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Characterizing circadian behaviour in the BTBR mouse model

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by

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Abstract

Circadian rhythms span across species and temporally co-ordinate behaviour and physiological processes to not only maintain a rhythm in the absence of cues but to also effectively coincide with external time giving cycles. A disruption in circadian rhythms causes a variety of psychological and physiological health impacts and can worsen already present disease states. Sleep and circadian issues are also comorbid with many disease and disorder states. Finding ways to alleviate the impact of circadian disruption is thus crucial. We characterized circadian behaviour in BTBR mice, that are used to model aspects of ASD. We found that BTBR mice differ drastically from C57BL/6J mice on many measures of circadian behaviour including, their free running period, their duration of activity, their total activity, their response to dark pulses in LL their entrainment patterns to shifted light dark cycle, their food anticipatory activity to schedules feeding and in the number of VIP and AVP cells in the SCN. Despite their short FRP BTBR mice entrained to ambient light cycles with stability and precision, a property that is not commonly observed. Additionally, they had more drastic changes in constant light conditions when compared to C57 controls. Despite this they maintained normal responses to light pulses, suggesting a conservation light input pathway, instead a possible dysregulation of arousal pathways, which aligns with what is known about BTBR physiology. We also found differences in VIP and AVP expressing cells in the BTBR SCN. Both these peptides have been implicated in the circadian entrainment to light cycle. Therefore, the BTBR mouse model provides not only the novel opportunity to study the mechanisms of circadian rhythms in a mouse with a drastically altered phenotype, but also can be used to study circadian rhythms when they are

dysregulated. Finally, their precise entrainment might provide clues of how to better deal with circadian disruption and how to optimize entrainment to changing light cycles.

Keywords: Circadian, mouse model, free-running period, entrainment, jet-lag, synchronization, SCN, VIP, AVP, food anticipation, feeding.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
FRP	Free running period
SCN	Suprachiasmatic nucleus
VIP	Vasoactive intestinal polypeptide
AVP	Arginine Vasopressin
GRP	Gastrin releasing peptide
FAA	Food anticipatory activity
FEO	Food entrainable oscillator
ASD	Autism Spectrum disorder
Per	Period
Cry	Cryptochrome
LD	Light /Dark
DD	Constant Darkness
LL	Constant light
CT	Circadian time
ZT	Zeitgeber time
Zeitgeber	Time cue (literally “time giver”)
5-HT	Serotonin

Chapter 1: General Introduction

1.1: Circadian rhythms: function and adaptation

Rhythms in behaviour and physiology are prevalent across species. Migration of birds, hibernation of mammals, and oestrous cycles are some of the many examples present around us. Biological rhythms are classified into categories dependent on the time scale on which they function. Seasonal cycles are those that drive behaviour according to seasonal variation, annual cycles are those cycle that occur once a year, ultradian cycles that occur more than once a day (e.g., feeding) and infradian cycles that occur less than once a day (e.g. oestrus cycles) . The focus of this dissertation will be on circadian cycles - those that occur daily. Circadian rhythms, from the Latin words "*circa"* meaning about and "*dies"* meaning day, are those biological rhythms, that are widely present in most behavioural and physiological processes and that that occur with a frequency of approximately a day (i.e. 24 hours; Aschoff 1984; Pittendrigh 1993; Pittendrigh and Daan 1976; Reppert and Weaver 2002; Antle and Silver 2005; Golombek and Rosenstein 2010; DeCoursey 2004). Circadian rhythms have been demonstrated in all kinds of organisms from unicellular organisms to human (Bell-Pedersen et al., 2005; Woelfe et al., 2004; DeCoursey 2004) and while they have evolved to adapt to the environmental ***light-dark (LD)*** cycle, they run endogenously with an approximately 24 hour period even in the absence of any cues (Pittendrigh 1993; Pittendrigh and Daan 1976). Rest-activity cycles are perhaps the most often considered example of circadian rhythms at the behaviour level, but numerous more examples of circadian architecture exist from rhythmic daily changes in hormone levels

(Kriegsfeld et al., 2002; Kalsbeek et al., 2012; Bailey et al., 2001), muscle strength (Drustet al., 2006), and metabolism (Panda 2016), to changes in the organism at the micron level such as cell maturation (Du Pre et al., 2014), cell division (Matsuo et al., 2003), extension and retraction of glial cell process (Chi-Castaneda et al., 2016) and immune cell function (Labrecque et al., 2015; Druzd et al., 2017). Recent work has provided evidence that that daily rhythms in oxidation of peroxiredoxin proteins may represent the universal marker for the circadian machinery across all domains of life (Edgar et al., 2012).

Recently what has become evident, and of critical importance to the human population, is the impact of circadian rhythms on health and disease. Many disease states both physiological and psychological are associated with circadian and sleep related issues or dysregulation of the circadian rhythms in the various tissues that underlie that particular disease. Circadian rhythms of various biological and physiological process are disrupted during periods of shift-work or irregular lighting schedules – commonplace in modern lifestyles. And circadian disruption is both associated with an increased incidence of certain diseases both physiological and psychological and a worsening of already present disease states. Circadian disruption is associated with a worsening of diabetes, hypertension, insulin resistance and high blood glucose (Eckle 2015; Depner et al., 2014). Sleep and circadian disorders are also highly comorbid with both psychological disorders (such as mood and anxiety disorder) and neurodevelopmental disorders (such as ASD and ADHD). These sleep and circadian problems range from insomnia, frequent night-time awakenings and poor sleep quality to sleep onset latency issues and circadian phase disorders (Glickman et al., 2010; Brainard et al., 2016;

Karatsoreos 2014). Conversely, and importantly, targeting and improving sleep and circadian related problem in these population can attenuate the negative symptoms and improve overall health and quality of life (Karatsoreos 2014).

Circadian rhythms are generated and maintained by central circadian clocks (Klein et al., 1991; Antle and Silver 2005; Schibler and Sassone-Corsi 2002; Mohawk et al., 2012). Circadian clocks work to ensure that divisions of activity of the organism are separated into specific phases of the 24h day. The daily peak and trough in behavioural and physiological processes, allows an animal to anticipate and prepare for changes in its environment and for its internal organization to be well co-ordinated (Bell-pedersen et al., 2005; Vaze and Sharma, 2013; Dunlap et al., 2004). In mammals, the daily rhythms in temperature, alertness, muscle strength and other physiological measures causes us to be active during the day and to rest at night (Moore-Ede et al., 1982; Roenneberg and Merrow 2016). The daily peak in melatonin, the hormone released by the pineal gland, ensures the organism is optimally suited for sleep (Hastings et al., 2007; Gnocchi et al., 2017). Muscle, tone and power and force production peak some hours after waking and ensure optimal efficiency in physical tasks during times of the 24h day when most organisms are presumably awake and these again decrease when the animal is ready to rest, to ensure that any unwanted or unnecessary movements are kept at bay (Drust et al., 2005; Michael et al., 2003; Duffy et al., 2009). Stress hormones and body temperature begin to rise just before the animal wakes and peaks a few hours later, to then fall and reach a nadir- to ensure proper priming of behaviour and physiological responses to deal with any events that might transpire during the animal's waking period (Young et al., 2018; Lamia et al.,

2008; Gnocchi et al., 2017). Thus, while rest-wake cycles are the prototypical examples, the coordinated circadian regulation of various physiological processes allow the organism to prime its behaviour according to whether it is awake or resting.

Without elaborate synchronization of an animal's physiology and behaviour to its environment, it risks at best its adaptivity to the environment and at worst survival. Cyanobacteria that are placed in LD schedules that are closest to their endogenous light cycles out-compete those strains that are placed in LD cycles that are vastly different from typical photoperiods (Ouyang et al., 1998). Nocturnal rodents have seemingly evolved their behaviour in this manner to avoid the high risk of predation during the day (Bhadra et al., 2017). Animals with lesions in circadian clock show a higher chance of leaving their burrow at inappropriate times, when risk of predation is high and consequently are exposed to higher level of predation (DeCoursey and Krulas, 1998). For humans, a lack of temporal synchrony between the internal and external environments that arise from jet lag, shift work, and light pollution are all consequences of increasingly modern lifestyles. This circadian disruption impacts both physiological and psychological states (e.g., impaired cognitive function, altered hormonal function, and gastrointestinal complaints) – the increasing risk of which has been shown in numerous studies over the past decade (Wulff et al., 2010). Misalignment between the external and internal environment is associated with a higher incidence of disease states such as cancer and cardiometabolic diseases, and higher incidence of immune system disorders and weakened immune responses (Castanon-Cervantes et al., 2010; Stevens et al., 2005; Karatsoreos et al., 2011; Shi et al; 2013).

The circadian system is a complex system- a central pacemaker is complemented by a myriad of subordinate oscillating units (Honma et al., 2018; Mohawk and Takahashi 2011). The whole circadian system, then, represents itself as a hierarchy of driving and driven coupled oscillators (Aschoff, 1981; Aschoff et al., 1982). Since, circadian disruption is an almost unavoidable consequence of modern lifestyles, it is important to thoroughly understand the mechanisms that both generate and maintain normal circadian behaviour to be able to best deal with the negative impact. Using models that either have natural variation in circadian function, or upon which such aberrations are induced, we can infer how the system behaves 1) under normal condition 2) why it behaves so 3) the changes that occur during perturbation and 4) how to re-organize the system so that these perturbations can be better dealt with.

1.2: Part 1: Overview of circadian Properties

There are three fundamental properties of circadian rhythms: 1) persistent free-running rhythms 2) entrainability and 3) temperature compensation (Pittendrigh and Daan 1976; Aschoff 1981; Johnson et al., 2003; Golden and Canales 2003). These three properties are universal to the circadian system and essentially determine how the system adapts to its external environment to allow the animal to adapt most effectively and efficiently to its environment.

1.2.1. Persistent free running rhythms

The first fundamental property of circadian rhythms is that all organisms have a circadian rhythm that is precise and continuously sustained in the absence of any cues that

signal temporal change. Thus, in constant conditions - environments where there are no changes in external cues such as light, temperature or humidity to signal cyclic changes in external time - rhythmic changes in behaviour and physiology persist with remarkable precision (Pittendrigh and Daan 1976). The circadian rhythms expressed in constant conditions are called free-running rhythms and the frequency of the oscillation to complete one circadian day is called the *free-running period* (**FRP**; where period can be defined as the length of time of the cycle). FRPs oscillate with a frequency of approximately 24 hours, with both inter-individual and inter-species variability (Pittendrigh and Daan 1976; Aschoff et al., 1981; Daan 2003). In many species, including humans and hamsters (a long-used model of circadian rhythms), the free running period is slightly over 24 hours. However, other species, like mice for example, show a shorter free running period.

Persistent circadian rhythmicity is observed experimentally by plunging various organisms and, even unicellular organisms, into constant conditions (Lakin-Thomas & Brody, 2004; Mergenhagen 1980; Mittag et al., 2005). If left in constant conditions without exposure to external cues a daily co-ordinated rhythm is still maintained. Thus, a plant will continue to cyclically produce photosynthetic machinery in conditions of ***constant light (LL***; i.e. 24 hours of constant and uninterrupted light ; Millar and Kay, 1991), humans in constant conditions continue to maintain circadian rhythms of rest and activity, as seen in the classic bunker experiments (Luce, 1971; Wever, 1989). Strictly speaking a diurnal rhythm can only be classified as circadian if it persists under constant conditions – a requirement that distinguishes it from diurnal rhythms that occur simply as a response to changing environments. Thus, a circadian

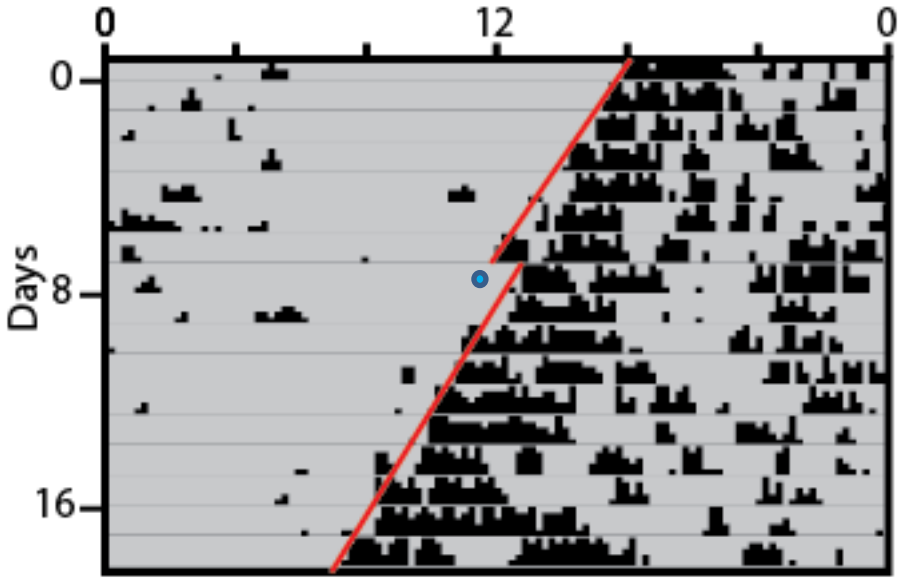
rhythm is endogenously generated and not just a passive response to a diurnal environment. However, in most cases, the literature does not distinguish between diurnal and circadian rhythms because almost all diurnal rhythms are circadian in nature and most circadian rhythms can synchronize to the external environment.

The closeness of the FRP to 24 hours is a consequence of the circadian system having evolved to coincide with the Earth's rotation about its axis, and, is a property that better allows synchronization to external cycles, that also conform to cycles of about a day. In general, stable and consistent synchronization of two oscillators to each other is most optimal if their inherent frequencies are nearly the same (Johnson et al., 2003). Compared to mechanical clocks, biological clocks are variable and not as accurate. FRPs change in their length over time. Sometimes the reasons for these changes are unclear and are called spontaneous changes (Pittendrigh and Daan 1976). However, FRPs can also change as a result of age (Nakamura et al., 2011, 2015; Duffy et al., 2002) and as a consequence of the light cycles it is synchronized to (termed aftereffects; Pittendrigh and Daan 1976; Scheer et al., 2007; Eskin, 1971).

The existence of the FRP is what betrays the presence of an endogenous oscillator that generates circadian rhythms, as opposed to the circadian rhythms being a passive reaction to the environment. In vertebrates, this oscillator was localized to the suprachiasmatic nucleus in the anterior hypothalamus and decades of research have now established it as the master pacemaker that generates circadian rhythms and synchronizes hierarchical oscillator networks throughout the organism, thus better adapting them to their environment (Klein et al., 1971; reviewed in Part 1.3.1).

Much of what we know about circadian rhythms emerged from the study of nocturnal rodents in constant darkness (Bittman et al., 2012; Jud et al., 2005). The most obvious overt indicator of the internal circadian system in an organism, is its rest-activity cycle **(Figure 1.1).** As can be seen in the actogram, in constant darkness the animal's onset of activity advances a little each day, due to this animal having a shorter FRP. The endogenous FRP is denoted by *tau or τ.* In laboratory rodents, wheel running is the most commonly used mode of collecting data, however infrared motion sensors and surgically implanted telemetry and temperature probes are also used (Jud et al., 2005). Based on rest-activity, each animal's circadian day can be graphed as a 24-hour plots. In constant conditions, the convention of ***circadian time (CT)*** is used to standardize time references between animals. **CT 12** is defined as that point in a nocturnal animal's daily cycle where the organism begins its activity (i.e. the onset of activity). Every other time point is then taken relative to CT 12 **(Figure 1.1).** This convention is also used to divide the animal's circadian cycle into "subjective day" and "subjective night", with CT0-CT12 generally forming the subjective day (when the animals is behaving as if it were day, so when a nocturnal animal is resting) and CT12-CT24/0 forming the subjective night (when the animal is behaving as if it were night, so when a nocturnal animal is awake). Of note here is that the subjective night and subjective day do not change depending on whether the organism in question is diurnal or nocturnal. The portions are divided equivalently. Thus, the rest activity cycle would be flipped in a diurnal animal – active during the subjective day and inactive during the subjective night.

Figure 1.1: **Representative actogram of a male adult mouse (C57Bl/j6) in constant darkness (DD).** The red line indicates a regression line fitted to onsets from which CT 12 (blue circle) can be calculated. By convention CT12 is the time of onset of the animal's activity. All other time points for manipulations are then calculated based on CT12 (therefore CT 6 is 6 hours prior to an animal's predicted activity onset and CT 18 is 6 hours after predicted activity onset). A phase shift is the difference between the two red lines (here showing a phase delay). This mouse also shows a free running period that is shorter than 24 hours with a consequent advancing of activity onset each day.


0
12
0
0
Days
8
16

1.2.2 Synchronization and entrainment

Circadian rhythms have evolved to ensure an organism's adaptive fitness. While able to free run in constant conditions, most organisms live in environments with a variety of time cues that, through their periodic variations, signal changes in time. The circadian system uses these cues to synchronize to these periodic changes and allow the animal to best adapt to its environment (Dunlap et al., 2004; Pittendrigh and Daan 1976; Aschoff 19811; Golombek and Rosenstein 2010). Synchronization between two oscillators (the internal timing system and the external timing system/cue) is the result of frequency of the internal system being aligned with that of an external system. In other words, synchronization occurs when the waveform of the driving rhythm (the environment) coincides with that of the synchronized rhythm (the internal clock rhythms or FRP; Johnson et al., 2003; Aschoff 1960; 1981; Daan and Aschoff, 2001; Pittendrigh, 1981). The external light/dark cycle is the most salient time cue and is the predominant synchronizing cue to which most organisms are synchronized and entrained to (Wright et al., 2013; Pittendrigh and Daan 1976; Dunlap et al., 2004; Golombek and Rosenstein 2010).

Synchronization and entrainment are not equivalent (Pittendrigh 1960; Johnson et al., 2003). With entrainment the internal rhythm fully conforms to the external rhythm, such that the properties of the internal rhythm are influenced by the external rhythm. When the internal rhythm is entrained to the external rhythm, the FRP of the internal rhythm (which deviates from 24 hours) is adjusted to match exactly period of the environmental cycle. In other words, when the circadian oscillator is entrained, a stable phase relationship is established between it

and the environmental cycle – such that the waveforms do not just run at the same frequency but that the conforming waveform is altered by controlling waveform- entrainment modifies the FRP of the internal system (Johnson et al., 2003; Pittendrigh and Daan 1976, 1981). During synchronization the frequency of the internal biological system matches that of the external cycle, however the external rhythm does not necessarily influence the FRP or phase of onset of activity (Pittendrigh and Daan 1976, 1981; Bittman et al., 2012). Thus, an organism can be synchronized without being entrained to the external environment when released back into constant condition. With entrainment, the phase of the onset of activity can be reliably predicted from the LD cycle (Aschoff 1960; 1981; Daan and Aschoff, 2001; Pittendrigh, 1981). Thus, to establish entrainment it is necessary that the period of the internal cycle equals that of the cycle of the cue and that upon "release" of the organism from the entraining cycle, the FRP resumes with the same phase as determined by the cycle of time giver (Aschoff 1960; 1981; Daan and Aschoff, 2001; Pittendrigh, 1981).

Environmental stimuli that synchronize and entrain the circadian system are termed ''zeitgebers,'' from the German word for ''time giver''. As mentioned, the environmental LD cycle is the most salient zeitgeber and predominate determinant of an organism's circadian rhythm. However other cues can also reset and entrain the clock, such as social cues, exercise, handling and pharmacological agents. In general, the circadian zeitgebers can be classified into two broad groups: Photic and Non-photic cues.

1.2.2.i: The Effect of a zeitgeber

In the presence of an oscillating environmental cue, the circadian system responds by conforming to the frequency of the environmental cue. A stable phase relationship is established between the two cycles (Aschoff 1960; 1981; Daan and Aschoff, 2001; Pittendrigh, 1981; Johnson et al., 2003). Thus, the FRP which generally deviates from 24 hours, needs to be reset daily in order to synchronize to the environmental cycle. Taking the example of the LD cycle for instance, if a diurnal organism with a FRP of 23 hours is exposed to 12 hours of light and 12 hours of dark, it will eventually be entrained so that the onset of waking period is well aligned to the onset of the light and its onset of rest coincides with that of the dark portion. If this organism were to fail to establish a stable phase relationship between its internal circadian cycle of rest and activity to the LD cycle, its rest activity cycle would be inappropriately phased to the environment. If it were to free run through the cycle, then this organism's rest-activity cycle would advance and be out of phase with the external cycle. In response to shifts in the cycle, the organism will attempt to re-entrain to the new shifted LD cycle.

There are "limits of entrainment" to the circadian system such that the circadian system is only able to entrain to non-24h cycles (T cycles) that do not differ too much from the endogenous FRP (Pittendrigh 1981; Pittendrigh and Daan 1876; Aschoff 1978, 1981; Johnson et al., 2003; Abraham et al., 2010). When the T-cycle differs by too much from the animals FRP, the animal will either free-run through the cycle or will alternate between being in a free-running state and being synchronized to the external cycle (a phenomenon termed relative co-ordination; Roenneberg and Merrow 2016). The circadian system generally entrains to T-cycles

that fall within the limits of entrainment (usually ~21-27hrs) . Behaviourally, this is done by "phase resetting" to the cue. Again, in the case of the LD cycle, an animal will reset the phase of its activity daily and either accordingly move its cycle earlier or later until the phases of the light cycle and rest-activity cycle match. Generally, with regards to a stable LD cycle, there tends to be a slight time difference between the two cycles called the "phase angle of entrainment" (Johnson et al., 2003; Aschoff et al., 1975; Aschoff and Pohl 1978). A positive phase angle occurs when the organism's activity begins in advance of the onset of darkness while a negative phase angle occurs when its activity occurs after the onset of darkness. Phase angle better allows us to encapsulate the efficiency with which an organism can organize its daily physiological rhythms (whether behaviour or physiology) with an appropriate phase angle to the daily environmental cycle (Aschoff et al., 1975; Aschoff and Pohl 1978). Thus, under an entraining or synchronising cycle the circadian system (via the circadian clock), resets daily so that it maintains a steady phase angle with the external cycle (one that is stable). Phase angle is generally influenced by FRP such that animals with FRPs shorter than the external cycle will tend towards a positive phase angle of entrainment and those with longer FRPs will tend to a negative phase angle (Aschoff 1960, 1981; Daan and Aschoff 2001; Pittendrigh 1981). When the organism is under a synchronising cycle, we follow the convention of mapping the time of day using ***zeitgeber time*** **(ZT; Figure 1.2)**. For an organism a LD cycle ***ZT 12*** is defined as the time lights go off whereas ***ZT 0*** is defined as the time lights come on.

Light also affects the clock discretely, under constant conditions such as constant darkness. As discussed, in constant conditions, animals will free run, based on which we can

deduce subjective day and night (CT12 being defined as the onset of the animals' activity). The response of the circadian system to different cues is phase dependent, i.e., the effect of a particular cue differs based on the time of day when it is presented. Practically this ensures that the circadian system is appropriately entrained to the external LD cycle. If the response was not temporally gated, the circadian system would respond to light, possibly, at random and this would impede appropriate entrainment (Pittendrigh and Daan 1976; Mrosovsky 1988; Boulous and Rusak 1982; Reebs and Mrsovsky 1989). This time of day (phase-dependent) effect, can be graphed and visualized using a phase response curve **(PRC, Figure 1.3).** A PRC is constructed by plotting the resetting response to the stimulus against the circadian time (CT) when the stimulus was delivered (Aschoff, 1965; Johnson, 1999; Pittendrigh and Minis, 1964). This resetting effect is termed a phase shift, whereby in response to a stimulus, the animal's activity either moves forward or backward as a result of light exposure **(Figure 1.1).** Light presented in the subjective day (CT 0-CT12) has little to no effect on the phase of activity and is often referred to as the dead zone on the PRC, a feature that is characteristic of the phase response curve across organisms. By contrast, light presented during the subjective night (CT12-CT24/0) ***phase shifts*** onset of activity. Light presented in the early subjective night (CT12-16) ***phase delays*** the activity rhythm (i.e., activity onset on subsequent days will begin later than on the day preceding the light pulse) and light presented in the latter half of the subjective night (CT 18-24) ***phase advances*** the activity rhythm (i.e. the activity on the subsequent days will start earlier than on days preceding the light pulse). Phase shifts have practical significance – light preceding expected dawn will induce a phase advance, while extension of light (or exposure to

light after dusk) will induce a phase delay (Aschoff et al., 1981; Daan 2003; Johnson et al., 2003).

Of note here is that most studies of circadian rhythms today, especially with regards to synchronization and entrainment follow the discrete, or non-parametric, entrainment model (Pittendrigh and Daan 1976). Light's effect on the clock is considered to be due to discrete time cues (i.e., brief light pulses) and, in nature, the transitions at dawn and dusk are what act as the discrete time cues. However, while in DD the PRC is rapidly reset in response to a light stimulus, overt rhythms require multiple cycles to attain a steady phase shift (Pittendrigh and Daan 1976; Aschoff 1960, 1981; Johnson et al., 2003). The discrete model makes two assumption 1) FRP measured in constant conditions accurately reflect the period under entrained conditions and 2) the stimuli used in entrainment and to derive the PRC are identical. The continuous, parametric, model in contrast, championed by Jürgen Aschoff (1960; Daan and Aschoff, 2001) states that the effect of light is not discrete, but present throughout the day - the differential effect on phase, at different points of day, is dependent on the intensity of light. Consequently, daily changes in light intensity is proposed to accelerate or decelerate FRP, allowing the circadian system to continuously adjust its cycle length to that of the environment. While the discrete model is more commonly used, the intensity dependent effects of light are important as they not only influence the magnitude of the phase shift but can influence how the FRP changes (Johnson et al., 2003; Jud et al., 2005). A change in either phase or period informs us of a change in the driving oscillators, either acutely, as with a phase shift, or chronically, such as a period change, that will then in turn affect entrainment to a cu

Figure 1.2: Representative actogram of a male adult mouse (C57Bl/j6) in 12hr:12hr light-dark (LD) cycle. Zeitgeber time (ZT) 12 is the time of lights off by convention and ZT 0 the time of lights on as is represented by the time scale at the top. Animals generally establish stable entrainment to the environmental LD cycle, with most of the activity taking place during the dark portion. To do so, organisms maintain a stable phase angle of entrainment (difference between the entraining and endogenous cycle), such that there is little variability day to day and activity onset coincides closely with the onset of darkness (in nocturnal animals).

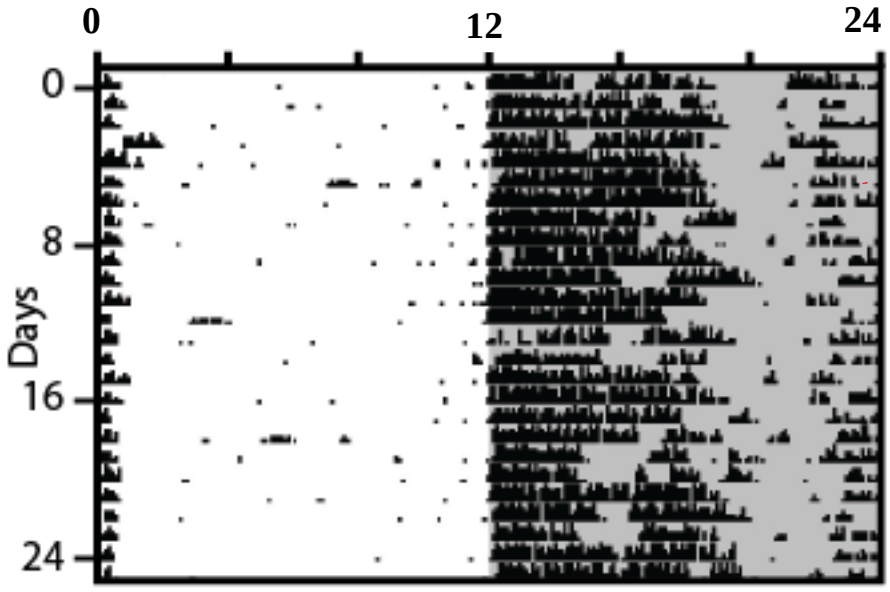
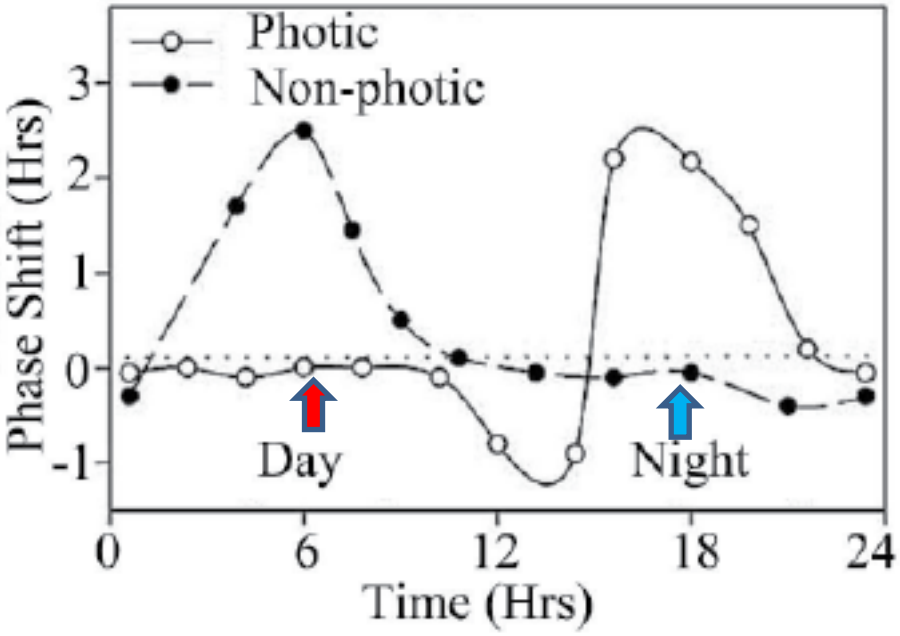

0
12
24
0
8
16
24
Days

Figure 1. 3: Graph showing PRCs for both photic and non-photic cues. The white and black dots show the magnitude of phase shift in hours at each time point. As can be seen photic and non-photic PRCs are non-overlapping, however both cue types can modulate the effects of the other. The light PRC has a dead zone during the subjective day (red arrow), whereas non-photic cues are ineffective at producing a phase shift during the subjective night (blue arrow).


Photic
Non-photic
Phase Shift (Hrs)
3
2
1
0
-1
Day
Night
0
6
12
18
24
Time (Hrs)

1.2.2ii: What determines a phase shift?

According to the discrete model an entrained pacemaker will be in equilibrium with an LD cycle – conceptually a collection or repetition of light pulses (Pittendrigh, 1966; 1981; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976). In nature these light pulses would be the dawn and dusk transitions. Thus, over repeated cycles organisms will shift their phase in response to these pulses and entrain to the cycle. If there was a shift in the entraining cycle, such as a change in time zone or change in length of the synchronizing cycle (i.e., seasonal photoperiod changes), then the internal circadian system, through the same process, re-aligns with that new cycle. This equilibrium is the result of light falling at phases to elicit appropriate phase shifting behaviour, i.e., a phase shift that is equal to the difference between the FRP and the entraining T cycle (Pittendrigh and Daan 1976; Johnson 2003; Pittendrigh, 1981; Pittendrigh and Minis, 1964). If the FRP is 25 hours, then the circadian system requires a net advance shift of 1 hour accomplished by a light falling on the PRC in the late night. If the FRP is 22 hours then the circadian system will need to experience a net delay shift of 2 hours, accomplished by light falling on the PRC in the early night. The phase angle between the entraining T cycle and the FRP will be different for different FRPs, with light pulses occurring at the specific phases necessary to correct the difference between the FRP and the environmental cycle. Both FRP and T are variable, and the phase angle and the appropriate phase shift will vary accordingly (Aschoff, 1965; Pittendrigh, 1981; Pittendrigh and Minis, 1964; Pittendrigh and Daan, 1976). These excellent predictive properties – necessitating only the expected phase shift and phase at the which it can be elicited as determined by the PRC, with the only additional information

being the FRP- is what makes this model so elegant (Aschoff, 1965; DeCoursey, 1960; Johnson, 1999; Pittendrigh, 1960; Pittendrigh and Minis, 1964).

This is not to mean that the continuous model has no place when studying the circadian system. The effects of light, while salient, are dependent on its intensity, duration and whether it is presented with other non-photic cues that can all modulate its effect on the circadian system (Aschoff 1981; Pittendrigh 1993). Factors that can most commonly influence the effects of light are (i) intensity (Aschoff, 1960; 1981) (ii) duration: long light pulses have experimentally been shown to have differential effects in both adult and ageing animals and lead to larger magnitude phase shifts as compared to short duration light pulses (Pittendrigh, 1981, Pittendrigh et al., 1984; Knoch et al., 2004; Antle et al., 2007; Meijer, Rusak, & Ganshirt, 1992; Nelson & Takahashi, 1991) and (iii) wavelength: During twilight transitions for example there is a more blue light over the dome of the sky, and red-light at the horizon (Roenneberg and Foster, 1997). Additionally, the effect of previous light cycles, age and other physiological factors can influence how the FRP reacts to a light stimulus. Light exposure in nature can vary depending on time of day and weather. Even twilight detection is more complex than previously thought and multiple photopigments help mediate how light affects entrainment (Foster and Helfrich-Forster, 2001).

Overall, while the effects of light on circadian behaviour can be precisely predicted, it does vary on the characteristic of the light exposure and can be modulated by a variety of factors both endogenous to the organism (like age) and exogenous (such as non -light mediated cues).

1.2.2.iii Non-photic cues

While light is the most salient cue, many other non-photic cues also affect the clock. These include, exercise, pharmacological substance, social cues, wheel access, restraint, and cage changes to name a few (Hastings et al., 1997, 1998; Yanielle and Harrington 2004; Mistlberger and Skene 2005). Non-photic cues also have phase dependent effects **(Figure 1.3)**. Additionally, non-photic cues also modulate the effects of light, both during the subjective day and during the subjective night (when they have no effect when presented alone). The PRC for non-photic stimuli is not as precise as the one for light, however it is posited that these cues have an adaptive relevance for specifying a temporal niche for locomotor activity and other behaviour (Challet and Pevet 2003; Caldelas et al., 2007).

Early recordings of human subjects suggested a role for social stimuli in circadian entrainment (Lund 1974). In animals, novelty induced wheel running, forced treadmill running, social cues, , dark pulses (where animals are subjected to a period of darkness when housed in constant light), cage changes, restraint, handling and even saline injections all have a similar effect of phase advancing the circadian rhythms when applied during the mid-subjective day (Reebs and Mrosovsky 1989; Wicklan and Turek 1991; Bobrzynska and Mrosovsky 1998; Marchant and Mistlberger 1996; Van reeth et al., 1989; Mistlberger et al., 2003). A number of pharmacological agents mimic these phase shifts such as systemic injections of triazolam, morphine or serotonergic (5-HT) agonists, or intra-SCN injections of NPY or muscarinic agonists. In the case of triazolam and morphine, it was discovered that their non-photic effects were not due to activity on the circadian clock itself, but rather by inducing an increase in locomotor

activity, as the phase shifts were prevented when activity was blocked (Mrosovsky 1996; Marchant and Mistlberger 1994ish). Arousal is often stated as an important common factor that underlies non-photic phase shifting (Maywood et al., 1997; Yamakawa et al., 2016; Mistlberger et al., 2003). Many non-photic stimuli are directly arousal eliciting (such as exercise, or wheel running), while others through their presentation induce locomotor activity (dark pulses, triazolam, cage changes etc). When this is activity is blocked, the phase shift is also blocked (Reebs et al., 1989; Mrosovsky 1995, 1996).

Non-photic cues also modulate phase shifting effects of light and light in turn can modulate the effects of non-photic factors (Yannielli and Harrington 2004; Maywood et al., 2002; Antle et al., 2007; Slotten et al., 2005; Challet et al., 2003; Lall et al., 2003). Wheel running activity or night-time injections of morphine can decrease the amplitude of phase advances induced by light but do not have any significant effects on phase delays (Ralph and Mrosovsky 1992; Mistlberger and Antle 1998; Mistlberger and Holmes 1999). Sleep deprivation during the subjective day can modify phase delays to light in both hamsters and mice (Challet et al., 2001; Mistlberger et al., 1997). Injection of 5-HT agonists and mixed agonists/antagonists can modulate the magnitude of light induced phase shifts (Rea et al., 1995; 2004; Mistlberger and Antle 1998). Conversely, non-photic phase shifts can be modified by subsequent light exposure (Mrosovsky 1991; Joy and Turek 1992). Some non-photic treatments like novelty-induced running (Mrosovsky and Salmon 1987), triazolam injections (Van Reeth and Turek 1987) or melatonin injections (Armstrong and Redman 1985) accelerate re-entrainment to a new LD cycle. Light can also potentiate 5-HT induced phase shifts (Knoch et al., 2004; Kaur et

al., 2009). Thus, photic and non-photic cues have non-linear, modulatory effects on each other that may help create a temporal niche, where an animals' behaviour and physiology is adapted optimally to a combination of environmental factors instead of just the most salient one (i.e., light).

1.2.2.iv: Feeding and the circadian system

Food is also a salient cue that can reset and influence circadian rhythms and while it can be classed as a non-photic stimulus, its influence on behavioural and physiological rhythms appears to be more independent of the central circadian clock and the circadian system (Stephan 2002; Mistlberger 2009, 2011; Pendergast and Yamazaki 2018) and therefore is reviewed here separately from other non-photic cues. Food restriction, both acute and chronic, elicits a range of behavioural and physiological responses in neural and peripheral circuits that help the organism maintain metabolic homeostasis (Muller et al., 2015; Duclos et al., 2013) and facilitate more efficient storage and release of nutrients and reduce energy expenditure. Experimentally the effects of food restriction are studied by restricting food availability in rodents during a 2 – 6 hour window during the subjective day (when a nocturnal animal is normal inactive and eats very little; Mistlberger 1994). This time restricted feeding induces feeding anticipatory activity (FAA) with a phase of onset that precedes the food presentation and shifts rhythms in the central and peripheral circadian system to coincide with anticipated mealtime (Boulos and Terman, 1980; Dibner et al., 2010). If a daily LD cycle is present, the central circadian clock itself is not shifted by daytime feeding schedules (Damiola et al., 2000; Stokkan et al., 2001).

While FAA can persist in the absence of a circadian clock **(Section 1.3.7)** it shows canonical properties of being under circadian control (Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2002). Once FAA is established it persists and continues to persist for several cycles with food deprivation (Davidson et al., 2001) which shows that it is a self -sustained clock mechanism. FAA appears to align with the limit cycle oscillator theory such that, if the feeding schedules greatly differs from 24 hours, FAA fails to emerge - showing "limits to entrainment" with an oscillator that runs with an intrinsic periodicity of 24 (Stephan 1982). However, when the feeding cycle is introduced slowly food anticipation does emerge to short and long cycles within the range of 22-31 hours (Mistlberger 1994). When the feeding schedule is shifted, it takes a few cycles for the FAA to re-align, causing transients (also a property observed with circadian entrainment; Davidson & Stephan 1998). FAA can re-appear months after the end of a food restriction schedule, at the correct phase matching when food deprivation was applied prior (Clarke & Coleman 1986; Rosenwasser et al., 1984), indicating that the system responsible for FAA can retain memory of food availability in constant conditions. These data therefore demonstrate that the anticipatory behaviour to scheduled feeding may be driven by an underlying circadian oscillator.

The food anticipatory rhythm is important because like other non-photic cues it shows that an animals' adaptation to the environment is not just solely dependent on one factor but is due to the combined adaption to all the factors in its environment. If food was scarce and not readily available, it is conceivable that an animal would more readily entrain to food availability rather than the light cycle. However, there is considerable interaction between FAA and the LD

entrained rhythm. While the known circadian clock is not required for the generation or maintenance of FAA (**Section 1.3.7),** animals with various circadian clock gene mutations do have modified FAA. Thus, studying FAA along with LD entrainment can inform us of the relative strength and co-ordination of the circadian system.

1.2.3 Temperature compensation

Finally, in addition to persistent free running rhythms and entrainment, the circadian system is also temperature compensated; in the face of day to day variations in temperature (generally an issue in poikilotherms), the speed of the clock (the period), remains stable to conserve the phase angle of entrainment to the environmental light cycle (Pittendrigh, 1993). This does not mean that temperature has no effect on the circadian system – temperature cycles can entrain the clock and temperature pulses can shift it (Pittendrigh, 1993). However, temperature compensation ensures that if a warm day was followed by a cool day, the circadian system would not run faster on the first day and then slower on the next, cool day. In other words, temperature compensation ensures that the rate of biochemical reactions (that generally speed up with progressively increasing temperature) do not changes with fluctuations in temperature.

1.2.4 Circadian properties and how they interact

The influence of light and other cues on the circadian system is non-linear and complex, dependent on duration, intensity and wavelength (Duffy et al., 1996; Duffy et al., 2009; Gorman et al., 2009; Warman et al., 2003). How different cues interact is also dependent on the kind of

cue given, the time point it is given at (before or after the light pulse), the intensity of either cue or the species in question. The phase responsivity of the circadian system allows it to compensate for small day-to- day variability in FRP (Johnson et al., 2003; Bittman et al., 2012; Piitendrigh and Daan 1976; Aschoff 1981). If FRP is shorter or longer on a particular day, or if the light cycle is shorter or longer on a particular day, light will strike the PRC at a phase that naturally elicits a phase shift that is smaller or larger than usual and therefore will counterbalance the effect of any environmental or endogenous variations (Pittendrigh and Daan, 1976). Light intensity can also modulate behavioural rhythms, and this is especially true in constant light. Brighter light intensities in diurnal animals leads to more activity and in nocturnal animals to less activity (Mrosovsky 1999, 2001; Redlin et al., 1999). Brighter light intensities can also lengthen the period in some species while shortening it in other species (Aschoff 1960). In LL especially, light differentially affects the phase, amplitude and FRP of diurnal and nocturnal animals. In nocturnal animals it supresses activity and lengthens FRP whereas in diurnal animals shortens FRP and increases activity, a correlation known as Aschoff's rule (Aschoff 1981).

How animals entrain to the governing light cycle is also influenced by the FRP. This is best elucidated in hamsters with the tau mutation (Ralph and Menaker, 1988; Ralph and Menaker 1990). Tau mutant hamsters can bear both a long period (26 hour) and short period (20 hours) phenotype. Homozygous tau mutant hamsters with 20 hr FRPs entrain to LD cycle with an earlier phase angle, while mutant hamsters with the long period mutation (and a FRP of 26 hours), entrain to the light cycle with a delayed phase angle (Ralph and Menaker, 1988). This

relationship between FRP and phase angle of entrainment is conserved across species (Johnson et al., 2003; Bitman et al., 2012). Drosophila flies with a similar mutation have phase angles that can be predicted by the FRP – evening activity peaks early in short period flies and later in long period flies when compared to wildtype flies (Hamblen-Coyle et al., 1992). Cyanobacteria also exhibit these same properties, indicating that these entrainment characteristics span phylogenetic divisions (Ouyang et al., 1998). Thus, while a stable phase angle is optimal, several factors can influence it.

Generally, after entrainment the FRP picks up at a phase that can be predicted from the entraining light cycle. However, in some cases, while rhythms appear entrained to the LD cycle, when released into constant darkness the FRP starts up from a phase that is not predictable from the exposure of periodic stimuli. This is termed masking, where the entraining stimuli "masks" or forces coordination of the two rhythms and masks the endogenous period of the internal clock, while not actually changing its properties. Thus, the internal organisation is synchronized without being entrained (Aschoff 1960; Mrosovsky, 1999). Masking can be broadly divided into positive and negative masking, where positive masking is a stimulus driven increase in clock output (for example – a light induced increase in locomotor activity) or negative masking which is a stimulus driven decrease in a clock controlled output (for example – a decrease in locomotor activity as a result of light exposure) . While masking tends to be considered a lack of entrainment, it can also be considered complementary to entrainment. Masking may encourage appropriate timing of behaviour and physiology, in cases of inadequate entrainment, or imprecise synchronization of the clock.

1.3 Part 2: Physiology of the circadian clock

1.3.1 The biological clock

The persistence of circadian rhythms in constant conditions, demonstrates the presence of an endogenous internal time-keeping system within organisms that generates the rhythms, as opposed to them being purely a result of reactive synchronization to the environment. The circadian system is considered a hierarchical network of cellular clocks in tissues of the brain and the periphery (Mohawk and Takahashi 2011), with the master circadian pacemaker situated in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Klein et al., 1991). The SCN functions like an orchestrator, co-ordinating the rhythms of the myriad body clocks (Yoo et al., 2004; Mohawk and Takahashi 2011; Welsh et al., 2010; Cuninkova and Brown et al., 2008). Structurally the SCN are superior to the optic chiasm and on either side of the third ventricle. The SCN consists of around 20000 neurons and measures around 400µm in the mouse (Reppert and Weaver 2002). Essentially the SCN has three main function 1) to act as a pacemaker and generate stable circadian oscillation in the absence of entraining agents 2) to synchronize these oscillations to the solar cycle 3) to convey time cues to the rest of the body

Decades of research has established the SCN as both necessary and sufficient to generate and sustain circadian rhythms (Weaver, 1998). Early studies showed that ablating the hypothalamus led to a loss of circadian rhythms indicating a hypothalamic site for the biological clock. Consistent with this, mice with a genetic mutation that caused varying degrees of the hypogenesis, showed a lack of entrainment to the LD cycle and were arrhythmic in wheel

running rhythms (Scheuch et al., 1982). Autoradiographic tracing methods showed that retinal afferents innervated the SCN and that the SCN is the principle termination site of the retinohypothalamic tract (RHT) that brings in light input (Kita and Oomura, 1982). Lesion studies localized the biological clock to the SCN : when this area of the anterior hypothalamus was lesioned it lead to a permanent loss in the circadian rhythms of behavioural (activity, sleep, drinking), endocrine (melatonin, cortisol), physiological parameters (body temperature, heart rate) and seasonal rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972; Rusak, 1977; Ibuka and Kawamura, 1975). Thus, while animals continued to present these behaviours, they no longer exhibited daily rhythms with distinct peaks and troughs.

This ability of the SCN to generate rhythms when the tissue is isolated in a brain slice is well established experimentally (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). In animals with bilateral enucleations (where the SCN would have receive no synchronizing light input) or that received knife cuts to create an island of hypothalamic tissue containing the SCN, the cells within it still maintained their spontaneous circadian rhythms of electrical activity (Inouye and Kawamura, 1979). Conversely, when an intact SCN from one animal is transplanted to the brain of a SCN lesioned animal (by in vivo, intracerebral grafting of foetal SCN into the brain of rodents carrying SCN lesions) it not only restores rhythmicity (Drucker-Colin et al., 1984; Sawaki et al., 1984; Lehman et al., 1987; DeCoursey and Buggy, 1989) but also determines the cycle length (i.e. the period) of the behavioural rhythm in the host organism (Ralph et al., 1990; Silver et al., 1996; Sujino et al., 2003). Thus, the consequent “new” endogenous circadian period (FRP) and rhythms of other physiological systems and

parameters, express the properties of the donor rather than the host, suggesting the SCN sets the pace for rhythmic behaviour in the organism.

These findings thus established that the SCN i) is an autonomous circadian pacemaker and is not simply driven by external stimuli ii) is a tissue-based clock iii) generates rhythms iv) synchronizes other tissue rhythms because these lose their rhythmicity when SCN is isolated but SCN does not and 5) enables rhythmicity onto other physiological and behavioural systems when transplanted – making it a “master pacemaker”. Thus, the SCN is viewed as a self-sustained oscillator that not only generates but also directs the rhythmicity and coordination of this rhythmicity in other central and peripheral clocks.

1.3.2 Inputs to SCN

Photic input reaches the SCN via a specialized set of retinal fibres called a retino-hypothalamic tract (Moore and Silver 1972; Hattar et al., 2). The RHT originates in the intrinsically photosensitive melanopsin ganglion cells in the retina, which are separate from retinal cells that comprise the classical image forming system (Foster et al., 1991; Freedman et al., 1990; Yamazaki et al., 1999; Hannibal et al., 2002; Hattar et al., 2002). While signals from both classic image forming and non-classic ganglion cells reach the SCN, rod and cone involvement in circadian entrainment is via synapses on ipRGCs (Guler et al., 2007; Schmidt et al., 2011; Drouyer et al., 2017). The pigment melanopsin that is found within IPRGCs, serves as the circadian photopigment and melanopsin is found exclusively in the retina (Hattar et al., 2002). Retinally degenerate mice, that lose all their classic photoreceptors by early adulthood,

exhibit normal circadian responses to light, i.e. they still show phase shifting responses and entrainment (Lucas et al., 2001; Foster et al., 1994). Genetically engineered mice that lack both rods and cones also show normal circadian light responses (Freedman et al., 1999). This maintenance of normal circadian responses has also been shown in the visually blind who entrain to environmental light cycles. Melanopsin gene knock-out attenuates circadian and other light responses and the RGCs lose their intrinsic photosensitivity (Hattar et al., 2002). Combined these results suggest that ipRGCs are an important input to the circadian system. However, melanopsin does not appear to be necessary for photic entrainment (Panda et al., 2003; Ruby et al., 2002; van Diepen et al., 2013; van Oosterhout et al., 2012), since melanopsin deficient animals entrain to light-dark cycles and phase shift to light (albeit with half the magnitude as WT animals; Panda et al., 2003; Ruby et al., 2002). Additionally, the SCN of melanopsin deficient mice (Opn4−/−) also shows sustained light responses (van Diepen et al., 2013; van Oosterhout et al., 2012a). Thus, the SCN does seem to have some degree of functional redundancy because entrainment is only completely lost when both the classic rod and cone receptors and melanopsin-based photoreceptors are eliminated (Hattar et al., 2003).

The ipRGCs form the retino-hypothalamic tract, that terminates mainly in the SCN onto the retinorecipient neurons in the ventral core (Fernandez et al., 2009). However, they also terminate more sparsely in the anterior hypothalamus, the sub paraventricular zone and the supraoptic region (Morin et al., 2013; Todd et al., 2012). RHT input also innervates the intergeniculate leaflet of the thalamus, which carries non-photic input to the SCN, suggesting a cross talk between photic and non-photic input and providing anatomical support for the

modulatory effects that photic and non-photic input have on each other (Pickard 1987; Morin et al., 2013). RHT axons release pituitary adenylate cyclase-activating polypeptide (PACAP) and glutamate, acting on NMDA-type glutamate receptors (NMDARs) and AMPA-type glutamate receptors to depolarize SCN neurons, increase intracellular calcium levels [Ca2+]i, activate the calcium/cAMP-dependent transcription factor CREB and increase PER expression via calcium/cAMP-dependent response elements (CRE; Irwin et al., 2007; Gooley and Saper 2017).

Non-photic input reaches the SCN via multiple pathways. A geniculo-hypothalamic tract (GHT) that originates in the intergeniculate leaflet of the thalamus uses neuropeptide Y (NPY) as its primary neurotransmitter to signal to the SCN, but also uses GABA and endorphins (Harrington 1997; Pickard et al., 1987; Maywood et al., 1997). The IGL in addition to the SCN projects to various regions in the forebrain and receives direct retinal projections (and therefore is also implicated in photic resetting (Harrington 1997; Horowitz et al., 2004). Projections from IGL contain NPY and NPY is critical to non-photic phase shifting (Marchant et al., 1997; Gamble et al., 2005; Edelstein et al., 1999; Challet et al., 2003). NPY injections in the SCN causes phase shifting responses that are similar to those caused by non-photic stimuli (Huhman et al., 1994, 1996; Biello and Mrosovsky 1996) and injections of NPY anti-serum attenuate phase shifts to non-photic stimuli (Biello 1995). Electrical stimulation of the IGL phase shift behavioural rhythms in a manner similar to non-photic cues (Rusak et al., 1989) and conversely, IGL lesions also alter circadian rhythms (Johnson et al., 1989) and attenuate phase shifts responses (Wickland 1994; Janik and Mrosovsky 1994). Animals with maximal phase shifting responses to novel wheel confinement, conversely, show FOS expression in the

geniculate and mainly in NPY containing neurons (Janik and Mrosovsky 1992). Loss of NPY immunoreactivity in the SCN eliminated phase shifts to arousal caused by saline injection and benzodiazepines (Maywood et al., 1997).

The SCN also receives projections from the dorsal and median raphe nuclei containing serotonin (5-HT; Meyer-Bernstein et al., 1996; Barassin et al., 2002; Dudley et al., 1998; Yamakawa and Antle 2010). 5-HT input causes phase shifts during the mid-day, underlies arousal induced shifting and can modulate light induced phase shift responses (Tominaga et al., 1992; Edgar et al., 1993; Cutrera et al., 1996). 5-HT effects are mediated though $5\text{-HT}_{1A/7}$ receptors and protein kinase A (PKA) activation (Prosser et al., 1993, 2000). Behavioural manipulations that cause non-photic phase shifts (such as wheel running), release serotonin and these behavioural shifts are supressed when 5-HT signalling is blocked or depleted (Edgar et al., 1997; Mintz et al., 1997). 5-HT agonists both in-vivo and in-vitro can not only reset circadian phase during the mid-subjective day, but also attenuate light induced phase shifts (Antle et al., 2003; Mistlberger and Antle 1998; Rea and Pickard 2000), via an attenuation of neurotransmitter release and firing rate of SCN neurons during the light pulse (Glass et al., 2003). Conversely, light responses are enhanced when 5-HT transmission is impaired through depletion of 5-HT, pharmacological blockade of the 5-HT receptor, or in animals with raphe lesions (Morin and Blanchard et al., 1991; Penev et al., 1993; Meyer-bernstein et al., 1996, 1997)

Recent evidence has also implicated the cholinergic arousal system as having an important role in non-photic phase shifting (Yamakawa and Antle 2016). Arousal induced phase

shifts are associated with FOS expression in the basal forebrain and many of the activated cells are cholinergic. These cells additionally project to the SCN and phase shifts are blocked with atropine injections to the SCN, showing that the cholinergic system is necessary in arousal induced phase shifts. Electrical stimulation of the basal forebrain in turn causes phase shifting in a manner similar to arousal procedures. i.e. phase advances in the middle of the subjective day.

1.3.3 SCN output pathways

Indirect and polysynaptic connections from the SCN to other hypothalamic sites and other centres in the brain drive endocrine and other physiological rhythms (Kalsbeek et al., 2002). The principal relay from the SCN runs from the sub-paraventricular zone adjacent to the SCN, dorsally and caudally into the dorsomedial hypothalamus (Saper and Scammel 2001). From there projections connect to arousal and sleep-regulatory centres of the orexinergic system and ventrolateral preoptic area thus mediating control of sleep dependent hormones (Kalsbeek et al., 2002). Projections also run to other autonomous centres and much of the viscera receive SCN input via their parasympathetic and/or sympathetic innervation. For example, the SCN drives glucocorticoid synthesis and release in the adrenal without accompanying hypothalamo-adenohypophysial activation (Ishida et al., 2005). Secretion of melatonin, a hormone that regulates seasonal rhythms and sleep efficiency, is also controlled by a polysynaptic pathway from the medial hypothalamus to the sympathetic afferents of the pineal gland (Ishida et al., 2005). Within the hypothalamus, projections connect the SCN to the

paraventricular nucleus, the preoptic area, and the medio basal nuclei and regulate daily rhythms of ACTH, gonadotropins, and metabolic hormones, respectively, by controlling the synthesis and release of relevant releasing factors (Kalsbeek et al., 2002).

1.3.4: SCN cell level networking and the TTFL

The intact SCN is a coherent network where cells maintain synchrony with each other, to produce coordinated rhythms that are more precise, of higher amplitude, and more robust than those displayed by isolated SCN neurons (Aton et al., 2005; Welsh et al., 2010; Liu et al., 2007). However, when SCN cells are disassociated from each other in culture, these cells lose their phase relationships and exhibit their own individual periodicities in firing rates and gene expression (Webb et al., 2009; Welsh et al., 2004; Herzog et al., 1998; Nakamura et al., 2001). At the molecular level, the rhythmicity of individual cells is dependent on autoregulatory transcriptional and translational feedback loops (TTFL) consisting of core clock genes within individual cells (**Figure 1.4;** Ueda et al., 2005; Zhang and Kay et al., 2010; Buhr and Takahashi 2013; Koike et al., 2012). Within each cell, transcription factors CLOCK and BMAL dimerize and act on E box elements of Period (Per) and Cryptochrome (Cry) genes to activate their transcription. PER and CRY protein then accumulate in the cytosol, are phosphorylated and translocated to the nucleus, where they inhibit activity of CLOCK and BMAL - thereby downregulating their own activation. In the absence of further gene expression, the PER and CRY dimers degrade, and by the end of circadian night, disappear, release CLOCK and BMAL from inhibition and the entire cycle is free to start again. This is augmented by a second level of

feedback loops mediated by transcription factors REV-ERB (reverse erythroblastic leukaemia viral oncogene homolog), RORA (retinoic acid related orphan receptor), and DEC1 (Drosophila hairy, enhancer of split, cAMP regulated; Partch et al., 2014; Preitner et al., 2002). For example, Bmal1 expression is induced after REVERBa, which acts as a repressor, is cleared in the later circadian night (Preitner et al., 2002). This then facilitates the re-initiation of the cycle coinciding with the degradation of PER and CRY complexes. These interlocked loops sustain a core oscillation and drive gene expression that in turn, drives the output of the clock - including diverse metabolic proteins, transcription factors, neuropeptide transmitters, and ion channels that determine and support the circadian properties of SCN neurons (Panda et al., 2002).

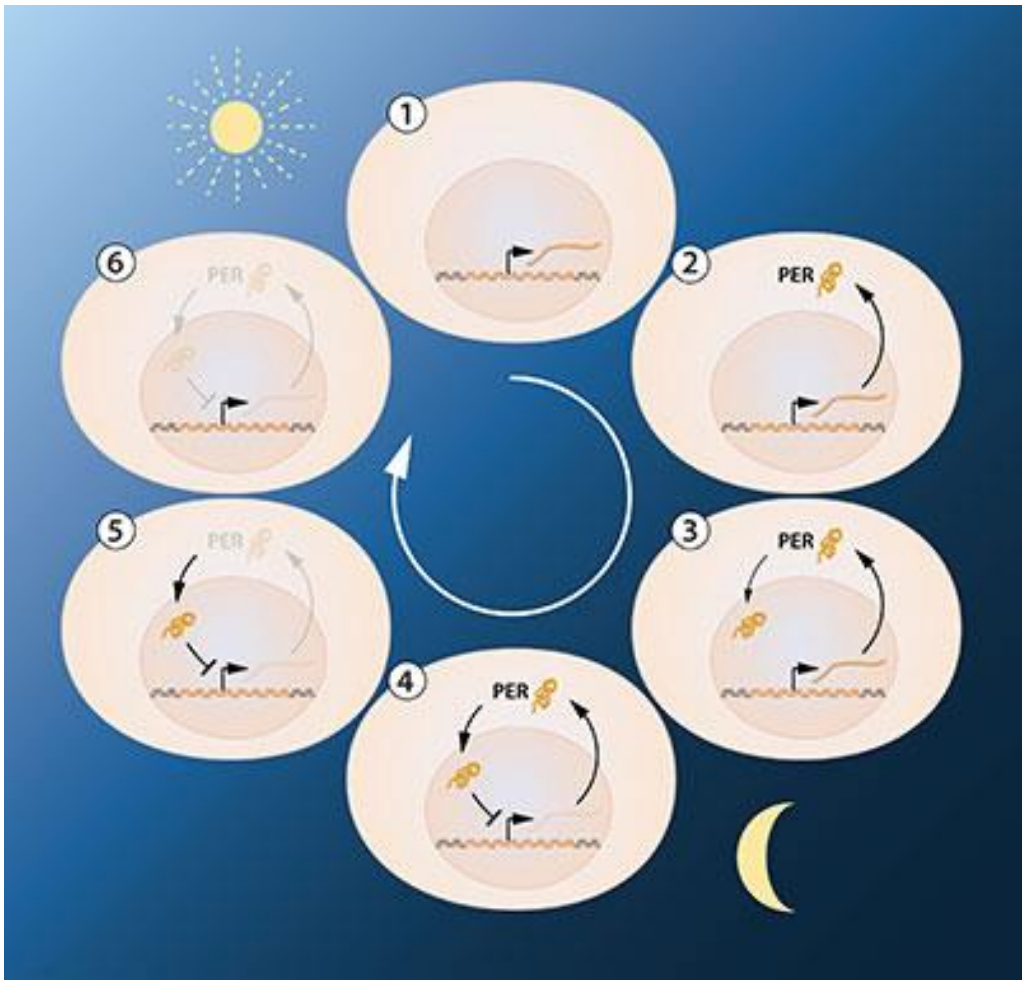
Identification of the genetic mechanism within SCN cells as a negative-feedback loop has helped identify the points in the cycle that contribute to setting period length. For example, *Clock* gene mutant mice have a long period phenotype that arises from impaired transactivation by the Clock protein lacking exon 19 (Lowrey and Takahashi 2011). The subsequently reduced *Per* and *Cry* target gene transcription rate leads to a slower progression of the cycle and longer behavioural (and other) rhythms. Similarly, mutations that delay the degradation of CRY, lengthen the circadian period due to a lengthening of phase of the negative feedback and those that accelerate it show a shortening of period (Godinho et al., 2007; Yoo et al., 2013). Mutations that destabilize PER, on the other hand accelerate the cycle and lengthen the period (Meng et al., 2008; Gallago and Virshup 2007). Thus, modification to any of the core clock genes appears to affect FRP. For example, the tau mutation in the hamsters is caused by a mutation in the CK1e enzyme that phosphorylates tau and thus influences the rate of its degradation (Meng

et al., 2008). Tau mutant mice have short (20-h) activity rest cycles and bioluminescence rhythms in SCN and peripheral tissues because of faster degradation PER protein that terminates the negative-feedback phase earlier than in wild-types (Meng et al., 2008). Similarly, loss of function mutations of E3 ubiquitin-ligases Fbxl3 and Fbxl21 delay or accelerate proteasomal degradation of Cry1 and Cry2 respectively, leading to correspondingly longer or shorter circadian period in vivo or in tissue and cell culture (Godinho et al., 2007; Yoo SH et al., 2013).

Cell cultures from animals that carry mutations in various clock genes, have similar periods to that of their behavioural rhythms in-vivo (Liu et al., 1997, 2007; Herzog et al., 1998; Nakamura et al., 2002). SCN neurons from Tau mutant hamsters that have short periods also have short periods (Liu et al., 2007). Animals with mutations in the signalling of Cry and Clock genes show long periods of activity and cultured cells from these animals show correspondingly long periods (Liu et al., 2007, Herzog et al., 1998). In one study SCN from genetically chimeric mice contained wild type cells (with ~ 23.7-hour rhythms) and Clock δ19 mutant cells (with ~24.5hour periods; Low-Zeddies et al., 2001). Locomotor activity of these animals were of intermediate phenotypes, i.e., between 23.7 and 24.5 hours. Thus, intrinsic properties of individual SCN neurons can be reflected in circadian behaviour and neurons of different periods can synchronize to each other to possibly set a mediatory period.

Figure 1. 4: Simplified version of the basic transcription translation feedback loop (TTFL) of clock genes. The figure demonstrates the TTFL using PER only. During the day, the *Bmal* and *Clock* gene expression leads their protein products to form heterodimers in the cytoplasm. These heterodimers translocate back into the nucleus where they then induce *Per* and *Cry* gene expression. During the night Per and Cry form heterodimers in the cytoplasm, translocate back into the nucleus and inhibit the expression of Clock and Bmal, thereby inhibiting their expression. Over the latter half of the night Per and Cry dimers degrade, which subsequently releases *Bmal* and *Clock* from inhibition and the cycle starts anew again.

Figure source: Press release. NobelPrize.org. Nobel Media AB 2019. Fri. 28 Jun 2019. <https://www.nobelprize.org/prizes/medicine/2017/press-release/>


1
2
PER
3
PER
4
PER
5
PER
6
PER

1.3.5: Limits of the TTFL

As mentioned TTFLs are not unique to the cells in the SCN but are found in a myriad of cellular systems. Fibroblasts, for example, are strong oscillators, but do not couple to each other- instead they desynchronize and lose population-level rhythms (Nagoshi et al., 2004, Leise et al., 2012; Welsh et al., 2004). What is unique to the SCN is thus the ability to adopt a common phase through synchronization and to maintain this population level synchrony even when the SCN is isolated (Herzog et al., 2017; Mieda et al., 2019). While the SCN is an intracellular network of genes and feedback loops, the prominence of the TTFL is questioned because of its unique ability to maintain this phase coherences amongst all of its constituent cells and maintain this persistent rhythm when isolated in culture (Balsalobre et al., 1998; Welsh et al., 2004). This is in combination with the findings that the effects of clock gene deletion are sometimes variable and with limited effects both within the SCN and in terms of the overall effect on circadian behaviour, while however having more serious consequences in some peripheral tissues and disassociated cells (Liu et al., 2007; Ko et al., 2010). Per1 and Cry1 deletions in disassociated SCN cells and fibroblasts severely impairs their rhythmicity (Ko et al., 2010). But the same deletion in SCN explants do not reflect this loss of synchrony - SCN explants from mice null for either of the two genes remain rhythmic as does the wheel running activity of these animals (Liu et al., 2007). This suggests that coupling mechanisms between cells of the SCN can maintain intracellular rhythmicity and compensate for losses in cell autonomous rhythmicity.

Further support for intercellular coupling mechanisms comes from the fact that even within the intact SCN, not all regions are intrinsically or uniformly rhythmic. The TTFLs of SCN cells, although synchronized, do not peak simultaneously. Circadian gene expression progresses as a spatio-temporal wave across the SCN. In each circadian day the clock genes Per1 and Per2 are first expressed in cells lying in the dorsomedial shell and then gradually spread out to the central and finally to the ventral region over the course of 4-8 hours, after which it recedes until expression is limited to the dorsomedial shell again (Doi et al., 2011; Yan and Okamura 2002; Quintero et al., 2003; Yamaguchi et al., 2003). The expression of clock genes in the shell region precedes that in the core by about 2-3 hours (Foley et al., 2011; Nakamura et al., 2001; Yan and Okamura 2002). These spatiotemporal patterns of gene expression are lost when synaptic communication is disrupted across the slice (Maywood et al., 2006; Yamaguchi et al., 2003; Deery et al., 2009), either by blocking with application of tetrodotoxin (TTX) or applying a protein synthesis inhibitor cycloheximide (CHX). When the CHX or TTX is removed, the cells gradually resynchronize to each other to re-establish their original phase relationships (Yamaguchi et al., 2003). Similar loss of gene expression patterns emerge after animals are exposed to constant light that causes behavioural arrythmia and is considered a model of circadian disorganization (Ohta et al., 2005). These waves of gene expression have been characterized in ex-vivo brain sections (Hastings et al., 1999; Koinuma et al., 2013) and in organotypic slices (Yamaguchi et al., 2003; Maywood e al 2011). These findings suggest that phase relationships among the cellular oscillators is an intrinsic property of SCN circuitry and that they are established and maintained via intracellular synaptic connectivity.

1.3.6: SCN Networking: in constant conditions and in response to the environment

The 20000 neurons in the SCN are primarily GABAergic (Moore et al., 2002) but can be divided into two discrete regions, a dorsomedial “shell” and ventrolateral “core” - based on afferents, gene expression patterns, location and neuro-peptidergic signalling (Abrahamson & Moore 2001; Antle and Silver 2005). The core receives environment input and is the site where terminals of the RHT neurons terminate (Hattar et al., 2006; McNeill et al., 2011). This information is then integrated and transmitted to the dorsomedial shell. Lesions of the SCN core eliminate circadian rhythms in running activity, drinking, body temperature and hormone secretion and this loss occurs even when a large portion of cells in the shell region survive the lesion (Lesauter and Silver 1999; Kriegsfield et al., 2004). While the core has numerous projections to the shell region, reverse projections from the shell to the core are sparse (Leak and Moore 2001; Abrahamson and Moore 2001). Accordingly, when the SCN core is separated from the SCN shell, only the shell loses its ability to maintain rhythmicity while core remains synchronized (Yamaguchi et al., 2003). Collectively these findings suggest that the core receives environmental input and transmits it to the shell and that this core to shell signalling is required to maintain synchronicity in the SCN. The core and shell also differ based on peptidergic content. The ventral core region is comprised of clusters of cells that express vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP). The shell on the other hand is dominated by arginine vasopressin (AVP) expressing neurons (Abrahamson & Moore 2001; Antle and Silver 2005). Projection from the retino- hypothalamic tract thus, terminate primarily on the VIP and GRP expressing cells (Lokshin et al., 2015). Both these cell groups are thus

implicated in photic entrainment and responsivity to light (Fan et al., 2015). VIP and GRP cells form dense ipsilateral and contralateral connections to AVP cells to relay photic information, but return connections are sparse (Albus et al., 2005). AVP cells also receive retinal innervation but to a much lower degree and receive a proportionally higher innervation from limbic structures (Lesauter and Silver 1999; Kriegsfield et al., 2004).

Cells in the SCN core exhibit low amplitude rhythms in electrical firing rate and clock gene expression. However, they do exhibit light-dependent increases in the expression of *Per1* and *Per2* genes (Yan and Silver, 2004; Hamada et al., 2004). Phase-shifts induced by light begin in the core, before spreading to the shell (Nagano et al., 2003; Yan & Okamura 2002, Yan & Silver 2004). Circadian rhythms of immediate early genes *c-fos* and *FRA-2*, that are thought to be necessary for the circadian system to reset in response to light, also show similar patterns of activation. Endogenous circadian expression of *c-fos* and *FRA-2* occur in the shell, but not in the core, whereas light-induced expression of *c-fos* and *FRA-2* occurs in the core, but not in the shell (Schwartz et al., 2000).

VIP, GRP and AVP neurons have been shown to play a key role in photic responsivity and entrainment. Over a decade of research has implicated VIP as a key player in both circadian behavioural rhythm patterns, response to light input and in maintaining and establishing cell to network level synchrony within the SCN (Colwell et al., 2003; Dragich et al., 2010; Vosko 2007; 2015; Reuss and Decker, 1997). Its effects in the SCN are primarily mediated through the VPAC2 receptors, encoded by the gene *Vipr2* (Cutler et al., 2003). While VIP mRNA levels are rhythmic in DD and VIP release in rhythmic in LD, VIP release in the SCN is arrhythmic in DD (Francl et al.,

2010; Dardente et al., 2004; Laemle et al., 1995). VIP administered to the SCN in-vivo and in-vitro resets circadian rhythms of behaviour, gene expression, and neuropeptide release, in a phase dependent manner that mimics light (Dardente et al., 2004; Piggins et al., 1995; Reed et al., 2001; Dragich et al., 2010; Meyer-Spasche et al., 2004; Cutler et al., 2003; Itri & Colwell 2003; Pakhotin et al., 2006; Kudo et al., 2013). During the subjective day and early subjective night, VIP dose dependently delays circadian rhythms of firing rates in the SCN, with a maximal effect around subjective dusk (Reed et al., 2001; An et al., 2011, 2012). During the late subjective night and early morning, VIP modestly advances the SCN. VIP has been shown to entrain *Per2* in both neurons and astrocytes (An et al., 2011, 2012; Nielsen et al., 2002; Marpergan et al., 2009; An et al., 2013) and dose dependently decrease expression and amplitude of *Per2* (An et al., 2013). VIP neurons also play a role in the SCN resynchronization after being disassociated from a change in photoperiod (Evans et al., 2013). Generally, VIP neurons respond rapidly to a shift in the light cycle whereas AVP neurons require days (Leak et al., 1999; Kalló et al., 2004; Albus et al., 2005). Transgenic animals with an overexpression of VPAC2 receptors, resynchronized faster to 8-hour phase advances and showed considerably shorter free running periods (Shen et al., 2000).

GRP's effects are mediated through BBR2 receptors (Aida et al., 2002; Karatasoreos et al., 2006). Like VIP, GRP cells in the SCN also receives retinal input and transmit this information to the dorsomedial shell (Tanaka et al., 1997; Abrahamson and Moore, 2001). Light induces immediate-early genes such as *c-Fos* and *Per1* mRNA in most of the GRP-producing SCN neurons (Antle et al., 2005; Hamada et al., 2004; Earnest et al., 1993; Romijn et al., 1996;

Gamble et al., 2007). GRP release in the SCN also shows circadian fluctuation in LD cycles but are non-rhythmic when placed in DD (Zoeller et al., 1992; Shinohara et al., 1993; Dardente et al., 2004; Karatsoreos et al., 2006). GRP cells are not intrinsically rhythmic and lack detectable clock gene expression (Karatsoreos et al., 2004). GRP-deficient mice display blunted phase shifts to light (Aida et al., 2002), however many other components of SCN synchrony appear to not be as strongly affected by GRP loss. While some studies report that GRP antagonists can block GRP induced phase shifts (McArthur et al., 2000) others show that antagonists do not affect GRP induced increases in Per1: GFP or C-fos (Gamble et al., 2007, 2011; Hughes 2005). There is some evidence that GRP induces expression of Per1, Per2, and C-fos in hamsters restricted to cells that form a cap dorsal to the CalB region and lateral to AVP cells (Antle et al., 2005).

AVP cells are intrinsically rhythmic and exhibit high amplitude day-night rhythms in electrical firing rate, clock gene expression, neuropeptide expression and release. They work through V1a/V1b receptors throughout the SCN (Li et al., 2009; An et al., 2012). Output from the shell influences visceral function (Ueyama et al., 1999), REM sleep (Lee et al., 2009), and timing of the luteinizing hormone surge (Kriegsfeld et al., 2012). Loss of V1a/V1b receptor signalling within the SCN enables faster entrainment to a shifted light cycle (Yamaguchi et al., 2013). Animals lacking this receptor re-entrained to 8-hour shifts in the LD cycle in half the time and pharmacological blockade of these receptors produced the same effect, consistent with a role for AVP signalling in influencing rhythmic behaviour (Yamaguchi et al., 2013). In other words, these animals seem resistant to jet-lag, suggesting

that normally AVP cells maintain some level of phase coherence within the SCN, therefore preventing immediate resetting (Yamaguchi et al., 2013). Mice with *Bmal1* deletion specifically in AVP neurons (Mieda et al., 2015), had a lengthened free running period, and duration of activity time. They also showed faster re-entrainment to shifts in the light cycle. The circadian expression of AVP was significantly reduced in the dorsal SCN. Cells in the dorsal SCN also showed PER2::LUC oscillation with highly variable and lengthened periods (Mieda et al., 2015). Similarly, deleting *casein kinase 1 delta (CK1δ)* in AVP neurons, lengthens PER2:LUC and behavioural rhythms, while overexpressing *CK1δ* shortens them (Mieda et al., 2016). The deletion of *Bmal1*, however, disrupted the expression of other neuropeptides in the SCN, not just AVP (Mieda et al., 2015), suggesting that the observed phenotype, is likely a result of effects that encompass more than just AVP neurons.

This functional redundancy between the different peptide expressing cell groups in the SCN is well supported. Firing rates in brain slices from VIPr2-/-mice (lacking VIP receptors), with compromised rhythmicity, is improved when GRP is added to the slice (Brown et al., 2005). Conversely, firing rates are further degraded by the application of GRP receptor antagonists (Brown et al., 2005). One study utilized a novel host/donor graft procedure (Maywood et al., 2011), in which circadian gene expression was first recorded from a mutant VIP-null SCN carrying a genetically encoded bioluminescence reporter, and then a wild-type graft SCN without the luciferase reporter was placed on top of the host SCN. The two were separated by presence of a 10-kDa molecular weight cut off membrane that would prevent neuronal contact and thus ensure that any signalling was paracrine. The host SCN lacked amplitude and

synchrony of circadian gene expression, which was restored by placing it in contact with a VIP intact graft. However, circadian gene expression could also be restored even when the host lacked the VPAC2 receptor and thus was functionally blind to VIP signalling. This restoration was blocked when antagonists against GRP or AVP were added, suggesting both peptides are sufficient to maintain some rhythmicity in the absence of VIP signalling. Behaviourally-compensation by other peptides is supported by evidence that shows that mice lacking VIP or VPAC2 receptors can still maintain entrainment to LD cycles- but when those mice are released into DD the phase of activity onset free runs from an advanced phase relative to wildtype animals (Harmar et al., 2002; Colwell et al., 2003; Piggins and Cutler, 2003; Hughes and Piggins, 2008), suggesting that normal circadian behaviour observed under the LD cycles might represent masking i.e., light suppresses the activity of the endogenous clock. This masking could thus be a result of compensation from GRP or another signalling system within the SCN that maintains gating to light input (Harmar et al., 2002; Colwell et al., 2003; Piggins and Cutler, 2003; Hughes and Piggins, 2008).

Thus, while at the individual cellular level the behaviour of every oscillator can be precisely mapped onto a genetic or proteomic mechanism, at the level of the network and more so at the level of the organism, this becomes more complicated and involves variability. While in many cases of circadian related mutations the behaviour observed is predictable from those factors, in other cases the deficits are less severe, absent or opposing what would be normally predicted. Thus, examining circadian characteristics and behaviours that are vastly different from what is typically recorded can be a useful tool to taking a top down approach to

studying the mechanisms that might underlie non-typical circadian rhythms, that will then potentially help inform us of 1) how certain factors determine circadian behaviour, and how their alteration can cause differences in phenotype, 2) how these factors might underlie alterations and effects observed in circadian challenged states 3) how the circadian network is organized in typical models.

1.3.7: Food anticipatory behaviour and the food entrainable oscillatory.

Finally, just like how food anticipatory activity (FAA) was reviewed separate from other non-photic circadian behaviour, the anatomy that underlies it will also be discussed separately as the loci for FAA appear to be independent of the light driven circadian clock for the most part. The loci that potentially control FAA have been under investigation for decades since it was first discovered. What has made FAA distinguishable, is that while it shows canonical properties of being the output of a circadian clock, it does not appear to be generated or controlled by the SCN. Animals with SCN lesions continue to show FAA behaviour, while losing general locomotor rhythms (Krieger et al., 1977). When the SCN is ablated and food is available ad-libitum, circadian organization is lost in behaviour and physiology. However, when food is restricted to once every 24 hours, for a 2-6 hour period, food anticipatory rhythms emerge and circadian rhythms of physiology are restored (Stephan et al., 1979; Boulos et al., 1980). Animals also anticipate food given on a 25-hour schedule while displaying a free-running period with a different rhythm (Edmonds and Adler 1977). Collectively these findings suggested that that FAA is mediated by a separate circadian oscillator. This is further supported by the fact that

generally FAA persists in animals with mutation in core clock genes including in clock mutant (Clk/Clk) mice, in mice null for Npas2 (a paralog of Clock), Cry1/Cry2, Bmal1 and Per1, Per2 and Per1/Per2 – with only modifications to certain parameters of FAA (such as length or amount; Pitts et al., 2003; Mistlberger et al., 2009; Dudley et al., 2003; Iijima et al., 2005; Van der Zee et al., 2008; Pendergast et al., 2009; Storch and Weitz 2009). Thus, unlike the classical view of the circadian hierarchy, which views the system as being organized into a master circadian clock that controls peripheral oscillators, evidence from feeding studies show that that the circadian system while mainly hierarchical also involves feedback from circadian oscillators downstream from the master, LD-entrained circadian pacemaker in the SCN.

While FAA doesn't require a fully functioning circadian clock, the role of a circadian clock cannot be discounted. For example, *Cryptochrome* (*Cry*)-deficient mice retain FAA however they show alterations in the kinetics and stability of FAA, thus suggesting some circadian control (Iijima et al., 2005). Similarly, animals with SCN lesions while retaining FAA, lose the limits of entrainment to feeding schedules – they entrain to food schedules outside the normal range of entrainment (Stephan et al., 1981; Takasu et al., 2012). Mice with cry deletion Cry1(-/-) mice, known to have short free running periods entrain under restricted feeding to shorter feeding cycles than to mice with mutation in Cry2(-/-) (Takasu et al., 2012). Thus, intrinsic period and entrainment ability of the FAA in these mice was affected similar to that of behavioural activity. These findings, that the FAA appears to be largely independent of the SCN, prompted decades of research to try and localize the food clock that is better known at the food entrainable oscillator **(FEO).** Early research focused on hypothalamic regions that were

known to influence other aspects of feeding and metabolism, including the ventromedial hypothalamus (VMH), paraventricular hypothalamus (PVN), arcuate nucleus (ARC) and lateral hypothalamus (LH; Mistlberger 1994; Mistlberger and Rusak 1988; Davidson et al., 2009). These studies were limited because lesions in this area significantly affected feeding and metabolism and caused weight loss or gain that interfered with movement. However, food anticipatory behaviour (as measured with nose pokes) did increase in anticipation of mealtime, suggesting persistence of FAA. Large lesions to the PVN causes significant obesity, but food anticipatory behaviour persisted in anticipation 2-3 hour before mealtime (Mistlberger and Rusak 1984). Similarly, despite significantly reduced movement throughout the day in animals with LH lesions, there was a proportionally comparable increase prior to expected mealtime to WT animals (Mistlberger and Rusak 1984). Thus, these brain regions were not supported as being necessary for FAA.

Similarly, lesions of the hippocampus and amygdala, the nucleus accumbens, the thalamic nuclei, the IGL, brainstem regions, preoptic area and the neocortex all appear to largely have little to no effect on food anticipatory rhythms (Mistlberger 1994; MIstlberger and Mumby 1992; Davidson et al., 2001; Landry et al., 2007). Some regions like the parabrachial nuclei (Gooley et al., 2006; Davidson et al., 2000), thalamic paraventricular nucleus (Nakahara et al., 2004) and nucleus accumbens (Mendoza et al., 2005) have offered mixed negative results. One study did report a reduction in food anticipatory activity in animals with cerebellum lesions (Mendoza et al., 2010). However, as the cerebellum is an important locus of control for movement, this might be due a general loss of movement ability rather than a

specific effect on mealtime anticipation or memory, especially since endocrine anticipatory activity was unaffected.

The dorsomedial hypothalamus (DMH) has been the focus of much research as a potential site for the FEO (See Mistlberger 2009). However, studies show conflicting results. Ablating cells in the DMH in one study showed an attenuation of FAA that correlated with the size of the lesion (Gooley et al., 2006). However, other studies failed to replicate this and noted fully intact FAA with DMH lesions (Landry et al., 2005, 2006). When the lesion extends, however, to include not only the DMH, but also the ventromedial hypothalamus and arcuate nucleus, an attenuation of FAA is noted (Tahara et al., 2010). But, again, a complete destruction of all these areas significantly impacts many other behaviours that contribute towards feeding behaviour without necessarily underlying anticipation or temporal memory. When these findings are combined with the behavioural findings that animals can entrain to multiple separate meal times (Coleman et al., 1982; Mistlberger et al., 2012; Silver et al., 2011), it suggests that the FEO is not one single locus and not only situated in the central nervous system but instead is a collection of loci throughout the CNS and the periphery that perhaps together co-ordinate feeding, mealtime memory and anticipatory activity.

This is supported by findings that peripheral oscillators that are involved in feeding have long been known to show circadian rhythmicity in-vivo and in-vitro and to be preferentially entrained to the feeding cycle (Mistlberger 2009). When food availability is restricted to a period in the middle of the light period (subjective day), a time when a nocturnal animal is normally inactive and eats little, clock gene rhythms in stomach, intestines, pancreas, liver,

adrenal gland, heart, lungs, muscle and other tissues are shifted, to resynchronize with the daily rhythm of food intake (Schibler et al., 2003; Damiola et al., 2000; Stokkan et al., 2001). A similar realignment with food availability is also observed in other brain areas (Verwey and Amir 2009). However, common feeding related hormones released in these organs are not required for FAA (Mistlberger 2009, 2011). FAA persists in animals lacking adrenal glands, insulin, leptin, ghrelin, cortisol or viscerosensory afferents of the vagus or splanchnic nerves (Stephan et al., 1979; Boulos et al., 1980; Davidson et al., 2002; Comperatore and Stephan 1990; Mistlberger and Marchant 1999; Davidson and Stephan 1998). Additionally, some aspects of FAA such as a memory of mealtime is incompatible with peripheral organ function. When food is restricted to a narrow temporal window FAA emerges. When food is then reintroduced ad-lib, activity returns to being primary nocturnal. If, after a period of time, food is the completely restricted for a few days, FAA emerges and this activity is phased with expected mealtimes on the prior food restriction protocol, i.e. a memory is retained for the food restriction schedule (Rosenwasser et al., 1984). However, clock gene rhythms in peripheral organs remain nocturnally phased (Davidson et al., 2003), suggesting that peripheral organs do not retain memory of a past daytime meal schedule, but instead appear to reflect only the most recent stable cycle of food intake. Another study found that *Per2* mutant mice failed to show behavioural food anticipation, while WT and *Per1* mutant mice retained FAA (Feillet et al., 2006). However, clock gene expression was comparable in peripheral organs. A mutation that abolishes *Per2* function thus appears to abolish anticipation of mealtime, without interfering with peripheral organ synchronization to the feeding cycles. Thus, food anticipation appears to

be the result of co-ordinated activity of multiple areas in the brain and periphery. Considering the importance of food and mealtime on an animal's survival there is conceivably considerable amount of redundancy within this system, such that deficiencies in any one (or multiple) locus will be compensated for by others in order to preserve feeding behaviour.

The anatomy of the SCN (and the extended circadian system) is complex. While circadian behaviour can be precisely mapped and the effects of different cues, lighting cycles and food can be predicted with reliable precision, the mechanisms that underlie these behaviours are either not completely known or in many cases the same loci can underlie different defects. Using models that show atypical circadian phenotypes can thus potentially better inform us of possible underlying differences in cellular heterogeneity, gene expression or networking.

1.4 Part 3: BTBR mouse model and rationale

1.4.1: Circadian disruption, health and the need for novel mouse models

Research has now implicated circadian disruption and disorganization as not only being correlated with a multitude of disease states, but also affecting, on a day-to-day basis, physical and cognitive function (Evans et al., 2013; Abbott et al., 2018). Circadian disruption occurs when the internal circadian clock is mis-aligned with the external environment. The most obvious example of this being the jet-lag experienced when an organism rapidly changes time zones. Circadian disruption can however occur as a result of shift work, irregular lighting

schedules and constant light exposure, which are all symptomatic of modern lifestyles. Workplace conditions increasingly use rotating shift work schedules where much of the population is, for extended periods of time, desynchronized with the external environment. Circadian disruption not only acutely affects productivity and mood, but also has long term health consequences (Karatsoreos 2012). Retired shift workers are reported to have poorer sleep quality, higher risk of diabetes, and hypertension (Guo et al., 2013). Pilots and flight attendants had higher levels of stress hormones, and female flight attendant were found more likely to be diagnosed with breast cancer compared to the general population (Mawson 1998; Kojo et al., 2005) and indiscriminate light exposure is associated with higher incidences of breast cancer (Bauer et al., 2013), temporal lobe atrophy, cardiovascular disease and diabetes (Karatosoreos 2011; Evans et al., 2013). Rotating shift workers more likely suffer from sleepiness and insomnia, decreases in productivity, diabetes, cancer, cardiovascular issues and more on the job accidents (Figueiro and White, 2013), an increase in oxidative stress from the production of free radicals (Faraut et al., 2013) and a dysregulation of stress hormones (Koch et al., 2017). Shift work also has negative effects on reproduction in women resulting in lower birth weights, and higher chances of miscarriage (Gamble et al., 2013). In otherwise healthy subjects' metabolism is diagnosed as pre-diabetic after only 10 days of disrupted light schedules (Nedeltcheva and Scheer 2014; Scheer et al., 2009). Circadian disruption also leads to weight gain and lowered resting metabolism and is implicated as one of the risk factors in obesity (Covassin et al., 2016; Engin et al., 2017) and results of experiments simulating shift work in mice also indicate that frequent shifts in the light cycle lead to weight gain (Karatsoreos

et al., 2011). Rodents maintained on disrupted circadian schedules show decreased life spans, extensive cardiovascular problems and severe renal problems (Martino et al., 2008; Hood et al., 2017). Not only are macrophages and other immune natural killer cells under circadian control, but immune challenges can also affect the circadian system (Cermakian et al., 2013). *Clock* gene mutant mice are obese and have metabolic disease (Turek et al., 2005; Oishi et al., 2006). Thus, both environmental disruption that "strains" the clock and a genetically 'broken' clock, can lead to similar phenotypes.

When an organism is exposed to a large shift in the light cycle (for example flying from north America to Europe or vice versa), the circadian system takes around 7-10 days to resynchronize to the external light cycle and entrain to it. It generally takes an animal longer to the entrain to a phase advance shift than it does to a phase delay (Yan and Silver 2002; Reddy et al., 2002). But in either case a stable phase angle is only established after some days. This is termed jetlag when the internal circadian system is out of sync with the external light cycle. In WT mice subjected to jet lag, *Per1* and *Per2* expression loses rhythmicity on day 1-2, oscillates with low amplitude on days 3-7 and recovers its original circadian expression pattern on day 8 (Yan and Silver 2002). After simulated multi time-zone travel, the SCN core resynchronizes to the shifted light cycle faster than the SCN shell (Nagano et al., 2003, Davidson et al., 2009, Rohling et al., 2011) and this desynchrony is thought to contribute to symptoms of jet-lag. *Per1* (Yamazaki et al., 2000) and *Per2* (Davidson et al., 2009) have both been found to re-entrain faster in the SCN than in any of the other tissues examined. There is also evidence that *Period genes* re-entrain faster than slower-adapting cryptochromes, which potentially acts as a rate-

limiting factor for behavioural adaptation (Reddy et al., 2002). These findings suggest that coordination of clock gene expression is globally disrupted during jet lag.

Sleep and circadian problems, however, also occur independently of induced environmental circadian disruption. Either as stand-alone circadian sleep wake disorders or co-morbid with a variety of neurodevelopmental (Barone et al. 2019; Carmassi et al., 2019) and psychological disorders (Asarnow et al., 2013). Circadian rhythm sleep disorders (CRSDs) are chronic or recurrent patterns of sleep and wake disturbance due to dysfunction of the circadian system, or misalignment between the timing of the endogenous circadian rhythm and external cycles (Zhu and Zee 2012). CRSDs are generally categorized according to the mechanisms that are evidenced to or potentially underlie them : 1) the endogenous circadian clock itself is altered (delayed sleep phase disorder (DSPD), advanced sleep phase disorder (ASPD), irregular sleep wake rhythm, and free-running disorder); 2) the external environment and social circumstances as a result, are altered relative to the endogenous circadian clock (jet lag and shift work disorder as previously reviewed). Genetic and environmental factors interact in the pathophysiology of these disorders. Studies in DSPD patients indicate polymorphisms in multiple clock genes including human(h) *Per3,* hCLOCK*,* h*PER1*, and h*PER2* (Katzenberg et al., 1998; Carpen et al., 2005, 2006). This along with changes entrainment patterns and a small advance portion of the PRC to light (thus changing resetting and entrainment) in DSPD patients appears to cause the sleep problems. A genetic basis has also been demonstrated in familial ASPD. Missense mutations in two different genes are reported in families with ASPD: S662G located within the casein kinase I (CKI) epsilon binding region in h*Per2 (thus controlling rate of*

per degradation) and T44A in *CKIδ* (Toh et al., 2001; Xu et al., 2005). Altering the function of both these enzymes alters the phosphorylation and rate of degradation of the Per genes and therefore changes the length of the period. These enzymes are also implicated in irregular sleep wake cycle disorder along with a decrease response to light. CRSDs (and associated sleep problems) are also found in neurodevelopmental and psychological disorders (Lamont et al., 2007; Jones et al., 2015) not only reduce quality of life and socio-cognitive functioning in patients, but also can exacerbate some of the other symptoms of these conditions themselves. Therefore, sleep and circadian problems either when occurring by themselves are prevalent and significantly impact the patient.

Thus, desynchrony and disruption in circadian function impacts the circadian system at a network and gene level and has chronic, often highly significant health impacts that contribute to severe diseases states. Considering a large proportion of the population partakes in shift - work or is otherwise exposed to irregular lighting schedules, it is pertinent that the negative effects of circadian disruption are recognized, understood and dealt with. In order to understand how a system functions when it is perturbed, it is important to fully understand how the circadian system functions normally. Additionally, endogenously occurring difference in certain behaviours and their underlying mechanisms can provide insight into both how the system functions normally and under stress. While there are many advantages to introducing mutations in specific strains of mice to specifically study the effects of circadian rhythms, in many cases, the mutation does not cause expected behavioural changes and might involve compensatory developmental changes due to the loss of the gene. Another option, however, is

to use models that endogenously (show) patterns in behaviour that are removed from what is normally or conventionally observed. These models can then be used to study adaptations of the system under varying conditions and possible mechanistic bases that contribute to these differences, that in turn can help understand how the system works under wild type conditions.

1.4.2: The BTBR mouse model

The BTBR mouse model was originally bred for studies on insulin-resistance, diabetes-induced nephropathy and phenyloketonuria (Bolivar et al., 2007, Moy et al., 2007, Nadler et al., 2006), but was recognized to display strong and consistent autism spectrum disorder (ASD) related behaviours. As will be described in Appendix 1, our lab originally started using the BTBR model to study the link between Sleep and ASD. Children and adolescents with ASD have many sleep related issues including increased sleep latency, disrupted sleep and insomnia and delayed phase of onset (Glickman et al., 2010; Maloe et al., 2005; Mazurek and Sohl 2016). These deficits not only impede function in patients with ASD but also significantly impact the quality of life and sleep of their caregivers (Glickman et al., 2010). These sleep issues are indicative of an underling circadian problem and a recent study found an increase in polymorphisms in circadian genes in ASD patients (Glickman et al., 2010; Yang et al., 2016). The BTBR mouse model has been used to specifically look at certain behavioural traits, revolving around sociability (Bolivar et al., 2007, Moy et al., 2007, Nadler et al., 2006). But outside of ASD they are used to model other physiological conditions, including other neuro-developmental disorders, learning and memory and insulin resistance and metabolism (See Meyza et al., 2017).

The BTBR strain (BTBR T^{+} *Itpr3*^{tf}/J) derives from the inbred strain BTBR (Black and Tan BRachyury) and carries the mutations a^{t}(nonagouti; black and tan), *Itpr3*^{tf} (inositol 1,4,5-triphosphate receptor 3; tufted), and *T* (brachyury). The strain was developed by L.C. Dunn from stock obtained from Dobrovolskaia-Zavadskaia, who brother-sister mated the stock at Columbia University and inserted *tufted* (*Itpr3*^{tf}) as a marker around 1956. The comparisons between BTBR and C57s was done a decade ago and several single nucleotide polymorphisms (SNPs) were found in the BTBR genetic background (McFarlane et al., 2008), including a nonsynonymous coding region polymorphism in the *Kmo* gene encoding kynurenine 3-hydroxylase - an enzyme that regulates the metabolism of kynurenine acid, a glutamate antagonist.

The BTBR mouse strain also shows a deficiency in cholinergic transmission and this combined with heightened level of kynurenic acid in the prefrontal cortex was correlated with decreased performance in the 5-choice serial reaction time task (McTighe et al., 2013). BTBR mice also almost completely lack a corpus callosum and have decreases in the hippocampal cortex but increases in the anterior commissure (Bohlen et al., 2012; Jones-Davis et al., 2013; Kusek et al., 2007; Wahlsten et al., 2003). This strain additionally shows high circulating baseline corticosterone levels (Benno et al., 2009, Frye and Llaneza, 2010, Silverman et al., 2010) and glucocorticoid receptor mRNA levels in the brain (Silverman et al., 2010) compared to C57Bl/6J mice and 129S1/SvImJ (129S) mice. They also have aberrant immune responses (Careaga et al., 2015) and immune system regulation including higher basal levels of plasma IgG, IgE and anti-brain antibodies (Abs) (Heo et al., 2011) and showed a higher number of mast cells and an

increased proportion of MHC class II-expressing microglial cells in the brain itself along with and upregulation of proinflammatory cytokines such as IL-33, IL-18 and IL-1b both at baseline and in response to environmental stressor (Bakheet et al., 2016 ; Onore et al., 2013) suggesting higher levels of baseline inflammation in this strain. Impairments have also been noted in monoaminergic neurotransmission (Gangi et al., 2016, Muller et al., 2016). BTBR mice have an increased density of 5-HT_{1A} receptor density in the hippocampus (Gould et al., 2011, 2014) and decreased [(3)H] cyanoimipramine and citalopram binding to the serotonin transporter (SERT, Gould et al., 2011). There is also an Increased 8-OH-DPAT-stimulated GTPγS binding in the BTBR hippocampus, which suggests a possible heightened capacity of 5-HT_{1A} receptors to activate G-proteins (Gould et al., 2011). Additionally, the density of hippocampal 5-HT axons was reduced in the BTBR mice (Guo and Commons, 2016), which is compensated by an increase in 5-HT cells in the media raphe. Modulating 5-HT signalling, using antagonists, or agonists, has shown to improve some of the repetitive behaviour and social cognitive deficits in BTBR mice (Gould et al., 2011; Amodeo et al., 2016; Chadman 2011). The reward system in BTBR mice also appears to be altered (Squillace et al., 2014). The study found that dopaminergic neurotransmission was altered - while D_1receptor activation appears intact, D_2 receptor function is impaired. This causes a hypoactivation of the reward system when measured in a functional magnetic resonance imaging (fMRI) scan of dopamine reuptake inhibitor challenge. Thus, BTBR mice differ on many aspects when compared to C57Bl/6J mice both centrally and peripherally. As the C57 strain is the most commonly used mouse model to study WT circadian phenotypes, the BTBR model provides the opportunity to study circadian phenotypes and

biology in the presence of all these differences. Importantly, many of the dysregulations found in the BTBR model are in systems (5-HT, HPA axis, Ach, insulin and metabolism) that are directly implicated in circadian entrainment, resetting and food entrainment, making the BTBR model potentially useful to isolate mechanisms underlying these behaviours.

One intervention that has had positive effects on multiple impairments in the BTBR is the ketogenic – high fat, high protein, low carbohydrate - diet (Smith et al., 2015; Ruskin et al., 2013). While looking at the effects of the ketogenic diet on changes in rest and activity in a LD cycle, in the context of ASD (Appendix1), we noticed that the endogenous FRP of BTBR mice was vastly different than the normally observed range of FRPs in C57 (and other strains of) mice. These endogenously occurring differences are extremely useful because it provides a bottom up approach into understanding the circadian system. Examining differences in behaviour and eventually the mechanistic causes of this differences can better help us understand factors the circadian system. This is opposed to the top-down approach of manipulating certain genes or other clock related factors. This combined with the aforementioned changes in signalling in systems in the BTBR mouse strain made it a promising model to study differences in circadian parameters and the possible mechanistic causes that might underlie them. Based on the initial findings we aimed to confirm circadian behaviour in constant darkness (DD) and then followed with an examination of circadian behaviour in constant light (LL) and circadian entrainment in stable and shifting LD cycles. We also tested food entrainment in the BTBR and finally examined SCN anatomy, looking at differences in VIP, AVP and GRP cells in the BTBR strain when compared to the C57Bl/6J strain.

Chapter 2: General Methods

2.1 Animals

A total of 50 adult male BTBR *T*$^{+}$ *Itpr3*^{tf}/J (BTBR; Alberta children's hospital and research institute; Jackson Laboratories, USA) and 55 C57Bl/6J (University of Calgary Life and Environmental Science Animal Resource Centre) mice were used for this study. Animals were at least 3 weeks old and ~25g upon arrival in the laboratory. All experiments were conducted on animals between 3-8 months of age and animals were age matched for all experiments. When not collecting data, mice were group housed in polycarbonate cages, with up to 4 mice per cage. When collecting wheel running data mice were individually housed in Nalgene Type L clear polycarbonate cages (30.3 cm long × 20.6cm wide × 26 cm high; Nalg Nunc International, Rochester, NY), equipped with a stainless-steel running wheel (diameter of 24.2 cm). Animals were maintained in a 12:12 light: dark cycle until the start of experiments, during which they were first exposed to the light cycle appropriate for that experiment for at least 3 weeks. Animals had ad-libitum access to food (Purina Lab Diet 5001) at all times, except during the schedules feeding studies, and were housed in temperature (21 +/- 1 °C) and humidity-controlled rooms. Cages were changed approximately every 14-18 days and at least a week prior to experimental manipulations. All manipulations and husbandry during dark periods were performed using night-vision goggles (General Starlight Company, Richmond Hill, Ontario, Canada). All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care for the ethical use of animals in research.

2.2 General procedure for circadian behaviour and data collection

Animals were housed individually in wheeled cages and when first put into wheels all animals were allowed at least 3 weeks to acclimatize before any data collection or manipulations were conducted. Between light cycles and between manipulations animals were allowed to entrain to a 12:12 light/dark cycle (LD) for at least 2 weeks and similarly were allowed to free run in constant conditions for at least 2 weeks before collecting data for analyses or performing any manipulations. Animals were housed in cages equipped with running wheels (diameter of 24.2 cm). Rotation of the running wheels was monitored by magnetic switches attached to the wheels and data collected and analysed using Clocklab acquisition and analysis software respectively (Actimetrics, Wilmette, IL, USA). Since arousal inducing procedures such as cages changes, wheel changes and handling can constitute as non-photic cues and cause phase shifts if they fall at the mid subjective day portion of the PRC (Tahara et al., 2015; Mrosovsky 1988). Cage and wheel changes were performed a minimum of 5- 7 days before any manipulation and at least 10 days after. Cage and wheel changes were never performed during a period of manipulation (for example: during a period of data collection after a phase shift). A light meter was also present in every room, which was also connected to the ClockLab collection set-up, and light status on recording rooms were monitored to ensure that there was no light pollution during dark periods.

2.3 Review of circadian parameters

2.3.1 Time points

For circadian research in rodents wheel running is the most commonly used to record rest/activity rhythms . Animals are most commonly housed either in a Light/Dark **(LD)** cycle, in constant darkness **(DD)** or constant light **(LL)** and these are the three lighting conditions employed in these experiments. When housed in a LD cycle, mice were maintained in a 12-hour light and 12-hour dark cycle **(12:12 LD)**. By conventions time points during a light cycle is referred to with Zeitgeber times **(ZT)** with **ZT 12** being time of lights off and **ZT 0** being the time of lights on. A nocturnal animals activity onset will thus coincide with **ZT 12** at time of lights off. ZT 18, for example, is 6 hours after lights off and **ZT 4** is 8 hours prior to light off. During periods of DD, the animals were housed in complete darkness with no dim light exposure or red-light exposure. By convention during DD the time points for circadian activity are based off an animal's own activity. By convention circadian times **(CT)** are used and **CT 12** is the onset of an animal's activity. **CT 18** would thus be 6 hours after activity onset and **CT 4** would 8 hours preceding an animal's activity onset. The same conventions that hold for CT time in DD also hold in LL. CT times are calculated based on an average of at least 7 days of activity using regression analyses and is performed using ClockLab.

2.3.2 Onset of activity

Activity counts were exported in 10 min bins and were only used if a bin had more than 50 revolutions and was followed by another 10-min bin with at least 100 revolutions. Activity onset is plotted to that bout of activity which is sustained for a minimum of 20 mins and has a

minimum of 150 revolution. These parameters for onset are used to calculate activity onset for all analyses. Activity offset is that bout of activity after which no further activity occurs for at least an hour. These parameters are pre-programmed into ClockLab and thus automatically used on all data. Generally, for all manipulations and analyses activity onset was taken as an average over at least 7 days, to account for day to day variability in phase of onset. When using activity onsets to the predict onsets for phase shifting manipulation (see below) a regression line is fitted to 7 days of activity onsets preceding the manipulation, using ClockLab. Similar procedures are followed for offset. However, when calculating the length /duration (Alpha) of an animal's activity either in LD or DD (see below), the difference between activity onset and activity offset is calculated for each day and that difference is then averaged over a period of at least a week.

2.3.3 Free running period

Free running period **(FRP)** refers to the frequency of the animal's day based off daily activity onset, the length of which is denoted by *tau*. If onset of activity is plotted daily and regression analysis performed on these onsets, we are given the length of the daily cycle. FRP is thus based on multiple days of activity, with the minimum being 7 days and is calculated in hours. FRP can tracked both in DD and in LL. While it can be measured in L:D cycles, this is not usually necessary except for those L:D cycles that are outside the "limits of entrainment", such that the animals FRP cannot synchronize to the external T cycle. For these the animal's FRP will either scallop (in repeated attempts to re-entrain to the governing light cycle) or its

endogenous FRP will take over and it will just free-run through the light cycle. For cycle within the range of entrainment however the FRP will coincide with that of the Zeitgeber cycle.

2.3.4 The phase angle of entrainment

Phase angle of entrainment is the value given to the difference (in time) between a defined phase of the internal rhythm (for example, the onset of an animal's activity or the peak blood concentration of an endocrine factor) and a defined phase of the entraining external rhythm (for example, onset of darkness in an LD cycle). In broader terms it is the difference between two cycles of the same frequency (in this case circadian). For example, the phase angle of entrainment to a light cycle is usually calculated as the average of the onset of the animals activity minus the onset of the dark or light period. A positive phase angle is one where the animal's activity preceded the defined zeitgeber phase, and a negative phase angle is one where follows the zeitgeber phase after a delay. Entrainment and phase angle go hand in hand – an animal's internal rhythm is said to be entrained to the external rhythm when a stable phase angle difference between two synchronized rhythms is established. To calculate phase angle, we calculated the difference between lights off and activity onset for each day, over a period of at least 7 days. This was then averaged to measure the average phase angle of entrainment for each animal to that particular entraining cycle.

2.3.5 The duration of the active phase - alpha

Alpha in the context of circadian rhythms **is** the duration of an animal's activity. This is time difference between onset and offset of the animal's activity. The alpha for each day is then averaged over a period of at least 10 days. The length of the circadian day can change over the life span or due to exposure to light cycles. Generally, a short FRP is correlated with a short alpha – a shorter day length coincides with a shorter frequency of occurrence.

2.3.6 Phase shifts

Photic and non-photic cues both reset the clock, this resetting takes form of a phase shift that involves a change in phase of the onset of activity on the days following exposure to the cue in question. Phase shifting responses are dependent on both the type of cue and the time at which it is given. Phase shifts can be graphed as a phase response curve with circadian phase along the x-axis and phase shift along the y-axis with advances being given positive values and delays negative values. Light has no effect during the day but can phase delay and phase advance the circadian clock during the early and late night, respectively. In mice, phase advancing light pulses are administered at CT 22 and phase delaying light pulses are delivered CT 16 (i.e. 10 and 4 hours after onset of activity respectively). Phase shifts are then calculated using Clocklab. A regression line is plotted to the activity onsets on the 5-7 days preceding the light pulses and another regression line to days 3-10 after the light pulses. Days 1 and 2 are generally not included to account for any transient effects. Phase shifts are calculated in circadian hours, to compensate for any changes in tau.

Same procedures are used for non-photic phase shifts. Dark pulses were administered to animals in LL to measure non-photic phase shifts (Chapter 4). Circadian times were calculated as they are in DD and dark pulses were administered from CT 5- CT 11 (i.e. for 6 hours starting 7 hours before predicted activity onset in LL). Phase shifts to dark pulses were then calculated using the procedures outlined above for light pulses in DD.

2.3.7 Total activity

Total activity is total number of wheel revolution for the animal either on a particular day, a particular time frame (for example: total activity during dark pulses) or over a range of days. For our experiments, total activity was calculated for the entire circadian day (as opposed to splitting activity into alpha (subjective night) and rho (subjective day) components). When calculating total activity as a general measure, used total daily wheel revolutions over at least 10 days collected in 10 min bins. When total activity is calculated for a portion of the day (light or dark cycle, animal's subject night etc), onsets and alphas (to determine the time to calculate wheel revolutions from) are calculated using the same parameters as above (Section 2.3.2).

2.3.8 Food anticipatory rhythms

Food anticipatory rhythms (FAA) are typically quantified by summing the amount of activity occurring 1–3 h prior to a daily meal and expressing this as a ratio relative to total 24 h activity (or to nocturnal activity), and then as a percent change from the ratio when food was available ad-libitum. This is an amplitude measure. The raw activity count or ratio may be significantly reduced in animals bearing a lesion or gene defect, but if the ratio is significantly

different from the ad-lib food access condition, then the timing mechanism is operating, and may be fully intact. To calculate onsets of activity during FAA (to measure total activity during that period and to measure alpha of FAA) same parameters were used as above for onsets, total activity and alpha.

Chapter 3: General circadian behaviour in constant darkness and constant light

3.1: Introduction

An animal's circadian behaviour in constant darkness is a non-invasive and reliable method of obtaining information about the endogenous clock. In the absence of any external cues, such as the external *Light/Dark (LD)* cycle, circadian behavioural rhythms are determined by the suprachiasmatic nucleus (SCN). In mammals, under conditions of constant darkness, the endogenous circadian period is approximately 24 hours. This results from one completed cycle of the transcriptional translational feedback loop (TTFL) that regulates clock gene expression, where transcription factors CLOCK and BMAL initiate transcription of clock genes Period *(Per)* and Cryptochrome *(Cry)*, whose protein products dimerize, translocate to the nucleus and inhibit the activity of CLOCK and BMAL – thereby inhibiting their own transcription. PER/CRY then degrade over the latter part of the 24-hour cycle, thereby releasing CLOCK and BMAL from inhibition and the cycle starts anew (Takahashi 2017). This endogenous molecular oscillation drives daily oscillations in physiology and behaviour. In constant environmental conditions, these rhythms show periods close to, but not equal to, 24h. Since the period expressed is that of a clock running free of time cues, this is referred to as the *free running period (FRP)*. The FRP exhibits species-typical patterns. Human FRPs are slighter longer than 24 hours, whereas mouse FRPs are slightly shorter (Pittendrigh and Daan 1976; Aschoff 1981). Other circadian parameters in constant darkness, such as duration of activity (alpha), total activity and phase shifts to brief periods of exposure to light, are also equally important because together with the FRP they

determine the temporal window of light responsivity, how the circadian system entrains to external cycles and how the circadian system re-entrains after a shift in the cycle (Johnson et al., 2003; Pittendrigh and Daan 1976)

Preliminary findings from a pilot study exploring the effects of a ketogenic diet (Appendix A) on circadian rhythmicity in BTBR mice showed, that compared to C57s, BTBR mice had very short FRPs of approximately 22 hours. Short FRPs have only been reported with targeted mutations of various components of the TTFL within the circadian clock and endogenously were found only as a result of a spontaneous tau mutation (Ralph and Menaker 1990; Cermakian et al., 2001; Lowrey et al., 2000; Shearmen et al., 2007 ; Colwell et al., 2003; van der Horst et al., 1999) and in most of these cases the shortening of the FRP was not as pronounced as what was observed in the BTBR strain. Thus, an animal model with a circadian period that is short without targeted engineering is a significant find, both from the perspective of characterizing the impact it may have on other circadian parameters, and from a mechanistic perspective - finding the mechanisms of this difference might inform us better about the nature of the circadian clock. Therefore, first: we wanted to replicate those findings to ensure it was not just a litter effect. Second: If the FRP in BTBRs was indeed as short as ~22 hours, their responsivity to light pulses might be modified.

While constant darkness allows for the expression of endogenous circadian rhythms, constant light (LL) disrupts then. LL is a commonly used model of disrupting the circadian system. LL desynchronizes individual cells within the SCN, disrupts phase relationships between cells, reduces SCN amplitude and weakens the SCN network (Ohta et al., 2005). In LL animals

show a lengthening of FRP, with a gradual loss of rhythmicity over a few weeks (Pittendrigh 1967; Ohta et al., 2005). How circadian behaviour manifests in LL can indirectly provide information about the stability of the circadian clock and its response to external perturbation. Additionally, in constant light animals can be exposed to a brief dark pulse. Dark pulses are interesting because they have both photic and non-photic components and thus can provide information about these components. Additionally, dark pulses inform about sensitivity to light, level of arousal after release from light and the impact of dark pulses on activity following the pulse, all of which helps us understand how the circadian system functions in response to light.

3.2 Methods

3.2.1 Animals

In total 24 BTBR mice and 23 C57Bl/6J mice were used for these series of experiments. Of these, 8 males and 8 female BTBR mice and 6 male and 7 female C57Bl/6J mice were used for DD experiments. Eight male BTBR and 8 Male C57Bl/6J were used for the LL experiments. All mice were received from Dr Rho at ACHRI, University of Calgary.

3.2.2 Procedures

i) General behaviour in constant conditions

Male and female BTBR (n= 8 (m), 8 (f)) and C57Bl/6J (n= 7(m); 8(f)) mice were used to examine circadian behaviour in constant darkness (DD). Animals were allowed to free run in DD for at least 2 weeks before data was used for analysis. Similar procedures were followed for

constant light (LL). For LL experiments male BTBR (n=8) and C57Bl/6J (n=8) were used. Light intensity for during LL experiments was maintained at ~ 200 lux (measured at cage level). Data for analysis were collected for at least 3 weeks and it was ensured that data to be analysed was collected at least a week after cage or wheel changes.

ii) Phase shifting to brief light pulses:

Male BTBR (n=8) and C57Bl/6J (n=7) were first allowed to free run in constant darkness (DD) for at least 3 weeks. They were then subjected, in separate experiments, to an early night light pulse 4 hours after activity onset (CT 16; with CT12 being defined as activity onset by convention) to induce phase delays, or to a late night light pulse 10 hours after activity onset (CT 22) to induce phase advances. Light pulses were 15 mins long and light intensity during the pulse was maintained at 40 lux. After exposure to the light pulse, animals were returned to DD and allowed to free run.

iii) Dark pulse manipulation:

Male BTBR (n=8) and C57Bl/6J (n=8) were allowed to free run in LL for at least 3 weeks. Following this they were subject to a 6-hour dark pulse from CT 5 – CT 11 (i.e. spanning from 7 – 1 hour before the onset of activity), during the animal's mid-subjective day. During the dark pulse animals were exposed to complete darkness (0 lux) and then returned to LL after. Wheel running was continuously monitored during the dark pulse.

iv) Data analysis

Animals were housed in cages equipped with running wheels (diameter of 24.2 cm). Rotation of the running wheels was monitored by magnetic switches attached to the wheels and data collected using Clocklab (Actimetrics, Wilmette, IL, USA). All experiments were timed relative to activity onset, which by convention is defined as circadian time (CT) 12 for nocturnal animals. Circadian period was calculated by fitting a regression line to daily onsets during a 7-day period. Phase shifts were calculated using the standard Clocklab routine as described previously (Sterniczuk et al., 2008). Briefly regression lines were fitted for activity onset a week before the light pulse and for days 3-10 after the light pulse. The first two days after the light pulse were not analysed to allow for transient effects (Pittendrigh and Daan 1976). The post manipulation regression line was then extrapolated back to the day of the light pulse. The difference between the two lines gives the value of the phase shift. Same procedures for predicting onset, calculating time of manipulation and calculating phase shifts were also used for dark pulses experiments and same conventions of CT were used in LL. Calculation of all other circadian parameters followed procedures detailed in Chapter 2. Briefly, total activity was calculated by taking the sum of daily total activity over a 10-day period using Clocklab analysis software (Actimetrics, Wilmette, IL, USA). Duration of activity (alpha) was analysed by comparing the time difference between activity onset and activity offset. Each individual alpha was then averaged over a 10-day period. Activity counts were exported in 10 min bins and were only used if a bin had more than 50 revolutions and was followed by another 10-min bin with at least 100 revolutions. Circadian parameters in DD were analysed using 2 x 2 ANOVAs, for sex

and strain and Phase shifting, LL and dark pulse shift data were analysed between groups(strains) using Independent t-tests and using SPSS software (IBM SPSS 2015).

3.3 Results

A 2x2 ANOVA was conducted to examine the effect of strain and sex on free running period. There was a statistically significant interaction between strain and sex on FRP ($F(1, 25) = 15.52$, $p=0.001$). A significant main effect was found for strain such that BTBR mice had significantly shorter FRPs when compared to C57Bl/6J mice (M= 22.94 +/- 0.29 vs 23.71 +/- 0.15; $F(1, 25)= 127.41$, $p< 0.0001$), but no significant main effect were found for sex, such that FRPs were comparable between male and female mice (M=23.12 +/- 0.55 vs 23.37 +/- 0.35 ; $F(1,25)= 3.29$; $p=0.87$). Planned contrasts revealed that BTBR male mice had shorter FRPs compared to C57Bl/6J males (M = 22.6 +/- 0.10 vs 23.80 +/- 0.07; $t(25)= 10.55$, $p <0.0001$), that BTBR males had significantly shorter FRPs compared to BTBR females (M= 22.6 +/- 0.10 vs 23.14 +/- 0.33; $t(25)= 4.30$, $p <0.0001$ and that BTBR females had significantly shorter FRPs compared to C57Bl/6J females (M = 23.14 +/- 0.33 vs 23.65 +/-0.14 ; $t(25)$ 5.31, $p <0.0001$) and C57Bl/6J males (M= 23.14 +/- 0.33 vs 23.80 +/- 0.07; $t(25)= 6.56$; $p< 0.0001$). However, no significant differences were found when comparing C57Bl/6J males and females (M = 23.80 +/- 0.07 vs 23.65 +/-0.14; $t (25) = 1.43$, $p= 0.165$).

A 2x2 ANOVA was conducted to examine the effect of strain and sex on duration of activity (alpha). There was a statistically significant interaction between strain and sex on alpha ($F (1, 25) = 19.18$, $p< 0.0001$). A significant main effect was found for strain such that BTBR mice had significantly shorter alphas of activity when compared to C57Bl/6J mice (M=7.80 +/- 0.48 v 12.70 +/- 0.68 ; $F (1, 25)= 61.62$, $p< 0.0001$), but no significant main effect were found for sex, such that alphas were comparable between male and female mice (M= 9.93 +/- 1.16 vs 10.22

+/- 0.49; F(1,25)= 0.14; p=0.71). Planned contrasts revealed that BTBR male mice had shorter alphas compared to C57Bl/6J (M = 6.63 +/- 0.51 vs 14.3 +/- 0.98 ; t(25)= 8.71, p <0.0001), that BTBR males had significantly shorter alphas compared to BTBR females (M= 6.63 +/- 0.51 vs 9.13 +/- 0.02 ; t(25)= 3.21, p=0.004), that BTBR females had significantly shorter alphas compared to C57Bl/6J females (M = 9.13 +/- 0.02 vs 11.3 +/- 0.60 ; t(25) 2.44, p =0.022) and that C57Bl/6J males had shorter alphas when compared to females (M = 14.3 +/- 0.98 vs 11.32 +/- 0.60 ; t(25)=2.98, p= 0.006).

A 2x2 ANOVA was conducted to examine the effect of strain and sex on total activity. No significant interaction was found between strain and sex on activity (F (1, 25) = 0.418, p=0.524). A significant main effect was found for strain such that BTBR mice had significantly higher total activity when compared to C57Bl/6J mice (M= 9955 +/- 838 vs 6172 +/- 898; F (1, 25)= 32.24, p< 0.0001), but no significant main effect were found for sex, such that total activity was comparable between male and female mice (M= 7577 +/- 464 vs 8719 +/- 753 ; F(1,25)= 3.66; p=0.67). Planned contrasts revealed that BTBR male mice had higher total activity when compared to C57Bl/6J male mice (M= 9558 +/- 453 vs 5313 +/- 464 ; t(25)= -4.56, p <0.0001), BTBR female mice had higher total activity when compared to C57Bl/6J female mice (M= 10408 +/- 838 vs 7031 +/- 898 ; t(25)= -3.05, p=0.002). No significant differences were found when comparing total activity between C57Bl/6J male and female mice (M= 5313 +/- 464 vs 7031 +/- 898; t (-1.79), p= 0.09), or comparing BTBR male and female mice (M= 9558 +/- 453 vs 10408 +/- 838; t(25)= -0.910, p= 0.37).

Phase shifting responses to late- and early-night light pulses between male BTBR and C57Bl/6J mice were compared using independent t-tests. Results showed that BTBR males mice had comparable phase delaying (M (hr) = 1.72 +/- 0.39 vs 1.25 +/- 0.20 ; t(11)= 1.02 ; p=0.32) and phase advancing responses (M (hr) = -2.30 +/- -0.25 vs -2.10 +/- -1.30 ; t (11) = 0.34, p = 0.73) to C57Bl/6J mice.

Circadian parameter in LL and phase shifts to dark pulses were also compared using independent t-tests. In LL both BTBR and C57 mice had a lengthening of period and FRPs between groups was comparable (M (hrs) = 25.27 +/- 0.19 vs 25.6 +/- 0.093, t (12) = 1.93, p=0.08), but the difference in lengthening was greater in BTBR mice (M (hr) = 2.50 +/- 0.21 vs 1.75 +/- 0.80 ; t (10) = 7.15, p =0.01). BTBR mice also had higher amounts of total activity during the dark pulse period (M= 4926.88 +/- 607.51 vs 2318.01 +/- 888.02; t (10) = 3.16, p =0.01). Results also showed that BTBR mice had no significant differences in phase shifts to dark pulses when compared to C57Bl/6J (M(hrs) = 0.792 +/- 4.65 vs 0.96 +/- 1.5; t (0.36, p = 0.73). However, BTBR animals had more robust phase shifting responses to dark pulses with higher variability in directionality of phase shifting responses. Some animals also showed large Type 0 type resetting responses in the range of 6-8 hours and in total had larger phase shifts to the dark pulses while lacking a directionality (Figure 3F). Qualitatively, the BTBR also had a more variable response to exposure to LL. Some animals lost rhythmicity completely immediately upon exposure, while others lost a significant amount of amplitude in activity while retaining rhythm

Figure 3. 1: **Representative actograms showing average activity profiles of Male C57Bl/6J and BTBR mice (top panel) and female C57Bl/6J and BTBR mice (bottom panel) housed in DD.** Scale represents 24 hours in real time. Actograms are double plotted i.e. each circadian day is represented twice. Day1 is thus followed by day 2 on the same line (right side of the actogram) and on the succeeding line (same side of actogram)

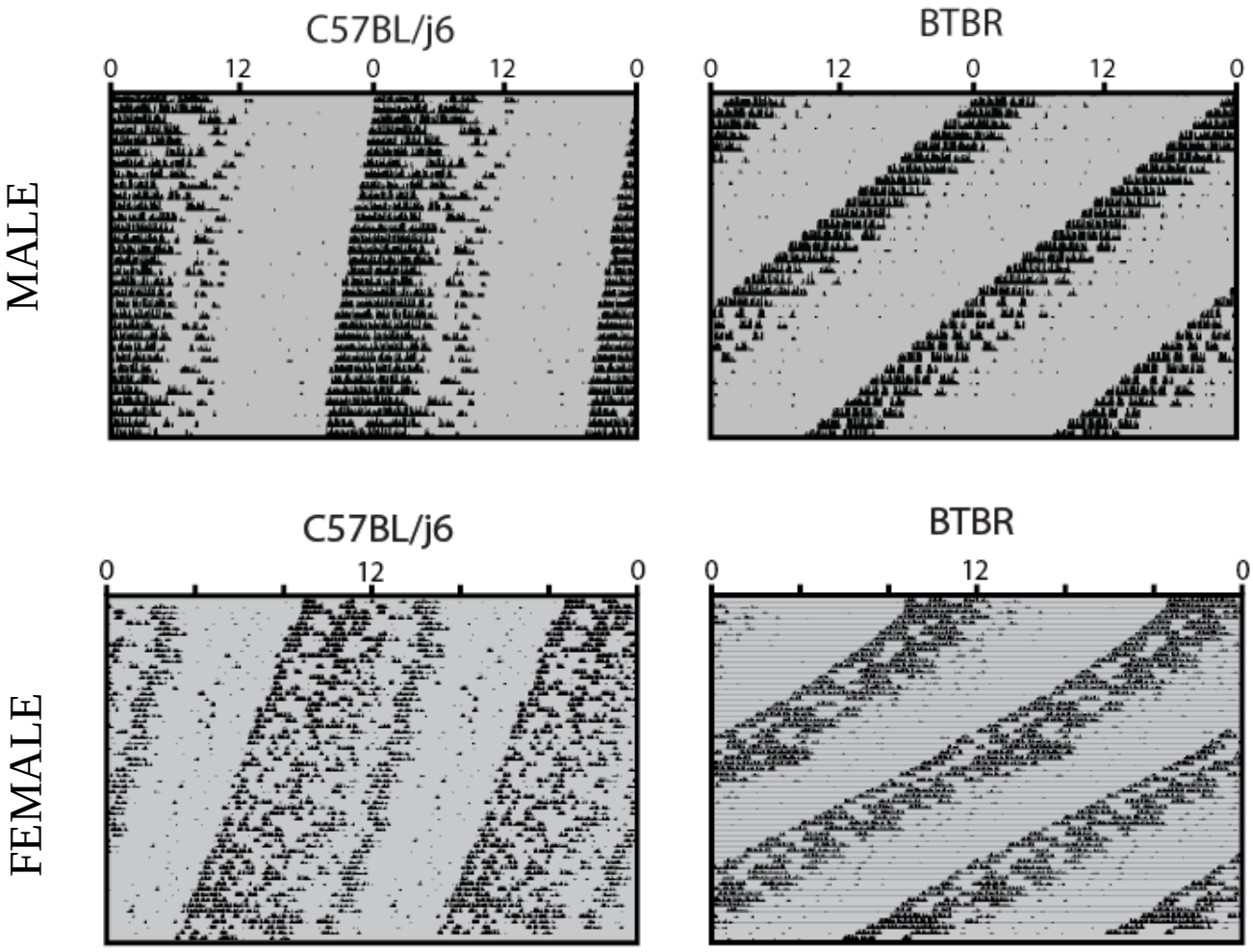

C57BL/j6
BTBR
C57BL/j6
BTBR
MALE
FEMALE
0
12
0
12
0
0
12
0
12
0
0
12
0
0
12
0

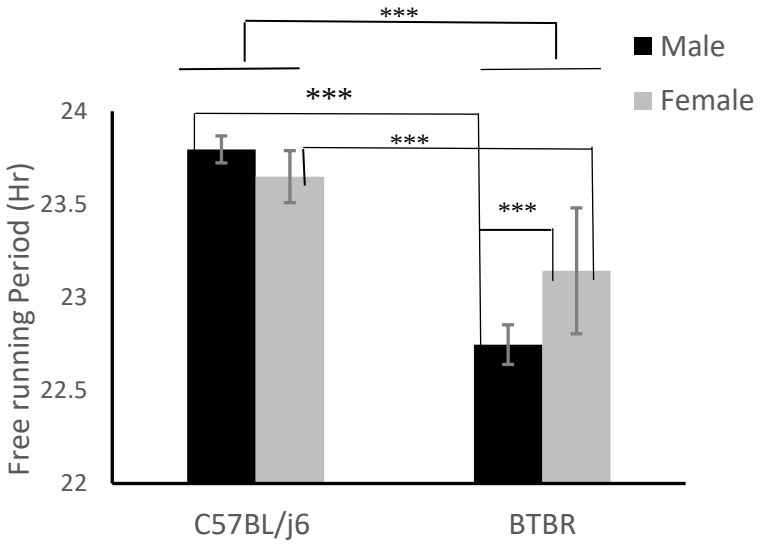
Figure 3. 2 A) bar graph showing differences in circadian Free running period (FRP) between male and female C57Bl/6J and BTBR mice. BTBR male mice had significantly shorter FRPs compared to C57Bl/6J ($p < 0.0001$) male mice and BTBR female mice ($p < 0.0001$). BTBR female mice had significantly shorter FRPs compared to C57Bl/6J male ($p < 0.0001$) and female mice ($p < 0.0001$. No differences were found between C57Bl/6J male and female mice ($p = 0.165$). An overall main effect was also found between strains, such that BTBR mice overall had lower FRPs compared to C57Bl/6J mice ($p < 0.0001$)

B) bar graph showing differences in duration of activity (alpha) between male and female C57Bl/6J and BTBR mice. BTBR male mice had significantly shorter alphas compared to C57Bl/6J ($p < 0.0001$) male mice and BTBR female mice ($p < 0.0001$). BTBR female mice had significantly shorter alphas compared to C57Bl/6J males ($p < 0.0001$) and female mice ($p < 0.0001$). An overall main effect was also found between strains, such that BTBR mice overall had shorter alphas compared to C57Bl/6J mice ($p < 0.0001$)

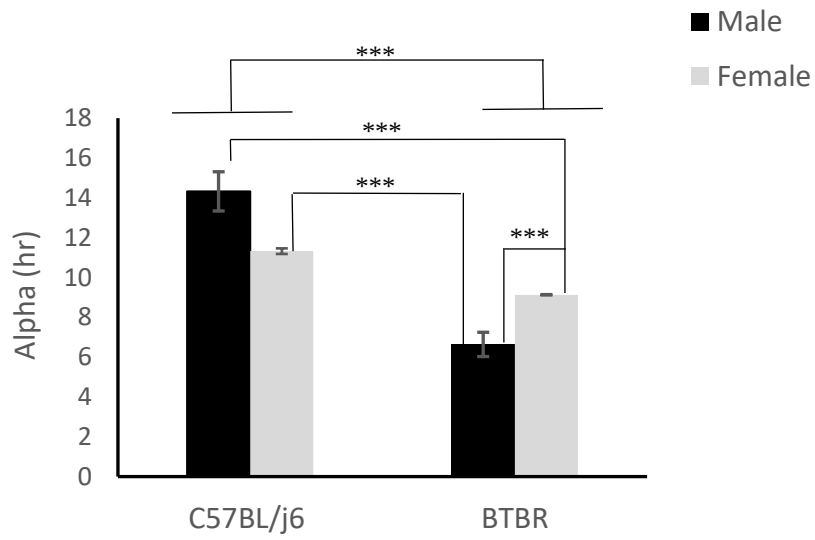
C) bar graph showing differences in total wheel running activity between male and female C57Bl/6J and BTBR mice. BTBR male mice had significantly higher total wheel running activity compared to C57Bl/6J ($p < 0.0001$) male mice and BTBR female mice had significantly higher total wheel running activity compared to C57Bl/6J female mice ($p=0.002$). But no differences were found when comparing BTBR male and female mice ($p = 0.37$) or C57Bl/6J male and female mice ($p = 0.09$). An overall main effect was also found between strains, such that BTBR mice overall had higher total wheel running activity compared to C57Bl/6J mice ($p < 0.0001$).

*** indicate $p<0.0001$, ** indicate $p<0.001$, * indicate $p<0$

A)


***
Male
Female
***
***
***
24
23.5
23
22.5
22
Free running Period (Hr)
C57BL/j6
BTBR

B)


Male
Female
***
***
***
***
18
16
14
12
10
8
6
4
2
0
Alpha (hr)
C57BL/j6
BTBR

C)

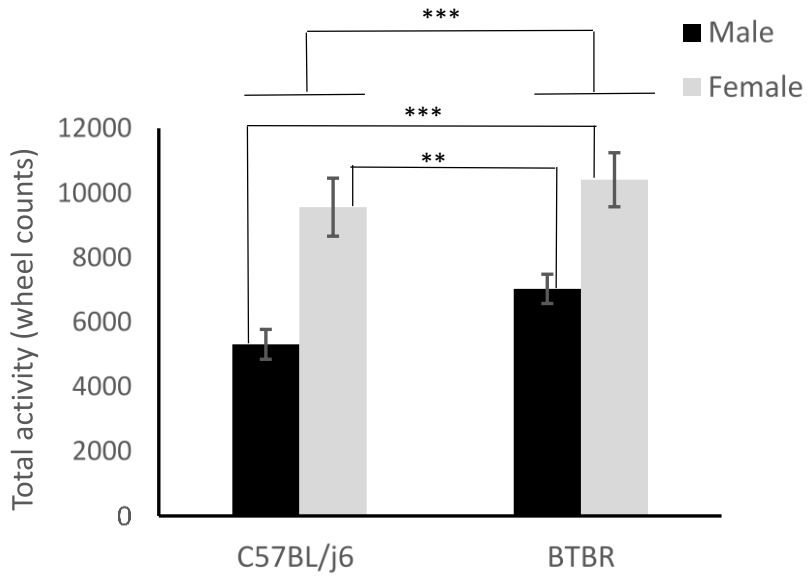

***
Male
Female
***
**
12000
10000
8000
6000
4000
2000
0
Total activity (wheel counts)
C57BL/j6
BTBR

Figure 3. 3: Representative actograms showing phase delays (Top panel) to a light pulse given at CT 16 (Yellow circle ; CT 12 by convention representing the times of activity onset) and phase advances (bottom panel) for a light pulse given at CT 22 – for C57Bl/6J and BTBR mice. The red and blue lines represent the regression lines fitted to activity onset before the light pulse and from days 3-10 after. The difference between the two represent the phase shift.

PHASE DELAYS

PHASE ADVANCES

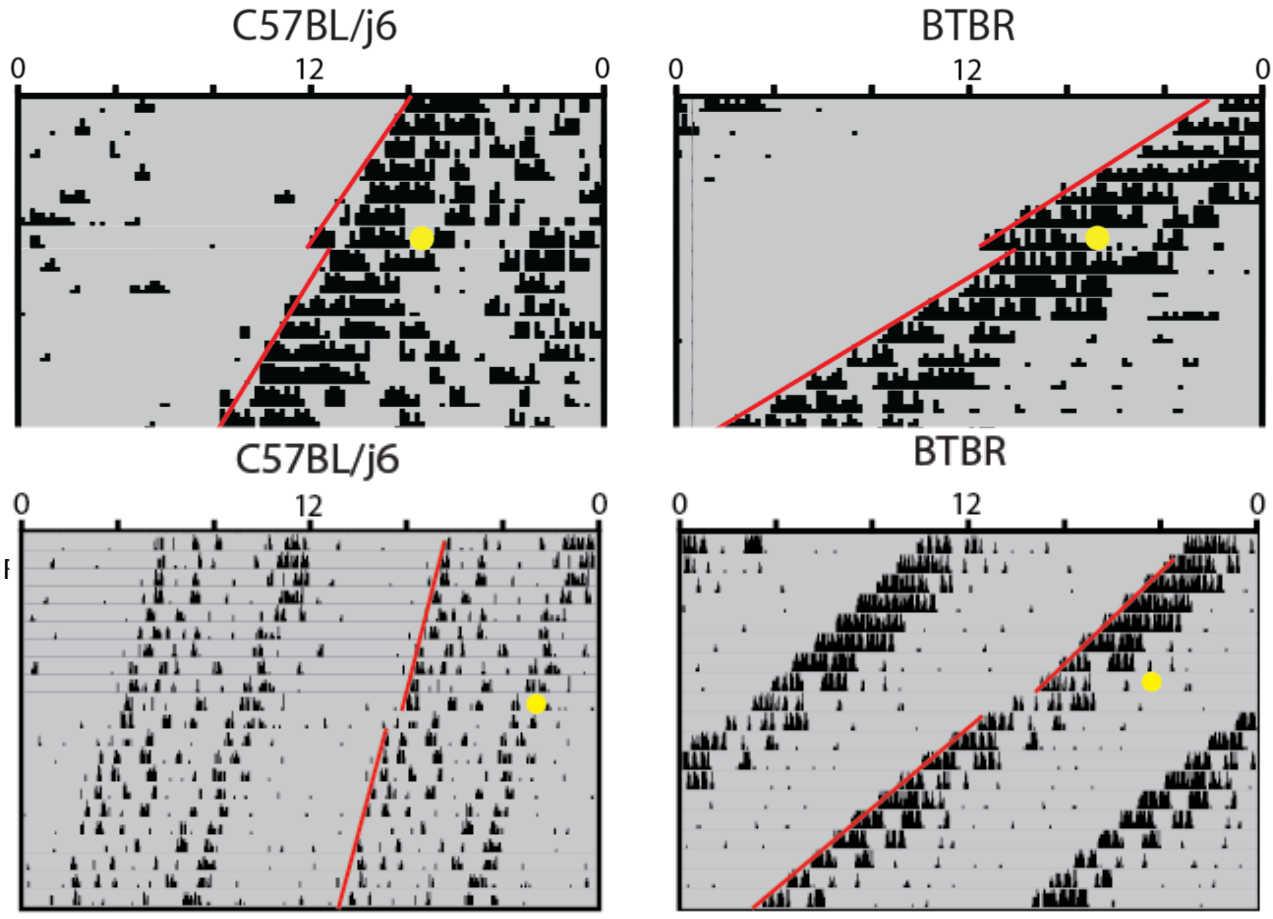
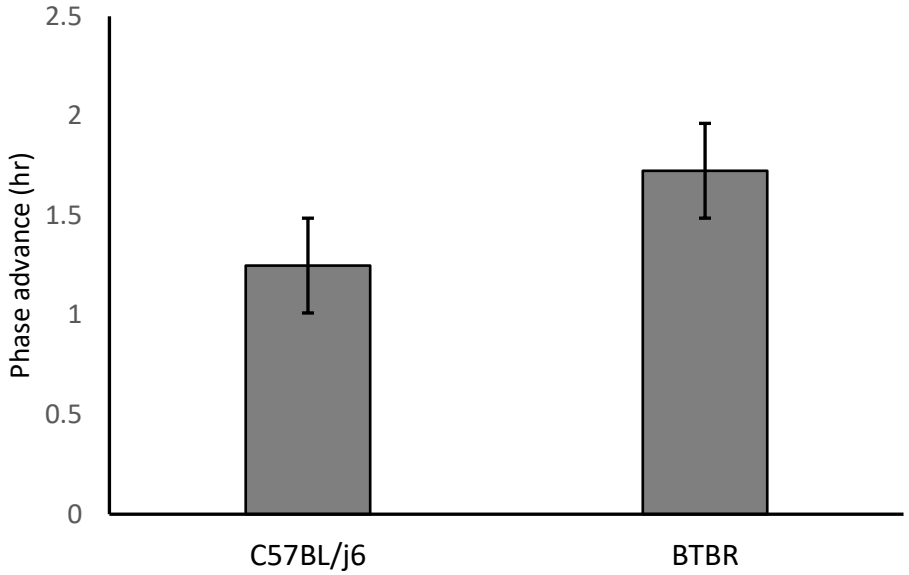


Figure 3. 4: Bar graphs showing phase delays (Figure 3.4 A) to a light pulse at CT 16 and phase advances (Figure 3.4 B) in C57Bl/6J and BTBR mice. BTBR mice and C57Bl/6J mice had comparable phase delays (p= 0.32 and phase advances (p= 0.73)

A)


2.5
2
1.5
1
0.5
0
Phase advance (hr)
C57BL/j6
BTBR

B)

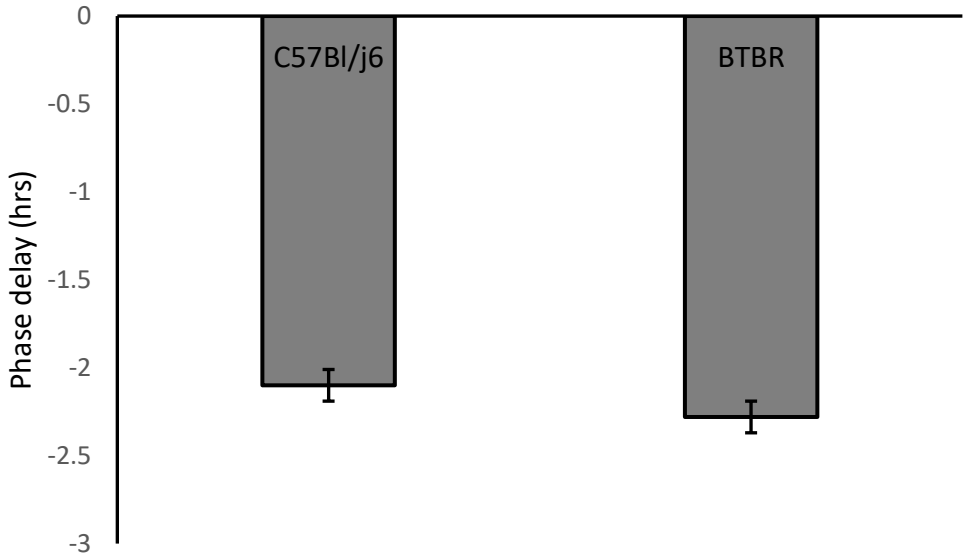

0
-0.5
-1
-1.5
-2
-2.5
-3
Phase delay (hrs)
C57Bl/j6
BTBR

Figure 3. 5: A) Representative double plotted actogram showing circadian behaviour in constant light LL. The scale represents 48 hours, with day 1 followed by day 2 on the same line and day 1 followed by day 2 on the succeeding line. The green box represents the timing of the dark pulse (6 hours). Phase shifts were calculated as they were for responses to brief light pulses.

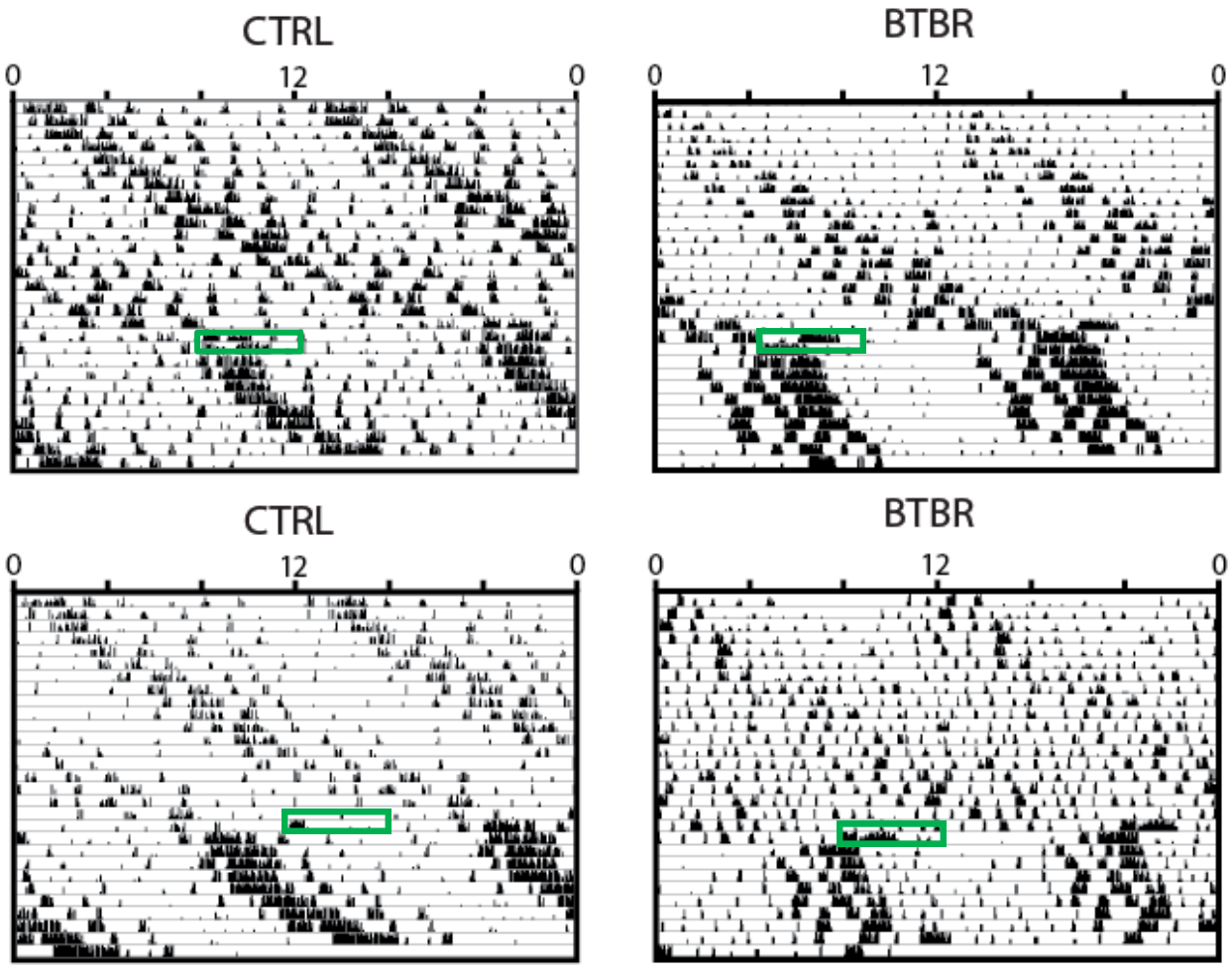
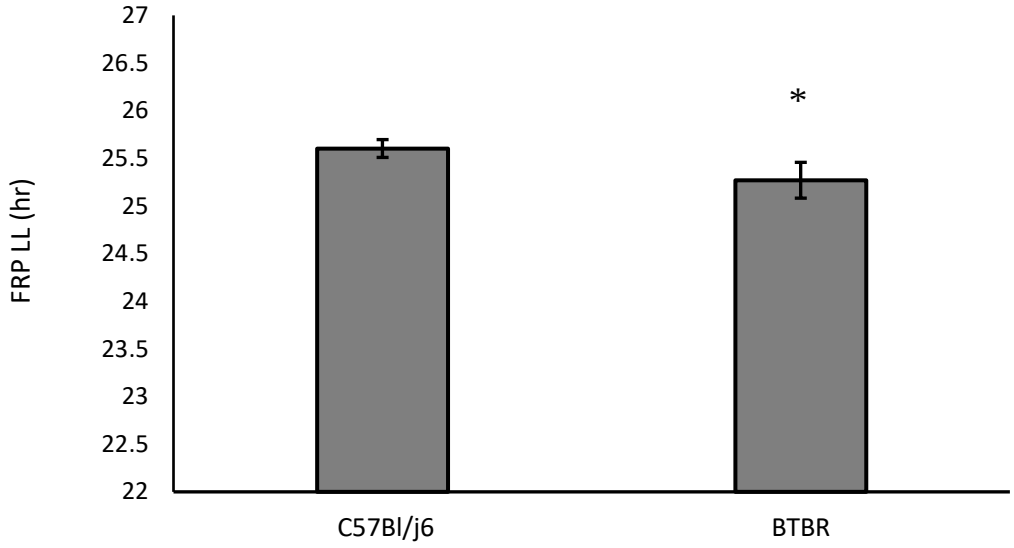
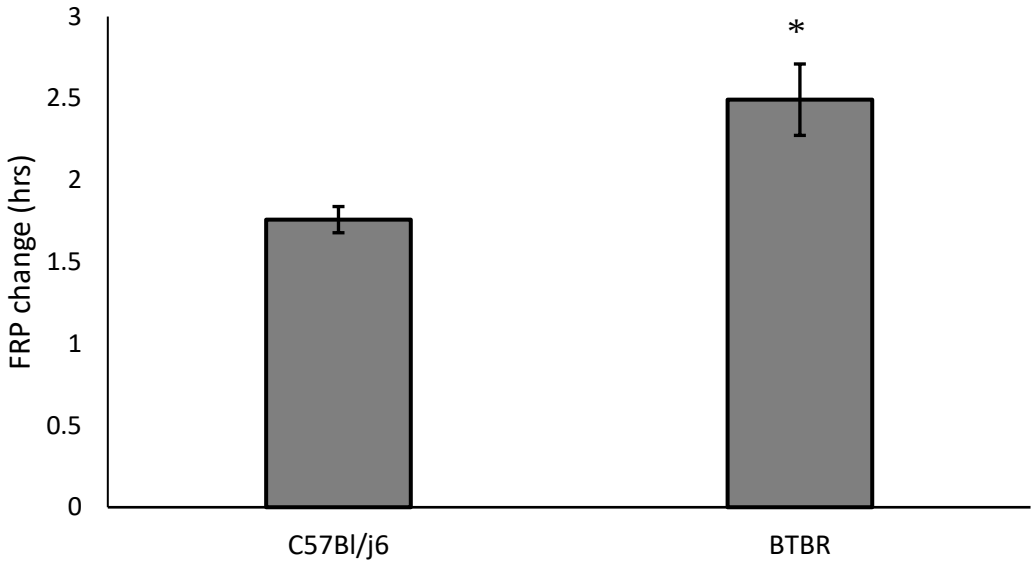

CTRL
0
12
0
BTBR
0
12
0
CTRL
0
12
0
BTBR
0
12
0

Figure 3. 6: **Graphs showing difference between BTBR mice and C57Bl/6J on circadian parameters in Constant light (LL).** Both groups mice had comparable FRPs in LL (**A,** p = 0.08), however BTBR mice showed a greater amount of period FRP lengthening (**B** ; p= 0.01), had greater amounts of activity during the dark pulses (**C**, **D**; p=0.01) and had more variability in their phase shifting responses but no significant differences when compared to C57 (**D**; p= 0.73)

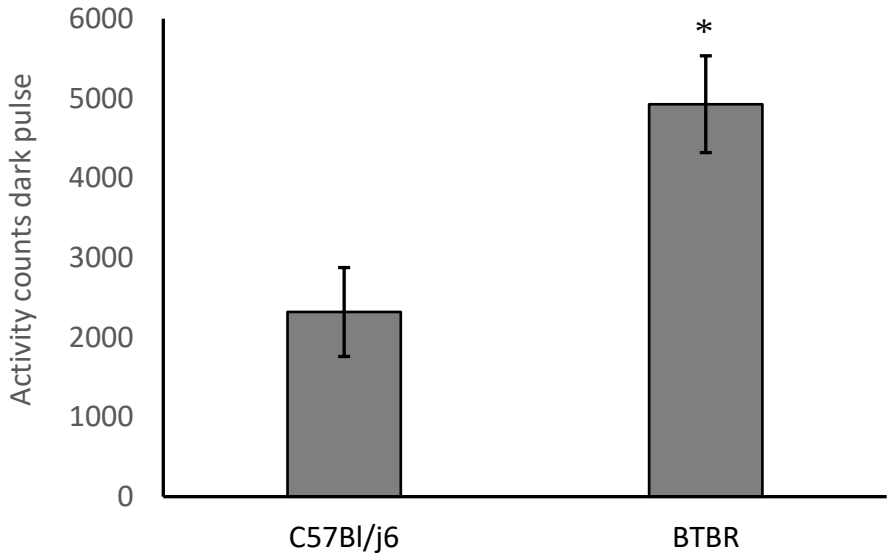
A


27
26.5
26
25.5
25
24.5
24
23.5
23
22.5
22
FRP LL (hr)
*
C57Bl/j6
BTBR

B


3
2.5
2
1.5
1
0.5
0
FRP change (hrs)
*
C57Bl/j6
BTBR

C)


6000
5000
4000
3000
2000
1000
0
Activity counts dark pulse
*
C57Bl/j6
BTBR

D)

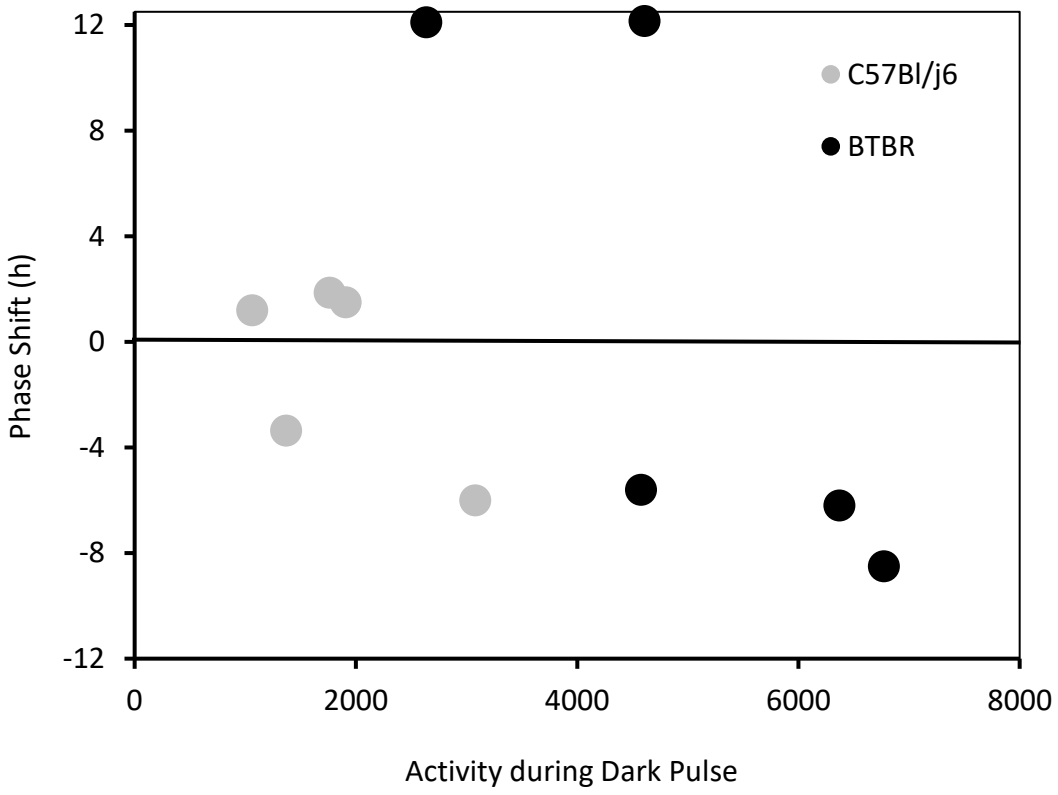

12
8
4
0
-4
-8
-12
Phase Shift (h)
C57Bl/j6
BTBR
0
2000
4000
6000
8000
Activity during Dark Pulse

Table 3. 1: Summary of values for all parameters that were compared in both BTBR and C57 mice under constant conditions. Table does not indicate interaction between sex and strain that was present. * indicates parameters that were examined in both males and females.

	C57BL/j6	BTBR	Significantly different
FRP male*	23.8 hr	22.6 hr	Yes
FRP female*	23.65 hr	23.14 hr	Yes
Total activity male*	5315 revolutions	9558 revolutions	Yes
Total activity female*	7031 revolutions	10408 revolutions	Yes
Alpha male*	14.3 hr	6.63 hr	Yes
Alpha female*	11.3hr	9.13 hr	Yes
Phase delay	1.25 hr	1.72 hr	No
Phase advance	-1.3 hr	-2.3 hr	No
FRP lengthening LL	1.7 hr	2.5 hr	Yes
FRP LL	25.6 hr	25.27hr	No
Dark Pulse activity	888 revolutions	4926 revolutions	Yes
Dark pulse phase shift	-1.5 hr	0.79 hr	No

4.4: Discussion

Most of what can be gathered about the endogenous functioning of the circadian clock and its role in behaviour and physiology can be gathered by characterizing circadian behaviour under constant conditions. In the absence of light, the activity observed is the endogenous activity driven solely by the circadian clock. In constant darkness the period, alpha, total activity, and responses to light pulses are good indicators of circadian function.

Generally circadian FRP, while variable, tends to be normally distributed around 23.7 hours in mice (Pittendrigh and Daan 1976). While all C57BL/6J mice showed circadian periods around this species typical mean, the BTBR strain consistently showed a short period phenotype, with an FRP of more than an hour shorter. This was consistent across all experiments and all groups of BTBR males and females. Additionally, the FRP observed had very low variability. At the molecular level, a short circadian period would theoretically indicate that some factor within the TTFL is accelerated, either in the positive or negative arm of the feedback loop. Thus, an acceleration of degradation of PER/CRY dimers would result in a faster release of the CLOCK/BMAL from inhibition and the cycle can start anew. The faster the degradation, the faster the frequency of the loop. Most commonly it is the rate of cycling of PER or CRY proteins and dimers that determines the cycles, such as is observed with the tau mutation (Ralph et al., 1990; Lowrey et al., 2000). However, genomic and transcriptomic studies have shown that multiple loci underlie a change in period and that the determination of FRP is complex and requires the relative co-ordination perhaps of many different factors (Shimomura et al., 2001; Takahashi et al., 2008).

Many candidate factors have been explored with regards to their effect on FRP. These include clock genes, enzymes that regulate clock genes and intracellular factors such as peptides in the SCN (Maywood et al., 2011; Hastings et al., 2019; Takahashi et al., 2008). However, with all these studies the findings are variable and in some cases contradictory. All these differences, indeed, may be due to subtle variation in the effect of the gene deletion, the level of redundancy in that particular system, or the amount of compensation available for that particular change in the gene. This would especially be the case for genetic deletions, whereby the loss of one particular gene can trigger compensatory functions from others and help develop alternative circuitry. What is most predominant is that no single factor appears to cause a shortening of free-running period, without a concomitant loss in rhythmicity over time in constant conditions, loss of responses to light or lack of maintenance of normal circadian parameters. However along with these other consequences, in many models a mutation or the deletion of a clock gene leads to a shorter or longer free-running period than found in wild types and this is observed with nearly all clock gene mutation models including Per1, Per2, Per3, Cry1, In Cry2, CKIε, Clock and Rev-erba mutants (Cermakian et al., 2001; Lowrey et al., 2000; Preitner et al., 2002; Shearman et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999). Some of these models will be briefly discussed below as they might indicate good target starting points when examining the circadian phenotype found in BTBR strain.

The mouse model mutant for core clock transcription factor Clock (termed *Clock*Δ19) shows a long period phenotype that results from impaired transactivation by the mutated Clock protein. This reduced transcription leads to a slower transcription of *Per* and *Cry* genes thus

delaying the TTFL and lengthening the period. These mice exhibit lengthened FRPs under constant conditions, lack a clear contrast in their activity consolidation during the light and dark periods and have larger phase responses to light (Low-Zeddies et al., 2001; Vitaterna et al., 1999). However, mice with homozygous deletion of the Clock genes show only slightly shorter free running periods (Debruyne *et al*., 2006). Thus, the type of mutation within a factor might determine the effect on FRP (and general circadian behaviour itself).

Another target includes the Cryptochrome (Cry) genes. As mentioned, *Cry* genes alongside *Per* genes are the targets of the Clock/Bmal transcription. A change in the rate of cycling of these proteins will thus affect the speed the clock. However, subtypes of Cry appear to have opposing effects on circadian period. *Cry1*- null mice have a short period phenotype whereas, *Cry2* null mice have a long period phenotype (Thresher *et al*., 1998; van der Horst *et al*., 1999; Vitaterna *et al*., 1999). These differences are mainlined at the in-vitro level and in the presence of a mutation in Fbxl*3*^{Afh} that stabilizes Cry proteins, thus showing that rate of cycling of these proteins can influence behavioural period (van der horst et al., 1999; Anand et al., 2013). In one experiment, SCNs null for the *Cry1* and *Cry2* genes were transduced with AAVs encoding either CRY1: EGFP or CRY2: EGFP (Edwards et al., 2016). Cry null SCNs transduced with CRY2 had a short period phenotype and those with CRY1 and a long period phenotype. Thus, each subtype of the gene has its own unique effect on FRP, suggesting perhaps that in a fully functional SCN the coordinated working of these genes and other clock genes might serve to determine a period that is an average of individual phenotypes.

Both *Per1* and *Per2* null alleles yield FRPs that are about 1 to 1.5 hours shorter than wild-type controls. Mice with homozygous *Per1*^{ldc} mutations have FRPs that are approximately 0.5 minutes shorter than wild-type controls and gradually lose rhythmicity after two weeks in DD (Bae *et al*., 2001). Similarly mice with *Per1*^{Brdm1} mutations have a FRP that is about 1 hr shorter than wild-type animals, but unlike *Per1*^{ldc} these animals maintain rhythmicity (Zheng *et al*., 2001). This is also consistent with the circadian behaviour of the *Per1*$^{-/-}$ mutant that exhibits a FRP that is about 0.7 hr with no loss of rhythmicity (Cermakian *et al*., 2001). However, there is a loss of rhythmicity in both these lines over time, thus consolidated rhythmicity tends to be temporary (even if long-term). Animal mutants for any of the core clock genes in any form tend to lose rhythmicity either immediately or after some cycles when transferred to DD (Thresher *et al*., 1998; Okamura *et al*., 1999; van der Horst *et al*., 1999; Vitaterna *et al*., 1999). BTBR mice, however, maintain rhythmicity and consolidated behaviour throughout their lifespan. Additionally, the shortening of FRP in these models is usually found not to be as drastic as what is observed in the BTBR strain.

Partial *Bmal1* knockout animals retain rhythmicity (Mieda et al., 2011 ; Husse et al., 2011). However, recent studies have found that deleting *Bmal1* in selective population of cells prevents the broad level desynchronization normally observed with global *Bmal1* knockouts (Hong et al., 2007; Izumo et al., 2014; Barca-Mayo et al., 2017), but instead causes more subtle changes in circadian function including a change in period. For example, *Bmal1* deletion in SCN astrocytes lengthens FRP (Tso et al., 2017) and similar results are observed with *Bmal1* deletion in AVP expressing cells (Mieda et al., 2015). This suggests that rather than control of FRP being

under one single locus, a combination of loci such as the gene and the cell subtype it is expressed in, may together determine basic circadian characteristics. This is supported by findings that have also identified factors involved in both transcriptional activation and repression and enzymes that affect Per and Cry stability in having a role in period changes (Chen et al., 2012; Maeir et al., 2009). As mentioned, E3 ubiquitin-ligases Fbxl3, and Fbxl21 modulate proteasomal degradation of CRY1 and CRY2 proteins and this would essentially change the speed of the clock. Casein kinase 1 enzymes (CK1 ε/δ) phosphorylate PER proteins , leading to proteasomal degradation and nuclear translocation (Camacho et al., 2001; Akashi et al., 2002; Eide et al., 2005). Mutations in *mCK1e*, *hCK1d* or in *hPer2* that accelerate degradation of PER also shorten the circadian period (Gallego et al., 2004), due to faster degradation of PER proteins which terminates the negative-feedback phase earlier. *CK1e* Tau mutant mice exhibit FRPs that are about 20-hours long and with bioluminescence rhythms in SCN and peripheral tissues of the same frequency (Meng et al., 2008). Thus, either Per and Cry or the enzymes that target them would serve as good targets to examine in BTBR mice.

What makes interpretations of our findings complicated, is that for most these models there is a loss of rhythmicity, altered entrainment or altered resetting responses in addition to the change in period. Even with mutations in enzymes that regulate clock gene function, the changes in circadian function are not limited to a change in period only (Xu et al., 2007; Meng et al., 2008; Etchegaray et al., 2009; Lee et al., 2009; Walton et al., 2009 Dey et al., 2005). Even in the tau mutant strain while rhythmicity persists both in LD and DD, a key finding (and what preceded the original discovery of these hamsters) is that they have an unusually early onset of

activity in an LD cycle compared to wildtype controls (Ralph et al., 1990). BTBR mice however (as elaborated on in the next chapter), show a stable and precise entrainment pattern to LD cycles and, as shown in this experiment, despite the short FRP and alpha maintain resetting responses that are comparable to wildtype animals.

One interesting finding was that the variability in FRP was very low in BTBR mice. It has been established, in multiple strains of multiple species, that as the FRP varies further from 24 hours, the interindividual variability increases (Pittendrigh and Daan 1976; Bittman 2012). Again, the variety of loci that may influence circadian period complicate this interpretation, however FRP is often variable both day-to day and over lifespan (Shimomura et al., 2001; Takahashi et al., 2008). This stability and precision in BTBR FRP is maintained throughout the lifespan and was observed in multiple BTBR litters over multiple years of experimentation. Robustness and stability of circadian rhythms may depend upon multiple clock genes, interacting transcriptional feedback loops and post-translational processes (Gonze et al., 2006; Mirsky et al., 2009; Stelling et al., 2004). Thus, stabilization of period may depend on optimal resonance between transcriptional and post-transcriptional events (Qin et al., 2010). Based on this it is possible that the high variability in many previous mutants result from the mutation being engineered (as opposed to be spontaneous). Mutations that produce a mismatch of time constants (e.g., for rates of turnover of constituent proteins that must interact) might decrease precision. However, the change in period in BTBR appears to be largely spontaneous, and this might have allowed for adaptations to any changes in the underlying transcriptional and post-

transcriptional processes, such that all factors that may determine circadian period have now adapted to provide a stable precise rhythm.

Based on short FRPs and short alphas we anticipated that BTBR mice would have altered responses to light pulses in the early and late subjective night. However, BTBR mice in our experiments showed normal responses to light when given light pulses, suggesting that the retinal inputs are conserved in these mice and that the retinorecipient cells in the SCN have enough conserved function to maintain average responses to light pulses. However, one limitation in our study is that we did not account for the change in alpha when looking at the response to light pulses. It is possible that the extremely short alphas modify the shape of the PRC to light pulses in these animals, such that based on CT 12 their early and late-night pulses time points will need to be moved in order to achieve comparable early and late time points. Future work will aim to establish phase response curves in these animals for the entire circadian cycle, by exposing animals to light pulses every 2 hours during their active phase.

Studies have looked at large scale transcriptome and genome analyses in BTBR mice. While these mice have several known polymorphisms and mutation in non-circadian related genes, the only clock implicated gene that is known to have a polymorphism in this strain in *Per3* (Jones-Davis et al., 2013). However *Per3* is generally not considered a core circadian core clock mechanism because loss of *Per3* has no effect on circadian rhythms either in *Per1/Per3* or *Per2/Per3* double mutant mice (Shearman et al., 2000; Bae *et al*., 2001). In some cases, *Per3* mutants show period phenotypes in cultured cells, peripheral tissues explants and in behaviour. However, the FRP is only about 30 mins shorter than wildtype. It is possible

that, *Per 3* might in some part contribute to the short period phenotype - either a result of a differential mutation of *Per3* compared to previous studies or in combination with other clock or clock-controlled genes. Thus, while previous studies looking at *Per3* polymorphisms have found that mutations tend to lengthen the circadian period (Archer et al., 2010; Ebisawa et al., 2001). However, this might potentially serve as a good starting point for future studies.

In addition to clock genes however it possible that one, or a combination, of the cellular subtypes within the SCN could underlie a short period. The SCN is a heterogenous nucleus that can be divided into a core and shell. The SCN core contains cells that express GRP and VIP that receive direct retinal input, whereas the shell region has cells that mainly contain AVP (Antle and silver 2005). Animals lacking VIP or its cognate receptor VPAC2R show a short period phenotype as do some animals with AVP mutations (Colwell et al., 2003; Yamaguchi et al., 2003). However, like mutations of clock genes, behavioural rhythms in these animals are of lower amplitude and show global losses in rhythmicity. Additionally, these mutations result in high variability in phenotypes. Some of the behaviour observed in the BTBR mice in our experiments (including to photic entrainment covered in the next chapter) indicated changes in VIP or AVP. However, our findings with SCN anatomy (Chapter 6) reveal a higher cell count and density of VIP and AVP staining, whereas studies that have previously studied the effect of VIP and AVP and related mutations on circadian behaviour found that it is a deficiency in the signalling of these peptides that causes these phenotypes we observed (short period, accelerated re-entrainment etc; elaborated on in chapter 6). Thus, further study will need to be undertaken to establish the role of these peptides in BTBR mice. The development of novel AAV

mediated technology (Brancaccio et al., 2017; Hastings et al., 2019; Mieda et al., 2015) provides a novel opportunity to perhaps target these cell subtypes within the SCN and thus observe how enhancement or reduction of their function might alter circadian function.

Like male BTBR mice, female BTBR mice also have shorter FRPs and shorter alphas, and higher total activity compared to C57BL/6j controls. However, we also found a significant interaction of sex in that the females have significantly longer FRPs and longer alphas when compared to male BTBR mice. Both male and female C57BL/6J mice in our experiment had comparable FRPs in line with previous findings that found no period difference between C57BL/6J male and female mice (Kuljis et al., 2013). Sex differences have been previously reported in other species such as the golden hamster (Davis et al., 1983; Schull et al., 1989) and Octodon degus (Labyak et al., 1995; Lee et al., 1997), however the male animals in these studies showed longer FRPs than females. This suggests further that perhaps there is an underlying locus of control in BTBR that changes their phenotype so significantly, including a change from the normally observed non-difference between sexes. Sex differences, for example, might be due to differences in gonadal steroids, which have previously been shown to be highly impactful on circadian period (Morin et al., 1997; Albers et al., 1981; Royston et al., 2014).

While constant darkness can indicate the working of the endogenous clock and its resetting responses, independent of any other external cues, we can also expose animals to LL conditions, whereby all animals are maintained under a constant and stable illumination at all times. While DD (and LD) help the expression of circadian rhythms, LL disrupts those rhythms. Over time, in constant light animals lose rhythmicity and amplitude of their behavioural

rhythms (Ohta et al., 2005). Thus, what is commonly observed within a couple of weeks of LL exposure is a dramatic decrease in the amount of running activity, a loss of consolidated behaviour and in many animals complete arrhythmicity after a few weeks. In nocturnal animals there is also a lengthening of FRP (Aschoff 1981). Both BTBR and C57BL/6J animals in our experiment, showed a loss of rhythmicity and lengthening of period. But BTBR mice had a proportionally greater increase of their free-running period and greater loss in amplitude of their circadian rhythms. A possible mechanism that can be examined further is the role of serotonin (5-HT). 5-HT modulates the effect of light – 5-HT agonists or procedures that induce arousal dependent on the 5-HT system, attenuate the phase shifts observed with light pulses (Mistlberger and Antle 1998), while 5-HT antagonists and mixed agonist/antagonists accentuate light induced phase shifts (Sterniczuk et al., 2008; Smith et al., 2010, 2015; Smart and Biello 2015). Thus, it is conceivable that in LL conditions, the 5-HT system could modulate the effect that light has on circadian function. Given that BTBR mice have deficiencies in 5-HT signalling (Guo et al., 2017; Gould et al., 2011, 2014; Guo and Commons 2016) it is possible that this might differentially regulate the effects that light has on the BTBR. Additionally, future work can aim to look at the role of clock genes. It has been reported that in LL, the FRP of *Per1* mutants lengthens with increasing illuminance, as expected by Aschoff's rule (Steinlechner et al., 2002). Thus, looking more closely at clock genes may provide some answers to the more dramatic dampening effect of LL in BTBR mice (and also provide clues to their atypical behaviours in DD).

Dark pulses are periods of exposure to complete darkness when the animal is housed in constant light. Dark pulses have both photic and non-photic components. Non-photic shifts can

be enhanced by LL- mediated by changes in 5-HT sensitivity- because LL results in low 5-HT levels (Mistlberger, Belcourt and Antle 2002). The effect of dark pulses is mediated by the amount of activity during the dark pulses and activity is necessary for a phase shift to occur and without activity commonly no phase shifts are observed (Reebs 1989). Dark pulses during the subjective day cause phase advances. But have also been recorded as shifting the circadian system in a manner similar to light pulses (in the early and late subjective night; Ellis et al., 1982). Dark pulses also have other effects, including transient as well as long-lasting changes in FRP, that depend on the phase at which the pulses are delivered (Burchard 1958; DeCoursey 1964; Elliott 1974; Daan and Pittendrigh 1976a). We found that BTBR mice had greater total activity during the dark pulse when compared to C57Bl/6J controls and had greater phase shifting responses. These phase shifts took form of moderately large phase delays and large phase advances. This is an interesting finding because these large phase shifts can be classified as type 0 phase shifts, not commonly induced in mice (Johnston et al., 2003). Type 0 resetting is potentially mediated by a weakened network- where the individual oscillators are no longer in sync with one and another, reducing the amplitude and strength of the system. As mentioned, LL weakens the network of oscillating cells in mammals (Ohta et al., 2005). The strength of the SCN as a network comes from the phase coherence of its individual oscillators, a loss of this would create a weaker system that more susceptible to perturbation and can be more easily reset. If behaviour in LL is taken as a reflection of underlying SCN network functioning, then it would appear that the BTBR mice have greater phase dispersion, mirroring the greater dampening of activity and the loss of rhythmicity. Thus, the BTBR potentially have more

weakened SCN networking that is more susceptible to perturbations, with cells unable to provide a sufficient combined output to maintain rhythmicity (Low-Zeddies and Takahashi, 2001). Additionally, the change in amount of activity in LL compared to the dark pulse could have potentially had an effect through changes in the 5-HT system. If there is dysregulation of 5-HT or attenuated 5-HT signalling in the BTBR (especially in terms of inputs to the SCN), a large period of activity following a dampened period of activity could cause a change in 5-HT signalling profound enough to have large arousal mediated non-photic phase shifts. It would be interesting and useful in future experiments, to examine further the role of 5-HT input to the SCN in the BTBR and establish if there is indeed a differential modulation of 5-HT. Future work can also examine non-photic phase shifting to 5-HT agonists and how 5-HT agonists and antagonists modulate light input in BTBR mice.

There were a few limitations with the dark pulse experiment. Firstly- the magnitude of dampening of activity made it difficult to establish precise onsets of activity, thus making the predictions of phase more difficult. This could have contributed to the variability in the resetting responses. Secondly- the control animals in this experiment did not phase shift as would be expected and in most cases had little to no activity during the dark-pulse. While a lack of phase shifting is expected with little to no activity, as previously described, it is unusual that almost none of the animals had any activity during the dark pulse.

Overall the circadian phenotype of BTBR mice appears to be drastically different from that of wild-type controls used in our experiments, but also in comparison to what is

generally observed in other strains of mice. Examining the underlying mechanisms might provide clues to the working of the SCN and the SCN within a wider network.

Chapter 4: Entrainment, re-entrainment and masking in BTBR mice

4.1: Introduction

While the circadian clock generates and maintains a daily rhythm in constant conditions, most organisms live in environments with a variety of time cues. Light is the predominant synchronizing cue and most animals have evolved a clock that can best adapt to the 24-hour cycle of Earth's rotation around the sun (Golombek and Rosenstein 2010). Optimal entrainment depends on an appropriate shift in phase of onset activity, that allows for minimal time difference between the onset of the internal rhythm and external light cycle (i.e. minimal phase angle of entrainment). When free running periods (FRPs) are much longer or shorter than the entraining T cycle, the phase angle of entrainment tends to be skewed either positively or negatively (Pittendrigh and Daan 1976; Aschoff 1966). Additionally, how an animal synchronizes to rapid shifts in the light cycle provide information about the stability and precision of the clock. During transcontinental travel behavioural re-entrianment appears after some days and is generally faster for phase delay shifts (Yan and Silver 2002; Reddy et al., 2002). The SCN re-entrains faster than peripheral tissues and at the level of the SCN, the core re-entrains faster than the shell (Nagano et al., 2003, Davidson et al., 2009, Rohling et al., 2011). At the molecular level Per genes re-entrain first in the SCN before other peripheral organs (Yamazaki et al., 2002; Davidson et al., 2009). It is known that FRP influences re-entrainment in a directional manner, i.e. longer FRPs allow for faster synchronization a delayed cycle and vice versa (Aschoff et al., 1975). However, much of this is based on subtle differences in FRP normally found across species (difference of up to an hour). Thus, we wanted to examine if the short FRP in BTBR mice

would allow for faster entrainment to advanced light cycles and slower entrainment delayed light compared to C57 controls. Animals with VIP and AVP mutations, for example, resynchronize to a shifted light dark cycle faster than wildtype controls, (Colwell et al., 2003; Yamaguchi et al., 2013). Thus, difference in re-entrainment patterns can indicate possible differences in the underlying networking of the SCN.

Like DD, LD cycles allow the expression of circadian rhythms and provide information about the adaptability of the clock. Circadian desynchrony that results from misalignment between the internal and external rhythms results in accelerated malignant growth (Filipski et al., 2009), temporal lobe atrophy combined with spatial cognitive deficits (Cho et al., 2001), can increase the risk of diseases, including cancers, diabetes, and hypertension (Kubo et al., 2006; Scheer et al., 2009; Buxton et al., 2012) and in rodents hastens death upon aging (Davidson et al., 2006) and alters immune responses (Castanon-Cervantes et al., 2010; Logan et al., 2012; Cuesta et al., 2016). This is in addition to the short term health impacts such decreased alertness, night-time insomnia, poor overall performance (Tapp et al., 1989), impaired cognitive skills (Cho et al., 2000), loss of appetite, depressed mood, reduced psychomotor coordination, and gastrointestinal disturbances (Waterhouse et al., 2007). Therefore, understanding the mechanisms that underlie circadian desynchrony, finding ways to reduce misalignment and understanding how entrainment can be accelerated are all important. We examined entrainment to ambient light cycles and resynchronization to shifted light cycles in BTBR mice. Based on the findings mentioned in the previous chapter (i.e., short FRP and short alphas), we anticipated entrainment to LD cycles with an advanced

phase angle, since such a short period would necessitate a larger daily phase delay and this exposure of more or the delay zone of the PRC to evening light. Additionally, we expected that such a short period would accelerated re-entrainment to an advance in the light cycle and delayed entrainment to a delaying shift in the LD cycle.

4.2: Methods

4.2.1: Animals

Adult male BTBR (n=13) and C57Bl/6J (n=17) mice were used for this study. All animals were between 3-6 months of age during the experiments. All mice were received from Dr Rho at ACHRI, University of Calgary. Mice were at least 3 weeks old when received and between 3-8 months of age during experiments. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care for the ethical use of animals in research.

4.2.2: Procedures

4.2.2 i) LD entrainment

13 BTBR mice and 17 C57Bl/6J mice were used to examine circadian parameters under an LD cycle. To examine circadian parameters and entrainment to a LD cycle, all animals were exposed to 12:12 LD. Animals were allowed to stably entrain for at least 3 weeks before collecting data to be analyses. LD entrainment was analysed on at least 10 days of data.

4.2.2 ii)) Jet lag manipulations

8 BTBR mice and 8 C57Bl/6J mice were tested on their ability to re-entrain to 8 hour shifted light dark cycles. All animals were initially allowed to entrain to a 12:12 L/D cycle. After 2 weeks, the light cycle was advanced by 8 hours. Animals were then allowed to entrain to this new light cycle. After at least 3 weeks the ambient light cycle was delayed by 8 hours. Again, the animals were allowed to re-entrain. Animals were considered to be completely entrained to the new light cycle the day on which there was a negligible phase angle difference (activity onset coincided with lights off) with no further change of the phase angle on the days following.

4.2.2 iii) Masking test

We aimed to determine if the synchronization the new light cycle after the jet-lag procedure was due entrainment or masking by light. Masking is the phenomenon where the frequency of the two cycles, locomotor activity and the ambient light cycle, match without the underlying clock conforming to the external light cycle. In nocturnal animals, light can mask the endogenous activity of the clock and thereby supress it. In this case the emergence of locomotor activity is not a reflection of the underlying biological clock, but rather a direct suppression from the light cycle. To conduct the masking experiments, animals were subjected to the 8-hour jet-lag procedure to mimic both eastward and west ward travel in separate experiments. After the light cycle was shifted, animals were maintained on the shifted LD cycle for 2 days before being released in DD. If animals were indeed entrained, their onset of activity

will begin at lights-off of the new cycle, however if light was primary driver of activity and masking was the cause of the activity patterns observed then onset of activity would begin closer to the previous LD cycle. After being released into DD animals were maintained in DD for at least 4 weeks following.

4.2.2 iv) Analysis

Alpha and total activity in LD were calculated as previously described (see chapter 2). Briefly alpha was the average over 10 days, of the difference between activity onset and activity offset and total activity was the total activity count over the span of 10 days. The phase angle of entrainment was determined first identifying the time of onset of activity and subtracting it from the time of dark onset. This was then averaged over a 7-day period. Positive phase angles indicate activity onsets that precede dark onset and negative phase angles are those that follow dark onset with a lag. For jet-lag experiments animals were considered entrained when a stable phase angle was established – i.e. activity onset coincided with lights off with a stable time difference that was maintained. For masking experiments the amount of masking was quantified using the phase angle difference of predicted light off (ZT 12) and onset of activity. Activity counts were exported in 10 min bins and were only used if a bin had more than 50 revolutions and was followed by another 10-min bin with at least a 100 revolutions. Data were analysed between groups using Independent t-tests and using SPSS software (IBM SPSS 2015).

4.3: Results

Findings were compared using independent t-tests. BTBR mice had a shorter alpha in LD when compared to C57Bl/6J mice (M= 8.43 hrs +/- 0.378 hrs vs 12.4962 +/- 0.272; t (28) = 8.20, p< 0.001). However, BTBR and C57Bl/6J mice had comparable total wheel running activity (M= 9828 +/- 388 vs 8491 +/- 625; t(28)= 1.90, p= 0.067) and phase angle of entrainment (M (mins)= 0.77 +/- 0.10 vs -5.67 +/- 0.21 ; t(28)= 0.15, p=0.165).

In response to jet-lag procedures, BTBR animals took significantly less time to retrain after an 8 hour phase advance (M = 5.5 +/- 0.26 days vs 9.5 +/- 0.46 days ; t(14)= 7.27, p<0.0001) and 8 hour phase delay in the LD cycle (M = 1.5 +/-0.27 days vs 4.88 +/- 0.61 days; +/- 0.26; t (14)= 5.06, p= 0.0002). However, during the masking test it was found that the phase of onset for BTBR mice was offset from what would be predicted of the ambient light cycle. However, due to the short FRPs in these animals, it is unclear if this advanced phase of onset is a reflection of failed entrainment or a natural consequence of the short FRP.

Figure 4. 1: Representative actograms showing average activity profiles of BTBR (a, right) and C57Bl/6J (a, left) in 12 hr:12 hr LD. Actograms are single plotted, and the scale bar represents 24 hours

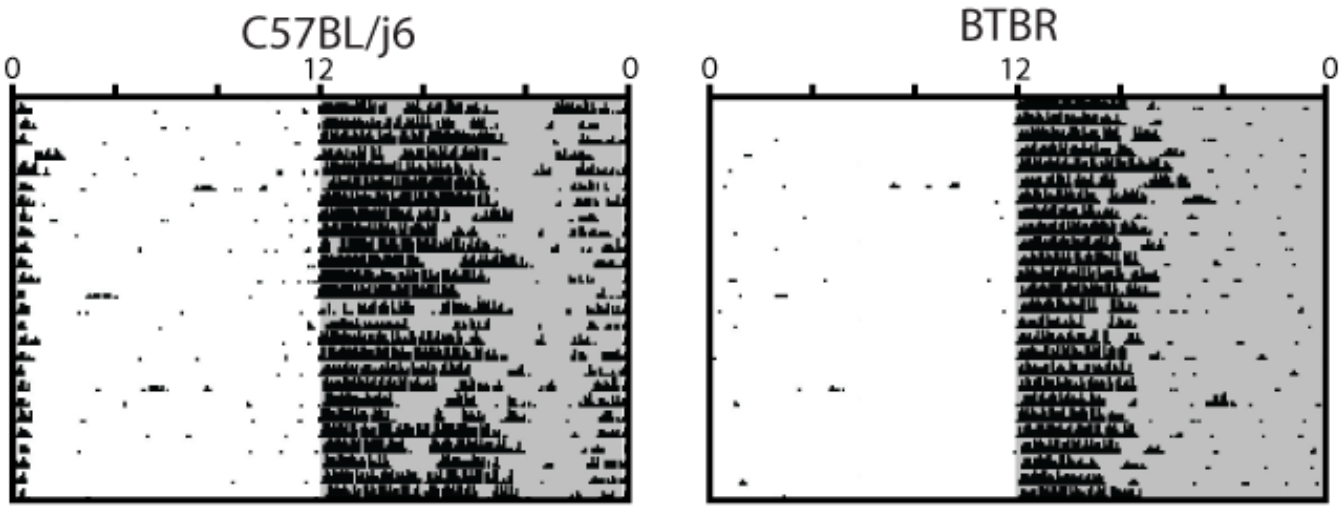
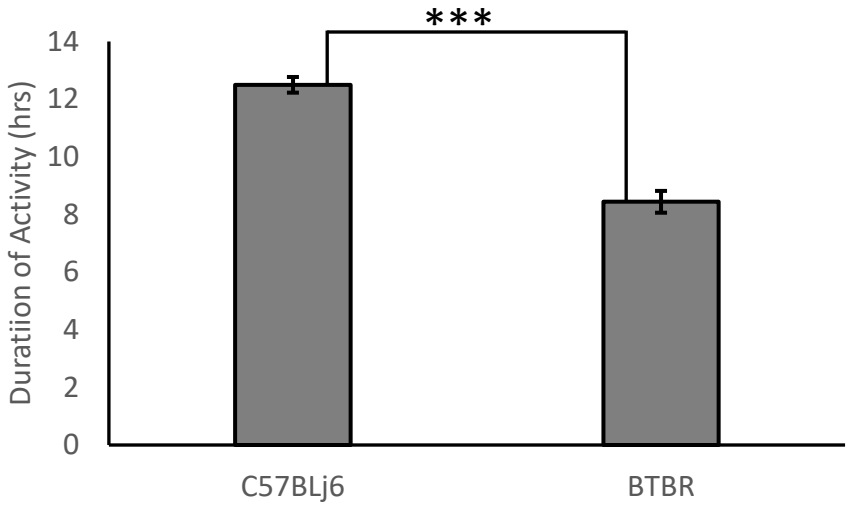
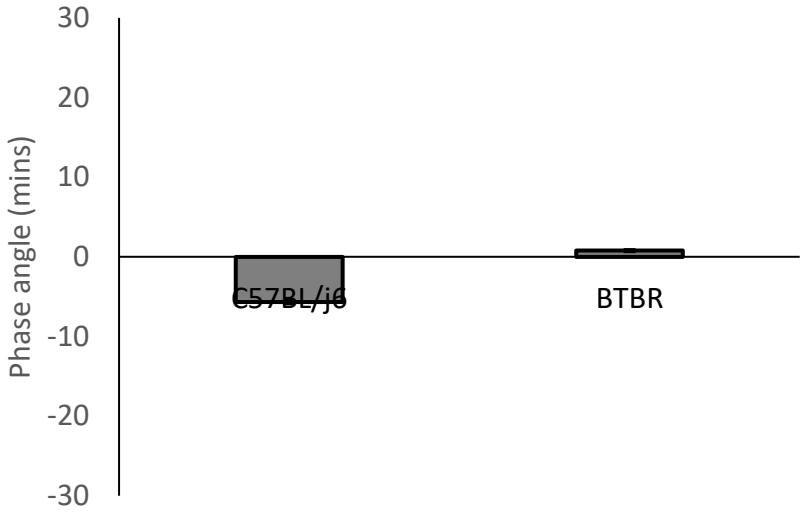

C57BL/j6
0
12
0
BTBR
0
12
0

Figure 4. 2: Bar graph showing entrainment behaviour in BTBR and C57Bl/6J mice in a 12:12 LD cycle. In LD conditions BTBR mice have significantly shorter alphas ($p<0.001$; **a**), but have comparable phase angles of entrainment ($p= 0.16$; **b**) and total activity ($p=0.07$, **c**)

A)


***
14
12
10
8
6
4
2
0
Duratiion of Activity (hrs)
C57BLj6
BTBR

B)


30
20
10
0
-10
-20
-30
Phase angle (mins)
C57BL/j6
BTBR

C)

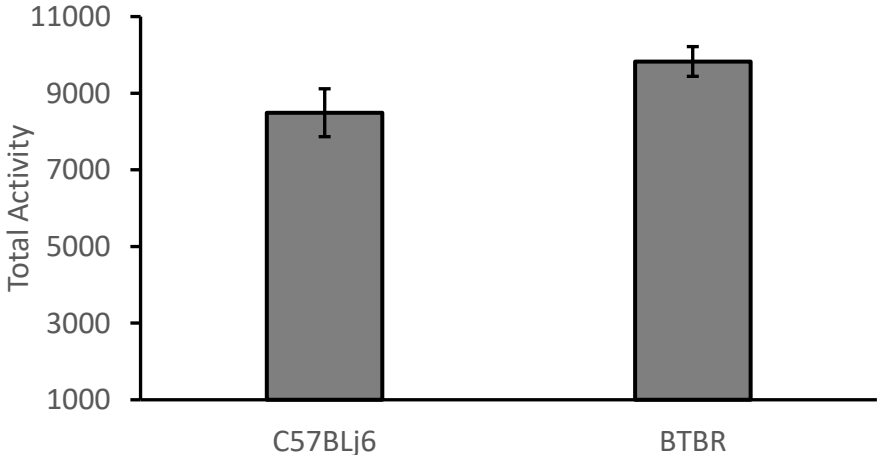

11000
9000
7000
5000
3000
1000
Total Activity
C57BLj6
BTBR

Figure 4. 3: Representative actograms showing average activity profiles of BTBR (a, right hand panels) and C57Bl/6J (a, left hand panels) when subjected to either an 8- hour advance (Top panels) or 8-hour phase delay (Bottom panels) in the entraining light cycle. Green arrows indicate the day of the shift.

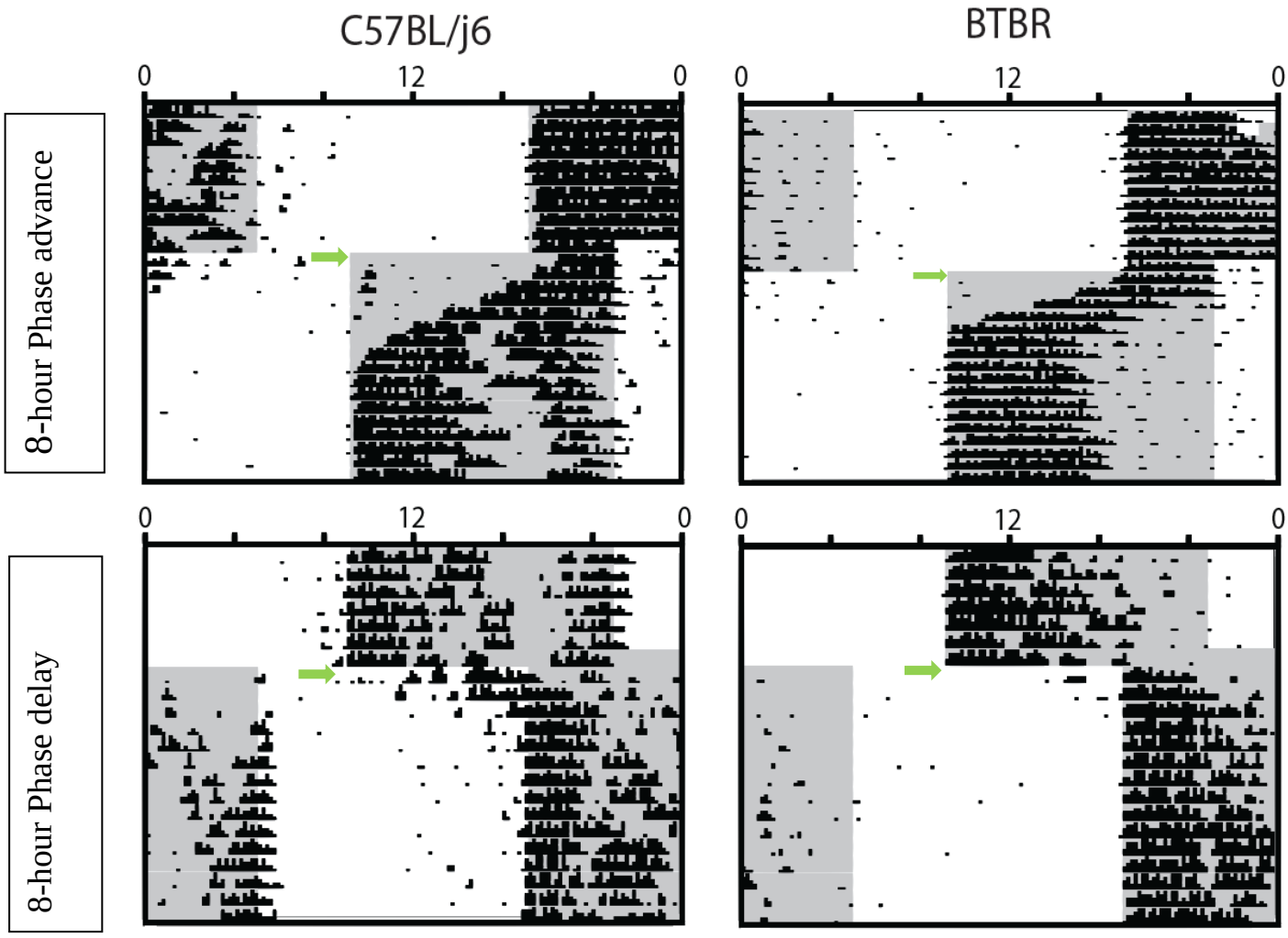
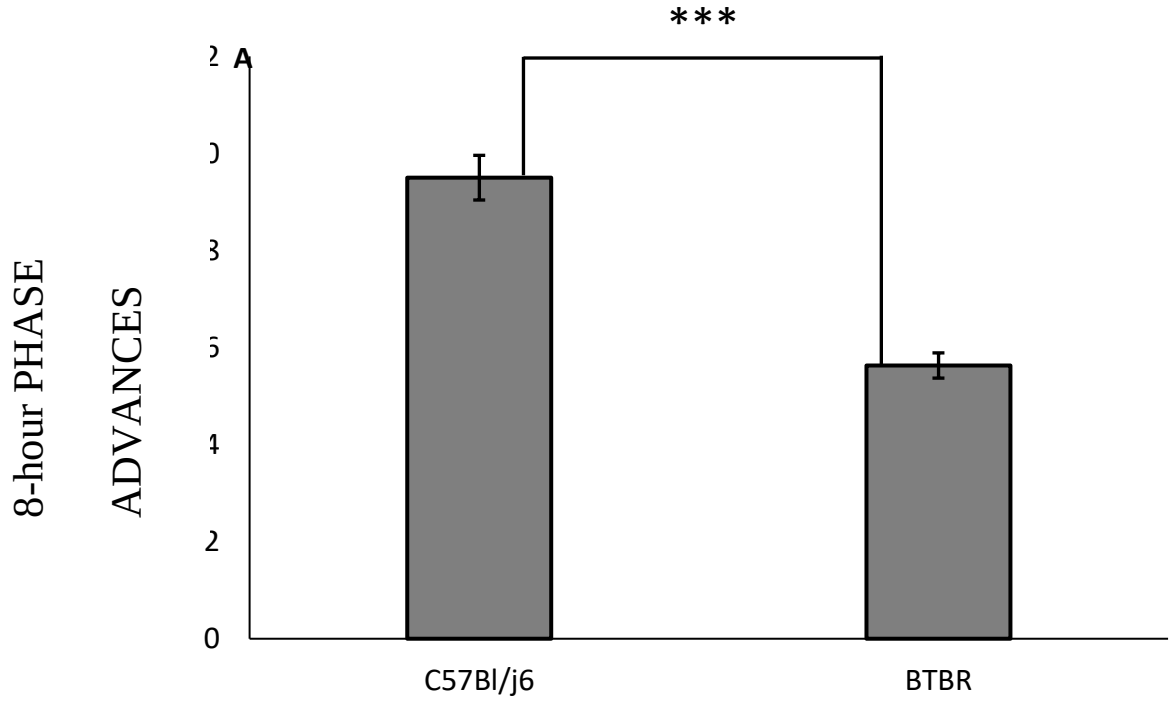

C57BL/j6
BTBR
8-hour Phase advance
8-hour Phase delay
0
12
0

Figure 4. 4 : Bar graphs showing differences between BTBR and C57Bl/6J mice when re-entraining to a either an 8-hour advance or delay in the ambient light cycle. BTBR mice re-entrained significantly faster to both phase advances ($p<0.001$, **A**) and phase delay shifts ($p<0.0001$, **B**), when compared to C57 controls.


A
***
8-hour PHASE ADVANCES
2
0
3
5
4
2
0
C57Bl/j6
BTBR

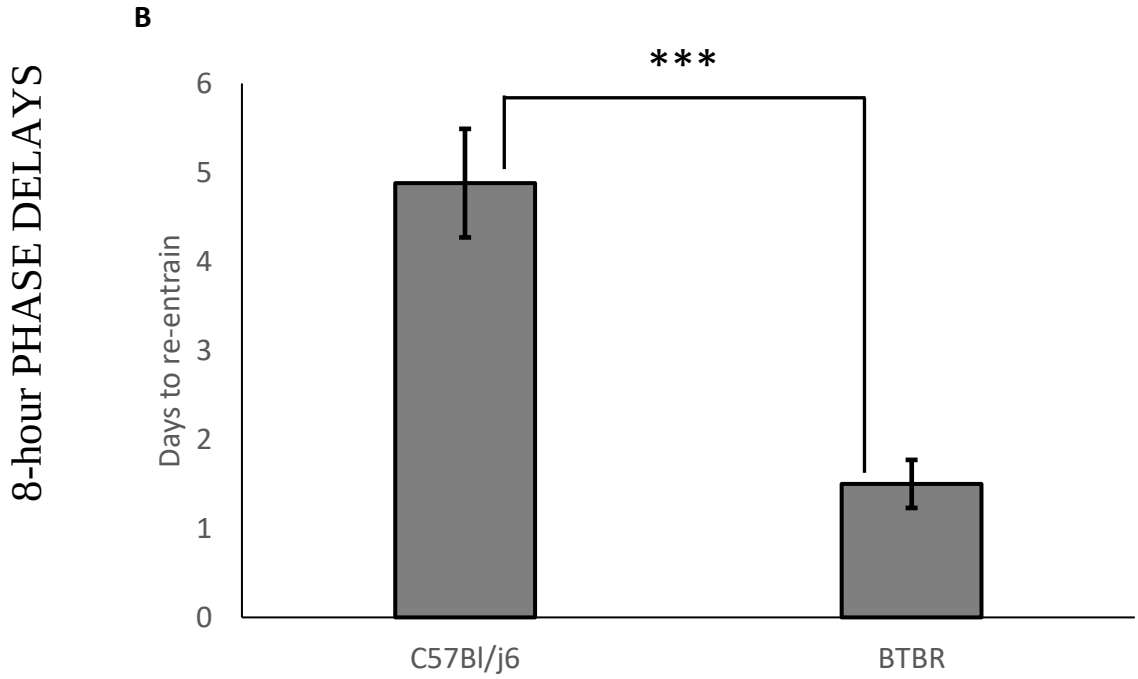
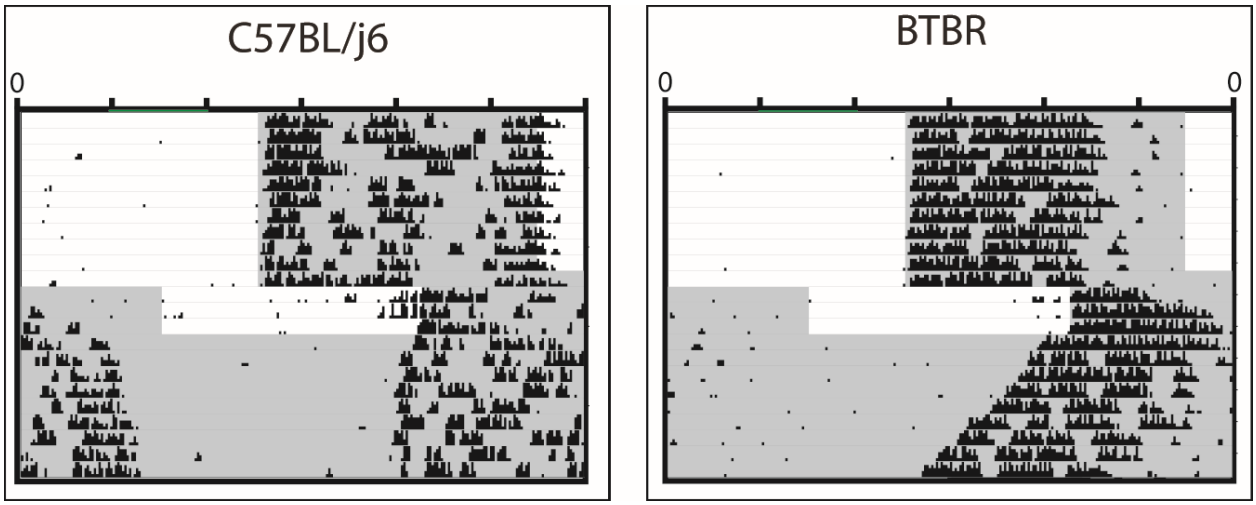

B
***
8-hour PHASE DELAYS
Days to re-entrain
6
5
4
3
2
1
0
C57Bl/j6
BTBR

Figure 4. 5: **Representative actogram of the test for masking, after an 8-hour phase delay.** Animals were subjected to 8-hour phase delay after establishing stable entrainment to a 12:12 LD entrainment. After 3 days in the shifted LD cycle, animals were released in DD to examine if phase of onset coincided with the light off in the shifted light dark cycle.


C57BL/j6
0
BTBR
0
0

4 4: Discussion

Most organisms live in environments with temporal cues, of which the light-dark cycle is the most predominant. Given the differences between BTBRs and C57BL/6J mice in DD we wanted to re-examine entrainment to a 12-hour LD cycle in BTBR mice. The short periods and short alphas led us to expect alterations in phase angle from wildtype mice, with a tendency towards advanced phase angles. However, BTBR mice show precise and stable entrainment to LD cycles, with little cycle to cycle variability and activity that was precisely consolidated to the timing of lights off. Similar to what we found in DD, BTBR animals also had higher total activity and shorter alphas in an LD cycle. We then subjected animals to acute jet-lag experiments where their ambient light cycles were either advanced or delayed by 8 hours in two separate experiments. Again, the FRP and endogenous circadian phenotype in BTBR mice led us to hypothesize that they would re-entrain to advances light cycle faster than C57BL/6J controls and re-entrain to delayed light cycles more slowly (because of the rapidly advancing FRP). However unexpectedly what we found was that BTBR mice re-entrained to both advanced and delayed light cycles faster than C57 controls – in both cases BTBR mice re-entrained in half the time it took control animals. Curiously, normal patterns of entrainment to shifted light-dark cycle were maintained in BTBR animals despite the short FRPs, i.e. resynchronization to a phase advance took longer than that to phase delay which is established in the literature (Yamazaki et al., 2000; Czeisler et al., 1990; Best et al., 1999; Reddy et al., 2002), but BTBR animals resynchronized to advances in approximately 4 days whereas they resynchronized to phase delays in approximately 1 day.

In general, the rate and direction of entrainment is said to be correlated positively with the direction of the FRP- thus shorter FRPs will cause easier resynchronization to advance and longer FRPs to delays. Advanced rhythms (corresponding to eastward flights) need more time to be re-entrained to when compared with delayed rhythms (which correspond to westward flights; Yamazaki et al., 2000; Czeisler et al., 1990; Best et al., 1999; Reddy et al., 2002). Re-entrainment rates are around 90 min per day for delayed phase shifts and 60 min per day for advanced phase shifts (Boulos et al., 1995; Aschoff et al., 1975). The rate of rapid re-entrainment observed with BTBR mice is not generally observed and has only been reported with certain mutations that affect SCN networking (Yamaguchi et al., 2013).

A few mechanisms have been proposed to underlie rapid re-entrainment to shifted light dark cycles. From a theoretical perspective, cells within the SCN are coupled together and maintain phase coherence to establish a strong network that is resistant to perturbation (Welsh et al., 2010; Herzog et al., 2017; Liu et al., 2007). This prevents the circadian system from resetting to small changes in the environment - such as small variations in day length, accidental light exposure or changes in light intensity. However, this same stability proves to be problematic when realigning to large shifts in light-dark cycles. Upon shifting, the SCN re-entrains faster and before peripheral oscillators (Vosko et al., 2010; Kiessling et al., 2010; Yamazaki et al., 2000; Oster et al., 2006; Sosniyenko et al., 2010). Indeed, what is considered jet lag is generally the result of the phase dispersion of the various body oscillators, which ends when oscillators re-establish synchrony with the SCN and the new light cycle (Lu et al., 2016; Vosko et al., 2010). Thus, mechanisms that underlie re-entrainment and modulation in re-

entrainment can be dependent on both SCN and extra SCN mechanisms. From a cellular perspective, a weakened oscillator, where cell phases are widely dispersed, would pre-dispose an animal to faster re-entrainment (Welsh et al., 2010; Abel et al., 2016). Based on this, one possible reason for accelerated re-entrainment in BTBR mice might be a weakened network – one where the cells are more desynchronized and dispersed, making these animals more susceptible to external perturbations but also allowing them to shift at a faster rate. Multiple scenarios could cause this network level desynchrony. Most relevant to BTBR mice would be the potential state of constant desynchrony with the external light environment- caused by the short FRP constantly realigning to a 24-hour cycle. However, even ignoring phase angle (which across studies was extremely stable and precise), BTBR animals had well consolidated activity that maintained a stable amplitude for an extended period of time. If indeed, the constant state of resynchronization to the light cycle was causing SCN circuit level desynchrony, these animals would be expected to show low amplitude rhythms or perhaps a change in characteristics of duration or bouts over time. However, future work could aim to establish characteristics of SCN network dynamics in BTBR mice, using multicellular recordings to look at SCN firing rates or bioluminescence recordings to look for at spatio-temporal organization of clock cells in SCN (Maywood et al., 2011), that could provide more detailed information about intra-SCN synchrony.

Both VIP and AVP have also been implicated in faster re-entrainment after a large shift in the light dark cycle. Mice lacking V1 receptors for AVP (V1a -/- and V1 b -/-) re-entrain faster to a shifted light dark cycle and pharmacological blockade of the same receptors in the SCN of

wild-type mice also resulted in accelerated recovery from jet lag (Yamaguchi e et al., 2013). Mice with a *BMAL1* deletion specific to AVP producing neurons showed marked lengthening in the free-running period and activity time of behaviour rhythms. When exposed to an abrupt 8-hr advance of the LD cycle, these mice re-entrained faster than control mice did (Mieda et al., 2015). Thus, it appears that a lack of AVP expressing cells or a deficit in AVP signalling allows for faster entrainment. However, in the latter study the circadian expression of other genes involved in intercellular communications, Prokineticin 2, and Rgs16, was also drastically reduced in the dorsal SCN, where AVP neurons dominate. Thus, whether the effects were due to the AVP alone, the combined reduction of AVP and these other genes or if the reduction of these genes were downstream targets of AVP deletion are not clear. However, from both studies it appears that either a lack of signalling in the dorsal SCN or a dysregulation of AVP signalling might underlie faster resetting. Theoretically this is supported by the fact that AVP neurons (and the dorsal SCN) serves as the output centre of the SCN.

The role of VIP is a little more complex. When added to SCN slice cultures, VIP induces Per 1 and Per 2 production and shifts behavioural and physiological rhythms (Piggins et al., 1995; Watanabe et al., 2000; Reed et al., 2001; Meyer-Spasche et al., 2004). *Per* genes are implicated in light resetting – light induced resetting induces expression of *Per 1* and *Per 2* (Akiyama et al., 1999; Tischkau et al., 2000, 2003; Kuhlman et al., 2003; Shigeyoshi et al., 1997; Albrecht et al., 2001) and both Per1 and Per2 mutants fail to phase advance and phase delay, respectively, to light pulses (Albrecht et al., 2001). Thus, VIP application induces effects similar to that of light induced resetting (Akiyama et al., 1999; Tischkau et al., 2000, 2003). In the

absence of VIP or its receptor, VPAC2R, neurons in the SCN fail to maintain synchrony SCN neurons fail to synchronize to each other and consequently many daily rhythms are lost (Harmar 2002; Colwell 2003; Aton et al., 2005; Brown et al., 2005; Maywood et al., 2005). Animals lacking the gene encoding for VIP appear to re-entrain faster to shifted light dark cycle (Colwell et al., 2003). However, the opposite is true as well- transgenic animals with an overexpression of VPAC2 receptors resynchronized fasters to 8-hour phase advances and show considerably shorter free running periods (Shen et al., 2000). And in another study, animals injected with VIP rapidly shifted their onset of activity and required fewer days to entrain when compared to vehicle-injected animals (An et al., 2013). This could be due to the fact that while a lack of VIP signalling causes loss of amplitude in SCN, higher phase dispersion of cells and reduces high amplitude cell firing (Maywood et al., 2016), so does enhancement of VIP signalling. The effects that VIP has on synchrony of SCN neurons appears to be dose dependent (An et al., 2013). At low doses VIP maintains or enhances synchrony in SCN cells, however at doses higher than 100nM, VIP induces cellular desynchrony and broadens the phase distribution. Thus, an increase in VIP levels might cause accelerated re-entrainment in a manner similar to what is observed with a deficiency in signalling: weakened coupling between cells that then predisposes the SCN to faster shifting responses to environmental perturbations. This indicates that perhaps rather than a directional change in signalling, it might be modulation of away from baseline (either a decrease or increase). Indeed, if it is just a change away from baseline that affects how the circadian system functions in terms of resetting and entrainment, then many previous results that appear contradictory might in fact be showing that that a loss

of stability within in the SCN is what underlies changes in circadian function. Thus, it is possible that a change away from a balance between VIP and AVP, or a change in either of these peptides is what enables BTBR mice to re-entrain faster. While our experiments have not aimed to establish a causal relationship between the accelerated re-entrainment and these peptide groups, as elaborated in chapter 7, BTBR mice do differ from wild types on both VIP and AVP, showing increases in number of VIP and AVP cells. Future work can aim to modulate levels of these peptides either pharmacologically or genetically and test if their entrainment properties change when SCN peptidergic signalling is enhanced or blocked.

Additionally, a number of pharmacological and external agents have also been indicated to accelerate re-entrainment to a shifted LD cycle, including triazolam, Sildenafil, adrenalectomy, scheduled activity during usual rest, and dim light at night (Evans et al., 2009; Mrosovsky et al., 1987; van Reeth et al., 1987; Agostino et al., 2007; Kiessling et al., 2010). One way these procedures help speed entrainment might be through the arousal or stress systems, that feedback onto the SCN. As mentioned, stability of circadian function is a result of the co-ordinating phase relationships of cells within the SCN and of the coordinated function of the myriad of peripheral oscillators and the feedback loops they form with the SCN. For example, when the adrenal clock is compromised either by transplanting a clock deficient adrenal or by adrenalectomy (Sage et al., 2004), adrenal-SCN feedback is affected. This may potentially desynchronize the circadian system, allowing for larger shifts. In support of this, disruption in glucocorticoid signalling such as adrenalectomies, genetic ablation of the adrenal clock, and inhibition of adrenal GC synthesis, all accelerate re-entrainment to a shifted light cycle.

Interestingly there is evidence of dysregulation of the stress axis in the BTBR strain. However, in general BTBR mice tend to have higher circulating corticosterone, higher basal glucocorticoid receptor mRNA and higher oxytocin peptide levels but no significant differences were detected in corticotrophin releasing factor (CRF) peptide or CRF mRNA (Benno et al., 2009, Frye and Llaneza, 2010, Silverman et al., 2010). It is important to note that these studies were not conducted in a circadian fashion. What maybe important is not the presence of adrenal or glucocorticoid signalling, but rather the rhythmicity of them or a change away from baseline, like what was proposed with SCN peptides. Thus, future studies could aim to further examine the circadian nature of stress hormones in BTBR mice, not only in DD but also in response to changing light cycles, to examine if a change in the stress axis may contribute to these entrainment patterns.

Like triazolam, many other 5-HT agents have also been implicated in faster entrainment to large shifts in the light cycle. A single administration of mixed agonist antagonist NAN-190 diminished the time needed to re-entrain to a shifted light dark cycle from a week to about 2 days (Kessler et al., 2008). Many compounds acting at the 5-HT_{1A} receptor potentiate the response to light (Gannon, 2003; Gannon and Millan, 2006; Moriya et al., 1998; Rea et al., 1995; Lall and Harrington, 2006). However, unlike the other studies which were conducted in animals housed in DD initially, the Kessler et al., (2008) study was conducted in animals that were housed in an LD cycle that was shifted- which exactly mimics our protocol. A few 5-HT signalling differences have been reported in BTBR mice. 5-HT tissue content and axonal coverage is reduced in the hippocampus of BTBR mice compared to C57BL/6J mice (Gould et al.,

2011, 2014). This was accompanied by compensatory changes in 5-HT neurons in regions providing innervation to the hippocampus (i.e. the dorsal and median raphe), including increased numbers of 5-HT neurons and hyperactivation of *Fos* expression (Gould et al., 2011, 2014). However, BTBR mice had higher GTPγS binding in the CA1 region of the hippocampus, in response to 8-OH-DPAT-and an elevated 5-HT_{1A} capacity to activate G-proteins (Gould et al., 2011, 2014) as well as lower [(3)H] cyanoimipramine and citalopram binding to the serotonin transporter (SERT, Gould et al., 2011). Future studies can aim to look at the density of serotonin cells and innervation in the SCN, from the raphe to the SCN and the response of the circadian system and the SCN to 5-HT agents. A dysregulation in the 5-HT input to the SCN could also potentially underlie the behaviour observed in LL (significant dampening of amplitude of activity) and the heightened activity during dark pulses with consequent large phase shifts.

While it is possible that re-entrainment is faster in these animals, another possibility also exists; perhaps BTBR mice are more light-sensitive and thus more subject to masking. There are two main ways to promote the occupation of a diurnal or a nocturnal niche by an animal: 1) synchronization to LD cycles of an endogenous biological clock that in turn controls activity and 2) a direct effect of light on activity without the internal rhythm conforming to the external rhythm. These are called entrainment and masking, respectively (Aschoff, 1960; Mrosovsky, 1999). During entrainment parameters of the internal rhythm are modified by the external light cycle, while this is generally not the case with masking (Mrosovsky 1999). In nocturnal mammals, negative masking refers to the suppression of activity by light and positive

masking refers to an enhancement of activity in the dark. We found evidence for both aspects in our experiments. In LL (Chapter 3), BTBR animals had a more dramatic suppression of activity, a greater loss of amplitude of activity and more activity during the dark pulse all suggesting a role for masking. In LD (In this experiment) the entrainment patterns show little variability from day to day with a precise albeit abrupt onset of activity with lights off, also an indicator of masking behaviour (Mrosovsky 1999). Thus, there is a chance that the rapid re-entrainment is not re-entrainment but negative masking by light – light supresses activity with a subsequent release in darkness. However, the masking test is complicated by the fact that BTBR mice have very short FRPs. While it looks like the onset of activity is not in sync with the offset of light, it is possible that the advanced onset is the result of a shorter FRP. Additionally, the onset is still much closer to the new ZT 12 than would be expected if masking were the predominant driving factor, indicating that a substantial amount of entrainment has already taken place. This combined with the comparable responses to night-time light pulses, we found, between BTBR and C57BL/6J mice would not indicate synchronization to result solely from masking. In any case, it is important to not undermine masking as underlying the phenotype observed in BTBR in response to or in the presence of light.

While some proposed mechanisms for masking have been elaborated on, results from these have proved inconclusive and the exact locus of control of masking is yet unidentified. Classical (rod/cone) and nonclassical (ipRGCs) account for all non-image-forming visual responses (Hattar et al., 2003; Mrosovsky and Hattar, 2003; Mrosovsky et al., 2001; Thompson et al., 2008) and conceivably the same pathways that underlie phase shifting and entrainment

also underlie a variety of other light related responses in the SCN, including masking and pupillary contraction (Hattar et al., 2003; Panda et al., 2003). This is further supported by the fact that light information from the classical photoreceptors must pass through ipRGCs to form those non-image-forming visual responses (Goz et al., 2008; Guler et al., 2008; Hatori et al., 2008). Additionally, both phase shifting and masking to light are conserved in mice with retinal degeneration (rd/rd; Foster et al., 1991; (Mrosovsky 1994). These mice are classically blind and lack image forming vision, but are sensitive to light, comparable to wildtypes, when it comes to phase shifting (Provencio et al., 1994) or to masking (Mrosovsky 1994; Mrosovsky et al., 1999). Thus, looking at retinal input pathways, changes in retinal protein or pigments, retinal or geniculate innervation (that is known to modulate light input) to the SCN or IGL, might help understand if the BTBR mouse strain is differentially sensitive to light.

Overall, BTBR mice have robust and precise entrainment to external LD cycles and re-entrain in half the time that is usually reported (Yan and Silver 2002; Reddy et al., 2002). Optimal entrainment to external cycles is necessary in terms of sufficient adaption to the environment to ensure an organism's fitness. Additionally, jet-lag and shift work, are common causes of circadian disruption that has both acute and chronic health impacts ranging from cardiometabolic diseases, cancer and impaired immune responses to psychological disorders and cognitive deficits. Thus, elucidating the mechanisms that could help abolish circadian disruption and establish faster re-entrainment to shifted light cycles is of importance. Examining these properties in BTBR mice and further studying how and why their entrainment and re-entrainment occurs with such precision and stability might help understand the

mechanisms that will help with circadian disruption. Future experiments can aim to look at retinal input pathways, SCN innervation from the retina, IGL or raphe or retinal innervation of the IGL to further examine if dysregulation of these pathways might underlie the enhanced responses to light. Currently we are running experiments on BTBR mice to further test masking by exposing them to skeleton photoperiods.

Chapter 5. Stronger food anticipatory behaviour in BTBR mice compared to C57Bl/6J mice

5.1: Introduction

While light is the predominant zeitgeber for the circadian clock, food and feeding time is also a potent zeitgeber for behavioural and physiological mechanisms (Feillet et al., 2006a; Stephan, 2002). Food restriction induces a variety of physiological responses to maintain metabolic homeostasis, to facilitate the extraction and storage of energy from ingested nutrients and to reduce energy expenditure (Challet et al., 2009; Johnston, 2014). In nocturnal rodents when food is restricted to the mid subjective day, behaviour and body temperature increase in anticipation of feeding time (Stephan et al., 2002; Mistlberger 2011).In rodents with wheel access, this presents as an anticipatory bout of activity prior to the window of food presentation termed Food Anticipatory Activity (FAA). FAA shows canonical properties of being circadian in nature because 1) it maintains a stable phase angle to food presentation 2) it persists over several cycles of constant conditions, which in this case would be complete food deprivation 2) it does not emerge under T cycles outside the limits of entrainment, i.e. no FAA emerges when food availability is restricted to ranges outside of 21-33 hours (Mistlberger et al., 1994) and 4) it can undergo phase shifts when the phase of food presentation is shifted (Boulos et al., 1980 ; Mistlberger et al., 1994 ; Stephan et al., 2002; Mistlberger et al., 2009). As all of these features are fundamental properties of a circadian clock, we know that FAA is a clock-controlled phenomenon..

FAA is proposed to be driven by the so-called Food Entrained Oscillator (FEO), which has been shown to be independent of the main circadian clock in mammals, the suprachiasmatic nucleus (SCN; Stephan et al., 1979, 2002). When food is restricted to the light portion of in LD cycle, the phase or other characteristics of general locomotor behaviour to the light cycle is not altered, regardless of mealtime (Damiola et al.,2000; Stokkan et al., 2001). In free-running and constant conditions mice continue to present with anticipatory food activity to a daily meal (Boulos et al., 1980; Marchant & Mistlberger, 1997). Importantly, this FAA not only persists in SCN ablated animals and but also restricted timed feeding encourages the expression of rhythmicity in these animals. SCN ablated animals lack circadian organization during ad-lib feeding and appear arrhythmic but show rhythmicity in behavioural and physiological rhythms when fed at the same time every day (Abe & Rusak, 1992; Boulos et al.,1980; Marchant & Mistlberger, 1997; Mistlberger, 1992; Stephan et al., 1979). Food restriction tends to affect clocks in peripheral tissues without affecting the SCN (Damiola et al.,2000; Hara et al., 2001). And while mutations in core clock genes can modulate aspects of FAA, core clock mechanisms do not appear to be necessary to generate or maintain FAA (Pitts et al., 2003; Dudley et al., 2003; Iijima et al., 2005; Van der Zee et al., 2008; Pendergast et al., 2009; Storch et al., 2009).

The exact location/s of the FEO are unknown. While decades of research have tried to identify the locus of FAA, the exact site of the food oscillator clock is still under contention. Lesion studies have failed to establish a single site (Mistlberger, 1994, 2011; Davidson, 2009), and daily feeding schedules synchronize circadian oscillators in many brain regions and almost every organ system (Feillet et al., 2006; Verwey and Amir, 2009; Dibner et al., 2010). However

various nuclei, brain regions and hormones have been implicated including the ventromedial hypothalamus (VMH), paraventricular hypothalamus (PVN), arcuate nucleus (ARC) and lateral hypothalamus (LH; Mistlberger 1994; Mistlberger and Rusak 1988; Davidson et al., 2009). What is currently accepted is that the FEO is perhaps not one specific site, but a collection of sites both in the brain and periphery that work together to regulate various aspects of feeding and metabolism.

We aimed to examine FAA in BTBR mice for two broad groups of reasons. First, from a circadian standpoint, FAA in animals exhibiting FRPs and other circadian properties significantly different from what is commonly noted in wildtypes, has been only studied in animals with engineered genetic mutations that target specific clock genes. However, due to the ubiquitous nature of circadian genes, these mutations might have extra circadian effects on responses directly (appetite, ingesting, metabolism etc) or indirectly (movement and locomotion) associated with feeding. Thus, the BTBR mouse strain gives us the novel opportunity of studying FAA in a mouse strain in which significant differences in core circadian properties are found endogenously. Second, the BTBR mouse strain has many abnormalities in metabolism and digestive related mechanisms, including insulin resistance and an altered sense of taste (Mc Tighe et al., 2013; Meyza et al., 2017). Differences in FAA in this strain can potentially elucidate how the underlying differences in gastric and metabolic process contribute to FAA and potentially the FEO itself.

5.2: Methods

5.2.1. Animals

Eight male BTBR and eight male C57Bl/6J were used in this study. All mice were received from Dr Rho at ACHRI, university of Calgary and were at least 3 weeks old when received and between 3-8 months of age during experiments. Mice weighed at least 20 grams on arrival. Animals had ad-libitum access to food (Purina Lab Diet 5001) at all times except for the during the scheduled feeding protocolAll procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care for the ethical use of animals in resear

5.2.2. Procedures

5.2.2.i Food restriction protocol

Animals were first allowed to stably entrain to 12: 12 LD cycle for 3 weeks. Following this food availability was slowly restricted to a 4-hour window over a few days (i.e. on day 1, food was made available for 16 hours, followed by 12 hours on day 2, 8 on day 3, 6 on day 4 and finally 4 on day 5). Food availability was then maintained from ZT 4- ZT 8 for 2 weeks, after which food was made available ad-libitum. For food restriction studies in DD, animals were allowed to free run for 3 weeks before food availability was restricted once again to a 4-hour window. In the DD experiment, the 4-hour window was chosen to coincide with a rough estimate of mid-day based on when the food restriction protocol in LD. However, as animals free ran through this, the temporal window was maintained throughout the experiment. This 4-hour window of daily food availability was maintained for 3 weeks, after which food was made available ad- lib

5.2.2.ii Data analysis

Total activity was calculated by taking the sum of daily total activity over a 7-day period using Clocklab analysis software (Actimetrics, Wilmette, IL, USA). Total activity during the food restriction was also compared between groups as ratio to the total activity during the night, using the same parameters. Duration of FAA, was analysed by comparing the time difference between activity onset and activity offset. Each individual alpha was then averaged over a 7-day period. Activity counts were exported in 10 min bins and were only used if a bin had more than 50 revolutions and was followed by another 10-min bin with at least a 100 revolutions. Data were analysed between groups using Independent t-tests and using SPSS software (IBM SPSS 2015).

5.3. Results

Independent t-tests were conducted to analyse differences in food anticipatory behaviours. BTBR animals had longer bouts of food anticipatory behaviour (M= 111.64 +/- 5.5 mins vs 50.35 +/- 11.4 mins; t (8) = 4.80, p= 0.0013), greater activity during the FAA (t(8)= 3.26, p=0.0114; M= 1242.6 +/- 82.2 vs 459.3 +/- 22.52) and higher anticipation ratios when comparing food anticipatory activity as a percent of total activity (M= 26.76 +/- 4.1 % vs 7.5 +/- 3 %; t (7)= 3.54,p = 0.009), compared to C57 controls.

In DD, qualitatively, two separate rhythms can be observed in the BTBR mice. Initially only one bout of locomotor activity was present – the daily circadian rhythm. During food restriction another bout of activity can be observed, with FAA bouts comparable to that observed during the LD protocol. This rhythm is synchronized to food presentation and maintains a stable phase angle with the presentation of food.

Figure 5. 1: R**epresentative actograms showing average activity during scheduled feeding for BTBR (right) and C57Bl/6J (left**). Scale represents 24 hours in real time. The green box indicates the 4-hour window of food presentation (ZT4-ZT8).

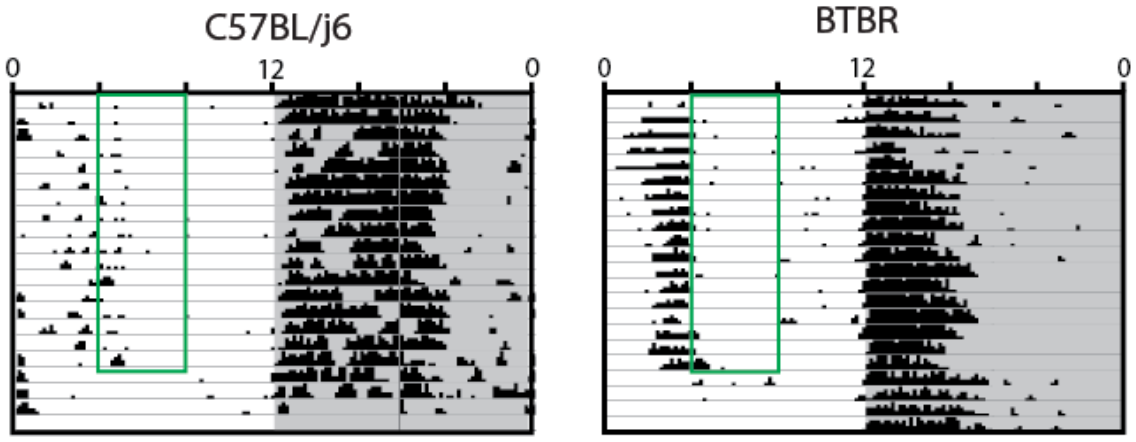
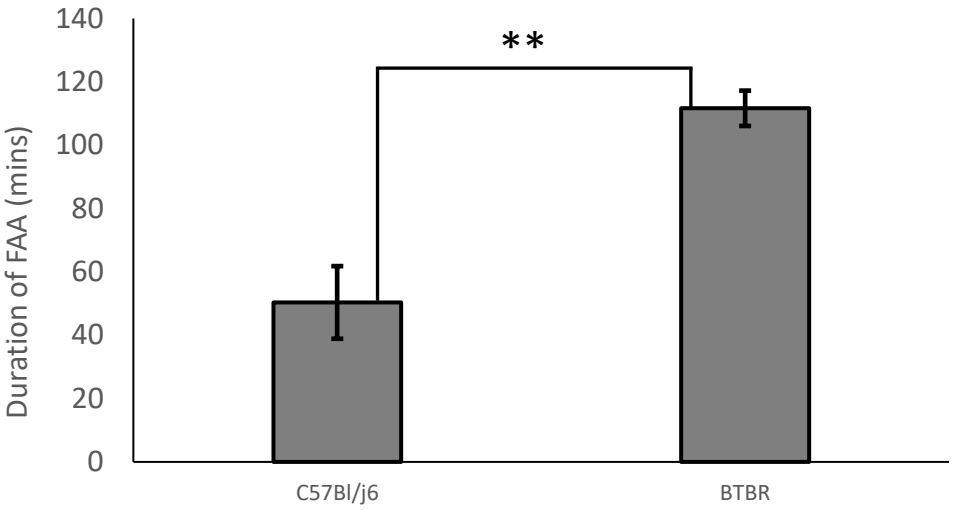
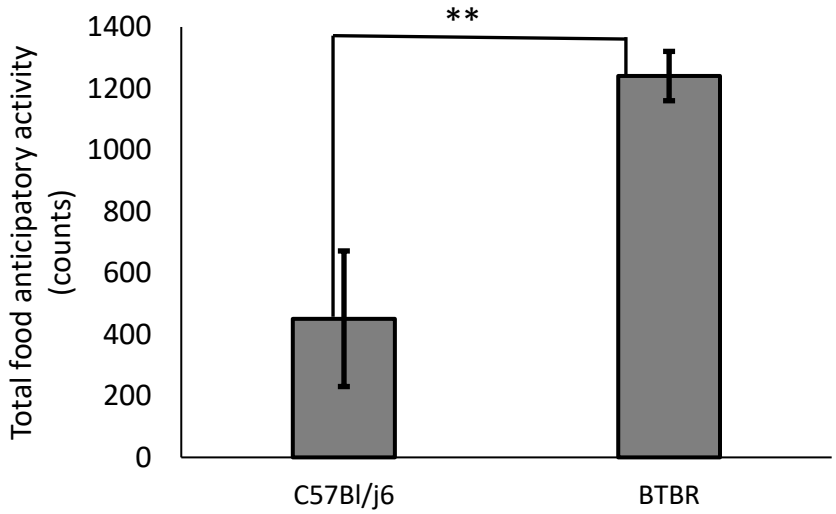

C57BL/j6
0
12
0
BTBR
0
12
0

Figure 5. 2: Bar graphs showing differences in FAA between BTBR and C57Bl/6J mice BTBR mice had significantly longer duration of FAA (A, p=0.0013),significantly more FAA compared to C57 controls (B, p=0.0014) and significantly higher ratios comparing FAA as a percent of total activity (C, p=0.009)

A)


140
120
100
80
60
40
20
0
Duration of FAA (mins)
**
C57Bl/j6
BTBR

B)


1400
1200
1000
800
600
400
200
0
Total food anticipatory activity (counts)
**
C57Bl/j6
BTBR

C)

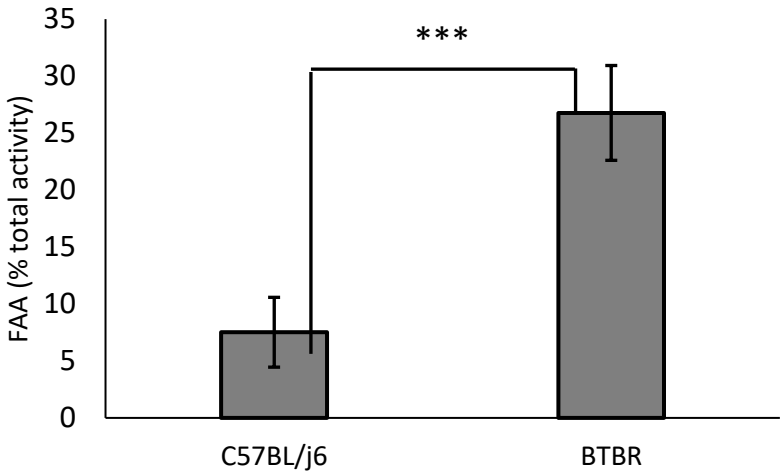
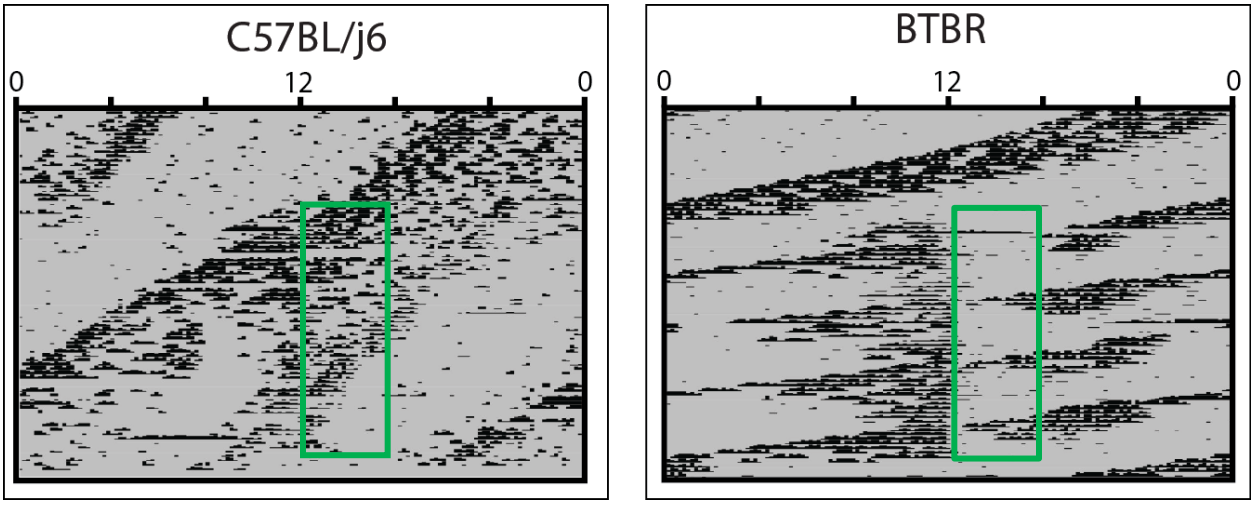


Figure 5. 3: **Representative actograms showing average activity during scheduled feeding for BTBR (right) and C57Bl/6J (left**) in DD. Scale represents 24 hours in real time. Food was provided at the same 4-hour window as during the 12:12 LD cycle, however this was arbitrarily chosen. The green box indicates the temporal window of food presentation.


C57BL/j6
0
12
0
BTBR
0
12
0

5.4: Discussion

Given that BTBR mice had significantly shorter FRPs and entrainment patterns, along with other known metabolic issues, we examined food anticipation in male BTBR mice and expected a change in amount of FAA. Results showed that BTBR mice have more robust FAA when compared C57Bl/6J mice. This robustness was evident as an increase in total activity during FAA and duration of FAA. Additionally, FAA of BTBR mice was proportionally greater than that of C57Bl/6J when the activity was expressed as a ratio of total activity during the day.

Both total activity and duration of FAA were almost 3 times higher in the BTBR mouse strain when compared to C57Bl/6J mice, even when total activity was comparable between the two. Given that the BTBR strain is used as model of metabolic issues and insulin resistance (Flowers et al., 2007; Ranheim et al., 1997) and circadian oscillators in peripheral organs, including the adrenal gland, liver, stomach, intestines, pancreas, kidney, heart, and lungs, entrain to daily feeding schedules (Damiola et al., 2000; Stokkan et al., 2001; Davidson et al., 2003) these differences may indicate a difference in peripheral organs associated with feeding and metabolism or with one of the many hormones associated with these processes.

Common feeding related hormones and peptides have been studied with regards to FAA and are found to have modulatory roles, while not being necessary or required for it. For example, the study of ghrelin has resulted in numerous but inconsistent results (Patton et al., 2013; 2014). Acyl-ghrelin and des-acyl ghrelin are peptide hormones derived from preproghrelin and are synthesized by oxyntic cells of the stomach (Kojima et al., 1999) and neurons in medial and lateral hypothalamic nuclei (Cowley et al., 2003). Studies have found that

FAA and rise in body temperature in anticipation of a daily meal, continue to be expressed in mice with knockouts either for the ghrelin ligand or receptor (Szentirmai et al., 2012; Gunapala et al., 2011; Gooley et al., 2006) and that KO mice do not differ from WT mice on parameters of FAA, such as total activity. Some other studies have, however, reported that while the FAA emerges and persists in KO animals, changes in FAA duration or length compared to WT are observed. For example, in one study the duration of FAA was decreased, with FAA starting closer to mealtime (LeSauter et al., 2009), while in another, the peak level of activity was different while there was no difference in the duration of FAA (Blum et al., 2009). Thus, it appears that while FAA persists in the absence of ghrelin signalling, certain aspects of it are modified by a lack of ghrelin.

Another feeding hormone that is shown to have modulatory effects, while not affecting the generation or persistence of FAA, is leptin. Leptin is an anorexigenic hormone released from adipose tissue in proportion to lipid stores (Zhang et al., 1994). Leptin opposes ghrelin by inhibiting NPY/AgRP neurons and stimulating POMC/CART neurons and causes a decrease in food intake and increase in metabolic rate (Kageyama et al., 2012). In animal models of leptin resistance or deficiency, ad-lib food access is associated with hypoactivity and obesity, but in both models strong FAA is observed when food availability is restricted to a single mid-day meal and the amount of this FAA is higher when compared to controls (Mistlberger and Marchant, 1999b; Ribeiro et al., 2011). Leptin administration on the other hand reduces pre-meal activity in food-restricted rats/mice (Verhagen et al., 2011). The inhibitory role of leptin on food-anticipatory activity has been inferred from its potentiation in food-restricted rodents with

impaired leptin signalling. This leptin and ghrelin modulate the expression of food anticipatory activity and food intake under ad lib feeding conditions. The augmented FAA in the BTBR mice in our experiment could thus suggest a dysregulation in either ghrelin or leptin. A decrease in either of these two hormones might cause a sharp increase in FAA. Interestingly BTBR animals deficient for leptin (BTBR ob/ob) develop diabetes faster than control animals (Hudkins et al., 2010) suggesting a dysregulation of either leptin mediated signalling pathways, or leptin independent metabolic pathways such as insulin. This may be particularly relevant since BTBR mice are also used as a model of increased obesity and type 2 diabetes susceptibility (O'Brein et al., 2015; Hudkins et al., 2010) and show insulin resistance (Meyza et al., 2017). However, insulin is not required for FAA and FAA persists in mice with insulin resistance (Davidson et al., 2002; Oishi et al., 2004).

Neuropeptide Y, that regulates non-photic and arousal mediated input to the SCN, has also been implicated in FAA, but like the other factors mentioned its role is also solely modulatory. In one study, the effect of NPY on FAA was shown to be transient - NPY KO mice showed reduced FAA on day 7 of food restriction, which however normalized by day 14, suggesting perhaps compensatory mechanisms from other systems over time (Gunapala et al., 2011). Additionally, a developmental lesion in the arcuate nucleus, induced by postnatal MSG exposure and confirmed by lack of NPY immunoreactivity, did not affect food anticipatory activity in rats (Mistlberger and Antle, 1999). Radiofrequency lesions of the hypothalamic paraventricular nucleus, that contains NPY, also do not impair food anticipatory activity in rats

(Mistlberger and Rusak, 1988). Thus, while NPY is considered a central feeding hormone, its role in FAA does not seem critical.

Thus, in relation to our findings, it appears that a single feeding related hormone or peptide cannot be taken to underly any particular difference in FAA. There is variability, and sometimes contrasting results, for each signalling peptide. Much like feeding behaviour during ad-lib, FAA during food restriction is likely regulated by multiples hormones underlying multiple discrete and joint mechanisms. A lack of signalling in one might not affect FAA due to considerable redundancy already present, or because it engages compensatory mechanisms. Therefore, potentially a combination of metabolic and feeding factors in BTBR mice causes the high levels of FAA we observed. Feeding hormones might serve as a good mechanism to further investigate FAA in BTBR mice, given their importance in modulating FAA and given the BTBR strain's predisposition to insulin resistance, diabetes and obesity. Overall this may suggest that a higher hunger driven motivation for food seeking in BTBR mice, due to imbalances in one or multiple feeding hormones.

Central neurotransmitters might also play a role in the feeding differences we observed. 5-HT signalling has been shown to modulate food anticipatory activity. 5-HT_{2C}R null mutant mice have enhanced food anticipatory activity and increased expression of c-fos to restricted feeding, in the nucleus accumbens and other extrahypothalamic regions (Hsu et al., 2010). Fluoxetine, a 5-HT reuptake inhibitor, does not affect locomotor activity under ad-lib condition, but does attenuate FAA under restricted feeding conditions - suggesting that an increase in 5-HT decreases FAA (Rosenblit- Susan et al., 2016). Additionally, 5-HT_2R antagonists and 5-HT_{2C}R

knockout lead to increased FAA (Hsu et al., 2010; Shibata et al., 1995). Differences in 5-HT signalling have been reported in BTBR mice. Specifically, BTBR mice have increased hippocampal $5HT_{1A}$ receptor density (Gould et al., 2011, 2014) and decreased binding to the serotonin transporter (SERT, Gould et al., 2011). Given the aforementioned findings on 5-HT modulation of FAA, altered 5-HT signalling might influence FAA in BTBR mice. A dysregulation in the 5-HT system will also potentially help explain the apparent greater negative masking of light on BTBR circadian activity in constant and entrained conditions. Thus, future studies can aim to study the 5-HT system in BTBR mice with respect to FAA.

Finally, there is a possibility that an underlying deficit in clock gene signalling could cause the difference in FAA we observed. However, research into the role of clock genes in FAA, much like peripheral hormones shows complex results - in most cases FAA persists in animals with clock gene mutations. While some studies report some alteration to some aspects of FAA, in many cases these are not consistent. For example in BMAL knockout animals there is a persistent attenuation of FAA (Mieda et al., 2011) and in *Per1/Per2/Per3* knockout mice, FAA is unstable and imprecise in a 24 h feeding schedule; yet, the animals are able to anticipate scheduled feeding (Pendergast et al., 2012). In one study using *Per2Brdm1 (*a deletion in the PAS domain of the Per2 protein), there is significant loss of expression of FAA (Feillet et al., 2006b; Zheng et al., 1999). However, when the mutation is a null allele of *Per* no obvious alteration in FAA seems to occur (Bae et al., 2001; Storch and Weitz, 2009). This difference in the result between the two studies could potentially be due to the fact that in the case of a null mutation paralogous genes might compensate. The distinct difference in FAA in the two *Per2*

mutant mouse models might be due to the redundant functions of paralogous genes that develop as compensatory mechanisms during development, which in the case of Per2Brdm1 would not occur because the mPer2 protein is partially functional. However, this is something that needs to be tested further. It does however emphasize an important point relevant to FAA- that it is the result of multiple systems within the brain and periphery. Owing to the importance that food availability and feeding has on an animal's survival, it is conceivable that considerable redundancy exists to ensure persistence of food seeking behaviour.

Overall, a variety of factors can underly the changes we observed in FAA in BTBR mice. But the complexity of the FEO and the myriad of factors that contribute to FAA make it difficult to establish clear criteria or factors that might be responsible for the changes in FAA that we observed. Future studies can look at both peripheral hormones and tissues and central neurotransmitters to help explain the changes observed in BTBR. Given the well-established difference in body weight, fat consumption, food seeking and metabolism in BTBR mice (Mc Tighe et al., 2013; Meyza et al., 2017) it is important to also look at the feeding and hunger related mechanisms first to better understand the mechanistic difference underlying these behavioural changes.

Finally, an interesting finding in our experiment, was that qualitatively in DD we observed two bouts of the activity rhythms. One for the circadian clock with a free running period of circa 23 hours and another that preceded that food presentation by a few hours and that had a period of 24 hours. This FAA rhythm emerged separately from the SCN controlled

rhythms, thus providing a visual representation of the fact these two rhythms are controlled, at least in part if not completely, by two separate oscillator systems.

Chapter 6. SCN anatomy differences between BTBR and C57Bl/6J mice

6.1. Introduction

The SCN in the anterior hypothalamus is the established site of the circadian master pacemaker. It receives light input from the retina (via the RHT) and non-photic input from the IGL (via the GHT), the raphe nuclei and the basal forebrain cholinergic system (Morin et al., 2013; Yamakawa and Antle 2016). The SCN can be divided further based on gene expression, peptidergic cell groups and innervation, into a ventrolateral core and a dorsomedial shell region (Antle and Silver 2005). The core region of the SCN is retinorecipient, and across species contains cells that express GRP and VIP (Hattar et al., 2006; McNeill et al., 2011). The SCN shell on the other hand is comprised of cells that express AVP. While the SCN shell is intrinsically rhythmic, some parts of the core are not. Period (Per) gene expression in constant darkness (DD) is highest in the SCN shell (Hamada et al., 2004). Projections from the core to the shell are dense while reverse projection are sparser (Leak and Moore 2001; Abrahamson and Moore 2001). Both the core and the shell send inputs to various hypothalamic and extra-hypothalamic areas, but to varying degrees (Kriegsfeld et al., 2004)

The core and shell and their constituent peptidergic cell groups have been implicated in circadian behaviour and entrainment to light. VIP has been demonstrated to be especially important for the maintenance and synchronization of cellular clocks in individual SCN neurons (Herzog et al., 2017). Loss of VIP or its cognate receptor VPAC2R (encoded by the *Vipr2* gene) results in a loss of daily rhythms in running-wheel behaviour and desynchronized rhythms in

spontaneous firing and gene expression among SCN neurons (Harmar et al., 2002; Colwell et al., 2003; Aton and Herzog, 2005; Brown et al., 2005; Maywood et al., 2006). Similarly, animals lacking AVP have been shown to have longer periods and to re-entrain to shifted light dark cycles faster (Mieda et al., 2015; Yamaguchi et al., 2013). There is a high degree of functional redundancy between these peptidergic groups. In co-culture experiments, explants from animals that lack VIP could still maintain synchronized cellular rhythms and population level synchrony due to compensation from GRP and AVP and only completely lost rhythmicity when both GRP and AVP signalling were blocked (Maywood et al., 2011).

Given the drastically different phenotypes of BTBR mice both in constant conditions and in response to light cycles, we examined if there were differences in VIP, GRP or AVP anatomy within the SCN. We expected that VIP and AVP cell numbers would be reduced in the BTBR SCN, as a lack of these peptidergic cells appears to most influence a change in period and enable faster re-entertainment.

6.2. Methods

6.2.1: Animals

A total of 25 adult mice were used for this experiment, comprising BTBR male (n=7) and female (n= 6) and C57Bl/6J male (n=6) and female (n=6; University of Calgary Life and Environmental Science Animal Resource Centre) mice. Animals were 25g upon arrival in the laboratory. Mice were either group housed in polycarbonate cages or individually housed in Nalgene Type L clear polycarbonate cages (30.3 cm long × 20.6cm wide × 26 cm high; Nalg

Nunc International, Rochester, NY) equipped with a stainless-steel running wheel (diameter of 24.2 cm). Animals were maintained in a 12:12 light: dark cycle until tissue collection. All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and adhered to the policies of the Canadian Council of Animal Care for the ethical use of animals in research.

6.2.2: SCN anatomy: Surgery, tissue collection and immunohistochemistry

All animals were housed in LD 12:12 and animals were given an overdose of pentobarbital and were perfused at ZT 12 (time of lights off). Animals received an overdose of sodium pentobarbital and were then perfused with 100 ml of phosphate buffered saline (PBS), followed by 100 ml of 4% paraformaldehyde in PBS. Brains were collected and post fixed in 4 % paraformaldehyde for 24 hours and cryoprotected in 30% sucrose for a further 48 hours. 35um sections were then collected through the anterior hypothalamus using a cryostat. Floating tissue sections were washed 3 times in PBS and blocked in PBSt (PBS with 0.1% triton-X100) containing 5% NDS for 1 hour. Tissue sections were then incubated in the primary antibodies – a guinea pig polyclonal antibody raised against vasoactive intestinal polypeptide (VIP ; Rabbit anti- VIP, 1: 5, 000, Immunostar) and a guinea pig polyclonal antibody raised against arginine vasopressin (AVP ; Guinea Pig anti-AVP, 1:5000 Peninsula) and a rabbit polyclonal antibody raised against gastrin releasing peptide (GRP ; Rabbit anti-GRP , 1:2000, Peninsula) for 48 hours at 4°C. Two separate series of tissue sections were used for the VIP and GRP antibodies and both tissue series were stained against AVP (thus each

series was either stained for VIP and AVP or GRP and AVP). Tissue sections were then washed again 6 times for 10 mins with PBSt before being incubated with the respective secondary antibodies (Cy3 Donkey Anti Guinea Pig, 1:200, Cy2 Donkey anti-rabbit, 1:200, Jackson Immuno) for 1 hour. Finally, tissues were washed with PBSt 3 times for 10 mins, mounted and cover slipped. Slices were then visualized with fluorescence microscopy to analyze cell density of VIP, AVP and GRP expressing cells between the BTBR and control SCN.

6.2.3: Microscopy procedures

Tissue sections were visualized using a microscope (Olympus BX51) equipped with filters for the different cyanine dyes used in the immunohistochemistry protocol. All tissue sections were visualized and photographed though a 20x objective. At this magnification, each hemisphere of the SCN can be visualized fully. All analysis and quantification were carried out bilaterally. The Cy2 filter (492-510nms) was used for visualizing GRP cells and the CY3 filter (550-570nms) was used for VIP and AVP cells on separate sections. All images were collected using imaging software (ImagePro Plus 5.1.2.59; Media Cybernetics, Inc) and analyzed using imagej software (ImageJ 1.42q; National Institutes of Health, Bethesda, MD).

6.3: Results

Differences in SCN cells numbers were compared between BTBR and C57 male and females were compared using 2x2 ANOVAs. For GRP cells, no significant main effects of strain ($F(1, 17) = 1.60$, $p = 0.224$) or sex ($F(1, 17) = 3.75$, $p = 0.07$) were found such that BTBR mice had comparable number of GRP expressing cells to C57Bl/6J mice (M = 22.69 +/- 1.18 vs 24.83 +/- 1.21) and male and female mice also had comparable GRP cell numbers (M = 25.40 +/- 1.05 vs 22.12 +/- 1.33). No significant interaction was found between strain and sex ($F(1, 17) = 0.05$, $p = 0.822$). Planned contrasts also revealed no significant differences across subgroups.

For VIP cells a significant main effect of strain ($F(1, 18) = 61.22$ $p < 0.0001$) and sex ($F(1, 18) = 6.32$, $p = 0.02$) was found, such that BTBR mice had significantly higher number VIP expressing cells in the SCN when compared to C57 mice (M = 86.08+/- 4.1 vs 41.26 +/- 3.98) and males had significantly higher number of VIP cells when compared to female (M = 70.88 +/- 3.65 vs 56.47 +/- 4.41). No significant interaction between strain and sex was found ($F(1, 18) = 3.21$, $p = 0.09$). Planned contrasts revealed that 1) BTBR males had significantly higher number of VIP cells compared to C57 males (M = 98.43 +/-4.96 vs 43.33 +/- 5.3; ($t(18) = -7.53$, $p<0.0001$) 2) BTBR males had significantly higher VIP expressing cells compared to BTBR females M = 98.43 +/- 4.96 vs 75.75 +/- 6.57; ($t(18) = 2.99$, $p = 0.008$) 3) BTBR females had significantly higher number of VIP cells compared to C57 females (M = 73.75 +/- 6.57 vs 39.20 +/- 5.87; $t(18) = -3.918$, $p=0.001$) 4) VIP cell number were comparable between C57 males and females (M = 43.33 +/- 5.3 vs. 39.20 +/- 5.87; $t(18) = -.519$, $p = 0.610$).

For AVP cells a significant main effect was for strain ($F(1, 19) = 9.67$, $p = 0.006$) and for sex ($F(1, 19) = 4.603$; $p = 0.044$) such that BTBR mice had higher number of AVP expressing cells when compared to C57 (M = 46.88 +/- 2.11 vs 37.75 +/- 2.51) mice and females had higher number of AVP cells compared males (M = 45.45 +/- vs 39.16 +/-). No significant interaction was found between strain and sex ($F (1, 19) = 2.75$, $p = 0.114$). Planned contrasts revealed that: 1) BTBR males had significantly higher number of AVP expressing cells compared to C57 males (M = 46.16 +/- 2.12 vs 32.16 +/- 2.02; $t(19) = -3.45$, $p = 0.003$) 2) that C57 females had significantly higher AVP expressing cells compared to C57 males (M = 43.33 +/- 2.86 vs 32.16 +/- 2.86 $t(19) = -2.775$, $p = 0.013$). 3) BTBR females and males had comparable number of AVP expressing cells (M = 47.60 +/- 3.13 vs 46.88 +/- 2.87 ; $t(19) = -1.01$, $p = 0.328$ 4) BTBR and C57 females had comparable number of AVP expressing cells (M = 47.60 +/- 3.13 vs 43.33 +/- 2.86; $t(19) = -0.337$, $p = 0.74$).

Figure 6. 1: Representative fluorescence microscopy images of GRP, VIP and AVP expressing cells in the SCN. Images were taken at 20x magnification. BTBR mice had significantly higher VIP and AVP cells compared to C57Bl/6J mice.

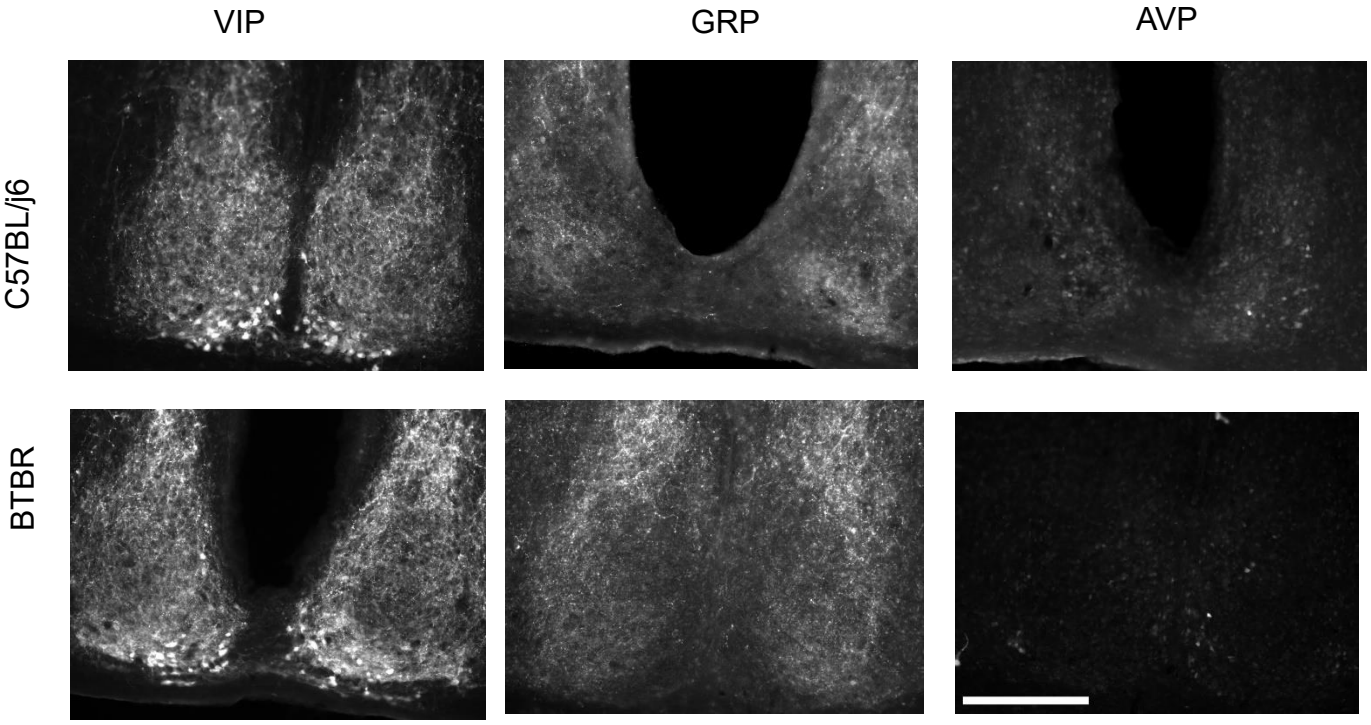
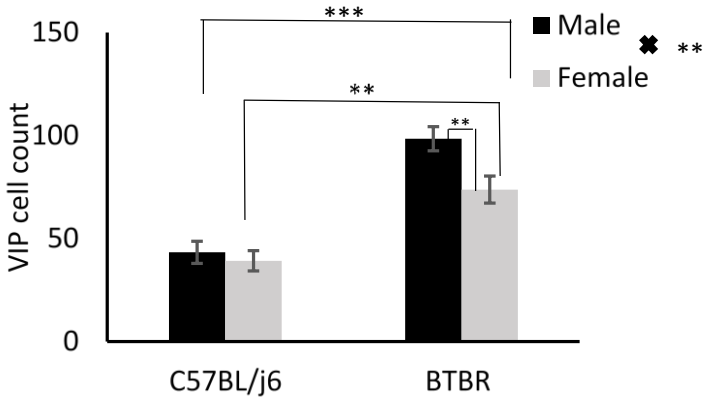

VIP
GRP
AVP
C57BL/j6
BTBR

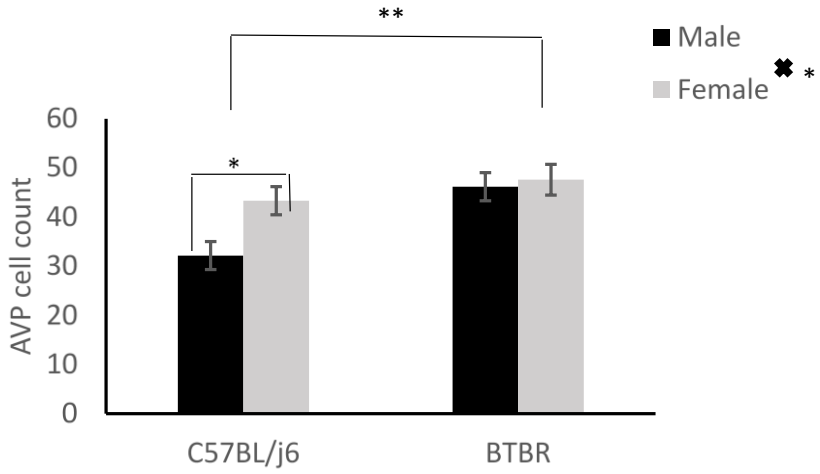
Figure 6. 2: Bar graph showing levels of VIP (A), AVP (B) and GRP (C) staining between male female BTBR and C57Bl/6J mice. * represents p < 0.01 ** represents p <0.001 and *** represents p<0.0001

A) VIP; BTBR mice had a significantly higher number of VIP cells when compared to C57Bl/6J mice (p<0.0001) and male mice overall had more VIP expressing cells compared to female mice (p = 0.02) as indicated by ✖, however no significant interaction was found (p=0.09). Planned contrasts showed that 1) BTBR males had more VIP cells than C57 males (p<0.0001) 2) BTBR males had more VIP cells when compared to BTBR females (p = 0.0083) 3) BTBR females had more VIP cells compared to C57 females (p = 0.001) and 4) VIP cell numbers were comparable between C57 males and females (p = 0.610)
B) AVP; BTBR mice had a significantly higher number of AVP cells when compared to C57Bl/6J mice (p = 0.006) and female mice overall had more VIP expressing cells compared to male mice (p = 0.044) as indicated by ✖Planned contrasts showed that 1) BTBR males had more AVP cells than C57 males (p = 0.003) 2) C57 females had significantly higher AVP expressing cells compared to C57 males (0.013) 3) AVP cell counts were comparable between BTBR males and females (p = 0.328) BTBR and C57 females (p = 0.74)
C) GRP; BTBR and C57 mice (p = 0.224) and males and female mice (p = 0.07) had comparable numbers of GRP expressing cells. No significant differences were found between any subgroups.

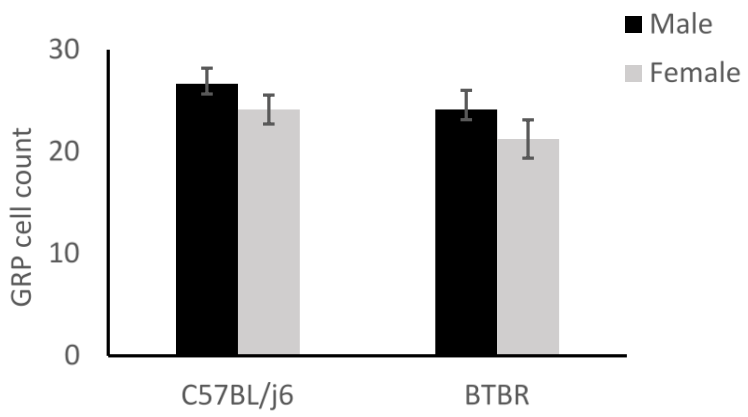
A)


***
**
**
Male
Female
**
150
100
50
0
VIP cell count
C57BL/j6
BTBR

B)


**
Male
Female
*
*
60
50
40
30
20
10
0
AVP cell count
C57BL/j6
BTBR

C)


Male
Female
30
20
10
0
GRP cell count
C57BL/j6
BTBR

6.4: Discussion

Given that BTBR mice had shorter periods and accelerated re-entrainment to shifted light dark cycles we expected that they would have fewer VIP and AVP expressing cells in the SCN. However, the BTBR mice in our experiment had higher numbers of both VIP and AVP expressing cells, while having a comparable number of GRP cells. While our experiment does not aim to establish a causal link between the differences in SCN anatomy and the circadian phenotype found in BTBR these results do show that there is an obvious difference in peptidergic content of the SCN core and shell, especially in VIP expressing cells and to a lesser extent in AVP expressing cells.

Both these peptides have been previously implicated in general circadian behaviour and in photic entrainment. Animals with mutant AVP receptors re-entrain to both phase advances and phase delays in half the time of wildtype controls (Yamaguchi et al., 2013). This resetting behaviour is similar to what we found in BTBR mice. The study, however, showed accelerated re-entrainment to shifted LD cycles following an attenuation in AVP signalling either genetically (using knockout for receptors to AVP) or by pharmacologically blocking the receptors, whereas we found an increase in the number of cells. While these findings seem discordant, it is possible that it is not a loss or enhancement of signalling specifically, that changes photic entrainment but rather a change from what is considered baseline. There is some support for this from studies done on VIP receptors (An et al., 2012). Animals lacking VIP receptors shown dysrhythmia and impaired responses to light (Colwell et al., 2003; Harmar et al., 2002; Dragich et al., 2010) and re-entrain to 8-hour shifts in light cycles almost immediately (Harmar et al.,

2002). On the other hand, animals in which the VIP receptors signalling was enhanced also showed faster entrainment to shifted light dark cycle (Shen et al., 2000; An et al., 2012). Application of VIP mimics the effect of light in-vivo and can phase shift behavioural and SCN rhythms (Akiyama et al., 1999; Tischkau et al., 2000, 2003). VIP pre-treatment approximately halved the time required for mice to re-entrain to an 8-hour shifted light schedule and for SCN cultures to re-entrain to a 10-h shifted temperature cycle. Additionally, however, the study also reported that VIP has a dose dependent effect on the SCN, with higher doses causing desynchrony in SCN neurons. This dose, while higher than the threshold required for VIP to cause phase shifts, is still 100-fold lower than what is required to saturate the response to VIP (therefore, still within physiological ranges). Thus, VIP dose dependently affects SCN synchrony.

There is some evidence that sustained activation of VIP neurons can reprogram the spatiotemporal wave within the SCN (Brancaccio et al., 2013). Spatiotemporal waves progress in a time dependent manner from the dorsal shell to the ventral core before receding back to the dorsal shell (Hastings et al., 2019). An alteration of the spatiotemporal wave could potentially change the network dynamics and how the SCN re-entrains to shifted cycles and responds to light. If VIP levels were maintained at the level recorded in our study for extended periods of time, it possibly could modulate the network dynamics in a similar manner. Additionally, high levels of VIP might, as suggested by the An et al., (2012 study), cause desynchrony in the SCN. As the strength of the SCN rhythm is dependent on the phase coherence of its individual oscillators, a loss of this will cause a loss of strength in this network. A weaker network is more sensitive to external perturbation (Herzog et al., 2017) and therefore

this would allow the SCN to shift faster. However there are some caveats with this 1) we only collected tissue at one time point and thus cannot conclude if levels of VIP in the BTBR are high throughout the day or for any extended period of time 2) in the An et al., (2012) study, VIP was applied to a slice preparation of the SCN, which is significantly different in mechanism from an endogenous increase as found in the our study. High endogenous levels might not elicit the same mechanisms of adaptation and might be confounded by other differential causative mechanisms, when compared to an acute application.

Interestingly, a recent study found increased levels of VIP signal intensity in SCN of animals that had the primary transcription factor *Bmal1* knocked out only in astrocytes (Mieda et al., 2015). This dysregulation of VIP expression could potentially alter circadian entrainment to light cycles. Future studies can aim to look at the role of glial cells in entrainment in BTBR mice, since there are reports of altered astrocytic and microglial histopathology in BTBR mice (Stephenson et al., 2011) and it has been reported that the differential day/night glial coverage of dendrites on VIP neurons is important to facilitate entrainment to light and to the pacemaker-resetting mechanism (Becquet et al., 2007; Girardet et al., 2010)

We also found significant sex differences between males and females in our experiment. It has been long known that sex differences are prevalent at the level of SCN and circadian behaviour (Bailey and Silver 2014). The BTBR strain potentially provides an opportunity to examine these sex differences in more detail, as generally no sex differences are reported when studying C57Bl/6J mice (Kuljis et al., 2013), indicating sex differences are

strain specific and therefore must be examined individually for each strain. Accordingly, while we found BTBR male and female mice showed sex differences in the number of VIP cells, no significant differences were found between control male and females. We found the opposite pattern for AVP cells however- no significant differences between BTBR males and females, but a significant difference was found between control male and females. It will be interesting to examine if the differential levels of these peptide expressing cells can modulate circadian period or other parameters.

Overall, BTBR male and female mice significantly differ from C57BL/6J controls in the number of VIP and AVP cells they express. Both these peptides have been repeatedly implicated in both circadian rhythmicity and photic entrainment and thus it is important to establish if there is a causal link between these cell groups and the circadian phenotype we observed. When looking at our results in DD conditions and the anatomy, we find that the more VIP and AVP cells expressed at ZT 12 the shorter the FRP of the animals. Thus, future studies can aim to modulate the level of these peptides and examine any causative links.

Chapter 7. General Discussion

7.1. Summary of major findings

This dissertation aimed to characterize 1) General circadian behaviour in constant conditions (Chapter 3) 2) Entrainment patterns to ambient lighting schedules (Chapter 4) 3) Food anticipatory behaviour (Chapter 5) and 4) SCN anatomy (Chapter 6) in BTBR mice compared to the C57BL/6j mouse strain, which is one of the most commonly used mouse strains to study wild-type circadian behaviour. We first looked at general circadian behaviour in constant darkness (DD). BTBR mice had very short FRPs (~22.5 hours) compared to what is normally observed in mice (~23.7 hours) and what was recorded in our control animals. Their duration of activity was also significantly shorter (~6 hours) when compared to C57BL/6J controls and they had higher levels of total wheel running activity. As wheel running behaviour is generally considered a good indicator of circadian function (Jud et al., 2005), these findings indicated a core dysregulation is the functioning of the circadian system. Based on these findings we expected that responses to light pulses in the late subjective night would be altered, however BTBR mice had responses to both phase advancing and delaying light pulses that were comparable to what was observed in C57BL/6J controls- suggesting that the differences in circadian behaviour were perhaps independent of light input pathways and restricted more to loci that provide arousal and locomotor activity feedback to the SCN (Webb et al., 2014; Hughes and Piggins 2012) or other systems regulating wheel running activity in constant darkness.

We then examined circadian behaviour under constant light conditions and found that the BTBR mice had an immediate dampening of locomotor activity, had greater lengthening of the FRP and had lower level of total activity in LL conditions. Thus, while their responses to light pulses were comparable, BTBR mice showed greater changes in activity in LL, suggesting that while there might not necessarily be an increase in sensitivity to light, BTBR mice may be more susceptible to masking (Mrosovsky 1999). When we tested responses to dark pulses, we found that BTBR mice had both a greater amount of wheel running activity during the pulses compared to controls and larger phase shifts to the dark pulses. While these dark pulses induced phase shifts were not directionally consistent (we observed both advances and delays) they were large in magnitude, suggesting that the exposure to dark pulse enhanced locomotor activity and potentially arousal induced shifting- which again aligns with these mice being more susceptible to masking.

The next set of experiments looked at entrainment patterns of BTBR mice under 12:12 LD cycle an in responses to shifting light dark cycles. We found that that BTBR mice, unexpectedly, had a stable and precise entrainment pattern to the LD cycle, with stable phase angles that varied less from zero compared to the control (i.e. their activity was more precisely aligned with lights off). BTBR mice still had shorter alphas under LD. Thus, despite the short FRPs these animals appear to entrain to ambient light cycles with high precision and stability. We then tested their re-entrainment patterns to shifting light cycles. Resynchronization after a shift in the light cycles generally takes multiple cycles, which arises from 1) the oscillators in the SCN establishing synchrony 2) the peripheral clocks resynchronizing with the SCN (Yan and

silver 2002; Reddy et al., 2002; Davidson et al., 2009). This resynchronization is directionally biased by the FRP- such that shorter FRPs allow for easier re-entrainment to advancing cycle and longer ones to delaying cycle (Aschoff 1975). However, entrainment is also reported to be direction dependent - animals take longer to re-entrain to phase advances than phase delays (Reddy et al., 2002; Davidson et al., 2009). Based on this we predicted that BTBR animals, due to their short FRPs, would re-entrain to phase advances faster than control mice and have slower re-entrainment to phase delays. We found however that BTBR mice re-entrain to both phase advances and phase delays in half the time of what is generally reported (and what we found with our control group). Re-entrainment to phase advances was established in ~3 days and to phase delays in ~1 day. Considering their greater dampening of activity in LL and possible effects of masking we performed a masking test, where animals were subject to a light cycle shift and then released into DD after 3 days- if re-entrainment did occur then the phase of onset of activity would coincide with lights off of the new zeitgeber cycle. Instead, if synchronization the new LD cycle was a result of masking, then upon release into DD the phase of onset would show a large phase angle difference from the zeitgeber cycle. We found that while BTBR animals were still offset from the zeitgeber cycle, the onset of activity was still close to that of the period of lights off in the new zeitgeber cycle suggesting that substantial re-entrainment had taken place within that time frame. Thus, while masking cannot be omitted, it does appear that BTBR mice 1) re-entrain faster to shifted light cycles 2) are more sensitive to segregating their behaviour into the light and dark portions.

We then looked at food anticipatory behaviour. This was based on both their altered circadian phenotype and the known metabolic dysregulation in BTBR mice (Flowers et al., 2007) and we expected that FAA would be altered consequently. We found that BTBR mice had higher amounts of total activity for FAA and a longer duration FAA when compared to C57BL/6j mice. This was maintained when the total amount of activity during the FAA was taken as a ratio of total activity during the activity. Qualitatively we also observed the emergence of two separate rhythms during the food restriction in DD. While both strains showed this, offsets in BTBR appear to be more aligned with the feeding cycle.

The final experiment examined anatomical differences between the two strains relating to specific peptidergic cell types in the core and shell regions of the SCN. Because BTBR animals re-entrain faster and have short FRPs, we expected an alteration in these cell groups, especially in VIP and AVP expressing cells, since both have been previously implicated in faster resetting and in influencing the FRP in mice. We found that the when sampled at ZT12, BTBR mice had significantly more VIP and AVP expressing cells in the core and shell respectively. Indicating that there might be a change in signalling from the core to the shell when compared to controls, however this will need to be further determined.

7.2. General discussion

Overall it appears that the BTBR mice differ drastically from C57BL/6J mice on multiple parameters of circadian behaviour and in the number of VIP ad AVP expressing cells in the SCN. What is especially important to note across all results in this set of studies, is the low variability

that is observed in the BTBR strain. Generally, shorter FRPs are associated with higher variability (Bittman 2012) however we found very little deviance from the mean in all measures in the BTBR mice. While this could be potentially influenced by the degree of inbreeding in this strain, this at the very least shows that the BTBR is a good model to study circadian parameters.

This is strengthened by the fact that the circadian phenotype we observed while different from WT behaviour, still aligned with theoretical and experimental predictions about circadian behaviour. For instance, a shorter FRP is correlated with a shorter duration of activity and higher total activity (Daan and Aschoff 2001; Pittendrigh and Daan 1976; Mrosovsky 1999) and BTBR mice consistently showed shorter duration of activity both in DD and LD conditions. Additionally, higher amount of locomotor activity, especially wheel running activity, is associated with shorter FRPs (Yamada et al., 1988; Harrington et al., 2007; Webb et al., 2014). BTBR mice again consistently showed consistently high levels of total running activity. This does however raise an important point. Total activity, alpha and FRP are all influenced by access to wheels. Animals tend to have longer FRPs, less total activity and longer durations of activity when they do not have access to wheels (Yamada et al., 1988). Additionally, there is some evidence that the there is an inverse correlation between FRP with wheel access and FRP without wheel access- i.e., animals with the shorter FRPs with wheel access have longer FRPs when not running in wheels (Yamada et al., 1988). Therefore, there is a possibility that the circadian phenotype we observed is restricted to wheel running behaviour and shows a dysregulation with the locomotor SCN feedback pathway (Webb et al., 2014; Hughes and Piggins 2012), rather than changes in the SCN itself. Future studies can aim to examine

circadian parameters while recording general activity and body temperature, instead of wheel running. However, most studies use wheel running to study circadian behaviour, and these changes are generally proportional across strains (that is across strains there is lengthening of FRPs with wheel access). Therefore, BTBR mice still function as a good model for studying altered circadian function.

These changes in circadian parameters indicate a core circadian deficit. Many studies have looked at the effects of clock genes on FRP, but no single locus has been identified that causes a change in FRP. Mice mutant for core clock genes, or the enzymes that control their transcription or phosphorylation, have all been shown to cause a change in period. These include *Per1, Per2, Per3, Cry1, In Cry2, CKIε, Clock* and *Rev-erba* mutants (Cermakian et al., 2001; Lowrey et al., 2000; Preitner et al., 2002; Shearman et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999). However, different mutations of the same locus can have varying effects. While some studies have reported a lengthening FRP with *Clock* mutations (Low-Zeddies et al., 2001; Vitaterna et al., 1999), other have reported FRPs only slightly shorter than normal with homozygous deletion of the *Clock* gene (Debruyne *et al*., 2006). *Bmal1* deletion in SCN astrocytes lengthens FRP (Tso et al., 2017) and similar results are observed with *Bmal1* deletion in AVP expressing cells (Mieda et al., 2015), however global *Bmal1* deletion causes arrhythmicity (Bunger et al., 2000). Similarly, *Cry1* null mice have a short period phenotype whereas, *Cry2* null mice have a long period phenotype (Thresher *et al*., 1998; van der Horst *et al*., 1999; Vitaterna *et al*., 1999). *Per1* and *Per2* null alleles both yield shorter periods, although this shortening varies depending on the mutation (Bae *et al*., 2001; Zheng *et*

al., 2001; Cermakian *et al*., 2001). Enzymes that control degradation of *Per* and *Cry* genes would also alter the speed of the clock. E3 ubiquitin-ligases Fbxl3, and Fbxl21 modulate proteasomal degradation of CRY1 and CRY2 and Casein kinase 1 enzymes (CK1δ/ε) phosphorylate PER proteins thereby altering the rate of the negative arm of the TTFL. Depending on the how they affect the rate of degradation (slowing it down vs speeding it up), they varyingly affect the FRP (lengthening vs shortening; Meng et al., 2000; Godinho et al., 2007; Yoo et al., 2013). These enzymes that target them would serve as good targets to examine in BTBR mice and a change as drastic as seen in the BTBR mice might arise due to a combination of factors.

Another potential source of regulation of FRP and entrainment could come from glial cells. Both astrocyte and microglia show circadian rhythms in their metabolism and function (Marpegan et al., 2011; Lavialle and Servière, 1993, Santos et al., 2005; Fonken et al., 2015). Astrocytes in addition have been implicated in directly influencing the circadian period. *Bmal1* knockouts in astrocytes lengthens the free-running period, as does CK1ε tau excision *in* astrocytes (Tso et al., 2017) and *Bmal1* knockout in astrocytes causes an increase in signal intensity of VIP cells (Barca- Mayo et al., 2017), suggesting that astrocytes potentially influence cellular signalling in the SCN. This may be potentially important in the BTBR strain, since there is evidence for a significant increase in staining for the astrocytic marker GFAP in BTBR brains. There were also some structural changes in astrocytic fibres and their orientation, combined with a decrease in NeuN (Stephenson et al., 2011). Therefore, modulation of SCN neuronal function by neighbouring astrocytes may potentially underlie some of the changes in the

circadian phenotype we recorded. Work in the lab is currently in the process of examining density and structure of astrocytes in the SCN.

A critical finding in our studies is the increased cell numbers of both VIP and AVP expressing cells. Both VIP and AVP have been implicated in photic resetting. As mentioned in chapter 6, a lack of AVP appears to accelerate re-entrainment to shifted light dark cycles, while VIP appears to facilitate re-entrainment both when its signalling is blocked and enhanced (An et al., 2012; Harmar et al., 2002; Shen et al., 2000). Both cell groups have also been shown to have an impact on FRP. Knocking out *Bmal1* in AVP cells lengthens the FRP (Mieda et al., 2015) as does knocking out *CK1δ* (Mieda et al., 2016). Overexpression of *CK1δ* in AVP cells, on the other hand, shortened the FRP. Future work can aim to modulate levels of either of these peptides in BTBR animals and examine if it changes general circadian behaviour or entrainment to light cycles. While each of these peptides may have their individual influence on the circadian phenotype inferred, it will be important to also consider how they interact. In-vitro, SCNs null for VIP only show complete arrhythmicity when signalling in AVP and GRP is blocked (Maywood et al., 2011), suggesting functional redundancy between these cell types. Additionally, AVP receptor V1a extends widely in the SCN, in both the core and the shell (Li et al., 2009). AVP mRNA is reduced in VPAC2 knockout mice and conversely AVP expression is induced by VIP or VPAC2 agonists (Rusnak et al., 2007). Therefore, both peptides may interact to impact circadian phenotype and should be examined accordingly.

The high levels of activity in DD and during the dark pulses (and the consequent large magnitude shifts) suggest a possible influence of the arousal system in the differential

behaviour in BTBR mice. From a circadian standpoint 5-HT, ACh and NPY all have crucial roles in arousal mediated activity and resetting of the clock. And arousal inducing procedures, including access to wheels and exercise, induce large phase shift responses and accelerate re-entrainment to shifted light dark cycles (Webb et al., 2014). In the SCN, 5-HT content has been shown to correlate positively with motor activity and negatively with the FRP (Shioiri et al., 1991), suggesting that 5-HT maybe associated with modification of circadian parameters. BTBR mice have been reported to have dysregulated 5-HT and cholinergic signalling, including potentially altered 5-HT content in the raphe (Gould et al., 2001, 2014). When combined with their response to light in LL condition and the jet-lag experiments and their higher total activity, a possible change in either the 5-HT input to the SCN or SCN regulation of arousal may underlie some of the phenotype we examined. Future work can aim to characterize 5-HT input to the SCN and the response to 5-HT agents in BTBR mice.

Finally, FAA was more robust in BTBR mice, which was expected from the known metabolic issues that this strain harbours (Meyza et al., 2017). However, while insulin, leptin and corticosterone are dysregulated in the BTBR strain, none of these factors have been implicated in FAA. It is important to keep in mind that many studies looking at FAA have aimed to characterize the necessity of different factors, whether this be clock genes, hormones, or signalling pathways. However, most studies did find some alterations in FAA when these factors were deleted or attenuated. Thus, the FAA patterns we observed might be a direct consequence of the differential expression of these hormones.

One interesting aspect in the BTBR that may be relevant to our set of studies is the role of the reward pathway. There is some evidence that dopaminergic neurotransmission in the BTBR mouse is altered (Squillace et al. 2014). While D_1 receptor activation appears intact, D_2 receptor function is impaired, resulting in hypoactivation of the reward system in a functional magnetic resonance imaging (fMRI) scan of a GBR 12909 (dopamine reuptake inhibitor) challenge. Both feeding behaviour and wheel running are associated with the reward pathway (Greenwood 2011; Dileone et al., 2012). Weeks of voluntary wheel running is associated with altered gene transcription in mesolimbic reward neurocircuitry and the regulation of food intake by the hypothalamus appears to rely on the reward and motivational neurocircuitry to modify eating behaviours. If there is a change in the reward pathway in BTBR mice, then this might carry over to wheel running and feeding behaviour. Future studies can aim to modulate levels of dopamine in the BTBR and re-examine wheel running behaviour and food anticipation. In one study, mice selectively bred to be high runners had several neurobiological changes in brain areas that are commonly affected in the BTBR mice (Meyza et al., 2017; Rhodes et al., 2005), including the hippocampus, prefrontal cortex, and nucleus accumbens. Given the link between reward and wheel running and arousal and wheel running and given how the BTBR mice have higher activation of the stress axis, dysregulation of the reward and arousal and 5-HT pathways may contribute to the differences observed. Understanding how these systems interact specifically with wheels and how they contribute to the circadian behaviour we observed may be critical to determining how these difference arise.

Finally, despite the short FRP and short alpha, BTBR mice have comparable responses to light and have stable entrainment. The core deficit in circadian disruption is the misalignment between the internal and the external cycles. This is then associated with the aforementioned health impacts and disease states. BTBR mice, despite what would be expected based on their circadian phenotype, entrained precisely to LD cycle and this was observed even after they were introduced to LD cycles after being maintained in constant conditions for an extended period of time. Therefore, future work can aim to quantify if this entrainment had any physiological impact (by looking at the stress response, organ health or response to toxins). It has been previously shown that when tau mutant hamsters with FRPs of 22 hours are placed in 24-hour T cycles they develop profound heart and kidney disease phenotypes and die earlier of cardiomyopathies. This cardiorenal phenotype is not present when they are housed in light cycles appropriate to their FRPs and can be reversed by the same (Martino et al., 2008). If BTBR mice are indeed entrained to external cycles with precision despite their phenotype and do so without any health impact, then understanding how their circadian system functions can help us understand how to better target the mechanisms and better deal with the symptoms of circadian disruption.

One important finding in our set of studies was that, when examined, there were significant sex differences between males and females (Table 2). We examined sex differences in DD parameters (FRP, alpha, total activity) and in SCN anatomy (VIP and AVP). Importantly, BTBR females were intermediate between C57BL/6J males and females, and BTBR males in all parameters, except for total activity (where BTBR females had highest amounts of activity). Sex

differences in circadian anatomy and behaviour are well established although there appears to be some species specific, behaviour specific and anatomy specific variations (Blattner et al., 2012; Bailey and Silver 2014; Kuljjis et al.,2013; Davis et al., 1983; Schull et al., 1989). In C57BL/6J mice, differences have been noted in free running period, in duration of activity, precision of onset of activity, PER:LUC bioluminescence and total activity (Kuljis et al 2013; Blattner et al 2012; Ogawa et al 2003). While patterns in duration of activity were maintained (female mice had longer durations of activity), the BTBR female mice in our study also had higher amounts of total activity and longer FRPs when compared to males. These differences however were not present when comparing C57BL/6J males and females. However previous studies have found contrasting patterns in total activity with some reporting females having higher amounts of activity (Blattner et al., 2012) and others reporting the opposite (Ogawa et al., 2003). Similarly, FRP differences between males and females are species specific- female hamsters and rats have been examined to have longer FRPs (Davis et al., 1983; Schull et al., 1989; Labyak et al., 1995), whereas in mice some studies have reported that females have shorter periods, others have reported comparable periods between males and females (Kuljis et al., 2013). We did not examine sex differences in other parameters, but future studies will aim to cover this gap as our findings suggest some other potentially interesting differences.

The circadian clock serves to anticipate and synchronize to daily changes in the light dark cycle. Not only is the disruption of the circadian rhythms as caused by rotating shift work linked to a variety of health and disease states, circadian rhythm disorders themselves affect a

significant proportion of the population and are co-morbid in patients with neurodevelopment, and psychological disease states. Sleep and circadian problems are noted in ASD, ADHD, and other neurodevelopmental disorders and also in mood and anxiety disorders, dementia and Alzheimer's, Parkinson's, chronic illnesses and mental health disorders (Jones et al 2015; Lamont et al 2007). There is evidence for a cyclical relationship between the two as the disease states themselves appear to cause sleep and circadian issues, but in turn the sleep and circadian issues exacerbate other symptoms of these disorders. Thus, not only circadian disruption, but sleep and circadian problems are also impactful on public health and it is thus important that therapeutics to correct them are effectively developed. Models like the BTBR can help not only understand potential sleep and circadian related problems in patients with these disease states but can also help us understand the mechanisms underlying human circadian disorders. Some clock genes or the respective enzymes that control the degradation of their proteins, have been implicated in human circadian disorders and sleep and circadian problems occurring as comorbidities. Models like the BTBR where there is a strong endogenous variance from the wildtype behaviour can be used to 1) find the underlying genetic or proteomic mechanisms that cause that difference 2) these genetic and proteomic factors can be modulated in these animals, to examine if these factors are completely causative in the human phenotype 3) findings can then be potentially used to find therapeutics to help treat or at least manage the symptoms of circadian disorders in the human population. Future work can further probe the molecular and network mechanisms of this differential phenotype in the

BTBR model and examine any similarities to already established phenotypes and mechanisms in sleep and circadian disorders, either as they appear alone or co-morbid with other diseases.

In conclusion, the BTBR mouse strain shows a novel circadian phenotype. Given the dysregulations in various signalling systems within this strain, delineating the causes of this change in phenotype may help us better understand circadian function. BTBR mice have deficiencies in many systems that are directly related to the circadian systems, whether in establishing circadian rhythms in constant darkness or in terms how it influences circadian photoentrainment. This could be used to help better target circadian disruption and how it is dealt with.

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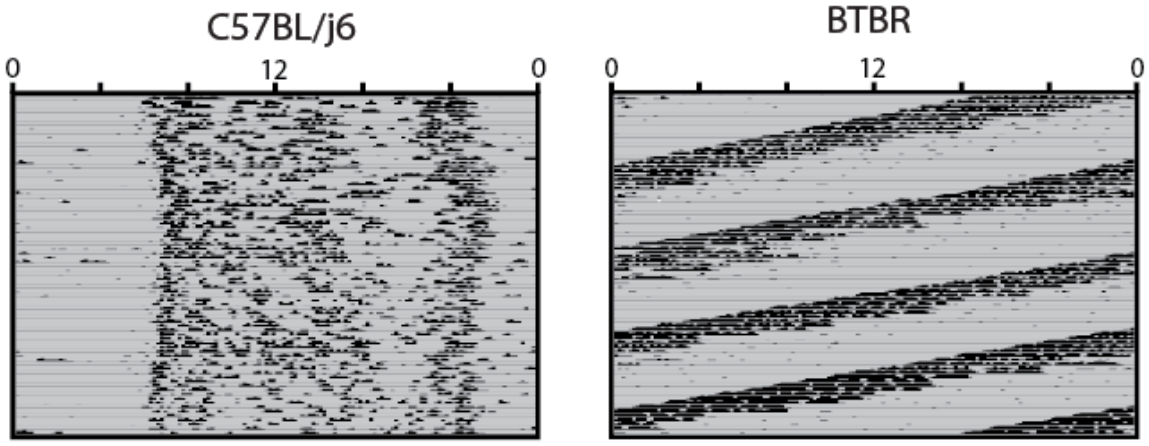
Appendix A

Previous research has shown that the ketogenic diet improves spontaneous seizures, abnormal EEG, and altered seizure threshold and reduces autism like behaviours in BTBR mice (Ruskin et al., 2016). As sleep problems are commonly reported in adolescents and adults with ASD, with a large negative impact on both patients and caregivers (Glickman et al., 2010), findings ways alleviate symptoms of ASD and sleep disturbances are critical to the patient's health. Given the success of the KD in improving socio-cognitive behaviours and epileptic symptoms, we used the ketogenic diet to examine if it would change any sleep related issues if present. While the KD had no impact on circadian rhythms we did find that BTBR mice had a drastically shorter FRP when compared controls (Figure A.1). Based on this we aimed to characterize circadian behaviours in constant conditions, photic entrainment, food entrainment and SCN anatomy in these mice.



Results of the independent t-test revealed that BTBR mice has a significantly shorter FRP when compared to C57Bl/6J mice (M= 22.6 +/- 0.14 vs 23.8 +/- 0.05 ; $t(10) = 6.87$, $p < 0.0001$). Considering the drastic differences from what is normally observed in C57 mice and its similarity to what is observed in tau mutant hamsters, we aimed to characterize their circadian phenotype.