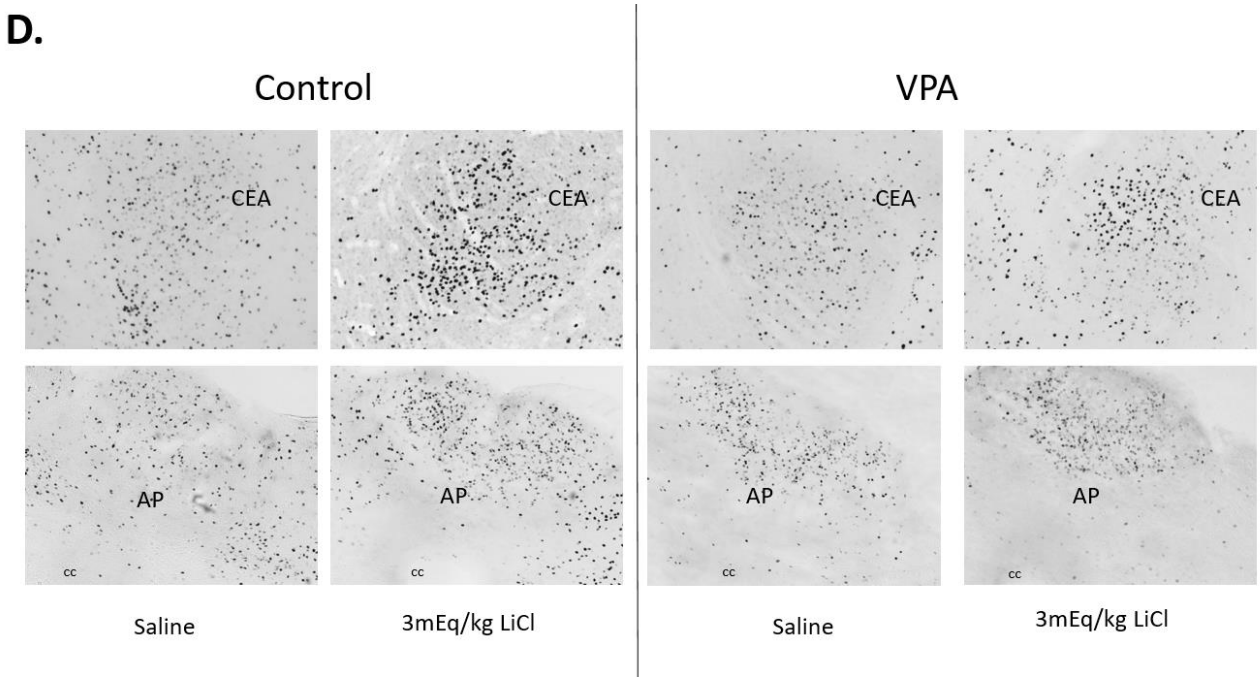

Figure 5- 3: c-Fos immunoreactivity in animals administered i.p. with saline and 3mEq/kg LiCl

(A) Control animals injected i.p. with saline vs 3mEq/kg LiCl show significant difference in c-Fos immunoreactivity in PVN, ARC, CEA and a strong trend of elevated c-Fos in the AP

(B) VPA animals injected i.p. with saline vs 3mEq/kg LiCl show no significant difference in c-Fos immunoreactivity

(C) Representative photomicrographs depicting sites that showed a significant difference in c-Fos levels in hypothalamus

(D) Representative photomicrographs depicting sites that showed a difference in c-Fos levels in CEA and AP

PVN—paraventricular nucleus of the hypothalamus, SON—supraoptic nucleus, ARC—arcuate nucleus, DMH—dorsomedial hypothalamus, VMH—ventromedial hypothalamus, NTS—nucleus tractus solitarius, AP—area postrema, BLA—basolateral amygdala, CEA—Central nucleus of the amygdala

Data are expressed as mean ± SEM, N = 6-10/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$

Control animals injected i.p. with 3mEq/kg LiCl showed a significant increase in the percentage of active OT cells in PVN ($t=2.052$, $df=7$, $P=0.040$) compared to the saline-

injected group, while the VPA animals displayed no statistically significant change after LiCl-exposure compared to saline. (Figure 5-4)

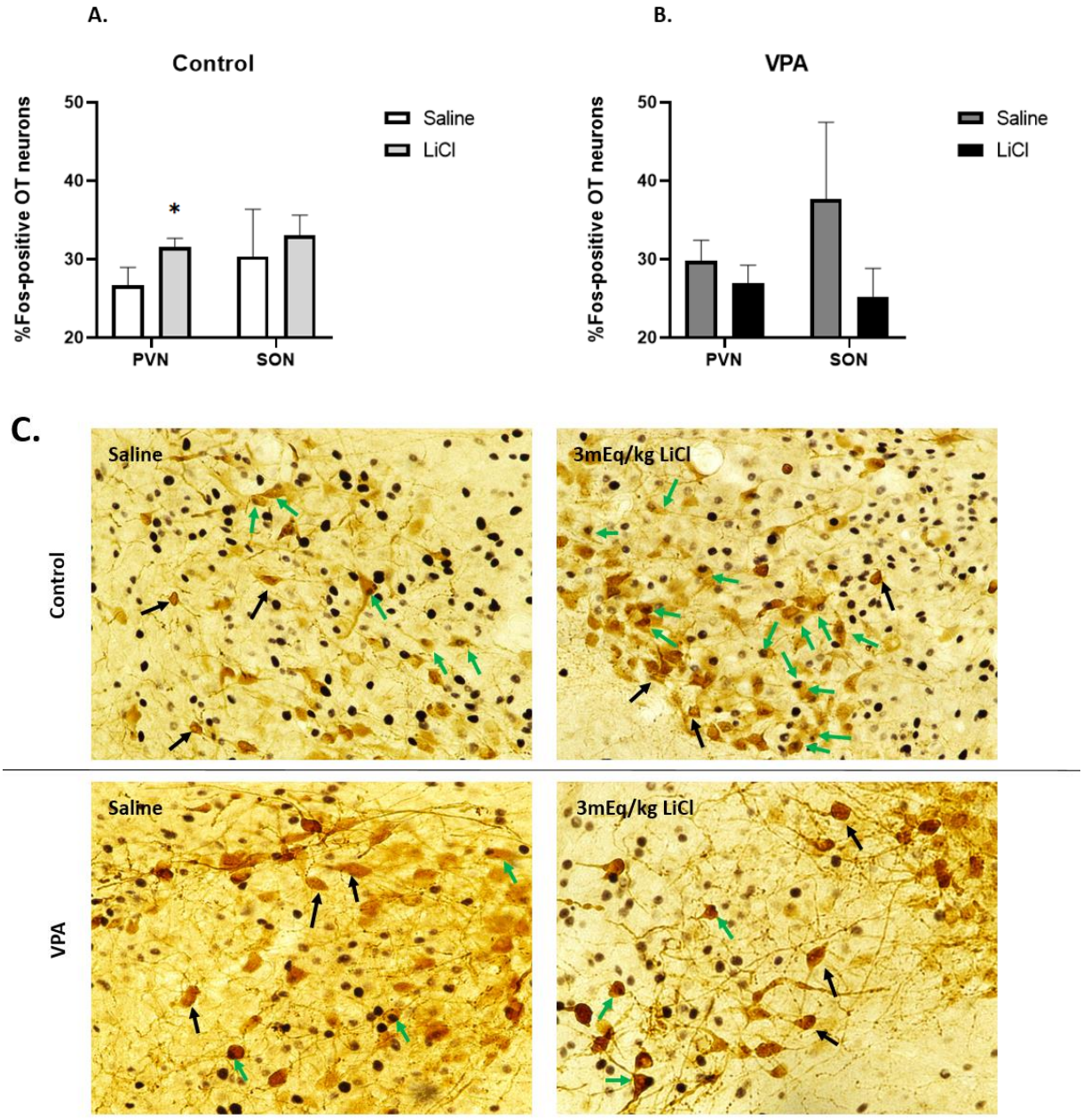

Figure 5- 4: Percentage of active OT neurons in animals administered i.p. with saline and 3mEq/kg i.p. LiCl administration

(A) Control animals showed a statistically significant increase in the percentage of active OT cells with 3mEq/kg LiCl in the PVN but no difference in the SON, (B) VPA animals showed no difference in percentage of active OT neurons in the PVN and SON of hypothalamus compared to saline-injected controls. (C) Photomicrographs

representing c-Fos-positive OT neurons in the control and VPA animals. Green arrowheads represent the active OT neurons; black arrowheads represent inactive OT-IR neurons.
Data are expressed as mean ± SEM, N = 5-8/group. * $p < 0.05$, ** $p < 0.01$, ***$p<0.001$.

5.5. Discussion

In Chapter 4 we established a relationship between the dysfunctional OT system in the VPA animals and aberrant feeding was observed in these animals. In this chapter, we examined the neuronal response to LiCl-induced aversion – or lack thereof – in the prenatally VPA-induced rat model of autism. We found that VPA rats do not acquire the typical aversive response to a novel tastant that is evoked by a standard dosage of i.p. LiCl in the control animals. LiCl decreases feeding behaviour in healthy control animals [21, 22]. Thus, we investigated the neuronal activation pattern by quantifying c-Fos-positive nuclei in the brain areas responsible to govern feeding behaviour. Our results reveal an elevated c-Fos immunoreactivity in the PVN, CEA and AP and diminished c-Fos immunoreactivity in the ARC in the control animals upon i.p. 3mEq/kg LiCl administration while the VPA animals showed no statistically significant difference in c-Fos immunoreactivity upon LiCl administration. Additionally, to assess whether LiCl administration causes any change specifically in the activity of OT neurons, we found that the percentage of active OT cells in the PVN were significantly higher in the control animals while no change in the percentage of active OT cells was observed in the VPA animals upon LiCl treatment.

OT neurons from parvocellular PVN projects to brainstem sites that regulate feeding (NTS, AP, DMNV). OT has been proposed to inhibit food intake in order to prevent the organism from ingestion of toxic substances. Central mechanisms responsible for the development and maintenance of aversion-induced hypophagia involve brainstem sites: the NTS, AP, DMNV and PBN which take part in the recognition and integration of peripheral aversive signals, including the presence of toxins in the circulation indicating the importance of not only the hypothalamic OT system, but also the relay sites that allow the OT neurons to act at the central target sites. The processes of nausea and vomiting result from continuous interactions between the gastrointestinal

tract (enterochromaffin cells of the GI mucosa, the enteric nervous system, vagus and splanchnic nerves), the CNS, and the autonomic nervous system. [23, 24].

Peripheral (i.p.) administration of LiCl has been shown to activate the chemosensitive neurons in the AP [25] and a lesion in the AP causes attenuated CTA-acquisition [26]. AP receives peripheral information through humoral routes as well as through splanchnic and vagal inputs. Visceral afferent areas: PBN, NTS also project to the AP. Adachi et al demonstrated that some AP neurons respond to direct application of isotonic LiCl suggests that induction of CTA is possible even without the activation of the vagal inputs [27]. This is in line with our finding that although there was an elevated number of c-Fos positive nuclei in the AP, there was no difference in the DMNV upon exposure to LiCl. The lack of activation of the AP in the VPA animals may indicate altered chemosensation and/or an anomalous vagal afferent activity involved in ASD [28].

The neuroanatomical positioning of the ARC enables it to sense various nutrients and hormones and convey neural information to other brain areas. Orexigenic AgRP administration to the lateral ventricles was shown to partially block the acquisition of LiCl-induced CTA [29] . The reduced c-Fos-IR nuclei in the ARC thus likely underpins the aversion-derived anorexia.

The PVN is a major site of neurogenic and humoral control of feeding behaviour - axons from the subdivisions of the PVN innervate different brain structures involved in various physiological responses. The elevated c-Fos positive nuclei in the hypothalamic PVN corroborates earlier findings [30-33]. The LiCl-sensitive neurons in the PVN are located in its rostral magnocellular division. The parvocellular dorsal subnucleus of the PVN is a part of the efferent pathway involved in mediating the response of LiCl administration [31]. Furthermore, the PVN is one of the main sources of OT. Verbalis et al [34, 35] demonstrated elevated plasma OT levels followed by LiCl administration. Therefore, activated OT cells contribute to the globally elevated c-Fos immunoreactivity in the PVN. The fact that the VPA animals showed no change in c-Fos immunoreactivity in the PVN after the treatment with LiCl, may indicate a lack of plasticity of the OT neurons [36]. Additionally, the stress response associated with the

LiCl administration by the CRH neurons may contribute to the elevated c-Fos in the PVN of the control animals [37] as well.

It is widely established that structural and functional connectivity of the amygdala is altered in individuals with ASD (discussed in the ASD aetiology section 1.2). Lesion studies in different subnuclei of the amygdala leads to inconclusive results. For example, Andre et al revealed that lesions of the CEA with Ibotenic acid were found to have no effect on CTA acquisition regardless of whether the taste CS was novel or familiar [50]. On the other hand, electrolytic lesion of the CEA showed attenuated CTA using saccharin–LiCl pairing [51, 52]. This is to note that our CTA paradigm involved novel tastant. Taste novelty strongly modulates the speed and strength of taste aversion conditioning. Koh et al reported that novel saccharin induced larger increases in c-Fos than familiar saccharin in CEA but not in BLA [53]. Our data further validates this observation. Knocking down CREB mRNA into the CEA several hours before CTA training revealed impaired CTA memory in rats even when tested 3–5 d later. CREB antisense in the amygdala had no effect on CTA memory retrieval once it had been formed. This result indicates that optimal functionality of CREB mRNA in the CEA is critical for the establishment of long-term CTA memory in rat [54]. c-Fos is a CRE-regulated immediate early gene. Hence, our data showing strongest interaction between VPA X treatment further validates the role of CEA in CTA acquisition in the control animals which is absent in the VPA animals (Supplementary Fig. 1).

Elevated c-Fos immunoreactivity in the CEA after LiCl treatment has been reported previously by many authors [38] [39] [40] [10, 26, 37, 41-43]. Blockade of OTR hinders the development of adequate aversive responses to LiCl. PVN-OT neurons project to the CEA that expresses OTR [44] which is crucial for discriminative tasks [40]. Olszewski et al reported that the pretreatment with the blood–brain barrier penetrant OTR antagonist L-368,899 significantly reduced the number of c-Fos- positive nuclei in the CEA upon LiCl administration. Additionally, i.p. treatment with the OTR antagonist abolishes the LiCl-induced aversion [10].These findings indicate that the central OT signalling in the amygdala is governs the development of CTA [45].

OT serves as a marker of aversive (including the post-aversion anorexia) responses [46, 47]. The elevated levels of the circulating OT and the corresponding neuronal

activity have been consistently demonstrated by many authors after LiCl administration [34]. Our data showing significantly higher percentage of active OT neurons in the PVN of the LiCl-treated control animals corroborates the findings of Kita et al who demonstrated that administration of LiCl increases the number of c-Fos-positive OT neurons [48]. The lack of change in the percentage of active OT neurons post-LiCl treatment in the PVN of the VPA rats is likely because of the dysregulated OT system. This is to note that in the above set of experiments CTA acquisition was measured. Although saccharin is commonly used in CTA paradigms, since it is a non-caloric solution, such will not elicit any response in the feeding circuit activity *in vivo*.

Kosaki et al reported that BTBR mice (an animal model of ASD) showed weaker latent inhibition of CTA [17] . In line with this finding Strekalova and colleagues reported St3gal5−/− mice with ASD-like behaviours to show impaired CTA. During the recall session of the CTA, LiCl-treated mutants exhibited no significant difference in sucrose preference compared to the saline group [18]. Our results for the first time delineate the neural bases of anomalous LiCl-induced aversion in the VPA model of ASD. OT is involved in mediating CTA via multiple mechanisms of CTA acquisition and aversion-derived anorexia [49]. The dysregulated OT system in the VPA animals is one of the underpinned reasons for the aberrant CTA observed in these animals.

5.6. Conclusion

In sum, our findings imply a lack of responsiveness of the *in utero* VPA-exposed rat model of autism - 3mEq/kg LiCl administered intraperitoneally showed no effect on food intake in the VPA animals while the matched controls exhibited aversive responses at the same dosage. This, together with a differential brain activity pattern in the VPA animals indicate a lack of central OT-tone in these animals is likely the underlying reason. In a broader context, this affects learning and memory and fear generalisation thereby failing to execute context specific inhibitory learning like CTA. ASD individuals often present pica, where these individuals consume non-edible substances even with a prior experience of pathophysiological consequences. Our results in animal models indicate possible neurohisological underpinnings of this behaviour however precise neural pathways employed in reward motivation-driven consumption of substances that causes malaise remain unexplored.

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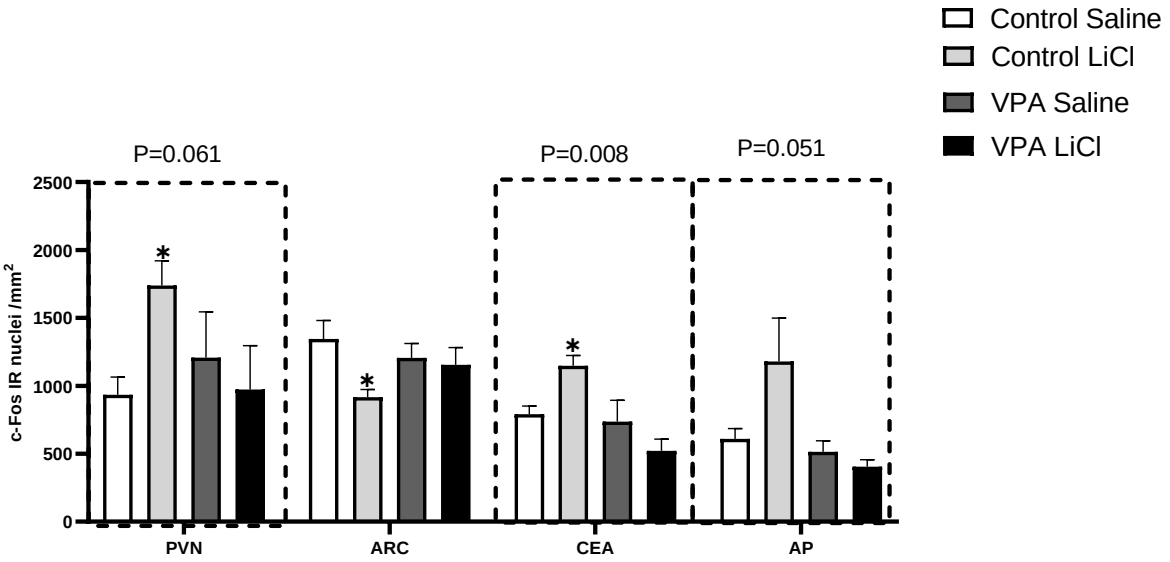
Supplementary Fig 1.



Two-Way ANOVA showing interaction between VPA X treatment (areas with dotted line). The CEA was the site where the highest level of VPA X treatment interaction (P=0.008) of all regions examined in our study was noted. * Significantly different from control saline group

Chapter 6
Discussion and Perspectives

Although for about past 80 years selective eating has been frequently documented to co-exist with ASD, there is a dearth of studies showing the neural basis of atypical food intake in ASD. The food selectivity, pica and all the other eating behavioral abnormalities associated with ASD are poorly understood. Fussy eating puts children with ASD at risk of nutritional deficits and dependence on specific type of food (e.g. French fries), ultimately affecting their growth (underweight or obesity), development and health. Parents and caregivers who feed ASD children often face more complex issues around food for a variety of reasons including food selectivity, refusal, pica, tantrum owing to mealtime change, etc. [1-3]. Additionally, dietary selectivity sometimes worsens with age, continuing its detrimental effect on health [4, 5]. Unless the causes of these behaviours are known, treatment cannot be effectively conceptualized.

Orexigenic and anorexigenic neuropeptidergic pathways form a part of a complex, dynamic network, whose balance shapes feeding behaviour of an animal. Selective eating stems from anomalous integration of energy homeostasis and reward processing. Chronic overconsumption of sugar not only leads to obesity, but also affects the neural system. For example, overstimulation of the OT system through repeated consumption of diets high in sugar leads to adaptations of receptor densities [6].

This research was aimed to investigate whether the VPA rats display abnormal feeding behaviour and to identify the aberrant neural processes that contribute to this phenomenon. Food intake behaviour involves hunger, satiation and reward processing. Through our series of experiments, we looked into these aspects of feeding in the VPA rat model of ASD. The study in which hunger processing in the VPA animals was investigated, the adult VPA rats consumed less ‘bland’ chow than that of the healthy controls. The lower intake of chow persisted even after acute food deprivation. There was an aberrant change in c-Fos immunoreactivity in key brain sites

that govern food intake in the VPA animals as opposed to the healthy controls upon acute food deprivation. This indicates differences in neural processing of hunger in autism. It is in fact in line with human ASD observations, in which ASD children have been described as ‘fussy eaters’ who are able to refuse non-preferred food during regular meal times, thus are capable to withstand energy deprivation [7]. Our data show typical changes in c-Fos immunoreactivity to hunger in the control animals as reported by other groups previously. For example, reduced number of c-Fos positive nuclei was reported in the PVN [8] and the SON [9] in healthy rats upon acute energy deprivation. Our finding showing no change in c-Fos immunoreactivity in these areas in the VPAs indicates that the VPA animals’ feeding circuits are differentially affected by the energy status of the animal, possibly leading to a more subdued drive to eat upon calorie deprivation.

This finding is further supported by a lack of change in expression patterns of feeding-related genes (such as OT, AgRP and MOR) upon hunger in the hypothalamus in the VPAs in contrast to the controls. Stomach distension leads to activation of hypothalamic OT neurons [10, 11] [12] [35, 36]. On the other hand, acute food deprivation leads to reduced brain OT concentration [13]. Our data indicating a downregulated OT mRNA expression in the hypothalamus of the food–deprived control animals further corroborates this finding. The lack of change in OT mRNA in the hypothalamus of the food-deprived VPA animals indicate dysregulated hunger processing.

AgRP, involved not only in mediating energy balance (reviewed in [14]) is also a key mediator of anxiolytic effects that drives an animal to seek for food when hungry [15]. Food deprivation activates AgRP neurons [16] triggering foraging and repetitive behaviours [17] [18]. Our data showing elevated AgRP mRNA expression in the hypothalamus of the control food-deprived group further validates these findings. In line with our result, acute food deprivation was shown to have resulted in increased MOR gene expression in the VMH, ARC and LH in healthy rats [19] indicating post-deprivation hyperphagia. In an fMRI-study, Stice et al reported that acute caloric deprivation correlated positively with activation of reward-, and motivation-areas (e.g., ACC, OFC) in response to images of palatable food [20]. Cumulatively, our results obtained from the healthy controls show typical dynamics of appetite-mediating

neuropeptides upon food-deprivation. The lack of changes in the expression levels of the genes governing hunger processing in the VPA animals indicate anomalous intake of the standard ‘bland’ chow in these animals.

Taking into account the aforementioned decrease in eating for hunger, what is particularly striking in the feeding response of the VPAs is the enhanced drive to consume palatable tastants. Thus, there is a mis-match in the hunger/satiety/reward continuum that determines a meal size. In the experiments described in Chapter 3, episodic intake of rewarding diets in the VPA rats was examined to address whether ASD affects reward-driven consumption. Foods that taste sweet are highly appealing in general. It was found that although our VPA animals consume an elevated amount of palatable foods, especially those that taste sweet, compared to their healthy controls, even without energy-deprivation. Considering the abnormal OT signalling in autism [21, 22], it can be hypothesized that the aberrant feeding for palatability stems from aberrant OT signalling (in Chapter 2, we showed the lack of change of the OT transcript upon acute food-deprivation in the VPA animals). Also, the fact that the MOR expression is affected in ASD (as shown in Chapter 2), changes in the opioid system, a well-known mediator of feeding reward, may play a role in overconsumption of palatable foods in ASD.

Evidence show an impaired OT signalling is involved the development of key ASD symptoms which could be a direct underlying reason for aberrant feeding as well. Swaab et al showed a reduction in the number of OT-positive neurons in Prader–Willi syndrome (genetic disorder that causes hyperphagia, obesity and intellectual deficit) patients exhibiting extreme overeating [23]. Hyperphagia and obesity also occur in case of mutations in single-minded-1 (SIM-1) gene that leads to insufficient development of the OT neurons. These symptoms can be reversed by exogenous OT administration [24]. Animals with central OT deficit (such as OT-KO mice) show elevated sugar consumption [25] [26] and exogenous OT showed an overall decrease in food intake in laboratory rodents [27]. With immunohistochemistry we showed reduced number of OT-IR cells in the hypothalamic SON corroborating the findings of Dai et al [21]. This, coupled with downregulated expression of OT and OTR mRNA in the hypothalamus and the brainstem in the VPA animals, point towards a dysregulated OT system that underpins the proclivity to sugar consumption in these animals. Hence,

we hypothesize that the effect of prosocial neuropeptide OT is worth investigating in the context of disrupted food intake in ASD.

Therefore, in Chapter 4, we investigated whether VPA rats exhibit heightened sensitivity to exogenously administered OT, a peptide known for its anorexigenic properties. Intraperitoneal (i.p.) OT treatment was found to reduce episodic intake of palatable diets as well as post-energy deprivation intake of standard laboratory chow; however, the dose needed to generate hypophagia was lower in VPAs than in controls. c-Fos immunohistochemistry revealed that the lower OT dose found to be anorexigenic in the VPA rats, was sufficient to affect brain activation in a way typical for hypophagia, whereas in healthy control animals, it did not induce any statistically significant c-Fos changes.

Upon acute i.p. OT administration, we observed that the VPA animals show elevated responsiveness compared to the controls. Animals with OT deficit show delayed clearance of exogenous OT from the system. For example, Smith et al demonstrated the plasma OT level remained significantly elevated for longer in OT-KO mice after acute i.p. administration [28]. The elevated level of OT in plasma for a longer period would lead to a sustained elevated level of intracerebral OT [28] [29] due to slower clearance. VPA animals showed downregulated OT mRNA in the hypothalamus coupled with low OT level in the CSF [21]. Thus, the combined effect of differential pharmacokinetics and slower clearance of OT from the brain leads to a sustained elevated sensitivity of the VPA animals to acute i.p. OT compared to the controls. This is further supported by the immunohistochemistry data where we showed that the VPA animals exhibit an elevated number of c-Fos-IR nuclei in brain regions that govern food intake such as PVN, ARC, DMH, BLA, CEA and the AP of the brainstem upon 0.3mg/kg i.p. OT administration while no difference in the brain activity pattern was observed in the controls at the same dose.

Our data further indicates elevated percentage of active OT neurons in the PVN of the VPA animals upon 0.3mg/kg acute i.p. OT administration, while no change was found in the controls. This results from the sustained elevated OT level in the system of the VPA animals that triggers central OT release. Peripherally administered OT induces a self-stimulation effect on its own hypothalamic circuitry [30, 31]. Activation of OTR

leads to elevated intracellular Ca^{2+} concentrations by mobilizing Ca^{2+} from the intracellular stores triggering exocytosis of OT packaged in large dense-core vesicles from the synaptic terminals of the OT neurons [31]. The OT neurons in the SON and PVN were shown to be strongly activated by peripheral OT [32]. These neurons contain OT receptors. Using integrate-and-fire model Rossoni et al suggested that the stimulation of OTR in these cells may lead to OT release in a feed-forward manner [33]. Furthermore, the central OT-circuit also regulates itself. Eliava et al identified a subset of PVN parvocellular OT neurons project onto magnocellular OT cells of the SON and regulate their function [34]. It can be argued that OT possesses poor permeability through the BBB. Ermisch et al measured the concentration of radiolabelled OT in the brain upon peripheral administration. Their results show that the BBB-free regions extracted up to 30 fold more peptide than BBB-protected regions. Although this peptide cannot cross the BBB in physiologically significant amounts, this does not exclude a possibility of a passive transport of miniscule but effective amounts upon a relatively high-dose of parenteral administration [35]. Furthermore, elevated BBB permeability has been observed in several animal models of ASD. For example, Cntnap2-/- rat model of ASD [36]. In Sema3F-KO mice (Semaphorin 3F KO mice, an ASD animal model,) Jagadapillai et al showed BBB disruption leading to changes in vascular permeability [37]. Taking these studies into account, the possibility of altered BBB permeability in the VPA animals needs investigation for future research; it cannot be ruled out that altered BBB permeability may lead to an elevated intracerebral OT in the VPA animals thereby feeding into the heightened responsiveness to the exogenous OT.

The aberrant feeding in ASD is not limited to food. Pica, which sometimes co-occurs with ASD is a feeding disorder defined by consumption of inedible substances like nails, hair, paper, soil and other potential sources of malaise [38-40]. Even with the experience of the risks associated with pica, ASD individuals tend to continue with this behaviour. Importantly, OT is involved in the process of avoidance of toxic foods, i.e., it facilitates the development of a conditioned taste aversion (CTA). Therefore, in Chapter 5, taste aversion acquisition was evaluated in the VPA animals.

In the laboratory setting, we used CTA to investigate whether the VPA animals respond to the aversive action of LiCl. We observed that when saccharin is coupled

with LiCl , VPA animals showed no effect while the dose of LiCl (3mEq/kg) is known to be sufficient to elicit CTA in the control animals [41] [42]. In line with this behavioural outcome, the immunohistochemical analysis revealed lower percentage of active OT cells in the PVN in the VPAs. This suggests that the responsiveness of the VPA animals to CTA is lower which is further indicated by the lack of change in response of the neural system to the aversive stimulus of LiCl. Elevated c-Fos immunoreactivity in the PVN, CEA and AP and diminished c-Fos immunoreactivity in the ARC was observed in the control animals while the VPA animals showed a lack of significant difference in c-Fos immunoreactivity at the same dose of LiCl indicating atypical CTA acquisition.

It was reported by several authors that LiCl treatment induces central OT release and OT serves as a marker of LiCl-induced aversion and post-aversion anorexia. The dysfunctional OT system in the VPA animals can be a possible reason for a blunted response to CTA, which is evident from our data that the VPA animals show no change in the activity of OT neurons in the PVN and SON upon LiCl treatment.

c-Fos immunoreactivity although generates valuable data, provides little information regarding the firing patterns of OT neurons in the VPA animals. Given these animals show reduced number of OT-ir cells in the SON, it is possible that the firing patterns are different in order to compensate. Performing slice recording may provide significant insight. VPA model is a great animal model for ASD, yet the effect of particular interaction of genotype, phenotype and neurobiology can only be established with genetic models. While staining for c-Fos is a valuable technique for examining neuronal activation patterns, it is important to be cautious when interpreting its absence. The lack of c-Fos staining does not definitively indicate a complete absence of neural activation. This is due to the existence of alternative molecular markers, and the complexity of functional connectivity within the brain. It would also be valuable in ASD research field to have *in vivo* data from freely behaving animals, employing high throughput techniques such as fibre photometry. For example, monitoring the activity of OTR expressing neurons in the VTA of the VPA animals under paradigms like social interactions, reward-driven feeding etc. Using a miniscope would provide valuable spatial resolution of the neurons.

Future directions:

Quattrocki and colleagues showed the role of OT in determining the precision of interoceptive signals [43]. Additionally, OT shows antinociceptive properties [44]. A subpopulation of PVN parvocellular neurons send projection to spinal cord. Evoked OT release from these neurons was reported to suppress nociception and to promote analgesia in an animal model of inflammatory pain [34]. The aberrant functionality of the OT system observed in the VPA animals may also lead to altered interoception (in this case, pain). Higher centres governing multimodal sensory, autonomic, motor, and cognition play important role in CTA acquisition and execution. Qian et al reported that the insular cortex unilaterally (right-side) responds to aversive visceral stimuli in an age-and vagal afferent-dependent way [45]. Gogolla et al reported altered multisensory integration in the insular cortices in BTBR mice [46]. Furthermore an fMRI study of BTBR mice revealed decreased bilateral functional connectivity for insular cortex [47]. This opens up the avenues for future research regarding involvement of higher-order brain areas in CTA development in the VPA animals.

The fact that the OT transcript show atypical dynamics upon food deprivation in the VPA animals could be a result of abnormalities in signalling of other appetite-regulating neuropeptides (as we have shown in Chapter 2, the VPA animals show a lack of change in other transcripts that regulate food intake upon food deprivation as opposed to the controls). OT neurons receive projections from multiple appetite regulating peptides such as alpha-MSH, GLP1 [48, 49], NPY etc. NPY promotes intake of high-carbohydrate diets more readily than that of fat [50]. The NPY neurons from the ARC project to the OT cells in the PVN governing the initiation of food intake. NPY inhibit OT neurons in the PVN via activation of GABAA receptors and NPY-Y1-mediated signalling reducing OT release leading to an increase in appetite [51]. Chronic treatment with VPA in early postnatal life has been shown to trigger NPY expression in rats [52]. Thus, an elevated baseline NPY itself may underpin elevated sugar intake or may affect the OT signalling in the VPA animals. Therefore, the elevated consumption of sugar and other diets rich in carbohydrate in the VPA animals as we have seen in Chapter 3, could be a result of aberrant signalling in broader appetite regulating circuits that, by itself can exert an effect or can employ the OT system.

Opioids regulate food intake - especially reward-dependent feeding and it acts via OT signalling. Opioid receptor ligands alters consumption of palatable diets more readily than that of non-palatable ones. Levine et al showed that the opioid receptor antagonist, naloxone, being more effective at reducing saccharin intake than water or quinine intake [53]. Yirmiya et al demonstrated that opioid receptor-deficient mice (CXBK strain) had lower preferences for saccharin-rich tastants [54]. Olszewski et al showed that opioid receptor agonist, butorphanol tartrate (BT) diminishes activity of the OT system. This implies that opioid agonists inhibit the anorexigenic effects of OT and promote consumption. On the other hand, opioid receptor antagonists exerts anorexigenic effect by activating OT neurons [55-57] [58]. It is well-known that OT promotes termination of feeding associated with generalised satiation [59] and an increase in the activity of OT signalling coincides with meal termination [60] . Therefore an elevated opioid signalling would delay the satiety signalling when palatable food are ingested. Hughes and colleagues reported that VPA-exposed rats exhibit upregulated expression of kappa opioid receptor in the PFC and amygdala [61]. Hence, dysregulated opioids could be a factor governing the drive for reward in the VPA animals by antagonizing the OT signalling. This is also relevant in the context of the interoception of pain in the CTA acquisition in the VPA animals. The role of anomalous functionality of broader circuits that affect the OT signalling needs investigation as well.

The fact that the activity of the OT neurons is differential in the VPA animals in response to i.p. OT or i.p. LiCl, does not negate the possibility of involvement of other neuropeptide(s). The anomalous responsiveness of OT could be a secondary consequence of another dysregulated neuropeptidergic process. For example, α-MSH (MC4R agonist) stimulates OT release [31]. Administration of MC4R agonist was reported to improve social interaction via OT signalling in Cntnap2-KO mouse model of ASD. [62]. Thus, a disrupted POMC epigenetics which has been described in offspring of dams fed with high fat diet [63] may affect optimal functionality of the OT system via α- MSH signalling. Since maternal obesity is associated with an increased risk of ASD [64] in the offspring, a dysregulated epigenetics of POMC may lead to altered α-MSH levels [65] thereby affecting OT signalling. Thus, OT may not be the sole causative factor of the differences in the dynamics of responsiveness, it may be a function of broader circuit abnormalities that warrants investigation.

In this thesis we investigated, using prenatal VPA-exposed rat model of ASD, the neural basis of aberrant food intake in autism. Our results indicate dysregulated hunger and satiety processing in these animals. OT and multiple other neuropeptides do not respond to internal homeostatic changes. For decades, OT has been reported to be disrupted in ASD in humans and animal models in the context of social deficits. It is also emerging as a promising molecule to improve the social behaviours in autism in humans and animal models. We found atypical response of OT transcript upon acute food deprivation in the VPA animals. The aberrant OT signalling in these animals underpins not only the elevated anxiety-like behaviour and social deficits but also, possibly, the overconsumption of palatable diets especially those taste sweet and/or rich in sugar. Our results provide the preliminary preclinical evidence of the effectiveness of OT as a pharmacological tool to curb overconsumption of sugar and other palatable diets with carbohydrate as the major compositional macronutrient in the context of autism. Furthermore, the VPA animals show blunted CTA acquisition. This is of particular interest in and the context of excessive intake of palatable substances that disrupt plasma osmolality (sugar) in the VPA animals - in spite of the unpleasant feeling of overconsumption of sugar, the ingestion does not cease. Even with the noxious pathophysiological consequences of pica (pain, infection), ASD individuals continue to consume inedible substances - anomalous OT signalling being an underpinning reason.

This work lays the foundation for future research to explore the involvement of the interaction of different neuropeptides in the context of feeding problems in ASD in addition to translation of data from animal studies to humans. Scattered reports claiming certain diets (such as gluten-free, casein-free diets) being beneficial for children with ASD do not suggest the basis of the ‘benefits’ let alone the sample size and the heterogeneity of the cohort under study. Understanding what might be behind rigid eating behaviours is the first step in helping ASD individuals to get more comfortable with new foods.

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Conclusion

The overarching aim of this doctoral thesis was to investigate the anomalous feeding in VPA rat model of ASD and to investigate the neural basis of that.

The key findings are:

- VPA animals show atypical feeding behaviour.
- VPA rats eat less standard ‘bland’ chow compared to the controls even when they are energy deprived. This indicates altered hunger processing in the VPA animals.
- VPA animals overconsume palatable diets especially those that taste sweet. This indicates consumption for reward-driven feeding.
- They are resistant to LiCl-induced CTA as shown by reduced sensitivity to i.p. LiCl.
- VPA rats show an elevated responsiveness to exogenous OT.
- c-Fos immunoreactivity in the VPA animals show anomalous response to food deprivation, upon anorexigenic OT treatment and to CTA acquisition.

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Appendices

Appendix I

DSM-5 criteria for ASD - Autism Spectrum Disorder 299.00 (F84.0) **Diagnostic Criteria**
A. Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history (examples are illustrative, not exhaustive, see text): 1. Deficits in social-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation; to reduced sharing of interests, emotions or affect; to failure to initiate or respond to social interactions. 2. Deficits in non-verbal communicative behaviours used for social interaction, ranging, for example, from poorly integrated verbal and non-verbal communication; to abnormalities in eye contact and body language or deficits in understanding and use of gestures; to a total lack of facial expressions and non-verbal communication. 3. Deficits in developing, maintaining and understanding relationships, ranging, for example, from difficulties adjusting behaviour to suit various social contexts; to difficulties in sharing imaginative play or in making friends; to absence of interest in peers. B. Restricted, repetitive patterns of behaviour, interests, or activities, as manifested by at least two of the following, currently or by history (examples are illustrative, not exhaustive; see text): 1. Stereotyped or repetitive motor movements, use of objects, or speech (e.g. simple motor stereotypies, lining up toys or flipping objects, echolalia, idiosyncratic phrases). 2. Insistence on sameness, inflexible adherence to routines, or ritualised patterns or verbal non-verbal behaviour (e.g. extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat food every day). 3. Highly restricted, fixated interests that are abnormal in intensity or focus (e.g. strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interest). 4. Hyper- or hyporeactivity to sensory input or unusual interests in sensory aspects of the environment (e.g. apparent indifference to pain/temperature, adverse response to specific sounds or textures, excessive smelling or touching of objects, visual fascination with lights or movement). C. Symptoms must be present in the early developmental period (but may not become fully manifested until social demands exceed limited capacities, or may be masked by learnt strategies in later life). D. Symptoms cause clinically significant impairment in social, occupational or other important areas of current functioning. E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and Autism Spectrum Disorder frequently co-occur; to make comorbid diagnoses of Autism Spectrum Disorder and intellectual disability, social communication should be below that expected for general developmental level. Specify if: With or without accompanying intellectual impairment With or without accompanying language impairment Associated with a known medical or genetic condition or environmental factor Associated with another neurodevelopmental, mental or behavioural disorder With catatonia

Table 5: Diagnostic criteria of ASD. Reprinted with permission from the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, (Copyright 2013). American Psychiatric Association. All Rights Reserved

Appendix II

Changes in feeding behaviour of ASD-animals with age

The difference in body weight between the control and the VPA animals was abolished post-40wk of age.

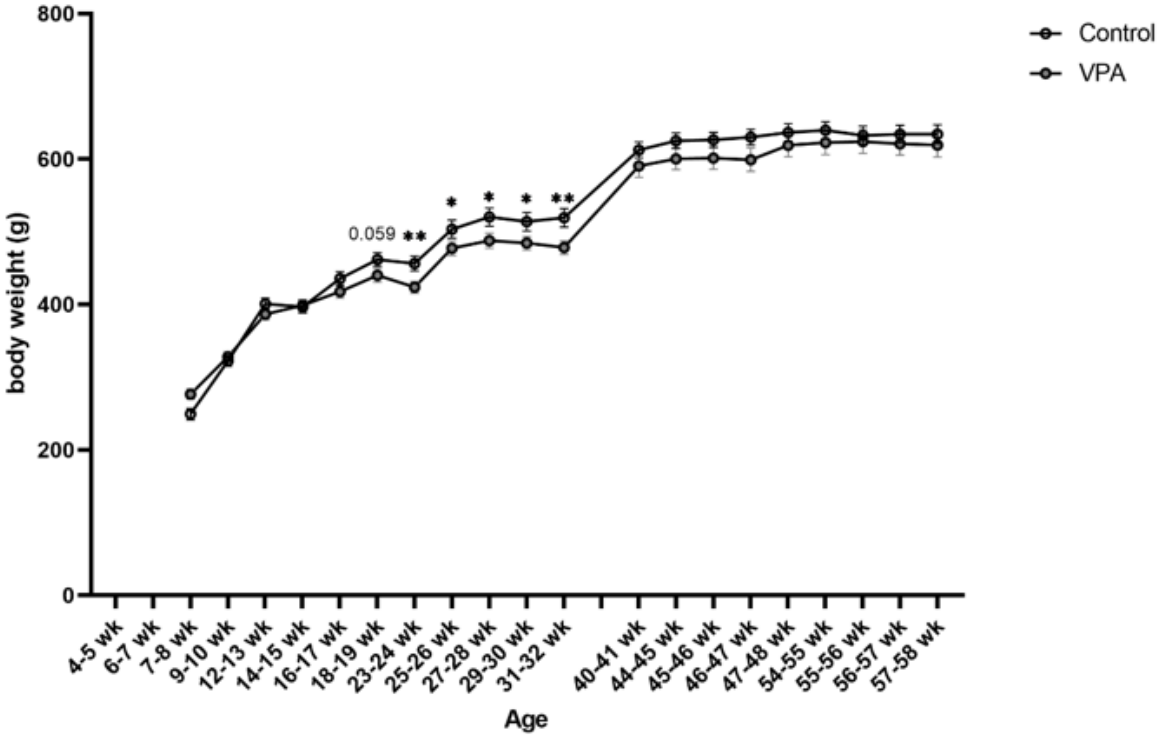

Figure 9- 1: Body weight trajectory of control and VPA animals

The consumption of palatable diets show opposite trend when the animals were 56-58wk old. VPA animals consume less sucrose (t=3.255, df=38, P=0.00119), Milk (t=1.4502, df=38, P=0.076), glucose (t=3.19268, df=37, P=0.001438), saccharine (t=2.60924, df=37,P=0.006509) , HFHS (t=1.522, df=37, P=0.068)

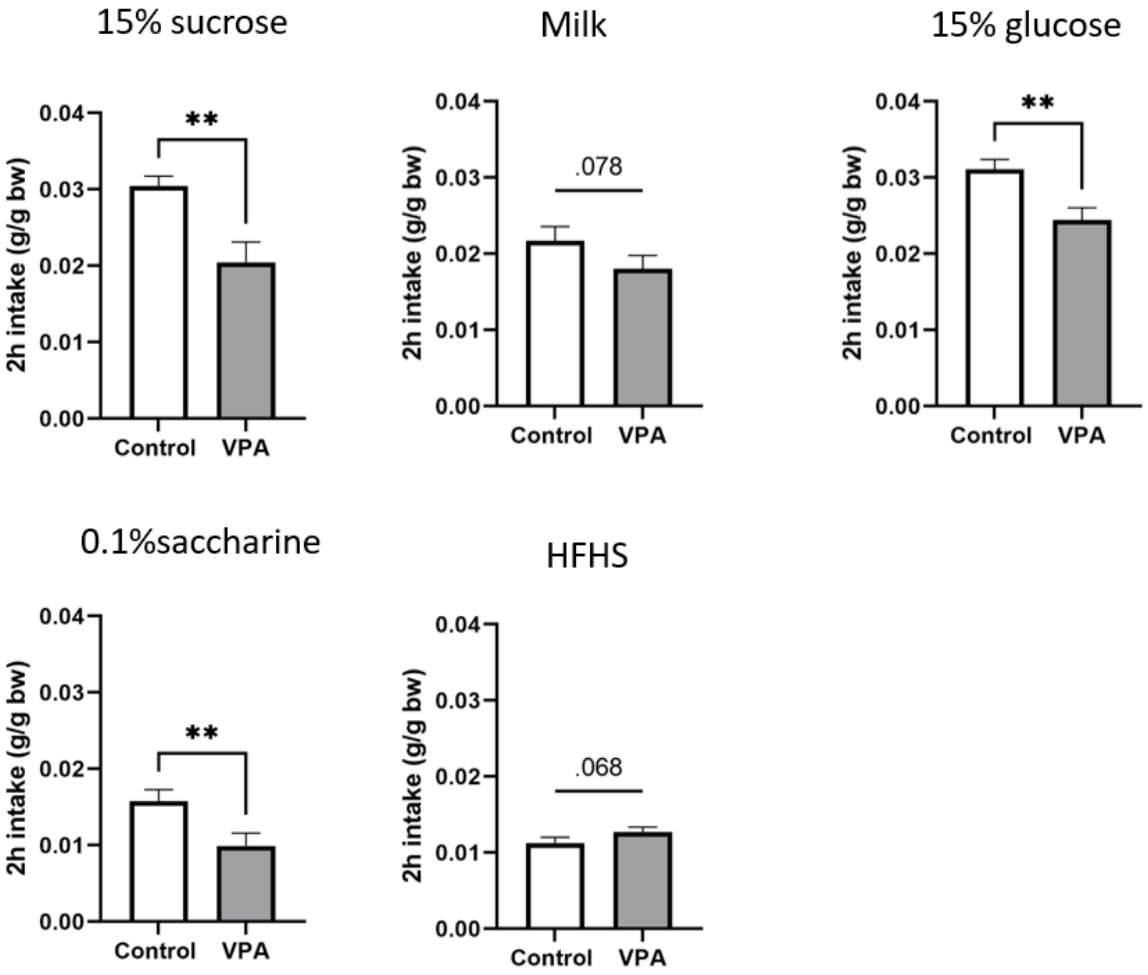

Figure 9- 2: Feeding behaviourof male VPA animals at 56-58wk of age compared with their matched controls

Feeding behaviours in VPA-animals far into adulthood is not known. ASD individuals tend to become more selective to the food preference with age.

Impaired at olfactory identification was identified in Asperger syndrome in males of about 30 years old [1-3]. Meta-analyses indicate an olfactory dysfunction in individuals with ASD [4]. Leekam et al further report impaired sensory processing in ASD [5].

Brewer et al showed that olfactory identification deficits of ASD may progressively deteriorate with age [6]. Age related changes in gene expression pattern involved in opioid signalling may also be implicated in early onset of anhedonia in VPA-animals

[7]. In addition, the level of Glutamate synthetase changes in VPA animals from P15 to P120 in the hippocampus [8]. The change observed is likely due to neurochemical changes with age and key neurodevelopmental genes may underline the differential trajectory of onset of anhedonia.

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Appendix III

This thesis is partially based on the following published and peer-reviewed papers:

Title: Neural Basis of Dysregulation of Palatability-Driven Appetite in Autism (2021)

Title: Mild Hypophagia and Associated Changes in Feeding-Related Gene Expression and c-Fos Immunoreactivity in Adult Male Rats with Sodium Valproate-Induced Autism (2022)