

Investigation of peripheral nerve sensitivity in two animal models of Autism Spectrum Disorder (ASD)

By

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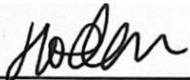
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Abstract

Introduction: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by deficits in social communication and restricted behaviours, and associated with sensory alterations, gastrointestinal dysfunction and disruptions in the circadian rhythm. This study utilised two animal models of ASD, the BTBR T+tf/J and the prenatal exposure to VPA mouse models of ASD, compared to the control C57 BL/6J strain. The aim of this study was to investigate changes in circadian rhythm and peripheral sensation in the two mouse models of ASD, and identify potential pathways involved.

Methods: Home-cage testing was conducted using LABORAS platforms, to record the animals' behaviour over 24 hours (C57 n = 8; BTBR = 9, VPA = 4). Cutaneous sensory thresholds were determined using the dynamic hot (DHP) and cold plate (DCP) tests (C57 n = 6; BTBR n = 6; VPA n = 4), and sensory function of the gastrointestinal tract was outlined using ex-vivo jejunum preparations, by recording the afferent nerve responses to mechanical and chemical stimuli. Values are mean +/- SEM analysed with two-way ANOVA using GraphPad Prism.

Results: the VPA mice exhibited altered circadian rhythm in the dark (active) phase compared to the BTBR and C57 mice ($P < 0.05$). In the DHP, the BTBR mice (n = 6) responded at a higher temperature (C57 - 38C; BTBR - 40C), responded significantly less to heat ($P < 0.0005$), while VPA mice (n = 4) showed similar responses to the C57 mice. In the DCP, VPA mice start to respond at a lower temperature (C57 - 16C; VPA - 2C), responded slightly less to cold ($P = 0.055$), while the BTBR mice showed similar responses to C57 mice.

In the afferent nerve recordings of the jejunum, the BTBR tissue exhibited significantly decreased responses to mechanical distension at a filling rate of 600 μ l/min ($P < 0.0006$; BTBR n = 9; C57 n = 10). Peak firing rate at 50 mmHg was 92.68 (+/- 12.80) imp/s⁻¹ in recordings from C57 (n = 10) tissue and 76.49 (+/- 15.44) imp/s⁻¹ in BTBR tissue (n = 9). BTBR afferents also showed an altered response profile to TRPV1 activation ($P < 0.0001$, BTBR n = 5; C57 n = 5), whereby nerve firing took significantly longer to desensitize compared to control afferents, suggesting altered function of TRPV1. Preparations from BTBR mice also exhibited significantly increased response to intraluminal application of an inflammatory soup ($P < 0.05$), BTBR n = 5; C57 n = 5).

Conclusion: the VPA model of ASD showed marked alterations in circadian rhythm and reduced response to cold stimuli, and the BTBR mouse model of ASD exhibited significantly decreased response to heat and significantly altered afferent nerve activity from the jejunum in response to various stimuli. Future studies should investigate whether these changes correlate with CNS dysfunction or whether altered peripheral sensation could drive some of the central deficits observed in ASD.

Glossary of terms and abbreviations

ASD	Autism Spectrum Disorder	DRG	Dorsal root ganglion
DSM - 5	Diagnostic and Statistical Manual of Mental Disorders Volume 5	IPAN	Intrinsic primary afferent neuron
ICD	International Classification of Diseases	DEC/ENaC	Degenerin/epithelial sodium channel
GABA	γ -aminobutyric acid	ASIC	Acid sensing ion channel
BTBR	BTBR T+ tf/J (BTBR) an inbred mouse strain	5-HT	5-hydroxytryptamine
BDNF	Brain derived neurotrophic factor	5-HT₃	5-hydroxytryptamine receptor 3
GI	Gastrointestinal	NGF	Nerve growth factor
VPA	Valproic acid	PGE₂	Prostaglandin E ₂
SAL	Saline	ATP	Adenosine triphosphate
mGluR	Metabotropic glutamate receptor	SEM	Standard error of the mean
SCN	Suprachiasmatic nucleus	ENS	Enteric nervous system
ASMT	Acetylserotonin Omethyltransferase	NTS	Nucleus tractus solitarius
TRP	Transient receptor protein	IBS	Irritable bowel syndrome
DHP	Dynamic hot plate	DCP	Dynamic cold plate

1 Introduction

1.1 What is ASD?

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by deficits in social communication and repetitive, restricted behaviours (American Psychiatric Association, 2013). ASD is one of the most heritable conditions of all the most complex neurodevelopmental disorders (Constantino, 2013), however, diagnosis is based on behavioural symptoms. The gold standards for diagnosis of mental disorders such as ASD are the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) and the International Classification of Diseases (ICD). Both manuals have classified Autism as a spectrum in their updates, incorporating all the subcategories in the previous versions. This allows all the different presentations of ASD to be considered in a more holistic view (Hayes, 2018).

Less than 1% of ASD cases have been confirmed to have a genetic basis (Chaste, 2015) and decades of studies on interactions of gene variants in the aetiology of ASD to predict risk of the condition have been unsuccessful. There is substantial genetic variability in ASD, which could cause the phenotypic heterogeneity characterising the spectrum (Asif, 2019). There has been a lot of effort to find biomarkers for diagnosis and personalising treatment, however this has been difficult due to the complexity of ASD (Klin, 2018).

One of the most prominent theories developed to explain brain abnormalities leading to ASD is alterations in connectivity, many groups have shown altered brain connectivity (Just, 2007; Kana, 2006, Villalobos, 2005; Kleinhans, 2008). Functional magnetic resonance imaging (fMRI) studies have shown abnormalities in the structure of brain regions such as the amygdala, cingulate anterior cortex and the cerebellum (Uddin, 2009). This altered connectivity impacts excitation-inhibition balances in ASD, Hegarty et al found altered cerebro-cerebellar functional connectivity was associated with cerebellar excitation-inhibition imbalances in individuals with ASD but not controls (Hegarty, 2018) which may suggest alteration in neurotransmitter levels. Neurotransmitters associated most commonly with ASD are the GABAergic, glutamatergic and serotonergic systems (Trottier, 1999).

ASD is almost 2-3 times more prevalent in males than females (Kim, 2011), however this may be due to difference in presentation which may explain why adult females with autistic disorder were diagnosed later in life (Begeer, 2013). ASD develops before children reach the age of 3, in some cases development is normal until the child 'regresses', by slowly losing language and refusing to make eye contact. This occurs in around 30% of cases (Parr, 2011).

The pathophysiology and aetiology of ASD is still not known, however, research has shown it is a multi-factorial disorder due to the variety of genes and environmental factors that contribute to its pathogenesis (Yenkoyan, 2017).

1.2 Animal models of ASD

To study the physiological manifestations of ASD, preclinical animal models have been developed (Erdogan, 2017). These models are based on laboratory rodents, as they are social animals, and any deficits in sociability can be detected easily (Cryan, 2005). Tests of rodent sociability include contact with known or unknown animals of the same species, social interaction, ultrasonic vocalisations (rodents vocalise below the frequency of human hearing) and aggressive behaviour (Grant, 1963; Carter, 1992; Terranova, 2005). Behavioural phenotyping of ASD models is based on the three domains affected in human ASDs, repetitive behaviour, communication and sociability.

To be considered a relevant model of human psychiatric disorders, animal models must fit pre-determined criteria, or validities. These are the construct, face and predictive validities (Willner, 1986). Construct validity is based on the causation of the studied phenotype, human ASDs are associated with genetic and environmental factors, so an animal model should be built using these factors (Belzung, 2005). If the animal model exhibits the same phenotypes or symptoms as the human disorder, it satisfies face validity. In this context, the animal model must show the behavioural diagnostic features of ASD, socio-communication deficits and repetitive restricted behaviours (Mabunga, 2015). Predictive validity means that any interventions to treat the symptoms in the human disorder should do the same in the animal model (Willner, 1986).

To generate these models, researchers replicate the genetic and environmental alterations that have been associated with development of ASD, such as knock out or humanised knock in of gene mutations and copy number variations reported in ASD such as fragile X syndrome and tuberous sclerosis (Gross, 2015; Baudouin, 2012). Other ASD-associated genetic alterations can occur spontaneously in some strains of mice such as the BTBR T+tf/J mice (McFarlane, 2008). Animal models of ASD can be induced through in utero exposure to various conditions associated with human ASD such as valproic acid (VPA) (Christensen, 2013).

BTBR T+tf/J (BTBR)

BTBR mice are an inbred strain, originally bred for studies on insulin resistance and phenylketonuria, it was discovered that these mice display consistent ASD-relevant

behaviours including increased repetitive grooming and deficits in sociability and communication (Bolivar, 2007; Moy, 2006; Nadler, 2006). Daimon et al conducted transcriptomic and proteomic analyses on hippocampal tissue of BTBR mice, finding reduced expression of brain derived neurotrophic factor (BDNF), Caskin1 and HOMER31 (Daimon, 2015), which have also been reported human ASD (Sheikh, 2010; Abrahams, 2008). These mice exhibit reduced size or complete destruction of the corpus callosum, a large bundle of nerve fibres that connect the two hemispheres of the brain which has been implicated in ASD, however the causal relationship is not clear (Paul, 2014). BTBR mice show gastrointestinal disruptions, altered GI motility and permeability, possibly due to disruptions in the serotonergic system (Golubeva, 2017).

The BTBR strain thus exhibits construct and face validity as a model of ASD, in order to determine whether it displays predictive validity, researchers conducted pharmacological intervention to improve the ASD-like symptoms. Silverman et al aimed to manipulate the excitatory neurotransmission of BTBR mice by administering methyl-6-phenylethynyl-pyridine (MPEP), an antagonist of the metabotropic glutamate receptor 5 (mGluR₅). They reported MPEP significantly reduced repetitive self-grooming in the BTBR mice (Silverman, 2010). Berry-Kravis et al used another mGluR₅ antagonist, fenobam, to treat adults with Fragile X syndrome, they found an improvement of 20% in the pre-pulse inhibition test, which measures acoustic sensory gating (Berry-Kravis, 2009)

Prenatal exposure to valproic acid (VPA)

Exposure to certain pharmacological agents in utero increases the risk of ASD, valproic acid (VPA) is an anti-epileptic drug and causes teratogenicity and neural tube defects (Choi, 2016). The prenatal VPA model of ASD is believed to be a better representation of autism caused by environmental and epigenetic causes (Nicolini, 2018) and shows good construct, face and predictive validity (Mabunga, 2015). VPA rodents exhibit alterations in sensory processing, Dendrinios et al reported alterations in development of the superior colliculus, a brain region essential for multisensory information processing (Dendrinios, 2011). Gastrointestinal alterations have also been reported in the VPA model of ASD, Kim et al found significant changes in the thickness of the mucosa and muscle layers of the stomach and ileum, abnormal epithelial cell morphology and impaired GI motility (Kim, 2013).

Use of VPA by pregnant women in the first trimester led to an increased risk of ASD (Christensen, 2013), and exposure to VPA on pregnant mice on embryonic day 11-12 results in increased stereotypic behaviour (Schneider, 2008) and decreased social interaction (Lucchina, 2014). Campolongo et al conducted a study comparing impact of housing in sociability of VPA mice, by housing VPA treated mice with saline treated mice (VPA-SAL) and

VPA mice with other VPA mice (VPA-VPA), with SAL-SAL as control. Their rationale was that housing VPA mice with other VPA mice contributed to reduced social interaction, and housing with control mice could act as a social enrichment exercise. Their results showed increased social interaction and play between VPA-VPA and VPA-SAL mice, however, repetitive and depression-like behaviours were not altered (Campolongo, 2010). Social enrichment has been reported to improve ASD symptoms in humans (Woo, 2013; Woo, 2015). MGLuR₅ antagonists have also been used to improve stereotypical behaviours in prenatal VPA mice (Mehta, 2011).

1.3 Co-morbidities in ASD

Many medical conditions appear to have higher prevalence in individuals in ASD compared to neuro-typical individuals, including epilepsy (Woolfenden, 2009), hypertension (Croen, 2015) and gastrointestinal dysfunction (Kohane, 2012). There is also increased rates of sleep problems in ASD, reports range between 50-80% (Richdale, 2009). Individuals with ASD are prescribed multiple psychotropic medications (Esbensen, 2009) which increase morbidity and disrupt healthy ageing (Bishop-Fitzpatrick, 2018).

Certain behaviours such as aggression and head banging have been considered part of the symptomology of ASD, however, may be a manifestation of untreated symptoms from other diseases, including gastrointestinal disorders (Buie, 2010). These reports support the theory that ASD is a whole-body disorder, and research is increasingly branching out into numerous biomedical fields. The focus of this study is to delineate the impact of circadian rhythm alterations and sensory disruption in the somatic and visceral transduction pathways in mouse models of ASD.

Circadian alterations in ASD

Children and adults with ASD exhibit increased sleep difficulty and altered circadian sleep rhythmicity compared to the general population (Carmassi, 2019). Studies have shown children with ASD may show sleeplessness defined by phase delay of sleep periods, and irregular sleep-wake patterns, Giannotti et al reported children with regressive ASD showed higher sleep onset delay, sleep duration and night awakening scores (Giannotti, 2008). The prenatal VPA animal model of ASD shows altered circadian rhythm, characterised by increased arousal in the sleep phase (Tsujino, 2007; Cusmano, 2014). Loohuis et al reported differential expression of genes involved in circadian regulation in VPA rats (Loohuis, 2017).

The suprachiasmatic nucleus (SCN) is the master biological clock of the hypothalamus (Moore, 1972; Stephan, 1972) and its neurons contain autonomous transcription-based clockwork (Kornhauser, 1996). It is controlled by the light-dark cycle, which is detected by the rod and cone cells in the retina, relaying the information to the SCN via a monosynaptic

pathway (Weng, 2009). Light also affects CLOCK genes which have approximately 24-hour gene transcription rhythms (Kornhauser, 1996), they are expressed in cells within bodily tissues and form translation/transcription negative feedback loops (Potter, 2016). Production of melatonin regulates the peripheral clock in a feed-forward mechanism (Meissl, 1990).

Altered circadian rhythm in ASD has been hypothesised to be due to polymorphisms in CLOCK genes (Yang, 2016) and genes involved in the production of melatonin (Jonsson, 2010). There has been a consistent finding of reduction in peripheral melatonin concentrations (Tordjman, 2005; Tordjman, 2012), potentially due to reduced activity of acetyl-serotonin Omethyltransferase (ASMT), an enzyme in the biosynthetic pathway from serotonin to melatonin (Melke, 2008). Reduced activity of ASMT could provide an explanation for the high peripheral serotonin and low melatonin concentrations observed in ASD (Missig, 2019).

To assess the circadian rhythm of the mouse models of ASD, we employed LABORAS (Laboratory Animal Behaviour Observation Registration and Analysis System), which is an automated system used for recognition of behaviours exhibited by mice and rats. It includes a platform, with a cage placed on top with bedding and food and water. By measuring the forces produced by the animal's movement on the platform, it scores behaviours such as grooming, locomotion, average speed, maximum speed and distance travelled (Van de Weerd, 2001). By recording the control C57, BTBR and prenatal VPA mice for 24 hours, we can observe their sleep-wake cycle and activity. The software also records eating behaviour, which could provide evidence for any differences in feeding exhibited by the mouse models of ASD, as altered feeding behaviour has been reported in ASD (Castro, 2016). This technique has been used previously by Pitzer et al, who conducted longitudinal home cage monitoring in their animal model of inflammatory pain (Pitzer, 2016).

Sensory disruption in ASD

Over 90% of individuals with ASD have atypical sensory processing (Chang, 2014) in all sensory modalities. Alterations in sensory processing have been described in literature reporting autism symptomology (Adrien et al., 1987, 1992, 1993; Baranek, 1999; Dahlgren & Gillberg, 1989; Kientz & Dunn, 1997; Ornitz, 1989; Ornitz et al., 1993) as well as by individuals with ASD themselves (Grandin, 1995). Sensory disruption in ASD can take three main forms, sensory hypo-responsiveness, hyper-responsiveness and sensory seeking, a behaviour characterised by a craving for a certain sensory experience (Hazen, 2014) and many individuals have a combination of the three.

Abnormalities in the processing and integration of sensory information can disrupt development intellectually and socially, for example, children with altered sensory processing

show delays in development of fine and gross motor skills, lack of coordination and poor balance (DeGangi, 1989). Disruption in auditory processing are of the more commonly reported sensory impairments in ASD, Greenspan et al found 100% of their participants (200 individuals) exhibited difficulties in responding to auditory stimuli (Greenspan, 1997). Avoidance of eye contact and gaze is a hallmark feature of ASD (Senju, 2009) and hypersensitivity to tactile stimuli has been associated with increased repetitive behaviours and verbalization, as well as abnormal attention focus (Baranek, 1997).

ASD has been associated with an indifference or hyposensitivity to pain stimuli, Nader et al compared responses to venepuncture in typically developing and ASD children, they found the children with ASD showed a delayed facial pain reaction in response to the procedure (Nader, 2004). They also reported discrepancies between parental reports of pain and the children's responses, putting into question the use of parental questionnaires in studies of autism (Nader, 2004).

Altered responses to thermal nociception have been reported in individuals with ASD (Cascio, 2008; Duerden, 2015) and in animal models of ASD, Schneider et al found increased response latency in the tail-flick test and paw withdrawal test by rats prenatally exposed to VPA, concluding they have increased nociceptive threshold (Schneider, 2001). These results were validated by further studies in prenatal VPA mice and rats (Dendrinou, 2011; Morakotsriwan, 2016; Mahmood, 2018). BTBR mice have also been found to display hypo-responsiveness to thermal stimuli (Wang, 2016) with an increased latency of paw withdrawal.

The sensation of temperature is mediated by the polymodal transient receptor potential (TRP) ion channels, which are expressed on sensory primary afferent neurons (Vriens, 2014). Their activation thresholds encompass the temperature ranges found in the environment (Bokinić, 2018). There are four TRP channels that are activated by increasing temperatures, TRPV3 (>34–38°C) and TRPV4 (> 27–35°C) mediate warm sensation, while TRPV1 (>43°C) and TRPV2 (>52°C) respond to hot stimuli, transducing painful sensations (Patapoutian, 2003; Tominaga, 2004; Dhaka, 2006). TRPM8 (<25°C) and TRPA1 (<18°C) mediate cold sensation (Peier, 2002; Bandell, 2004; Dhaka, 2006). TRPM8 knock out mice show deficits in cold sensitivity (Bautista, 2007; Dhaka, 2007; Colburn, 2007). The cell bodies of primary afferent neurons in the skin are in the dorsal root and trigeminal ganglia. The afferent nerves responsive to thermal stimuli are split into two types, the thinly myelinated A δ fibres and the unmyelinated C fibres (Bokinić, 2018). Figure 1.1 below shows the neural pathway of thermal sensing in the mouse, from the primary afferents in the skin to the spinal cord and thalamus, primary somatosensory cortex (S1), secondary somatosensory cortex (S2) and the insular cortex (IC).

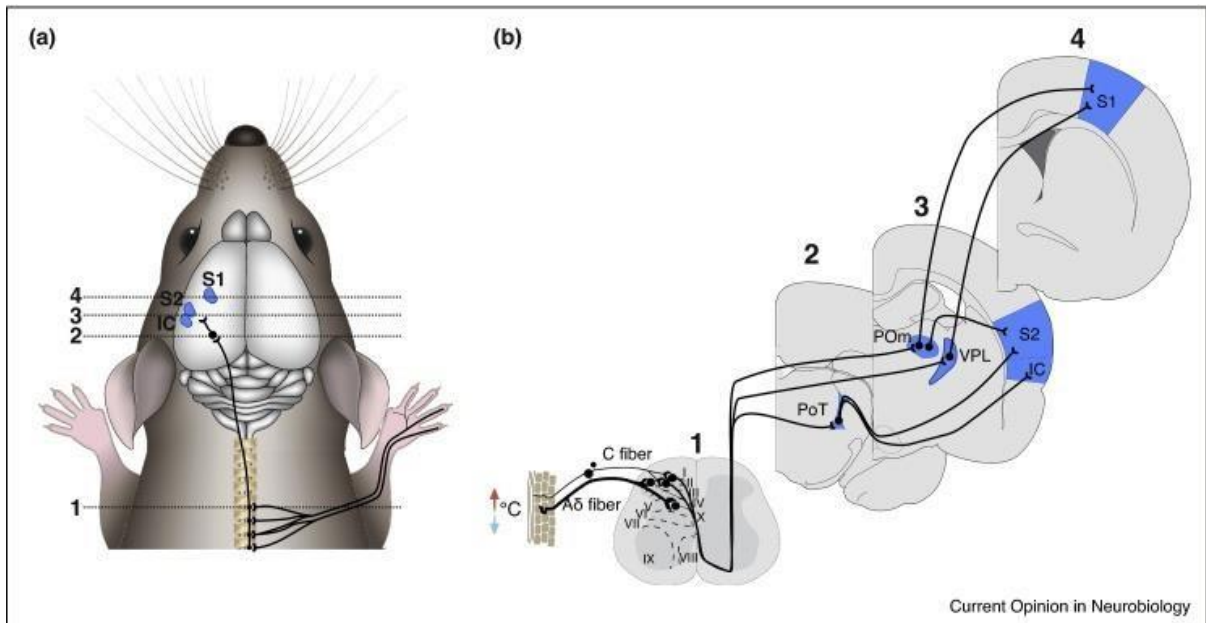


Figure 1.1: a schematic representation of the neural pathways transducing thermal sensation from paw to somatosensory cortex in mice. A) A diagram of a mouse showing the thermal pathway from the paw to the brain, B) cross sections of the murine nervous system, the numbers correspond to the points shown in A). Adapted from Bokinić et al (2018).

Han et al conducted a study investigating the role of TRPV1 in ASD thermosensation, SHANK3 is part of a family of proteins responsible for synaptic scaffolding (Sheng, 2000), and mutations are found in around 2% of human ASDs (Leblon, 2014; Yi, 2016). Using different lines of SHANK3 mutant mice, they showed SHANK3 plays an important role in pain transduction as it regulates the expression of TRPV1, haploinsufficiency of SHANK3 leads to defects in function of TRPV1 in mouse and human dorsal root ganglion (DRG) neurons (Han, 2016). In the present study, we aimed to characterise the function of TRPV1 in two other mouse models of ASD, to determine any changes in function and to compare the somatic and visceral transduction pathways of pain.

Thermosensation studies in mouse models of ASD have been conducted on hot plates set at a nociceptive temperature, where the time for the animal to withdraw their paw is measured in order to identify presence of hyperalgesia. However, the possibility of thermal allodynia has not been investigated yet. According to Yalcin et al, this can be done through use of the Dynamic Hot Plate (DHP) or Dynamic Cold Plate (DCP) tests, where the animal is placed on the plate set at an innocuous temperature, then raised or lowered slowly until nociceptive temperatures are reached (Yalcin, 2009). The point at which the animal starts exhibit escape behaviour, defined as jumping, is used as a measure of response to nociceptive temperatures. Thus, if the animal starts jumping at temperatures that are considered innocuous, they may have thermal allodynia. This test allows a bigger picture of thermal sensitivity in rodents to be drawn, especially in models of ASD, where sensory disruption is reported widely. In this study

especially, by studying visceral sensory pathways of pain through recording of gastrointestinal afferents, a comparison can be made between somatic and visceral pain responses in ASD.

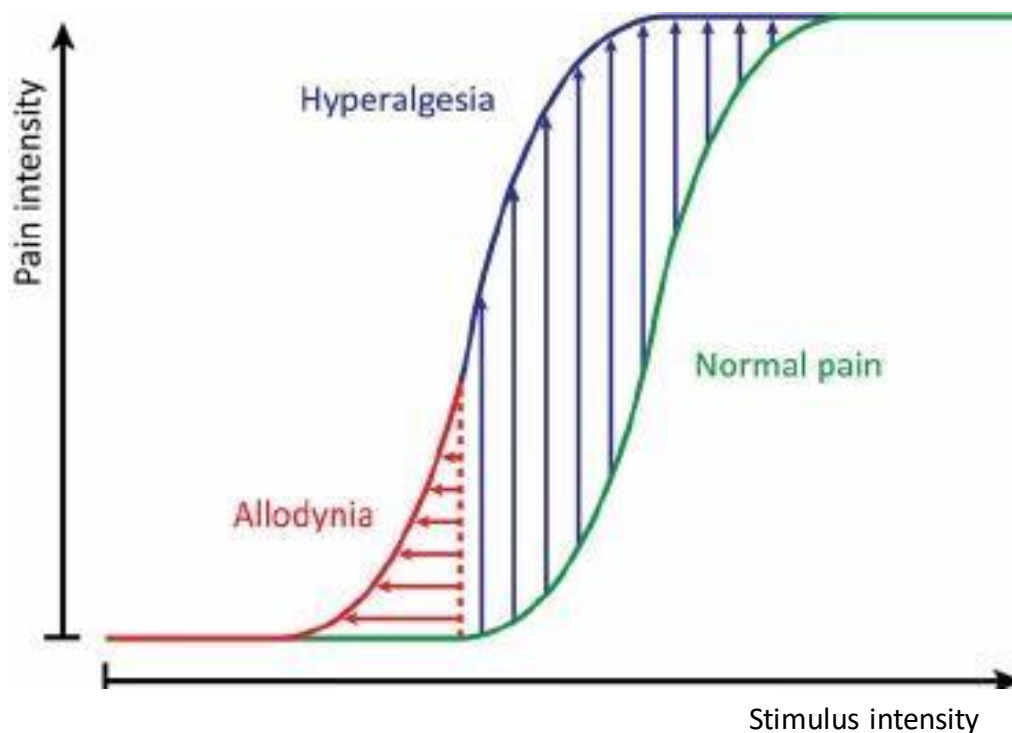


Figure 1.2: a graphical representation of the relationship between stimulus intensity and pain intensity, in normal pain and two types of hypersensitivity, allodynia and hyperalgesia (adapted from Lolignier, 2014)

Gastrointestinal disruptions in ASD

Over the past few decades, an increase in anecdotal reports of GI disturbances in individuals with ASD has sparked much research interest. The most commonly reported symptoms of GI disruption are chronic constipation, diarrhoea and abdominal pain (Hsiao, 2014). A 2014 meta-analysis of gastrointestinal symptoms in individuals with ASD showed an overall higher prevalence than neurotypical individuals, higher rates of diarrhoea, constipation and abdominal pain (McElhanon, 2014). They also highlighted the disparity in methodology and sample sizes, and how very few studies investigated the actual pathophysiology of GI disruption in ASD.

Children with ASD who exhibit GI symptoms are often undiagnosed, reports of altered bowel habits are unreliable generally, and considered more so when the population has communication deficits (Mayer, 2014). Autism and functional GI disorders have similar imaging abnormalities in the brain regions regulating sensory stimuli and emotions (Ellingson, 2013; Green, 2013). Children with irritable bowel syndrome (IBS) and functional abdominal

pain (FAP) show increased intestinal permeability (Shulman, 2008), in ASD this was first reported by de Magistris et al, who found increased permeability in 37% of patients with ASD and 21% of their relatives (de Magistris, 2010)

Visceral hypersensitivity is the 'enhanced intestinal perception converting otherwise physiologic stimuli into discomfort' (Barshop, 2016). ASD has been linked to functional abdominal pain (FAP), abdominal pain without other clinical signs such as abnormal laboratory tests (Wasilewska, 2015). However, there have been no studies on the state of visceral sensitivity in ASD, even though increased abdominal pain is a commonly reported symptom. To measure the afferent sensory response to distension, we used an in vitro multi-unit afferent nerve recording technique, which has been extensively used in the literature (Rong, 2004; Keating, 2008; Rong, 2008; Wang, 2012; Daly, 2011)

1.4 Neuroanatomy of the gut

The autonomic nervous system controls the function of the GI tract motility and secretion. It is split into two main branches, the enteric nervous system (ENS) and the extrinsic neural elements that form the connections between the gut, brain and spinal cord (Grundy, 2008).

The ENS is a complex independent nervous system comprised of the myenteric and submucosal plexuses that regulate reflex contractions and relaxations of the gut, as well as sensing the chemical contents of the lumen and controlling secretion and blood flow (Chandrasekharan, 2013).

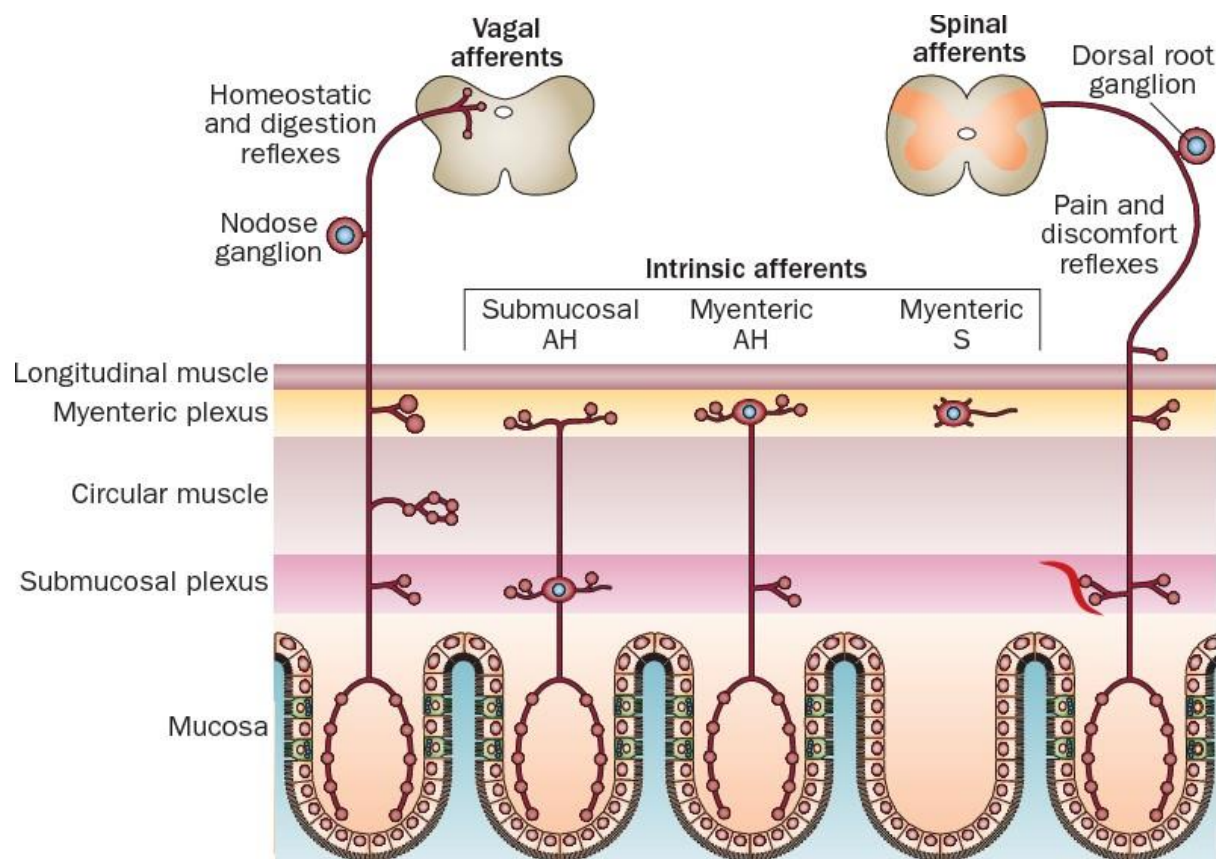


Figure 1.3: a schematic representation of the intrinsic and extrinsic afferent innervation of the small intestine, which extend into the lamina propria. Extrinsic vagal afferents synapse at the nodose ganglion and further into the NTS, spinal afferents synapse at the DRG and travel into the spinal cord. Intrinsic AH (after-hyperpolarisation) neurons from the submucosal and myenteric plexuses. There are also S (synapse) neurons which are mechanosensitive in the myenteric plexus, they don't project into the mucosa (Furness, 1998).

Extrinsic innervation of the gut comes in the forms of vagal and spinal afferent fibres. The cell bodies of vagal afferents are in the nodose and intracranial jugular ganglia, innervating from the pharynx to the proximal colon (Berthoud & Neuhuber, 2000). Spinal afferent cell bodies are in the dorsal root ganglia (DRG) to the spinal cord (Sengupta and Gebhart, 1994), they are composed of splanchnic and pelvic nerves, which follow the sympathetic and parasympathetic pathways to the gut wall (Grundy, 2006). These afferent fibres innervate the gut wall at various levels including the muscle, mucosa and the enteric ganglia (Blackshaw, 2007) and are near the intrinsic primary afferent neurons (IPANs) of the ENS. Extrinsic nerves run in mixed nerve fibres containing both afferent and efferent nerves (Sengupta and Gebhart, 1994). The nerve terminals of vagal and spinal afferents are localised to various layers within the gastrointestinal tracts. In the mucosal layers and the smooth muscle, as well as the serosal and mesenteric attachments, and the response profile is consistent with the placement (Berthoud, 2000). Vagal afferents innervate mainly the upper gastrointestinal tract, and

regulate gastric reflexes and perception of hunger, fullness, bloating, and nausea (Vermeulen, 2014), whereas spinal afferents mediate primarily pain signalling from the gut (Kyloh, 2011).

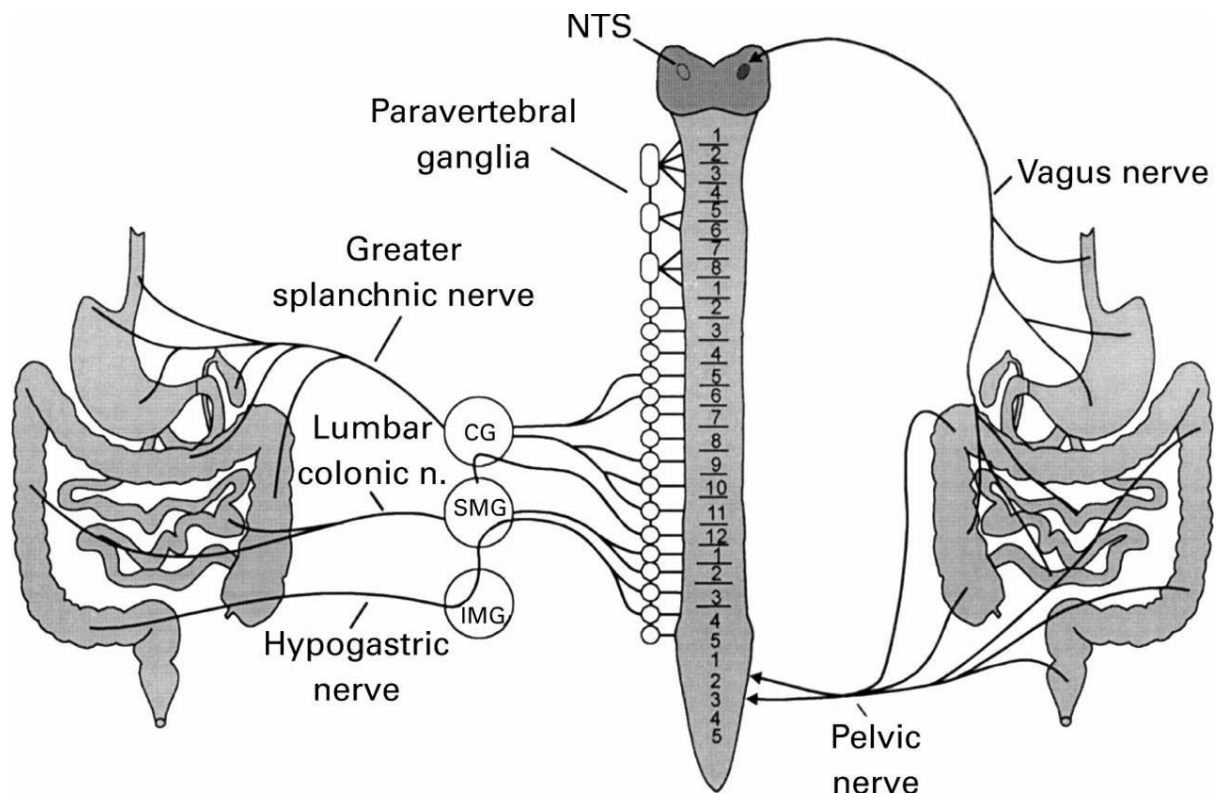


Figure 1.4: a schematic representation of extrinsic afferent innervation to the gastrointestinal tract. On the left are the sensory nerves associated with the sympathetic nervous system. The spinal visceral nerve fibres pass through prevertebral (CG – coeliac ganglion, IMG – inferior mesenteric ganglion, SMG – superior mesenteric ganglion) as well as paravertebral ganglia on the way to the spinal cord. On the right, the vagus nerves to the nucleus tractus solitarius (NTS) of the brainstem, and the pelvic nerves innervating the sacral spinal cord (Gebhart, 2000)

1.5 What activates visceral afferents?

Vagal and spinal afferents terminate in various levels within the gut wall and are responsible for the transduction of sensory information to the CNS. They are equipped with various receptors that respond to mechanical and chemical stimuli (Grundy, 2004).

Mechanosensation

The GI tract has a primary mechanical function, and ability to detect mechanical stimuli is critical for healthy function of the gut, for example in the stomach, distension is important for satiety and motility (Alcaino, 2017). Alterations in mechanosensation are associated with disorders such as constipation (Neshatian, 2015), obesity (Daly, 2011; Acosta, 2015) and the pathogenesis of colon cancer (Fernandez-Sanchez, 2015).

Mechanosensitive extrinsic afferent fibres terminate within the longitudinal and circular muscle, the mesentery, within enteric ganglia or close to the interstitial cells of Cajal (Lynn, 2005). Serosal and mesenteric afferents are activated by distortion of the mesenteric attachment, not the gut wall, meaning distension must be substantial in order to elicit afferent firing (Berthoud, 2004). Muscular afferents respond to distension of the gut wall, vagal afferents exhibit sustained responses, whereas splanchnic afferents are quicker adapting (Blumberg, 1983; Janig, 1991; Blackshaw, 1987). Mucosal afferents of both vagal and splanchnic origin are not activated by distension or contraction of the gut wall, they are more sensitive to mechanical deformation of the mucosa and adapt rapidly to continuous stimuli (Lynn, 1999; Cottrel & Iggo, 1984).

The stimulus-response profiles of extrinsic afferents to mechanical stimuli depend on their origin, vagal afferents transduce stimuli within the physiological range, and splanchnic afferents mediate mostly pain (Cervero, 1994; Blackshaw, 2002). There are also afferents which only respond to mechanical stimuli after inflammatory insult, known as silent afferents/nociceptors (Feng, 2010; Gold, 2010).

The identities of the mechanosensitive ion channels in the gastrointestinal tract are still elusive (Alcaino, 2017). The ion channel families that have so far been associated with mechanosensation are the degenerin/epithelial sodium channels (DEC/ENaC), the transient potential (TRP) channels and the acid sensing ion channel (ASIC) families (Page, 2005). Recently, the Piezo ion channel family was discovered (Coste, 2010), and research groups have described their function in gut mechanosensation (Wang, 2016). Through studies on sensory nerves from TRPA1^{-/-} mice, Brierley et al found a role for TRPA1 in mechanosensitivity. The response to noxious colonic distension was significantly reduced compared to control mice, and TRPA1 agonists led to mechanical hypersensitivity, which was exacerbated in colitis (Brierley, 2009). TRPV1^{-/-} mice exhibit significantly attenuated afferent responses to jejunal (Rong, 2004) and bladder (Daly, 2007) distension.

Chemosensation

A large range of chemical mediators can directly or indirectly influence the activity of visceral afferent nerves (Grundy, 2004). This can occur through direct activation of ligand gated ion channels, and through sensitization, which involves altering the activation threshold of another ion channel for example bradykinin lowers the heat activation threshold of TRPV1 (Sugiura, 2002). There is another mechanism of modulating the sensitivity to chemical stimuli, by altering the expression or activity of channels and receptors, for example, cholecystokinin is a neuropeptide that can regulate the expression of endocannabinoid receptors, in fasting state

the cannabinoid CB1 receptor is upregulated and after feeding, expression is lowered (Burdyga, 2004).

In the small intestine, serosal and mesenteric afferent nerves are chemosensitive, facilitated by their close contact with blood vessels (Lynn, 1999; Blumberg, 1983), responding to circulating or locally released factors such as adenosine triphosphate (ATP), bradykinin, capsaicin, histamine and 5-HT (Hicks, 2002; Coldwell, 2007; Barbara, 2007). These mediators are released in inflammatory states, by activated mast cells, and act directly on sensory nerves, or indirectly by initiating release of other agents, which in turn activate the sensory nerves. The presence of inflammatory mediators around the nerve terminal alters the expression of inflammatory receptors, in a process known as sensory neuronal plasticity (Kirkup, 2001). During local tissue injury, there is release of chemical mediators such as ATP, bradykinin, and prostaglandin E₂ (PGE₂), substances that can activate the afferent neurons directly or indirectly through triggering release of algescic mediators such as histamine, 5-HT and nerve growth factor (NGF) (Bueno, 2002).

Mechanical stimulation of the gut mucosa leads to release of serotonin (5-HT) by enterochromaffin cells (Bulbring, 1959; Bertrand, 2004) resulting in secretion of fluid (Cooke, 1997) and stimulation of motility (Heredia, 2009). 5-HT receptors compose a large group of structurally and pharmacologically distinct families of which there is one, 5-HT₃, classed as a ligand gated ion channel (Barnes, 1999). 5-HT₃ is expressed on vagal afferent terminals (LealCardoso, 1993; Hillsley, 1998; Kreis, 2002) and application of 5-HT causes an increase in afferent firing, which is significantly attenuated by 5-HT₃ antagonists (Hillsley, 1998). 5-HT₃ activation leads to increased glutamatergic transmission in the NTS of the brain (Glaum, 1992; Takenaka, 2011), which modulates various autonomic functions (Andresen, 1994).

TRPV1 is a ligand gated ion channel activated by capsaicin, noxious heat and acid (Caterina, 1997; Huang, 2002). It is expressed on sensory nerves that project to the trigeminal, nodose and dorsal root ganglia, preferentially on unmyelinated fibres (Helliwell, 1998; Michael, 1999; Ward, 2003), as well as in the brain (Mezey, 2000). In the gut, TRPV1 activation on sensory nerves causes release of peptide transmitters, which alter functions of the smooth muscle, vasculature and immune responses (Barthó, 2004; Mózsik, 2007). While TRPV1 plays a major role in exacerbating the inflammatory response in colitis, ileitis and pancreatitis (Eijelkamp, 2007; McVey, 2001; Nathan, 2001), it is also involved in gastrointestinal pain in the context of functional disorders of the gut such as irritable bowel syndrome (IBS) (Akbar, 2008). TRPV1 aids in the modification of vascular, immune and smooth muscle function of the gut (Barthó, 2004; Mózsik, 2007), through the release of transmitters such as substance P and neurokinin A by neurons during periods of inflammation (Holzer, 1998). Inflammatory conditions lead to

recruitment of intracellular TRPV1 vesicles being translocated to the plasma membrane by exocytosis (Morenilla-Palao, 2004). This provides a rationale for investigation of TRPV1's role in gastrointestinal dysfunction in ASD through recording of mesenteric afferent nerves of the jejunum, as both inflammatory (Lee, 2018) and functional (Penzol, 2019) gut disorders have been associated with ASD.

Another member of the TRP channel family is the TRPA1 channel, which is expressed on primary afferent neurons which project to the trigeminal, nodose and dorsal root ganglia (Zhang, 2004; Kobayashi, 2005; Anand; 2008), as well as in the ENS, enterochromaffin and enteroendocrine cells (Penuelas, 2007; Purhonen, 2008; Nozawa, 2009). Primarily, TRPA1 was identified as a cold temperature sensor (Story, 2003), but it is also a polymodal receptor sensitive to endogenous and exogenous irritants (Kang, 2010). In the GI tract, TRPA1 is necessary for mechano- and chemosensitivity, activation by agonists increases the response to mechanical stimuli (Brierley, 2009; Cattaruzza, 2010). There is evidence that this phenomenon occurs in the context of GI inflammation, in a rat model of colitis there is upregulation of TRPA1 in DRGs, increasing the visceromotor response to distension (Yang, 2008), which was abolished in TRPA1 knockout mice (Cattaruzza, 2010). Stress is associated with upregulation of TRPA1 in the DRG (Yu, 2010). This is interesting in the context of ASD, as GI symptoms has been linked to stress (Mazurek, 2013; Fulceri, 2016; Ferguson, 2017; Bishop-Fitzpatrick, 2015), TRPA1 may play a role in the increased hyperalgesia.

Sensory modality	Sensory elements	Stimulus	Response and function
Mechanosensitivity (Low threshold – innocuous stimuli)	Vagal and spinal sensory neurones	Neuron deformation	Modulation of reflex activity, eating behaviour, pain, immune functions. Crosstalk with ENS
Mechanosensitivity (High threshold – painful stimuli)	Spinal sensory neurones	Neuron deformation	Modulation of reflex activity, nociception (pain), blood flow. Crosstalk with ENS
Chemosensitivity	Vagal and spinal sensory neurones	Acid, Short chain fatty acids, ischaemia, inflammation and injury	Modulation of reflex activity, nociception (pain), cytoprotection

Figure 1.5: a table summarizing the sensory aspects of visceral afferent nerves innervating the jejunum, the sensory elements, the stimuli they respond to and the subsequent function activation serves. Adapted from Blackshaw et al, 2007

1.6 Research aims

This thesis focused on the alterations in circadian rhythms and sensory signalling in the somatic and visceral transduction pathways in two mouse models of ASD. The objectives included

1. Inducing the valproic acid mouse model of ASD in a C57 BL/6J background
2. Investigating the changes in behaviour over 24 hours, as a measure of circadian rhythm
3. Investigating the thermosensation of two mouse models of ASD, prenatal VPA and BTBR, using the Dynamic Hot and Cold plate test
4. Determining the visceral afferent sensitivity to chemical and mechanical stimuli to characterise the sensory function of the gut.

Hypotheses

1. The circadian rhythm of behaviour, included sleeping and eating behaviour is altered in the mouse models of ASD
2. The sensitivity to noxious thermal stimuli is reduced
3. Afferent nerve sensitivity to mechanical distension as well as 5-HT, TRPA1 and TRPV1 agonists is altered
4. Afferent nerve sensitivity to inflammatory mediators including bradykinin, prostaglandin E₂, 5-HT and histamine is altered

2 Materials and Methods

2.1 Animals

Mice used in these experiments were males of three groups, control C57 BL/6J, BTBR T+tf/J purchased from the Jackson Laboratory, and prenatal VPA treated mice, which were induced from a C57 BL/6J background. All mice were housed with littermates, and received ad libitum food and water, in accordance with UK Home Office regulations for animal welfare. Experiments were conducted while the animals were between 3 and 6 months old (adult), the LABORAS study first, followed by the dynamic hot/cold plate tests and the jejunum recordings. All animals were humanely sacrificed by cervical dislocation in accordance with UK Home Office regulations with Schedule 1 procedures of the UK Animals (Scientific Procedures) Act 1986.

2.2 Induction of VPA model

On the two days before mating, bedding from the male cages were added to the female cages. Breeding pairs of C57 BL/6J background were set up, female mice were placed in the male stud breeding cage at approximately 7 am and taken out at approximately 4 pm (9 hours). They were weighed and examined, then placed back into their original cage with their cage mates, this was taken to be embryonic day 0 (E0). On E11, the females were weighed again and examined, and pregnancy was determined by weight gain and abdominal palpation. The mice determined to be pregnant received a single subcutaneous injection of 500 mg/kg VPA.

2.3 LABORAS (Laboratory Animal Behaviour Observation Registration and Analysis System)

Tracking information provided by LABORAS include average speed, maximum speed and distance travelled. Habituation was started on the two days before the test. On day 1, mice were habituated to the experimenter and the LABORAS cage/room. Each mouse is placed in a separate cage with bedding, food and water, for 15 minutes. On day 2 mice were handled again by the experimenter and placed the LABORAS cage/room, for 30 minutes. On test day, the bedding, water, food and mice were weighed, and the mice were placed into their cages and left for 24 hours. At the end of the testing period, the mice were weighed again, and placed back into their home cage. The bedding, water and food were weighed again also.

Data analysis

The LABORAS software produced an excel file containing all the data collected over the 24 hours, which was used in this analysis. The weight change of the animals, the bedding and water were negligible, so were not included into analysis. The data was analysed in GraphPad Prism and expressed as mean +/- SEM.

2.4 Dynamic Hot/Cold Plate

All animals were habituated to the test room and to the Plexiglas Ugo Basile Hot-Cold Plate equipment, to reduce the stress response and allow for accurate analysis.

Day 1	Day 2	Day 3	Day 4	Day 5
Habituation day 1 Home cage is placed in Behavioural lab for one hour Each mouse is placed on the plate set at 30 °C for 15 minutes	Habituation day 2 Home cage is placed in Behavioural Analysis lab for one hour. Each mouse is placed in the plate set at 30 °C for 30 minutes	Hot plate test	Habituation day 3 Home cage is placed in Behavioural lab for one hour Each mouse is placed in the plate set at 20 °C for 15 minutes	Cold plate test

Figure 2.1: the schedule followed for the Dynamic Hot/Cold plate experiments. The habituation is conducted on days 1, 2 and 4, to reduce the stress response of the animals on test day.

Hot plate test

The video camera was started a few seconds before the test began. The animal was placed on the plate set at 30 °C and its identification and the details of the test were flashed on the screen. The hot plate was started, increasing the temperature to 41 °C in 1 °C/min increments. Once this was reached, the test was stopped, the animal placed into an interim cage, the camera stopped, and the video saved. The plate and surrounding areas were cleaned, before starting the next experiment.

Cold plate test

This test was conducted in an identical way as to the hot plate test, except the starting temperature was 20°C and declined to 1°C. As the Ugo Basile plate only allows for a total of 999 seconds between ramp changes in temperature, the temperature decrease occurred at a rate of 1°C/0.8 mins.

Data analysis

The videos were analysed by the experimenter, the number of escape behaviours (jumps) counted at the temperature at which they occurred. Results are expressed as mean +/- SEM with the N values, which refer to the number of animals.

2.5 In vitro recording of jejunum afferent nerves

After the mice were sacrificed, a midline incision of the abdomen was performed, the entire intestine from the duodenum to the colon was excised and harvested in carbogenated Krebs solution (composition, in mM: NaCl 118.4, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11.7). Fine dissection of the tissue was completed to leave a 2 cm segment of jejunum with the mesenteric neurovascular bundle attached in the recording chamber, which was continuously perfused with oxygenated Krebs and kept at 35°C to prevent tissue degradation

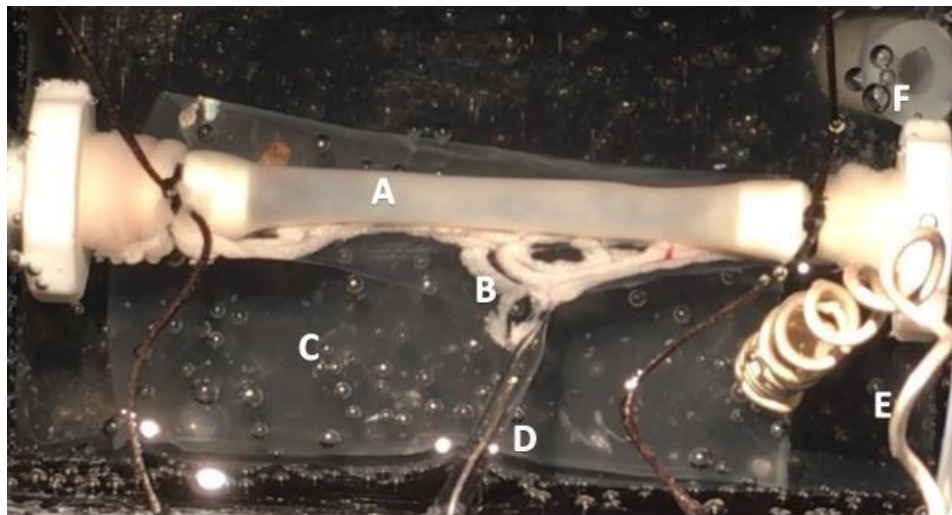
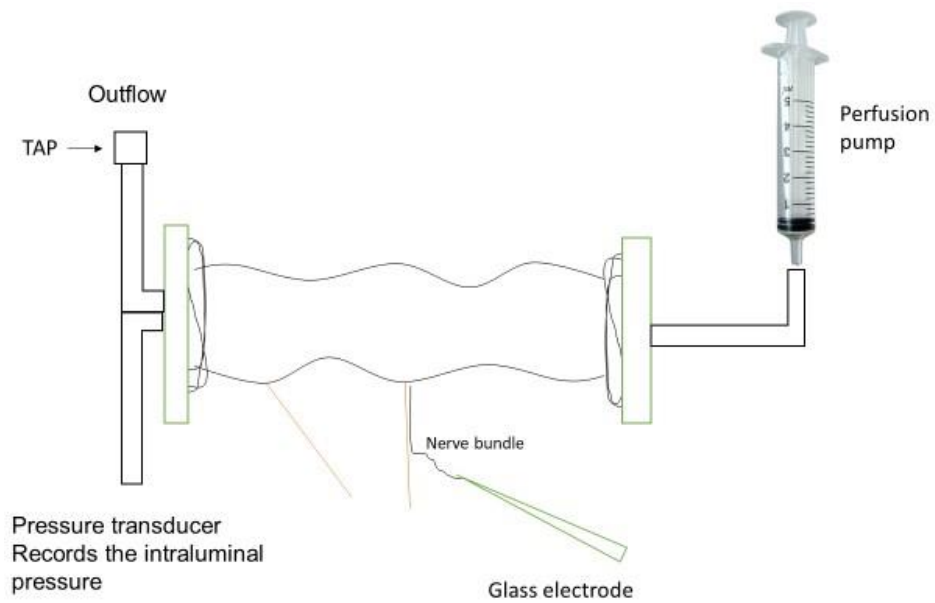


Figure 1.7: in vitro preparation to measure jejunum afferent nerve activity and intraluminal pressure. Schematic diagram and picture of the preparation. A – 2 cm segment of jejunum, B – mesenteric attachment pinned to C – sylgard platform of the organ bath, D – glass electrode with a nerve bundle inserted. E – the ground wire and F – the inflow of oxygenated Krebs solution.

As shown on figure 1.7, the segment was cannulated to both ends of the recording chamber and tied firmly, one end connected to the infusion pump to allow filling of the segment, the

other end connected to a pressure transducer and an outflow with a three-way tap. By closing the tap and turning on the perfusion pump, the segment can be filled to 50 mmHg.

The mesenteric attachment was pinned to a sylgard-lined platform to aid dissection of the nerve bundle. Using a dissection microscope, the surrounding adipose tissue was removed, and the nerve bundle identified with fine forceps. The nerve bundle was sucked into a suction electrode and sealed with adipose tissue to insulate the signal. The electrode was attached to a Neurolog (NL 100, Digitimer Ltd, UK) and an AC amplifier (NL104). Signals were amplified by 10, 000 times, filtered (NL125, pass band filter) and recorded on a computer via a power 1401 analogue to digital interface and Spike2 software (Version 7.10, Cambridge Electronic Design, UK). Multi-unit afferent nerve activity was quantified by a Spike processor (Digitimer D130) to count the number of spikes over a pre-determined threshold, which was usually set at twice the baseline noise level.

Experimental protocols

Control distensions

At the start of each experiment the jejunum was distended to 50 mmHg three times, to ensure there were no holes or leakages and distensions were reproducible, after which the nerve bundle dissection was started.

Altering the filling rate

Experiments were conducted at either 600 μ l/min or 200 μ l/min, filling to 50 mmHg at 10-minute intervals.

Extraluminal application of pharmacological agents

All pharmacological agents were diluted with Krebs buffer and perfused into the organ bath using a peristaltic pump or applied directly on the bath as a bolus.

Intraluminal application of pharmacological agents

Pharmacological agents to be applied intraluminally were diluted in Krebs and perfused continually into the lumen using the perfusion pump.

Compound	Concentration	Target	Manufacturer
2-methyl 5-hydroxytryptamine (2-methyl 5-HT)	100 μ M (Keating, 2008)	5-HT ₃	Tocris
Capsaicin	1 μ M (Keating, 2006)	TRPV1	Tocris
Allyl isothiocyanate (AITC)	100 μ M (Yu, 2014)	TRPA1	Sigma
5-hydroxytryptamine (5-HT)	5 μ M	5-HT receptors	Sigma
Bradykinin	5 μ M	B ₁ and B ₂	Tocris
Histamine	5 μ M	H ₁	Sigma
Prostaglandin E ₂	5 μ M	EP ₂	Sigma

] (Jones, 2005)

Figure 1.8: a table summarizing the compounds used in the jejunum afferent nerve recording, concentrations and the literature they were derived from. 5-HT, bradykinin, histamine and prostaglandin E₂ were included in the inflammatory soup as previously described by Jones et al (2005).

Data analysis

Distension results were analysed using a Spike2 script, all electrophysiological data was presented as change in frequency of afferent discharge in response to increasing intraluminal pressure or application of pharmacological agent. All data was expressed as mean +/- SEM and n number, which refers to number of animals.

3 Results

3.1.1 Prenatal VPA exposed mice show altered circadian rhythm

Rodents are more active in the night-time as they are nocturnal, and all the groups studied showed an increase in immobility in the light phase. LABORAS defines immobility as a period of uninterrupted resting while the animal is in a lying or sitting position. In this analysis, the 24-hour test period is split into 4-hour intervals to show the change over time. In the dark phase (shaded area on figure 2), the BTBR and c57 mice exhibit a decrease in immobility as they become more active. The VPA mice show an increase in immobility which levels out over the dark phase, which may be a manifestation of altered circadian rhythm.

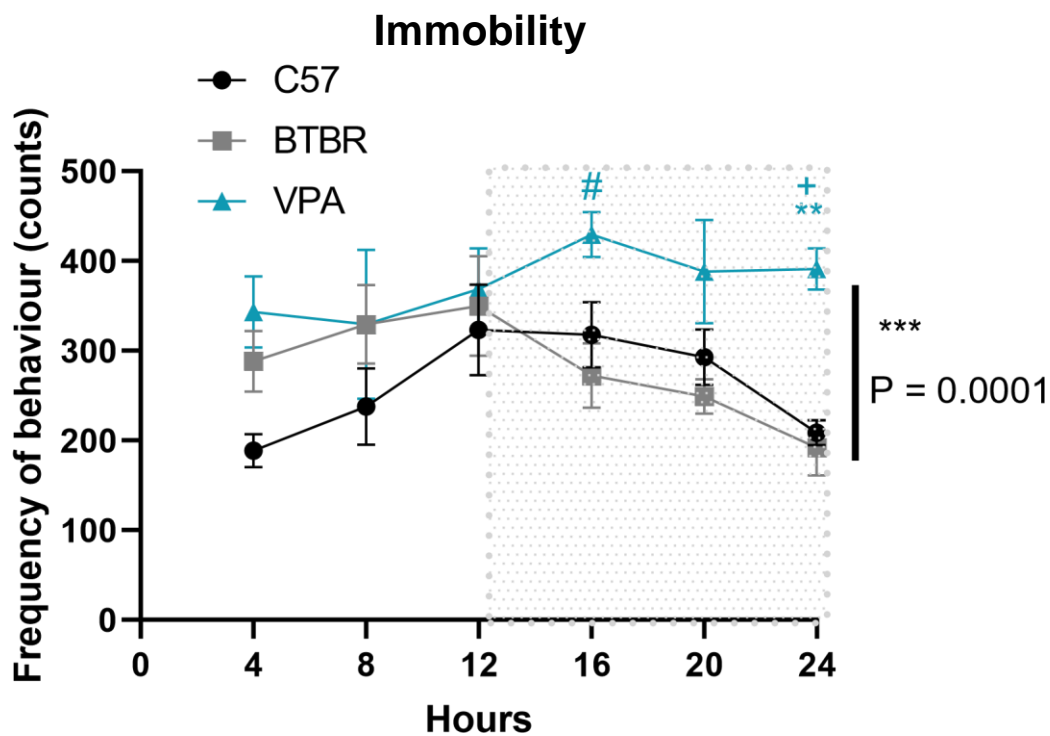


Figure 2: The alterations in immobility (a measure for sleep) shown by C57, BTBR and VPA mice over 24 hours, the BTBR and C57 mice show a gradual increase in immobility in the light (sleep) phase and a gradual decrease in the dark (wake) phase. The VPA mice show increased immobility in the light phase, which stays high in the dark phase. VPA mice were significantly more immobile than the BTBR mice in the 12- to 16- hour interval ($P = 0.0458$) and the 20- to 24- hour interval ($P = 0.0068$) and the C57 mice in the 20- to 24- hour interval ($P = 0.0176$). C57 $n = 8$; BTBR = 9, VPA = 4

All three strains showed normal circadian alterations of feeding, as eating count was low in the light phase and increased in the dark phase. The time spent eating was higher in the light phase, they spent longer eating a smaller amount of food, while it reduced in the dark phase as they ate more in a shorter time period. However, the VPA mice deviate from this trend as, although the pattern of eating and total amount eaten was unchanged (figure 3A and 3B), there were variations in time taken to eat, as shown on figure 3C. This is interesting as the total time eaten is unchanged, as shown on figure 3D. In the light phase, the VPA mice took a significantly shorter time to eat the small amount, then in the dark phase it took them much longer to eat the larger amount. This may be due to differences in satiety, as the meal in the light phase was smaller, they ate it quickly, however a larger amount of food may take them longer to eat, they may have eaten in smaller bursts as they got hungry soon after they ate.

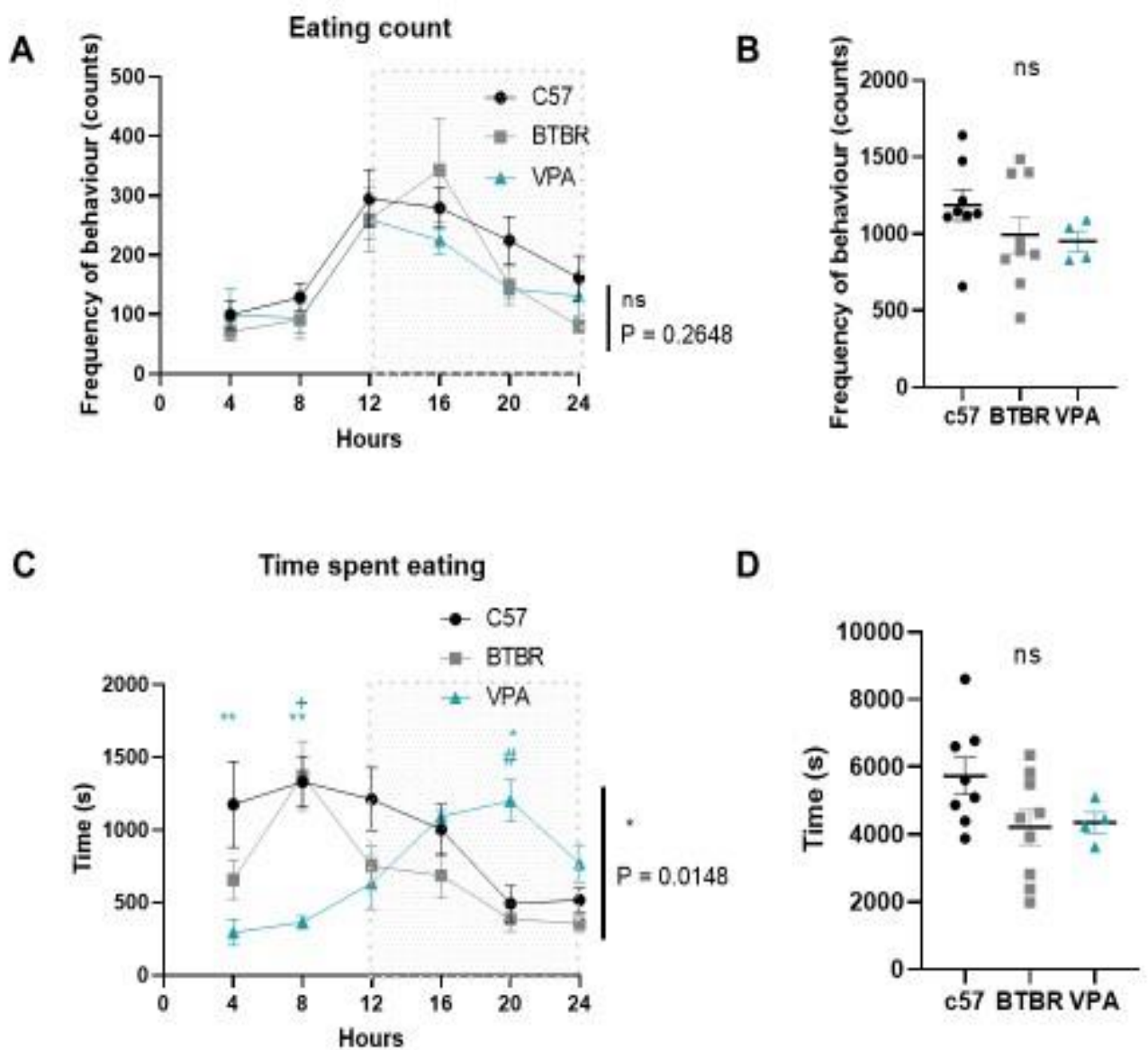


Figure 3: the eating behaviour of C57, BTBR and VPA mice over 24 hours. **3A:** the frequency of eating behaviour over 24 hours split into 4- hour intervals. In the light phase, eating count was low in all groups, increasing in the 8- to 12- hour interval, peaking in the 12- to 16- hour interval, then gradually decreases. There was no significant difference between the three groups ($P = 0.2648$). **3B:** the total frequency of eating over 24 hours, there was no significant difference between the three groups ($P = 0.3346$). **3C:** the amount of time spent eating over 24 hours, split into 4-hour intervals. The C57 and BTBR showed increased eating time in the light phase, peaking between the 4- and 8- hour interval, and decreased in the dark phase. VPA mice spent significantly decreased time eating compared to C57 in the first four hours ($P = 0.0060$), between the 4- to 8- hour interval ($P = 0.0022$) and significantly increased time between the 16- and 20- hour interval ($P = 0.0373$). compared to the BTBR mice, VPA mice spent significantly decreased time eating between the 4- to 8- hour interval ($P = 0.0011$) and significantly increased time between the 16- and 20- hour interval ($P = 0.0109$) **3D:** total time spent eating over 24 hours. There was no significant difference between the strains, $P = 0.0987$. analysed with one-way ANOVA with Bonferroni post-test. C57 $n = 8$; BTBR = 9, VPA = 4.

3.1.2 The BTBR and VPA mice displayed significantly decreased grooming than the c57 mice

Excessive grooming behaviour by the BTBR and VPA mice is reported extensively in the literature, however in this study these mice groomed significantly less than the c57 mice throughout the 24 hours recorded. Grooming increased initially, then gradually decreased in all strains, however the c57s groomed consistently higher across the 24 hours recorded. In terms of total grooming, BTBR mice showed significantly less grooming behaviour than c57 mice ($P = 0.0090$), VPAs also groomed less, but not significantly ($P = 0.1158$).

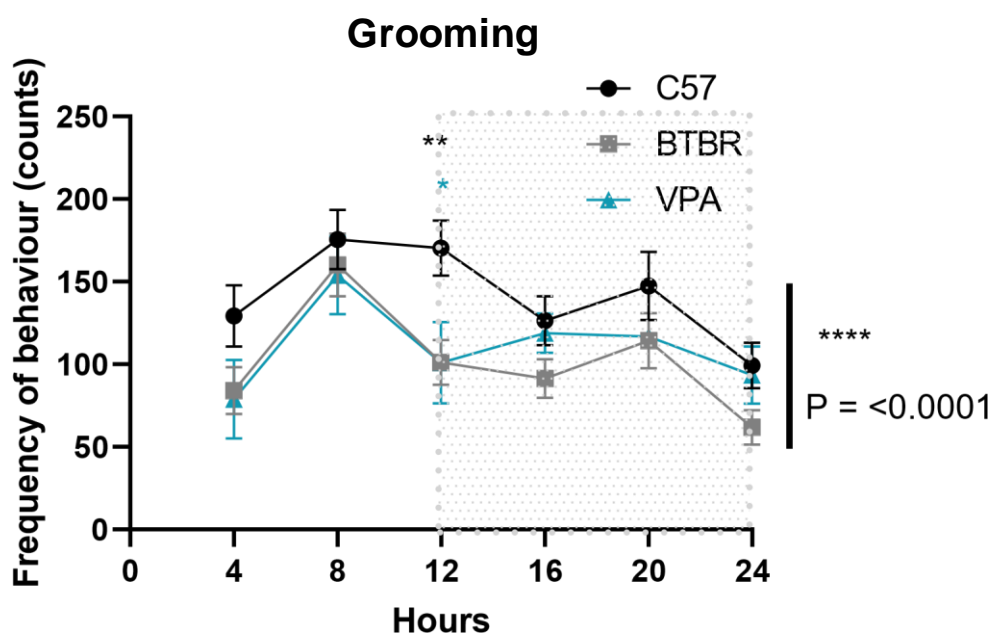


Figure 4: grooming behaviour exhibited by the three groups over 24 hours, there didn't seem to be variations in grooming between the light and dark phases. The VPA and BTBR mice groomed significantly less compared to C57 mice over 24 hours ($P = <0.0001$). The BTBR mice groomed significantly less compared to C57 mice in the 8- to 12- hour interval ($P = 0.0060$), VPA mice groomed significantly less compared to C57 mice in the 8- to 12- hour interval ($P = 0.0399$). analysed with two-way ANOVA with Bonferroni post-test. C57 $n = 8$; BTBR = 9, VPA = 4.

3.1.3 VPA mice show slightly altered circadian changes in locomotion

Exploratory behaviour was measured over 24 hours in the LABORAS cages, which are placed on sensory platforms able to detect and score rodent movements. LABORAS specifies distance as the total distance travelled (in metres) by the animal throughout the test. Locomotion is the general activity of the animal while it is mobile, and the average speed is defined as the speed the animal travels in millimetres per second. As the animals explore the new environment, all measures of locomotion decrease in all three groups over time as they become habituated (figures 5A, 5B and 5C)

This data shows the circadian alterations of activity of rodents, as they are nocturnal activity significantly increases in the dark phase (figure 5A), however, the VPA mice do not appear to show these circadian alterations as their activity decreases in the dark phase. Between the 12- and 16- hour interval, the activity of the VPA mice is significantly lower than the BTBR mice ($P = 0.0420$). BTBR mice show almost identical circadian changes in locomotion to c57s, but significantly increased average speed and distance travelled. Between the 8- and 12- hour interval, the VPA mice show significantly decreased distance travelled ($P = 0.0005$) and average speed ($P = 0.0499$) compared to the BTBRs.

The distance travelled is an interesting metric as the distance increases as the animals explore the new environment, decreases over time as they become habituated, this trend is also shown in their average speed.

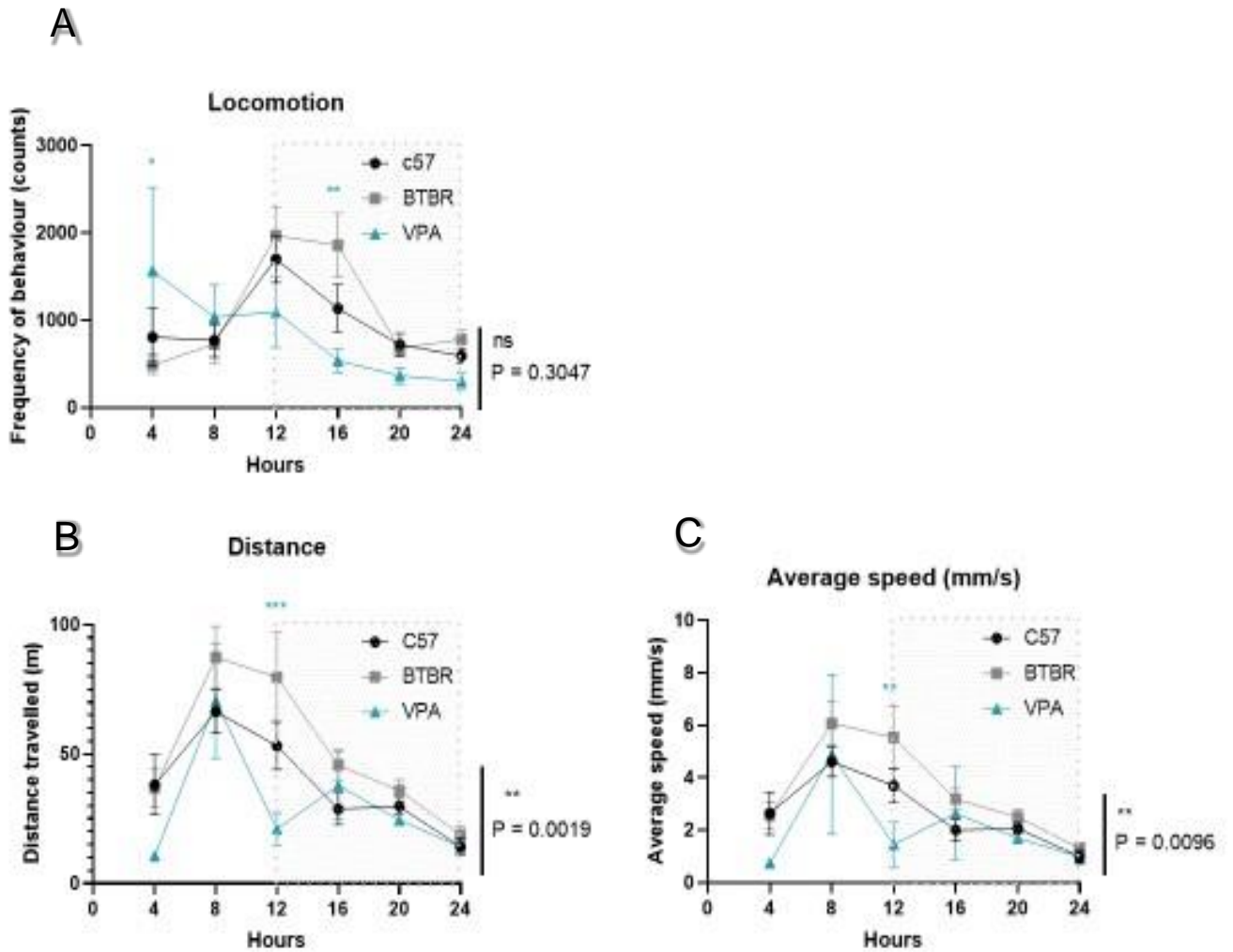


Figure 5: changes in activity over 24 hours exhibited by the C57, BTBR and VPA mice. **5A)** a graph showing the general locomotion of the mice. The BTBR and C57 mice show clear circadian variations in locomotion, as it starts low in the light (inactive) phase and increases in the dark (active) phase. VPA mice show a significant decrease in activity in the dark phase, especially in the 12- and 16-hour interval. ($P = 0.0085$). **5B)** a graph showing the distance travelled in metres, the three curves were significantly different ($P=0.019$). The BTBR mice travel significantly further than the C57 and VPA mice throughout the test, whereas the VPA mice travel significantly less than the BTBR mice between the 8- and 12-hour interval ($P = 0.0005$). **5C)** A graph showing the average speed travelled over 24 hours in mm/s, the three curves were significantly different ($P=0.0096$). As with distance, the BTBR mice travelled significantly faster than the C57 and VPA mice, the VPA mice travelled significantly less than the BTBR mice in the 8- and 12- hour interval ($P = 0.0499$). C57 $n = 8$; BTBR = 9, VPA = 4, analysed with two-way ANOVA with Bonferroni post-test for multiple comparisons

3.2 Alterations in sensitivity to thermal stimuli in BTBR and VPA mouse models of ASD

BTBR and prenatal VPA mice exhibit significantly different responses to changes in temperature

The dynamic hot plate is a method of assessing thermosensation in rodents, as described by Yalcin et al (2009). As the temperature increases slowly from 30°C to 41°C in 1 degree/minute intervals, the sensory threshold, or the point at which the animal identifies as pain and exhibits escape behaviour (jumps) was recorded. In this study, the BTBR mice showed significantly reduced sensitivity to heat, response began at a higher C57 mice (40°C vs 38°C), the number of escape behaviours was lower at any given temperature compared to the behaviour exhibited by the C57 or VPA animals ($P= 0.0068$; $n = 6$). VPA mice also started responding at 40°C also, however the number of jumps was higher than BTBRs and by 41°C, matched that of the C57 mice.

The dynamic cold plate works in a similar way to the dynamic hot plate, the temperature is decreased slowly from 20°C to 1°C (1°C/0.8min) and the point at which the animal responds by an escape behaviour (jumps) was again recorded. In this case, the c57s and BTBRs show similar responses to decreasing temperature, at around 16°C the animals start jumping, but rate of jumping is highest from 2°C. The VPAs show very little response to cold compared to the other groups, response started at 2°C and number of jumps was lower compared to c57s but not to a significant level ($p = 0.0559$).

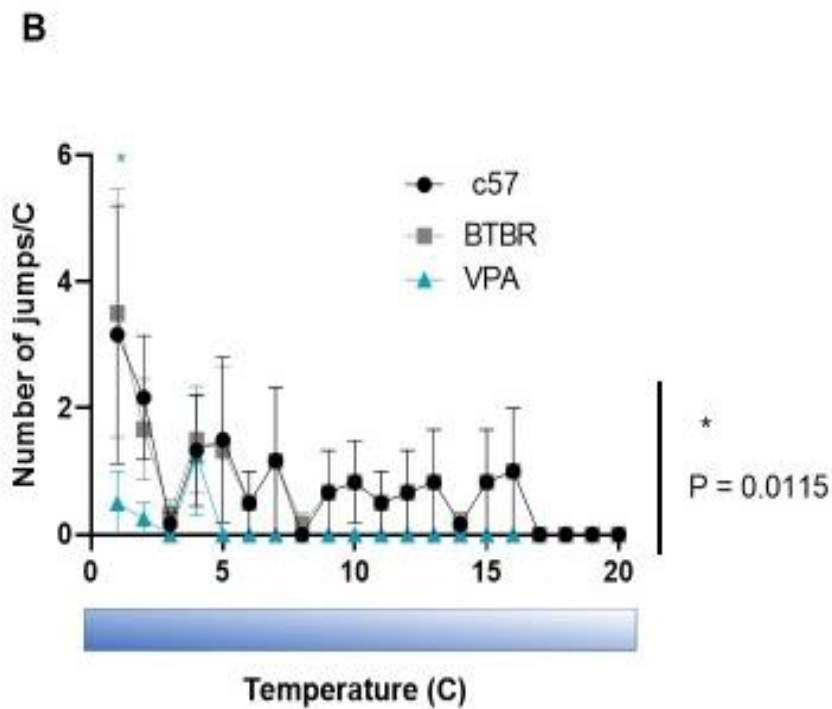
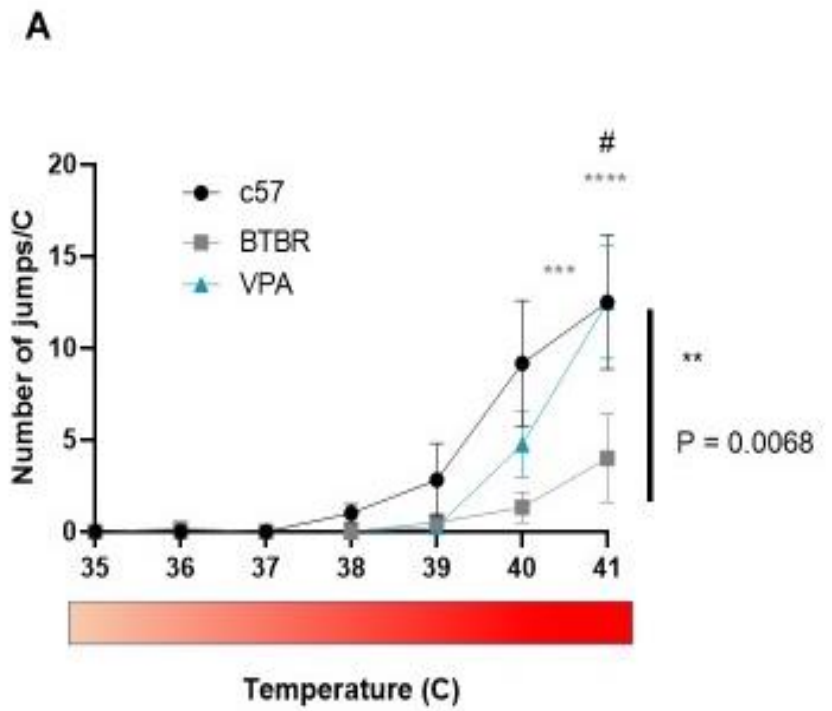


Figure 6: all animals displayed escape behaviour in response to alterations in temperature, which was measured as number of jumps per degree interval. Figure **6A**: the graph starts at 35°C as there was no

response in any of the animals between 30-35°C. Increasing temperature caused all three groups to increase escape behaviour, at the top temperature of 41°C, the C57 group jumped 12.5 (+/- 3.66) times, the VPA group 12.5 (+/- 3.07) times, whereas the BTBR group jumped significantly less at 4 (+/- 2.41) times (BTBR vs C57 $p = <0.0001$; BTBR vs VPA $p = 0.0003$)

Figure **6B**: Decreasing temperature also resulted in jumps, which starts for the C57 and BTBR mice at 16°C where they jump around 1 time per degree. Peak escape behaviour occurs at 1°C, the C57 group jump 3.17 (+/- 2.04) times, the BTBR group 3.5 (+/- 1.96) times, whereas the VPA group jumped significantly less at compared to BTBRs at 0.5 (+/- 0.5) times ($p = 0.0246$) and less compared to c57, but not significant ($p = 0.0559$). C57 $n = 6$; BTBR $n = 6$; VPA $n = 4$.

Analysed with two-way ANOVA with Bonferroni post-test

3.3 Mechanosensitivity in BTBR mouse model of ASD

Mechanosensitivity of the jejunum was assessed in tissue taken from BTBR and c57 control mice through an in vitro preparation which allowed multi-unit recording of afferent nerves, as previously described by Rong et al (2004). Ramp distension was performed by intraluminal perfusion of oxygenated Krebs buffer to a maximum pressure of 50 mmHg, in order to stimulate the mechanoreceptors responsive to intraluminal filling. Two filling rates were chosen to assess the impact of different mechanical stimuli on the afferent response, the jejunum was distended at either 200 $\mu\text{l}/\text{min}$ (slow) and 600 $\mu\text{l}/\text{min}$ (fast). Below is a sample trace from control c57 tissue at 200 $\mu\text{l}/\text{min}$.

The afferent nerve response profile to increasing intraluminal pressure to 50 mmHg was similar in the c57 and BTBR tissues. There was a bi-phasic response evidenced by moderate rise in firing concomitant with rises in pressure until approximately 20 mmHg, at which point afferent firing increased in a linear fashion. This is a finding reported commonly (Rong, 2004; Keating, 2008; Rong, 2008; Wang, 2012; Daly, 2011)

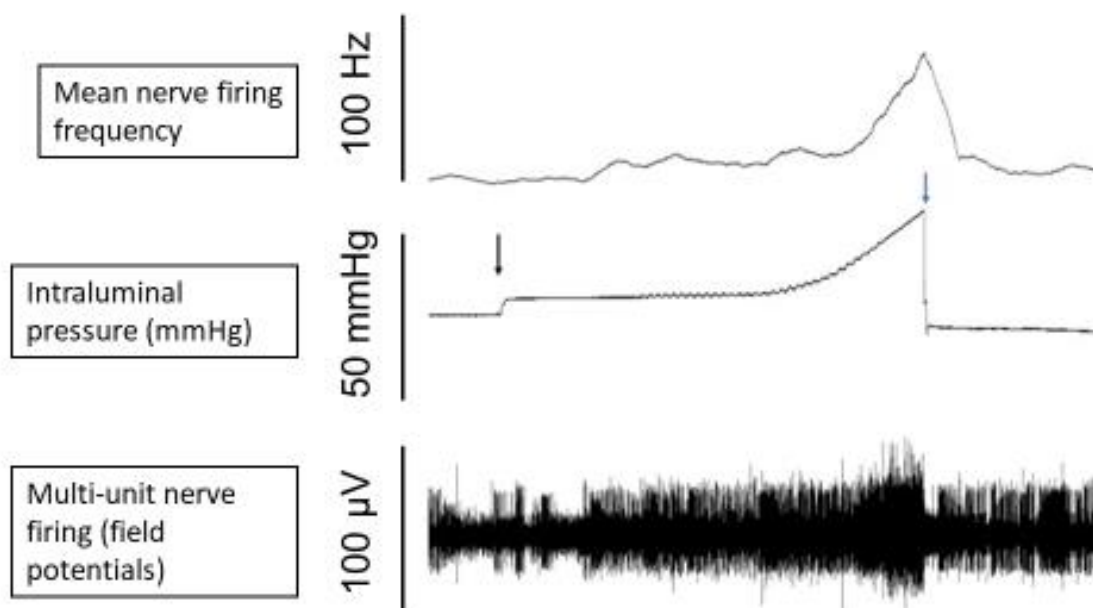


Figure 7: a sample trace showing the response of mesenteric nerve fibres to increasing intraluminal pressure. The multi-unit nerve firing corresponds to the field potentials captured by the electrode and visualised as spikes which vary in amplitudes. The frequency of firing is shown in the mean firing rate (mean spikes per 10 seconds) which shows the biphasic nature of nerve firing response. The black arrow represents the start of perfusion, and the blue arrow represents the opening of the three-way tap, allowing the gut to empty. The mean nerve frequency shows the increase rate in spike frequency per 10 seconds, and shows a bi-phasic response, the peak nerve response at 50mmHg.

3.3.1 Afferent firing and compliance is altered by rate of distension

An important aspect of mechanosensitivity is neurogenic adaptation, Yang et al published an interesting study where they showed a faster filling rate leads to increased afferent firing, as the increased rate does not allow for the afferent nerves to adapt as quickly as the tissue stretches to accommodate the increasing volume (Yang, 2012). We also observed this phenomenon, in control tissue the 600 $\mu\text{l}/\text{min}$ filling rate ($n = 10$) resulted in a higher afferent response, the firing rate increased by 69.66% compared to the 200 $\mu\text{l}/\text{min}$ filling rate ($n = 5$, $P = <0.0001$). This pattern was observed in the BTBR tissue ($P = 0.0017$) however less pronounced, afferent firing increased by 51.65% (600 $\mu\text{l}/\text{min}$ $n = 9$; 200 $\mu\text{l}/\text{min}$ $n = 5$), as shown on figure 8B).

We observed significantly decreased pressure-volume relationship at 600 $\mu\text{l}/\text{min}$ in the c57 tissue (figure 8C). The 200 $\mu\text{l}/\text{min}$ filling rate accommodated more liquid than the 600 $\mu\text{l}/\text{min}$ rate ($P = 0.0045$). Strikingly, in the BTBR tissue, the opposite is true, the 600 $\mu\text{l}/\text{min}$ filling rate allowed a significantly increased accommodation of liquid from 5 mmHg, which suggests alterations in the biomechanical properties of the tissue.

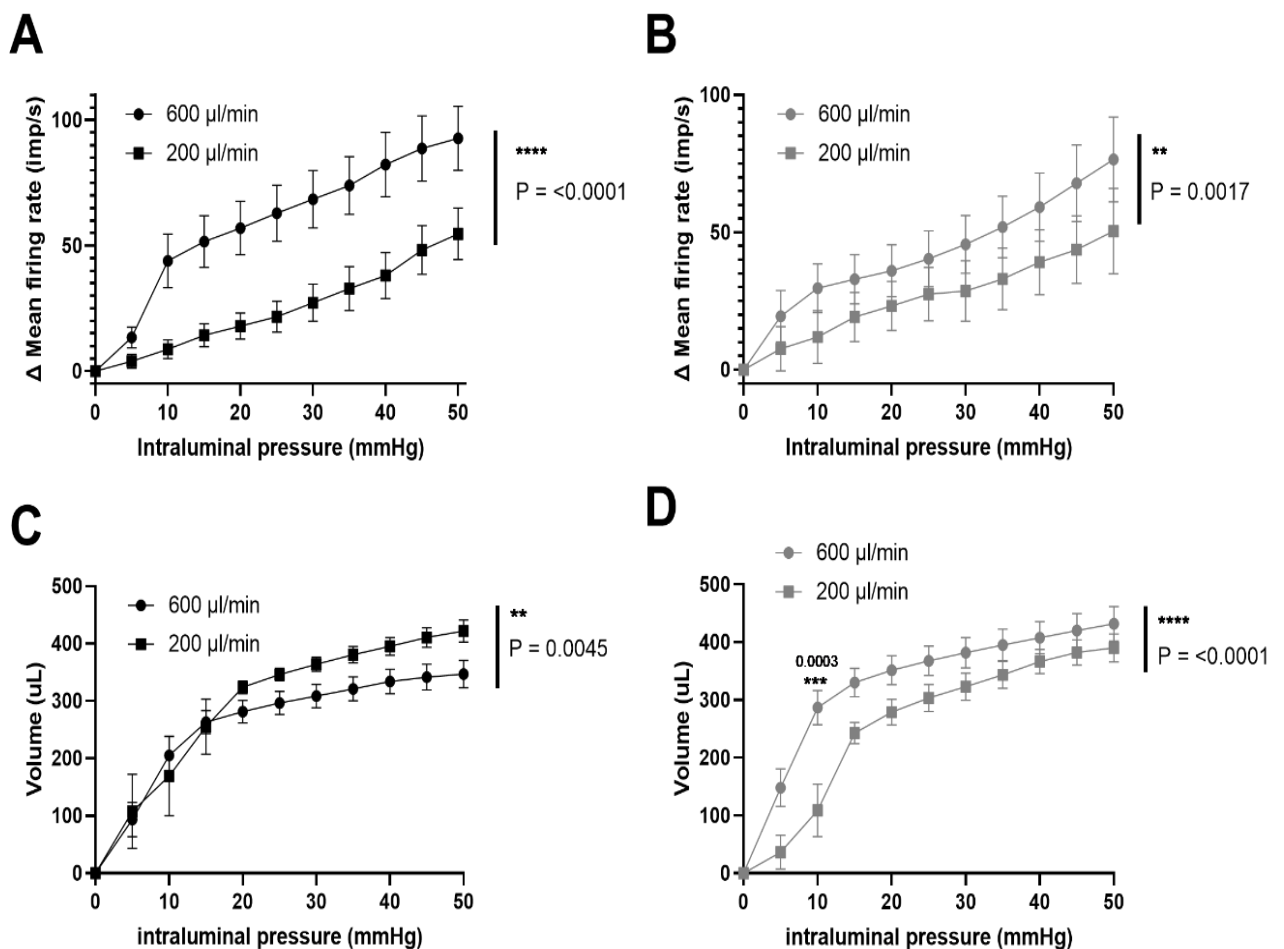


Figure 8: the impact of altered filling rates on afferent firing and compliance. The graphs on the left

correspond to C57 data and the graphs on the right correspond to BTBR data. **8A** shows the distension response to 200 and 600 $\mu\text{l}/\text{min}$ in c57 tissue, the faster rate produces a significantly increased afferent response compared to the slower rate ($P = <0.0001$, 600 $\mu\text{l}/\text{min}$ $n = 10$; 200 $\mu\text{l}/\text{min}$ $n = 5$). This pattern was also observed in the BTBR tissue, in figure **8B** the faster rate produced a significantly increased response ($P = 0.0017$; 600 $\mu\text{l}/\text{min}$ $n = 9$; 200 $\mu\text{l}/\text{min}$ $n = 5$) however the difference was not as pronounced. Figure **8C** shows the pressure-volume relationship of c57 tissue, the 200 $\mu\text{l}/\text{min}$ filling rate led to accommodation of more volume ($P = 0.0045$; 600 $\mu\text{l}/\text{min}$ $n = 10$; 200 $\mu\text{l}/\text{min}$ $n = 5$) Figure **8D** shows the pressure-volume relationship of BTBR tissue, here the increased rate led to accommodation of more volume ($P = <0.0001$; 600 $\mu\text{l}/\text{min}$ $n = 9$; 200 $\mu\text{l}/\text{min}$ $n = 5$) and at 10 mmHg the volume was significantly higher at the rate of 600 $\mu\text{l}/\text{min}$ ($P=0.0003$). Analysed with two-way ANOVA with Bonferroni post-test.

3.3.2 There is no significant difference in afferent response to 50 mmHg distension at 200 $\mu\text{l}/\text{min}$

The mesenteric afferent response to intraluminal filling at a rate of 200 $\mu\text{l}/\text{min}$ was not different between tissue from BTBR and C57 mice, figure 9C shows the two curves are almost overlapping ($P= 0.6897$; C57 $n = 5$; BTBR $n = 5$). Figure 9D illustrates the pressure-volume relationship which is a measure of compliance. The BTBR tissue showed a significant decrease in the pressure-volume relationship ($P= 0.0056$), suggesting reduced accommodation of volume as the pressure increased.

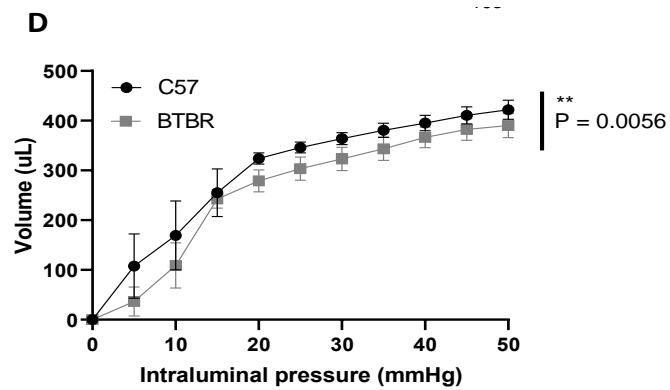
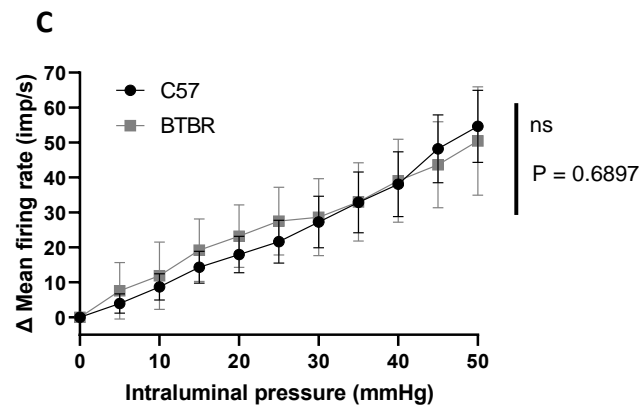
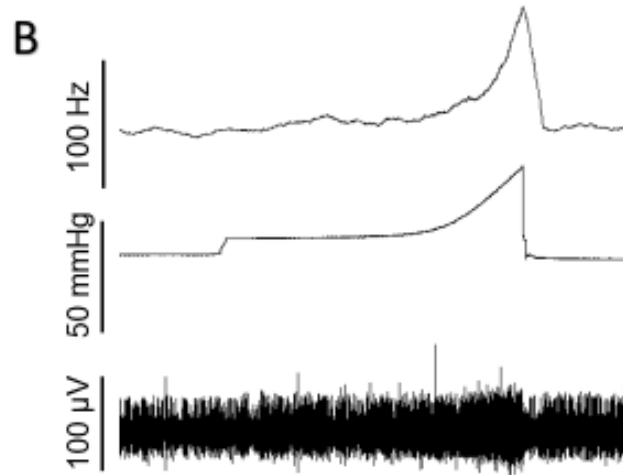
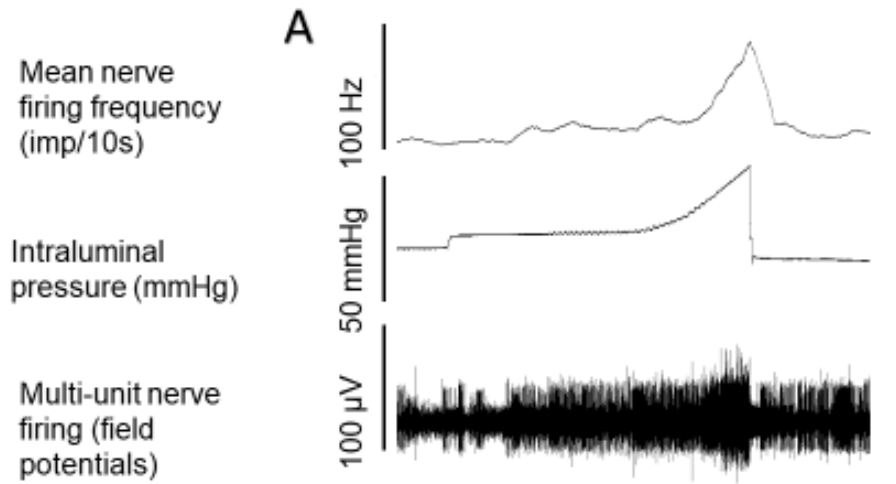
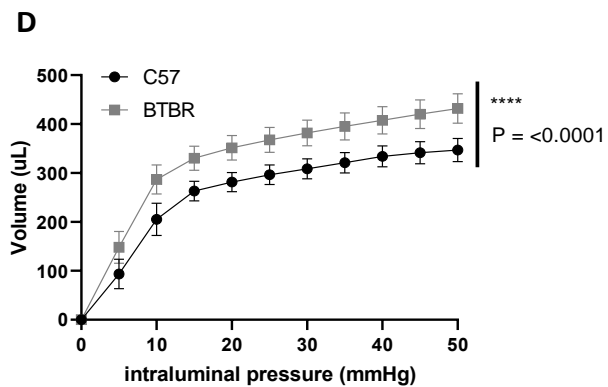
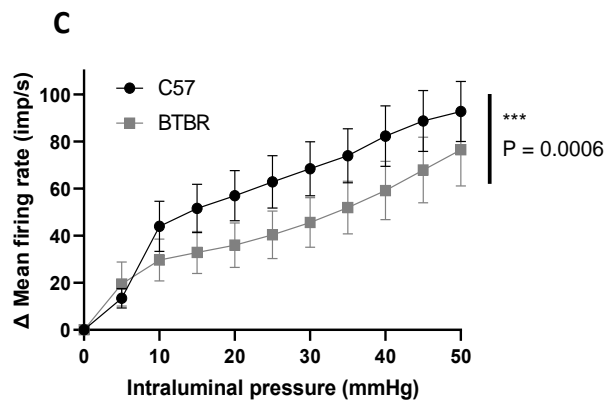
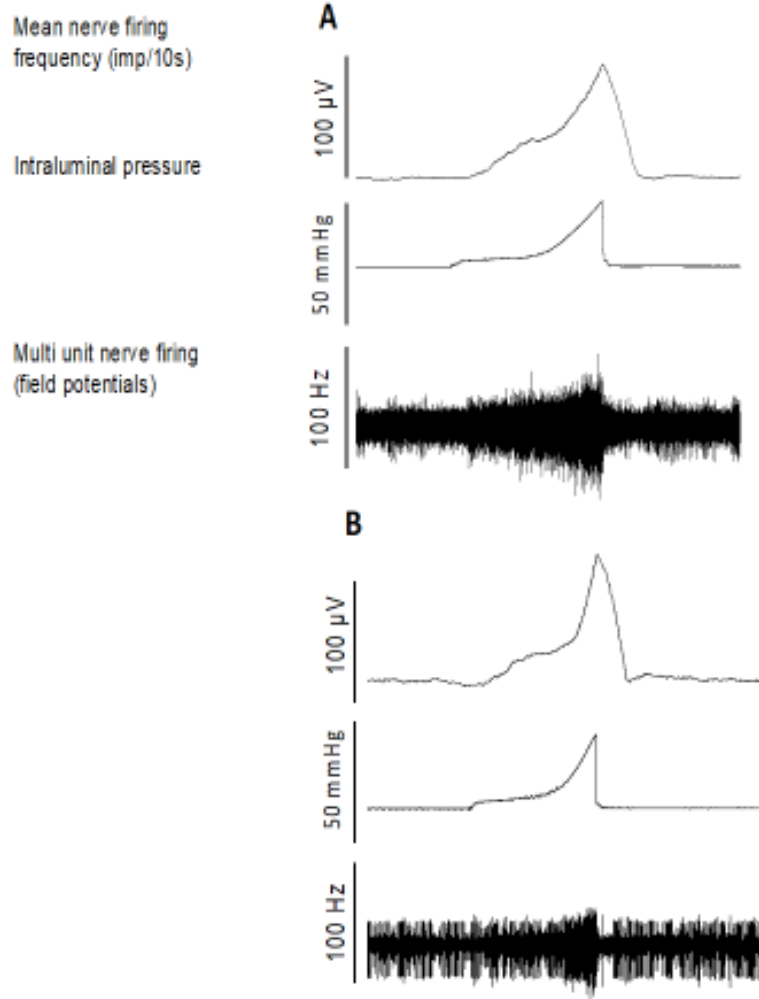


Figure 9: The figure above shows sample traces for the C57 tissue (**9A**), BTBR tissue (**9B**), the graph illustrating the data collected from all the preparations (**9C**) and the pressure-volume relationship (**9D**). The slope of the curve of figure **9C** overlaps roughly, as there was no difference in firing between the two groups ($P= 0.6897$). Peak change in firing was $54.62 (+/- 10.33)$ imp/s⁻¹ in the c57 tissue and $50.44 (+/- 15.53)$ in the BTBR tissue. **9D** illustrates the pressure-volume relationship during the ramp distensions, the BTBR tissue showed significantly reduced volume at the higher intraluminal pressures. C57 n = 5; BTBR n = 5, analysed with two-way ANOVA with Bonferroni post-test.

3.3.3 Faster distension rate causes significantly lower afferent response in BTBR tissue

In this study, the rate of distension was increased to 600 μ l/min in order to assess the responses of the afferents in the BTBR and C57 tissue, and if there are any differences. As shown on figure 10C, the BTBR tissue exhibited significantly decreased afferent firing after 10 mmHg compared to the C57 tissue ($P= 0.0006$; C57 n = 10; BTBR n = 9). We summarised the response profiles to distension on figures 10E and 10F, the 'low threshold' firing was taken as change in afferent firing occurring between 0 – 20 mmHg and 'high threshold' firing was taken between 20 – 50 mmHg. This gives a rough measure as to which component of the ramp distension profile is affected in the BTBR tissue. The high threshold firing was similar between the C57 and BTBR tissue (figure 10F; $P = 0.6254$), and the low threshold firing was attenuated in the BTBR tissue, although not significant (figure 10E; $P = 0.1628$).

The compliance was also significantly altered, at 600 μ l/min, the BTBR tissue accommodates significantly more volume than the C57 tissue ($P = <0.0001$). It is striking to see that the BTBR tissue can accommodate more volume, but the afferent firing is lower, this may reflect alterations in the function of the mechanosensitive receptors in the gut wall, changes in the biomechanical properties or structure of the tissue. This is compounded by the alterations in response depending on the rate of filling.



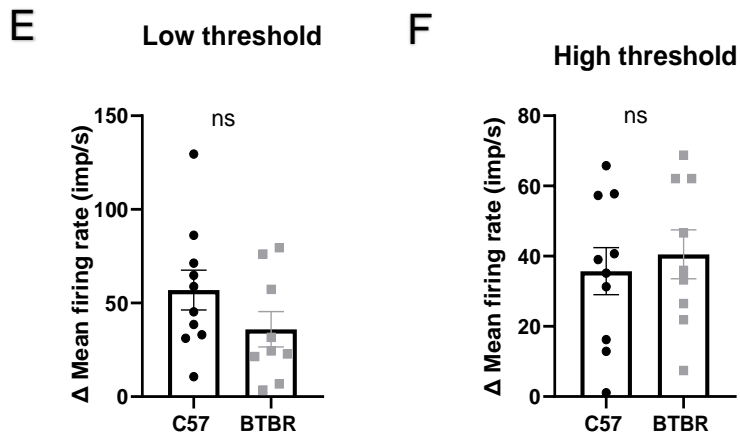


Figure 10: The figure above shows sample traces for the C57 tissue (**10A**), BTBR tissue (**10B**), the graph illustrating the data collected from all the preparations (**10C**) and the pressure-volume relationship (**10D**). **10C** shows the BTBR tissue exhibits a significantly reduced response to distension, peak change in firing was 92.68 (+/- 12.80) imp/s⁻¹ in the c57 tissue and 76.49 (+/- 15.44) in the BTBR tissue. **10D** illustrates the pressure-volume relationship during the ramp distensions, the BTBR tissue showed significantly increased volume at the higher intraluminal pressures. C57 n = 10; BTBR n = 9, analysed with two-way ANOVA with Bonferroni post-test. **10E** shows a comparison between the afferent discharge rates between 0 – 20 mmHg (low threshold), and **10F** shows the change in afferent firing between 20 – 50 mmHg (high threshold) between the C57 and BTBR tissue. The low threshold analysis shows a decreased response in the BTBR tissue, but not to a significant degree (P = 0.1628) whereas there was no difference in the high threshold analysis (P = 0.6254). Analysed with unpaired t-test.

3.4 Chemosensitivity in BTBR mouse model of ASD

Chemosensitivity was measured by the afferent nerve response to application of various drugs, including 100 μM 2-methyl 5-hydroxytryptamine, 1 μM capsaicin and 100 μM AITC in order to stimulate the 5-HT₃, TRPV1 and TRPA1 receptors respectively. We also used a cocktail of inflammatory mediators termed 'inflammatory soup', consisting of 5-HT, prostaglandin E₂, histamine and bradykinin, all at 5 μM concentrations. The aim was to simulate an inflammatory insult to the gut and record the afferent nerve response to such stimulation.

3.4.1 No significant difference in response to 5-HT₃ agonist 2-methyl 5-HT

5-HT is a neurotransmitter most abundant in the gut and modulates a wide variety of different processes. In the ASD literature, there has been reports of both increased and decreased production of 5-HT, which is then attributed to the increased rates of gastrointestinal dysfunction in ASD (Israelyan, 2019). We first used 100 μM 5-HT, however failed to obtain reproducible results so chose to use the synthetic 5-HT₃ receptor agonist 2-methyl 5-HT instead, which did give good results. In this study, the organ bath perfusion was paused, 100 μM 2-methyl-5HT applied as a bolus dose into the bath, left for 60 seconds and the perfusion was then restarted. There was no difference in response to 2-methyl 5-HT, although the general response was quite moderate in both C57 and BTBR tissue ($P = 0.4070$; C57 $n = 8$; BTBR $n = 5$).

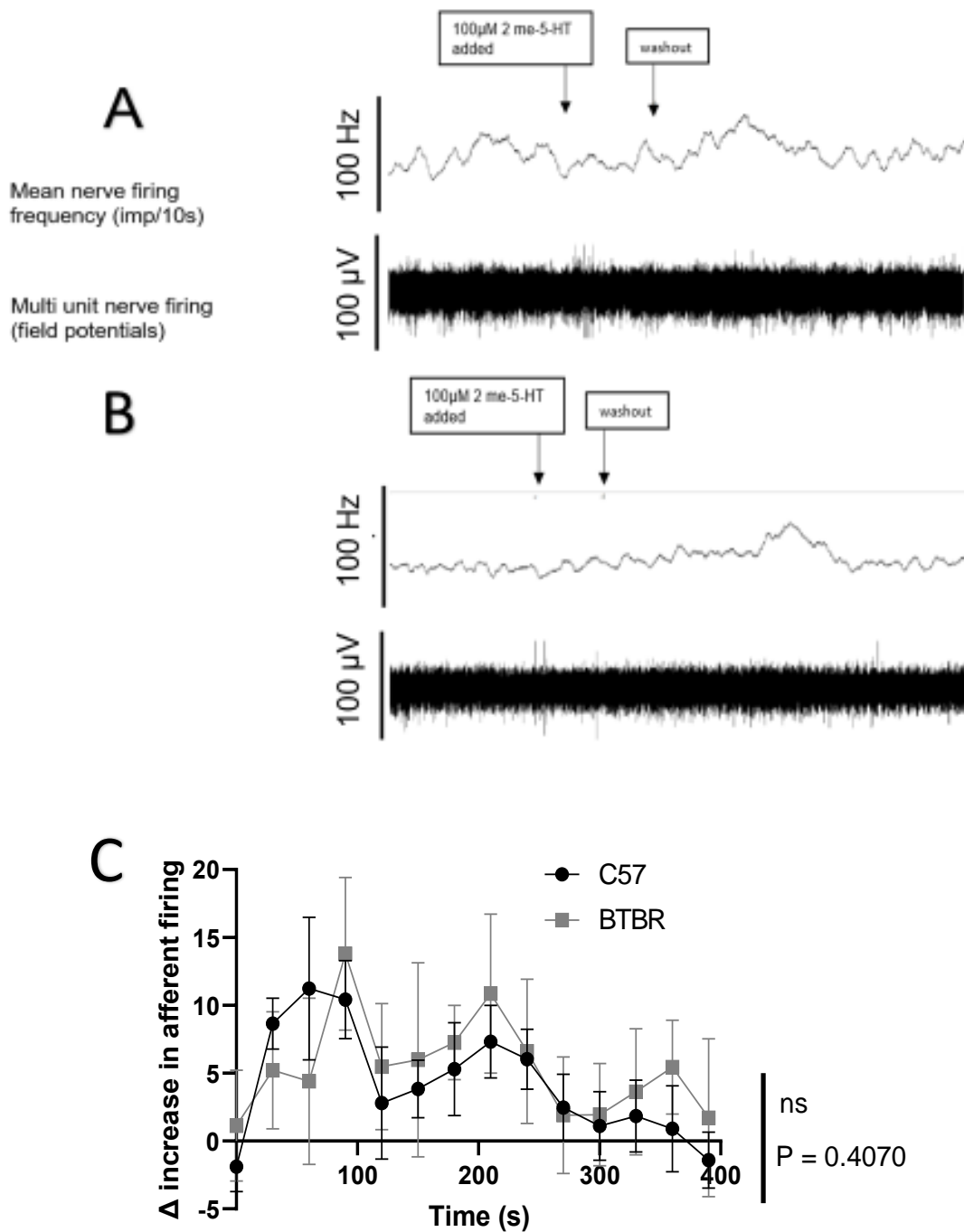
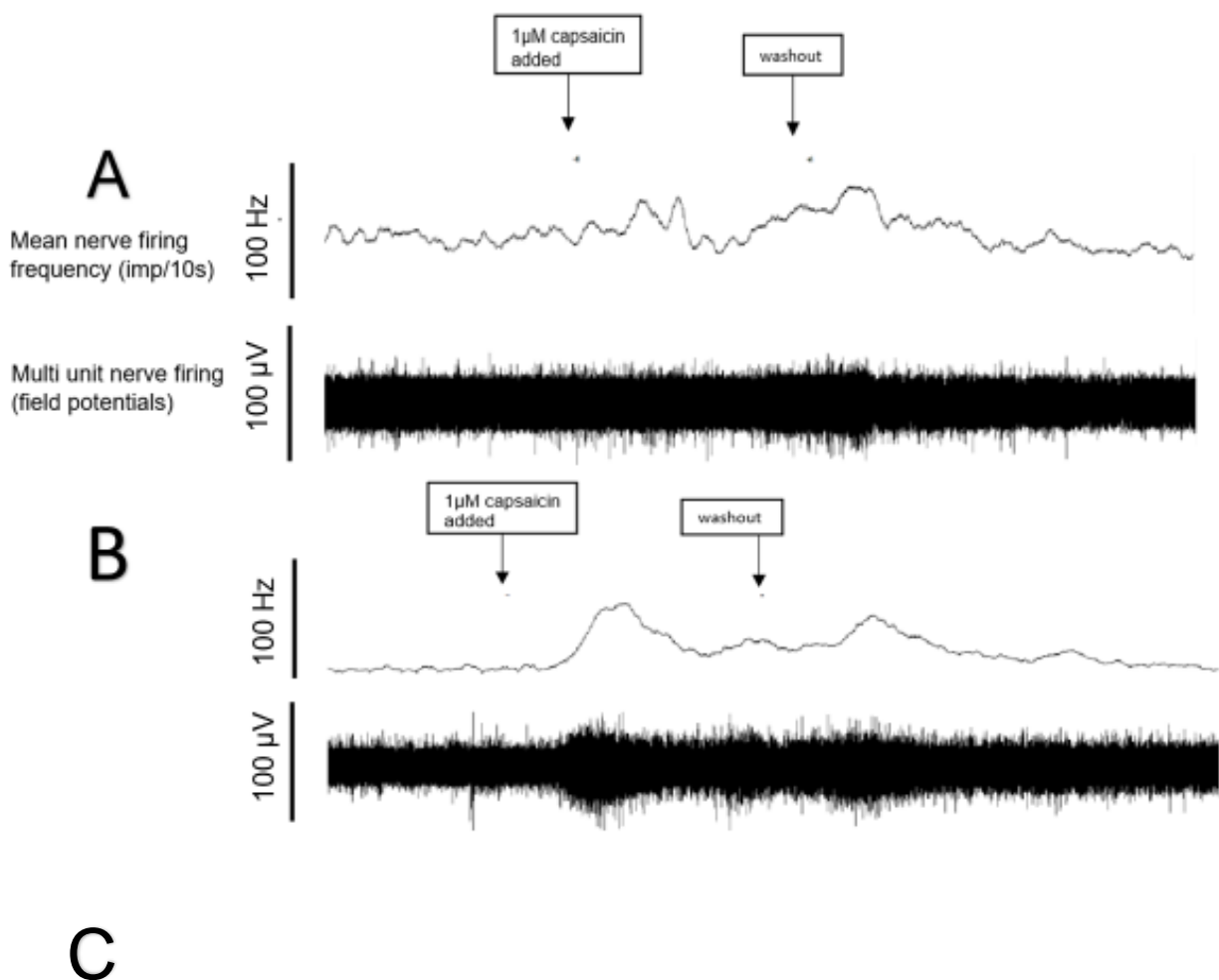


Figure 11: this figure consists of two sample traces, which show the afferent response of **11A)** C57 and **11B)** BTBR tissue to 100 μ M 2 methyl-5-HT. On each trace there are two arrows, which represent the point at which the compound was added and washed out. In both cases, the responses are quite moderate compared to the baseline before application. **11C** shows comparison of the afferent nerve response to 5-HT₃ activation in c57 and BTBR tissue over 400 seconds. There is no significant difference between the groups in response to 2-methyl 5-HT application (P = 0.4070). C57 n = 8; BTBR n = 5. All points are mean +/- SEM. Analysed with two-way ANOVA with Bonferroni post-test

3.4.2 There was a significant alteration in TRPV1 response in BTBR tissue

Altered TRPV1 function has been reported in the dorsal root ganglion (DRG) neurons in an animal model of ASD (Han, 2016), in this study the aim was to characterise TRPV1 function at the primary afferent level. 1 μM capsaicin was applied extraluminally through the perfusion pump, the arrows on the sample traces below show when the capsaicin reached the bath (observed through bubbles in the organ bath). The second arrow is at the washout point, when fresh Krebs entered the bath (again observation of bubbles) which was changed when the capsaicin solution had fully perfused. In both traces, there is an initial increase in firing which is followed by a decrease, then another increase, which perhaps shows the activation of different populations of nerves. Figure 12C shows a significantly prolonged response in the BTBR tissue ($P = <0.0001$; C57 $n = 5$; BTBR $n = 5$). It takes a longer time to reach the desensitization stage which may mean altered function of TRPV1 at the nerve terminals.



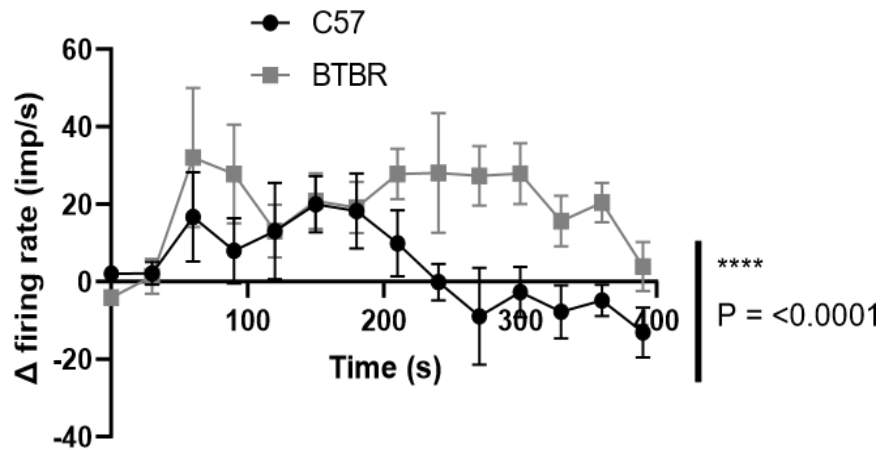


Figure 12: this figure consists of two sample traces, which show the afferent response of **12A)** C57 and **12B)** BTBR tissue to 1 μ M capsaicin. On each trace there are two arrows, which represent the point at which the compound was added and washed out. **12C** shows comparison of the afferent nerve response to TRPV1 in c57 and BTBR tissue over 400 seconds. The BTBR tissue shows delayed desensitization, as the response is maintained past the point at which the C57 tissue desensitizes, this may suggest altered function or expression of TRPV1. C57 n = 5; BTBR n = 5. All points are mean +/- SEM. Analysed with two-way ANOVA with Bonferroni post-test

3.4.3 There was no significant difference in response to TRPA1 activation

TRPA1 is another member of the TRP family which is expressed on the primary afferent terminals of the jejunum (Brierley, 2009; Yu, 2009). It is activated by chemicals such as mustard oil and noxious cold (Jordt, 2004), and has not been evaluated in ASD models. In this study, 100 μ M AITC was applied through the perfusion pump in the same protocol as described above with capsaicin. Figure 13A is a sample trace of C57 tissue, there is a significant increase in firing which persists for a relatively long time, which is also shown in 13B, a sample trace of BTBR tissue.

In this study, there was no significant differences between the two groups in afferent response to application of 100 μ M AITC (P = 0.5557; C57 n = 5; BTBR n = 5). This may suggest TRPA1 is not relevant in the gastrointestinal dysfunction in ASD.

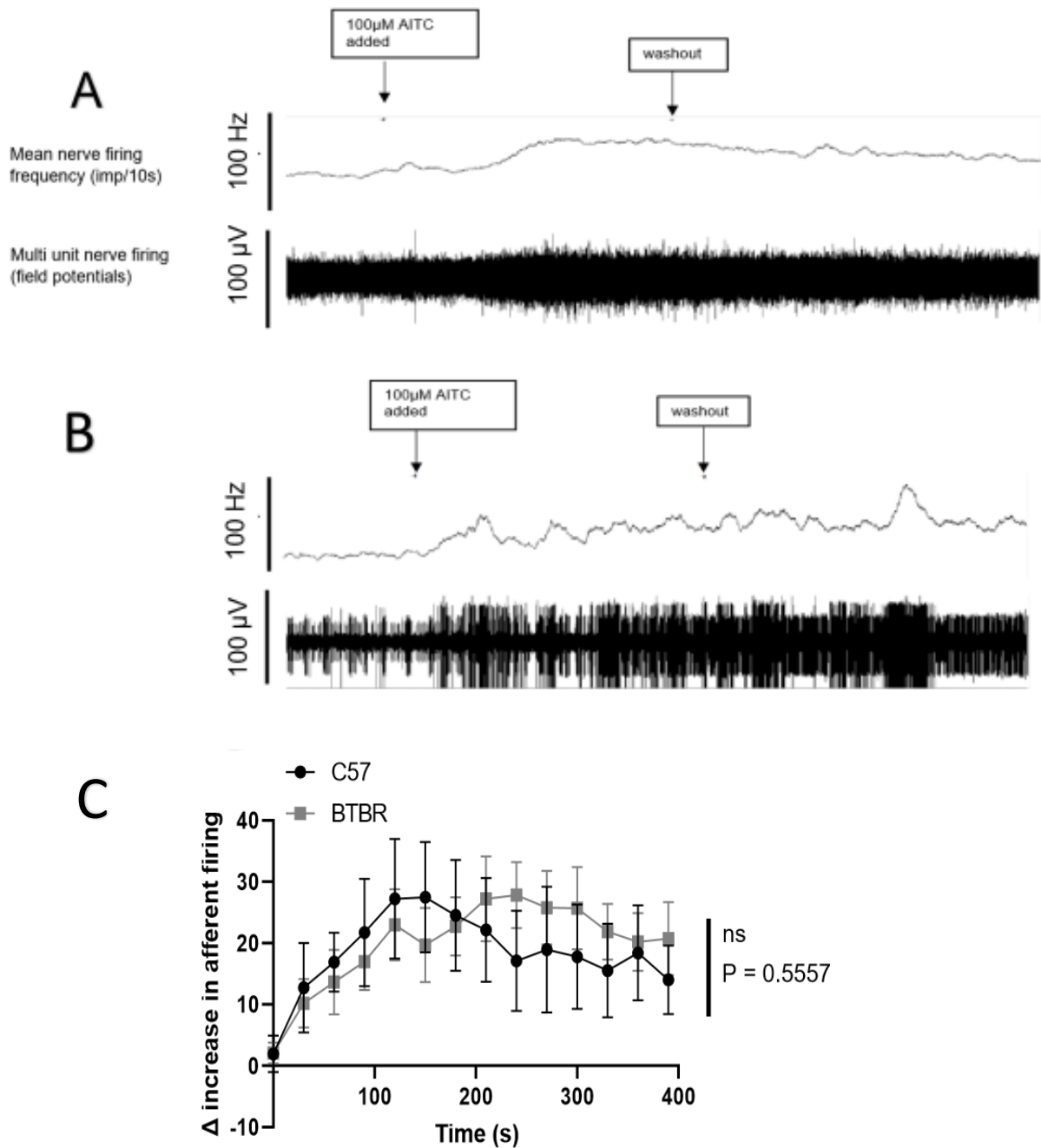
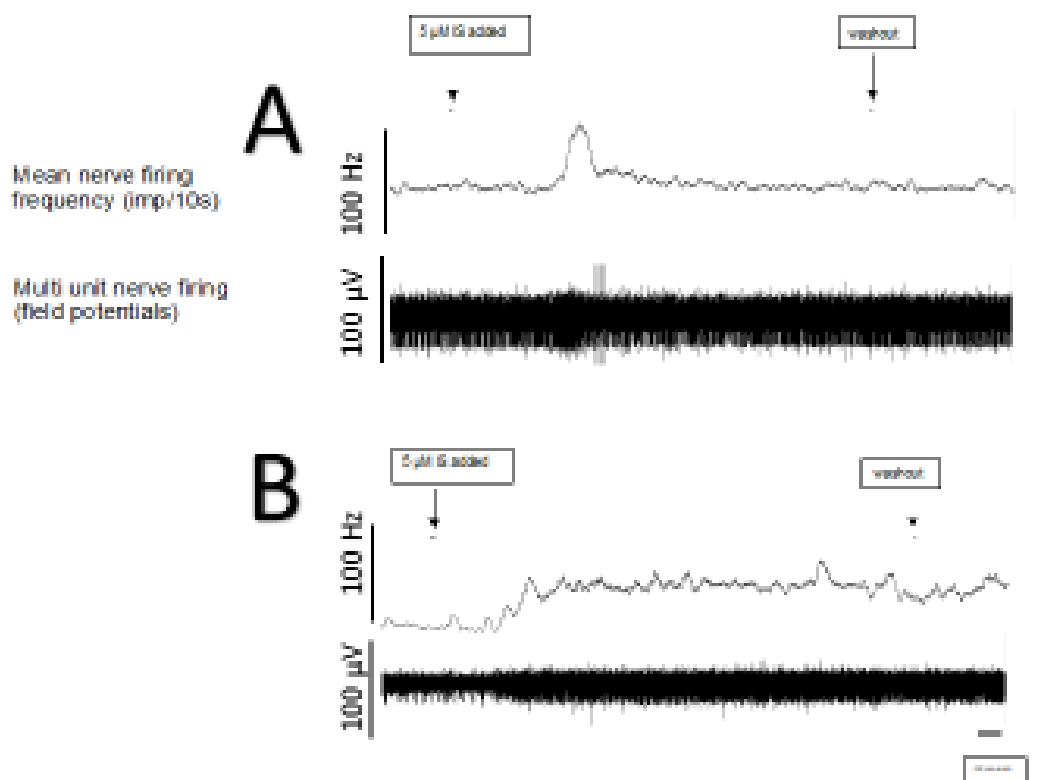


Figure 13: this figure consists of two sample traces, which show the afferent response of **13A)** C57 and **13B)** BTBR tissue to 100 μ M AITC. On each trace there are two arrows, which represent the point at which the compound was added and washed out. Figure **13C** shows a comparison of the afferent nerve responses to TRPA1 activation in c57 and BTBR tissue over 400 seconds. There is no significant difference between the groups in response to AITC application ($P = 0.5557$). C57 $n = 5$; BTBR $n = 5$. All points are mean \pm SEM. Analysed with two-way ANOVA with Bonferroni post-test

3.4.4 Application of inflammatory soup elicited a significantly increased response in BTBR tissue

Inflammatory soup is a term describing the mediators that are often associated with inflammatory pain transduction. In this study, the recipe was taken from Jones et al (2005), including 5 μ M final concentrations of bradykinin, serotonin, histamine and prostaglandin E₂ to sensitize chemosensitive afferents. The inflammatory soup was applied intraluminally, at a rate of 600 μ l/min, then replaced with fresh Krebs buffer to wash out. As shown in figure 14A, the C57 afferents responded with a peak response, returning to baseline shortly after, whereas in 14B the BTBR afferents show a prolonged response. 14C illustrates BTBR tissue shows significantly increased response to inflammatory mediators compared to C57 tissue ($P = 0.0138$; C57 $n = 5$; BTBR $n = 5$).



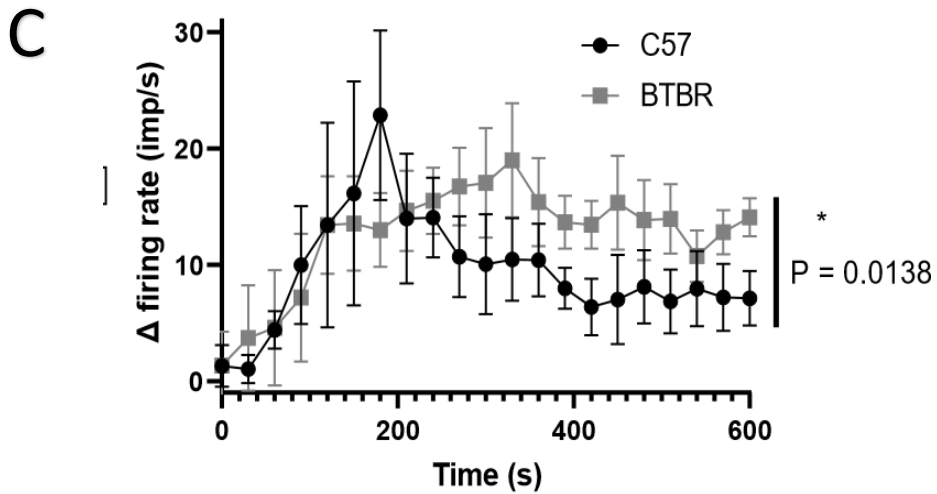


Figure 14: this figure consists of two sample traces, which show the afferent response of **14A)** C57 and **14B)** BTBR tissue to inflammatory soup. On each trace there are two arrows, which represent the point at which intraluminal perfusion is started, and when it is replaced with fresh Krebs buffer to wash out. Figure **14C** shows a comparison of the afferent nerve responses to inflammatory mediators in c57 and BTBR tissue over 400 seconds. BTBR tissue shows significantly increased response to inflammatory mediators compared to C57 tissue ($P=0.0138$). C57 $n = 5$; BTBR $n = 5$. All points are mean \pm SEM. Analysed with two-way ANOVA with Bonferroni post-test

4 Discussion

4.1.1 Circadian alterations of sleeping and feeding in mouse models of ASD

The circadian rhythm is biological timing, composed of the suprachiasmatic nuclei (SCN) of the hypothalamus which regulates the 24-hour rhythm of locomotion, hormones and feeding (Potter, 2016). In this study, the Laboras test was conducted as a non-invasive method of analysing the circadian rhythm of the control c57, BTBR, and prenatal VPA mice. The Laboras software gives many measures of activity relevant for this analysis, including locomotion, eating count, time spent eating and immobility.

In figure 5, the VPA mice showed significantly decreased activity at certain points of the dark (wake) phase, while the BTBR mice had similar changes in activity as the c57 mice. This pattern was also observed in the immobility exhibited by the three groups, figure 2 shows VPA mice showed significantly increased inactivity in the dark phase compared to the BTBR and c57 mice, which show comparable inactivity. This could mean that circadian rhythm of activity is normal in the BTBR mice but altered in the VPA exposed mice.

Tsujino et al also found altered circadian rhythms in VPA exposed rats, however the pattern is different, the locomotor activity was normal in the dark (wake) phase but higher in the light (sleep) phase compared to the control rats (Tsujino, 2007). Their results showed that the VPA exposed rats had shorter sleep times and more frequent arousal, however in this study, the VPA exposed mice had greater sleep times and less arousal in the dark phase. Cusmano et al conducted electroencephalogram (EEG) and electromyographic (EMG) recordings in VPA exposed rats to analyse the sleep times and time spent in non-rapid eye movement (NREM) and rapid eye movement (REM) in the dark and light phases. Their results showed VPA exposed rats spent significantly more time in wake and significantly less time in NREM sleep compared to controls, they also exhibited increased high frequency activity and disruptions in the GABAergic system which modulates sleep states (Cusmano, 2014). The discrepancies in finding could be due to species differences, the present study used a c57 background, however Tsujino et al and Cusmano et al both used Wistar and Sprague-Dawley rats respectively. The prenatal VPA exposure could have different effects on the circadian rhythm in each species. Nonetheless, the n number for VPA exposed mice was quite small, four mice from two litters, so a bigger n number is required before any definitive conclusions can be made.

Tsujino et al also investigated circadian rhythm of feeding, their results show increased counts of feeding in the light (sleep) phase, while in this study, we found the time taken to eat was

altered, not the counts of eating. This is a very important distinction, as time taken to eat denotes feeding frequency and satiety, longer duration of meals could indicate an alteration in the feedback mechanism controlling satiation and fullness. This suggests altered vagal chemosensation and perhaps mechanosensation, however, the ex-vivo gut recordings could not be conducted in prenatal VPA mice due to lack of time.

4.1.2 Grooming in ASD mouse models

Grooming is an innate behaviour exhibited by animals and serves multiple purposes, for hygiene, thermoregulation and social communication, especially in the context of social grooming or allogrooming (Spruijt, 1992). Grooming also serves the function of de-arousal, in rodents grooming can occur after a period of anxiety after a stressful situation has passed (Kalueff, 2016).

A striking finding of this study was the significantly decreased grooming presented by the BTBR and VPA mice. This stands in clear opposition to the vast catalogue of research presenting increased grooming in BTBR (Amodeo, 2014; Brodtkin, 2013; Cai, 2019; Nadeem, 2019; Amodeo, 2018, Wu, 2017) and prenatal VPA (Du, 2017; Campolongo, 2018; Mehta, 2011; Wang, 2018) models of ASD. These studies were all conducted in an open field, or similar test where an animal is placed in a lightly bedded, or unbedded, box and recorded for 10 or 20 minutes. In these tests, the VPA and BTBR mice groom significantly more than the control mice. Kang et al conducted a study where they used both methods, a self-grooming test where they recorded the animals for 10 minutes, and a LABORAS test where they recorded the animals for 48 hours. In the self-grooming test, the VPA mice groomed significantly more than control mice, however, in the LABORAS test, they found no significant difference in grooming time (Kang, 2015). They explained that the increased self grooming exhibited by VPA mice in the self-grooming tests conducted widely in the literature, may be novelty-induced grooming. The home cage-like environment of the LABORAS cage is more familiar to the mice, and decreased novelty may result in decreased stress.

Individuals with ASD have reduced novelty processing or enhanced familiarity preference (Maes, 2011), and VPA treated rats explore a novel environment less than controls as reported by Schneider & Przewlocki and Olexová et al (Schneider & Przewlocki, 2005; Olexová, 2013), BTBR mice also exhibit novelty aversion (McTighe, 2013).

To conclude, this evidence suggests that mouse models of ASD may exhibit novelty-induced grooming in self-grooming or open field tests, if these animals are tested in a home cage-like environment, they exhibit significantly reduced grooming compared to control mice. This may be due to a reduced capacity for novelty processing and rigidity and may be another example

of validation of these models for ASD, as fears of novelty and resistance to change is a symptom often associated with ASD (Kerns, 2017).

4.1.3 Locomotion in ASD mouse models

In this study, there were differences in locomotion exhibited by the three strains, as defined by counts of locomotion, distance travelled, and average speed of movement measured over 24 hours in the LABORAS cages. The BTBR mice exhibited mild hyperactivity, as there was a steeper increase in distance travelled in the first 8 hours of the test, and significantly increased average speed. This is consistent with results reported by Faraji et al who, through an open field test, found BTBR mice explored significantly faster than C57 mice. They conclude this phenotype is a manifestation of hyperactivity, which has been reported in children with ASD (Faraji, 2018; Johnson, 2011). In contrast, Jasien et al found no difference in distance travelled in their open field test (Jasien, 2014). It is important to note the difference in methodology of these studies, Faraji et al conducted a 20-minute test, compared to Jasien et al, where the test was 10 minutes long, which is very short compared to the 24 hours of data collected in this study. The increase in recording time provides a more complete picture of the behaviour of the mice, allowing for habituation and adaptation to the environment.

The VPA mice exhibited significantly increased locomotion in the first 4 hours of the LABORAS test, however this decreased rapidly. In general, the VPA mice exhibited decreased locomotion than BTBR or c57 mice, however not significantly. They travelled significantly less distance and average speed, compared to BTBR mice. Peralta et al also observed rats prenatally treated with VPA showed significantly reduced mean speed and total distance travelled, describing it as 'hypokinetic' (Peralta, 2016). However, Schneider and Przewlocki, found adult VPA-treated rats exhibited normal locomotion in an open field test and reduced exploratory behaviours such as rearing (Schneider and Przewlocki, 2005). These differences may not be due to methodology, as Kang et al also found no significant difference in distance travelled by the VPA rats in a 48-hour LABORAS test (Kang, 2015). In this study, rearing was normal in both BTBR and VPA mice.

There are studies linking ASD to Attention Deficit Hyperactivity Disorder (ADHD), in studies of emotional recognition and theory of mind, Buitelaar et al found no differences between ASD and ADHD individuals, and Sinzig et al found a high phenotypical overlap between the two disorders (Sinzig, 2009; Buitelaar, 1999). In the DSM-V, there is a diagnostic category of ADHD comorbid with ASD (American Psychiatric Association, 2013). In the context of this study, the increased distance travelled and increased average speed shown by the BTBR mice could be evidence for hyperactivity in this strain, however further research is needed.

4.2 Thermosensing in ASD mouse models

Most studies on thermal thresholds and sensitivity in ASD have been based on the traditional hot plate test, where the plate is set to a known nociceptive temperature and the animal is placed on it, the time taken for the animal to withdraw its paws from the hot surface is recorded. This is a common, reproducible test, however it does not give a wide picture of nociceptive thresholds, as different manifestations of sensory disruptions cannot be measured. In the context of ASD, this is an important field as tactile allodynia has been reported in children with ASD, where innocuous mechanical stimuli were considered painful and related to deficits in self-regulation (Silva, 2016). The dynamic hot/cold plate test was performed to investigate any potential changes in thermal sensitivity using a technique which allows identification of any changes in response to gradual changes in thermal stimuli.

As shown on figure 6A, BTBRs has a significantly reduced response to the dynamic hot plate, as shown by delayed start to jumping, and a reduced number of jumps in total. This suggests that the BTBR animals have a hyposensitivity to heat. However, in figure 6B, BTBRs respond comparably to c57s to decreasing temperature, suggesting a normal sensitivity to cold. VPA mice responded in an opposite manner to the BTBRs, as they showed normal responses to heat, but hyposensitivity to cold. This is a striking result and could mean differences in the central or peripheral nociceptive pain pathway in both models. The altered temperature perception and response in the mouse models of ASD could be due to aberrations in function of any point of the sensory transduction pathway, from the afferent nerves that detect the stimuli, to the spinal cord where the sensory information is processed, and further up to the somatosensory cortex of the brain (Bokiniec, 2018). Wang et al suggest that although CNS processing is vital for sensory transduction, it is only as good as the information collected by the primary afferents. Most neurons are polymodal, they are activated by a range of stimuli. By conducting in vivo calcium imaging studies on anaesthetised mice and stimulating their paws with different temperatures, their study revealed that there are fundamental differences in the way hot and cold stimuli is encoded by the dorsal root ganglion (DRG) neurons. (Wang, 2018).

In the context of ASD, there is very sparse data on the function of thermosensitive afferents in the skin. There have been many studies finding alterations in C-tactile afferent fibres, which mediate low force stroking, such as hypo and hyperfunction and fewer numbers of fibres (Kaiser, 2016; Riquelme, 2016; Silva, 2016), however, fewer studies on A δ or C fibres which mediate nociceptive signalling. Wang and Almeida et al found hyposensitivity to electrical stimulation of C fibres, and normal responses to stimulation of A β and A δ in BTBR mice (Wang

and Almeida, 2016). This is significant as C fibres are important for thermal and mechanical nociception (Alizadeh, 2014), and is concordant with the results of this study where there was hyposensitivity to heat nociception in BTBR. A possible explanation of the data in this study might be that in the BTBR animals there is a reduction in C fibre sensitivity, which translates to altered thermosensitivity.

In Han et al's study, Shank3 ^{-/-} and Shank3^{+/-} mice also showed reduced responses to spontaneous pain, as measured by reduced time spent licking paws injected with 10 µg capsaicin. Further, homozygous conditional knock outs of Shank3 in the peripheral nociceptive sensory neurons showed heat deficits that were like those observed in TRPV1 knock out mice (Han, 2016). Altered TRPV1 function in the somatic afferent nerves of BTBR mice may explain the hyposensitivity observed in study.

To date, there have been no studies on cold sensitivity in VPA mice in the literature. The hyposensitivity to cold nociceptive stimuli is thus a novel finding. There may be alterations in function of afferent fibres, expression of sensory ion channels such as TRP channels or abnormalities in integration and processing at the level of the CNS. Alterations in the somatosensory and insular cortices of VPA mice is a commonly reported finding (Dendrinis, 2011; Kataoka, 2013; Hara, 2017; Campolongo, 2018). As shown in figure 1.2, these brain regions are essential in the thermal transduction pathway. It is important to note that this data shows VPA mice exhibit normal responses to increasing temperature, the deficit only occurs in the decreasing temperature, which may suggest deficits specific to cold sensation. This may be due to altered TRPM8 or TRPA1 expression at the somatic primary afferents. There is clearly a need for further study in this field, as the altered thermal nociception in ASD has significant clinical implications.

4.3 Alterations in visceral signalling in ASD models

Sensory information from the visceral organs are conveyed through afferent nerves to the central nervous system. Changes in afferent signalling may be evidence of pathophysiological processes, in cases of visceral hypersensitivity (Mayer, 1994). Gastrointestinal disturbances are well characterised in ASD symptomology, however there has been little inquiry in the neural element of the gut-brain axis despite its influence in health and disease

4.3.1 Afferent nerve response to intraluminal distension

Different distension rates were chosen to assess the impact of altered mechanical stimuli on the afferent nerves, in figure 8A, the afferent firing in response to a filling rate of 600 µl/min was significantly higher than at 200 µl/min in control mice. This phenomenon was also observed by Yang et al, who found that filling rates of 500 µl/min and 1 ml/min induced higher afferent firing compared to 300 µl/min. They attribute it to increased neural adaptation of the

mechano-receptors, where the responsiveness of the afferent nerve decreases at constant stimuli, and faster rates correspond with increased dynamic changes in stress and strain (Yang, 2012). However, they ruled out any involvement of the adaptation of smooth muscle as they observed no difference in the stress and strain at the different rates. Multi-unit recordings of mesenteric nerves give a general picture of mechano-sensitivity, but also allow further analysis of specific field potentials (spikes) within the recordings that are identifiable and specific in wavelength and amplitude (Hillsley, 1998). In the context of a distension experiment, this allows the analysis of the relationship between stimulus and response of the individual fibres and classify them according to the point at which afferent activity begins, at low pressure (low threshold mechanoreceptors) or high pressure (high threshold mechanoreceptors) (Daly, 2011). There are also fibres that start firing at low pressures but increase in a linear pattern as the pressure increases, which have been termed wide dynamic range fibres (Keating, 2008). Yang et al conducted this analysis and found some fibres that previously did not fire at low pressures were activated when the rate was increased, and low-threshold units behaved like wide dynamic range fibres at the higher rate. This suggests that there may be a temporal element to the thresholds of afferent firing.

We observed that at the faster rate of 600 $\mu\text{l}/\text{min}$, the BTBR tissue displayed a significantly attenuated response to distension compared to the C57 tissue. This is striking as the firing rates are almost identical at 200 $\mu\text{l}/\text{min}$. At the 200 $\mu\text{l}/\text{min}$ rate, it took approximately 138 seconds to reach 50 mmHg, whereas at 600 $\mu\text{l}/\text{min}$ it took approximately 45 seconds to reach the same pressure, which shows it is a more intense mechanical stimuli to the gut. The reduced response by the BTBR afferents may suggest attenuated response to nociceptive stimuli. It is interesting to regard this data alongside the TRPV1 data, as it considered a mechanically active ion channel (Jones, 2005), mechanical stimulation of TRPV1 appears to show reduced activation, whereas chemical stimulation shows prolonged sensitization of the receptor. In Rong et al's study of jejunal mechanosensitivity in TRPV1-null mice, the afferent firing was significantly reduced in compared to control mice (Rong, 2004), noting that they used a distension rate of approximately 200 $\mu\text{l}/\text{min}$. In the present study, TRPV1 may be functionally altered in BTBR afferents, impacting the response to both sensory modalities in different ways. As this alteration is only clear in the increased rate of distension, it may mean TRPV1 function is specifically altered in pathophysiological states. This was also reported by Daly et al, who in their study on bladder mechanosensitivity in TRPV1-null mice, found that at 50 $\mu\text{l}/\text{min}$, the afferent response to distension is like that of control mice (Daly, 2007). However, at 200 $\mu\text{l}/\text{min}$ the afferent response was significantly attenuated in the TRPV1-null mice, through single unit analysis they found only low-threshold afferents were affected and concluded TRPV1 is important for nociceptive states than normal physiological function. These findings were very similar to those in the present study, where altered function only

occurred in response to increased mechanical stimulation. In-vivo, this may mean that TRPV1 function is normal in the BTBR mice if the gut is in a healthy state, however in pathophysiological states its function is attenuated. This result may also be due to properties of the nerves, there may be increased adaptation, there may be differences in the morphology of the tissue which influence the biomechanical properties, for example muscle thickness or collagen production.

We conducted further analysis of the 600 $\mu\text{l}/\text{min}$ data to define whether the attenuation in afferent response to distension is due to the low threshold or high threshold components. Between 0 – 20 mmHg, the change in afferent firing was lower in the BTBR tissue, but not to a significant degree (figure 10E; $P = 0.1628$). While there was no difference in the change in afferent firing between 20 – 50 mmHg (figure 10F; $P = 0.6254$). This analysis, however, is not the most accurate representation of the firing rates of low and high threshold units, as it is just an approximation. Due to time constraints, we could not do a complete single unit analysis, which would be very interesting to do in further studies as it would show whether there are alterations in the low threshold units, of mostly vagal origin, or the high threshold units responsive to nociceptive stimuli projecting to the spinal cord. There is also the contribution of wide dynamic range units, which we couldn't take into consideration in this study, but would be clear to see in single unit analysis.

In this study, the measured parameters were pressure and volume, however in biomechanical terms, mechanoreceptors do not respond to forces acting within the lumen because the receptors are located further into the gut wall (Gregersen, 2013). Recently published literature investigating gastrointestinal mechanosensitivity have used stress and strain as parameters (Yang, 2015; Siri, 2019). These methods allow quantification of the impact of forces on the intestinal wall have on afferent nerves and would be useful to provide evidence for the functionality of the mechanoreceptors in health and disease. However, in this study, stress and strain were not used to measure mechanical force as it is technically difficult requiring extra equipment and software. There are many measurements required for this analysis, including length of the tissue, circumference and mid-wall radius (Gregersen, 2003). Although not perfect, pressure is a constant that can be measured directly in the lab with a pressure transducer and not dependent on calculations after the fact. This way, the experimenter can be sure they applied the same mechanical stimuli to each tissue, regardless of its length or other properties which would affect stress and strain measurements.

4.3.2 Compliance of the jejunum

Compliance is defined as the ratio between volume change and pressure change measured through a ramp distension (Gregersen, 2003). In figures 8C and 8D, there is first a linear phase

then a plateau, as the higher pressures are reached. Not only are there changes in compliance between tissue from the C57 and BTBR mice (figures 9D and 10D), there are also changes depending on rate of distension. In the c57 tissue, the 200 $\mu\text{l}/\text{min}$ rate led to increased volume accommodation than at the 600 $\mu\text{l}/\text{min}$ rate. In the BTBR tissue, the opposite is true, the faster rate led to the jejunum to accommodate a higher volume than the slower rate. This may be due to changes in the biomechanical properties of the tissue, and more in-depth analysis of the stress-strain properties of the BTBR tissue may provide some answers. As discussed above, biomechanics was beyond the scope of this study, however, this data is compelling and provides motivation for further studies. Changes in the viscoelasticity or composition of the gut could have serious effects on motility and normal function.

4.3.3 Response to 5-HT₃ activation

Serotonin (5-HT) is a neurotransmitter abundant in the GI tract (Feldberg, 1953), the enterochromaffin cells in the gut mucosa contain the largest store of 5-HT in the body (Bülbring, 1959; Pan, 2000; Bertrand, 2004). As such, it is essential for many processes, including secretion and peristalsis through modulation of the ENS, as well as sensitizing afferent sensory fibres that project to the CNS (Grundy, 2008). The 5-HT receptor family is composed mostly of G-protein coupled receptors, except 5-HT₃, which is a ligand gated ion channel that rapidly depolarizes neurons (Frazer, 1999). The present study aimed to characterise the mesenteric afferent nerve response to 5-HT stimulation, which has been shown to be mediated in a direct and indirect mechanism, depending on the population of fibres responding.

Previous studies have suggested that the direct afferent response to 5HT is mediated through 5-HT₃ – expressing fibres, which generate a quick, transient burst of firing, and an indirect response which is mediated through 5-HT_{2A} – expressing fibres, producing a delayed onset but prolonged nerve firing (Hillsley, 1998). 5-HT₃ – expressing fibres are of vagal origin, as vagotomy abolishes the 5-HT₃ mediated response (Miller, 1992), however the secondary, 5HT_{2A} mediated response remained (Hillsley & Grundy, 1998). As the present study was utilising multi-unit recording of extrinsic afferent nerves, we chose to use the synthetic 5-HT₃ agonist 2-methyl 5-HT as it gave more reproducible responses than 5-HT. There may have been issues with the method of administration, we put the 5-HT through the perfusion pump which may have been too slow, causing the nerves to be desensitized.

5-HT dysregulation is implicated in ASD due to elevated blood 5-HT concentration, which is considered the first biomarker in ASD, reinforced by a 2014 meta-analysis by Gabriele et al

who concluded that peripheral elevation of 5-HT has a genetic basis, and can be used as an endophenotype to aid classification of ASDs (Gabriele, 2014). The BTBR model of ASD also exhibits hyperserotonemia, BTBRs have decreased baseline 5-HT transporter (SERT), which transports 5-HT into the pre-synaptic neuron to modulate the concentrations in the synaptic cleft, leading to increased concentrations throughout the brain (Gould, 2014). There is also increased 5-HT_{1A} activity in the hippocampus, administration of its partial agonist buspirone increased sociability in BTBR mice (Gould, 2011). Golubeva et al recently found dysregulated 5-HT production in BTBRs, the availability of 5-HT was halved in the small intestine and colon, due to a downregulation of *TPH1* and *SERT* genes (Golubeva, 2017). This provided a rationale for investigating the serotonergic system in the context of sensory nerves in the BTBR mice.

As shown in figure 11, there was no significant difference in afferent nerve response to 5-HT₃ agonist 2-methyl-5-HT between tissue from the BTBR and C57 mice. However, this does not give a complete picture of serotonergic signalling in the mesenteric afferent nerves, as the other receptor types involved were not interrogated. 5-HT is released by the enterochromaffin (EC) cells and secreted into the lamina propria, where it can reach neurons and activate specific receptors (Gershon, 2003). In our preparation, the 2-methyl-5-HT was applied into the organ bath, meaning it had to penetrate multiple layers of muscle and connective tissue. In this study, data suggesting alterations in biomechanical composition of the jejunum was obtained, meaning that between the C57 and BTBR tissue, there may have been variations in the amount of drug that reached the nerve terminal. In further studies, responses to intraluminal and extraluminal application could be compared to deduce which method provides the most reproducible responses.

This study characterised only one pathway of 5-HT transmission, it is important to keep in mind 5-HT's role in extrinsic sensory neurotransmission in the gut is not limited to the 5-HT₃ pathway. Hicks et al found 30% of spinal afferent fibres responsive to 5-HT also responded to 2-methyl-5-HT (Hicks, 2002), suggesting other non-5-HT₃ mediated mechanisms.

4.3.4 Response to TRPV1 activation

TRPV1 is highly expressed in nodose ganglion neurons projecting to the stomach and small intestine, to dorsal root ganglion neurons that travel to the colon, as well as within the enteric nervous system (Guo, 1999; Michael, 1999; Robinson, 2004, Kadowaki, 2004). TRPV1 signalling in ASD was implicated by Han et al's paper showing deletions in the *Shank3* gene leads to diminished TRPV1 function in human DRG neurons (Han, 2016). In the present study,

we aimed to characterise TRPV1 function in the visceral primary afferents in a mouse model of ASD.

In this study, 1 μ M concentration of capsaicin was used to assess the TRPV1 activity in BTBR and C57 mice, shown in figure 12. The afferent response was prolonged in the BTBR group, and it took longer to reach the desensitization stage. There are many possible mechanisms behind this altered response profile of TRPV1. Capsaicin-induced desensitization is mediated by the Ca²⁺/calmodulin pathway, as TRPV1 has two calmodulin binding domains (CaMBD), one in the distal C terminus (Numazaki, 2003) and one in the first ankyrin repeat domain (ARD) in the N terminus which also binds to ATP (Lishko, 2007). TRPV1 activation leads to an influx of Ca²⁺ ions, which bind to and activate calmodulin, further binding to ARD N terminal domain replacing ATP resulting in inactivation of TRPV1 and desensitization (Lishko, 2007). This mechanism is supported by evidence showing disrupting ATP binding by mutations in the ARD stopped binding of Ca²⁺/calmodulin and thus inhibits capsaicin induced desensitization (Lau, 2012).

The gene encoding calmodulin, *CALM1*, has been identified as a candidate gene for ASDs (Hadley, 2014) and proteomic analysis showed a decrease in calmodulin in a small population of children with ASD (Shen, 2017). BTBR mice exhibit a significant decrease in phosphorylated Ca²⁺/calmodulin protein kinase II in the hippocampus, which was increased by administration of folic acid, leading to improvements in spatial learning (Zhang, 2019). Considering this data, and the importance of the Ca²⁺/calmodulin unit in the capsaicin induced desensitization of TRPV1, reduced production of calmodulin could lead to the slower rate of desensitization. This could provide an explanation as to why the BTBR afferent nerve response to capsaicin showed slower desensitization of TRPV1.

TRPV1 interacts with metabotropic glutamate receptors (mGluRs), which are G protein coupled receptors with seven transmembrane domains and classified into three groups based on pharmacological activity and signal transduction mechanisms (Jin, 2011; Conn, 1997). Group I mGluRs, especially mGluR₅ is co-expressed with TRPV1 on DRG neurons and activation enhances TRPV1 function and increases thermal sensitivity (Hu, 2002). mGluR₅ activation results in mechanical and thermal hypersensitivity via the protein kinase C (PKC) signalling pathway, which can, along with protein kinase A (PKA) and Ca²⁺/calmodulin dependent protein kinase 2 (CaMK2) activate TRPV1 through its many phosphorylation sites (Varga, 2006; Jung, 2004). Group II/III are inhibitory receptors and are expressed on primary afferent neurons (Carlton, 2001) and act by inhibiting peripheral nociceptors, including TRPV1 induced excitation (Carlton, 2009). TRPV1 activation leads to influx of Ca²⁺ ions which triggers glutamate release, the more Ca²⁺ is taken up by the mitochondria through the stimulus, the

longer the duration of glutamate release and activity at the synapse (Medvedeva, 2008). $mGluR_{2/3}$ has been identified as potentially analgesic, activation leads to suppressed sensitization in response to inflammatory mediators (Du, 2008). Inhibition of $mGluR_{2/3}$ increases activity of sensory neurons (Carlton, 2011) and pain-like behaviour (Yang, 2003) providing further evidence for its role in nociceptive transmission.

There has been evidence of glutamate dysfunction in ASD, significantly elevated serum glutamate was reported in adults with ASD (Shinohe, 2006), as glutamate does not pass the blood-brain barrier readily (Rojas, 2014), this points to peripheral effects. Chen et al reported a significantly decreased levels of $mGluR_{2/3}$ protein and mRNA levels in the prenatal VPA model of ASD, and through administration of N-acetylcysteine which acts on the $mGluR_{2/3}$, there was improvement of anxiety-like behaviour and increased social interaction (Chen, 2014). There has been much work delineating the impact of $mGluR_5$ mediated synaptic dysfunction in ASD, in fact, $mGluR_5$ antagonists are in clinical trials for treating ASD patients as they have found to reduce the repetitive grooming behaviour in multiple animal models including BTBRs (Silverman, 2010), prenatal VPA (Mehta, 2011) and C58/J (Silverman, 2012).

There have been no studies yet on the relationship between TRPV1 and $mGluRs$ in nociceptive primary afferent signalling in ASD models, which would be a very interesting avenue for research. In the context of this study, dysregulation in $mGluR_5$ activation of TRPV1 or $mGluR_{2/3}$ mediated inhibition may be behind the delayed desensitization observed following TRPV1 activation.

The dynamic hot plate results show BTBR mice exhibit a hyposensitivity to nociceptive heat. Comparing the two sets of data, it may suggest TRPV1 function may differ depending on whether it is a somatic or visceral transduction pathway.

4.3.5 Response to TRPA1 activation

TRPA1 is another member of the TRP family of sensory receptors, and is activated by chemicals such as mustard oil, environmental toxins and noxious cold (Bautista, 2006; Bandell, 2004). The sensory disruptions experienced by individuals with ASD are well known, however the potential impact of TRPA1 has yet to be investigated. TRPA1 also detects inflammatory agents within the gut and is also expressed in intrinsic neurons as well as enterochromaffin cells of the gut, which suggests it has many different roles as well as sensory transduction (Bautista, 2013). TRPA1 has also been identified as important for mechanosensitivity, as intra-colonic administration of mustard oil in neonatal mice increases expression of TRPA1 in the DRG, which in turn leads to hypersensitivity in adulthood without the introduction of inflammation (Christianson, 2010).

The results of TRPA1 activation in this study show no significant difference between the c57 and BTBR mice, as shown in figure 13. This suggests there is no difference in TRPA1 function between the two strains, as the curves were almost over-lapping. It is important to note that we only used one concentration of AITC, and research has shown AITC is able to activate TRPV1 (Alpizar, 2014). Future studies should include dose response experiments using a more specific agonist of TRPA1.

It is interesting to compare these results to the response to the dynamic cold plate as TRPA1 mediates cold sensitivity (Miyake, 2016). As there is no difference in either case, it may suggest that TRPA1 may not be involved in sensory dysfunction in BTBR mice. The cold hyposensitivity shown by VPA mice suggests that there may be abnormalities in TRPA1 function in that model, further study could provide interesting results.

However, in this study, desensitization of TRPA1 appeared to take much longer to occur than TRPV1 desensitization, and the experimental design did not take this into account. TRPA1 also undergoes the same Ca^{2+} mediated inactivation as TRPV1, through the Ca^{2+} /calmodulin complex (Hasan, 2017). Further studies could include longer wash-out times and show the rate of deactivation of TRPA1 in the BTBR and C57 tissue, and whether this coincides with the delayed desensitization observed in TRPV1. This could be taken further into molecular studies of calmodulin and its interactions with TRPA1 and TRPV1, to prove whether it is this mechanism that is perturbed in the BTBR mice.

4.3.6 Response to intraluminal application of inflammatory soup

After studying the functions of 5HT and TRP channels in the BTBR model of ASD, we decided to investigate the effect of a localised inflammatory insult on the gut. By applying an 'inflammatory soup' a cocktail of mediators composed of bradykinin, prostaglandin E_2 (PGE_2), histamine and serotonin, as described by Jones and colleagues (Jones, 2005). The rationale for these experiments was to assess the nerve activation triggered by these mediators, which would give a measure of the inflammatory state of the gut in the control c57 and BTBR mice. The afferent nerve recording thus gives a bigger picture of the inflammatory response as analysing the expression of certain receptors using molecular mechanisms does not reflect the function of these receptors.

In this study, the BTBR tissue showed a significantly increased response to intraluminal application of inflammatory soup than the c57 tissue (figure 14). This may show that there may

be an increased presence of inflammatory mediators in the gut lumen, resulting in increased afferent sensory transduction potentially leading to increased visceral pain. This data supports the literature describing enhanced systemic inflammation (Wang, 2019), and increased expression of inflammatory cytokines (Cristiano, 2018) in the BTBR model of ASD.

The inflamed state of the gut in the BTBR tissue could have a wide variety of effects, in the structure and function of the gastrointestinal system. The enteric nervous system is organized into the submucosal and myenteric plexuses, and abnormalities in neuronal morphology and survival have been reported in IBD (Geboes, 1998). This has also been described in the colon of BTBR mice, Golubeva et al found the myenteric plexus showed a reduced spatial density and increased intra-ganglionic space, there was a 50% reduction in inhibitory motor neurons and total myenteric neurons (Golubeva, 2017). They deduced this may be due to abnormal development of excitatory and inhibitory neurons in BTBR mice, however, it also could be due to neuronal loss due to inflammation. In a rat model of experimental colitis induced by intrarectal application of dinitrobenzene sulfonic acid (DNBS), Sanovic et al found significant neuronal loss in the myenteric plexus, 50% reduction of neuronal number in six days (Sanovic, 1999). Losses of myenteric neurons have also been described in models of acute colitis in mice and guinea pigs (Boyer, 2005; Linden, 2005).

These changes in gut innervation impacts on motor function, models of colitis show reductions in propulsive motility and migrating motor complexes which are responsible for movements of faecal pellets (Hofma, 2018). There are also changes in mucosal barrier function in IBD, increased paracellular permeability and tight junction protein abnormalities have been shown in studies (Söderholm, 1999; Salim, 2010). Golubeva et al's study also described delayed intestinal transit and reduced velocity of oral to anal propulsion in the BTBR mice, as well as increased epithelial permeability of the distal ileum and colon (Golubeva, 2017). This evidence adds to the theory of impaired gastrointestinal structure and function due to increased inflammation in a model of ASD, as well as the link between IBD and human ASD (Lee, 2018).

Importantly, these impairments of the colon have effects on the small intestine, there is literature concluding abnormalities in the structure and function of the small intestine in cases of IBD and IBS. Nutrient and fluid malabsorption in the jejunum in humans (Soule, 1984) and various animal models (Empey, 1993; Pantzar, 1994), changes in permeability (Buning, 2012) and dysmotility (Rao, 1987). In this study, the inflammatory response of mesenteric afferents of the jejunum was investigated, and the data shows there may be increased inflammation in tissue from BTBR mice. If BTBR mice exhibit symptoms like those in IBD, afferent responses to inflammatory mediators may be enhanced in the colon. This may be another avenue of research which is worth following up on, this study provided a small window into gastrointestinal health in BTBR mice.

An increase in response of the sensory nerves to inflammatory mediators could mean an upregulation in expression or sensitization of the receptors, which functionally result in visceral pain. In this study, responses to capsaicin and inflammatory soup application were both increased in BTBR tissue, which could be linked as inflammatory mediators sensitize TRPV1 and reduce its activation thresholds (Shin, 2002; Moriyama, 2005)

5 Conclusion

This study was designed to be a general outlook on the circadian and sensory alterations that are found in ASD, to identify any research avenues that could be explored more closely in subsequent studies and published in the future. It means that we were not able to identify a mechanism causing the differences identified, however this may not have been achievable in a one-year masters project. The jejunum was chosen due to vagal afferent input and ease of identification and recording of nerves. Also, following the 3Rs guidance set by the Home Office for animal research, the colon tissue harvested from the mice in this study was used by another masters student in our lab. However, in the context of gut dysfunction in ASD, the most common symptoms are chronic constipation and diarrhoea (Wasilewski, 2015) which manifest from the colon. Colon recordings would be the next step to complete the full picture of gastrointestinal health in the BTBR mice. In this study, the focus was kept on the neural element of the gut-brain axis, however it is important to keep in mind the myriad of functions and roles the microbiota plays in health and disease.

Limitations of the study

This study contained limitations, the most prominent is the time restraints. With more time, further experiments could be conducted to elucidate the mechanisms behind the findings. We were also unable to conduct the gut recording experiments in the prenatal VPA mice, there is significant evidence in the literature describing GI alterations in this mouse model. Further studies could provide interesting evidence towards this hypothesis. The n numbers were also small, in some experiments there were 5 of each strain, which is not a representative figure. This was especially evident in the prenatal VPA n numbers, which were very small, however this was unavoidable as we had to induce the model in-house and due to the time constraints, it was difficult to generate a robust n number. Also, there is the contribution of the litter effect, where animals from the same litter exhibit more similarities than animals from different litters, in the cases of maternal treatment such as prenatal administration of VPA, the n number refers

to the number of litters not individual mice (Lazic, 2013). Thus, the n number of prenatal VPA is two, with four individual mice, which is very underpowered.

Future directions

This study identified many potential avenues of research. Further studies in the prenatal VPA mouse model of ASD could provide many interesting insights, especially in the gut recordings as due to time limitations we could not complete those experiments in this study. The dynamic hot/cold plate showed the alterations in thermal signalling in both the VPA and BTBR mice, further studies could include direct recordings of the cutaneous afferent nerves in response to both thermal stimuli and other agonists of the TRP channels expressed in those terminals. The BTBR experiments provided many striking findings in this study, all of which could be taken further to find the causative mechanisms such as biomechanical alterations of the gut, altered afferent response to mechanical stimuli, application of capsaicin and inflammatory soup. Molecular studies and proteomics could add to the understanding behind the function of TRPV1 in BTBR mice. The inflammatory soup experiments were conducted as a general look into the response to inflammatory mediators, pharmacological experiments including individual mediators could show which pathway is altered in the gut of BTBR mice. Afferent nerve recordings from the colon and bladder could be conducted to complete the story of visceral sensation in this model, compiling evidence for a future publication.

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