Effects of Prenatal Administration of Valproic Acid on Circadian Organization and Clock-Gene Expression: Implication for Autism Spectrum Disorders

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ABSTRACT

Effects of prenatal administration of valproic acid on circadian organization and clock-gene expression: implication for Autism Spectrum Disorders

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Autism Spectrum Disorder (ASD) is a significant lifelong neurodevelopmental disorder characterized by alterations in social-emotional communication and the presence of restrictive and repetitive behaviours. Circadian rhythm disruption is one of the most common comorbidities in ASD, affecting up to 80% of individuals. However, despite the increasing prevalence of the disorder over the past decade, insights into the nature of circadian rhythm disruption in the disorder remain poorly investigated and understood.

This objective of this thesis was to characterize the circadian system in a valproic acid (VPA)-induced rodent model of ASD. First, we examined wheel-running behaviour – the current gold standard for describing functional changes within the circadian system – in both male and female Wistar rats exposed to either saline or VPA *in utero*. We demonstrated the existence of a diminished and unstable master clock in VPA-exposed animals in which aberrant behaviour was driven by alterations in photic-entrainment capacity. Secondly, we investigated the daily expression profile of BMAL1, a core clock-gene necessary for circadian behaviour, in neural structures involved in the pathogenesis of ASD behaviours. Here, we showed altered temporal dynamics of BMAL1 in peripheral tissues involved in the regulation of social behaviours, motivation, reward and monoaminergic output and suggested a potential role for the clock in the emergence of maladaptive behaviours seen in ASD. Finally, we showed that the emergence of circadian disturbances post *in utero* exposure to VPA can be passed onto the second and third

generations, opening the possibility that circadian rhythm disruption can be passed onto future generations through the germline. Collectively, this body of research emphasizes the urgent need for novel perspectives in the treatment of ASD-associated comorbidities and highlights the nature of circadian dysregulation in the disorder.

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"The question is not what you look at,
but what you see."
Henry David Thoreau

CONTRIBUTION OF AUTHORS

Chapter 1:

The experiments were designed by Sarah Ferraro and Dr. Shimon Amir. Experiments were conducted by Sarah Ferraro and assisted by Dr. Nuria de Zavalia for experiments five and six. Data was analyzed and interpreted by Sarah Ferraro with input from Dr. Shimon Amir. Data visualization was done by Dr. Nuria de Zavalia and Sarah Ferraro. Manuscript production was written by Sarah Ferraro, with editing by Dr. Shimon Amir.

Chapter 2:

The experiments were designed by Sarah Ferraro and Dr. Shimon Amir. Experiments were conducted by Sarah Ferraro and assisted by Dr. Nuria de Zavalia. Data acquisition, analysis, interpretation, and visualization was done by Sarah Ferraro. The manuscript was written by Sarah Ferraro, with editing by Dr. Shimon Amir.

Chapter 3:

The experiments were conceived by Sarah Ferraro and Dr. Shimon Amir. Breeding and caretaking of the animal colony was done by Sarah Ferraro. Animal caretaking for the duration of the behavioural experiments was done by Nour Quteishat. Data analysis, interpretation and visualization was done by Sarah Ferraro. The manuscript was written by Sarah Ferraro, with input from Dr. Shimon Amir.

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ACC: Anterior cingulate cortex

ACTH: Adrenocorticotropic hormone

ADHD: Attention deficit hyperactivity disorder

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA: Analysis of variance

ASD: Autism Spectrum Disorder

BLA: Basolateral nucleus of the amygdala

BMAL1, *Bmal1*: Brain and muscle arnt-like protein-1, or *gene*

CLOCK, *Clock*: Circadian locomotor output cycles kaput protein, or *gene*

CORT: Corticosterone

CRE: cAMP/Ca2+ responsive element

CREB: calcium/cAMP response element binding protein

CHR: Corticotropin releasing hormone

CRY, *Cry:* Cryptochrome protein, or *gene*

DA: Dopamine

DD: Constant dark

DS: Dorsal striatum

FOS, *c-Fos*: FOS protein or *gene*

FVS: Fetal valproate syndrome

GABA: ɣ-amino-butyric acid

GD: Gestational day

GR: Glucocorticoid receptors

GSK, *Gsk*: Glycogen-synthase kinase

HDAC: Histone deacetylase activity

HPA: Hypothalamic-pituitary-adrenal

IR: Immunoreactive

iPRGC: Intrinsically photosensitive retinal ganglion cells

LA: Lateral nucleus of the amygdala

LD: Light/dark cycle

LHb: Lateral habenula

LL: Constant light

mPFC: Medial prefrontal cortex

MRI: Magnetic resonance imaging

fMRI: Functional magnetic resonance imaging

NDD: Neurodevelopmental disorder

NMDA: N-methyl-D-aspartate

PER, *Per*: Period protein or *gene*

PND: Postnatal day

PRC: Phase-response curve

PVN: Paraventricular nucleus

REV-ERB, *Rev-Erb*: REV-ERB protein or *gene*

RHT: Retinohypothalamic tract

ROR, *Ror*: RAR-related orphan receptor protein or *gene*

SCN: Suprachiasmatic Nucleus

SFR: Spontaneous firing rate

TTFL: Transcription-translation feedback loop

VPA: Valproic acid

VIP: Vasoactive intestinal peptide

VPAC2: Vasoactive intestinal peptide receptor type 2

ZT: Zeitgeber time

GENERAL INTRODUCTION

Preamble

Autism spectrum disorder (ASD) is a polygenic neurodevelopmental disorder characterized by deficits in social interaction and communication, and the presence of restrictive and repetitive patterns of behaviour. Circadian rhythm disruption is a prevalent accompaniment of ASD but insight into the functional links is limited. The work presented in this thesis aims to characterize circadian organization and clock-gene expression in an animal model of ASD, ultimately shedding light on the nature of circadian disturbances associated with the disorder.

The Circadian System: A hierarchy of clocks

Living beings possess an astonishingly accurate internal time-keeping system that is responsible for daily rhythmic events such as sleep and wakefulness, feeding, metabolism and hormone regulation, among others (Takahashi et al., 2008). In mammals, these rhythms are controlled by a distributed network of central and peripheral clocks governed by a master pacemaker located in the suprachiasmatic nucleus of the hypothalamus (SCN) (Welsh et al., 2010). These cellular clocks anticipate and prepare our physiology and behaviour to align with predictable environmental changes; notably, that of the earth's 24-hour day-night cycle. In turn, much of our behavioural and physiological functions display predictable diurnal variation; thus, it comes as little surprise that disruption within this system has been associated with a host of pathologies, including diabetes, cancer, affective disorders and Autism Spectrum Disorder (ASD) (Glickman, 2010; Takahashi et al., 2008).

Circadian rhythms possess important properties that distinguish them from diurnal rhythms. First, circadian rhythms persist in the absence of any exogenous time-cue (zeitgeber) and in constant conditions, differentiating these rhythms from ones that occur simply in response to environmental changes (Vitaterna et al., 2001). Secondly, while circadian rhythms are not driven by the environment, they can however, align themselves to the day-night cycle (S. Pittendrigh, 1960). Should shifts in the external day-night cycle occur, as in the case of trans-meridian travel, these rhythms will also be accordingly phase-shifted. This process of alignment, or synchronization with external time-cues, is known as entrainment. Finally, the length of the circadian cycle (i.e. period) is generally insensitive to physiological fluctuations in temperature (Menaker & Wisner, 1983; C. S. Pittendrigh, 1954). In this sense, the circadian clock differs from the majority of biochemical reactions, whose kinetics are greatly influenced by temperature. However, the molecular clock's capacity to remain temperature compensated is integral; if temperature fluctuations easily step up or slow down biological processes, then the internal pacemaker would not be able to predict and adapt to environmental changes (Hong et al., 2006).

Intracellular Mechanisms: The Molecular Clock

Circadian timekeeping within the SCN and peripheral tissues begins with self-sustaining, autoregulatory transcriptional-translational feedback loops (TTFLs). In their simplest form, the protein products of circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1) heterodimerize and form the positive arm of the feedback loop. CLOCK/BMAL1 bind to enhancer-box (E-box) regulatory sequences of their target genes, *Period (Per1, Per2, Per3)* and *Cryptochrome (Cry1, Cry2)* and promote transcription, increasing

intracellular level of their protein products, PER1/2/3 and CRY1/2. After some time, PER and CRY heterodimerize and translocate into the nucleus where they suppress the transcriptional activity of the CLOCK/BMAL1 complex, forming the negative arm of the TTFL (as reviewed in Hastings et al., 2018; Takahashi, 2017). Existing PER/CRY complexes are marked for degradation by ubiquitination, and intracellular PER/CRY concentration levels decrease. The degradation of PER/CRY relieves the transcriptional repression of CLOCK/BMAL1, allowing the cycle to begin anew. The complete transcription-translation process of the positive and negative arms of the feedback loop takes approximately 24 hours to complete (Gallego & Virshup, 2007; Partch et al., 2006).

The core of the TTFL is further stabilized through ancillary feedback loops. The CLOCK/BMAL1 complex initiates transcription and translation of two nuclear receptors, REV-ERBα/β and RAR-related Orphan Receptor (ROR α/β), which bind REV-response element (RRE) sequences on the *Bmal1* promoter that activate and supress *BMAL1* transcription, respectively (Guillaumond et al., 2015; Preitner et al., 2002). This ancillary loop confers added precision to the core TTFL, as altered periodicity and amplitude of activity rhythms occurs in response to genetic mutations of these nuclear receptors (Cho et al., 2012). Additionally, synthetic manipulations with REV-ERB α/β agonists also alters the amplitude of the core TTFL, suggesting that an output of the TTFL is simultaneously an input back into it (Solt et al., 2012).

The output of the molecular clock acts to provide temporal regulation and coordination of cellular activity. Many clock proteins encode transcription factors and act at promoters for genes that contain E-boxes and RRE sequences (Hastings et al., 2018). This functionality allows them

to contribute to the regulation of downstream gene targets, setting off a hierarchical cascade of gene expression specific to the cell in question, thus resulting in circadian control of systems-level physiology (Akhtar et al., 2002; Panda et al., 2002). Moreover, systemic signals under the influence of the SCN, including hormone secretion, fluctuations in core body temperature and feeding can also act as non-photic zeitgebers in regions outside of the SCN and exert time-of-day dependent effects on tissue and cellular functions (Buhr & Takahashi, 2013).

The Master Pacemaker

All nucleated cells in the mammalian body contain the genes necessary to constitute the circadian clock and together form a hierarchically structured network known as the circadian system. At the top of this hierarchy is a region within the mammalian brain, adjacent to the third ventricle of the anterior hypothalamus, known as the suprachiasmatic nucleus (SCN) (Rusak & Zucker, 1979). Seminal research has highlighted the SCN's role as the body's master pacemaker; surgical lesions of the SCN in rodents result in severe disruption or abolishment of endocrine and locomotor rhythms (Stephan & Zucker, 1972). Moreover, grafts of fetal SCN tissue into SCN-lesioned animals restores activity rhythms with a periodicity determined by the genotype of the SCN donor (Ralph et al., 1990; Sujino et al., 2003). SCN lesions also result in arrhythmic, or desynchronized clock-gene expression in peripheral tissues, highlighting its role as a single master clock responsible for the coordination and synchronization of rhythms among subordinate oscillators and to the external environment (Sakamoto et al., 1998). These discoveries demonstrate that the SCN is both a sufficient and necessary region for circadian behaviour.

In order to accurately entrain our behavioural and physiological functions to the correct time-of-day, the SCN must be predictive of solar time. Thus, the primary time-telling cue, or zeitgeber (ZT), for the SCN is light (S. Pittendrigh, 1960). Entrainment to the day-night cycle is due to direct innervation of the SCN by intrinsically photosensitive retinal ganglion cells (ipRGCs), forming the retinohypothalamic tract (RHT) (Berson et al., 2002). These photoreceptor cell-types express the photopigment melanopsin and depolarize in response to short-wavelength irradiation, releasing glutamate and pituitary adenylate cyclase-activating polypeptide onto SCN neurons (Hannibal, 2002). Glutamate release, acting at N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors located on SCN neurons, results in membrane depolarization and intracellular Ca^{2+} influx (Ebling, 1996; Meijer & Schwartz, 2003). Ca^{2+} ion influx leads to the phosphorylation and activation of calcium/cAMP response element binding protein (CREB), that binds to calcium/cAMP response elements (CREs) on the promoter of target genes, promoting transcription (Ebling, 1996). This CRE-dependent function represents a mechanism where clock genes with functional CREs can be expressed rapidly and aid in resetting or entraining the circadian clock, as is the case with *Per1* (Albrecht et al., 1997; Shigeyoshi et al., 1997). In this way, light has the capacity to phase-shift rhythmic behaviour and the molecular clock of an organism. The presentation of light can phase advance or delay, as well as leave molecular and behavioural rhythms unchanged, and is dependent on the phase in which light is presented (S. Pittendrigh, 1960). Thus, light-sensitivity, or the phase-response curve (PRC) of light, predicts an organism's response to changes in light-dark cycles. Additionally, oscillatory clock-gene expression in the SCN persists in the absence of photic or non-photic cues, as well as in constant conditions (i.e.: constant dark or light), suggesting that this is due to the endogenous properties of the SCN (Welsh et al., 2010).

Traditionally, the SCN can be divided into two regions; the ventral retinorecipient core, and the dorsal shell (Abrahamson & Moore, 2001). The ventral core of the SCN sends dense projections to the shell region, but few projections emanate from the shell to the core (Leak et al., 1999). Thus, photic information conveyed through the RHT excites neurons within the core and is relayed via ɣ-amino-butyric acid (GABA)-ergic signalling from the core to the shell (Moore & Speh, 1993). Notably, the SCN is distinct from other oscillatory cells and tissues due to its emergent property as a network of coupled oscillators (Antle & Silver, 2005; Hastings & Herzog, 2004). This neuronal network confers additional resilience and robustness to the SCN clock and allows neuronal populations within the SCN to respond in flexible ways to both incoming photic information and outgoing signals (Kunz & Achermann, 2003; VanderLeest et al., 2009). In part, neuropeptides play a large role in the synchronization - or coupling - of individual SCN neuronal oscillators to one another (Brown et al., 2007). Vasoactive intestinal polypeptide (VIP) is the most abundant neuropeptide expressed within the core and plays a critical role in the coupling of SCN neuronal oscillators. Loss of VIP or its receptor, VPAC2, results in disorganized locomotor behaviour and desynchrony among SCN neurons (Colwell et al., 2003; Cutler et al., 2003; Harmar et al., 2002). Other neuropeptides, such as arginine vasopressin and gastrin releasing peptide, are also believed to play a role in neuronal coupling and in the regulation of SCN output signals (Abrahamson & Moore, 2001; Antle et al., 2005; R. Y. Moore et al., 2002).

In order to transmit temporal information to the rest of the body, the SCN must emit a coherent output signal. One critical output of the SCN is the cyclical nature of the spontaneous firing rate (SFR) of neurons. Neuronal firing is higher during the circadian day and is low during the circadian night (Houben et al., 2014). Moreover, behavioural rhythms are coupled to the

oscillatory nature of the SFR, as the onset and offset of activity are tightly linked to the midpoints of the fall and rise of neuronal ensemble firing (Houben et al., 2014). However, there is also evidence for both humoral and neural signals as possible outputs of the SCN clock. The surgical placement of an SCN graft encapsulated in a semi-permeable membrane in SCN-lesioned animals has been shown to modestly rescue behavioural rhythmicity, but not other types of rhythms (Silver et al., 1996). Additionally, ablation of the efferent axons from the SCN results in the complete loss of circadian rhythmicity, suggesting that neuronal outputs from the SCN play a major role in the transmission of temporal information (Inouye & Kawamura, 1979). It should be noted, however, that the full extent by which the SCN emits its temporal code is still largely unknown.

Peripheral Oscillators: Synchronizing central and local time

Rhythms within the SCN are autonomous and produce a rhythmic output of clock genes, designating this region of the brain as the master autonomous timekeeper. However, the SCN is also responsible for coordinating and synchronizing rhythms in peripheral - or subordinate - clocks found in other tissues. Numerous cells and tissues throughout the body display rhythmic clock gene/protein expression, and these properties have been shown to persist in culture (Damiola et al., 1998; Yamazaki et al., 2000). Peripheral clocks demonstrate rhythmicity at the genetic level, as up to 10% of all mRNA transcripts within a given tissue will show circadian rhythmicity, regulating functions relevant to each tissue type (Akhtar et al., 2002; Storch et al., 2002). These findings reflect the role of the circadian clock in temporally regulating numerous biological functions.

While peripheral clocks demonstrate endogenous rhythmicity, damage or loss of the SCN results in desynchrony of these rhythms (Kornmann et al., 2007). The SCN exerts coordination and synchrony of peripheral clocks primarily through rhythmic control of neuroendocrine and autonomic signals, and to a lesser extent, through regulation of feeding behaviour and core body temperature (Brown et al., 2002; Damiola et al., 2000; Stokkan et al., 2001). Consequently, peripheral oscillators respond to a variety of non-photic cues, such as the timing of food delivery, glucocorticoids, drugs of abuse and temperature (Dibner et al., 2010).

Although rhythmicity in peripheral tissues is mainly driven by local circadian oscillators, there remains contribution from the SCN (Kornmann et al., 2007). Selective manipulation of genes responsible for liver clock functionality results in arrhythmic transcription of hepatic mRNA when the local hepatic oscillator was arrested. However, oscillations were still detected in a subset of transcripts, notably, those related to the core clockwork (ex. *Per2)* (Kornmann et al., 2007). These results demonstrate that rhythms within peripheral tissues are driven by intracellular local clocks, but also from signals coming from the SCN, suggesting that the SCN not only coordinates rhythmicity in subordinate tissues, but also drives them.

Peripheral clocks have also been found throughout the central nervous system, outside of the SCN. These extra-SCN oscillators are unique from other peripheral clocks found in the body due to the presence of the blood-brain barrier. Specifically, output signals from the SCN and other entrainment cues do not necessarily exert the same coordinating and synchronizing effect on extra-SCN oscillations as they do in other tissues, due to the highly selective, semi-permeable nature of the blood-brain barrier. As such, only neural signals and lipophilic molecules present in

the circulation, such as glucocorticoids, have the potential to affect extra-SCN clocks (Dibner et al., 2010). Rhythmic clock-gene expression has been identified in nuclei of the hypothalamus, olfactory bulb, amygdala, hippocampus, and cerebral cortex, among other areas (Abe et al., 2002; Granados-fuentes et al., 2012; Harbour et al., 2013; Lamont et al., 2005; Wakamatsu et al., 2001). These local clocks act in the tissue-specific regulation of neuronal functions, including neuroendocrine output, emotional regulation, and motivation (as reviewed in Begemann et al., 2020). However, it is important to note that the functional relevance of every extra-SCN clock has not yet been determined and remains an important area of future study. Regardless, alterations in the phase-relationship or loss of specific clock-genes result in the emergence of abnormal physiology and behaviour, and have been linked to the incidence of depression, bipolar disorder, Alzheimer's disease, and schizophrenia (Li et al., 2013; Oliver et al., 2012; Timothy et al., 2018; Zhou et al., 2016).

Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a heterogeneous group of neurodevelopmental disorders (NDD) affecting 1 in 66 Canadian children. These statistics represent more than a 175-fold increase since the year 2000, although the reasons for such remain elusive (Maenner et al., 2020). The Diagnostic and Statistical Manual of Mental Disorders stipulates that an ASD diagnosis must be accompanied by deficits in social interaction and communication, including difficulty engaging in nonverbal behaviours used for communication, developing relationships, and in reciprocating social-emotional approaches (American Psychology Association, 2013). Moreover, individuals must show stereotyped speech or motor movements, strict adherence to

routines, or ritualistic behaviours. Together, these symptoms must be present in early childhood and limit day-to-day functioning (American Psychiatric Association, 2013).

The etiology of ASD has not yet been fully discerned; however, it is known that the majority of occurring cases are idiopathic and have no clear genetic or environmental link (Varghese et al., 2017). Three main categories of genetic abnormalities have been associated with ASD: single gene mutations, copy number variations and polygenic risk factors. These genetic abnormalities are typically *de novo* mutations found in approximately 20-30% of individuals with ASD (De Rubeis & Buxbaum, 2015; Iossifov et al., 2012). Rarely, single gene mutations with a clear genetic link to ASD are identified and account for 5% of ASD diagnoses. Often, these include mutations in genes encoding for synaptic cell adhesion proteins and synaptic development (e.g.: *Neuroligin1/2/3/4, Neurexin-1α, Shank3*) (De Rubeis et al., 2014). Copy number variations, such as inversions, deletions, translocation of chromosomes and duplications account for 10% of ASD cases (Glessner et al., 2009). Finally, accumulation of many common genetic variants can contribute to the risk of developing ASD and are referred to as polygenic risk factors (Neale et al., 2012). Heritability studies demonstrate 90% concordance for monozygotic twins, but less than 10% concordance among dizygotic twins and siblings, although these findings have been highly disputed since their reporting (A. Bailey et al., 1995; Hallmayer et al., 2011; Sandin et al., 2014). Nevertheless, the development of ASD cannot be attributed to changes within any one single gene.

Perhaps one of the most striking features of ASD is the male preponderance of the disorder, wherein males are affected approximately four times more than their female counterparts

(Maenner et al., 2020). Moreover, many of the comorbidities associated with ASD such as Attention Deficit Hyperactivity Disorder (ADHD) and intellectual disability also demonstrate a male bias (Werling & Geschwind, 2013). However, while males are more likely to be diagnosed with ASD, they also have a higher propensity to display maladaptive behaviours that are less severe in nature. These findings are in sharp contrast to that of females; those who meet the criteria for ASD often carry a higher mutational load and tend to demonstrate more severe ASD symptomatology (Tsai et al., 1981). Additionally, recent evidence suggests that females with ASD often fall below the threshold for detection and go undiagnosed due to a sexually dimorphic presentation of ASD behaviours, thus resulting in an overstatement of the male/female disease ratio (Kreiser & White, 2014). Nevertheless, these studies are complicated by sociological factors and findings are often contradictory owing to a poor understanding of the mechanistic underpinnings delineating sex differences in ASD. These differences may hinge upon multiple factors such as genetic susceptibility, alterations in sex-specific pathways, vulnerability to environmental insults, and epigenetic mechanisms (Ferri et al., 2018).

ASD, a disorder of neural connectivity?

Historically, research on ASD has aimed to identify brain regions and characterize alterations in neural connectivity to explain the emergence of abnormal behaviours seen in ASD individuals (as reviewed in Minshew & Keller, 2010; Schumann & Nordahl, 2011). These seminal studies were accomplished via use of magnetic resonance imaging (MRI), and later, functional MRI (fMRI), affording the investigation of gross macroscopic changes in the brain, including alterations in the volumetric size of defined regions, cortical thickness, and white and grey-matter alterations. From these studies emerged a common theme; the development of the brain in

children with ASD undergoes two stages: a period of accelerated growth during early childhood, and a period of arrested or slowed growth later in childhood (Courchesne et al., 2007). In particular, the frontal cortices and amygdala are regions of both peak overgrowth and arrested development in ASD and are believed to play a major role in the pathogenesis of ASD-like behaviour (Courchesne et al., 2007). More recently, it was found that cortical surface enlargement in infants is predictive of ASD onset, whereby the rate of volumetric change is correlated with an increased severity of the disorder (Hazlett et al., 2017). Additionally, newer forms of fMRI permitted functional connectivity studies to be conducted, allowing researchers to define alterations in neural connectivity and therefore define the capacity for the brain to coordinate information processing. Studies utilizing these tools have redefined ASD from a disorder of 'social relatedness', to a disorder of altered connectivity, whereby underdevelopment of critical neural pathways delineate the emergence of abnormal information processing in these individuals (Just et al., 2004, 2007). Consistently, findings of decreased neural connectivity in frontal-posterior cortical pathways were reported and are consistent with the core deficits seen in ASD, as these regions are exceedingly involved in the regulation of higher order functions. Moreover, while the processing of information typically follows a predictable sequence of cue-dependent activation events, individuals with ASD show disorganized activation in disparate structures, suggesting global neuronal dysfunction affecting many structures and pathways (Hasson et al., 2009). It should be noted, however, that alterations within structures and in connectivity pathways are not solely confined to cortical regions. Increasingly, research has highlighted the role of frontal-striatal pathways, affecting regions such as the anterior cingulate cortex (ACC) and the striatum, a subcortical structure, in the abnormal processing of reward and

motivated behaviours, further emphasizing the broad nature of neuronal dysfunction in ASD (Schmitz et al., 2008).

One question that remains is the origin of the abnormal neural developmental trajectory seen in children with ASD. To answer this question, researchers have turned to post-mortem tissue analysis to investigate potential morphological and neurobiological changes in individuals with ASD. It has been postulated that the increase in overall head circumference and brain size is potentially due to an increased number of neurons, or neuropil (i.e. the space between neurons). However, findings from these studies have demonstrated a far murkier picture and reflect a myriad of disturbances, including alterations in neuronal maturation and migration, an increased number of neurons, or increased neuropil, and are found to be region-specific (Buxhoeveden et al., 2006; Casanova et al., 2003; Mukaetova-Ladinska et al., 2004). One region that has consistently shown neurobiological alterations is the ACC, which had previously been implicated in the generation of ASD-like behaviours from fMRI studies. Specifically, decreased density of GABA(A) receptors within this region has been reported, leading to the suggestion that GABAergic innervation may be disturbed in ASD, thereby altering the delicate excitatory/inhibitory balance in the ACC (Oblak et al., 2009). Similar findings have also been reported in the cerebellum, further emphasizing that alterations in the balance of neuronal excitation/inhibition may play a role in the developmental abnormalities seen in ASD (Yip et al., 2009). Recently, region-specific glial abnormalities have also been observed, suggesting that in part, neuroinflammation may contribute to, or be a consequence of, abnormal neuronal function in ASD (Morgan et al., 2010; Vargas et al., 2005).

ASD and its comorbidities: a new field of study

ASD, as it is currently classified, is a relatively young disorder whose clinical perception has changed from one classically considered a "childhood disorder" to one that persists into adulthood, presenting lifelong challenges for the affected individual. Perhaps younger still is the acknowledgement that ASD is found to be highly comorbid with other physiological and psychological alterations, as previous classifications of the disorder stipulated that ASD and ADHD could not be diagnosed concurrently. It is now understood that up to 50% of individuals with ASD also present with ADHD and are found to be exceedingly concomitant with anxiety disorder, depression, epilepsy, and intellectual disability (LoVullo & Matson, 2009; Masi et al., 2006). These findings have major clinical implications; as the number of comorbidities increases in individuals with ASD, so does the need for individualized treatment (Matson & Goldin, 2013). Furthermore, many questions remain yet unanswered: How can clinicians best diagnose comorbid conditions in ASD? To what extent will existing therapies help with these comorbid conditions? Do these comorbidities arise in ASD because they share common etiological pathways? Further research in this area is critical, as addressing the heterogeneity of symptoms and their responsiveness to treatment remains a challenge for clinicians, individuals with ASD, and their families alike.

Sleep-wake cycle alterations and ASD

Among the most commonly observed set of comorbid symptoms in individuals with ASD is sleep-wake cycle disturbances (Richdale, 1999; Richdale & Prior, 1995). Children with ASD most often present with sleep maintenance insomnia, difficulty arising in the morning, and increased sleep latencies, all of which tend to be severe in nature (Richdale & Prior, 1995;

Wiggs, 2004). Recent estimates suggest that 50-80% of children with ASD exhibit sleep-wake cycle alterations, occurring in stark contrast to the 20-30% of typically developing children (Couturier et al., 2005; Maski & Owens, 2016; Richdale, 1999). Interestingly, sleep disturbances in children with ASD are not found to be a product of intellectual dysfunction, as is seen in most NDDs, and are observed at higher rates in ASD than any other NDD (Cotton & Richdale, 2006; Patzold et al., 1998; Richdale & Prior, 1995). While the teleological reasons for sleep remain unknown, it is widely accepted that sleep is important for developing and developed organisms alike. A growing body of evidence has shown that sleep is important for normal brain development in children and that disturbances in this process during childhood may have an impact on the neural connectivity of the adult brain (Billeh et al., 2016; Kayser et al., 2014). Moreover, research in children with ASD has highlighted that sleep-wake cycle disturbances exacerbate ASD symptomatology, increasing repetitive behaviours, further decreasing their capacity for social interactions, and decreasing seizure threshold (Cohen et al., 2014; Richdale & Schreck, 2009). These sleep-wake cycle alterations have been reported to emerge in infancy, long before an ASD diagnosis has been made, and persist through adolescence and into adulthood, posing lifelong challenges (Baker & Richdale, 2015; Karaivazoglou & Assimakopoulos, 2018; Richdale et al., 2014). However, sleep and circadian disturbances often go unassessed and untreated in individuals with ASD, as current practice prioritizes treatment for maladaptive behaviours. This clinical perspective is not without issue, as evidence also suggests that attempts at treating the core ASD deficits without treating comorbid sleep and circadian disturbances will likely lead to ineffective outcomes (Richdale et al., 2014).

THE PRESENT THESIS

Rationale

Increased sleep latencies, sleep maintenance insomnia and difficulty awakening in the morning are among the most frequently reported comorbid symptoms in individuals with ASD (Glickman, 2010; Matson & Goldin, 2013). In addition to clinical assessments, genome-wide association studies implicating clock-gene polymorphisms as a risk-factor for the disorder, and abnormal temporal secretion of melatonin and cortisol in children with ASD, collectively suggest an underlying impairment of the circadian timing system (Corbett et al., 2008; Richdale & Prior, 1992; Veatch et al., 2015; Yang et al., 2016). Research has documented that sleep-wake cycle disturbances play a role in exacerbating ASD symptomatology, affecting daytime behaviour, seizure threshold, aggression and overall quality of life (Cohen et al., 2014; Richdale et al., 2014). As such, sleep and circadian disorders are not solely an adverse consequence of ASD, but ones that can be targeted for future clinical interventions. Thus, identification of the molecular abnormalities underlying circadian dysregulation in ASD would allow for the development of targeted strategies for reinstating rhythmicity and its associated behaviours. However, research directly evaluating the circadian system in ASD remains sparse. The present thesis aims to address this issue through three main objectives; (1) the characterization of the circadian system in a valproic acid (VPA)-treated animal model of ASD, (2) the characterization of clock-gene expression within the SCN and peripheral tissues, and (3) the investigation of transgenerational inheritance of circadian disturbances.

Valproic Acid: A teratogenic link to ASD

Valproic acid (VPA) is an anticonvulsant and mood-stabilizing medication, commonly prescribed for the management of epilepsy and bipolar disorder (Favre et al., 2013). VPA has been shown to act both as a neuromodulator and as a histone deacetylase inhibitor (HDAC), regulating gene expression through chromatin remodelling (Johannessen & Johannessen, 2003; Phiel et al., 2001). However, VPA is also a potent teratogen; chronic administration of VPA within the first trimester of pregnancy has been linked to neural tube defects, structural cardiac abnormalities, polydactyly, neurodevelopmental delay and craniosynostosis. Abnormalities due to *in utero* VPA exposure can be grouped under the diagnosis of Fetal Valproate Syndrome (FVS) (Clayton-Smith & Donnai, 1995). FVS triples the risk of having a child with ASD; among children exposed to VPA *in utero*, 60% display at least two diagnostic features of ASD, and 11% are given a full diagnosis (S. J. Moore et al., 2000). While the emergence of other NDDs can occur after *in utero* VPA exposure, the development of ASD is most prevalent (Bromley et al., 2013).

VPA Mechanisms-of-action

Although the full mechanistic extent by which VPA exerts its effects *in utero* is still unknown, it is believed that VPA plays a role in the pathogenesis of ASD through its activity as an HDAC inhibitor. Exposure to VPA on gestational day (GD) 12.5 in rodents induces a transient hyperacetylation of H3 and H4 – two main histone proteins involved in the structure of chromatin in eukaryotic cells – a transient increase in cellular apoptosis, and a decrease in cellular proliferation and migration in the embryonic brain (Kataoka et al., 2013). Moreover, offspring exposed to VPA on GD 12.5 display behavioural alterations akin to ASD-like

behaviours, including deficits in social interactions, increased anxiety-like behaviour, and learning impairments. Importantly, these findings were not observed when valpromide, a VPA analog lacking HDAC inhibition activity, was used, suggesting that alterations in the embryonic brain were due to VPA's HDAC inhibitor potential. However, it has been hypothesized that the emergence of ASD-like behaviours after *in utero* VPA exposure may also be due to other mechanisms-of-action. In particular, VPA has been shown to inhibit glycogen-synthase kinase 3 (GSK-3β), a negative regulator of the Wnt-signalling pathway. This pathway is highly implicated in axonal remodelling, cellular proliferation, embryonic patterning, and organogenesis (Chenn, 2008). Alterations in the activation of the Wnt pathway may lead to abnormalities in cellular proliferation and migration of neuronal cells critical for the formation of neuronal networks in the developing embryo (Chen et al., 1999; Jung et al., 2008). Finally, VPA is believed to alter the delicate excitatory/inhibitory balance in the developing brain. Injection of VPA on GD 12 in rodents leads to the overexpression of glutamatergic transcriptional promoters, resulting in an increase of excitatory glutamatergic neuronal markers (Kim et al., 2014). Additionally, treatment with VPA leads to the downregulation of genes necessary for GABAergic inhibitory neuronal development (Fukuchi et al., 2009). These findings are consistent with the current hypothesis that a shift towards excitatory functioning in the brains of individuals with ASD underlies the generation of autistic behaviours (Casanova et al., 2003). Collectively, these findings suggest that *in utero* administration of VPA during the critical period of embryonic development may play a multifaceted role in the development of ASD-like behaviours.

VPA: An animal model of ASD

ASD is a complex disorder in humans for which animal models can only approximate some of its symptoms. Thus, in order to effectively utilize animal models, a systematic approach was developed to evaluate the commonalities between these models and human disease, and is based upon three criteria: construct validity, face validity, and predictive validity (Chadman et al., 2009; van der Staay, 2006). The degree of mechanistic similarity underlying the animal model and the human disorder is known as construct validity. While ASD is a multifactorial disorder, most cases are believed to be idiopathic, wherein environmental factors, such as VPA exposure, act as a developmental risk factor for the disorder. Based on the knowledge that teratogenic compounds such as VPA are linked to increased rates of ASD, researchers developed a rodent model wherein maternal challenge with VPA on GD 12.5 [500mg/kg] resulted in pups that displayed congenital malformations, as well as behavioural and neuroanatomical characteristics seen in human ASD populations (Rodier et al., 1996; Schneider & Przewłocki, 2005). By this line of reasoning, the VPA animal model effectively mimics one aspect of human etiology.

Face validity refers to the recapitulation of disease endophenotypes in the models themselves. By this definition, ASD models must possess behavioural, neuroanatomical, or neurophysiological alterations analogous to what is seen in the human disorder. Individuals with ASD are diagnosed based on the presence of two core deficits: (1) social communication and interaction impairments, and (2) behavioural inflexibility (American Psychiatric Association, 2013). Indeed, animals exposed to VPA *in utero* were shown to display behaviours akin to the core deficits seen in the human population, demonstrating decreased social preference and social play, and increased stereotypic behaviours (Kim et al., 2011; Markram et al., 2008; Roullet et al., 2010;

Schneider et al., 2008; Schneider & Przewłocki, 2005). Moreover, these animals also demonstrated behaviours akin to the comorbid psychopathologies seen in ASD individuals, such as increased anxiety-like behaviours, exacerbated fear-memories, decreased seizure threshold, and hypo-and-hyper sensitivity to sensory stimuli, perhaps suggesting that the pathways that underlie ASD-like behaviours also underlie the generation of comorbid behaviours (Kim et al., 2011; Markram et al., 2008; Schneider & Przewłocki, 2005). Collectively, these findings suggest that the VPA model does indeed replicate the core deficits seen in ASD individuals and offers the opportunity for the study of psychopathologies concomitantly observed with the disorder.

Predictive validity, or the capacity for animal models to respond similarly to known treatments for human disorders, is perhaps the most widely debated in ASD animal research. This is largely due to the absence of treatments for ASD, making any treatment response comparison between the model and the human disorder difficult. Behavioural interventions are the first-line treatment for individuals with ASD, with an emphasis on early intervention (Bryson et al., 2003). While there are no pharmacological treatments for the core deficits seen in ASD, treatment does exist to help with aggression, self-injurious behaviour, and certain comorbid symptoms, such as anxiety (Doyle & McDougle, 2012). In an effort to mimic early behavioural interventions for children with ASD, studies have implemented environmental enrichment for VPA-exposed rats as a means to satisfy predictive validity (Schneider et al., 2006). These studies revealed that multisensory stimulation and environmental enrichment pre-and-post weaning resulted in decreased anxiety and stereotypic-like behaviours, as well as enhanced social behaviours. These findings are believed to parallel the success often seen in early childhood behavioural therapies for children with ASD. In line with the hypothesis that a shifted excitatory/inhibitory balance

through increased NMDA receptor (NMDAR)-mediated excitatory transmission underlies the generation of abnormal behaviour in individuals with ASD, the NMDA antagonist, memantine, was administered to VPA-exposed animals. This study reported that memantine increased social preference and decreased repetitive behaviours, such as digging and self-grooming, suggesting that a dampening of NMDAR-mediated transmission alleviates social deficits and stereotypic behaviours (Kang & Kim, 2015). While memantine is currently only used for the treatment of Alzheimer's disease in Canada, predictive validity can also serve a secondary function, which is to utilize animal models to identify pharmacological interventions that may prove to be efficacious in humans (Gauthier et al., 2006). The results from this study suggest that memantine adds to the predictive validity of the VPA model and may alleviate the core deficits in individuals with ASD, but this remains to be seen in human clinical trials.

Advantages and limitations of the VPA-model

MRI technology and post-mortem studies have imparted a wealth of data describing volumetric, functional, and morphological changes in the brains of individuals with ASD. However, these tools are not without their limitations. While the use of MRI can be used track volumetric changes over the course of an individual's life, it also requires that participants remain still for up to an hour. This limitation means that it is difficult to include young children in these studies, an age where tracking the developmental trajectory of the brain is of utmost importance. Although there are a few methods to surmount this issue, the bulk of MRI studies have generally focused on adolescents and adults with ASD (as reviewed in Schumann & Nordahl, 2011). Additionally, post-mortem tissue analysis has been critical in understanding the neuronal changes that arise in ASD, such as alterations in cellular morphology and functionality. However, these studies also

have many limitations, such as extremely small sample sizes, tissue quality and age at the time of death. Perhaps the most confounding of all, however, is that these studies are influenced by the individual's comorbid symptoms, medication regimen, cause of death and environmental factors, making inferences about the abnormal development of the brain in ASD difficult. The current limitations surrounding the analysis of human ASD populations necessitates animal models, as they provide the opportunity to identify the biological underpinnings of specific disorders, while tightly controlling for confounding factors. Although the VPA model appropriately replicates the behavioural and molecular abnormalities associated with idiopathic ASD, most autistic individuals have not been exposed to this drug *in utero,* suggesting that findings from this model may be limited to ASD individuals who have been exposed to HDAC inhibitors during gestation. Nonetheless, there is consensus among experts that the etiology of ASD is likely multifactorial in nature, and that these factors converge onto similar molecular pathways leading to the development of the disorder. Therefore, the use of a VPA-model offers an excellent opportunity for the study of circadian disturbances in ASD. Interestingly, whereas diurnal disturbances are well documented in ASD individuals, minimal research into these circadian disturbances has been documented in the VPA-model. As such, our current proposed project also seeks to lend face validity to this animal model.

Objectives

Characterization of the circadian system in an animal model of ASD

Although there is ample evidence that ASD individuals exhibit alterations in diurnal processes, research evaluating the circadian system as a contributing factor remains sparse. Locomotor activity rhythms are among the most commonly studied behavioural output of the SCN clock and

recordings of these rhythms are currently the gold standard for describing functional changes within the circadian system in rodents. The findings from this study will identify the core characteristics of circadian alterations in the VPA-model and highlight the nature of these disruptions. Moreover, given the illness ratio of the disorder and the suggestion of sexually dimorphic maladaptive behaviours in ASD, we have chosen to include both male and female animals in our study. This decision offers the opportunity to determine whether the potential presence of circadian disturbances post *in utero* exposure to VPA also demonstrates sexual dimorphism. Finally, analysis of corticosterone (CORT) will also be assessed as its production is largely under influence of the SCN clock. Alterations in the temporal secretion profile of CORT may reveal the characteristics of circadian dysfunction on downstream physiological functions under control of the master clock.

It should be noted that it is currently unknown whether VPA exerts any teratogenic effect on the retinas of developing rodent pups. Since specialized cells of the retina convey information directly to the SCN, morphological and function analyses will be conducted to reveal any changes within the retina itself, or in its connection to the SCN. These results will ascertain whether retinal dysfunction is present and potentially contributing to VPA-induced circadian disturbances.

The effect of *in utero* VPA administration on clock-gene rhythms

The effect of *in utero* VPA administration on clock gene rhythms within the SCN and peripheral tissues has not yet been investigated. In order to further understand circadian alterations at the behavioural level, it is necessary to characterize the expression of clock-genes within the SCN

clock. Immunostaining for BMAL, a clock-gene necessary for normal rhythmic behaviour, will be performed on SCN slices using previously established methods. In addition, other brain regions that have been implicated in ASD, including the amygdala, habenula, prefrontal cortex (PFC) and cortex, will also undergo immunostaining for BMAL1 (as reviewed in Varghese et al., 2017). These regions are largely involved in emotional regulation, memory formation, and social behaviours – all of which have been shown to be altered in ASD individuals. Local clocks act in the regulation of tissue-specific functions and alteration of clock-gene expression within these regions may highlight a putative basis for the behavioural abnormalities seen in ASD, including the generation of comorbid psychopathology.

Transgenerational inheritance of circadian disturbances in VPA-exposed animals

ASD diagnosis occurs at more than a hundred-fold increase since the year 2000 (Choi et al., 2016). While a portion of this increase is due to better awareness, increased testing and a broader clinical definition, many researchers hypothesize that epigenetic mechanisms may underlie the increased prevalence of this disorder within the population. Previous research has found that the subsequent F2 and F3 generations born to animals exposed to VPA *in utero* display similar ASD-like behaviours, despite never having been exposed to the drug themselves (Choi et al., 2016). These results suggest that environmental insults can be epigenetically transmitted and may explain the increase in ASD prevalence. We are interested in whether the circadian disturbances found in VPA-exposed animals are also transmitted to their F2 and F3 generations. All F2 and F3 animals will undergo circadian behavioural analysis. The results from these experiments are important and unprecedented, as transmission of circadian phenotypes has not yet been identified and may represent a novel understanding of the functioning of the canonical

clock. Moreover, it would delineate whether circadian disturbances, which have many negative consequences on behavioural and physiological functioning, can be passed on to future generations. This would further emphasize the urgent need for novel targeted therapies in ASD comorbidities.

CHAPTER 1

Prenatal administration of valproic-acid alters circadian organization and clock-gene expression: Implications for Autism Spectrum Disorders.

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Abstract

Autism Spectrum Disorder (ASD) is a pervasive neurodevelopmental disorder characterized by restrictive patterns of behaviour and alterations in social interaction and communication. Up to 80% of children with ASD exhibit sleep-wake cycle disturbances, emphasizing the pressing need for novel approaches in the treatment of ASD-associated comorbidities. While sleep disturbances have been identified in ASD individuals, little has been done to assess the contribution of the circadian system to these findings. The objective of this study is to characterize circadian behaviour and clock-gene expression in a valproic acid (VPA)-induced animal model of autism to highlight perturbations potentially contributing to these disturbances.

Male and female VPA-exposed offspring underwent circadian challenges, including baseline light-dark cycles, constant dark/light and light pulse protocols. Baseline analysis showed that male, but not female, VPA-animals had a greater distribution of wheel-running behaviour across light-dark phases, while controls showed greater activity confinement to the dark phase ($p = 0.0256$). Moreover, males exposed to VPA showed a later activity offset ($p < 0.0001$). Constant light analysis indicated an attenuated masking response ($p < 0.0001$) and an increase in the number of days to reach arrhythmicity ($p < 0.0001$). A 1-hour light pulse (150 lux) at CT 15 after six days of constant dark showed that both sexes exposed to VPA exhibited a lesser phase-shift when compared to controls ($p = 0.0043$). Immunohistochemical and western-blot assays

reveal no alterations in retinal organization or function. However, immunohistochemical assay of the SCN revealed altered temporal dynamics of BMAL-1 expression in VPA-exposed males (p = 0.0016), and a shifted rhythm in females (p = 0.0053). These findings suggest alterations within the core clockwork of the SCN and photic-entrainment capacity, independent of retinal dysfunction. Furthermore, we have demonstrated sexually dimorphic alterations in circadian behaviour in VPA-animals, consistent with human epidemiological data.

Introduction

Autism Spectrum Disorder (ASD) is a pervasive neurodevelopmental disorder affecting approximately 1-2% of individuals in Western societies, where males are disproportionately affected 4 times more than females (Maenner et al., 2020). ASD is characterized by restrictive and repetitive patterns of behaviour and variances in social communication and reciprocity, though the etiology and development of the disorder remain unclear (American Psychiatric Association, 2013). Individuals with ASD often present concomitantly with disorders such as epilepsy, depression, attention deficit-hyperactivity disorder and anxiety (Crawley, 2012; Varghese et al., 2017). However, among the most frequently observed comorbidities is that of sleep-wake cycle disturbances, affecting up to 80% of children with ASD (Glickman, 2010; Richdale, 1999). Most often, these alterations manifest as increased sleep latencies, waking during the night and difficulty arising in the morning (Baker & Richdale, 2015; Richdale & Prior, 1995; Wiggs, 2004). Additionally, evidence points to abnormal temporal secretion profiles of cortisol and melatonin in children with ASD, collectively suggesting an underlying impairment of the circadian timing system (Corbett et al., 2008; Richdale & Prior, 1992).

In mammals, circadian rhythms are controlled by a distributed network of central and peripheral clocks governed by a master pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Rosbash, 1995; Takahashi et al., 2008). These cellular clocks anticipate and prepare physiological and behavioural processes to align with predictable environmental changes

but most notably, that of the earth's 24-hour light-dark cycle. This entrainment to solar time results from direct innervation of the SCN through intrinsically photosensitive retinal ganglion cells (ipRGC's) that express the photopigment melanopsin (Berson et al., 2002). Entrainment to the light-dark cycle and synchronization of peripheral clocks by the SCN is necessary for physiological and psychological wellbeing; thus, it comes as little surprise that disruption within this system has been associated with a host of pathologies, including cancer, diabetes, and affective disorders (Logan & McClung, 2019; Takahashi et al., 2008). At the cellular level, an evolutionarily conserved circadian timekeeping mechanism within the SCN and peripheral tissues begins with self-sustaining, autoregulatory transcriptional-translational feedback loops (TTFLs) (Hastings et al., 2018). Within the core loop, the CLOCK/BMAL1 transcription factors are involved in the rhythmic transcription of their own inhibitors, *Period* (*Per1, Per2*) and *Cryptochrome* (*Cry1, Cry2*), which form the negative arm of the TTFL. Subsequent degradation of the PER/CRY complexes abrogates the inhibitory effect on CLOCK/BMAL1, allowing the cycle to begin anew (Takahashi, 2017). Taken together, normal circadian functioning is therefore dependent on three components: input pathways that allow environmental stimuli to entrain the clock, the central pacemaker, and output pathways which regulate physiological and behavioural processes.

Circadian disturbances in ASD individuals have been reported to emerge in infancy, long before a diagnosis has been made, and persist throughout adolescence and adulthood, potentially posing lifelong detrimental consequences on physiological and psychological functioning (Baker & Richdale, 2015; Karaivazoglou & Assimakopoulos, 2018). In specific, alterations in sleep and circadian rhythms have been associated with a decreased capacity for social interactions, seizure

threshold, and overall quality of life in children with ASD (Cohen et al., 2014; Richdale & Schreck, 2009). As such, sleep and circadian disorders are not solely an adverse consequence of ASD, but ones that can be targeted for future clinical interventions. Thus, identification of the molecular abnormalities underlying circadian dysregulation in ASD would allow for the development of targeted strategies for reinstating rhythmicity and its associated behaviours. However, research evaluating the circadian system as a contributing factor in diurnal alterations in ASD individuals remains sparse.

To gain insight into these questions, we utilized a valproate (VPA)-animal model of ASD. While VPA is frequently prescribed as an anticonvulsant and mood stabilizing medication, it is also a known human teratogen with well-documented links to the development of ASD after *in utero* exposure (Clayton-Smith & Donnai, 1995). Administration of VPA on gestational day 12.5 in rodents results in pups that display behavioural and neuroanatomical characteristics akin to what is seen in human ASD populations, including decreased engagement in social behaviours, hypo-and-hypersensitivity to sensory stimuli and increased stereotypic behaviours (Rodier et al., 1996; Schneider & Przewłocki, 2005). Moreover, male rodents are found to exhibit distinct ASD-like behaviours more frequently than their female littermates, recapitulating the sex differences seen in human ASD populations (Kataoka et al., 2013; Schneider et al., 2008). As such, the VPA model offers an excellent opportunity to study circadian disturbances in these animals. We report that VPA-exposed males, but not females, display a diminished and unstable circadian oscillator, in which behavioural instability is further driven by aberrant photic integration. Critically, we have found that circadian alterations in VPA-exposed animals occur independently of input pathway disturbances and arise due to alterations within the core-clockwork of the SCN and

potential downstream output pathways. Finally, we have found sexually dimorphic alterations in circadian behaviour in VPA-animals, whereby females can entrain to standard conditions but show alterations under challenge conditions. Taken together, our results highlight that the VPA-model provides new insights into the etiology of circadian alterations in ASD.

Materials and Methods

Animals and Housing

All experimental procedures followed the guidelines set by the Canadian Council on Animal Care and approved by the Animal Care Committee of Concordia University. Time-pregnant naïve female Wistar rats (4 months of age) were purchased from Charles River Canada (Charles River, St-Constant, QC) and were delivered on gestational day (GD) ten. Dams were individually housed in clear plastic cages with *ad libitum* access to food and water and kept under a 12h light/dark (LD) cycle in the animal care facility. On GD 12, dams received an intraperitoneal injection of either VPA dissolved in saline (500 mg/kg, 1 mg/kg) or 0.9% saline solution (1 mg/kg). The resulting litters were not culled, and mothers could tend to their pups with minimal interference. Pups were subjected to behavioural assays to measure developmental markers between PND 2-10. Weaning occurred on PND 23.

Between PND 24-30, pups were introduced to sound-attenuated isolation boxes equipped with running wheels and *ad libitum* access to food and water. Wheel-running activity was collected continuously by VitalView software (Mini-Mitter, Starr Life Sciences Corp., Oakmont, PA, USA). Graphical representation of wheel-running behaviour was acquired by using Actiview Biological Rhythm Analysis software (Mini-Mitter) and analysed using Clocklab (Actimetrics, Evanston, IL, USA).

Righting Reflex Task

On PND 2,3,4 and 5 pups were briefly placed in a supine position on a table. The time to fully right themselves by 180̊ was recorded, up to a maximum of 30 seconds. Promptly after assessment, pups were reintroduced to their home cages to decrease any potential effect of stress on maternal behaviour or within the pups themselves. This task was used to measure postnatal locomotor development in saline and VPA-exposed pups and as a marker of overall postnatal health and development.

Olfactory Discrimination Task

Between PND 9-10 pups were placed in the centre of a clean, transparent standard housing cage with two olfactory stimuli at opposite ends of the cage. The two olfactory stimuli used were clean SaniChip bedding (neutral odor) or bedding from the home cage (nest odor) and were placed in two separate weighing boats. The bedding from the home cage had been in use for approximately 3 days prior to the day of the task. Latency to reach the home bedding was recorded up to a maximum of 3 minutes. The cage was sanitized with a 70% ethanol solution between subjects.

Circadian Analysis

Initially, male, and female offspring were placed in a standard 12:12 LD cycle for two weeks to determine the following parameters: stability/variability (IS/IV), onset/offset of activity, amplitude and alpha. Moreover, activity during the light and dark phases were determined by activity bout analysis (threshold set at: 1-wheel revolution/min/10 consecutive minutes). Animals

were then placed in constant dark for three weeks. The first week was used to determine the endogenous free-running period (tau) of each animal. Subsequently, an Aschoff type-1 light pulse protocol was employed to determine the behavioural phase shifting response. In brief, a 1-hour 150 lux light pulse was conducted three hours after the onset of running-wheel activity (CT 15). CT 15 was determined for each animal on the day of the light pulse by fitting a regression line to the actogram obtained from wheel running. The subsequent phase shift (in hours) was monitored and averaged over five days. Finally, a constant light protocol was employed for one month before the termination of the experiment. The number of days needed to achieve arrhythmicity and masking behaviour was calculated. Results were obtained through use of the Clocklab software (Actimetrics, Evanston, IL, USA) analysis tool. In the case of results obtained through visual inspection, such as the number of days needed to achieve arrhythmicity, two individuals (one blind to experimental conditions) cross-verified their results to prevent the potential for bias in the analysis.

Tissue preparation and Immunohistochemistry

BMAL1 Expression

In order to discern rhythmic expression differences between groups, four time points for tissue harvesting were selected (Zeitgeber time (ZT) 1, 7, 13 and 19). 14-18 weeks old rats were deeply anesthetized with sodium pentobarbital (100mg/kg, intraperitoneally) and transcardially perfused with 300 ml of cold saline (0.9% NaCl), followed by 300 ml of cold paraformaldehyde (4% in a 0.1M phosphate buffer, pH 7.3). Brains were extracted and post-fixed for 24h in paraformaldehyde at 4°C, then sliced coronally at 30µm or 50µm for immunofluorescence or

immunohistochemistry assays respectively. These slices were then stored at -20°C in Watson's Cryoprotectant.

Sections containing the SCN were used for immunohistochemical staining and a polyclonal antibody against BMAL1 (1:30,000; made in rabbit, Novus Biologicals) was used. Primary antibody solution was made with a milk buffered (5%) triton-TBS solution and 2% normal goat serum and incubated for 48h at 4°C. After rinsing the free-floating tissue in TBS, a secondary incubation solution containing biotinylated anti-rabbit IgG made in goat (1:200 dilution; Vector Laboratories, Burlington, ON, Canada) was used. Brain sections were rinsed in fresh TBS and then incubated in an avidin-biotin solution for 2h (4°C; Vectastain Elite ABC Kit; Vector Laboratories). Finally, for visualization of immunoreactive cells, sections were rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution (10 mins), followed by a solution of 0.5% 3,3-DAB, 0.01% H202 and 8% NiCl2 (10 mins). Subsequently, sections were mounted onto gel-coated glass slides and underwent serial alcohol dehydration, cleared with Citrisolv (Fisher Scientific, Pittsburgh, PA, USA) and coverslipped. Examination of the sections of interest was conducted via the use of a light microscope (Leica, DMR) and images were captured with a Sony XC-77 video camera, usually with a minimum of 8 images per brain region captured. Using ImageJ software (http://imagej.nih.gov), a set dimension was chosen (100x100) for the brain region of interest and background staining (40x40); the density of the background was subtracted from the density of the area of interest to yield our result.

p75NTR Expression

To investigate proper connectivity from the retina to the SCN after in utero VPA exposure, immunoreactivity for p75NTR was performed. Tissue sections containing the SCN were obtained as described above. Polyclonal antibodies for p75NTR (1:8000; made in rabbit, Millipore Sigma #07-476) were used. All steps described above including primary, secondary, and tertiary descriptions, were followed as detailed.

Immunofluorescence

14 weeks old rats were subjected to a 1-hour light pulse (150 lux) at CT 15 and were sacrificed 1h after the termination of the light pulse. Double labeling was performed for c-FOS (1/800 dilution, PhosphoSolutions) and VIP (1/10000 dilution, Immunostar). Free-floating sections were rinsed for 10 minutes in phosphate-buffered saline (PBS, pH 7.4), followed by 3x10 minute rinses in 0.3% Triton-X in PBS (PBS-TX). Tissue was pre-blocked for 1h at room temperature with gentle agitation in a solution of PBS-TX with 3% milk powder and 6% normal donkey serum. The tissue was then incubated at room temperature overnight with gentle agitation in the primary antibody solution. The primary antibody solution was composed of the primary antibody diluted in 2% NDS and 3% milk powder buffer. Finally, a secondary antibody solution containing Alexa-488 (1/1000 dilution, anti-rabbit) and Alexa-594 (1/1000 dilution, anti-mouse. Life Technologies, Carlsbad, CA, USA) was used for signal amplification. Tissue was rinsed 3x10 minutes in PBS-TX, and a final 10 minutes in PBS before being mounted onto slides and allowed to dry. Slides were coverslipped using VECTASHIELD® Antifade Mounting Media (Vector Laboratories, Inc., Burlingame, CA, USA) for fluorescence with DAPI (Vector Laboratories, Inc.). Confocal images were taken using the Olympus FV10i automated confocal laser scanning microscope using a 60x objective at the Centre for Microscopy and Cell Imaging

at Concordia University, Montreal, Canada. The location of the SCN was determined based on landmarks from "The Rat Brain in Stereotaxic Coordinates" (Paxinos & Watson, 1997). All confocal parameters (capture area, depth, contrast, brightness, etc.) were kept constant and laser intensity was set automatically and then adjusted for each brain area.

CORT ELISA

Trunk blood was collected from animals after a terminal live decapitation in heparinized blood collection tubes. Samples were centrifuged for 10 minutes at 4℃, 13,000 r.p.m and plasma were extracted and stored at -80℃. Plasma CORT levels were assessed using a CORT Enzyme Immunoassay (ELISA) (Thermofisher Scientific) and the assay was run per manufacturers guidelines.

Western blot analysis

14-16 weeks old rats were entrained for 2 weeks to a 12:12 h LD cycle and were sacrificed shortly after lights on (between ZT 1-3). The eye was isolated and both retinas were dissected out and frozen on dry ice. The tissue was then homogenized (two retinas per tube from the same sample) in 200 ul of lysis buffer [1M Tris-HCL pH 6.8, 10% sodium dodecyl sulfate, 0.1 ml phosphatase inhibitor cocktail 2 (Sigma #P5726, Burlington, MA, USA)), 0.1 ml phosphatase inhibitor cocktail 3 (Millipore Sigma, #P0044, Burlington, MA, USA), 1× protease inhibitor cocktail (Roche, Basel, Switzerland)]. The extracted proteins (100 ug/lane) were electrophoresed into a 10% SDS-PAGE gel and blotted into a nitrocellulose membrane (Bio-Rad, # 1620112, Hercules, CA, USA). The subsequent membrane was blocked with a 5% milk buffer, 0.3% Tween-PBS solution and incubated overnight at 4°C in 0.3% Tween-TBS with the anti-

Melanopsin (1:500 dilution) antibody (ThermoFisher, #PA1-780, Waltham, MA, USA) and anti-actin (1/80000 dilution) antibody (Millipore Sigma, #A5441, Burlington, MA, USA). Finally, the membrane was incubated in 0.3% Tween-PBS, 5% milk buffer solution with the addition of a goat anti-rabbit and anti-mouse IgG horseradish peroxidase-conjugated antibody (1/2500 dilution; Millipore Sigma, #AP132P). Signal visualization was achieved via Western Lighting Chemiluminescence light-emitting system (PerkinElmer Life Sciences, Waltham, MA, USA). Between each antibody incubation, membranes were washed in three, ten-minute intervals in 0.3% Tween-PBS.

Statistical Analysis

For activity parameters, differences between groups were analysed with unpaired two-tailed t-test (actogram analysis) or one-way analysis of variance (ANOVA). A two-way ANOVA was utilized for analysing BMAL1 expression within the SCN and CORT secretion. A significance threshold was set at $\alpha = 0.05$ using GraphPad Prism 9 (GraphPad software LLC, San Diego, CA, USA). Planned comparisons were conducted with a Bonferroni correction when appropriate. Corrections for unequal variances were used when appropriate and detailed in the figure captions.

Results

Development of VPA-offspring

To investigate the effect of prenatal VPA-exposure on the development of the resulting offspring, animals were subjected to various screening assays to assess motor development, ASD-like behaviours, and general health. The weight of male offspring shortly after birth

(Supplementary Fig.S1a), after weaning and into adulthood (Fig. S1b) were recorded and were indistinguishable between groups. Since VPA had little effect on overall weight gain in males, the weight of female offspring was recorded shortly after birth and weaning equally and revealed no distinguishable differences between groups (Fig. S1c). A righting reflex task was performed shortly after birth where VPA and saline-exposed offspring were placed in a supine position and the time to right themselves ventrally was recorded. No significant differences between groups were detected, indicating normal motor development in VPA-exposed pups (Fig. S1d).

Nest-seeking responses mediated by the olfactory system are important for the development of social behaviour in rodent pups (Terry & Johanson, 1996). Deficits in nest-seeking behaviour have been documented in VPA-exposed males but not in VPA-exposed females and are indicative of olfactory discrimination deficits later in development (Favre et al., 2013; Roullet et al., 2010). To verify the presence of known ASD-like behaviours in offspring generated in our laboratory, the olfactory discrimination task (ODT) was conducted on PND 9 and is detailed in the methodology. Consistent with previous reports, males exposed to VPA demonstrated a longer latency to engage with home bedding when compared to controls (Fig. S1e) ($p < 0.0005$). However, this effect was not seen in VPA-exposed females (Fig. S1f). These results indicate the presence of ASD-like behaviours in offspring while the general health and development of VPA-exposed animals is otherwise intact.

Baseline Conditions

Non-image forming pathways and circadian timekeeping are important for the generation of rhythmic behaviours but are equally integral in emotion regulation and metabolic processes; all

of which are altered to some degree in ASD individuals (Legates et al., 2012). Therefore, we sought to determine whether *in utero* VPA-exposure altered circadian rhythmicity and if so, the extent of such alterations. To address whether VPA-exposed animals display circadian disturbances, we first examined wheel-running behaviour in rats housed under standard 12-hour light/dark (LD) conditions. Under baseline conditions, VPA-exposed males displayed an inability to confine wheel-running activity to the dark phase - behaviour typical of nocturnal animals (Fig 1a). Rather, VPA-exposed males increased their ratio of day/night activity events, showing less diurnal variation in comparison to controls ($p = 0.0256$) (Fig 1b.). As a result, the duration of the active phase (i.e. alpha) in VPA-exposed males was significantly extended by 2.2 hours ($p = 0.0149$) (Supplementary Fig. S2b) and resulted in activity offsets occurring ~1.43 hours into the light phase (one-way ANOVA, $F(3, 15.24) = 15.51$, $p < 0.0001$) (Fig. 1c). Stable activity onsets and offsets are considered a marker of normal circadian functioning, while variability in this parameter is an indicator of circadian disruption. VPA-exposed males showed increased variability in their activity onsets in comparison to controls, indicating rhythm instability (Fig. S2a). Moreover, the amplitude of the behavioural rhythm was significantly lower in VPA-males, indicating decreased strength of the internal clock output ($p = 0.0073$) (Fig. 1d). Analysis of intra-daily variability revealed a significant increase in VPA-exposed males, suggesting increased fragmentation in daily rhythms ($p < 0.0001$) (Fig. 1e). Furthermore, inter-daily stability – a marker of synchronization strength between the internal rhythm and the external LD cycle – was decreased in VPA-exposed males, alluding to alterations in entrainment capacity ($p = 0.0047$) (Fig. 1f). Notably, these circadian alterations were not seen in VPA-exposed females, suggesting sexually dimorphic behaviour in circadian rhythmicity post-*in utero* treatment under baseline conditions. Collectively, these results suggest marked rhythm instability

and dysregulation of the SCN clock output under baseline conditions in VPA-exposed males but not in VPA-exposed females.

Figure 1.

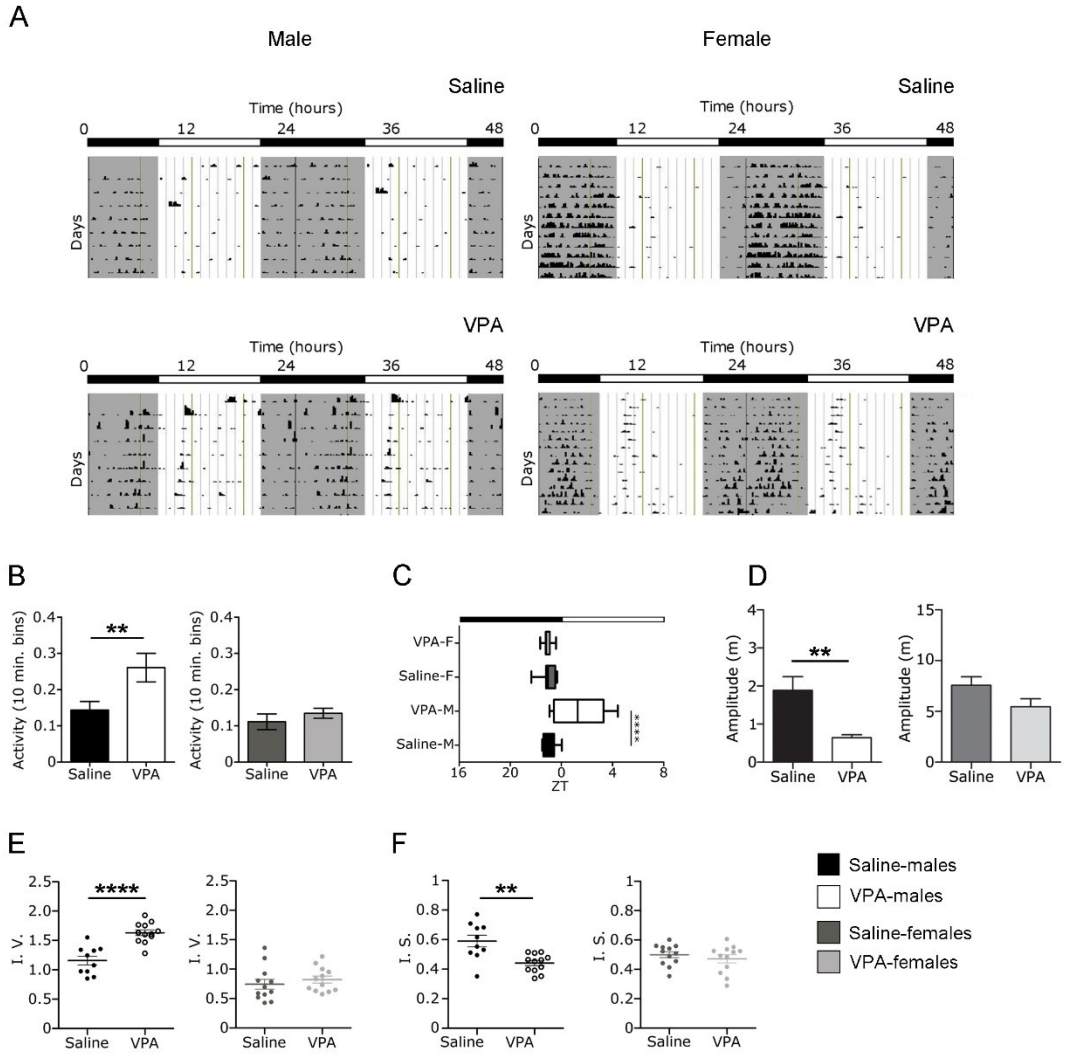


Figure 1. Prenatal VPA exposure alters wheel-running behaviour under baseline conditions.

(A) Representative double-plotted actograms from (left panel) saline-exposed males (n = 10) and VPA-exposed males (n = 12). Right panels represent double-plotted actograms from saline-exposed females (n = 12) and VPA-exposed females (n = 12), maintained under 12-hour light/dark conditions. Gray shaded areas indicate lights off (Zeitgeber time (ZT) 0 = lights on, ZT 12 = lights off). **(B)** VPA-exposed males, but not females, show less circadian variation in wheel-running activity under baseline conditions (saline: M = 0.1438 ± 0.02346, VPA: M = 0.2607 ± 0.03958, p = 0.0256). Graphs depict a ratio of activity bouts during the light phase/ activity bouts during the dark phase (bout = 1count of activity/minute/ten minutes). **(C)** Activity offsets in VPA-exposed males occur approximately 1.43 hours into the light phase (1-way ANOVA, F(3, 15.24) = 15.51, p < 0.0001. Dunnett's multiple comparisons revealed a significant

difference in offset between VPA-exposed and saline-exposed males (saline, M= 7.062 ± 0.1655, VPA: M = 9.433 ± 0.5861 $p < 0.005$, where 7:00h corresponds to ZT 1). **(D)** Amplitude (m) is diminished in VPA-exposed males in comparison to controls, but not in VPA-exposed females (saline: M = 1.885 ±0.3610, VPA: M = 0.6412 ±0.07913, $p = 0.0015$). **(E)** VPA-exposed males show fragmented daily activity rhythms (intra-daily variability) (saline: M = 1.157 ±0.07543, VPA: M = 1.631 ±0.1735). **(F)** Inter-daily stability is decreased in VPA-exposed males (saline: M = 0.5899 ±0.03950, VPA: M = 0.4413 ± 0.01779) indicating decreased photic-entrainment capacity. Data were analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM.

Dysregulation under constant conditions

To further understand the circadian disturbances seen under baseline conditions in VPA-exposed animals, we examined the effects of constant conditions (constant dark (DD) and constant light (LL)) on rhythmicity. Under DD conditions, neither males nor females displayed differences in their intrinsic free-running period (Fig. 2b), suggesting that the observed circadian disturbances are not due to a lengthening or shortening of the molecular clock. Furthermore, alpha was unchanged between groups under DD conditions (Supplementary Fig. 3b). This finding suggests that under baseline conditions, rhythm instability occurs through aberrant photic entrainment, consequently driving an increase in alpha. However, the amplitude of the intrinsic rhythm in DD was lowered in VPA-exposed males ($p < 0.0001$), suggesting decreased strength of the SCN clock output independent of light-induced effects (Fig. 2c). As observed under baseline conditions, females exposed to VPA did not show any differences under DD conditions when compared to controls.

Negative masking, or a decrease in locomotor activity in response to light, is commonly observed in response to constant light conditions in nocturnal animals (Mrosovsky & Hattar, 2003) (Fig. 3a). However, under LL both males ($p < 0.0001$) and females ($p = 0.0024$) exposed to VPA demonstrated an attenuated negative masking response (Fig. 3c). The LL paradigm also elicits loss of rhythmicity after prolonged exposure – an effect believed to occur due to uncoupling of SCN neurons. Strikingly, in both VPA-exposed males ($p < 0.0001$) and females ($p < 0.0001$), the number of days needed to achieve arrhythmicity was significantly longer compared to controls, again suggesting an underlying impairment of photic integration in these animals (Fig. 3b). Notably, while females did not display circadian alterations under baseline

conditions, the results obtained during LL demonstrate circadian dysregulation under challenge conditions.

Figure 2

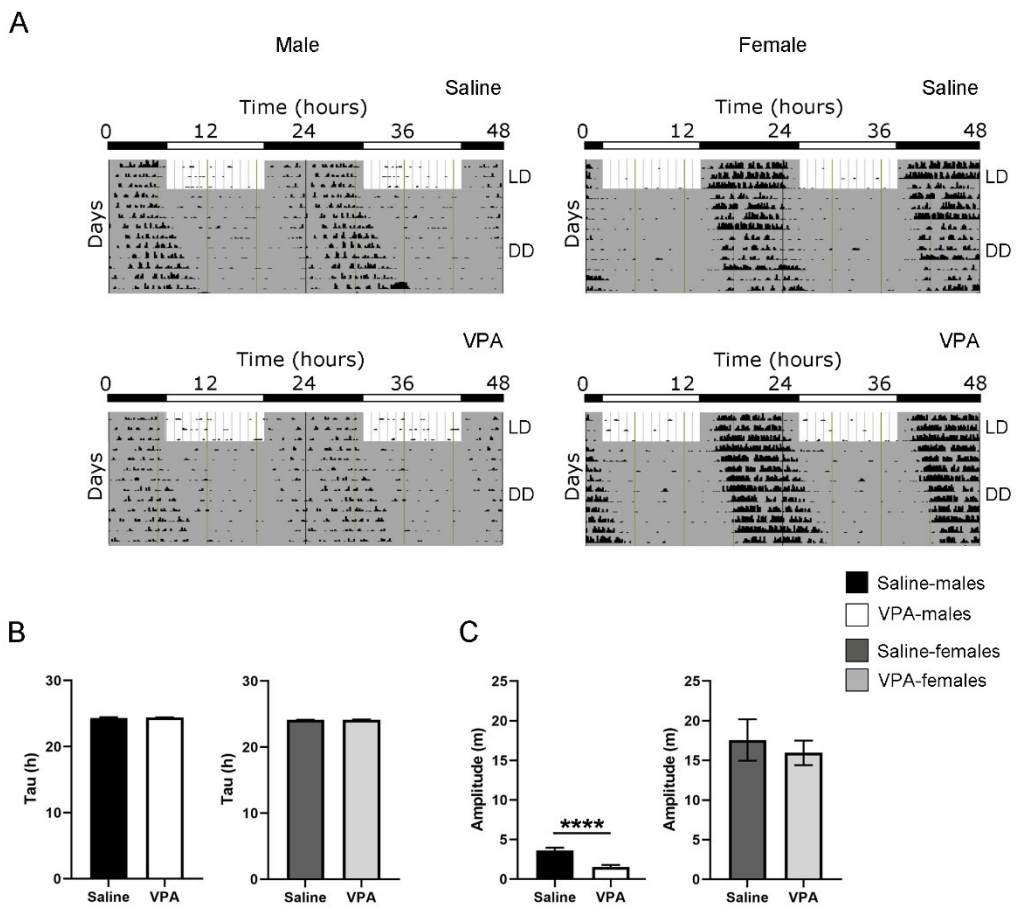


Figure 2. Behavioural rhythms are diminished in VPA-exposed males under constant dark (DD) conditions.

(A) Representative double-plotted actograms from (upper panel) saline-exposed males and VPA-exposed males. Lower panels represent double-plotted actograms from saline-exposed females and VPA-exposed females, maintained under constant dark conditions. **(B)** Tau (hours), or the period of the endogenous free-running rhythm, is unchanged between VPA-exposed males/females and saline-exposed males/females. **(C)** Amplitude (m) is lessened under DD conditions in VPA-exposed males (saline: M = 3.618 ±0.3464, VPA: M = 1.545 ±0.8215, $p <$ 0.0001). Data were analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM.

Figure 3.

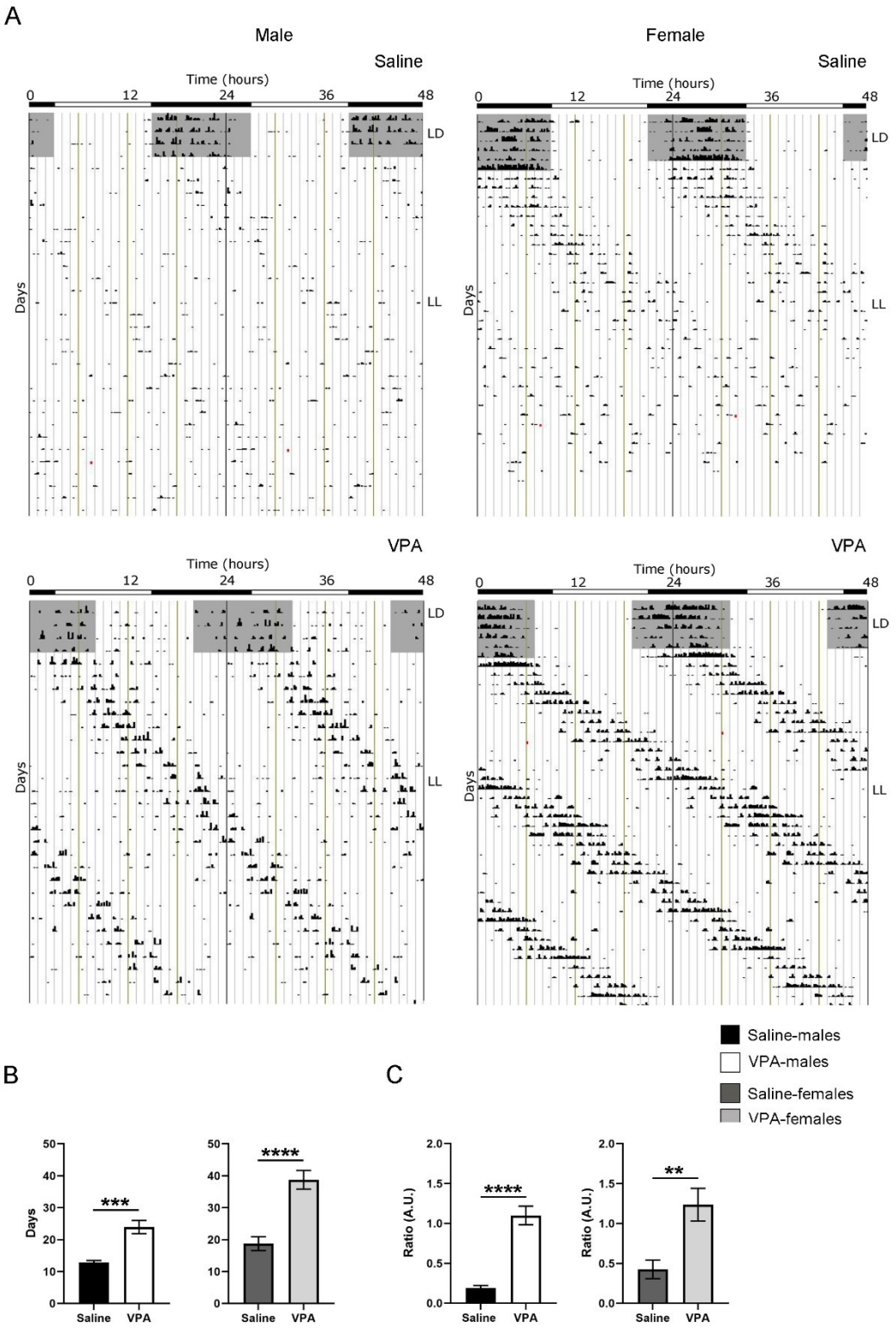

A
Male
Saline
Female
Saline
VPA
VPA
Time (hours)
0
12
24
36
48
LD
LL
Days
Saline-males
VPA-males
Saline-females
VPA-females
B
C
Days
Ratio (A.U.)
***
****
**
Saline
VPA

Figure 3. Prenatal exposure to VPA alters locomotor suppression under constant light (LL) conditions in both sexes.

(A) Representative double-plotted actograms from (upper panel) saline-exposed males and VPA-exposed males. Lower panels represent double-plotted actograms from saline-exposed females and VPA-exposed females, maintained under constant light conditions. **(B)** Both VPA-exposed males and females demonstrate an increase in the number of days needed to reach arrhythmic behaviour, in comparison to their respective control counter parts. (Males; saline: M = 12.89 ± 0.5638, VPA: M = 23.93 ±2.069, $p < 0.0001$. Females: saline: M = 18.75 ±2.171, VPA: M = 38.75 ±2.926, $p < 0.0001$). **(C)** Both VPA-exposed males and females show an attenuated negative masking response under LL conditions, represented by a ratio of (10d average of total activity in LL)/(10d total activity in LD) (Males; saline: M = 0.1895 ±0.03219, VPA: M = 1.100 ±0.1169, $p < 0.0001$. Females; saline: M = 0.4248 ±0.1167, VPA: M = 1.236 ±0.2055 $p = 0.0024$). Data were analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM.

Impaired response to phase-shifting light pulse

Based on the results obtained from baseline and constant conditions, we sought to determine whether the integration of photic information was impaired in VPA-exposed animals. The administration of a light pulse (LP) during the early subjective night or late subjective night elicits a phase delay or advance, respectively. This phenomenon known as the phase-response curve to light (PRC) can be utilized to monitor plasticity of the SCN. Both saline and VPA-exposed males and females were released into DD conditions for six days before a 1-hour, 150 lux LP was employed 3 hours after the onset of running-wheel activity (Circadian Time, CT 15. Aschoff type 1 protocol) (Fig. 4a). The subsequent phase shift in activity, measured over a 5-day period, was obtained for both groups (graphical representation found in S3). When exposed to light at CT 15, VPA-treated males displayed an attenuated phase-shifting response when compared to controls ($p = 0.0043$) (Fig. 4b). Interestingly, a similar scenario was observed for VPA-exposed females when compared to controls ($p = 0.0044$). These results indicate that VPA-exposed animals display altered plasticity of the SCN in response to a phase-shifting light pulse, further suggesting aberrant photic-entrainment in these animals.

Figure 4.

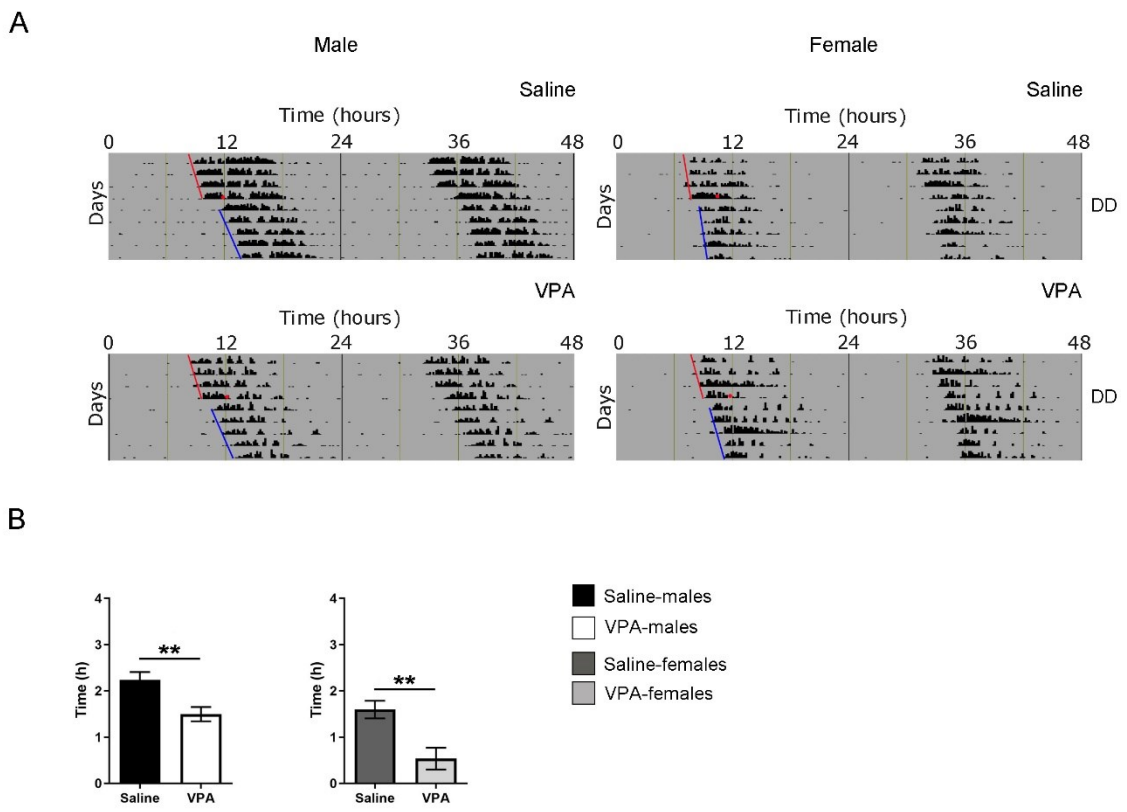


Figure 4. Altered responses to an Aschoff type-1 light pulse in VPA treated animals.

(A) Representative double-plotted actograms from (upper panel) saline-exposed males and VPA-exposed males. Lower panels represent double-plotted actograms from saline-exposed females and VPA-exposed females, maintained under DD conditions for 6 days before a 1h light pulse at CT 15 (3 hours after the onset of wheel-running activity) was elicited (represented by the black circle). The subsequent phase shift in activity can be observed. **(B)** The averaged phase shift (h) over 5 days, for VPA and saline-exposed males/females. Both VPA-exposed male/female animals demonstrate a lessened phase-shifting response (Males; saline: M = 2.238 ±0.1688, VPA: M = 1.495 ±0.1549, $p = 0.0043$. Females; saline: M = 1.595 ±0.1900, VPA: M = 0.5351 ±0.2368, $p = 0.0044$). Data were analyzed using a two-tailed unpaired t-test and are plotted as mean ± SEM

Circadian dysregulation in VPA-animals occurs independently of retinal dysfunction

One area of potential contribution to the observed phenotype may be due to impact of *in utero* VPA exposure on the development of the retina. It is currently unknown whether VPA affects normal development of the retina and/or if it affects retinal input to the SCN, which is necessary for normal entrainment to LD cycles. To address the question of retinal development, an immunofluorescent (IF) staining of the retinal cell layers was performed using the nuclear marker DAPI. IF staining of the retina revealed no overt effect of *in utero* VPA exposure on the presence and normal organization of all five layers of the retina (Fig. 5a).

Previous research has documented the expression of p75-neurotrophin receptor (p75NTR), a neurotrophic factor, in retinal afferents projecting to the SCN and within the retinorecipient core of the SCN itself (Bina et al., 1997; Kiss et al., 1993). Retinal neurotoxic agents such as monosodium glutamate (a primary disruptor of retinal ganglion cells (RGCs), when administered during the neonatal period, abolishes p75NTR immunoreactivity within the SCN (Beaulé & Amir, 2001). To gain further insight into the origins of circadian disturbances in VPA-animals, we investigated the presence of intact RGC projection to the SCN through IHC assay for p75NTR. Within the SCN, p75NTR immunoreactivity was found in both groups, indicating that a normal connection between the retina and the SCN had been established despite exposure to VPA *in utero* (Fig. 5b).

iPRGCs express the specialized photopigment melanopsin and depolarize in response to short-wavelength irradiation (Berson et al., 2002; Hattar et al., 2006; Provencio et al., 1998). Seminal studies have documented that melanopsin knockout animals (*Opn 4 -/-*) display attenuated

negative masking response (Mrosovsky & Hattar, 2003). A western-blot analysis was conducted to determine whether a lack and/or reduction of melanopsin expression contributes to our observed phenotype. The assay revealed no significant differences in the expression of melanopsin in both males and females exposed to saline or VPA *in utero*, indicating that a decrease or lack of melanopsin within iPRGCs does not underlie the attenuated negative masking response observed in VPA-exposed animals.

Expression of the immediate-early gene *c-fos*, can be used as a marker of neuronal activation in the SCN in response to light exposure during the early subjective night (Kornhauser et al., 1990, 1996; Rusak et al., 1990). To gain insight into whether light elicits the same neuronal response within the SCN, a 1-hour, 150 lux LP at CT 15 was performed. Animals were sacrificed 1-hour post LP and tissue was sectioned to obtain the SCN, followed by IF staining for c-FOS and vasoactive intestinal peptide (VIP) within the SCN. No significant differences in the number of c-FOS positive cells in the SCN of either VPA or saline-treated animals were detected, suggesting that photic information was properly relayed from the retina to the SCN and the same local response was elicited, highlighting potential downstream perturbations.

Immunoreactivity for VIP was used to distinguish the ventral "core" of the SCN; however, VIP is equally important for the coupling of cellular oscillators and proper entrainment (Aton et al., 2005). Mice that lack VIP display weak behavioural rhythms, thus marked changes in VIP expression may contribute to the circadian disturbances in seen in VPA animals (T. M. Brown et al., 2007; Colwell et al., 2003). However, the expression pattern of VIP did not appear to differ between groups in mid-coronal SCN slices, suggesting that changes in VIP expression are

unlikely to contribute to circadian alterations in our model (Supplemental figure, S4). Collectively, the results from these assays highlight that circadian dysregulation in VPA-treated animals occurs independently of retinal dysfunction.

Figure 5.

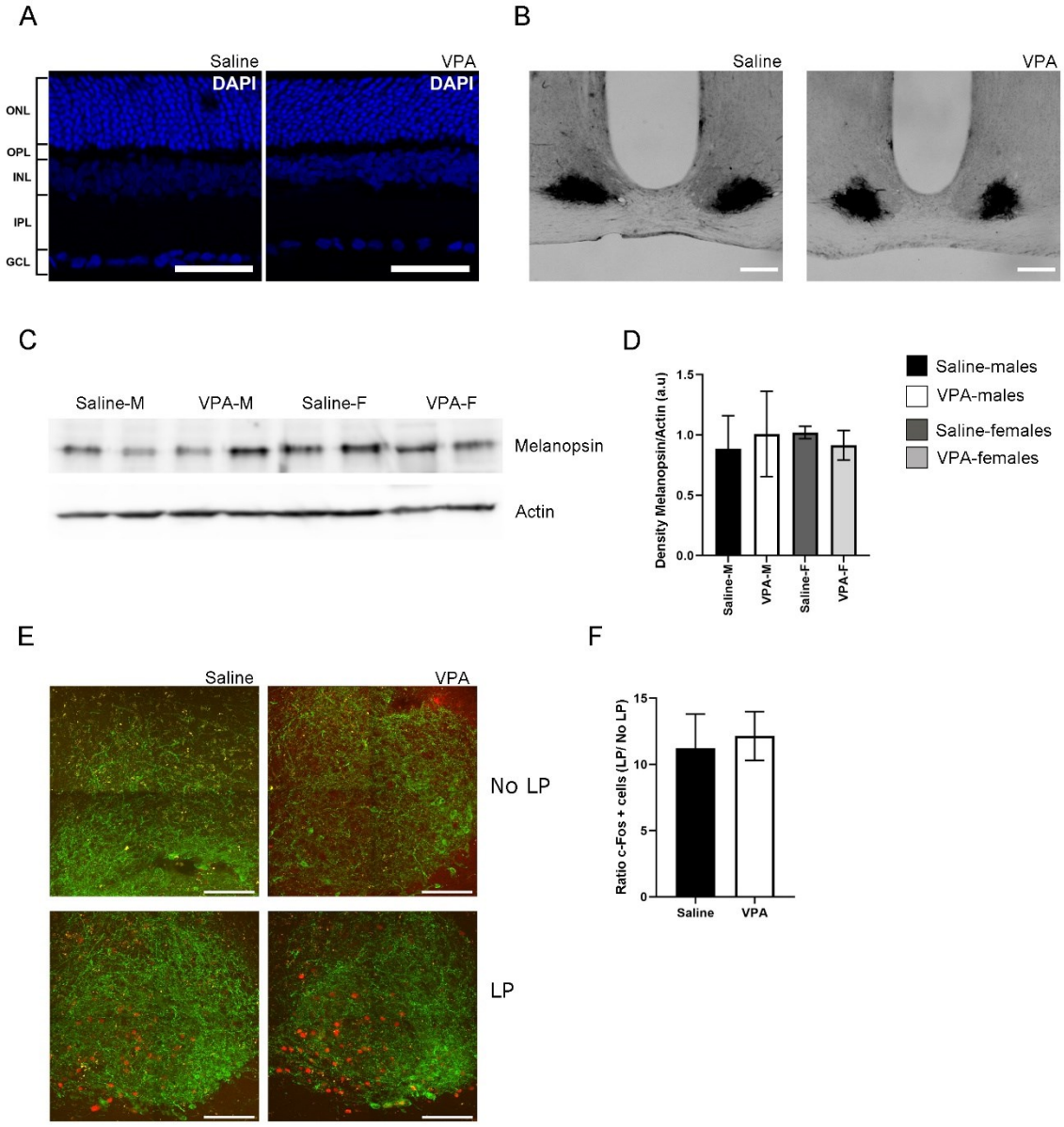


Figure 5. Prenatal exposure to VPA does not alter retinal organization.

(A) Immunofluorescent staining for the nuclear marker, DAPI in saline and VPA-exposed animals. Note the normal organization of all five layers of the retina (outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and ganglion cell layer (GCL)) Scale bar = 100µm. (B) p75-NTR immunoreactivity in the SCN of saline (n = 3) and VPA (n = 3) exposed animals. (C) Western blot from retinal lysates from saline and VPA-exposed male/female animals collected between ZT1-3 under standard 12:12 conditions. Right: densitometry measurements for melanopsin relative to actin controls (one-way ANOVA, ns). (D) Immunofluorescent images of c-FOS (red) and VIP (green) in light pulse (LP) or no LP conditions. Scale bar = 100µm. Right: number of c-FOS immunoreactive cells (student's t-test, ns).

BMAL1 expression in VPA-exposed animals

Circadian timekeeping within the master clock is achieved through the rhythmic expression of clock-genes within neurons of the SCN (Takahashi, 2017). Global or region-specific loss of the clock-gene *Bmal1* has been shown elicit a loss of behavioural rhythms and negatively affects overall physiological functioning (Bunger et al., 2000; Kennaway et al., 2013; Sutton et al., 2017). As such, BMAL1 positions itself as a necessary component of the core molecular clock. To gain mechanistic insight into our behavioural phenotype, we characterized the expression of BMAL1 at four time points over a 24-hour period within the SCN under 12:12 conditions. In VPA-exposed males, BMAL1 followed an abnormal temporal expression pattern compared to controls (significant interaction, $F(1, 14) = 15.10$, $p = 0.0016$), peaking at the beginning of the dark phase (ZT 13), whereas BMAL1 expression in controls peaked at the beginning of the light phase (ZT 1). Moreover, elevated expression of BMAL1 was detected in VPA-exposed males relative to their control counterparts. Surprisingly, while VPA-exposed females did not show circadian dysregulation under baseline conditions, BMAL1 demonstrated abnormal temporal expression in comparison to controls (significant interaction, $F(3,16) = 6.227$, $p = 0.0053$). Expression of BMAL1 in VPA-exposed females was maximal towards the end of the dark phase (ZT 19), in contrast to the beginning of the light phase (ZT 1) in controls. These results highlight that altered behavioural rhythmicity in VPA-exposed animals is reproduced at the level of the molecular clock and is reflective of desynchronization between external zeitgebers and clock-gene expression.

Figure 6.

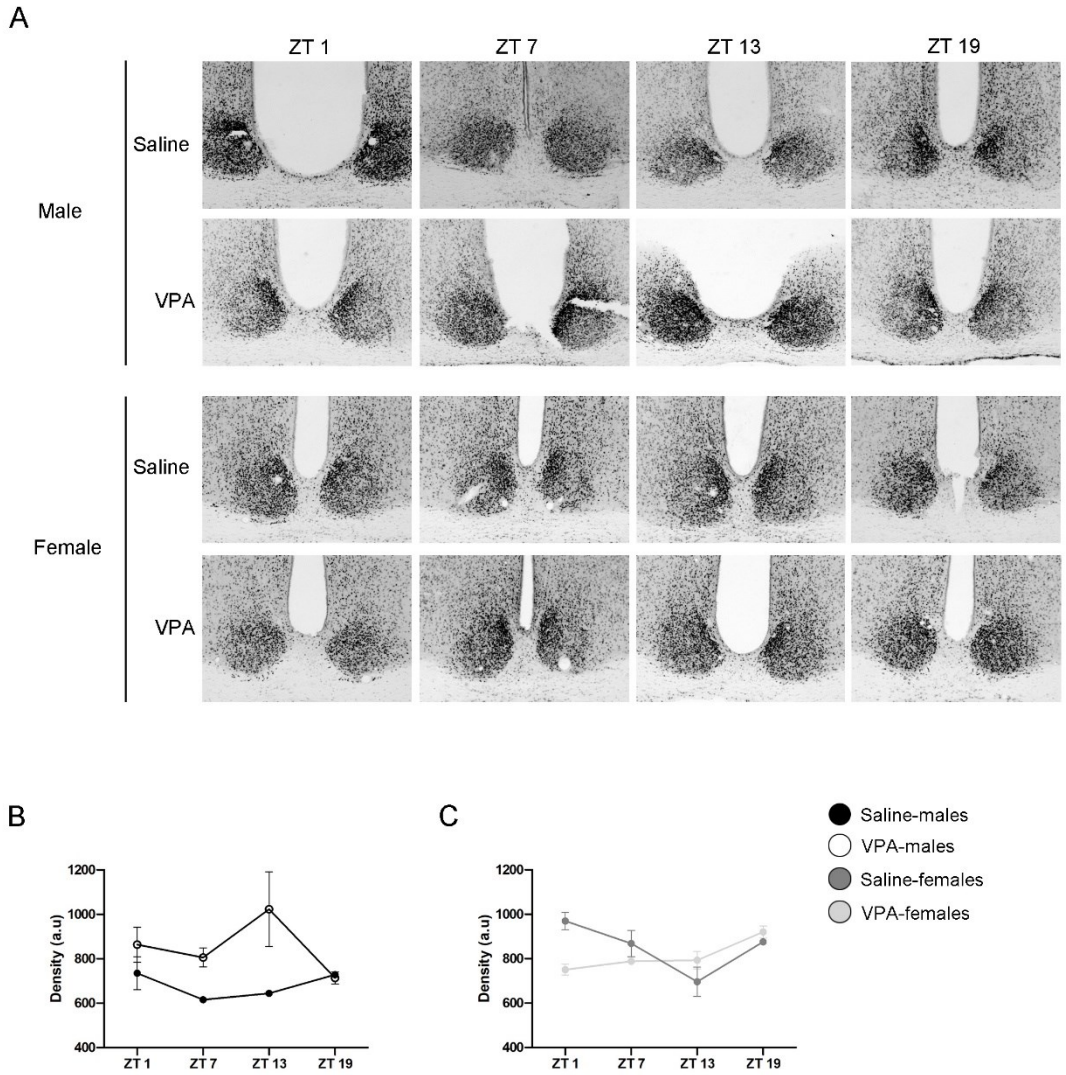


Figure 6. Prenatal exposure to VPA alters the temporal dynamics of BMAL1 expression.

(A) Representative images of BMAL1 immunoreactivity within the SCN of saline and VPA-exposed females (upper panels) and males (lower panels) across a 24h period (ZT 1, 7, 13, 19). **(B)** BMAL1 expression within the SCN of VPA-exposed males shows anti-phasic properties (two-way ANOVA, significant main effect of treatment. $F(1, 14) = 15.10$, $p = 0.0016$). Planned comparisons revealed a significant difference at ZT 13 between groups ($p = 0.0044$). **(C)** Temporal BMAL1 expression within the SCN of VPA-exposed females is altered compared to saline-exposed females (two-way ANOVA, significant interaction of treatment x time. $F(3,16)= 6.227$, $p = 0.0053$). Planned comparisons revealed a significant difference in BMAL1 expression at ZT 1 between groups ($p = 0.0053$). Data were analyzed using a two-way ANOVA and a Bonferroni planned comparison of time points.

VPA animals display altered temporal profiles of CORT

Cortisol in humans and corticosterone (CORT) in rodents display distinct temporal secretion profiles that are largely under the control of the master clock (Spiga et al., 2014). Cortisol secretion in humans' peaks in the morning hours shortly after awakening, declines rapidly over the course of the morning and finally decreases to its lowest point during the evening. However, in rodents the peak of CORT secretion occurs near the onset of the active phase. Since VPA-exposed males display circadian disturbances at both the behavioural and molecular level, we sought to determine whether downstream physiological functioning under the control of the SCN clock was also disturbed. An enzyme-linked immunosorbent assay (ELISA) was conducted for CORT in plasma samples taken at four timepoints over 24 hours. Similar to the results obtained from BMAL1 expression, CORT secretion in VPA-exposed males showed a marked difference in its daily secretion profiles (interaction effect, $F(1, 13) = 14.35$, $p = 0.0023$), peaking hours after the onset of the dark phase (ZT 19), whereas peak expression was seen at the beginning of the dark phase (ZT 13) in controls. Moreover, the secretion of CORT appears to be elevated in VPA-exposed males. However, in VPA-exposed females, no significant differences in secretion level, or temporal profiles were detected (n.s, $F(3,15) = 0.7024$), despite the observed alterations within the core clockwork.

Figure 7.

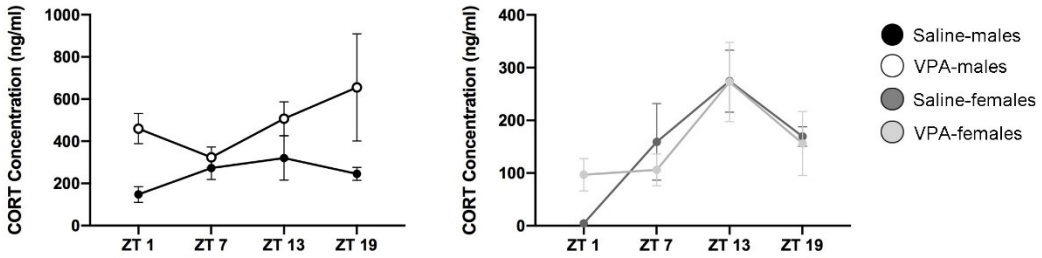


Figure 7. Altered temporal secretion profiles of CORT in VPA-exposed males.

(A) Plasma CORT levels for saline and VPA-exposed males and **(B)** females, under standard 12:12 LD conditions, collected over a 24h period (ZT 1, 7, 13, 19). Prenatal exposure to VPA alters secretion of CORT (pg/ml) in males, but not females (two-way ANOVA, significant main effect of treatment, $F(1,13) = 14.35$, $p = 0.0023$. Planned comparisons revealed a significant difference in CORT secretion at ZT 19 between groups (saline: M = 1251, VPA: M = 3281, $p = 0.0321$). Means ± SEM are shown. Data were analyzed using a two-way ANOVA and a Bonferroni planned comparison of time points.

Discussion

Here, we have provided several lines of evidence that demonstrate that VPA-induced animal model of Autism Spectrum Disorder shows circadian dysregulation of behaviour and physiology, likely through aberrant photic entrainment. We found that VPA-exposed males, but not females, demonstrate unstable and diminished output from the central circadian clock under baseline conditions. However, under constant conditions (DD) we observe no changes in the free-running period of these animals, albeit the fact that output strength of the clock remains lessened in DD. These results suggest that alterations in these animals are driven, in part, through abnormal integration of photic information. To further this point, under constant light conditions (LL) we observe, in both males and females exposed to VPA, an increase in the number of days needed to achieve arrhythmicity and an attenuated negative masking response. In response to an Aschoff type-1 light pulse, VPA-exposed animals also demonstrate an attenuated phase shift response when compared to controls. Taken together, these results suggest the existence of a diminished central circadian oscillator in VPA-exposed animals, in which decreased photic entrainment capacity further drives instability of behavioural rhythms under baseline and LL conditions.

We have demonstrated that circadian alterations in VPA-animals occur independently of retinal dysfunction. Exposure to VPA *in utero* has no effect on the organization of retinal layers, nor on the integrity of the retinohypothalamic tract, responsible for relaying photic information from the retina to the SCN (Berson et al., 2002). Melanopsin, a specialized photopigment that aids in the depolarization of retinal ganglion cells in response to short-wavelength irradiation (Hattar et al., 2006), was found to be present and did not differ in expression levels when compared to controls. We have also reported that local neuronal responses, examined through c-FOS

expression within the SCN in response to a 1-hour LP at CT 15, did not differ between groups. Collectively, these data suggests that retinal dysfunction, either at the level of the retina itself or through its connection to the SCN, does not drive circadian alterations in VPA-animals. Moreover, since localized responses to the 1-hour LP in VPA animals did not differ between groups, we propose a downstream mechanism to be responsible for the disturbances seen in these animals.

Input pathways that allow environmental stimuli to reset the clock, the master pacemaker, and output pathways that regulate physiological and behavioural processes are the necessary components that govern circadian rhythmicity. Global loss of *Bmal1* elicits an immediate loss of rhythmic behaviours in DD and decreased entrainment capacity under 12:12 conditions, indicating that *Bmal1* is necessary for the generation and maintenance of normal circadian behaviour (Bunger et al., 2000). Interestingly, BMAL1 not only showed markedly different temporal dynamics in comparison to saline-treated animals, but also showed increased expression profiles. While we have determined that the input pathway to the SCN remains intact, these findings suggest alterations within the core-clockwork itself.

SCN output signals are diverse, including humoral output and neuronal projections emanating from the SCN to distinct hypothalamic and thalamic regions (Inouye & Kawamura, 1979; Saper et al., 2005; Silver et al., 1996). However, the full mechanistic extent by which the SCN exerts its control over downstream physiological rhythmicity is not entirely known. One key output of the master clock is the cyclical nature of the spontaneous firing rate of SCN neuronal ensembles (SFR), which encodes solar time through increased neuronal firing during the circadian day, and

decreased activity during the circadian night. Downstream rhythmic behaviour is thought to be driven by the SCN's SFR, since behavioural cycles are tightly linked to rhythmic changes in SFR (Houben et al., 2014). However, changes to the SFR are also known to reset the phase of the TTFL, highlighting the reciprocal nature of input/output influences on rhythmicity of the molecular clockwork (Jones et al., 2015). Whether alterations in SFR of VPA exposed animals are present and contributed to disorganized circadian behaviour in VPA-exposed animals is currently unknown. Additionally, VPA has been shown to alter neuronal excitatory/inhibitory balances, increasing glutamatergic tone, and decreasing GABAergic neuronal development (Fukuchi et al., 2009; Kim et al., 2014). Communication between neurons within the SCN is largely GABAergic (R. Y. Moore & Speh, 1993), thus a shift in the excitatory/inhibitory balance within the SCN may also contribute to alterations in rhythmic behaviour and clock-gene expression, although this remains to be investigated.

While cortisol secretion in humans' peaks shortly after wakening, peak cortisol secretion in children with ASD has been shown to drift later relative to the LD cycle and is often found in increased concentrations, particularly during the evening hours (Corbett et al., 2008; Richdale & Prior, 1992). Elevated trough concentrations of cortisol have been associated with chronic stress, depression, decreased responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis, and adverse metabolic consequences (Dallman et al., 2000). Corticosterone secretion – a downstream physiological marker under circadian control – is also altered in VPA-animals and resembles the abnormal cortisol secretion seen in ASD children, demonstrating a much later peak time relative to the LD cycle. Moreover, plasma CORT was found to be elevated especially in relation to the nadir of controls, suggesting alterations within the HPA-axis in these animals. While the HPA-

axis is involved in stress responses in mammals, crosstalk between the HPA axis and the circadian clock has been well documented (Rao & Androulakis, 2019). In part, the SCN exerts circadian control of glucocorticoid (GC) secretion by relaying photic information directly to the hypothalamic paraventricular nucleus, a region important in the GC secretion cascade (Kalsbeek et al., 2012). However, to achieve a state of internal synchrony, appropriate entrainment between the SCN and peripheral tissues is thought to occur partially through GC rhythmicity. Robust clock-gene expression in peripheral tissues is driven in part by rhythmic GC secretion and is dependent on the presence of a functional GC-receptor, highlighting the bi-directional relationship between both systems (Balsalobre et al., 2000). Chronic circadian misalignment between the external light-dark cycle and the SCN and consequently, between the SCN and peripheral tissues, has been associated with a host of adverse metabolic outcomes and alterations in neuronal complexity in brain regions involved in higher-order functioning (Karatsoreos, Bhagat, et al., 2011). Clearly, the interaction between both systems not only affects downstream physiological functions, but also the adaptive capacity to modulate physiology and behaviour in the face of stressful situations, all of which have been shown to be altered in ASD individuals.

ASD is found to occur at much higher rates in males than in females, although the biological origins of this discrepancy are still unclear. While there are many theories that aim to explain the presence of this sex bias, the proposition that many factors including risk genes and environmental insults, interact to increase the risk of ASD development in males, remains common to most hypotheses (Ferri et al., 2018) . Recent findings within the field of ASD also delineate the complicated nature of studying sexually dimorphic behaviour in ASD, as it has been proposed that many females with ASD go undiagnosed due to a male-conceptualized model

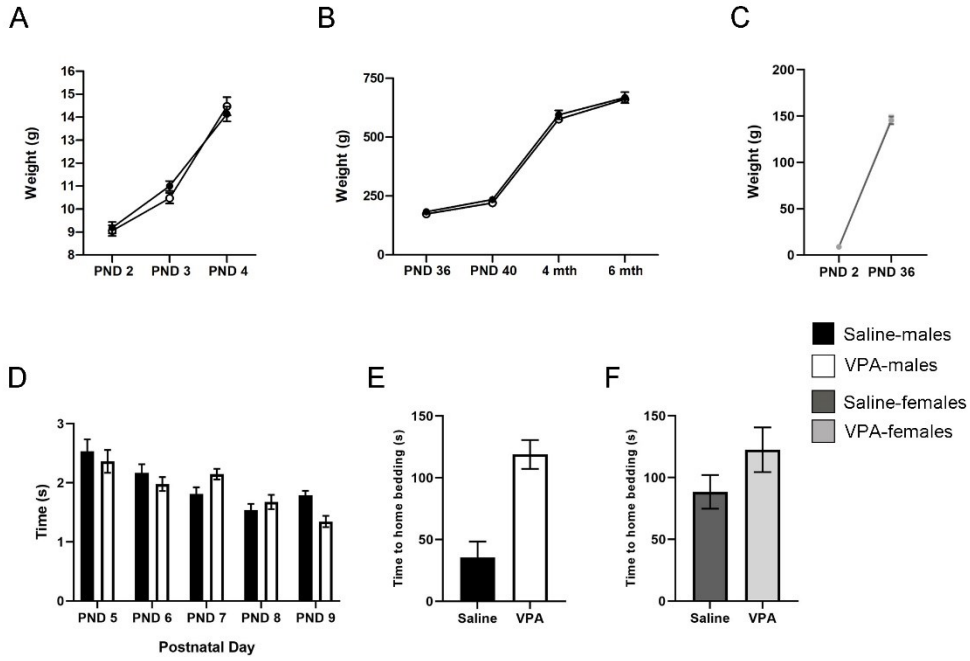
of ASD behaviour, thereby overestimating the male: female ASD ratio (Kreiser & White, 2014). Although understanding this sex bias in ASD is complicated by both biological and sociological factors, it remains an important factor in understanding the underlying biology of ASD. Moreover, there are known differences in circadian behaviour between the sexes both in humans and laboratory animals. Gonadal hormones influence the intrinsic free-running period in rats, but it is unknown if their effect is exerted at the level of the central clock, or on downstream regions which govern locomotor behaviour (Albers, 1981; Schull et al., 1989). In our study, VPA-exposed females did not display abnormal behavioural rhythmicity under 12:12 conditions but displayed overt circadian alterations under challenge conditions. Moreover, they demonstrated an attenuated phase-shifting response to an Aschoff-type 1 light pulse, indicating decreased plasticity of the central pacemaker. Despite normal behavioural rhythmicity under 12:12 conditions, the daily profile of BMAL1 expression within the SCN demonstrated abnormal temporal dynamics, suggesting a sexually dimorphic downstream compensatory mechanism that regulates normal rhythmic locomotor behaviour under baseline conditions.

Characteristics of circadian disorders include difficulty awakening at an appropriate time, difficulty initiating and maintaining sleep, and decreased alertness during the day; all of which are common complaints in ASD individuals. Key challenges surrounding data collection in children with ASD and reliance on parental reports highlight the suitability of the VPA model, which allows for further understanding of the biological mechanisms that underlie alterations in these individuals. Notably, we have demonstrated the existence of circadian alterations in our model highly reminiscent of sleep-wake cycle disturbances seen in ASD populations. Our results indicate that while input pathways remain intact and functional, the central pacemaker and

possibly downstream output pathways are involved in the etiology of circadian disturbances in this ASD model. Future experiments investigating the electrical output of SCN neuronal ensembles would help elucidate whether known alterations in the excitatory/inhibitory balance contributes to diminished and unstable output from the master clock. Our results and the necessity of future investigations are critical, as they may lead to new therapeutic approaches for treating circadian disorders concomitantly observed with a variety of neurodevelopmental disorders.

Supplementary Information

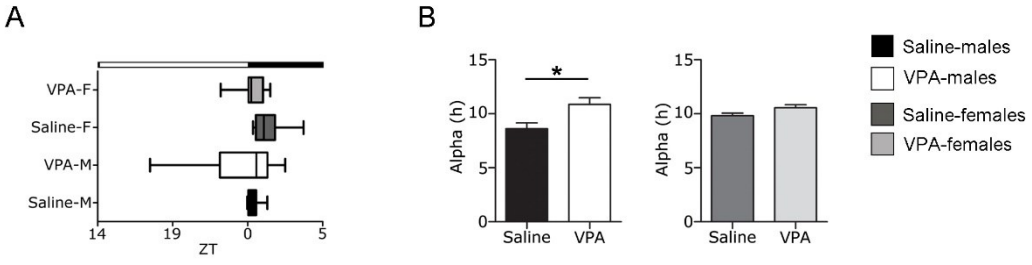
Supplemental Figure 1.



Supplemental Figure 1. General development and health in saline and VPA-exposed animals.

(A) Weight (g) of saline (n = 16) and VPA-exposed (n= 17) male pups shortly after birth on PND 2, 3 and 4 (two-way ANOVA, significant main effect of time, $F(2,91) = 175.4$, $p < 0.0001$). **(B)** Weight (g) of saline (n = 5) and VPA-exposed male pups (n = 6) after weaning and into adulthood (two-way ANOVA, significant main effect of time $F(3,36) = 761.4$, $p < 0.0001$). **(C)** Postnatal and post-weaned weight of saline (n = 13) and VPA (n = 24) exposed animals (two-way ANOVA, significant main effect of time, $F(1, 56) = 8269$, $p < 0.0001$). **(D)** Righting reflex task to assess motor development on PND 5, 6, 7, 8, 9 in saline and VPA exposed animals. Time to right themselves ventrally was recorded in seconds (two-way ANOVA, significant main effect of time, $F(4,284) = 13.02$, $p < 0.0001$). **(E)** Time to engage with home bedding (s) in saline (n = 18) and VPA (n = 13) exposed animals (saline: M = 35.50 ±12.86, VPA: M = 118.9 ±11.66, $p = 0.0005$). **(F)** Time to engage with home bedding (s) in saline (n = 10) and VPA (n = 31) exposed animals. No significant differences were detected. Means ± SEM are shown. Data was analyzed using a two-way ANOVA, or an unpaired two-tailed t-test.

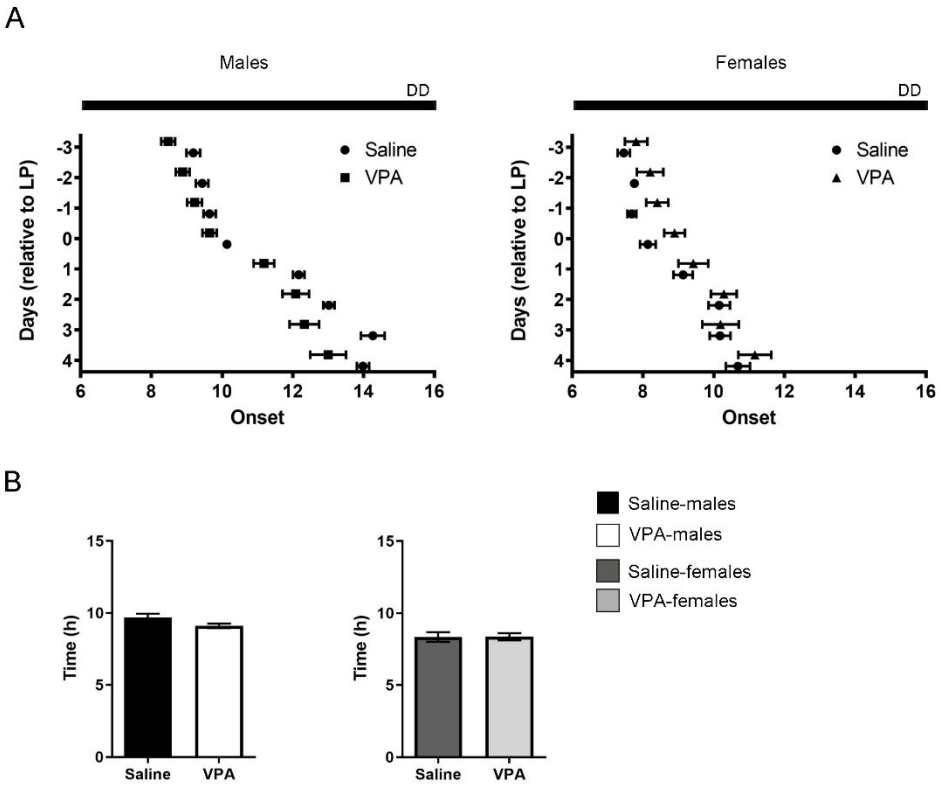
Supplemental Figure 2.



Supplemental Figure 2. Behavioural rhythms are diminished in VPA-exposed males under constant dark (DD) conditions.

(A) Onset (h) analysis for saline and VPA-exposed animals. **(B)** Alpha (h) is longer in VPA-exposed males under 12h:12h conditions (saline: M = 8.623 ±0.5374, VPA: M = 10.86 ±0.6222, $p = 0.0149$), but not in VPA-exposed females (right). Means ± SEM are shown. Data was analyzed using a one-way ANOVA, or an unpaired two-tailed t-test.

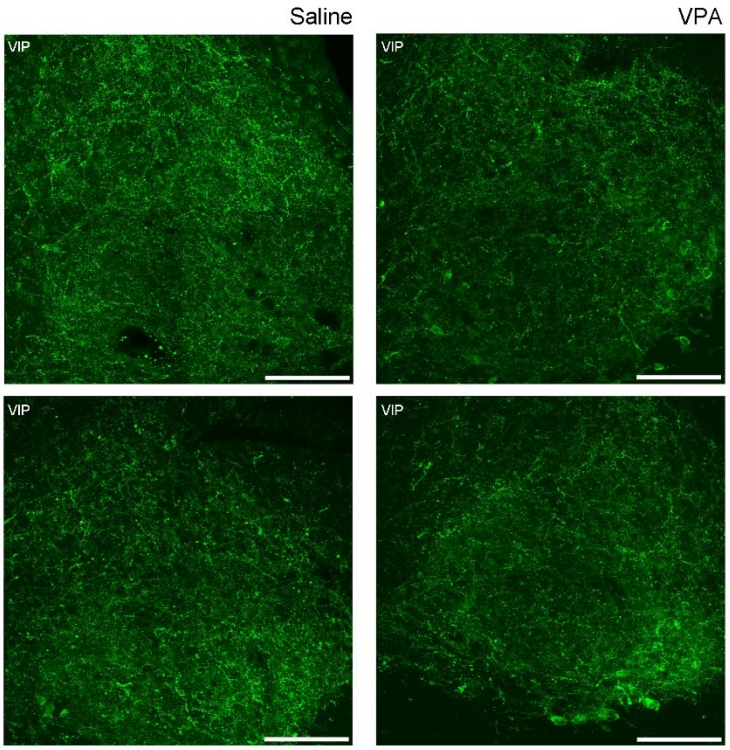
Supplemental Figure 3.



Supplemental Figure 3. Altered responses to an Aschoff type-1 light pulse in VPA treated animals.

(A) Group mean onsets prior to the light pulse given at CT 15 (denoted as day 0) and after. Note that VPA exposed males and females show an attenuated phase shifting response to the light pulse. **(B)** Alpha (h) in DD is not different between groups.

Supplemental Figure 4.



Supplemental Figure 4. The distribution of a key neuropeptide, VIP, does not differ in the SCN of saline or VPA-exposed animals.

(A) Mid-coronal SCN slices from saline (left panels) and VPA (right panels) exposed animals (60x). Green immunohistochemical staining represents with an anti-vasoactive intestinal polypeptide (VIP) immunoreactivity. Note that VIP is localized within the processes in the dorsal SCN.

CHAPTER 2

Valproic acid (VPA) alters circadian clock-gene BMAL1 expression in Autism Spectrum Disorder (ASD)-related brain structures.

Sarah Ferraro, Nuria de Zavalia, Shimon Amir

Author Contributions :

- Conceptualization: Sarah Ferraro, Shimon Amir.
- Performed research: Sarah Ferraro, assisted by Nuria de Zavalia.
- Data Analysis: Sarah Ferraro
- Visualization: Sarah Ferraro
- Manuscript production: Sarah Ferraro, editing by Shimon Amir

Abstract

Circadian oscillators downstream of the suprachiasmatic nucleus (SCN) have been observed in numerous brain structures and are suggested to play a role in the regulation of neuronal functions such as mood, neurohormonal output and cognitive behaviours. Reports of altered clock-gene rhythmicity in specific brain regions have been associated with the emergence of abnormal behaviour and linked to various neuropsychiatric disorders. The present study seeks to determine whether alterations in the daily expression profile of the core clock-gene BMAL-1, is altered in extra-SCN regions implicated in the pathogenesis of Autism Spectrum Disorder (ASD) post *in utero* exposure to valproic acid (VPA). Prenatal exposure to VPA has been well-validated as an animal model of ASD in rodents, resulting in morphological and behavioural alterations akin to human ASD populations. Brain tissue from male and female animals exposed to either VPA or saline *in utero* was collected over a 24h period at a 6h sampling rate. The expression of BMAL1 was quantified in brain sections containing the medial prefrontal cortex, anterior cingulate cortex, dorsal striatum, lateral and basolateral nuclei of the amygdala and the lateral habenula after immunohistochemical staining. These regions were selected due to their roles in social-emotional processing, motivation, anxiety-like behaviours, and modulation of monoaminergic transmission; all of which are disturbed in individuals with ASD. Here, we find an effect of VPA-exposure on BMAL1 expression in almost all regions except for the lateral habenula in males. Moreover, we also find this effect in VPA-exposed females, albeit only within the dorsal striatum, lateral amygdala and lateral habenula, potentially suggesting sexually dimorphic effects on the expression of BMAL-1 in specific structures post *in utero* exposure to VPA. Finally, we contextualize these findings in light of emerging data in the fields of ASD and circadian biology

and suggest a possible role for BMAL1 in the manifestation of ASD-behaviours in VPA-exposed animals.

Introduction

The circadian system is an evolutionarily conserved biological prediction tool that synchronizes physiological and behavioural processes with the earth's light-dark cycle, optimizing organismal function and survival. These biological oscillations are generated and maintained through a hierarchical network in which the suprachiasmatic nucleus (SCN) of the hypothalamus is the master autonomous pacemaker (Stephan & Zucker, 1972; Welsh et al., 2010) . At the molecular level, circadian timekeeping is achieved through the expression of autoregulatory feedback loops with transcriptional, translational, and post-translational elements (Takahashi, 2017). The heterodimerization of two core clock-genes, *Clock* and *Bmal1*, promote transcription of their own inhibitors, the *Period* and *Cryptochrome* gene families, whose protein products act to supress CLOCK/BMAL1 transcriptional activity (Hastings et al., 2018). Ubiquitination and subsequent degradation of these gene families alleviates the inhibitory effect on CLOCK/BMAL1, allowing the cycle to reinitiate (Gallego & Virshup, 2007; Partch et al., 2006).

The presence of oscillating clock-genes downstream of the SCN has been observed in both neural and non-neural tissues including the liver, adrenal glands, olfactory bulb, amygdala and habenula, among others (Abe et al., 2002; Bittman et al., 2003; Granados-fuentes et al., 2012; Harbour et al., 2013; Kornmann et al., 2007; Lamont et al., 2005). The maintenance of synchrony within these regions is largely dependent on the presence of a functional master pacemaker, designating several extra-SCN clocks as "subordinate oscillators". However, the phase and robustness of clock-gene expression in peripheral tissues differs between regions and in relation to the SCN (Abe et al., 2002; Chun et al., 2015). In this role, the SCN coordinates and synchronizes rhythms in downstream peripheral clocks, achieving a state of synchrony among oscillators through its humoral and neuronal output signals (Inouye & Kawamura, 1979; Silver et

al., 1996). Evidence exists to suggest that cellular clocks within the central nervous system (CNS) regulate specific neuronal functions including mood, cognitive behaviours and neurohumoral output, although the functional relevance of every rhythmic extra-SCN region has not yet been ascertained (Begemann et al., 2020).

Autism Spectrum Disorder (ASD) is a heterogenous group of disorders with similar and overlapping symptomology but differential clinical progression and severity. Currently, ASD affects 1 in 66 individuals in the United States and Canada and is observed at a male: female ratio of 4:1 (Maenner et al., 2020). Behavioural variances in social-emotional communication and repetitive patterns of behaviour represent the core clinical features of the disorder. In specific, difficulties engaging in nonverbal behaviours used for communication, developing relationships, and reciprocating social-emotional approaches are typically observed in individuals with ASD. Moreover, stereotyped speech or movements, adherence to routines and insistence on sameness underlie aspects of the behavioural inflexibility seen in the disorder (American Psychiatric Association, 2013). However, ASD is found to be highly concomitant with other neurophysiological and psychiatric comorbidities, including depression, attention deficit-hyperactivity disorder, anxiety, epilepsy and circadian dysfunction (LoVullo & Matson, 2009; Masi et al., 2006). Between 40-80% of children with ASD experience sleep-wake cycle alterations which, in contrast to other neurodevelopmental disorders, occur independently of intellectual dysfunction and negatively impacts daytime behaviour, highlighting the pervasive nature of sleep and circadian disturbances on overall functioning (Cohen et al., 2014; Cotton & Richdale, 2006; Patzold et al., 1998; Richdale & Schreck, 2009). Genome-wide association studies implicating genes within the core molecular clockwork, clinical findings and results from

animal models suggest that in part, the circadian timing system is responsible for mediating sleep-wake cycle alterations in ASD (Delorme et al., 2021; Richdale & Prior, 1992; Veatch et al., 2015; Yang et al., 2016).

Neuroimaging, post-mortem neuropathological studies, and clinical assessments have aimed to characterize the neuroanatomical basis of ASD-behaviours. Consistently, reports of abnormal brain development during early life have been observed, followed by slowed or arrested growth later in childhood (Courchesne et al., 2007). Brain regions reliably reported across studies include the cerebellum, limbic structures, and frontal lobe and are involved in social, communication, cognitive and motor abilities; all of which are affected to some degree in ASD (Minshew & Keller, 2010; Pardo & Eberhart, 2007). Interestingly, there is considerable overlap between regions believed to be involved in the generation of ASD behaviours and regions in which rhythmic clock-gene expression has been detected. Therefore, it stands to reason that alterations within extra-SCN clocks may highlight a putative basis for the behavioural abnormalities seen in ASD.

Previously, we have reported the existence of an unstable and diminished central oscillator in a valproic-acid (VPA) animal model of ASD (Ferraro et al., 2021a). VPA is commonly prescribed for the management of epilepsy and bipolar disorder, however treatment with VPA during gestation is associated with increased fetal risks, including a higher incidence of ASD diagnosis later in childhood (Clayton-Smith & Donnai, 1995). Rodents exposed to VPA prenatally demonstrate behavioural alterations common to individuals with ASD, including reduced social engagement and exploration, decreased social preference, and increased repetitive behaviours

(Favre et al., 2013; Roullet et al., 2010; Schneider & Przewłocki, 2005). Furthermore, VPA-exposed rodents also display behaviours akin to the observed comorbidities in ASD, including decreased seizure threshold, hypo and hyper-sensitivity to sensory stimuli, and increased anxiety and depressive-like behaviours (Kataoka et al., 2013; Markram et al., 2008). Thus, the VPA model offers the opportunity for the study of ASD-like behaviours and its associated comorbidities.

In the present study, we sought to characterize the daily expression profiles of BMAL1 in brain regions implicated in the generation of ASD-like behaviours in the VPA-model. Our recent work has characterized alterations in behavioural rhythmicity and in the temporal expression of BMAL1 within the SCN of animals exposed to VPA prenatally, demonstrating changes within the master pacemaker itself (Ferraro et al., 2021a). Given the abundance of research highlighting the SCN's role in coordinating and synchronizing rhythms within peripheral tissues and extra-SCN regions, we predict that the daily expression of BMAL1 within brain regions implicated in ASD pathogenesis also demonstrates altered rhythmicity. Further investigations into the role that circadian dysfunction plays in the generation or exacerbation of symptoms is warranted, as addressing the heterogeneity of ASD behaviours and comorbid symptoms remains paramount.

Materials and Methods

Generation of the VPA-model

Time-pregnant Wistar female dams were obtained from Charles River Canada (Charles River, St-Constant, QC) on gestational day (GD) ten. Dams were placed under a standard 12h:12h light/dark (LD) cycle, individually housed with free access to food and water. Intraperitoneal

injection of VPA (500 mg/kg, 1 mg/kg) or a 0.9% saline solution (1 mg/kg) was administered on GD 12. Gestation occurred without any other significant manipulations and the resulting litters were not culled. Weaning was recorded on postnatal day (PND) 23. All procedures conducted in this experiment followed the guidelines set forth by the Canadian Council of Animal Care and by the Animal Care Committee of Concordia University.

Animals and Housing

VPA and saline-exposed male (N= 24) and female (N=24) animals were individually housed in sound-attenuated isolation chambers equipped with running-wheel apparatuses and computer controlled light sources between 8-10 weeks of age. Animals were kept under a 12h:12h LD cycle for approximately 3-weeks to monitor baseline activity under standard conditions. Running-wheel activity was recorded using VitalView software (Mini-Mitter, Starr Life Sciences Corp., Oakmont, PA, USA) and the graphical representation of activity was constructed using Actiview software (Mini Mitter).

Tissue preparation and Immunohistochemistry

To investigate the daily profile of BMAL1 expression in structures implicated in ASD pathogenesis, saline and VPA-exposed animals were perfused and sacrificed at four time points across a 24-h period (Zeitgeber time (ZT) 1, ZT 7, ZT 13, and ZT 19. ZT 0 denotes time of lights on and ZT 12 denotes time of lights off). Perfusions, tissue harvesting and immunohistochemical analysis were conducted as previously reported (Ferraro et al., 2021a). In brief, 11-13 weeks old animals were transcardially perfused with cold saline (0.9% NaCl), followed by a cold paraformaldehyde solution (4 % in a 0.1 M phosphate buffer, pH 7.3). Brain tissue was

subsequently extracted, sections were obtained (coronal, 50 μm) using a Leica vibratome and stored for future use. The collected tissue sections included the anterior cingulate cortex (ACC), medial prefrontal cortex (mPFC), dorsal striatum (DS), amygdala, and habenular regions. Regions of interest were located using "The Rat Brain, in Stereotaxic Coordinates" (Paxinos & Watson, 1997).

Free-floating immunohistochemistry for BMAL1 (1:30,000, polyclonal made in rabbit, Novus Biologicals #NB100-2288, Littleton, CO, USA) was performed by incubating tissue sections in a primary solution containing the BMAL1 antibody and 2% normal goat serum in a 5% milk-TBST solution (TBST: 0.3% Triton, 50 mM Trizma buffer, 0.9% saline) for 48h at 4°C. Sections were then rinsed in TBS and incubated in a secondary solution, containing biotinylated anti-rabbit IgG, raised in goat (1:200, Vector Laboratories, Burlington, ON, Canada) for 1h at 4°C. Finally, sections were incubated in a tertiary Avidin-Biotin-Peroxidase solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlington, ON, Canada) for 2h at at 4°C. Staining of immunoreactive cells was achieved through a 0.5 % DAB, 0.01 % H_2O_2 and 8 % $NiCl_2$ solution, rinsed for 10 minutes and then mounted onto gel-coated slides. Serial alcohol dehydration and Citrisolv clearing (Fisher Scientific, Pittsburgh, PA, USA) were performed and slides were coverslipped. Sections were identified under a Leica, DMR light microscope. Image capture was achieved by using a Sony XC-77 camera, Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA) and analyzed Image J (http://imagej.nih.gov).

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism 9 software (GraphPad software LLC, San Diego, CA, USA). Two-way analysis of variance (ANOVA) without repeated measures was performed to compare differences between groups. Planned comparisons were made for significant interactions using the Bonferroni correction unless stated otherwise. Alpha was sent at $P<0.05$. Statistical descriptions and findings are given in the text and summarized in **Table 1** and **Table 2**.

Results

Rhythmic clock-gene expression has been well documented within the rodent cerebral cortex and is dependent on a functional SCN pacemaker (Rath et al., 2013). The medial prefrontal cortex (mPFC) is region intimately involved with higher-order processes, including sensory and motor integration and mentalizing capacities. Mentalizing, or the capacity to infer the mental states of others, is known to be severely affected in ASD. BMAL1 expression was found to be rhythmic in control males, however temporal dynamics differed between groups (significant interaction $F_{(3, 16)} = 8.030$, $p = 0.0017$) (Fig.1a). Planned comparisons revealed increased expression at ZT 19 in controls compared to VPA-exposed males (M= 361.9 vs. VPA M= 191.4, $p = 0.0023$). Moreover, no rhythmicity was detected within the mPFC of VPA-exposed males, suggesting a blunting of the rhythmic expression of BMAL1 within this region.

The anterior cingulate cortex (ACC) is a limbic region responsible for the regulation of a myriad of higher-order functions, including impulse control, cognitive and emotional processing, and executive functioning. Alterations in cell morphology and decreased $GABA_A$ receptor density

within the ACC have been described in post-mortem ASD individuals and are believed to mediate decreased social-emotional and verbal processing in ASD (Oblak et al., 2009; Simms et al., 2009). Within the ACC, the expression of BMAL1 across time between control and VPA-exposed males also differed (two-way ANOVA, significant interaction, $F_{(3,16)} = 4.630$, $p = 0.0163$) (Fig. 1b). Planned comparisons revealed maximal expression of BMAL1 at ZT 19 in controls (M= 364.7), in contrast to VPA-exposed males in which ZT 19 corresponded to the nadir of expression (M= 176.5 $p < 0.0001$), suggesting an inverted rhythm within this region. BMAL1 expression within the ACC in saline and VPA-exposed females was not found to be rhythmic and no significant differences between groups across time were detected ($F_{(3,16)} = 2.272$, $p = 0.1194$) (Fig. 2a).

Topographically, insistence on sameness, repetitive motor movements or inflexible adherence to specific routines represent a core feature of ASD (Turner, 1999). However, research investigating the neurobiological underpinnings of these behaviours remain sparse. Recently, the striatum has been implicated in the generation of stereotypic behaviours in ASD as increased striatal volume, particularly in the caudate nucleus of the dorsal striatum (DS), has been positively correlated with an increase in repetitive behaviours (Hollander et al., 2005; Langen et al., 2009). Expression of BMAL1 within the DS of saline and VPA-exposed males showed altered temporal dynamics (two-way ANOVA, significant interaction, $F_{(3,16)} = 4.802$, $p = 0.0143$) (Fig. 1c). We observed a blunted BMAL1 rhythm across time in VPA-exposed males, while rhythmicity was detected in control males. Interestingly, BMAL1 expression did not appear to be blunted in VPA-exposed females and differed in comparison to controls (two-way ANOVA, significant interaction, $F_{(3, 15)} = 4.284$, $p = 0.0226$) (Fig. 2b). Specifically, expression of BMAL1

within the striatum appears to be shifted in VPA-exposed females in comparison to controls, with peak expression occurring at ZT 19 (controls, M= 269.6 vs. VPA, M= 402.9, $p = 0.0023$).

One region of the limbic forebrain important for the regulation of social behaviour, memory and emotional processing is the amygdala. Notably, this region is a site of substantial overgrowth in young children with ASD and is implicated in the decreased social behaviours and exacerbated anxiety responses commonly observed in ASD individuals (Courchesne et al., 2007). While the amygdala is composed of several distinct neuroanatomical nuclei, two regions, the basolateral nuclei (BLA) and the lateral nuclei (LA), were selected to investigate BMAL1 rhythms in the VPA model due to their roles in sensory processing and generation of anxiety-like behaviours (Ledoux, 2004). Rhythmic expression of BMAL1 within the BLA was detected in both saline and VPA-exposed males although marked differences in the temporal profile were observed between groups (two-way ANOVA, significant interaction, $F_{(3,16)} = 16.56$ $p < 0.0001$) (Fig. 1e). Planned comparisons revealed a significant difference in BMAL1 expression at ZT 13 ($p = 0.0032$), which corresponded to the time of peak expression in controls (M=181.1), in contrast to VPA-exposed males, which represented the nadir of expression (M= 103.5). Moreover, peak expression in VPA-exposed males occurred towards the end of the dark phase (ZT 19) (controls, M= 146 vs. VPA, M= 205.6, $p = 0.0238$), a few hours before the nadir of expression for control males, suggesting a near inversion of the expression rhythm between groups. Similarly, expression of BMAL1 within the LA of VPA-exposed males also showed altered temporal dynamics (two-way ANOVA, significant interaction, $F_{(3,16)} = 5.024$ $p = 0.0121$) (Fig. 1d). Planned multiple comparisons revealed a significant difference in BMAL1 expression at the beginning of the light phase (ZT 1) ($p = 0.0128$), which corresponded to the time of maximal

expression in VPA-exposed males (M=226.2), in contrast to the nadir of expression for control males (M=111.8). Furthermore, despite the appearance of a blunted BMAL1 rhythm in the BLA of VPA-exposed females, no significant differences in the temporal expression of BMAL1 were detected in comparison to female controls ($F_{(3, 15)} = 1.824$ $p = 0.1860$) (Fig. 2d). However, BMAL1 rhythms within the LA of VPA-exposed females appear to be phase advanced (two-way ANOVA, significant interaction, $F_{(3, 14)} = 25.77$ $p < 0.0001$), as peak expression occurred near the beginning of the dark phase (ZT 13) in controls (controls, M= 320.4 vs. VPA, M= 235.1, $p = 0.0068$), in comparison to the middle of the light phase (ZT 7) in VPA-exposed females (controls, M=232.1 vs. VPA, M = 366.5 $p < 0.0001$) (Fig. 2c). Taken together, expression of BMAL1 shows differential temporal expression within the BLA and LA in VPA-exposed males and in the LA of VPA-exposed females.

The SCN sends neuronal projections to a small number of structures such as the lateral habenula (LHb) (Buijs, 1978; De Vries et al., 1981). The LHb has been of recent interest due to its role in the modulation of monoaminergic transmission, negative social processing and aggression (Flanigan et al., 2020; van Kerkhof et al., 2013). Moreover, the LHb shows properties of a self-sustained clock and has been proposed to play a role in the complex integration between the circadian system and its modulation of monoaminergic output. A significant difference in BMAL1 expression within the LHb of VPA-exposed males was detected in comparison to controls (two-way ANOVA, main effect of treatment, $F_{(1,16)} = 5.071$, $p = 0.0387$), and appeared to be dampened (Fig. 1f). In VPA-exposed females, rhythmic expression of BMAL1 appeared to be inverted in comparison to controls (two-way ANOVA, significant interaction, $F_{(3,13)} = 17.58$, $p < 0.0001$) (Fig. 2e). Planned comparisons revealed peak expression at ZT 13 in VPA-exposed

females (M= 227.4, $p < 0.0002$), which corresponded to the nadir of expression for controls (M= 55.24). A detailed statistical summary of these findings can be found in Table 1 and Table 2.

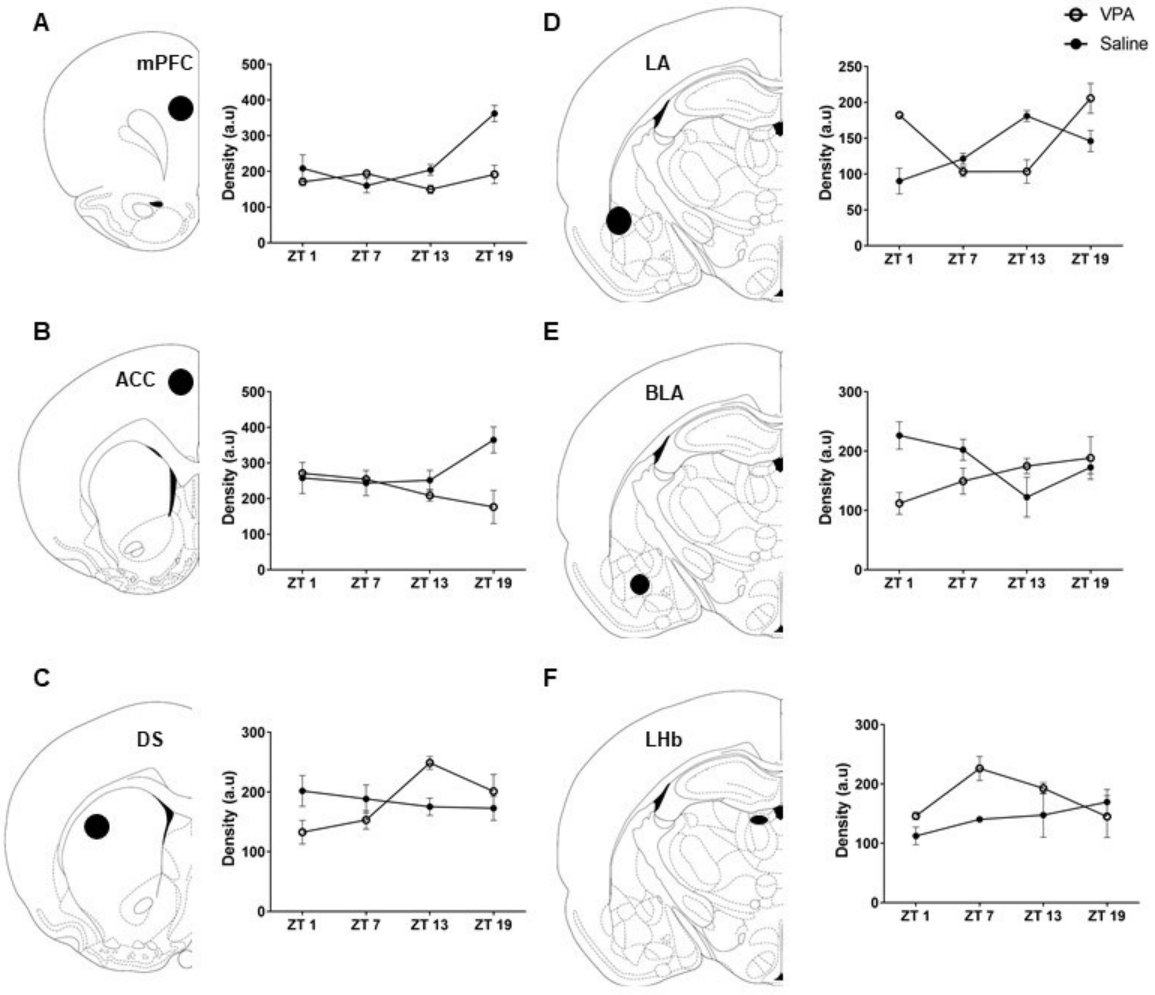


Figure 1. Prenatal exposure to VPA alters expression of BMAL1 in cortical, striatal, limbic and epithalamic structures in male rats

The effect of *in utero* VPA exposure on the diurnal expression pattern of BMAL1 in the **(A)** medial prefrontal cortex (mPFC), **(B)** anterior cingulate cortex (ACC), **(C)** dorsal striatum (DS), **(D)** lateral nuclei of the amygdala (LA), **(E)** basolateral nuclei of the amygdala (BLA), and **(F)** lateral habenula (LHb) in saline (closed circles, n=12) or VPA-exposed males (open circles, n=12) under 12h:12h conditions. Sampling time is denoted as zeitgeber time (ZT), where ZT 0 is defined as time of lights on and ZT 12 as time of lights off. Data was analyzed using a two-way analysis of variance (ANOVA) with planned comparisons for significant interactions (Bonferroni correction). Data is plotted as mean ± SEM. Statistical details can be found in **Table 1**. Black dots indicate approximate location of the region of interest.

Table 1. *In utero* exposure to VPA alters BMAL1 expression in cortical, limbic, striatial and epithalamic regions: Males

Structure	ZT	Treatment	ZT x Treatment
BLA	F(3, 16) = 7.744, P=0.0020	F(1, 16) = 2.222, ns	F(3, 16) = 16.56, P<0.0001
LA	F(3, 16) = 0.7245, ns	F(1, 16) = 2.247, ns	F(3, 16) = 5.024, P=0.0121
mPFC	F(3, 16) = 10.36, P=0.0005	F(1, 14) = 14.70, P=0.0015	F(3, 16) = 8.030, P=0.0017
ACC	F(3, 16) = 0.6823, ns	F(1, 16) = 5.555, P=0.0315	F(3, 16) = 4.4630, P=0.0163
LHb	F(3, 16) = 2.207, ns	F(1, 16) = 5.071, P = 0.0387	F(3, 16) = 2.143, ns
DS	F(3, 16) = 1.968, ns	F(1, 16) = 0.0017, ns	F(3, 16) = 4.802, P=0.0143

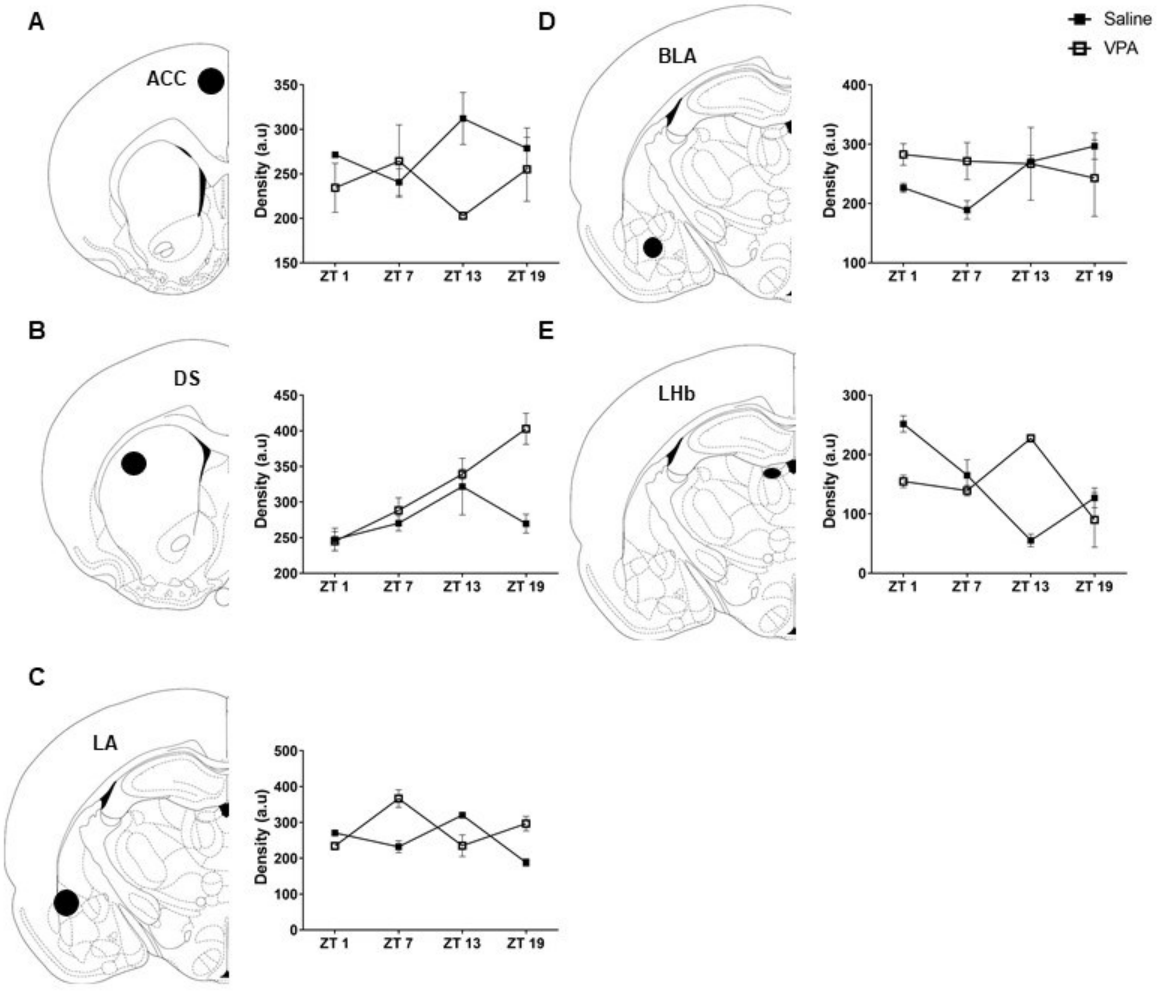


Figure 2. Prenatal exposure to VPA alters expression of BMAL1 in cortical, striatal, limbic and epithalamic structures in female rats

The effect of *in utero* VPA exposure on the diurnal expression pattern of BMAL1 in the **(A)** anterior cingulate cortex (ACC), **(B)** dorsal striatum (DS), **(C)** lateral nuclei of the amygdala (LA), **(D)** basolateral nuclei of the amygdala (BLA), and **(E)** lateral habenula (LHb) in saline (closed squares, n=12) or VPA-exposed females (open squares, n=12) under 12h:12h conditions. Sampling time is denoted as zeitgeber time (ZT), where ZT 0 is defined as time of lights on and ZT 12 as time of lights off. Data was analyzed using a two-way analysis of variance (ANOVA) with planned comparisons for significant interactions (Bonferroni correction). Data is plotted as mean ± SEM. Statistical details can be found in **Table 2**. Black dots indicate approximate location of the region of interest.

Table 2. *In utero* exposure to VPA alters BMAL1 expression in limbic, striatial and epithalamic regions: Females

Structure	ZT	Treatment	ZT x Treatment
BLA	F(3,15) = 0.6422, ns	F(1, 15) = 0.7567, ns	F(3, 15) = 1.824, ns
LA	F(3, 14) = 5.978, P=0.0077	F (1, 14) = 8.426, P=0.0116	F(3, 14) = 25.77, P<0.0001
ACC	F(3, 16) = 0.1354, ns	F(1, 16) = 4.008, ns	F(3, 16) = 2.272, ns
LHb	F(3, 13) = 8.515, P=0.0022	F(1, 13) = 0.05031, ns	F(1, 13) = 17.58, P<0.0001
DS	F(3, 15) = 9.9263, P=0.0010	F(1, 15) = 8.620, P=0.0102	F(3, 15) = 4.284, P=0.0226

Discussion

Our results demonstrate for the first time, that prenatal exposure to VPA alters BMAL1 expression within cortical, limbic and epithalamic regions implicated in the pathogenesis of ASD behaviours. Previously, we have reported altered temporal dynamics of BMAL1 expression within the SCN of VPA-exposed males and females, and demonstrated that SCN output, through analysis of locomotor activity and corticosterone (CORT) secretion, is disturbed, suggesting the presence of an unstable central oscillator (Ferraro et al., 2021a). Under normal conditions, the SCN integrates information from the environment and generates coherent rhythms among oscillators within peripheral tissues. Desynchrony between the external environment and the internal clock due to modern living conditions such as trans-meridian travel, shiftwork, and exposure to artificial light at night, have been associated with increased insulin-resistance, obesity, depression and decreased higher-order cognitive capacities, to name a few (Cuesta et al., 2021; Karatsoreos, Bhagat, et al., 2011; Legates et al., 2012; Leproult & Cauter, 2014). Thus, the maintenance of a normal phase-relationship between the external environment and the SCN, but equally between the SCN and peripheral tissues, remains critical for physiological and psychological wellbeing. The results from our study indicate that temporal synchronization among oscillators is altered in brain regions implicated in ASD pathogenesis in VPA-exposed animals and this effect differs between the sexes. Lastly, we will discuss the potential implication of our results in the context of emerging data in the fields of ASD and circadian biology.

Prenatal VPA exposure alters the expression of BMAL1 in limbic structures involved in the expression of anxiety behaviours

The LA of the amygdala receives major inputs from somatosensory, olfactory, taste, visual and auditory systems, acting as a sensory gatekeeper responsible for downstream communication to areas within the amygdalar structure, including the BLA (Ledoux, 2004). Somatosensory processing is largely disturbed in individuals with ASD, and both hyper-and-hypoactivity of the amygdala have been reported in response to visual and social stimuli in individuals with ASD (Kilroy et al., 2019). Additionally, the encoding of fear-memories and expression of anxiety-related behaviours is attributed to the functionality of the BLA and its output projections to the central nucleus of the amygdala, the striatum and the hippocampus, and have been shown to be exacerbated in both VPA-exposed rodents and in human ASD populations (Gale et al., 2004; Ledoux, 2004; Markram et al., 2008). It is important to note that many of these behaviours occur in a time-of-day dependent manner under normal conditions (Nakano et al., 2016). By extension, it is unsurprising that robust rhythms of clock-gene expression have been observed in the amygdala and are dependent on the presence of a functional SCN clock (Lamont et al., 2005). Strikingly, the expression of BMAL1 within the BLA of VPA-exposed males showed anti-phasic properties compared to controls, while VPA-exposed females did not show significant expression differences within this structure when compared to controls. However, BMAL1 expression within the LA of VPA-exposed females appeared to be phase advanced with respect to controls, demonstrating sexually dimorphic alterations in BMAL1 expression post *in utero* exposure to VPA. Altered temporal expression of BMAL1 within the BLA may contribute to the exacerbated anxiety response seen in VPA-exposed males; disturbances in the expression of BMAL1 within this structure result in the loss of the normal diurnal variation seen in anxiety-like behaviours. Consequently, these behaviours remain consistently elevated over 24 hours (Nakano et al., 2016). These findings emphasize that rhythmic BMAL1 expression is important

for the regulation of anxiety-responses and alterations in its expression lead to amplified anxiogenic responses.

Subregions of the PFC display blunted rhythms across 24h in VPA-exposed animals

Regions of the cerebral cortex, including the PFC and somatosensory cortexes have been highly implicated in ASD-behaviour, due to their roles language, decision making, social-emotional and social-cue processing (Baron-Cohen et al., 1994). Previous studies have documented the rhythmic expression of core clock-genes within neurons of the cerebral cortices, suggesting the existence of a circadian oscillator within this region. However, loss of SCN function results in dampened clock-gene rhythmicity within cortical neurons, highlighting its role as a subordinate oscillator under control of the master pacemaker (Rath et al., 2013). Intriguingly, phase-relationship dysregulation between individual clock-genes in the cerebral cortex has been documented in post-mortem tissue from patients with major depressive disorder (MDD) (Li et al., 2013). Moreover, loss of BMAL1 within cortical neurons induces the emergence of depressive-like behaviours in mice and is accompanied by reductions in norepinephrine levels within the cortex (Bering et al., 2018). These findings suggest that alterations in clock-gene expression within cortical neurons may be involved in the etiology of depressive-behaviours. In this study, we have shown blunted expression of BMAL1 in the mPFC and altered temporal dynamics within the ACC of VPA-exposed males in comparison to controls but not in VPA-exposed females. These observations highlight that the altered expression of BMAL1 previously observed in the SCN of VPA-exposed animals also affects temporal expression of BMAL1 in downstream extra-SCN oscillators. Taken together, the presence of a blunted BMAL1 rhythm within the PFC of VPA-exposed males may highlight a basis for not only the core behavioural

abnormalities seen in the VPA model, but also in the generation of comorbid depressive-like behaviours commonly seen in ASD. These findings are important, as dissecting the heterogeneity and complexity of ASD-behaviours and their comorbidities remains critical.

Alterations in striatal BMAL1 expression may mediate the dysregulation of motivated behaviours and increased stereotypic behaviours

Motor control, learning, motivation, and reward-driven behaviours are partly mediated by a large subcortical structure known as the striatum. Developmental abnormalities within the striatum have been reported in individuals with ASD, where enlargement of the right caudate nucleus is positively correlated with repetitive behaviours (Hollander et al., 2005). Notably, increased size of the right caudate has also been observed in adults with OCD, suggesting common pathways mediating these repetitive behaviours (Scarone et al., 1992). Rhythmic clock-gene expression has been observed in the dorsal striatum and is sensitive to dopamine (DA) release (Hood et al., 2010). In addition, striatal specific deletion of BMAL1 causes sexually dimorphic alterations in alcohol-drinking behaviour, potentially suggesting that the striatal clock is involved in motivated and reward-seeking behaviours (de Zavalia et al., 2020). Hypo-activation of the striatum has been observed in reward-motivated paradigms in ASD individuals and is believed to facilitate atypical motivational functioning seen in the disorder, likely mediated through dysfunctional circuitry between subregions of the striatum and the ACC (Kohls et al., 2013). In the present study we show that prenatal exposure to VPA results in blunted BMAL1 expression in the DS of males, and a shifted rhythm in females in comparison to saline-exposed animals. Due to the striatal clock's intimate involvement with DA and the modulation of reward-seeking behaviours, it is not beyond reason to suggest that alterations in the temporal expression of BMAL1 within

the DS may partially mediate the atypical motivational functioning in ASD. While it is also reasonable to argue that disturbances in the phase, or expression of striatal clock-genes may be involved in the increased prevalence of stereotypic behaviours, this association remains to be seen and warrants further investigation.

Prenatal exposure to VPA alters BMAL1 rhythmicity in regions involved in monoaminergic transmission

One region that shows properties of a self-sustained circadian oscillator is the lateral habenula (LHb) (Zhao & Rusak, 2005). The LHb acts as a critical relay node between the limbic forebrain and midbrain structures and is involved in the modulation of monoaminergic transmission and reward circuitry through its projections to the dopaminergic ventral tegmental area (VTA) (Goncalves et al., 2012; Lecourtier & Kelly, 2007). Neurons of the LHb demonstrate rhythmic changes in their firing rate leading some to suggest that the LHb may modulate time-of-day regulation of motivated behaviours through daily changes in its intrinsic firing frequency, directing affecting the firing of DA neurons within the VTA (Zhao & Rusak, 2005). Unsurprisingly, the LHb has been implicated in the dysregulation of monoaminergic transmission, central to numerous neuropsychiatric conditions including depression, ADHD, and schizophrenia; all of which are known to demonstrate alterations in motivated behaviours. However, recently the LHb has been implicated in the pathogenesis of ASD-like behaviours, as normal functioning of this region is necessary for the generation of social play and modulation of aggressive behaviours in rats (Flanigan et al., 2020; van Kerkhof et al., 2013). In the present study, VPA-exposed males displayed alterations in BMAL1 rhythmicity in the LHb and appear to be blunted in comparison to controls. Remarkably, VPA-exposed females demonstrate anti-phasic properties in BMAL1 expression. Given the fact that the LHb projects to many

monoaminergic modulatory centers, it is conceivable that alterations in clock-gene rhythmicity within the LHb may influence time-of-day regulation of monoaminergic production and transmission, affecting a myriad of physiological and psychological processes. How alterations in the phasic-expression of clock-genes within the LHb affect the modulation of aggression and social behaviour are still unknown.

Glucocorticoids entrain peripheral clocks

The primary zeitgeber for the SCN is light. However, many subordinate oscillators lack direct retinal input and rely on other signals to remain synchronized. Clock-gene expression in peripheral tissues can be entrained by several zeitgebers, such as food, locomotor activity and glucocorticoid (GC) secretion (Dibner et al., 2010). While the SCN is devoid of GC receptors and therefore insensitive to the effects of circulating GC, the amygdala is rich in GC receptors and can be entrained through glucocorticoid signaling, albeit that only specific sub-nuclei are sensitive to the effects of adrenalectomy on clock-gene expression (Lamont et al., 2005; Segall & Amir, 2010). In addition, the PFC is largely sensitive to untimely GC signalling, as anti-phasic administration of corticosterone (CORT) in adrenalectomized, but otherwise SCN intact, animals result in blunted clock-gene rhythmicity (Woodruff et al., 2016). Collectively, these findings suggest that normal GC secretion plays a modulatory role in clock-gene expression within the PFC and in subregions of the amygdala. Previously, we have reported shifted CORT expression in VPA-exposed males, and its increased expression relative to the nadir of controls (Ferraro et al., 2021a). These observations bare weight on our present findings, as inappropriate temporal secretion of glucocorticoids from a diminished central oscillator may serve as another factor which destabilizes rhythms in peripheral tissues. Though the mechanism by which desynchrony

between the SCN and peripheral oscillators occurs has not been disentangled in our study, we cannot disregard the potential contribution of inappropriately phased CORT secretion in our results.

Given the heterogeneity of ASD symptoms, research has since focused on alterations in the brain's connectivity network, as focalized disturbances are unlikely to completely account for the complex symptomatology seen in ASD. Subregions of the PFC, including the ACC, are known to have dense connections to key limbic structures such as the amygdala and hippocampus (Petrides & Pandya, 2007). Thus, it is also possible that alterations in the rhythmic expression of clock-genes in one region may impact either the functionality, such as the potential case between the LHb and the VTA, or the rhythmic oscillation of clock-genes within another region. In specific, we propose that changes induced by *in utero* VPA-exposure affect the rhythmic expression of BMAL-1 in specific neural structures and such changes may be causally related to the emergence of ASD behaviours through a BMAL-1 mediated mechanism. However, how and if this occurs remains unclear. One way to answer these questions would be to utilize viral-mediated approaches to manipulate the expression of BMAL-1 locally in select regions. These studies offer the opportunity to investigate the direct effects of altered BMAL-1 expression on the emergence of specific ASD-related behaviours. Moreover, they represent a warranted and necessary set of future experiments, as it is fundamentally unclear how local clocks affect neural circuits that underlie the emergence of specific behaviours. Ultimately, these studies may provide clues for novel therapeutic targets in the treatment of ASD-related behaviours.

CHAPTER THREE

The transgenerational inheritance of circadian disturbances in a valproic acid (VPA)-model of Autism Spectrum Disorder

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Author Contributions :

- Conceptualization: Sarah Ferraro, Shimon Amir.
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- Data Analysis: Sarah Ferraro
- Visualization: Sarah Ferraro
- Manuscript production: Sarah Ferraro, editing by Shimon Amir

Abstract

Autism Spectrum Disorder (ASD) is a heterogenous group of disorders with similar and overlapping symptomology but differential clinical progression and severity. Disturbances within the circadian system are a prevalent pathological accompaniment of ASD but remain unaddressed in ASD individuals, presenting significant lifelong challenges. Over the past two decades the incidence of ASD diagnoses has continued to rise, leading to the suggestion that epigenetic mechanisms may underlie both the emergence of maladaptive behaviours seen in the disorder and the increased prevalence of ASD within the population. Previous research has documented that both the ASD-like behaviours and the neurobiological alterations resulting from *in utero* exposure to valproic acid (VPA), persist into the F3 generation. In this study we investigated whether comorbid circadian disturbances previously described in the VPA-exposed F1 generation were also present in the F2 and F3 generations. We produced a patrilineal breeding scheme wherein males exposed to either saline or VPA *in utero* were mated with naïve females to produce the F2 generation. Males from the F2 generation were similarly mated to produce the F3 line and all animals underwent circadian locomotor analysis. Here, we show that the emergence of disorganized circadian behaviour arising from altered photic-entrainment capacity are present in F2 and F3 VPA-exposed lineages. Moreover, we show a sexually dimorphic persistence of these behaviours wherein males, but not females, demonstrate a longer and more stable presentation of circadian rhythm disruption across all three generations. These findings demonstrate that deleterious comorbid disturbances in the circadian timing-system can also be transmitted through generations and emphasize the urgent need for novel therapies addressing ASD comorbidities.

Introduction

Autism spectrum disorder (ASD) is a significant, lifelong neurodevelopmental disorder typically diagnosed during childhood. The core diagnostic features of the disorder include impairments in social interaction and communication, and the presence of inflexible and repetitive behaviours (American Psychiatric Association, 2013). Additionally, ASD is found to be increasingly associated with various physiological and psychiatric comorbidities, emphasizing the pressing need for individualized therapies to address both the core symptoms of the disorder and the individual's specific comorbidities (Matson & Goldin, 2013). Remarkably, sleep and circadian disturbances are among the most common and burdensome comorbid symptom of the disorder, affecting up to 80% of individuals with ASD (Richdale & Schreck, 2009). These disturbances have been linked to an exacerbated ASD symptomatology and present as a major pathological component of the disorder (Cohen et al., 2014). Previous work in our laboratory has characterized the circadian system in a valproic acid (VPA) animal model of ASD, revealing the presence of disorganized and diminished circadian behaviour driven by alterations in the entrainment capacity (i.e. the synchronization of behaviour with the external environment) of the master clock (Ferraro et al., 2021a). These findings further stress the need for novel perspectives in the treatment of ASD-associated comorbidities as current research has highlighted that untreated sleep and circadian disturbances in these individuals will lead to ineffective attempts at treating the core behavioural deficits seen in the disorder (Richdale et al., 2014).

Currently, 1 in 54 children in the United States are diagnosed with ASD, representing more than a 175% increase since the year 2000 (Maenner et al., 2020). These reports of increased prevalence are exceedingly alarming due to the inherent lack of pharmacological treatments

targeting the core deficits in ASD due to an unclear understanding of the etiology and pathogenesis of the disorder. Whole-exome sequencing has revealed up to 1000 genes implicated in ASD susceptibility, clearly suggesting that genetic abnormalities play a role in the pathogenesis of the disorder (Geschwind & State, 2015). However, despite the increasing number of identified susceptibility genes, ASD is considered to be a moderately heritable disorder with a growing emphasis on the contribution of shared and non-shared environmental influences (Hallmayer et al., 2011; Sandin et al., 2014). Moreover, the majority of ASD cases are idiopathic in nature and are believed to arise due to interactions between susceptibility genes and environmental insults (Varghese et al., 2017). Specifically, maternal immune activation and prenatal exposure to certain drugs have been identified as possible environmental risk factors contributing to the pathogenesis of the disorder (Lee et al., 2015; Rodier et al., 1996; Strömland et al., 1994). Given the fact that only 5% of ASD cases involve fully penetrant genetic mutations and that environmental influences play a larger role than previously believed, the development of the disorder cannot be fully attributed to changes within one single gene. Understanding the etiological mechanisms underlying the disorder remains paramount, as the increasing prevalence of ASD and a lack of effective treatment strategies are among the greatest challenges facing pediatric medicine today.

Undeniably, the prevalence of ASD has risen steadily over the years, although the reasons for this increase remain a topic of debate (Prior, 2003). Nevertheless, while better awareness of the disorder, increased clinical evaluations, and a broader clinical definition may explain a portion of this increase, many researchers hypothesize that epigenetic mechanisms may also play a role in the rising prevalence of ASD within the population (Choi et al., 2016). Epigenetics is the process

by which environmental factors and experiences can create long-lasting changes in gene expression through the regulation of chromatin structure (Nestler, 2009). It has been well documented that both pre-and-postnatal environmental factors, such as maternal stress, nutrition, exposure to endocrine disruptors, and other chemicals, can lead to epigenetic changes in developing offspring, affecting phenotypic outcomes (Champagne & Curley, 2009; Csaba & Inczefi-Gonda, 1998; Hales & Barker, 2013; Sharpe et al., 1995). Additionally, deleterious epigenetic alterations have been deeply implicated in the development of some psychiatric disorders and are believed to affect the development of critical neuronal pathways, ultimately shaping behavioural phenotypes (Kraan et al., 2019; Monteggia & Kavalali, 2009; Nestler & Lüscher, 2019). Moreover, these phenotypic alterations are often present in subsequent generations through a process referred to as transgenerational epigenetic inheritance (Anway et al., 2005; Csaba & Inczefi-Gonda, 1998; Roemer et al., 1997; Szutorisz et al., 2014). This mechanism is of particular importance, as it suggests that environmental factors and life-experiences can modify not only the epigenetic status of the present individual, but ultimately that of subsequent generations, potentially altering the development of key pathways necessary for the generation of normal behaviour and physiology.

Recently, Choi et al., observed that the behavioural and neurobiological consequences of prenatal exposure to valproic acid (VPA) – a well known environmental risk factor for the development of ASD – are effectively transmitted from the first to the third generation (Choi et al., 2016). VPA is a frequently prescribed anticonvulsant and mood stabilizing medication; however, it is also a potent teratogen whose mechanism-of-action partly arises through its property as a histone deacetylase (HDAC) inhibitor (Johannessen & Johannessen, 2003; Phiel et

al., 2001). Prenatal VPA exposure in rodents is a well established and validated animal model of ASD that effectively recapitulates both the behavioural and neurobiological alterations seen in the disorder (Rodier et al., 1996; Roullet et al., 2010; Schneider et al., 2008). The observation that both the behavioural and molecular consequences due to *in utero* exposure to VPA can be transmitted from the first to the third generation suggests that VPA may affect the germline and result in alterations affecting neuronal development, leading to the emergence of ASD behaviours. Moreover, the authors posit that given VPA's intrinsic HDAC inhibitor activity, their findings may support a role for epigenetic modifications in the increasing prevalence of the disorder.

Given the increasing prevalence of ASD in the population and the previous results from Choi et al., we wondered whether the circadian disturbances previously described in the VPA model could be transmitted from the F1 to the F3 generation. In brief, the circadian system is a biological prediction tool important for the generation of rhythmic patterns of behaviour and physiology (Takahashi et al., 2008). In mammals, these rhythms are generated and maintained by a hierarchical network of clocks governed by a master pacemaker located in the suprachiasmatic nucleus of the hypothalamus (SCN) (Welsh et al., 2010). Photic information from the environment is conveyed to the SCN through specialized retinal ganglion cells and can align (entrain) rhythms to the external light-dark cycle (Welsh et al.). Additionally, cellular clocks also exist in peripheral tissues and are involved in the temporal regulation of tissue-specific functions (Dibner et al., 2010). Given the ubiquitous nature of the circadian system, disturbances in its functioning have been associated with depression, diabetes, cancer, schizophrenia, and autism, among others (Li et al., 2013; Oliver et al., 2012; Takahashi et al., 2008; Timothy et al., 2018).

The findings from the present study would be unprecedented as transmission of circadian behavioural phenotypes, to our knowledge, has not yet been identified. More importantly however, these results would reveal whether circadian disturbances that are associated with deleterious consequences on behavioural and physiological functioning can be passed on to future generations, further emphasizing the need for novel approaches in the treatment of ASD-associated comorbidities.

Materials and Methods

Generation of the VPA model

Female Wistar dams (4-months of age) were ordered from Charles River Canada (Charles River, St-Constant, QC) and delivered on gestational day (GD) 10. Animals were placed in clear plastic cages under standard laboratory lighting conditions (12h:12h light/dark), with free access to food and water. On GD 12.5, pregnant females were intraperitoneally injected with VPA, dissolved in sterile saline [500mg/kg]. Dams used for the generation of control animals were injected with a sterile saline solution. At least 4 dams per condition were used to breed each generation (F0 to F2) to minimize any litter specific effects. Females were left to whelp naturally and no culling occurred. Animals born from VPA-injected females are referred to as VPA-exposed, while animals born from saline-injected females are referred to as saline-exposed. Collectively, these animals are referred to as the F1 generation.

On PND 23 animals were separated by sex and weaned from their mothers. From the cohort of saline and VPA-exposed animals, 12 males and 12 females (a maximum of 2-3 rats per litter), per condition, were selected for further circadian analysis (F1). Additionally, 12 male rats were

chosen (6 VPA-exposed and 6 saline-exposed) and set aside for breeding purposes. The male rats left aside for breeding purposes were group-housed with their littermate until breeding occurred. Any additional animals were euthanized. All experimental procedures and animal use were in accordance with the Canadian Council on Animal Care and approved by the Animal Care Committee of Concordia University.

Breeding scheme

5-month-old saline and VPA-exposed males (N = 12) were used for the generation of the F2 cohort. Males were separated from their cage-mate 10 days prior to the introduction of a female, and individually housed. 4-month-old naïve female Wistars (N=12) were purchased from Charles River and introduced to the F1 saline and VPA-exposed males. Male and female animals were left alone for a 2-week period, at which point females were removed, individually housed, and allowed to whelp naturally, producing the F2 generation. Similarly, 12 male and 12 female rats/per condition, were selected from this cohort (a maximum of 2 rats per litter), to undergo further circadian analysis. An additional 12 male rats (6/condition) were selected to breed the F3 generation. Breeding of the F3 cohort occurred in the same fashion as described above. While only the F1 animals have been exposed to VPA or saline directly, F2 and F3 decedents from either lineage will be referred to as “VPA F2/F3”, or “saline F2/F3” for simplicity reasons.

Circadian Analysis

Between 4-6 weeks of age, F1, F2 and F3 animals selected for circadian analysis were individually housed in running-wheel cages situated in light-proof, sound-attenuated, and ventilated cabinets. Rats were exposed to a 12h:12h light/dark (LD) cycle for 3 weeks before

being placed in constant dark (DD) for 2 weeks. After the completion of DD, animals were placed back into 12h:12h conditions for 2 weeks, and then exposed to constant light (LL) for a period of 35 days. After 35 days, animals were placed back into a 12h:12h cycle for two weeks and subsequently euthanized.

Animal activity was continuously captured by VitalView software (Mini-Mitter, Starr Life Sciences Corp., Oakmont, PA, USA) and graphically represented using the Actiview Biological Rhythm Analysis software (Mini-Mitter). The data was analysed using Clocklab software (Actimetrics, Evanston, IL, USA). Various circadian variables of interest were calculated, including tau (endogenous circadian period), alpha (duration of activity period), activity bouts across 24h (bout threshold was set at 1 count of activity/min/ten min), intradaily and interdaily stability (quantification of rhythm fragmentation and strength of synchronization to the external LD cycle) and amplitude (robustness of the rhythm). Moreover, onset and offset of activity were determined and further speak to the robustness of the SCN clock. Under LL conditions, the number of days needed to become arrhythmic and masking behaviour (a ratio of total activity during LL/ total activity during LD) were calculated.

Statistics

Data were analysed using GraphPad Prism 9 (GraphPad software LLC, San Diego, CA, USA). For all activity parameters, the difference between groups were analysed with an unpaired two-tailed t-test. A significance threshold was set at $\alpha = 0.05$ and corrections for unequal variances were used when appropriate and detailed in the figure captions.

Results

Baseline Activity

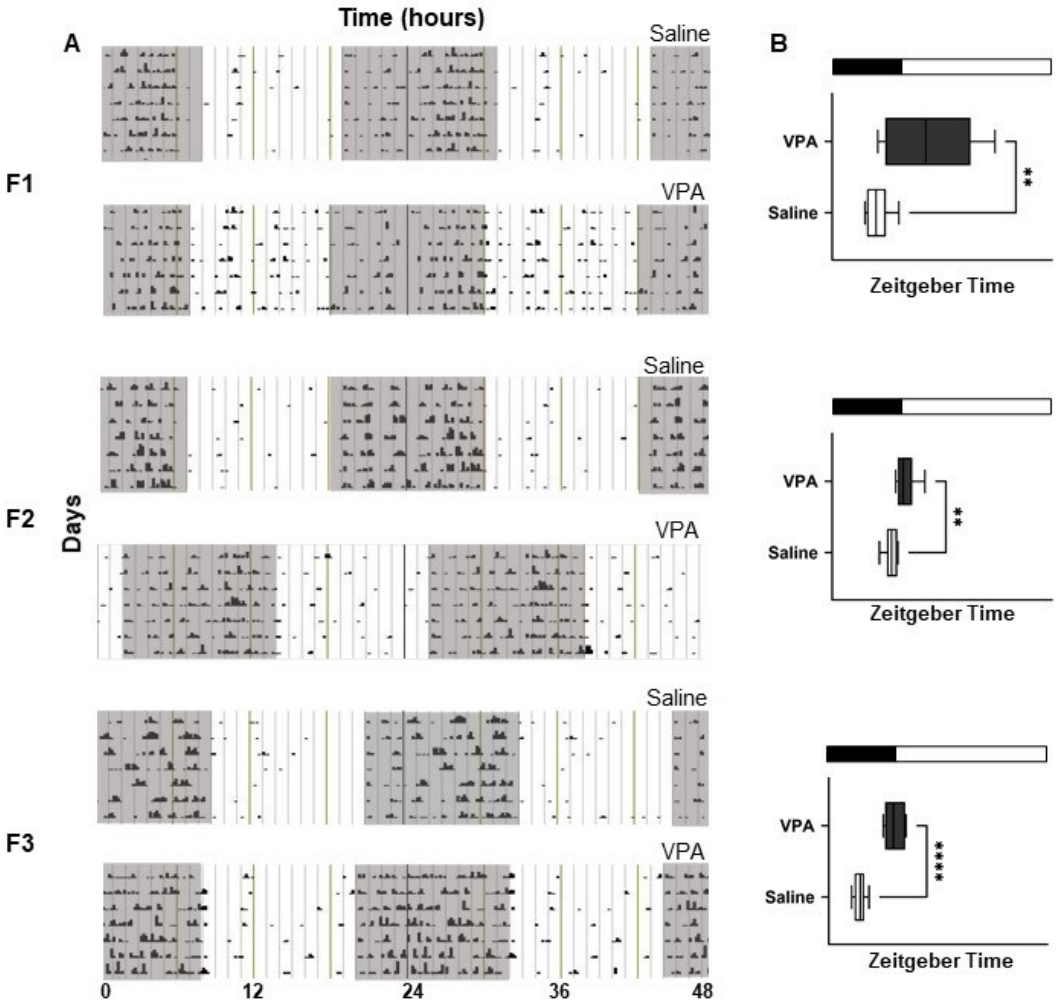
Prenatal exposure to VPA results in profound disturbances in rhythmic locomotor behaviour due to the presence of an unstable and diminished central oscillator (Ferraro et al., 2021a). In this study, we explored whether circadian dysregulation post *in utero* exposure to VPA can be transmitted to subsequent generations by monitoring locomotor activity rhythms under various lighting conditions. To address these questions, we first examined wheel-running behaviour in saline and VPA-exposed male and female rats, across all three generations, under standard 12h:12h light-dark (LD) conditions (Fig. 1a). As previously reported, under baseline conditions F1 VPA-exposed males displayed increased activity events during the light phase – a behaviour atypical of nocturnal animals – when compared to F1 saline-exposed males ($p = 0.0323$) (Fig. 1c). Moreover, the activity offsets of F1 VPA-exposed males occur well into the light-phase ($p = 0.0015$) (Fig.1 b) and consequently result in a lengthened activity phase (i.e. alpha) when compared to controls ($p = 0.0149$) (Fig.1d), hinting at potential alterations in the photic-entrainment capacity of the SCN. Additionally, F1 VPA-exposed males display increased fragmentation in their rhythmic locomotor activity (i.e. intra-daily variability) ($p < 0.0001$), decreased synchronization to the external 24h LD cycle (i.e. inter-daily stability) ($p = 0.0016$) and a lessened amplitude of the behavioural rhythms ($p = 0.0073$) when compared to controls (Fig. 1e-g). Collectively, these data from the F1 generation suggest marked disorganization of the circadian timing system, specifically alluding to alterations within the photic entrainment capacity of the master clock.

Interestingly, similar results were also detected in the F2 male generation, wherein F2-VPA exposed males demonstrated increased light-phase activity (p = 00426), later activity offsets (p = 0.0015), and an increase in alpha (p = 0.0157) (Fig. 1b-d). Notably, F2 VPA-exposed males also demonstrated a significantly lower behavioural amplitude (p = 0.0056) compared to saline-exposed males, suggesting the presence of a diminished central oscillator under standard conditions (Fig. 1g). However, they did not show significant differences in their intra-daily variability, or inter-daily stability (Fig. 1e-f).

To determine whether transgenerational inheritance of circadian disturbances post *in utero* exposure to VPA can occur, it was also necessary to determine whether circadian dysregulation was present in the F3 generation. Indeed, VPA-exposed males also demonstrated results akin to the disturbances seen in the F1 and F2 generation. In specific, these animals also demonstrate augmented light-phase activity (p = 0.0149), a later activity offset (p < 0.0001), and a longer activity phase (p = 0.0022) (Fig. 1b-d). However, like the effects observed in the F2 generation, F3 VPA-exposed males did not show alterations in inter-daily stability, intra-daily variability or in amplitude, perhaps suggesting a decay in the behavioural phenotype across generations (Fig. 1e-g).

Previously, we have reported that F1 VPA-exposed females were spared from the behavioural phenotype observed under standard conditions (Fig. 2a). In this study, it was observed that neither the F2, nor F3 female VPA lineage, demonstrated alterations under 12h:12h conditions, consistent with our prior observations from the F1 generation. Taken together, these findings indicate the presence of circadian dysregulation in F1, F2 and F3 VPA-exposed males, but not

females. Moreover, these results specifically allude to alterations in the entrainment capacity of the master pacemaker.


A
Time (hours)
Saline
VPA
F1
F2
F3
Days
0
12
24
36
48
B
VPA
Saline
**
****
Zeitgeber Time

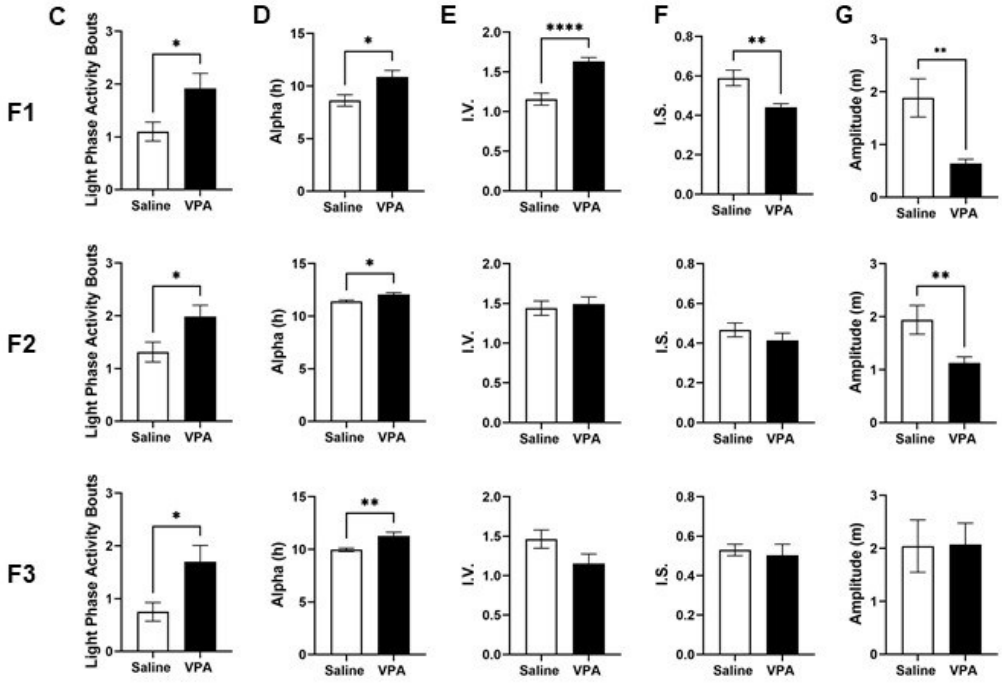
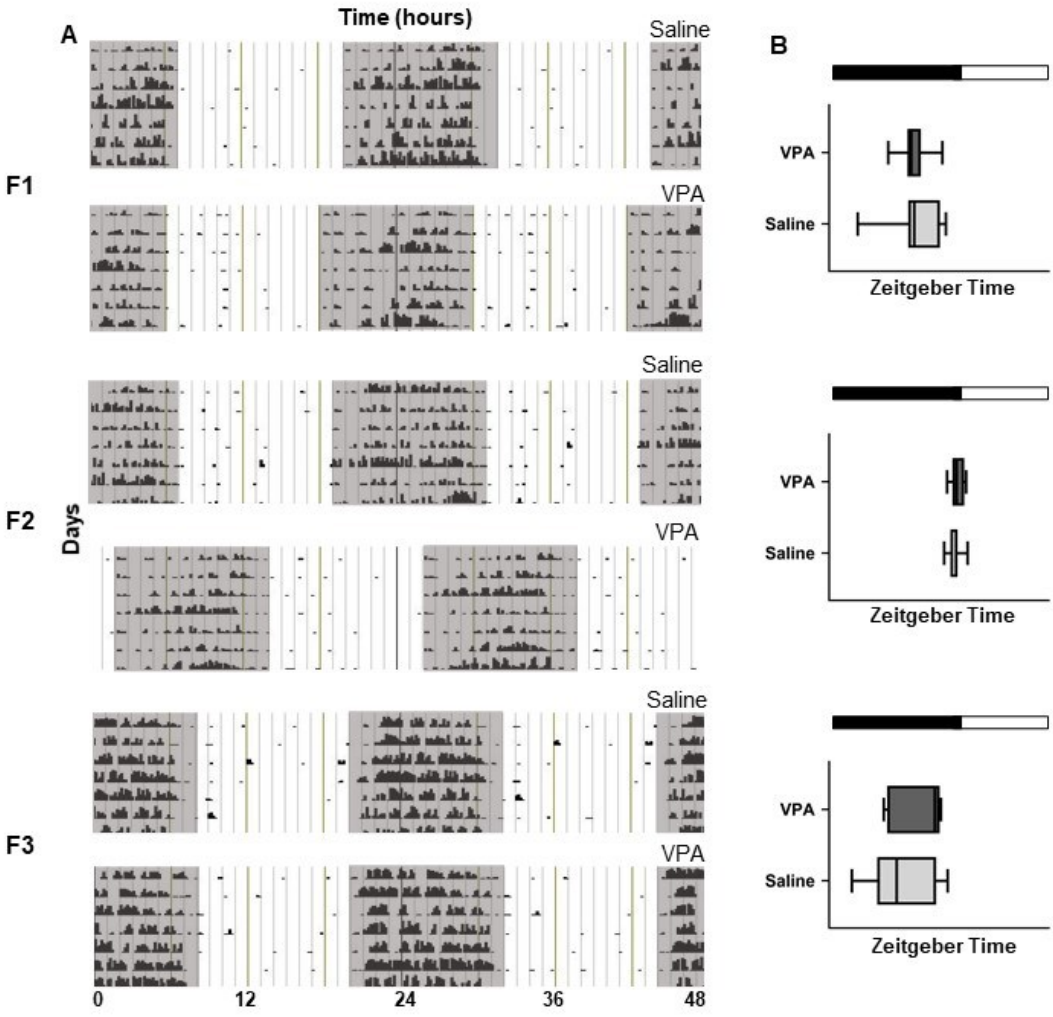

C
D
E
F
G
Light Phase Activity Bouts
Alpha (h)
I.V.
I.S.
Amplitude (m)
Saline
VPA
F1
F2
F3

Figure 1. Wheel-running behaviour under baseline conditions in F1, F2 and F3 males.

(A) Representative double-plotted actograms from saline and VPA-exposed males across F1, F2, and F3 generations. Animals were maintained under 12-hour light/dark conditions. Gray shaded areas indicate time of lights off (Zeitgeber time (ZT) 0 = lights on, ZT 12 = lights off). **(B)** Activity offsets for saline and VPA-exposed males across all three generations (F1: Saline n = 10, VPA n = 12. F2: Saline n = 8, VPA n = 12. F3: n = 8, VPA n = 6). Activity offsets were significantly later in VPA-exposed animals across all three generations. Data is depicted by a box plot diagram. **(C)** Light-phase activity bouts (bout threshold: 1 count/min/10 min) remain consistently elevated across all three generations in VPA males compared to saline males. **(D)** Length of the active phase (alpha: h) is extended in F1, F2 and F3 VPA males. **(E)** F1 VPA-exposed males demonstrate fragmented daily activity rhythms (intra-daily variability) but not F2 or F3 males when compared to controls. (**F)** Inter-daily stability is altered in F1 VPA-exposed males in comparison to F1 saline-exposed males. No significant difference between groups was detected in F2 or F3 males. **(G)** F1 and F2 VPA males demonstrate a lessened behavioural amplitude (m) when compared to saline males. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.


A
Time (hours)
Saline
VPA
F1
F2
F3
Days
0
12
24
36
48
B
VPA
Saline
Zeitgeber Time

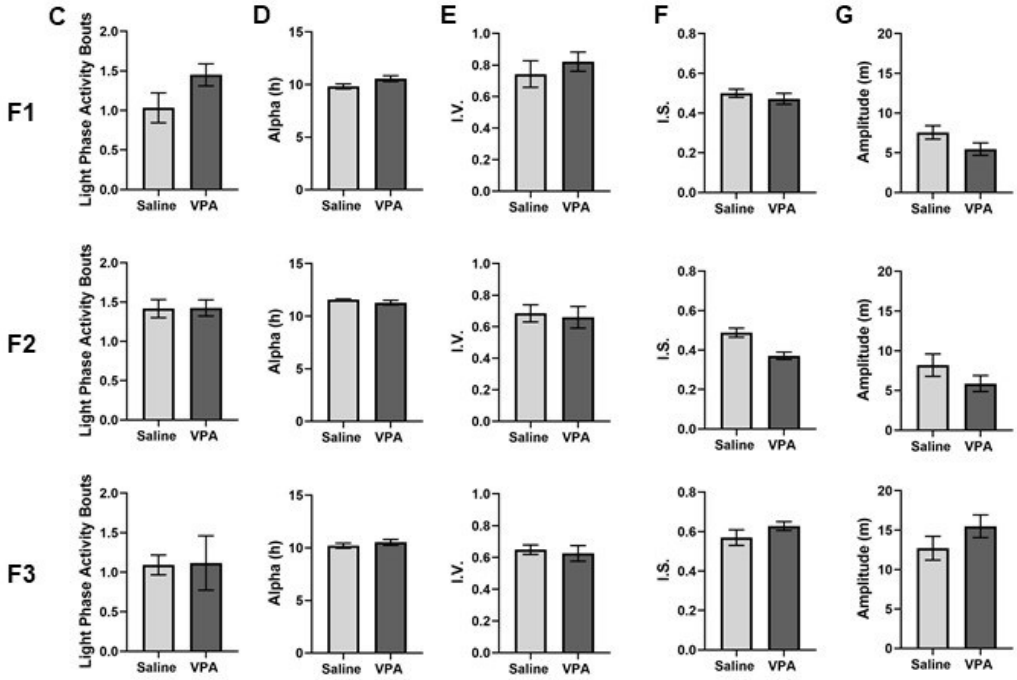

C
D
E
F
G
F1
F2
F3
Light Phase Activity Bouts
Alpha (h)
I.V.
I.S.
Amplitude (m)
Saline
VPA

Figure 2. Wheel-running behaviour under baseline conditions in F1, F2 and F3 females.

(A) Representative double-plotted actograms from saline and VPA-exposed females across F1, F2, and F3 generations. Animals were maintained under 12-hour light/dark conditions. Gray shaded areas indicate time of lights off (Zeitgeber time (ZT) 0 = lights on, ZT 12 = lights off). **(B)** Activity offsets for saline and VPA-exposed females across all three generations (F1: Saline n = 12, VPA n = 12. F2: Saline n = 12, VPA n = 12. F3: n = 11, VPA n = 6). Data is depicted by a box plot diagram. **(C)** Light-phase activity bouts (bout threshold: 1 count/min/10 min). **(D)** Alpha (h) in F1, F2 and F3 saline and VPA females. **(E)** Intra-daily variability (**F)** Inter-daily stability. **(G)** Amplitude (m). No significant differences were revealed between saline and VPA females across all three generations for any of the above parameters. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM.

Constant Conditions

Constant Dark

In order to further understand the nature of the circadian disturbances in the F2 and F3 VPA lineage, we examined the effects of both constant dark (DD) and constant light (LL) on rhythmicity. In the absence of photic input to the SCN, the endogenous free-running period (tau) of the animal can be determined (LeGates & Altimus, 2010). When placed under DD conditions for a period of two weeks, F1 VPA-exposed males did not demonstrate alterations in their intrinsic free-running period, suggesting that the observed phenotype under 12h:12h conditions is not due to changes in the length of the circadian cycle (Fig. 3b). However, a significant decrease in the amplitude of their intrinsic rhythm under DD conditions was observed, indicating the presence of a weakened central oscillator ($p < 0.0001$) (Fig. 3c). Moreover, the increased activity phase seen under 12h:12h conditions did not persist under DD conditions (Fig. 3d). These findings imply that the lengthening of alpha under standard conditions in F1-VPA exposed males is driven by aberrant photic-entrainment and a weakened oscillator, leading to the emergence of disorganized circadian behaviour.

Under DD conditions F2 VPA-exposed males did not show alterations in tau, consistent with our findings from the F1 generation (Fig. 3b). However, while F2 VPA-exposed males demonstrated a weakened amplitude under 12h:12h conditions, the amplitude of the endogenous rhythm under DD conditions was not significantly different when compared to controls (Fig. 3c). Similar to the results obtained from the F1 generation, F2 VPA-exposed males also did not show alterations in alpha under DD conditions despite a significant increase in this parameter under baseline conditions (Fig. 3d). Additionally, results from the F3 VPA-exposed generation reveal no

significant differences in tau, amplitude, or alpha under DD conditions when compared to controls (Fig. 3b-d). Notably, there were no alterations in any of the aforementioned parameters in F1, or F2 VPA-exposed females when compared to controls (Fig. 4b-d). Unfortunately, due to the SARS-CoV-2 pandemic, the results from the DD condition were not obtained for the F3 saline-treated females. However, given that neither the F1 nor F2 VPA-exposed females differed in any significant way from their saline treated counterparts under DD conditions, we do not believe this to be a confounding factor in the interpretation of our data.

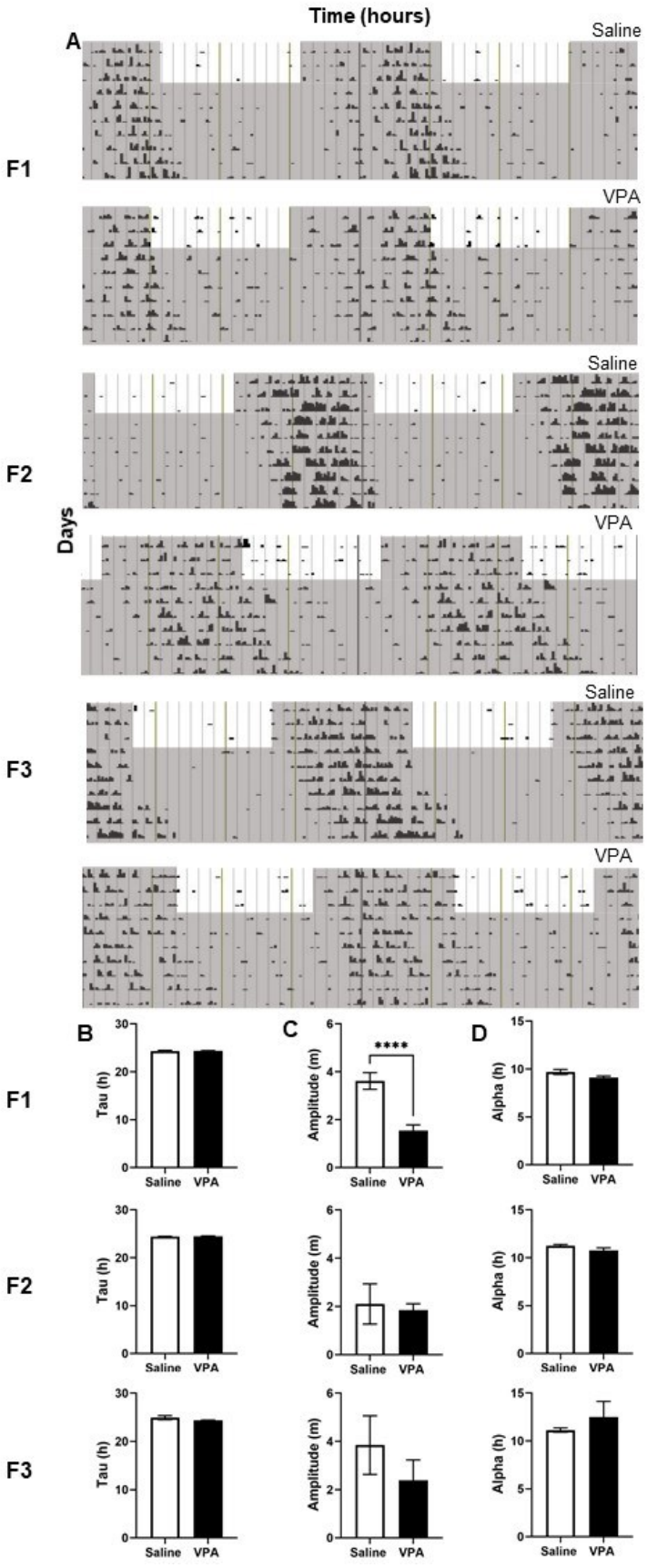

A
Time (hours)
Saline
VPA
F1
F2
F3
Days
B
C
D
Tau (h)
Amplitude (m)
Alpha (h)
****
Saline
VPA

Figure 3. Wheel-running activity under constant dark conditions in F1, F2 and F3 males.

(A) Representative double-plotted actograms from saline and VPA-exposed males across F1, F2, and F3 generations. Animals were maintained under constant dark conditions. **(B)** Tau (h) is unchanged between saline and VPA animals across all three generations. **(C)** The amplitude (m) of the endogenous rhythm is altered in F1 VPA-exposed males. No significant differences in amplitude were detected in F2 or F3 males between groups. **(D)** Alpha is unaltered under DD conditions between groups, across all three generations. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM. **** $p < 0.0001$

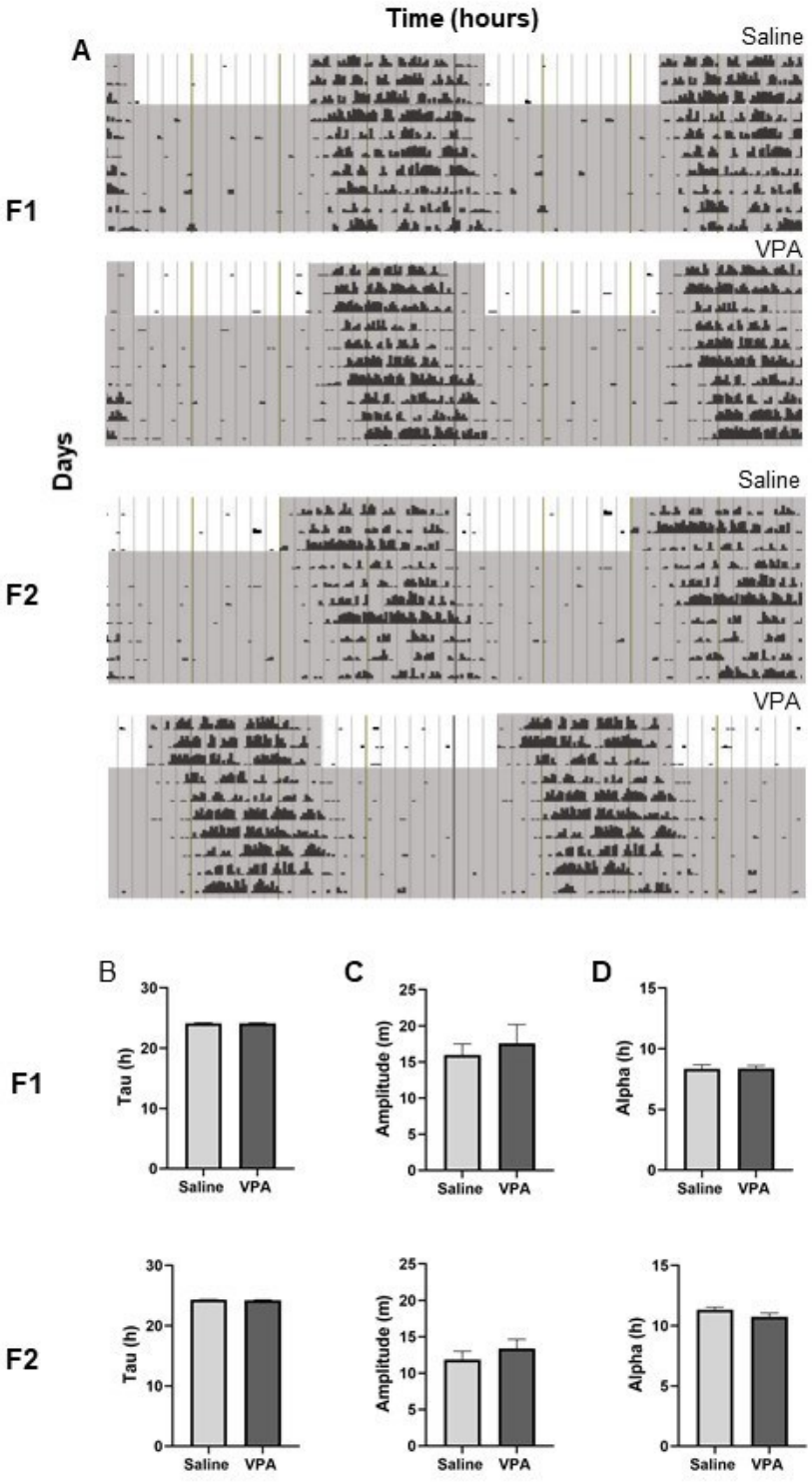

Time (hours)
A
Saline
F1
VPA
Days
Saline
F2
VPA
B
C
D
F1
Tau (h)
Amplitude (m)
Alpha (h)
Saline
VPA
F2
Tau (h)
Amplitude (m)
Alpha (h)
Saline
VPA

Figure 4. Wheel-running activity under constant dark conditions in F1 and F2 females.

(A) Representative double-plotted actograms from saline and VPA-exposed females across F1 and F2 generations. Animals were maintained under constant dark conditions. **(B)** Tau (h) of the endogenous free-running rhythm **(C)** Amplitude (m) **(D)** Alpha (h). No significant differences between saline and VPA females were detected under DD conditions across generations. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM.

Constant Light

Exposure to light results in a suppression of locomotor behaviour (i.e. negative masking) in nocturnal animals (Mrosovsky, 1999). However, prolonged exposure to constant light has been documented to disrupt the circadian clock by affecting the neuronal coupling of the SCN, resulting in the emergence of arrhythmic behaviour (Ohta et al., 2005). Given the findings obtained from the two previous conditions, we hypothesized that the integration of photic information was aberrant in F1 VPA-exposed males. Remarkably, under LL conditions F1 VPA-exposed males demonstrated an impaired negative masking response when compared to controls ($p < 0.0001$), again suggesting alterations in the integration of photic information in these animals (Fig. 5b). Moreover, the number of days needed to reach arrhythmicity in F1 VPA-exposed males was significantly increased when compared to controls ($p < 0.0001$) (Fig. 5c). Strikingly, under LL conditions F2 VPA-exposed males also demonstrated an attenuated masking response ($p < 0.0001$) and an increase in the number of days needed to reach arrhythmicity ($p = 0.0344$) when compared to controls (Fig. 5b-c). F3 VPA-exposed males did not demonstrate a diminished locomotor masking response, however they did demonstrate a significant increase in the number of days to reach arrhythmicity ($p < 0.6399$) (Fig. 5b-c).

Surprisingly, under LL conditions F1 VPA-exposed females also demonstrated an attenuated negative masking response ($p < 0.0024$) and an increase in the number of days to reach arrhythmicity ($p < 0.0001$) in comparison to F1 saline-exposed females (Fig. 6b-c). These findings suggest that while females do not demonstrate alterations under baseline conditions, they do demonstrate dysregulated circadian behaviour under challenge conditions. Moreover, F2 VPA-exposed females also demonstrate a significant impairment in their negative masking

response ($p < 0.0010$) but demonstrated no significant differences in the number of days to reach arrhythmicity (Fig. 6b-c). F3 VPA-exposed females did not show significant differences in either parameter (Fig. 6b-c). Collectively, these findings suggest the presence of altered circadian behaviour from the F1 to the F3 generation in VPA-exposed males, but not females.

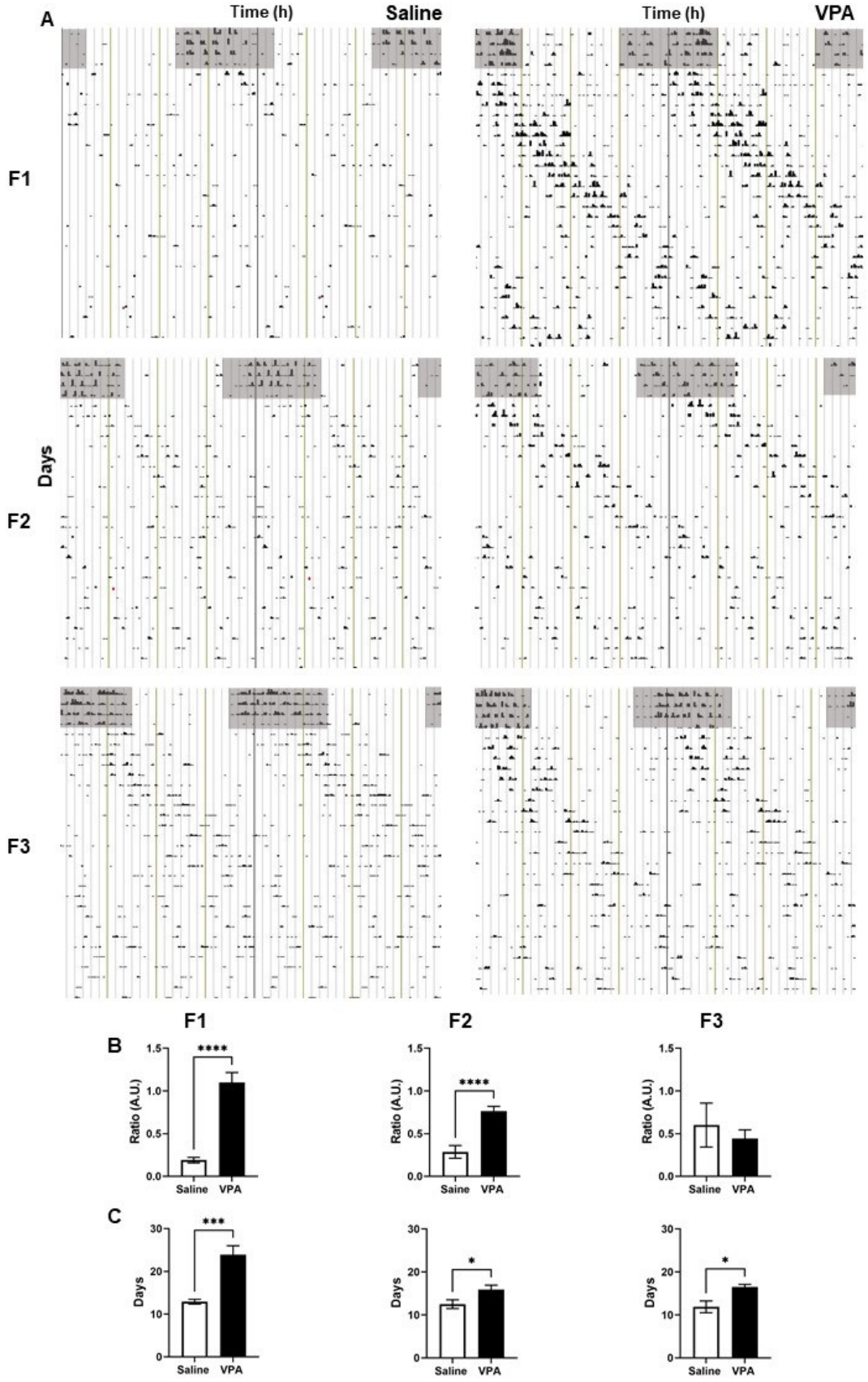

A
Time (h)
Saline
Time (h)
VPA
F1
F2
F3
Days
F1
F2
F3
B
Ratio (A.U.)
****
****
C
Days
***
*
*
Saline
VPA

Figure 5. Wheel-running behaviour under constant light conditions in F1, F2 and F3 males.

(A) Representative double-plotted actograms from saline and VPA-exposed males across F1, F2, and F3 generations. Animals were maintained under constant light (LL) conditions. **(B)** F1 and F2 VPA-exposed males demonstrate an attenuated negative masking response under LL conditions. Data is represented as a ratio (10d average of total activity in LL/10d average of total activity in LD). Negative masking behaviour appeared to be normal in F3 VPA-exposed males when compared to controls. **(C)** The number of days needed to achieve arrhythmicity was significantly longer in VPA-exposed males in comparison to their respective controls across all three generations. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM. * $p < 0.05$,** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

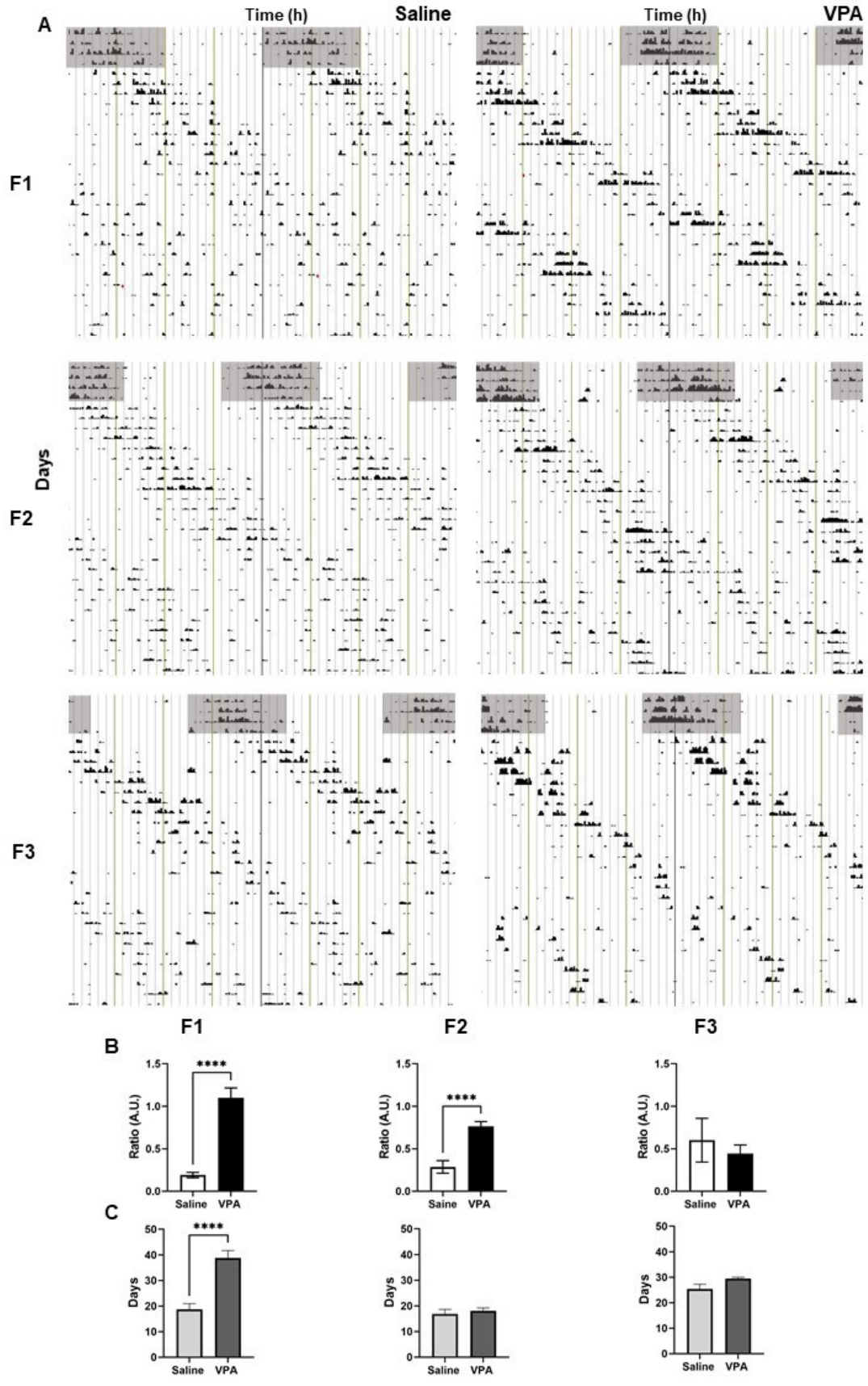

A
Time (h)
Saline
Time (h)
VPA
F1
Days
F2
F3
F1
F2
F3
B
Ratio (A.U.)
****
Saine
VPA
C
Days
****
Saline
VPA

Figure 6. Wheel-running behaviour under constant light conditions in F1, F2 and F3 females.

(A) Representative double-plotted actograms from saline and VPA-exposed females across F1, F2, and F3 generations. Animals were maintained under constant light (LL) conditions. **(B)** F1 and F2 VPA-exposed females demonstrate an attenuated negative masking response under LL conditions. Data is represented as a ratio (10d average of total activity in LL/10d average of total activity in LD). No significant differences were detected in F3 VPA-exposed females relative to controls. **(C)** The number of days needed to achieve arrhythmicity was significantly longer in F1 VPA-exposed females in comparison to their respective controls. This effect did not persist into the F2 and F3 cohorts. Data was analyzed using a two-tailed unpaired t-test (or Welch's correction when appropriate) and are plotted as mean ± SEM. **** $p < 0.0001$.

Discussion

In this study, we show for the first time that circadian disturbances present in the F1 generation post *in utero* exposure to VPA are also present in the F2 and F3 generations in male, but not female animals. Moreover, we show that this effect is mediated by the paternal lineage from F1 VPA-exposed males, which circumvents the potential for abnormal maternal behaviour to contribute to our results (Weaver et al., 2004). Previous findings from our laboratory highlight the nature of circadian disturbances in F1 VPA-exposed males, revealing alterations in the master clock's capacity to generate robust and stable rhythmic locomotor behaviour. Additionally, we have previously found that these alterations do not arise from any potential effect of *in utero* VPA toxicity on the developing retina, thereby indicating disturbances at the level of the master-clock itself (Ferraro et al., 2021a). Remarkably, our findings of light-phase activity offsets, increased alpha in standard conditions, and the persistence of the constant light phenotype in the F3 VPA-lineage lead us to conclude that it is specifically alterations in the photic entrainment capacity of the SCN that persist transgenerationally. However, while we have found circadian disturbances under challenge conditions in F1 VPA-exposed females, we did not see these effects in the F3 generation. Intriguingly, these observations suggest that males may be more vulnerable to the long-term transgenerational effects of VPA and the persistence of circadian dysfunction.

Disturbances in sleep-wake cycles are among the most common comorbid symptom in individuals with ASD and have been found to endure throughout childhood and into adulthood, presenting major lifelong challenges (Baker & Richdale, 2015; Karaivazoglou & Assimakopoulos, 2018; Richdale et al., 2014). Research has highlighted the association between

circadian disturbances and worsened behavioural outcomes in children with ASD, including increased repetitive behaviours, decreased social interaction capacity, and a decreased seizure threshold (Cohen et al., 2014; Richdale & Schreck, 2009). Moreover, a growing body of evidence has shown that sleep is important for normal brain development in children and that disturbances in this process during childhood may have an impact on the neural connectivity of the adult brain (Billeh et al., 2016; Kayser et al., 2014). Unfortunately, sleep and circadian disturbances often go unassessed and untreated in individuals with ASD, as current practice prioritizes treatment for maladaptive behaviours. However, this clinical standard is exceedingly counter-effective; attempts at treating the core deficits in ASD without treating comorbid circadian disruption leads to poor outcomes, partly due to the importance of normal sleep on learning processes (Richdale et al., 2014). Our data from this study adds to the findings of Choi et al., emphasizing that transgenerational inheritance post *in utero* exposure to VPA is not strictly limited to the core deficits seen in ASD, but also encompasses the transmission of concomitant circadian disturbances. These findings are exceptionally alarming; given the detrimental effects of circadian alterations in individuals with ASD and the current lack of effective treatment, these findings emphasize the pressing need for novel therapies targeting comorbid circadian dysfunction in the disorder.

Previously, we have reported changes within the SCN's molecular clockwork in F1 VPA-exposed animals, reflecting that the observed alterations at the behavioural level were indeed mirrored at the level of the molecular clock (Ferraro et al., 2021a). However, we do not know whether these biochemical alterations persist into F2 and F3 generations, necessitating the need for further studies. Additionally, we have found similar disturbances within local clocks present

in neural structures associated with ASD and comorbid behaviours (Ferraro et al., 2021b). Whether these observations also extend to the F2 and F3 generation remain to be seen and are of value, as disruption of the molecular clock in various extra-SCN regions has been associated with the emergence of abnormal behaviour (Bering et al., 2018; Karatsoreos, Bhagat, et al., 2011; Li et al., 2013; Nakano et al., 2016). Finally, although the findings of this study are unprecedented and represent a way in which circadian disruption in ASD can be passed on to subsequent generations, transgenerational epigenetic inheritance of this kind has not yet been tested in humans. Moreover, research evaluating sleep and circadian disturbances in children born to ASD individuals, to our knowledge, has not yet been conducted or evaluated. Therefore, these transgenerational possibilities in humans remains to be seen. Nevertheless, our findings offer insight into the insidious means by which circadian dysfunction can be passed on to future generations, potentially perpetuating their deleterious consequences onto generations to come.

GENERAL DISCUSSION

Prenatal exposure to VPA alters circadian rhythms

Circadian rhythm disruption is a hallmark of many neurodevelopmental disorders. The work presented in this thesis sheds light on the nature of the circadian system in the VPA-exposed animal model of ASD. In our first study we show that *in utero* exposure to VPA – an environmental risk-factor for the development of ASD – results in disorganized and diminished circadian behaviour under baseline conditions in males, but not in females exposed to the drug. Moreover, the findings from the baseline 12h:12h conditions suggested that the circadian alterations present in VPA-exposed males seem to arise from aberrant photic entrainment. We came to this conclusion based on these specific findings; 1) the occurrence of activity offsets in the light phase, 2) the distribution of running-wheel behaviour (i.e. an increase in daytime activity events) and 3) an increase in alpha that is not present under constant dark conditions. However, to ascertain whether alterations in the integration of photic information was driving our behavioural phenotype, we conducted constant dark, constant light, and light-pulse experiments in these animals. Here, we have demonstrated that VPA-exposed males do not demonstrate normal negative masking behaviour in response to chronic constant light and necessitate an increase in the number of days to become arrhythmic. Additionally, these animals also display altered phase-shifting responses to Aschoff type-1 light pulses, confirming our hypothesis that alterations in photic integration likely underlie our behavioural phenotype. However, in constant dark conditions VPA-exposed male animals also demonstrate a lessened intrinsic behavioural amplitude, indicating the existence of a weakened oscillator. Together, these findings led us to suggest that *in utero* VPA exposure leads to the existence of a diminished and unstable central circadian oscillator, resulting in disorganized circadian behaviour.

It is important to highlight that abnormal circadian behaviour in VPA-exposed animals is not driven by alterations within the retina itself, nor in its inputs to the SCN; a finding previously reported in other animal models of psychiatric disorders (Timothy et al., 2018). Rather, our current data supports the idea that alterations at the behavioural level reflect disturbances in the phase-relationship of BMAL1 expression within the master clock itself in both male and female VPA-exposed animals. Behavioural locomotor rhythms are tightly linked to the rhythmic nature of the spontaneous firing rate (SFR) of SCN neurons, largely considered to be a major output of the SCN clock (Houben et al., 2014). Interestingly, alterations in SFR have been shown to reset the phase of the molecular clock, highlighting the bidirectional nature of input/output influences on the TTFL (Jones et al., 2015). However, whether alterations in the SFR of the SCN are present in VPA animals and contribute to the generation of disorganized locomotor behaviour remains an area of future research.

Circadian disturbances post *in utero* exposure to VPA demonstrate sexual dimorphism

Interestingly, while females did not demonstrate alterations under baseline conditions, they did demonstrate disturbances akin to VPA-exposed males under constant light and light-pulse experiments. These findings were surprising, as they suggest the presence of a compensatory mechanism under 12h:12h conditions, but the emergence of circadian dysregulation under challenge conditions. As previously mentioned, the preponderance of ASD is largely shifted towards males (Maenner et al., 2020). However, recent data suggests that females often go misdiagnosed or are diagnosed later in life and ultimately display ASD-like behaviours differently than their male counterparts, suggesting the existence of sexually dimorphic behaviour in ASD (Ferri et al., 2018). Our findings would lead us to suggest that circadian

dysregulation in the VPA-model also demonstrates sexual dimorphism. Moreover, we wonder whether the circadian disturbances seen in VPA-females are perhaps insidious in nature, as these findings suggest that circadian dysregulation remains 'hidden' or 'masked' so to speak, under baseline conditions but emerges under challenge conditions. Of course, our experiments were conducted in a controlled environment, but chronic circadian disruption is common in today's industrialized world. Aberrant light at night, artificial lighting, trans-meridian travel, and shiftwork are a common reality and act as daily circadian challenges. It is possible that under perfect standard conditions, ASD females might be spared from these effects, but realistically, are at risk of circadian dysregulation and its impacts due to modern living challenges. While this remains a hypothesis, it is an important consideration for studies to come.

While one the goals of our research was to include female animals, the ultimate objective was not to compare the sexes. In other words, we were interested in whether circadian alterations were also present in VPA-exposed females rather than in how VPA-exposed females differed from their male counterparts. However, it is important to acknowledge that sex differences in circadian rhythmicity have been reported between the sexes (M. Bailey & Silver, 2014). Importantly, these sex differences are extremely species dependent and even vary between strains of the same species (Krizo & Mintz, 2015). Female rats demonstrate a slightly shorter intrinsic free-running period when compared to males and ovariectomy results in a lengthening of this period (Albers, 1981; Schull et al., 1989). Additionally, female mice show larger phase shifts in response to light pulses than their male counterparts (Kuljis et al., 2013). However, genetic ablation of estrogen receptor alpha in female mice results in an amplified phase-shifting response to light, suggesting that estrogen works to decrease responsiveness to the effects of a

phase shifting light pulse (Blattner & Mahoney, 2013). Similar results have been found for testosterone, as male gonadectomized mice also demonstrate larger phase shifting responses than male mice who have not been gonadectomized (Karatsoreos, Butler, et al., 2011). Given these findings, it is likely that gonadal hormones play a role in mediating sexually dimorphic circadian behavior. However, whether gonadal hormones play both an organizational and activational role at the level of the SCN, or whether they exert their effect on downstream regions responsible for the regulation of locomotion remains unclear.

Excitation/inhibitory balance: a role in altered circadian behaviour?

Disturbances in the delicate balance between neural excitation and neural inhibition has been proposed as a major contributor in the pathogenesis of ASD (Rubenstein & Merzenich, 2003). Prenatal VPA exposure has been shown to increase glutamatergic neuronal markers, but decrease those related to GABA-ergic signalling (Fukuchi et al., 2009; Kim et al., 2014). Communication within the SCN and to hypothalamic regions responsible for the generation of locomotor behaviour is largely GABA-ergic (Colwell, 2015; R. Y. Moore & Speh, 1993), thus a shift in the excitatory/inhibitory balance within the SCN may contribute to alterations in the neuronal communication necessary for the generation of normal rhythmic behaviour. Moreover, *in vitro* treatment with GABA, but not glutamate, can entrain the rhythmic expression of *Bmal1* in cortical neurons (Barca-Mayo et al., 2017), leading us to wonder whether alterations in E/I balance in VPA treated animals may have a direct impact on BMAL1 rhythmicity in peripheral tissues. However, the status of the GABA-ergic system within the SCN in VPA treated animals is currently unknown but presents as an opportunity for future studies in our laboratory.

***in utero* VPA-exposure alters local clocks in extra-SCN regions: A clock-mediated role in the generation of ASD-behaviours?**

Given the ubiquitous nature of the circadian system and the SCN's role in synchronizing rhythms amongst peripheral clocks, we wondered whether alterations at the level of the master clock were also reflected in extra-SCN oscillators in neural structures implicated in the pathogenesis of ASD behaviours. A thorough review of the literature describing volumetric, morphological and functional changes in the brains of ASD individuals led us to the selection of six regions of interest: the medial prefrontal cortex, anterior cingulate cortex, dorsal striatum, lateral habenula, lateral nuclei and basolateral nuclei of the amygdala. These regions were selected due to their roles in higher-order cognitive behaviours, motivation, reward, and emotion regulation. Moreover, cortical regions such as the medial prefrontal cortex and anterior cingulate cortex have also been implicated in the generation of depressive-like behaviours; a common comorbid psychopathology in individuals with ASD. Similarly, the basolateral nucleus of the amygdala was also chosen due to its role in the generation of anxiety-like behaviours, which had previously been shown to be altered in the VPA-model and exhibits robust circadian variation (Markram et al., 2008; Nakano et al., 2016). Although the statistical description of these findings is thoroughly detailed in Chapter 2, all regions, with the exception of the lateral habenula in VPA-exposed males, demonstrated altered temporal dynamics of BMAL1 expression. We postulate that there may be a BMAL1-mediated role in the generation of ASD-like behaviours and its associated comorbidities based on the wealth of research detailing how alterations in local circadian clocks lead to the emergence of abnormal behaviour. It is also true however, that these are specific associations that we have made, and these theories have not been tested directly. It is within this statement that the true challenge in the field of ASD research resides; despite

increased research efforts aiming to explaining the neurobiological underpinnings of the disorder, there is still an unclear understanding of the etiology and development of the disorder. These efforts have yielded an abundance of valuable data, however linking genetic or environmental insults to the emergence of ASD behaviours necessitates studies which aim to connect synapse functionality, cell types, specific circuits, and ultimately neural networks. This is a daunting, but important task for research to come.

While the data we have described in our second study is observational, it highlights that alterations in local clocks, believed to mediate time-of-day regulation of tissue-specific functions, is ultimately altered in brain regions strongly associated with ASD-behaviours. These findings can perhaps be best described as a form of local circadian misalignment, and moreover, lend well to the proposed "social-timing hypothesis" in ASD. Social-timing, or the capacity for children to engage in turn-taking during prototypical human social interactions, is an obvious core deficit in children with ASD. Specifically, it has been proposed that biological clocks, both circadian and ultradian, are necessarily for neuronal information and communication processing. Alterations in any of these oscillators are suggested to result in physiological and psychological consequences, affecting information processing and leading to the emergence of abnormal social-timing (Wimpory et al., 2002). Other suggestions, such as the weak coherence hypothesis, posit that coherent information processing is present in typically developing children, but is misaligned in children with ASD, leading to issues in temporal processing ultimately affecting social behaviour (Happé & Frith, 2006). While both hypotheses are based on clinical assessment, they both implicate alterations in the circadian timing system in the development of abnormal social behaviour in ASD.

Glucocorticoids entrain peripheral rhythms

One interesting finding from the first study that further lends face validity to the VPA model is the presence of phase-shifted and elevated blood plasma CORT levels. While the SCN is devoid of glucocorticoid receptors (GR) and therefore insensitive to the effects of circulating glucocorticoid, GR are expressed extensively throughout the brain and periphery (Balsalobre et al., 2000). Appropriate entrainment between the SCN and peripheral tissues is thought to arise primarily through rhythmic glucocorticoid secretion, but also due to glucocorticoid receptor-mediated effects in peripheral tissues. Exogenous treatment with the synthetic glucocorticoid dexamethasone in both fibroblasts and liver cells induces robust circadian gene expression of clock and clock-controlled genes, and are dependent on the presence of a functional GR (Balsalobre et al., 2000). Moreover, treatment with dexamethasone induces time-of-day dependent phase shifts of clock-gene expression in peripheral tissues, establishing a distinct phase-response curve (PRC) to glucocorticoid secretion in peripheral tissues. Additionally, glucocorticoid secretion is important for clock-gene expression in extra-SCN structures. Adrenalectomy abolishes the daily rhythm of PER2 within the central nucleus of the amygdala and the oval nucleus of the bed nucleus of the stria terminalis (Amir et al., 2004; Lamont et al., 2005). Importantly and perhaps most pertinent to our findings is that alterations in the temporal secretion profile of circulating glucocorticoid via chronic or acute injections of CORT alters clock-protein rhythms in the same brain regions, highlighting the importance of rhythmic and appropriately phased glucocorticoid secretion (Segall & Amir, 2010).

The findings from our first study suggest disturbances both within the circadian control of CORT secretion, but also within the hypothalamic-pituitary-adrenal (HPA) axis itself. The bidirectional relationship between the circadian system and the HPA-axis is not only important for

downstream physiological functions, but also for the adaptive capacity to modulate physiology and behaviour in face of stressful situations. Furthermore, it is necessary to interpret our results in light of the bigger picture; the maintenance of the appropriate phase of CORT secretion is necessary for the temporal regulation of downstream tissue-specific functions mediated by glucocorticoids. Inappropriate temporal secretion of CORT, through its capacity to act as a zeitgeber for numerous peripheral tissues, may serve as another factor which further destabilizes rhythms in peripheral tissues, such as some of the brain regions explored in our studies. Though desynchrony between the SCN and peripheral oscillators has not been disentangled in our study, we cannot disregard the potential contribution of inappropriately phased CORT secretion in our results.

Circadian dysregulation post *in utero* exposure to VPA persists transgenerationally

In our last experiment we explored whether the circadian disturbances described in F1 VPA-exposed males could be passed on to F2 and F3 generations. This experiment was exploratory in nature as research by another group had demonstrated that the ASD-like behaviours seen in VPA-exposed animals could be passed on from the directly exposed F1 generation to the F3 generation. To our knowledge, no other study had shown that circadian disturbances could be passed onto subsequent generations. To answer this question, we utilized a patrilineal breeding scheme wherein VPA-exposed males from the F1 generation were used to breed the F2 generation and so on. This choice mitigated the potential for abnormal maternal behaviour from VPA-exposed females to affect our behavioural outcomes.

Our results from the third study were surprising; we found that the circadian disturbances present in the F1 generation were also present within the F3 generation. Although we did observe a decaying or 'washout' of the phenotype over each subsequent generation – a common finding in many transgenerational studies (Burggren, 2015) – we were surprised to find that the transmission of this behaviour was specific to the alterations in photic-entrainment capacity. Changes in the strength of the master clock (measured through amplitude and rhythmic stability), while present in the F2 generation, did not persist to the F3 generation, suggesting that the underlying mechanism regulating light-induced circadian dysfunction may perhaps be mediated by some stable epigenetic marker that evades germline reprogramming. Interestingly, we found that the impaired negative masking phenotype present in F1 VPA-exposed females was also present in the F2 generation, but not the F3. However, the increase in the number of days to reach arrhythmicity seen in the F1 VPA-exposed females was not seen in the F2 generation. These findings suggest that there may be differences in the epigenetic transgenerational dynamics between VPA-exposed males and females. In other words, these sex differences may arise due to some sex-dependent resilience to various epigenetic modifications (i.e. DNA methylation, histone modification, etc). In this case, the 'washout' effect may occur more rapidly in female offspring, suggesting that males may be more susceptible to long-term transgenerational effects of VPA and the persistence of circadian dysfunction.

Pitfalls and alternatives

Our findings from the third study are preliminary as numerous challenges were presented while testing the F3 generation. Firstly, the SARS-CoV2 2019 pandemic impacted our breeding scheme for the F3 generation due to the university shutdown and a pause on all research projects.

This led to breeding the F2 males at an older age (6 months vs. 4 months) than what had previously been done for the generation of the F2 cohort. Moreover, the F3 VPA-males were also placed into running wheels at an older age than the previous cohort (PND 24 vs. 3-4 months). This discrepancy was not only impacted by the current pandemic, but also from the major structural damage that occurred in our lab due to flooding within the university. Unfortunately, we lost much of our equipment/testing space that we used to characterize circadian running-wheel behaviour. As a result, the characterization of the F3-VPA lineage could not be conducted until damage control, safety inspections, and a rebuilding of the testing room was completed. Testing the F3-VPA lineage at 3-4 months of age vs. shortly after weaning may have impacted our data; epigenetic modifications within a single animal are often age-dependent and subject to change (Burggren, 2015; Smith & Meissner, 2013). It is possible that a time-dependent decay in the epigenetic modifications responsible for the presence of our phenotype within the F3 male animal might have occurred, decreasing the chance of observing aberrant circadian behaviour.

While we had been collecting tissue to look at clock-gene expression within the SCN in all three generations, we encountered major issues when trying to collect tissue from the F3 cohort. The flooding occurred during the testing period for the F3-saline group; due to the location of the flood, we had water coming into our testing rooms, making for a potentially dangerous situation for our animals. After discussion with my supervisor and per the instructions from Environmental Health and Safety for the CSBN, we chose to sacrifice our animals to prevent any harm due to the flood. Sadly, this decision prevented us from acquiring the tissue needed to conduct those experiments. The length of time from the beginning of the transgenerational

project to the end was approximately two years, therefore re-starting this project was not possible. However, we believe that this portion of our research has yielded extremely interesting data and warrants future follow-up on the expression of BMAL1 within the SCN of all three generations, and potentially investigating the existence of stable epigenetic markers underlying this transmission.

CONCLUDING REMARKS

While it is attractive to contend that circadian rhythm dysfunction actively contributes to the pathogenesis of ASD, it is still unknown whether neuronal programs important for the development of the brain are also involved in the ontogeny of the circadian system. In other words, is the emergence of circadian dysregulation in ASD due to some abnormal pathophysiological development of the brain, or does it actively contribute to the pathogenesis of the disorder? How and if the circadian system is important for normal development remains an area of the field that is poorly understood, but also offers the opportunity for immense growth. How does the rhythmic expression of genes and protein synthesis change as a function of development and are these changes also altered in individuals with ASD? These are fundamental questions that remain to be answered. Nonetheless, alterations in the circadian system have been associated with a host of pathological conditions and a worsened quality of life (Karatsoreos et al., 2011; Logan & McClung, 2019; Takahashi et al., 2008). Additionally, it has been well documented that normal sleep and circadian rhythmicity play significant roles in learning and mood regulation (Karatsoreos et al., 2011; Watson & Buzsáki, 2015). Unsurprisingly and perhaps most sinister, is the finding that attempts at treating the core deficits seen in ASD without treating comorbid circadian dysfunction leads to ineffective outcomes, likely due to the

importance of normal circadian functioning on learning processes. As such, circadian dysfunction is not simply an adverse consequence of ASD, but one that can and should be targeted for future clinical interventions.

Although fully penetrant single gene mutations only account for 5% of ASD cases, many of the identified genes encode proteins important for synaptic organization and function (e.g.: *Neuroligin1/2/3/4, Neurexin-1α, Shank3*) (De Rubeis et al., 2014; Sahin & Sur, 2015). Moreover, alterations in synaptic morphology, synaptic number and the suggestion of E/I imbalance has led to the reconceptualization of ASD as a 'synaptopathy'(Ebrahimi-Fakhari & Sahin, 2015). This begs the question: are the dysregulation of synaptic mechanisms and circadian disturbances in ASD mechanistically connected? Temporal regulation of synaptic plasticity, neuronal gene expression, as well as affect and cognition have all been linked to the circadian system (Eckel-Mahan, 2012; Li et al., 2013; Lipton et al., 2017). However, it is fundamentally unknown how this global organizing system known as the circadian clock regulates local control of synapses and neuronal circuits underlying specific behaviours relevant to ASD. Ultimately, the aim of future experiments would be to mechanistically link how alterations in circadian timing affect specific behavioural circuits to better therapeutic targets in ASD individuals.

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