CIRCADIAN MISALIGNMENT HAS HARMFUL EFFECTS ON THE BRAIN AND BEHAVIOUR

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Abstract

With circadian rhythm disruption being a huge component of modern life, it is paramount to understand how it affects the brain and body. Circadian rhythm disruption impairs memory in humans and rodents. We use phase advances of the light dark cycle to challenge circadian rhythms in rats and observe concomitant memory impairments. While this has been extensively documented, it is unclear exactly how it occurs. The goals of this thesis were threefold: 1) Determine if our phase-shifting paradigm induces circadian misalignment. 2) Determine if the effects of our paradigm are long lasting. 3) Investigate three of the most theorized mechanisms for how circadian rhythm disruption elicits memory impairments. I found that our paradigm induces circadian misalignment that has long lasting effects on activity rhythms and memory. I also determined that sleep deprivation, elevated corticosterone, and hippocampal cell death are not responsible for the memory impairment induced by circadian rhythm disruption.

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List of Abbreviations

EEG – electroencephalogram

ELISA – enzyme-linked immunosorbent assay

EMG – electromyogram

FJ – Fluorojade-B

GABA – y-aminobutyric acid type A

FBN – Fisher brown Norway

LE – Long Evans

LFP – Local field potential

LTP – Long term potentiation

MWT – Morris water task

mPFC – medial prefrontal cortex

NREM – Non-rapid eye movement

REM – Rapid eye movement

SCN – suprachiasmatic nucleus

SWS – Slow wave sleep

ZT – Zeitgeber time

Chapter 1

General Introduction[1]

Circadian Rhythms

Circadian rhythms are endogenously generated oscillations in physiology and behaviour that are found in virtually all organisms ranging from bacteria to vertebrates (Young & Kay, 2001). The sleep-wake cycle, activity-rest cycle, hormone secretion, body temperature, and metabolism are all examples of circadian rhythms (Barnard & Nolan, 2008; Moore, 1999). Environmental cues (zeitgebers) synchronize circadian rhythms with the environment so that organisms are able to anticipate daily events and adjust their physiology and/or behaviour accordingly to better take advantage of their environment (Merrow, Spoelstra, & Roenneberg, 2005). Light is the strongest zeitgeber for circadian rhythms, but food, exercise, social interaction, and learning can entrain circadian rhythms in certain circumstances (Krishnan & Lyons, 2015; Merrow et al., 2005; Mistlberger, 2011; Ralph, Sam, Rawashdeh, Cain, & Ko, 2013).

The circadian system is one of the most ideal to study the link between genes and behaviour as they are generated at the cellular level by autoregulatory transcription/posttranscription/translation/post-translational feedback loops containing a concert of genes (clock genes) (Okamura, 2004; Reppert & Weaver, 2001). In short, CLOCK and BMAL1 form heterodimers that activate the transcription *of cry and per* homologues which then dimerize and move into the nucleus where they interfere with the binding of CLOCK-BMAL1 dimers to *cry* and

[1] This introduction in largely this form features in: (Deibel & McDonald, 2017).

per promoters (Okamura, 2004; Reppert & Weaver, 2001). Another regulatory loop influences the core loop by promoting or suppressing transcription of BMAL1 via the binding of REV-ERBα and RORAα, respectively, to ROR elements in the BMAL1 promoter region (Okamura, 2004; Reppert & Weaver, 2001). Post-translational modifications are involved in the fine-tuning of these feedback loops (Ko & Takahashi, 2006).

Amazingly, depending on the tissue and cell type, the expression of up to 30% of transcripts display diurnal variations (Aguilar-Arnal & Sassone-Corsi, 2014; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002). Although, in vertebrates molecular oscillations are occurring in virtually all tissues, a master clock located in the anterior hypothalamus, the suprachiasmatic nucleus (SCN), is necessary for the generation of most rhythms and more importantly the synchronization of the peripheral brain and body clocks (Antle & Silver, 2009; Dibner, Schibler, & Albrecht, 2010; Reppert & Weaver, 2001). It is thought that the SCN uses neuropeptides, direct neuronal efferents, and hormones to coordinate rhythms in other tissues (Antle & Silver, 2009; Guilding & Piggins, 2007; Mohawk, Green, & Takahashi, 2012). SCN activity and dependent rhythms persist in the absence of zeitgebers, whereas the oscillations and synchronization of most peripheral clocks is dependent on the SCN (Antle & Silver, 2009; Guilding & Piggins, 2007; Mohawk et al., 2012; Yoo et al., 2004).

Circadian Rhythm Disruption

"I have lost my rhythm. I can't Sleep. I can't Eat." Charles Bukowski, Metamorphosis.

Circadian rhythms are ingenious adaptations that allow organisms to be at their best at times when it is most likely to be beneficial. This is achieved by synchronizing our internal clocks with the environmental clock. But, as suggested by Bukowski, bad things happen when the time represented by the internal and environmental clocks is different.

This lack of sync between our external environment and internal pacemaker is more pronounced in our society today than ever before. Realities of life today such as exposure to artificial light at night, jet lag, and shift work are particularly challenging for circadian rhythms (Banks, Nolan, & Peirson, 2016; Haus & Smolensky, 2013; Zelinski, Deibel, & McDonald, 2014). 15% of the global work force in developed countries are shift workers, not to mention that working longer hours and at times outside of the normal day shift is increasing in the United states (Alterman, Luckhaupt, Dahlhamer, Ward, & Calvert, 2013; Krishnan & Lyons, 2015; K. P. Wright, Bogan, & Wyatt, 2013). Regardless of whether one is working a rotating schedule consisting of night and day shifts or even just working night shifts, circadian rhythms are not entrained to the new schedules (Folkard, 2008; Gibbs, Hampton, Morgan, & Arendt, 2007; Haus & Smolensky, 2006; Simon, Weibel, & Brandenberger, 2000). Major problems that affect virtually all aspects of physiology arise when circadian rhythms are not synchronized with the environment (Kuhn, 2001; Zelinski et al., 2014). Chronic circadian rhythm misalignment has been associated with cognitive impairments, heart disease, metabolic syndrome, shortened lifespan, and cancer (Banks et al., 2016; Haus & Smolensky, 2006; Zelinski et al., 2014).

Physiology has been well documented in different populations of shift workers (Folkard, 2008). However, many of these studies investigating the health of shift workers are inconsistent (Antunes, Levandovski, Dantas, Caumo, & Hidalgo, 2010; Lowden, Moreno, Holmbäck, Lennernäs, & Tucker, 2010). Take diet for instance, some studies report increased caloric intake (Maa A A de Assis, Nahas, Bellisle, & Kupek, 2003; Maria Alice Altenburg De Assis, Kupek, Nahas, & Bellisle, 2003; Reinberg et al., 1979) while others report no change (Lennernas, Hambraeus, & Akerstedt, 1995; Pasqua & Moreno, 2004; Sudo & Ohtsuka, 2001). Findings like these are likely due to the fact that epidemiology studies are prone to cofounds such as self-selection bias, self-report measures, and cultural or regional differences (Lowden et al., 2010). As a result it is very hard in some cases to determine the symptoms of chronic circadian rhythm disruption, let alone the mechanisms involved.

Animal Models of Circadian Rhythm Disruption

Thankfully there are many different animal models of circadian rhythm disruption that can be used to investigate the mechanisms and pathology of chronic circadian rhythm disruption (for review see (Deibel, Zelinski, Keeley, Kovalchuk, & McDonald, 2015; Krishnan & Lyons, 2015; Smarr, Jennings, Driscoll, & Kriegsfeld, 2014; Zelinski et al., 2014). There are many different flavours of circadian rhythm disruption that are embodied in different models (for review see (Deibel et al., 2015; Krishnan & Lyons, 2015; Smarr et al., 2014; Zelinski et al., 2014). Rhythms can stop oscillating with expression of that process being constitutive instead of displaying daily variations. This occurs in manipulations that affect the central clock such as SCN lesions, or transgenic mutations of core clock genes such as *Bmal1*. These techniques are imperative when deciphering how the master clock affects

downstream or peripheral processes. However, with these techniques it is hard to determine the locus of the dysfunction. SCN lesions affect oscillations in virtually all other brain and body clocks, while most transgenic mutations affect the entire brain.

Alternatively, rhythms can continue to oscillate but are no longer synchronized with the environment or other oscillators in the brain and body. It is thought that during shiftwork circadian rhythms become misaligned[2] from the cues entraining them (Haus & Smolensky, 2006). Animal models that manipulate zeitgebers often produce circadian rhythm misalignment. One method is to expose the animals to constant illumination paradigms. The endogenous clock takes over in the absence of zeitgebers and thus rhythms drift over time because the period or length of time it takes to do one oscillation is slightly different from 24h in rodents (Zelinski et al., 2014).

In shift work or jet lag situations there are zeitgebers, they are just either variable or occurring at abnormal times. Inserting light or dark pulses where they shouldn't appear in the cycle, or large deviations in when the lights should come on or off are more akin to what is occurring during shift work and jet lag. Advancing or delaying the onset or offset of lights on or off is a great way to misalign rhythms from the environment. T-cycles – non-24 hour light cycles – are used to challenge the circadian system in this way. T-cycles can be symmetrical, meaning that there is an equal amount of light and dark within the cycle, or they can be asymmetrical with different amounts of light and dark in the cycle. We use a T21 cycle, which is

[2] As circadian misalignment is a form of circadian rhythm disruption, throughout this thesis unless specifically differentiated, these terms are to be interpreted as one and the same.

thought to be outside the range of entrainment for a rat (Campuzano, 1998; Stephan, 1983). This results in either a free-running circadian rhythm of approximately 24 hours (Stephan, 1983), which would look similar to that in constant conditions, or a free-running rhythm and another rhythm with a period closer to 21 hours (Campuzano, 1998). Thus, these paradigms induce circadian misalignment because circadian rhythmicity is maintained, but these rhythms are not entrained with the light dark cycle.

Circadian Rhythms and Memory

Circadian rhythms and memory are inextricably linked. The time that something occurs or is learned is an important part of both memory acquisition and retrieval. The animal literature is rich with observations of how these two processes interact.

Time-place-learning

Stemming from observations in the wild that organisms visit locations at times of day when it is most beneficial for them to forage, animal's ability to encode time has been studied extensively in the lab (Daan & Koene, 1981; Moore-Ede, Sulzman, & Fuller, 1982; C M Thorpe & Wilkie, 2006). In daily time-place-learning studies, animals must learn to go to certain locations at a specific time of day to receive a reward or avoid an aversive stimulus (Mulder, Gerkema, & Van der Zee, 2013; C M Thorpe & Wilkie, 2006). In some of these paradigms, rats' use a circadian timer to associate a specific time of day with a location (Deibel & Thorpe, 2012; Mistlberger, De Groot, Bossert, & Marchant, 1996a; Christina M Thorpe & Wilkie, 2007; Van der Zee et al., 2008a). However, the story in rats is a complex one as performance in time-place-learning tasks might vary depending on the type of task

used, or the strain of rat used (Deibel, Ingram, et al., 2014; C M Thorpe, Deibel, Reddigan, & Fontaine, 2012). Mechanistically, time-place-learning requires Cry 1 & 2 (Van der Zee et al., 2008a), but not Per 1 & 2 genes (Mulder, Van Der Zee, Hut, & Gerkema, 2013), nor does it require an intact SCN (Mistlberger et al., 1996a). These data suggest that a peripheral circadian oscillator is likely mediating performance, but it remains to be seen the exact nature of the mechanisms underlying time-place-learning.

Time Stamping and Circadian Variations in Cognition

The time-stamping phenomenon is another classic example of how time is involved in memory. As pioneered by our research group, some rodents only display memory for a context when their memory is tested at the same time that learning occurred. This is true for hamsters' memory of both aversive and appetitive contexts (Cain, Chou, & Ralph, 2004; Ralph et al., 2002). Similar to the circadian modulation of time-place-learning, the relationship between circadian rhythms and time-stamping is complex as only some rat strains show evidence of time-stamping (Cain, Ko, Chalmers, & Ralph, 2004; McDonald, Hong, Ray, & Ralph, 2002). Similarly in hamsters, time-stamping also does not require an intact SCN (Cain, Chalmers, & Ralph, 2012; Ko, McDonald, & Ralph, 2003). As with time-place learning, a peripheral oscillator appears to be modulating the temporal gating of context memory inherent to time stamping. Ralph and colleagues provided evidence suggesting that there is a context-entrainable oscillator mediating performance, however the locus of said oscillator is unknown (Ralph et al., 2013).

An extension of time stamping is the finding that some rodents learn better at different phases of the circadian cycle. In rodents, acquisition and/or retention of

an operant task, or hippocampal dependent tasks such as contextual fear conditioning, spatial radial arm maze task, novel object location task, and spatial learning in the Morris water task (MWT) is better during the night time (Gritton, Kantorowski, Sarter, & Lee, 2012; Hauber & Bareiß, 2001; Takahashi, Sawa, & Okada, 2013; Valentinuzzi et al., 2001; Valentinuzzi, Menna-Barreto, & Xavier, 2004). Amazingly, in the MWT, while there were no time-of-day effects in acquisition on a recent probe trial performance, spatial memory in a probe trial two weeks after the cessation of training was stronger for rats that were trained during the night time (Gritton et al., 2012). In contrast, in mice, some studies suggests that recall for various paradigms such as contextual fear conditioning, fear conditioning to tone, and spatial working memory is better during the day-time (Chaudhury & Colwell, 2002; Eckel-Mahan et al., 2008; Rawashdeh et al., 2014).

Circadian Disruption and Memory

Time-place-learning, time-stamping, and circadian variations in cognition are examples of how circadian rhythms influence learning and memory under entrained conditions, however as discussed earlier, there are many circumstances in which circadian rhythms are not entrained. Memory impairments have been associated with long periods of time working shift work or in the air travel industry (Cho, 2001; Cho, Ennaceur, Cole, & Suh, 2000; Marquié, Tucker, Folkard, Gentil, & Ansiau, 2014; Rouch, Wild, Ansiau, & Marquié, 2005).

Many laboratories have investigated the effects of circadian rhythm disruption on learning and memory in rodents. It was first discovered that memory retention in active and passive avoidance tasks was impaired by changing the light dark cycle (Davies, Navaratnam, & Redfern, 1974; Fekete, Van Ree, & De Wied,

1986; Fekete, van Ree, Niesink, & de Wied, 1985; Stone, Rudd, Ragozzino, & Gold, 1992; Tapp & Holloway, 1981). However, these studies had several design flaws. First, these studies used strains of rats that display time-stamping, thus it was unclear if the memory impairment was due to circadian rhythm disruption, or training and testing occurring at different times of day. Second, their pattern of impairments suggested that the animals were not re-entrained during the memory test, which could mean that impairments were due to state-dependency differences between training and testing. Similarly, it was thought that the deficit was due to a retrieval failure as impairments were only detected if testing occurred within 24 hours from the cessation of phase shifting. Finally and perhaps most importantly, it was unclear if other types of memory, such as episodic memory would be affected.

The hippocampus is thought to mediate episodic memory in humans and thus has been extensively modeled in rodents (Tulving, 1993; Tulving & Markowitsch, 1998). The MWT is the gold standard measure of hippocampal dependent memory in rodents (McDonald, Hong, & Devan, 2004). In the standard version, rats learn to navigate to a hidden platform in a large pool of water over eight trials administered each day for four or five consecutive days (McDonald et al., 2004). It is thought that rats use an internal compass (path integrator) in conjunction with the arrangement of distal cues in the room to navigate to the platform (McDonald et al., 2004). Memory is then tested in a probe trial that involves returning the animal to the pool without the platform present with the idea that an animal with a spatial memory will spend more time in the area of the pool that contained the platform during training (McDonald et al., 2004).

Given that circadian rhythms and hippocampal dependent memory both decline with age, we hypothesized that circadian rhythm disruption would impair hippocampal dependent memory. Our lab has created a rat circadian rhythm disruption paradigm that induces circadian rhythm misalignment by changing the light-dark cycle for a brief or lengthy period of time (Deibel et al., 2015; Zelinski et al., 2014). As mentioned above, we use a T21 cycle, which in the acute version involves light onset or offset three hours earlier than anticipated (3 hour phase advance) for six consecutive days (Craig & McDonald, 2008; Devan et al., 2001; Zelinski, Hong, & McDonald, 2014). It takes approximately 20 days to re-entrain to a 24h LD after the acute paradigm (Craig & McDonald, 2008; Devan et al., 2001). In our chronic 64 day paradigm, there are four cycles of the acute paradigm, with each cycle separated by 10 days of re-entrainment to a normal 12:12 LD cycle (Craig & McDonald, 2008; Deibel, Hong, Himmler, & McDonald, 2014; Zelinski, Tyndall, Hong, & McDonald, 2013). This paradigm continuously challenges the circadian system, as rhythms are not able to entrain during these brief 10 day periods (Craig & McDonald, 2008). Re-entrainment to a 24h LD had not occurred days after the cessation of phase advances in the chronic paradigm (Craig & McDonald, 2008).

Rats that received five days of training in the standard version of the MWT during the acute paradigm were able to learn the location of the platform during training as evidenced by decreasing latencies and path lengths to the platform (Devan et al., 2001). However, when memory retention was tested in probe tests seven and 17 days after the end of acquisition, the circadian rhythm disrupted rats did not show evidence of remembering the platform location (Devan et al., 2001). In contrast to the interpretation from the early demonstrations in active and passive

avoidance tasks, we suggested that the retention deficit appeared to be due to a failure to consolidate the memory, as learning was not affected (Devan et al., 2001).

While consolidation of a hippocampal dependent memory was impaired if acquisition occurred during circadian rhythm disruption, it was unclear if circadian rhythm disruption could have anterograde and retrograde amnestic effects on memory. In the anterograde assessment we used a more sensitive MWT variant that could tease apart distributed and massed training deficits (Craig & McDonald, 2008). Training was started 10 days after the end of the phase advances in both the acute and chronic paradigms (Craig & McDonald, 2008). The chronically circadian disrupted rats had impaired: distributed acquisition, rapid acquisition of a novel platform location, and impaired retention of either of these spatial locations (Craig & McDonald, 2008). The rats that experienced acute circadian rhythm disruption and then training were indistinguishable from controls, which suggests the consolidation deficit observed previously in the acute paradigm is a result of phase advances occurring during learning (Craig & McDonald, 2008; Devan et al., 2001). That being said, chronic circadian rhythm disruption has anterograde amnestic effects on hippocampal dependent memory that affect both acquisition and retention of the information.

The next series of experiments from our lab determined if acute or chronic circadian rhythm disruption has a retrograde amnestic effect on hippocampal dependent memory by exposing the animals to circadian rhythm disruption after memory acquisition. Interestingly, both acute and chronic circadian rhythm disruption induced retrograde amnesia in the MWT (Zelinski et al., 2014, 2013). These studies also investigated if sex or access to a running wheel influenced the

memory impairment elicited by circadian rhythm disruption. Two main findings emerged: 1) Memory impairments were more severe in males. 2) The memory impairment was more pronounced for animals without wheels (Zelinski et al., 2014, 2013). In tandem with our previous studies, these data as a whole suggest that circadian rhythm disruption has more severe effects on hippocampal dependent memory when acquisition occurs during or before circadian rhythm disruption.

While the studies mentioned above clearly suggest that the hippocampus is sensitive to circadian rhythm disruption as assessed via the MWT, what about other tasks that assess hippocampal function? Both acute and chronically circadian disrupted rats were unimpaired in both tone and contextual fear conditioning (Craig & McDonald, 2008). The fear conditioning paradigm used, likely requires minimal hippocampal involvement, however it remains a possibility that fear memory could be susceptible to circadian rhythm disruption if acquisition occurs during or before disruption (Craig & McDonald, 2008). For example, in mice, single phase advances right before or after contextual fear conditioning impair retention but not acquisition of the fear memory (Loh et al., 2010). We also assessed the effects of our chronic circadian rhythm disruption paradigm in a version of fear conditioning that relies more on the hippocampus because it involves the discrimination of several contexts (Antoniadis & McDonald, 2000). Remarkably, rats that experienced chronic circadian rhythm disruption actually performed better in a measure of this task (Deibel, Hong, et al., 2014). Similarly, mice that experienced multiple phase shifts were indistinguishable from controls and better than animals that received a single phase shift in the recall of contextual fear conditioning (Loh et al., 2010). As will be discussed below, it is possible that a potentiated stress response from chronic phase

shifting is resulting in facilitation of the fear memory in these instances (Deibel, Hong, et al., 2014).

While the fear conditioning tasks mentioned above require the amygdala, the hippocampus is also a key player in fear conditioning (Antoniadis & McDonald, 2000). Thus, we wanted to investigate the effects of our circadian rhythm disruption paradigms on other behaviours that do not involve the hippocampus. We have developed a stimulus-response visual discrimination task in the radial-arm-maze that requires the dorsal striatum, but not the hippocampus (McDonald & Hong, 2004; McDonald & White, 1993). This task is particularly useful because cognitive flexibility, which is reliant on the prefrontal cortex can be assessed by reversing the reward contingencies (Zelinski et al., 2014, 2013). Performance in the visual discrimination task and the cognitive flexibility reversal of the reward contingencies was impaired in the chronic, but not acute circadian rhythm disruption paradigm (Zelinski et al., 2014, 2013). As with the MWT data, males and animals without wheels also performed poorer in these tasks. These data as a whole suggest that circadian rhythm disruption does not just affect hippocampal dependent memory, but can affect behaviours reliant on the dorsal striatum and prefrontal cortex. It is also possible that this retrograde amnesia effect for both hippocampal and non-hippocampal-dependent tasks is due to a change in the hierarchical interaction of various brain circuits that is a result of impaired hippocampal functioning induced by circadian rhythm disruption. Readers are encouraged to see a recent theoretical paper from our group on the interaction of various neural circuits in anterograde and retrograde amnesia (Lee, Zelinski, McDonald, & Sutherland, 2016).

As with the time-place-learning and time-stamping, the effect of circadian rhythm disruption on memory is a complex one that depends on sex, when the circadian rhythm disruption occurs, task used, and whether the animals had access to a wheel. It is unclear why some tasks are more susceptible to the effects of circadian rhythm disruption, nor why females might be more protected than males under some conditions. However, undeniably, data from our lab, and other labs in various rodent species (Fekete et al., 1986, 1985; Fernandez et al., 2014; Fujioka et al., 2011; Gibson, Wang, Tjho, Khattar, & Kriegsfeld, 2010; Loh et al., 2010, 2015; Ruby et al., 2008) unanimously suggest that circadian rhythm disruption can impair memory.

Research Question

The million dollar question regarding circadian rhythm disruption is how and why does it affect the brain and the body? While we have demonstrated that circadian rhythm disruption impairs memory in rats, in order to know how and why this happens many questions have to be answered. This thesis will seek to answer some of these questions. 1) In order to learn more about why circadian rhythm disruption impairs memory, we first need to know exactly what our circadian misalignment paradigm does to circadian rhythms acutely and chronically. This will be addressed in Chapters 2 and 3. 2) Is the memory impairment elicited by circadian rhythm disruption transient? This will be investigated in Chapter 3. 3) What are some of the mechanisms for how circadian rhythm disruption leads to hippocampal dysfunction? Chapter 4 will investigate three of the most commonly proposed explanations for how circadian rhythm disruption affects the hippocampus.

Chapter 2

Does Acute T21 Exposure Elicit Circadian Misalignment?

Introduction

While previous studies have assessed the effects of T21 cycles on rats, these studies typically use much more T21 exposure and either use symmetrical or very asymmetrical cycles. In the past we have demonstrated in Long Evans (LE) male and female rats that six days of asymmetrical T21 cycle produces more activity during the light period of the cycle and can alter the phase of activity onset (Craig & McDonald, 2008; Zelinski et al., 2014). We have also demonstrated that six days of T21 exposure did not affect period length in the next four days after this exposure (Craig, Hong, Kopp, & McDonald, 2009). However, many questions still remain when characterizing how six days of T21 exposure affects circadian rhythms. First, we cannot say that true circadian misalignment occurred because we did not assess the period or prominence of the rhythm during T21 exposure. Similarly, while entrainment has been altered in the past, we have never assessed the precision or variability associated with this entrainment.

Second, a bane of rat research is that all strains are not created equally. Memory tasks that involve the discrimination of time are particularly susceptible to strain differences (Cain, Ko, et al., 2004; McDonald et al., 2002; C M Thorpe et al., 2012). Thus it is possible that some rat strains might be more affected by circadian rhythm disruption than others. Although we have conducted many studies investigating the effect of circadian rhythm disruption on memory in the rat we have

exclusively used only the LE rat, which is known for its superiority in spatial learning and memory tasks.

The present study investigated the effects of six days of asymmetrical T21 exposure on running wheel activity in two rat strains. We also tested both males and females in one of the strains. We hypothesized that T21 exposure would misalign activity and with the light-dark cycle, while still maintaining rhythmicity with a period of approximately 24 hours.

Methods

Animals

FBN males and females

Nine seven-month old male and nine six-month old female Fischer brown Norway (FBN) rats bred at the University of Lethbridge were used. The rats were initially pair housed in clear Plexiglas cages lined with beta cob bedding. The animals were separated and singly housed in cages with wheels (diameter of 24cm), in which they remained for the rest of the experiment. Food and water were available ad libitum for the duration of the experiment. The rooms were maintained with a temperature of 21°C and a humidity of 35% and had a red light bulb (< 5 lux) that always remained on.

LE males

Twelve six-month old male LE rats were surgically implanted with local field potential (LFP) electrodes (described below) and the seven rats with the best local field potential recordings were selected for all subsequent procedures. After surgical implantation the rats were given one to two weeks of recovery time before being housed in the recording cages. The subjects had food and water available ad libitum

during the recovery period and in the recording cages. Additionally, the rats had access to a running wheel (diameter of 30.5cm) in the recording cages. This and all subsequent procedures in this thesis were conducted in accordance with the Canadian Council for Animal Care and approved by the University of Lethbridge Animal Welfare Committee.

T21 Light-dark Cycle

The rats had at least a minimum of 10 days of exposure to a normal 12:12 light-dark cycle before exposure to the T21 cycle. The FBN and LE male rats were put on a reverse light-dark cycle and were given time to entrain to this cycle before exposure to the T21 cycle. Room entrances were minimized to once daily at ~Zeitgeber time two (ZT2) for the FBN rats and ~ZT10 for the LE rats. Cages were cleaned once weekly.

The rats were exposed to a T21 light dark cycle for six days. Thus there were seven three-hour phase advances in these six days. The T cycles were asymmetrical with the FBN rats receiving 57% light (12L:9D) and the LE rats receiving 43% light (9L:12D). See Tables 1 & 2 for examples of these schedules.

Table 1. T21 schedules used. First time is lights off and second time is lights on. So 19:30 – 4:30 means that the lights went off at 19:30 and came back on again at 4:30.

DAY	12L:9D	9L:12D
	19:30 – 7:30	9:00 – 21:00
1	19:30 – 4:30	6:00 – 18:00
2	16:30 – 01:30	3:00 – 15:00
3	13:30 – 22:30	0:00 – 12:00
3	10:30 – 19:30	21:00 – 9:00
4	7:30 – 16:30	18:00 – 6:00
5	4:30 – 13:30	15:00 – 3:00
6	1:30 – 10:30	12:00 – 00:00
	22:30 – 10:30	12:00-00:00

Data Analysis and Statistics

ClockLab (Actimetrics, Wilmette, IL, USA) was used to collect and analyze the wheel data. ClockLab was used to calculate a variety of measures that characterize circadian rhythmicity such as, period of oscillation calculated with the Chi squared test (periods ranging from 20-26 were tested), rhythm prominence (amplitude of Chi squared test), percentage of activity that occurs during the dark (nocturnality) or light phase of the light/dark cycle, onset of activity, precision of activity onset (error associated with least squares fit of activity onsets), and phase of activity in relation to lights off. For phase, the time of lights off were subtracted from the onsets of activity, so a negative number indicates that the onset of activity

occurred before lights off. ClockLab was also used to make normalized actograms with one-minute bins.

Parametric two-tailed statistics were used in all instances and p was 0.05 for all statistical tests. All of the statistics were conducted with SPSS 21 (IBM, Armonk, New York) and GraphPad Prism software (GraphPad, La Jolla, CA) was used to make all of the graphs.

For the wheel running data, repeated measures ANOVAs were used to determine if activity changed throughout the shift. Thus, the data were averaged into five six-day blocks: 6 days preceding the shift (b1); six days of the shift (b2); six days following the shift (b3); next six days (b4); next six days (b5). Due to *a priori* hypotheses that the shift would impact circadian rhythms we conducted two contrasts: 1) comparing the measures before and during the T21 cycle (b1 vs. b2); 2) comparing the measures before and after the T21 cycle (assessed in one contrast: b1 vs. b3, b4, b5). For the sake of brevity, we only report the statistics for these planned contrasts. The first contrast was slightly different (b1 vs. b3) in the phase calculations for the control shift, as it does not make sense to calculate phase during the T21 cycle as the time of light onset changes each day.

In regards to the ANOVAs, when Mauchly's sphericity was violated, the Greenhouse-Geisser correction was applied. For the independent samples t-tests, corrected degrees of freedom were used if Levene' test was violated.

Results

Wheel Running

FBN Males

As indicated in Figures 1A & 2 and Table 2, although there was no change in period length during the shift (b2) compared to b1, the period was significantly shorter after the shift compared to before the shift ($F(1, 8) = 17.189$, $p = .003$). While amplitude was not significantly decreased during the shift, it was after the shift ($F(1, 8) = 27.282$, $p = .001$). Activity onset error did not change during the shift, but was significantly smaller after the shift ($F(1, 8) = 14.290$, $p = .005$). Nocturnality was only significantly reduced during the shift ($F(1, 8) = 30.018$, $p = .001$), and not after. Finally, there was no change in the phase of activity onset.

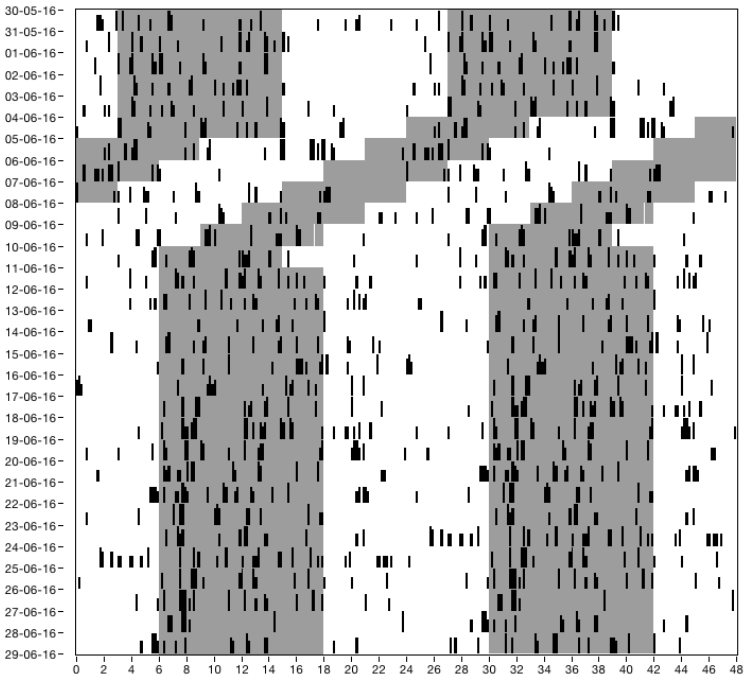
FBN Females

As indicated in Figures 1B & 2 and Table 2, there was a non-significant trend for a elongation of period length during the shift ($F(1, 7) = 5.153$, $p = .057$), however there was no change in period after the shift. The shift did not affect rhythm amplitude. Activity onset error increased during the shift ($F(1, 7) = 10.171$, $p = .015$) and this increase was maintained ($F(1, 7) = 13.815$, $p = .007$). Nocturnality decreased during the shift ($F(1, 7) = 29.686$, $p = .001$), but was actually greater after the shift compared to before ($F(1, 7) = 9.566$, $p = .017$). Finally, there was no change in the phase of activity onset.

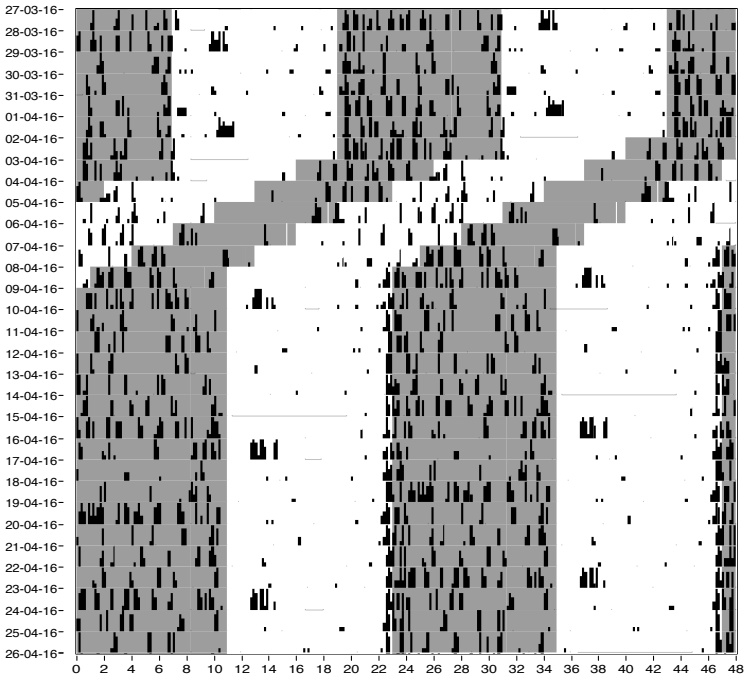
LE Males

As indicated in Figures 1C & 2 and Table 2, the shift had no effect on period length, rhythm amplitude, or activity onset error. Nocturnality significantly decreased during ($F(1, 6) = 104.887$, $p < .001$) and after the shift ($F(1, 6) = 9.610$, $p =$

.021). Finally, the phase was only significantly advanced after the shift ($F(1, 6) = 7.994$, $p = .030$).



A)



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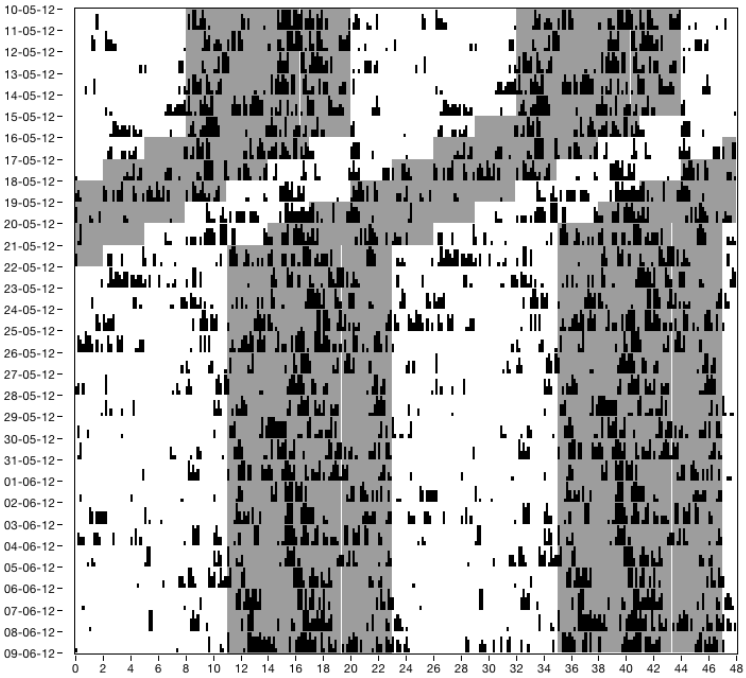

Figure 1 Actograms[3] of wheel activity during exposure to a T21 light dark cycle. A) Male FBN rats (n = 9). Representative actogram from one rat. B) Female FBN rats (n = 8). Representative actogram from one rat. C) Male LE rats (n = 7). Representative actogram from one rat. Shaded area indicates periods in which the lights were off.

[3] Refer to appendices for actogram's of every rat.

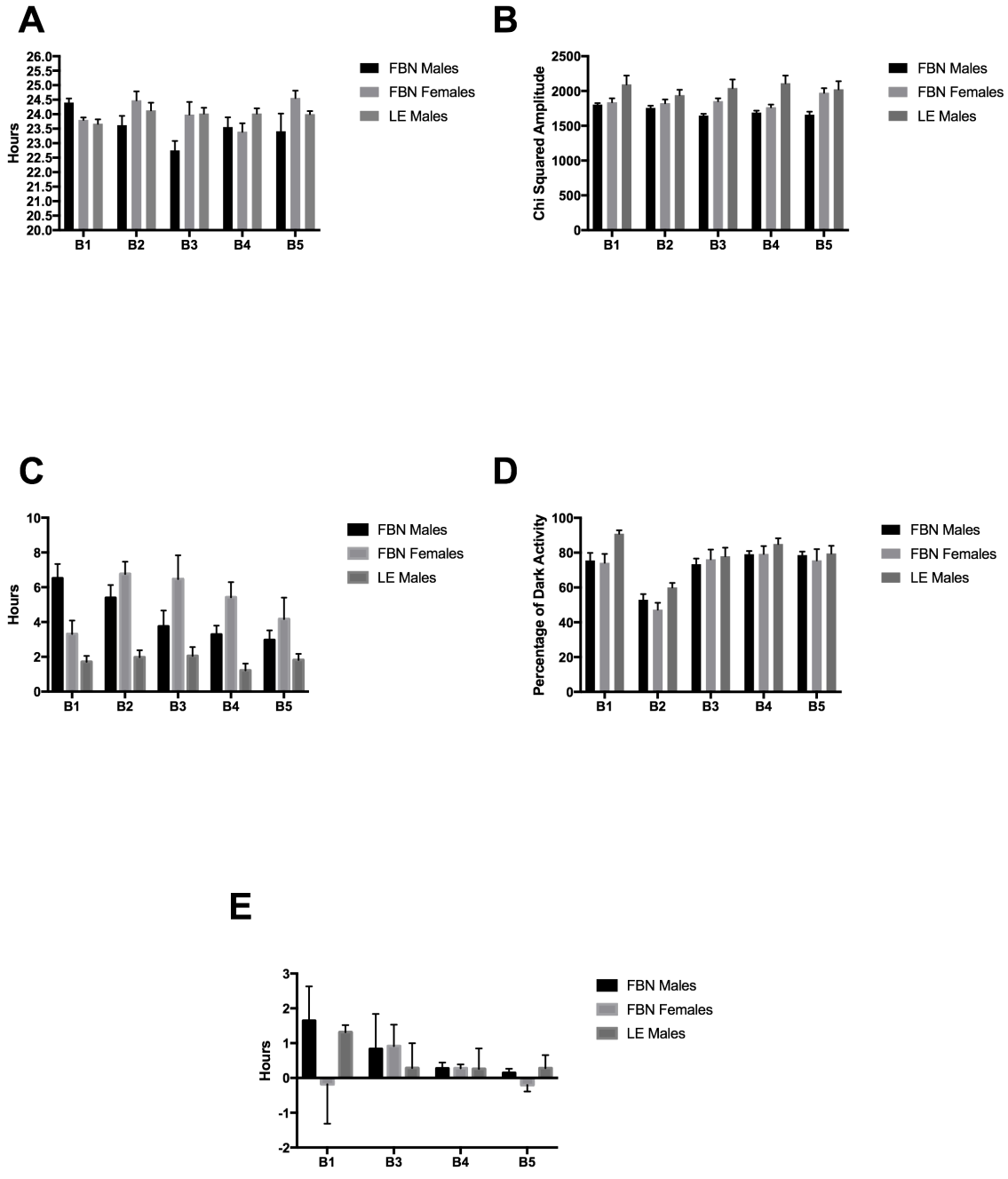

Figure 2. The effects of a T21 light dark cycle on measures of circadian activity rhythmicity in rats.
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Nocturnality. E.) Phase of activity onset.

Table 2. Summary of the effects of a T21 light dark cycle on wheel running activity of rats.

Circadian Parameter	FBN Males	FBN Females	LE Males
Period	Shorter Post Shift	No Change	No Change
Amplitude	Decreased Post Shift	No Change	No Change
Onset Activity Error	Decreased Post Shift	Increased During & After Shift	No Change
Nocturnality	Decreased During Shift	Decreased During Shift and was Increased Post Shift	Decreased During & After Shift
Phase	No Change	No Change	Significantly Advanced After Shift

Discussion

The T21 cycle had an impact on running wheel activity in all rats, with all groups having decreased nocturnality during the shift. Interestingly the effects of the T21 cycle on other circadian parameters differed depending on the sex and strain of the rats. The FBN male rats were the most affected, with decreased period length and rhythm amplitude after the shift. However, the onsets of activity were actually more precise or less variable post-shit, and there was no change in the phase of entrainment. While the FBN female rats did not have any change in period length, rhythm amplitude, or phase of entrainment, the onsets of activity were much more variable during the shift and remained that way post-shift. Period length, rhythm amplitude, and activity onset error were unaffected in the male LE rats. However

nocturnality remained decreased post shift, and the phase was also significantly advanced post-shift.

Generally the rats were not able to entrain to the T21. This was evidenced by no change in period length, and much less activity during the dark phase of the cycle during T21 exposure. Furthermore, the failure to find a change in phase suggests that the animals primarily free-run during the T21 and then entrained quickly once a normal 12:12 LD was introduced. The male FBN rats had a decreased period length post-shift, which suggests that these animals attempted to entrain to the T21 and did not quite re-adjust to the 12L:12D by the end of block five.

The finding that period is largely unaffected by the T21 cycle is consistent with T21 cycles with similar photoperiods (Cambras, Chiesa, Araujo, & Díez-Noguera, 2004; Campuzano, 1998). The lack of another rhythm with a shorter period of approximately 21h could be a result of using wheels (Campuzano, 1998; Stephan, 1983), and also due to very minimal T21 exposure compared to other studies (Cambras et al., 2004; Campuzano, 1998; Stephan, 1983). In the FBN female rats, the increased variability in the activity onsets suggests that rhythms in these animals might have been showing signs of relative coordination. In other words activity onset was more variable because the clock was attempting but failing to entrain to the T21.

As the animals were primarily free running during the T21, it is not surprising that there was much more activity during the light-phase of the cycle. As previously mentioned this circadian misalignment is thought to occur during shiftwork and have deleterious effects on the brain and the body (Escobar et al., 2011; Park, Cheon, Son, Cho, & Kim, 2012; Zelinski et al., 2014). Increased activity

during the light phase of the cycle means that more feeding was also likely occurring during this phase of the cycle. This has been shown to have grave consequences on rhythms, memory, and body weight (Loh et al., 2015; Salgado-Delgado, Angeles-Castellanos, Buijs, & Escobar, 2008; Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010).

Qualitatively the FBN female rats appeared to be less affected than the FBN male rats. This fits with our past data, suggesting that females might be more protected to the harmful effects of circadian rhythm disruption on memory. In similar paradigms we found that female LE rats had much better memory in the distributed MWT after either acute or chronic T21 exposure compared to males (Zelinski et al., 2014, 2013). Not surprisingly, females often have stronger rhythms that are more driven by the endogenous clock rather than zeitgebers (Cambras et al., 2004; Campuzano, 1998). The data from this chapter, support this by suggesting that females are more likely to free-run than males. This could be attributed to better/stronger SCN functioning in females, as female Fischer (F344/n) rats did not show a decline in SCN neurons in adulthood whereas FBN males did (Tsukahara, Tanaka, Ishida, Hoshi, & Kitagawa, 2005). As will be discussed in detail later in this thesis, it remains to be seen whether entrainment, partial entrainment, or free-running during T21 exposure is more harmful for memory.

Additionally, male LE rats generally appeared to be less affected by the T21 cycle compared to the FBN rats. However, it should be noted that the FBN rats received 57% light within the T21, whereas the LE rats received 43% light. This could explain the fact that the FBN rats had more deviations in circadian measures then the LE rats, as more light increases the chances of a rhythm that corresponds

to the T21 cycle. Thus free running would be more likely with a photoperiod with less light.

With the different photoperiods in mind, and the lack of LE females, the present thesis is not intended as an explicit comparison of strain and sex differences. Rather the data simply suggest that such factors need to be considered. For example, some strains have better circadian rhythms, with LE rats having activity rhythms of higher intensity (Bauer, 1990) and more stable DD activity rhythms (Stryjek, Modlińska, Turlejski, & Pisula, 2013) than other common strains. Unfortunately, to our knowledge, activity rhythms have never been characterized in FBN male or female rats.

Conclusions

The finding that activity rhythms become misaligned from the light-dark cycle are consistent with other studies in rodents that challenge the circadian system with phase advances (Altimus et al., 2008; Castanon-Cervantes et al., 2010; Craig & McDonald, 2008; Gibson et al., 2010; Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011; Legates et al., 2012; Loh et al., 2010; Sei, Kiuchi, Chang, & Morita, 1992; Van Dycke et al., 2015), forced activity during the light period (Salgado-Delgado et al., 2008; Salgado-Delgado, Angeles-Castellanos, et al., 2010; Salgado-Delgado, Nadia, Angeles-Castellanos, Buijs, & Escobar, 2010), or feeding that is restricted to the light phase (Loh et al., 2015). However, these studies typically involve much more exposure than six days. My results clearly show that six days of T21 exposure rapidly decouples activity from the external light dark cycle and the severity might depend on sex and rat strain.

Chapter 3

Are The Effects of T21 exposure Long Lasting?

Introduction

As discussed in Chapter 1, our research group were the first to demonstrate in rodents that circadian misalignment can impair hippocampal dependent memory (Devan et al., 2001). This impairment was believed to be due to a failure to consolidate the memory as learning was unaffected (Devan et al., 2001). Following this discovery, we have come to several conclusions: 1) circadian misalignment can have anterograde and retrograde effects on memory (Craig & McDonald, 2008; Zelinski et al., 2014, 2013) 2) impairments are more severe when learning occurs during circadian misalignment (Craig & McDonald, 2008; Devan et al., 2001) 3) males and animals without wheels are most affected (Zelinski et al., 2014, 2013). Similarly, other labs in various rodent species (Fekete et al., 1986, 1985; Fernandez et al., 2014; Fujioka et al., 2011; Gibson et al., 2010; Loh et al., 2010, 2015; Ruby et al., 2008) unanimously suggest that circadian rhythm misalignment can impair memory.

In humans the effects of chronic shift work on cognition can be long lasting, with one study observing that cognitive impairments remained up to four years after cessation of shift work (Rouch et al., 2005). Other than concluding that entrainment of the activity rhythm to a 24h LD after six days of T21 exposure takes approximately 20 days (Devan et al., 2001), we have never determined whether this T21 exposure has any chronic effects on circadian rhythms or memory. The present

chapter investigated the long-term effects of six days of T21 exposure of the activity rhythm and hippocampal dependent memory in FBN female rats.

Methods

Animals

Twenty six-month old FBN female rats bred at the University of Lethbridge were used. Eight of the rats were used in the previous chapter, but served as the control rats in the present chapter because their exposure to the T21 cycle was after the completion of this experiment. The rats were initially pair housed in clear Plexiglas cages lined with beta cob bedding. Food and water were available ad libitum for the duration of the experiment. The rats were divided into two groups of 10 and housed in two separate colony rooms that were maintained with a temperature of 21°C and a humidity of 35%. Both groups of animals were exposed to a 12:12 light-dark cycle for 10 days. Each room had a red light bulb (< 5 lux) that always remained on.

T21

The rats in one of the rooms, henceforth referred to as the shifted rats, were exposed to a T21 cycle with 12 hours of light and 9 hours of dark (57% light) for six consecutive days (see Table 3). From this point on shift and T21 are synonymous. On day seven a 12:12 light-dark cycle was reintroduced. The other group of rats served as the control group and continued to be maintained on a 12:12 light-dark cycle. The animals were not housed in wheels at this point as wheel running can improve circadian entrainment and performance in learning and memory tasks.

Table 3. T21 Schedule.
Train time refers to the time of MWT training.

Day	Light Schedule	Train Time
	Acclimation: Lights on: 07:30 Lights off: 19:30	-
1	Lights on: 04:30 Lights off: 16:30	-
2	Lights on: 01:30 Lights off: 13:30	2:30
2	Lights on: 22:30 Lights off: 10:30	1:30
3	Lights on: 19:30 Lights off: 07:30	00:30
4	Lights on: 16:30 Lights off: 04:30	23:30
5	Lights on: 13:30 Lights off: 01:30	22:30
6	Lights on: 10:30 Lights off: 22:30	-
	Re-entrainment: Lights on: 10:30 Lights off: 22:30	21:30 (retention probe)

Distributed Morris Water Task

MWT training was conducted as previously (Devan et al., 2001). On the second day of exposure to the T21 cycle, both groups of rats started distributed MWT training. The shifted animals were trained once every 23 hours (see "Train Time" in

Table 3) to minimize the efficacy of training as a ZT. The control animals were trained every 24 hours one hour before lights off (ZT11).

A circular pool with a diameter of 1.4 m and depth of 40 cm was filled to 80% of capacity with 21°C water that was rendered opaque with nontoxic white acrylic paint. A clear plastic platform (10 × 10cm) sat two centimeters below the surface of water and was always in the same position in the southwest quadrant. The experimenter's position and the position of extra-maze cues such as posters, stool etc. remained static throughout training.

Batches of rats were placed in clear Plexiglas cages and transported to the training room. The rats were started from four different start locations (N, W, E, S) and the starting locations were determined in a pseudo random way such that each day a different order was used and within a day the rats received an equal number of trials from each starting location and never received two consecutive trials from the same starting location. As in Devan and colleagues (Devan et al., 2001), the animals received eight trials a day with an inter-trial interval of approximately six minutes for five consecutive days. The rats were given 60 seconds to find the platform and latency to reach the platform was recorded manually and with Noldus Ethovision 3.1 (VP118). Once on the platform the rats remained there for 10 seconds before they were removed and placed in the holding cages. After every trial fecal material was removed and the water was agitated to mitigate scent cues. 17 days after MWT training the rats received a single 60 second probe trial in which the platform was removed and the amount of time the animals spent in each quadrant of the pool was recorded. The percentage of time spent in the target and non-target quadrants was calculated. The average time spent in the non-target quadrants was then calculated

by adding up the percentage of time spent in the three non-target quadrants and dividing by three.

Wheels

To assess the long-term effects of the T21 cycle on circadian rhythms 65 days after the T21 cycle, the animals were separated and singly housed in cages with wheels (diameter of 24cm), in which they remained for the rest of the experiment. Due to a limited number of wheels, an animal was removed from each group, so from this point on there were nine animals in each group. The rooms were only entered once daily at ZT2 and the cages were cleaned once a week also at ZT2. ClockLab (Actimetrics, Wilmette, IL, USA) was used to collect and analyze the wheel data in the same manner that was described in Chapter 2.

Rapid Acquisition Morris Water Task

96 days after the shift, the animals received a single massed MWT training session in a new room. This task is thought to be a more sensitive measure of hippocampal function (Bolding & Rudy, 2006; Deibel & Skinner, 2014). The pool was the exact same dimensions as the pool in which distributed MWT training occurred. The exact same MWT procedure was used as described above except for several changes. First, a new platform location was used (Northwest quadrant). Second, the rats only received a single session of 16 trials at ZT9 and the next day at the same time were given a 60 second probe trial, in which the platform was removed.

Statistics

As in the previous chapter, parametric two-tailed statistics were used in all instances and p was 0.05 for all statistical tests. All of the statistics were conducted

with SPSS 21 (IBM, Armonk, New York) and GraphPad Prism software (GraphPad, La Jolla, CA) was used to make all of the graphs.

For the MWT distributed acquisition latency data, a mixed model ANOVA with day and trial as repeated measures factors and group as a between measures factor was used. For the MWT massed acquisition data a mixed model ANOVA with block (average of two trials) as the repeated measures factor and group as the between measures factor. For the probe trials, a mixed model ANOVA with quadrant (target vs. other) as the repeated measures ANOVA and group as the between measures factor was used. As *a priori* we were interested in knowing if each group remembered the location of the platform, regardless of the whether or not there was a significant quadrant × group interaction, for each group, planned comparison with Bonnferroni corrections comparing time spent in the target quadrant compared to the other quadrants was conducted. In regards to the ANOVAs, when Mauchly's sphericity was violated, the Greenhouse-Geisser correction was applied. For the independent samples t-tests, corrected degrees of freedom were used if Levene' test was violated.

For the long-term circadian rhythm analyses, independent samples t-tests were used to compare the various measures between the two groups. For the error associated with the least squares regression fit, two outliers, one from each group (>2 standard deviations from the mean) were removed, giving n's of 8 for each group.

Results

Distributed Morris Water Task

Latencies significantly decreased across the training days ($F(2.296, 41.326) = 61.493$, $p < .001$) suggesting that learning occurred and there was no difference in

performance between the groups (Figure 3A). In the 60 second probe trial given 17 days after the end of acquisition each group spent significantly more time in the target quadrant compared to the non-target quadrants (Figure 1B; control: $p = .008$; shifted: $p = .010$). These data indicate that both groups of animals acquired and remembered the distributed version of the MWT. This is surprisingly given that the shifted animals experienced a T21 cycle that induces circadian misalignment during MWT training.

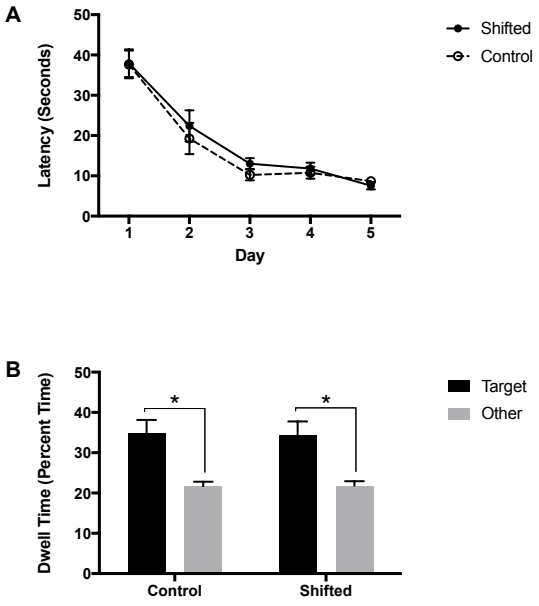

Figure 3. Distributed Morris water task.
A) Training was distributed across five days and both groups acquired the task similarly. B) Both the control (n =10; $p = .008$) and shifted (n = 10; $p = .010$) groups remembered the location of the platform in 60s probe trial given 17 days after the end of acquisition. Error bars represent standard error of the mean and * indicated $p < .05$.

Long-Term Circadian Rhythm Assessment After a Shift

Despite there not being any memory impairments, the animals were put into wheels 65 days after the T21 cycle to confirm that entrainment had occurred. As

depicted in actograms for the control and shifted animals, data were collected for 30 days (Figure 4). There were no differences between the groups in regards to period with both groups oscillating very close to 24h (Figure 5A). Nor was there a difference between the groups in total amount of activity (data not shown), rhythm prominence, or the percent of activity that occurred during the dark phase of the light-dark cycle (B). However, surprisingly several measures suggested that the shifted animals were not quite in phase with the light dark cycle. First the phase was significantly delayed in the shifted animals (Figure 5C; $T(15) = -3.257$, $p = .005$). Along these lines, the shifted animals also had more error associated with a least squares regression fit to the onsets of activity, which suggests that onset time was much more variable in these animals compared to controls (Figure 5D; $T(14) = -3.806$, $p = .002$). These data suggest that while the shifted animals' activity was circadian, the phase of this rhythm never quite entrained to the new light dark cycle even up to 95 days after exposure to the T21 cycle.

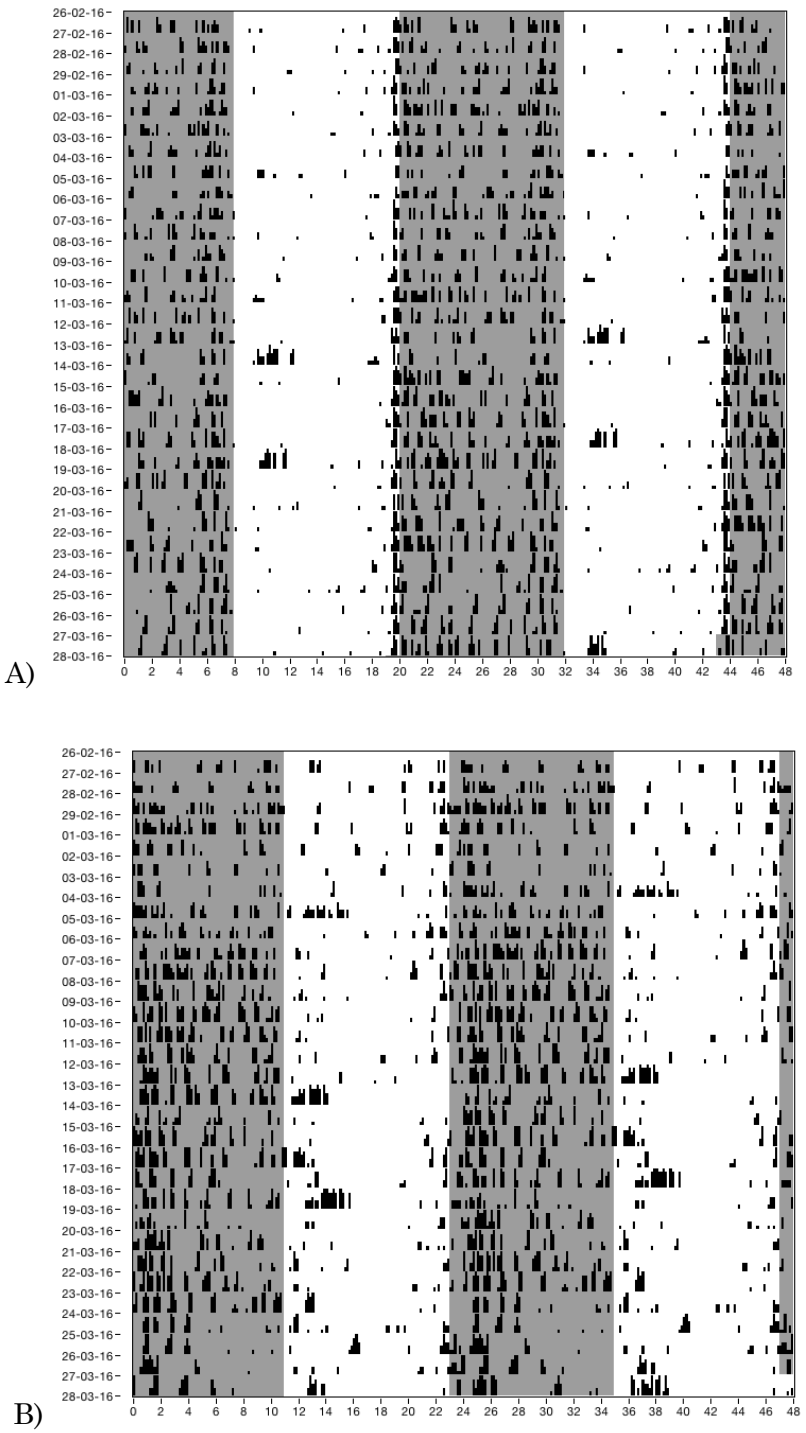

Figure 4. Double plotted actograms of wheel running activity.

Actograms[4] represent a 30 day period that started 65 days after exposure to the T21 cycle (control (A; n = 9); shifted (B; n = 9)). These actograms represent activity for one animal in each group.

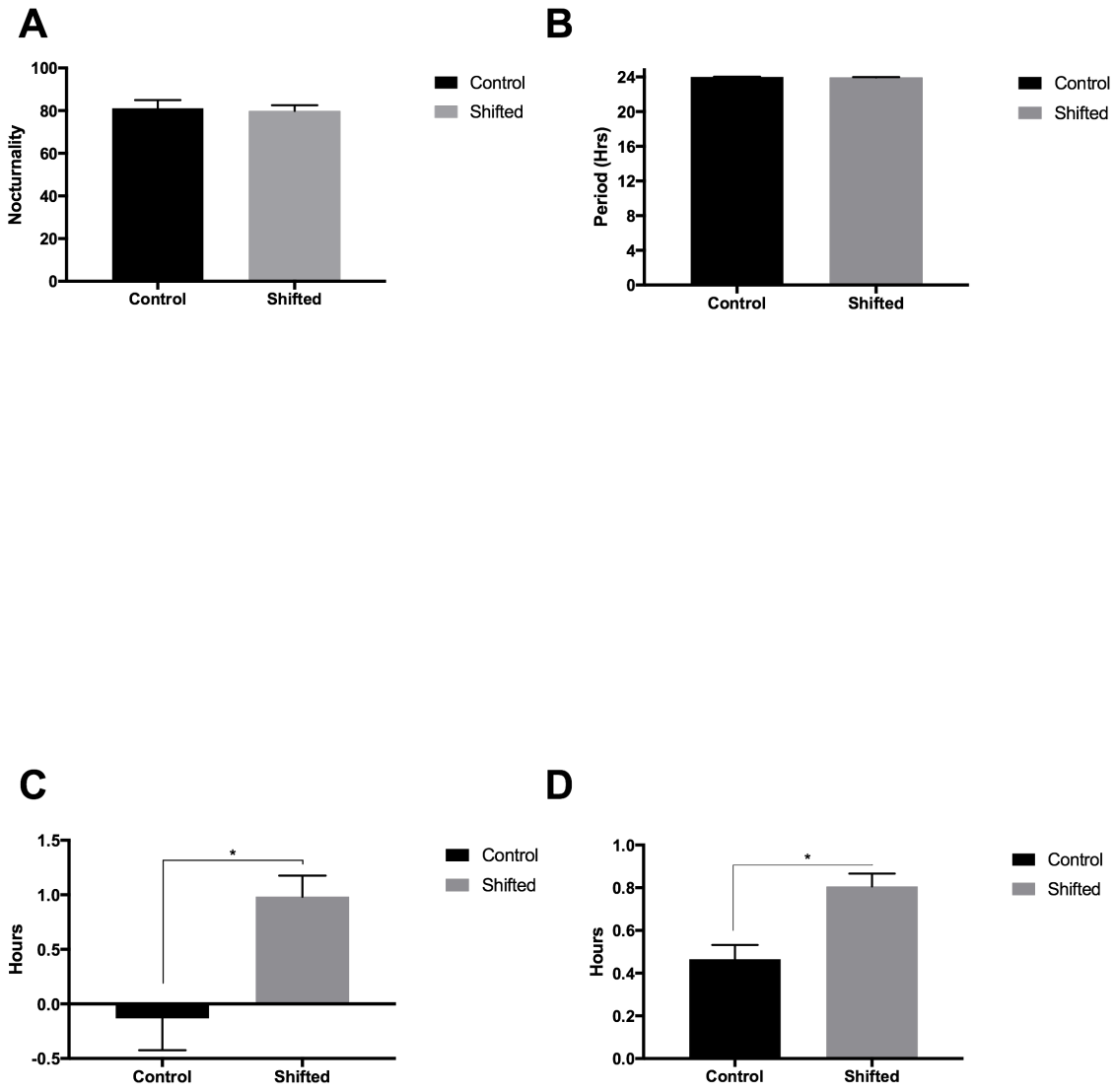

Figure 5. Circadian rhythm analyses.
The shifted animals had a similar period (A) of oscillation and percentage of activity that occurred during the dark phase compared to control animals (nocturnality) (B). However, surprisingly, the shifted animals had a significantly delayed rhythm compared to the control animals (C; $p = .002$). The shifted animals also had more error (D) associated with a least squares regression for the onset of activity. Error bars represent standard error of the mean and * indicated $p < .05$.

[4] Refer to appendices for actogram's of every rat.

Rapid Acquisition Morris Water Task

With the altered phase in the wheel running activity in mind, 96 days after exposure to the T21 cycle, the animals received a single massed MWT training session consisting of 16 trials to a new platform location in a completely new room. As depicted in Figure 6A, both groups of animals learned the location of the new platform as indicated by significantly decreased latencies to the platform ($F(1.919, 30.700) = 17.179$, $p < .001$) and there was no differences between the groups in performance. Shockingly, however, in a 60 second probe trial administered the next day, only the control animals spent significantly more time in the target quadrant compared to the other quadrants (Figure 4B; $p = .001$). These data suggest that six days of exposure to a T21 light dark cycle affects hippocampal-dependent memory even when assessed 96 days later.

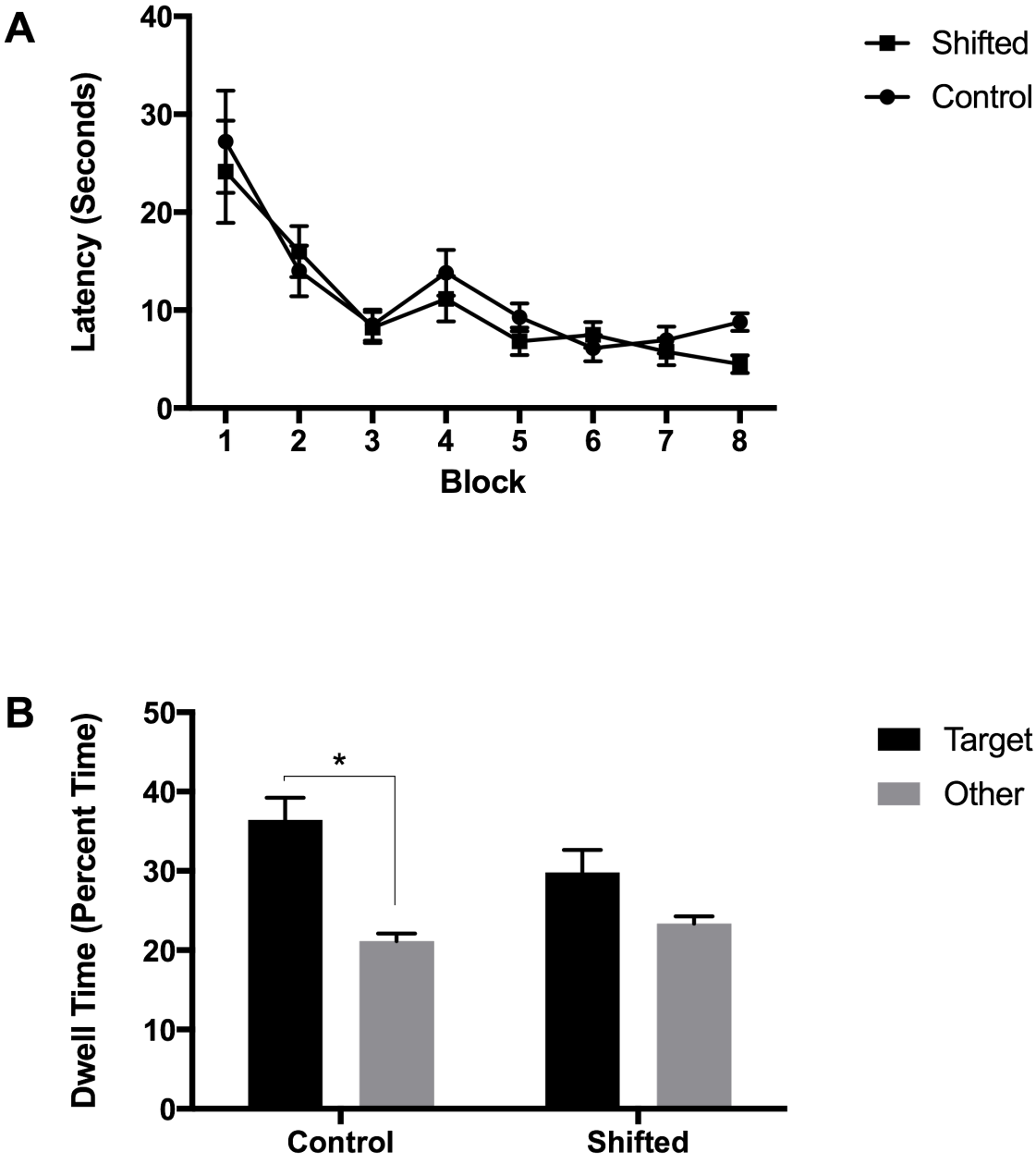

Figure 6. Rapid Acquisition Morris water task.
A) Both the control (n = 9) and shifted (n = 9) animals acquired the location of a novel platform location in a novel room, during a single massed training session consisting of 16 trials. B) Surprisingly, only the control animals spent significantly more time in the target quadrant compared to the non-target quadrants in a 60 second probe trial given the day after the massed training session (p = .001). Error bars represent standard error of the mean and * indicated $p < .05$.

Discussion

Summary

Unexpectedly, the female FBN rats remembered a spatial location acquired in the distributed version of the MWT 17 days after learning occurred. As there was intact memory it was very surprising that we found evidence that the shifted rats were not quite aligned with the light-dark cycle several months after the T21 cycle. With this circadian misalignment in mind, we thought we would try the more sensitive rapid acquisition training version of the MWT that is thought to place a higher demand on hippocampal processing. Despite the shift occurring almost 100 days before the massed MWT, the shifted rats did not retain the acquired memory when tested the day after massed training. As a whole these data suggest six days of T21 cycle exposure produces persistent circadian rhythm misalignment that likely produces subtle but long lasting hippocampal dysfunction.

T21 affects Circadian Rhythms Chronically

We chose not to have the originally shifted animals in wheels during the T21 exposure because wheel running promotes both memory and circadian rhythm entrainment (Zelinski et al., 2014, 2013). Although in different animals, the long-term wheel data suggests that six days of T21 exposure can have long lasting effects on circadian rhythms. As in the FBN female rats from Chapter 2 that were exposed to the T21 cycle, the onset of activity was less precise over the long-term as well. In contrast, to these animals, the phase was significantly delayed in the shifted animals well after T21 exposure. It is unclear why the phase was still delayed in these animals. In the control animals that were shifted, the phase became more aligned with the light-dark cycle as time elapsed since T21 exposure. Similarly, we have shown in the past that phase usually entrains within a month after T21 exposure

(Devan et al., 2001; Zelinski et al., 2014, 2013). Nonetheless the higher variability associated with the time of activity onset (precision) also suggests that both the control shifted and long term shifted animals were not quite in phase with the light dark cycle after T21 exposure.

In summary these data suggest a T21 cycle elicits long term perturbations in the circadian rhythms of female FBN rats. Sex, strain, and duration of both T21 exposure and data collection need to be investigated in this paradigm. To our knowledge, this is the first characterization of the activity rhythm in FBN rats in control and T21 conditions.

Why Not Impairments in the Distributed MWT?

In contrast to our previous demonstration with male LE rats in this paradigm (Devan et al., 2001), it was surprising to find that the female FBN rats did not have impaired retention of the distributed MWT. In the current study as we did not test FBN males or other rat strains, we can only hypothesize about possible mechanisms

As discussed in Chapter 2, one possibility is that females are partially protected from the harmful effects of T21 exposure on memory (Zelinski et al., 2014, 2013). This could be a result of rhythms that are more influenced by the endogenous clock rather than the light dark cycle (Cambras et al., 2004; Campuzano, 1998; Tsukahara et al., 2005). Another possibility that arises from the data in Chapter 2 is that T21 exposure might affect rat strains differently. To our knowledge activity rhythms in 12:12 and T21 cycles have never been characterized in FBN rats. It appears that FBN rats are more affected by the T21 cycle than LE rats, however it remains to be seen whether free-running or partial entrainment to a T21 is more detrimental to memory.

It could be that the protection was due to improved MWT performance by the FBN female rats. It seems unlikely that sex ameliorated MWT performance as male rats typically perform better in the MWT than females (Keeley, Bye, Trow, & McDonald, 2015). Along these lines, strain also was likely not improving MWT performance, as although similar, FBN male rats were slightly inferior to LE male rats in the distributed MWT (Harker & Whishaw, 2002).

Why Impairments in the massed MWT?

Distributed MWT and massed MWT appear to place different demands on the hippocampus, with massed training MWT paradigms being more sensitive or placing a higher demand on the hippocampus than distributed versions. It is possible that the T21 exposure elicited subtle hippocampal dysfunction that was either not strong enough to be detected in the distributed MWT or only localized to part of the hippocampal circuit. Distributed MWT can be solved with only the dorsal hippocampus (Moser et al., 1995; Ruediger, Spirig, Donato, & Caroni, 2012), whereas versions of the massed MWT require both dorsal and ventral hippocampus (Ferbinteanu, Ray, & McDonald, 2003). Similarly we have observed that brain damaging agents can only produce impairments in the massed and not distributed versions of the MWT (Craig, Hong, Kopp, & McDonald, 2008; Sutherland, McDonald, & Savage, 2000). Along these lines, retention of the massed MWT is typically very transient in the span of hours (Bolding & Rudy, 2006; Deibel & Skinner, 2014) compared to many days for the distributed version (17 days in the current study) (Spreng, Rossier, & Schenk, 2002; Zelinski et al., 2014, 2013).

It is unlikely that the prior distributed MWT training interfered with massed MWT for several reasons. First, a new room and different relative platform location

were used in the massed MWT compared to the distributed version. Second, the massed MWT training occurred 96 days after distributed MWT training. It is likely that this interval was sufficient for the degradation of information acquired in the distributed MWT, as prior massed MWT experience 10 days previously did not influence subsequent massed MWT performance to a new platform location in the same room (Deibel & Skinner, 2014).

In addition to the massed MWT being a more sensitive paradigm than the distributed version it is possible that the long period of time between the massed MWT training and T21 exposure allowed circadian misalignment to incubate. The long term circadian rhythm data suggests that the rats that were exposed to the T21 had a delayed phase and less precise onsets of activity compared to control rats even 65-95 days after the T21. Thus these animals were experiencing circadian misalignment for a very long period of time. Whereas, when they received the distributed MWT training they had only experienced 1-6 days of circadian misalignment. Impairments possibly could have been detected if the rats were trained in the distributed MWT many days after T21 exposure.

Conclusions

The findings from this study suggest that the relationship between circadian rhythm disruption and memory is more complex than expected. I was surprised to find there were evidence of circadian rhythm disruption and hippocampal dysfunction approximately 100 days after only six days of exposure to a T21 cycle. As I only had one rat strain and only tested females this study is aiming to serve solely as an exploratory investigation that hopes to ignite further investigations into the possible chronic effects of relatively transient periods of circadian rhythm disruption.

Nonetheless to our knowledge this is the first study to report activity circadian rhythms in this relatively new hybrid strain of rat and employ this rapid acquisition training MWT paradigm in circadian rhythm disrupted animals.

Chapter 4

How Might T21 Cycle Elicit Hippocampal Dysfunction?

General Introduction

Chapters 1 & 2 demonstrated that six days of T21 exposure can cause circadian misalignment that can be long lasting and correspond with chronic hippocampal dysfunction. But, what might be the possible mechanisms for this dysfunction? Generally it is unclear how circadian dysfunction induces hippocampal dysfunction (Deibel et al., 2015; Eckel-Mahan & Storm, 2009; Keeley, Zelinski, Fehr, & McDonald, 2014; Smarr et al., 2014).

Clock genes and molecules involved in hippocampal plasticity display circadian oscillations in the hippocampus (Eckel-Mahan et al., 2008; Feillet, Mendoza, Albrecht, Pévet, & Challet, 2008; Jilg et al., 2010; Peixoto et al., 2015; Phan, Chan, Sindreu, Eckel-Mahan, & Storm, 2011; Rawashdeh et al., 2014; Wakamatsu et al., 2001; Wang et al., 2009). This likely precipitates circadian oscillations in long term potentiation (LTP) facilitation and decay (Barnes, McNaughton, Goddard, Douglas, & Adamec, 1977; Chaudhury, Wang, & Colwell, 2005). Not surprisingly, animals with mutations of the core clock genes often have impaired memory in hippocampal dependent tasks that is thought to be due to the abolition of oscillations in plasticity molecules and the resultant LTP failure (Jilg et al., 2010; Kondratova, Dubrovsky, Antoch, & Kondratov, 2010b; Rawashdeh et al., 2014; Van der Zee et al., 2008b; Wang et al., 2009; Wardlaw, Phan, Saraf, Chen, & Storm, 2014).

While impaired LTP is most likely involved in the memory impairment elicited by circadian misalignment, this is probably the most downstream player in this story. Many different factors set into motion by circadian misalignment could impair hippocampal plasticity (Deibel & McDonald, 2017; Deibel et al., 2015; Zelinski et al., 2014).

Three of the most common are sleep disruption, increased stress response, and hippocampal atrophy. The present chapter investigates the impact of six days of T21 exposure on sleep, stress response, and hippocampal morphology.

Experiment 4.1. Sleep[5]

Introduction

Sleep is regulated by both circadian and homeostatic mechanisms (Cirelli, 2009) and good sleep has been associated with good memory function (Diekelmann & Born, 2010). Sleep is generally categorized into two states, rapid eye movement (REM) sleep and non-REM (NREM) sleep. REM sleep is characterized by high frequency and low amplitude electrical activity, whereas NREM sleep, which includes slow wave sleep (SWS), is characterized by low frequency and high amplitude electrical activity (Diekelmann & Born, 2010). Slow wave neocortical oscillations, which occur during SWS, are characterized by a large amplitude waveform (K-complex), which are then followed by low amplitude high frequency bursts of activity (sleep-spindles) (Crowley, Trinder, Kim, Carrington, & Colrain, 2002; Diekelmann & Born, 2010). Among other things, it has been hypothesized that K-complexes and sleep-spindles might be involved in the transfer of information to

[5] This dataset was collected by Ryan Rota.

the cortex that is thought to occur during memory consolidation (Diekelmann & Born, 2010).

In mice and rats, phase shifting the light-dark cycle (Altimus et al., 2008; Castanon-Cervantes et al., 2010; Loh et al., 2010; Sei et al., 1992; Van Dycke et al., 2015), or restricting feeding to the light phase of the cycle (Loh et al., 2015) desynchronizes the sleep cycle, but generally does not change the total amount of sleep, or sleep stages (Castanon-Cervantes et al., 2010; Loh et al., 2010; Sei et al., 1992). Interestingly, in a symmetrical T22 (L11:D11) cycle some rhythms become dissociated from each other, meaning that some rhythms (REM sleep and core body temperature) are not able to entrain to the T-cycle and free-run, whereas other rhythms (activity, slow wave sleep, and sleep) show evidence of free-running and entrainment (Cambras et al., 2007). However, to our knowledge in the rat, no study has assessed the effect of an asymmetrical T21 light cycle on sleep, nor has any circadian disruption paradigm assessed K-complexes and sleep-spindles.

Methods

Animals and T21

These were the same seven male LE rats in which we reported wheel-running behaviour in Chapter 1. Therefore these animals received the same six days of T21 exposure with 43% light as described in Chapter 2.

Local Field Potential Recording

Bipolar electrodes were constructed for LFP recording. The electrodes consisted of medical grade 40 (Sigmund Cohn, MT Vernon, NY, USA, part #316SS3T) stainless steel wire coated in polytetrafluoroethylene (Teflon). Pre-surgery electrode impedances ranged from 50K to 100K ohms. Two pairs of

electrodes were implanted; the first twisted pair was implanted to the hippocampus (HC, -3.84 mm from Bregma, 2.4 mm lateral and 2.4 mm depth; tip separation of 0.4 mm). The second twisted pair was implanted to medial prefrontal cortex (mPFC, 2.76 mm from Bregma, 2.8 mm lateral and 4.25 mm depth at a 55 degree angle from dura; tip separation of 1.8mm). In addition, two mono-electrodes (Cooner Wire, Chatsworth, CA, USA, part #AS 631) was sutured bilaterally to the neck (nuchal) muscle for EMG (electromyogram) recordings. A single stainless steel ground screw was also implanted in each rat's cranium (-2 mm from Lambda). Depth and positions of electrodes were confirmed by histological analysis.

For the first group of six rats, a first generation Avatar system (Electrical Geodesics Inc., Eugene, OR) was adapted for rat use. This system was capable of recording at a 256Hz sampling rate with up to six referenced channels. The recording signals were transmitted in real time to a host computer by Bluetooth with 16bit sample size and a 6.5mV dynamic range. The second group of six rats were recorded on a second generation Avatar 3000 (Electrical Geodesics Inc., Eugene, OR) series. It allowed recording at a 2000Hz sampling rate to an internal microSD card, which was replaced each week. It also had an average 250Hz Bluetooth transmission rate that was used for monitoring LFP activity during continuous recording. The sampling size and dynamic range were expanded to 24 bit sample size and 2.25V, respectively.

A spectrogram of the hippocampal electroencephalogram (EEG) was calculated using a Hamming window of two seconds with no overlap. EMG power was calculated by filtering the EMG signal with a high-pass filter at 5 Hz to get fine muscle tone, rectified and then smoothed with a moving average filter with a 10

second window. Three researchers were presented with this spectrogram data and associated EMG power for each rat and stage of the experiment, and they manually scored the data for the brain states of motionless, REM sleep and slow wave sleep. This was used to fine-tune the parameters for the automated scoring that was used to analyze the rest of the data.

The first step for the automated scoring algorithm is to identify behavioral states (motion and motionless states) using the EMG power signal that was calculated for the manual scoring. A threshold on the EMG power was adjusted so that the agreement between manual scoring and automated scoring is maximized (Supplemental Fig. 1).

The second step is to detect REM sleep and slow wave sleep in motionless episodes using the spectrogram of the hippocampal EEG signal used for the manual scoring. Because slow wave sleep and REM sleep are characterized by strong delta oscillation (1-4 Hz) and theta oscillation (6-10 Hz) in the hippocampus respectively, mean delta power and theta power were calculated from the spectrogram. A ratio of theta to delta power was used to find REM sleep epochs, and a ratio of delta to theta power was used to find slow wave sleep epochs (Louie & Wilson, 2001). A threshold for each of these ratios was determined by comparing the resulting epochs against manually scored spectrogram data for each animal and stage of recording.

K-complexes are characterized by a drop in the cortical LFP trace when a down-state occurs, followed by a gradual rise when a subsequent up-state happens. K-complex detection is performed by looking for this shape within the cortical LFP trace. First, the cortical LFP trace was filtered between 0.75Hz and 6Hz during motionless episodes. The instantaneous slope (derivative) was computed by

calculating the difference between adjacent signal values in the filtered LFP trace. Using this slope two thresholds were determined: the 98th percentile of the negative slope values and the 95th percentile of the positive slope values for the downward and upward slope thresholds, respectively. These thresholds were selected since the downward slope value needs to be greater than the upward slope value to make sure the correct shape is found and were adjusted later using information from the manually scored data. Local minima less than the negative slope threshold were used to mark potential start of K-complex times and local maxima values greater than the positive slope threshold were used to mark potential end times. Initial detection of K-complexes was performed by matching start and end times which occur within one second of each other. Start times were then adjusted by finding the first time point in the calculated slope value before the local minima which has a value greater than or equal to zero to ensure the entire length of the K-complex event is contained within the timestamps. A similar procedure was performed for end time points as well, finding the first value less than or equal to zero**.** For each rat one hour of cortical LFP data were manually scored for K-complexes. This was used to adjust the upward and downward slope values to ensure maximal agreement.

In order to detect spindles, the cortical LFP trace during motionless periods was filtered between 10-20 Hz. To improve quality and continuity of the spindle detection, a minimum duration of spindles (200 ms) and minimum gap between spindles (700 ms) were imposed. This ensured that the spindles we analyzed had at least two oscillations for lower spindle frequencies and that any momentary loss in spindle power did not count the same spindle multiple times. Spindle power was

calculated by rectifying the filtered cortical LFP signal. Peaks in the spindle power above a threshold that was manually set were used to find potential spindles. A boundary threshold set to be one quarter the peak power gave us the duration of the spindle episode. Like the K-complex data, spindles were manually scored using one hour of data for each rat, and this manual scoring was used to adjust the peak threshold used.

Statistics

The same parameters as those used for the wheel-running data in Chapter 1 were applied to the sleep data, however there were only three six day blocks: 6 days preceding the shift (b1); six days of the shift (b2); six days following the shift (b3). Thus, the following planned contrasts were used: b1 vs. b3 & b1 vs. b2. For all of the sleep measures there was not a change in the total amount of these processes, thus these statistics are not reported.

Results

Total Sleep

As depicted in Figure 7 and Table 4, the shift did not affect period length or amplitude of the rhythm. However, the activity onset error increased after the shift ($F(1, 6) = 7.014$, $p = .038$), but not during. Light expression decreased during the shift ($F(1, 6) = 24.675$, $p = .003$), but was not different after the shift when compared to b1. Finally, the phase of activity onset did not change.

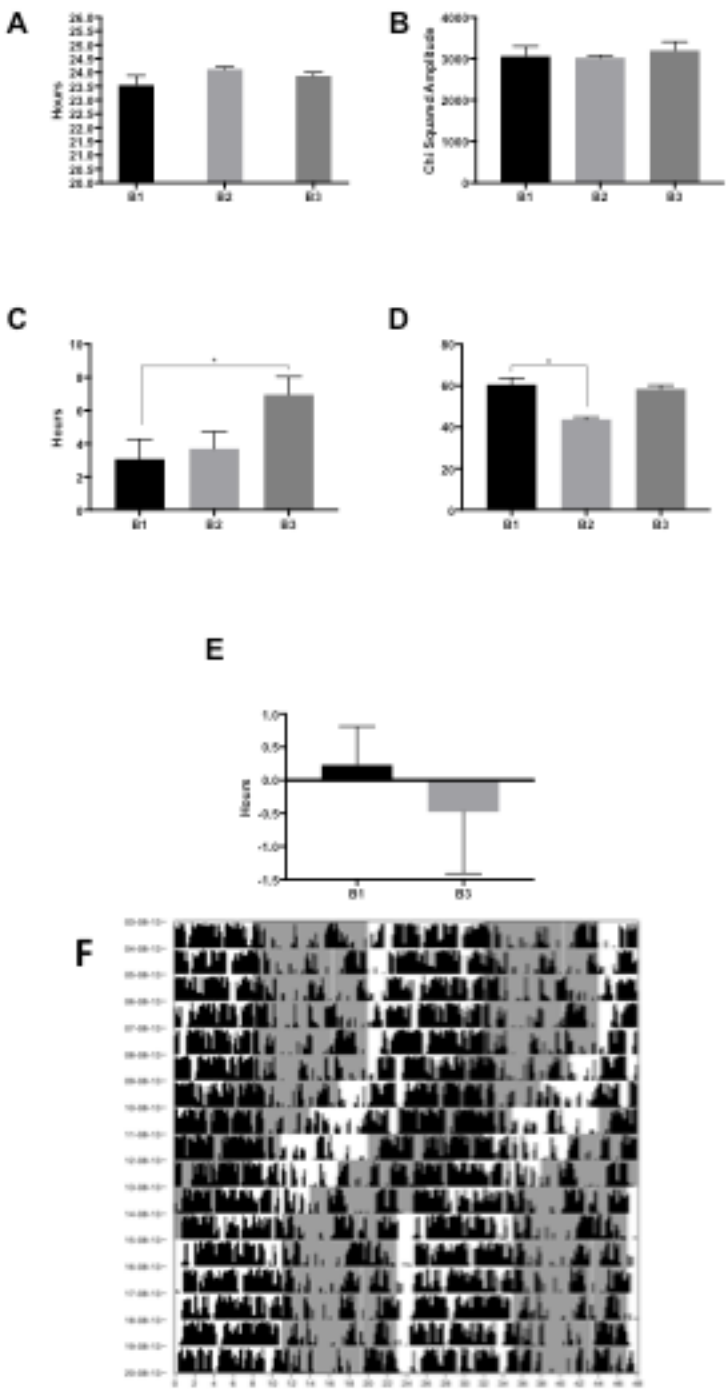

Figure 7. Total sleep in male LE rats (n = 7).
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Light activity. E.) Phase of activity onset. F) Actogram like representation of total sleep. Normalized representative actogram-like representation from one animal. Shaded area indicates periods in which the lights were off. Error bars represent standard error of the mean and * indicates a *p* value of less than .05.

REM Sleep

As depicted in Figure 8 and Table 4, period length was not greater after the shift and there was only a non-significant trend for a longer period length during the shift ($F(1, 6) = 5.509$, $p = .057$). The shift did not affect rhythm amplitude, nor did it affect activity onset error. Light activity decreased during the shift ($F(1, 6) = 22.091$, $p = .003$), and but was not different after the shift compared to b1. Finally, the phase of activity onset was not affected by the shift.

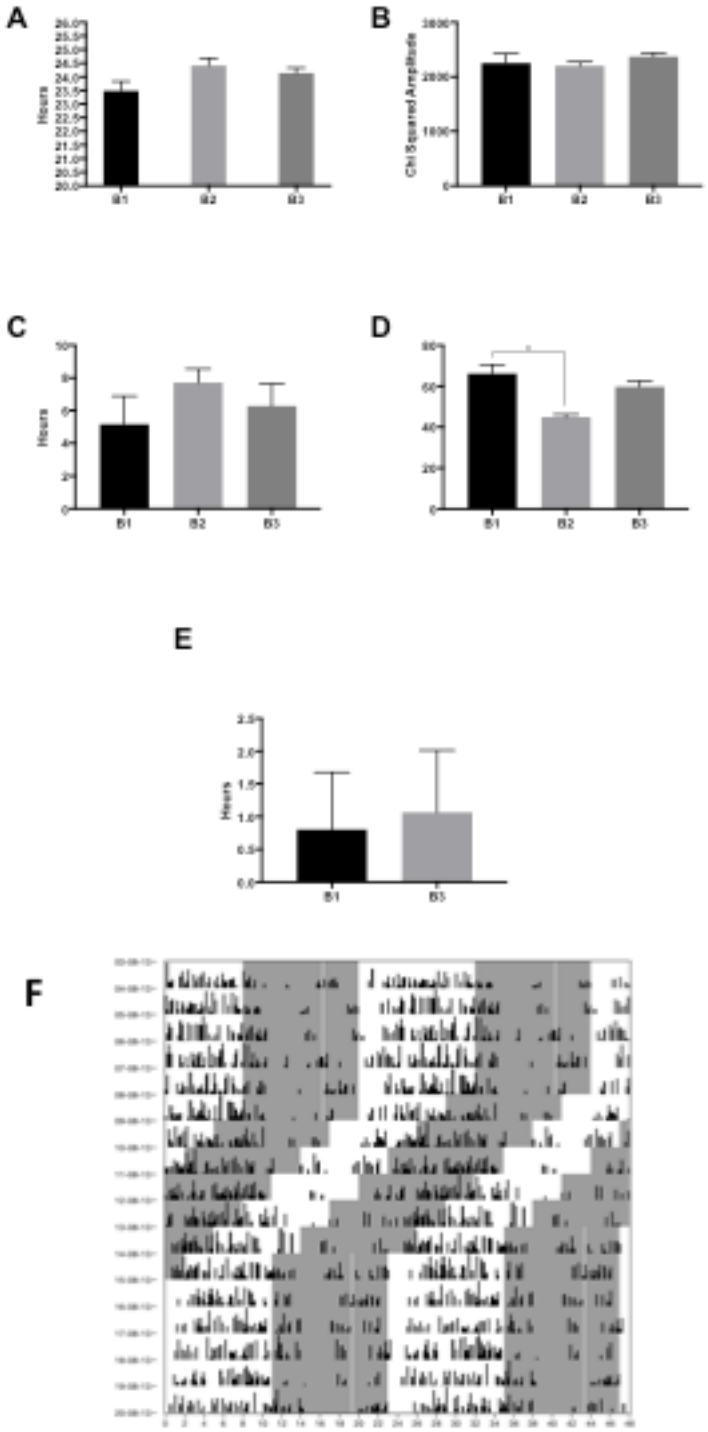

Figure 8. REM sleep in male LE rats (n = 7).
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Light activity. E.) Phase of activity onset. F) Actogram like representation of REM sleep. Normalized representative actogram-like representation from one animal. Shaded area indicates periods in which the lights were off. Error bars represent standard error of the mean and * indicates a *p* value of less than .05.

SWS Sleep

As depicted in Figure 9 and Table 4, period length and rhythm amplitude were not affected by the shift, however activity onset error was greater after ($F(1, 6) = 13.340$, $p = .011$), but not during the shift. In contrast, light activity was only less during the shift ($F(1, 6) = 58.00$, $p < .001$) and not after. Finally, the phase of activity onset was not affected by the shift.

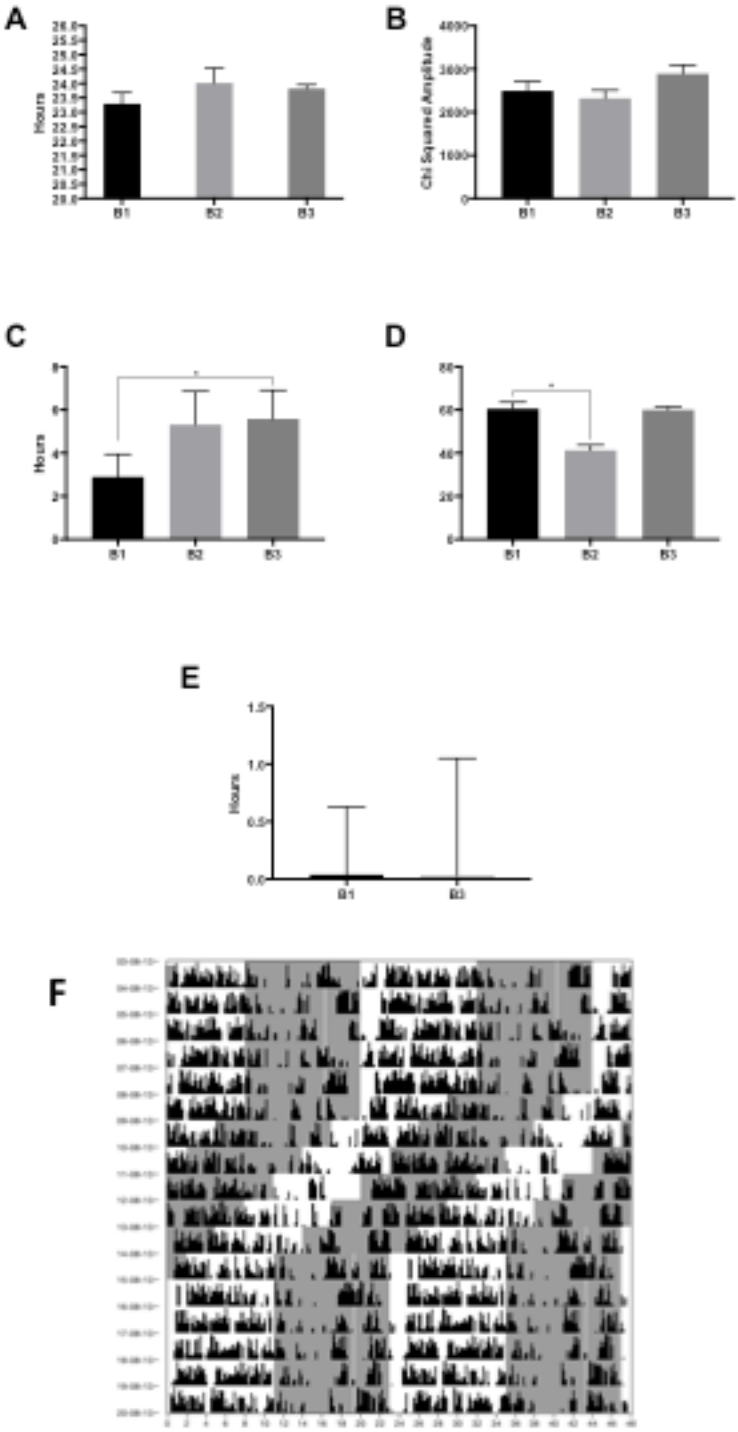

Figure 9. SWS sleep in male LE rats (n = 7).
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Light activity. E.) Phase of activity onset. F) Actogram-like representation of SWS sleep. Normalized representative actogram-like representation from one animal. Shaded area indicates periods in which the lights were off. Error bars represent standard error of the mean and * indicates a *p* value of less than .05.

K-Complexes

As depicted in Figure 10 and Table 4, period length, rhythm amplitude, activity onset error, and the phase of activity onset were unaffected by the shift. Light activity decreased during the shift ($F(1, 5) = 30.282$, $p = .003$), and was also decreased in b3 compared to b1 ($F(1, 5) = 8.457$, $p = .033$).

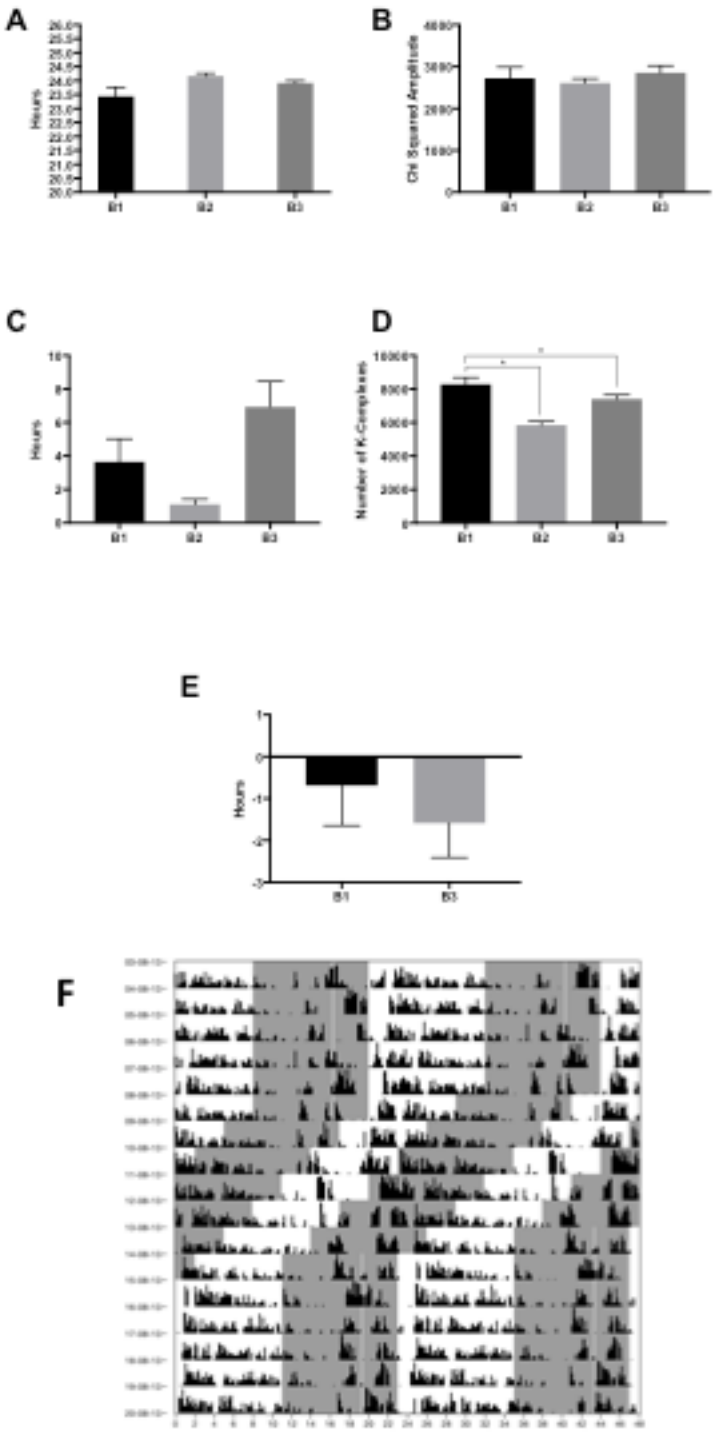

Figure 10. K-complexes in male LE rats (n = 7).
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Light activity. E.) Phase of activity onset. F) Actogram-like representation of K-complexes. Normalized representative actogram-like representation from one animal. Shaded area indicates periods in which the lights were off. Error bars represent standard error of the mean and * indicates a *p* value of less than .05.

Spindles

As depicted in Figure 11 and Table 4**,** period length was elongated during the shift ($F(1, 5) = 7.664$, $p = .039$), but was not different after the shift when compared to b1. Rhythm amplitude did not change, but the activity onset error was increased after ($F(1, 5) = 18.337$, $p = .008$), but not during the shift. Light activity was decreased only during the shift ($F(1, 5) = 20.659$, $p = .006$). Finally, the phase of activity onset was not affected by the shift.

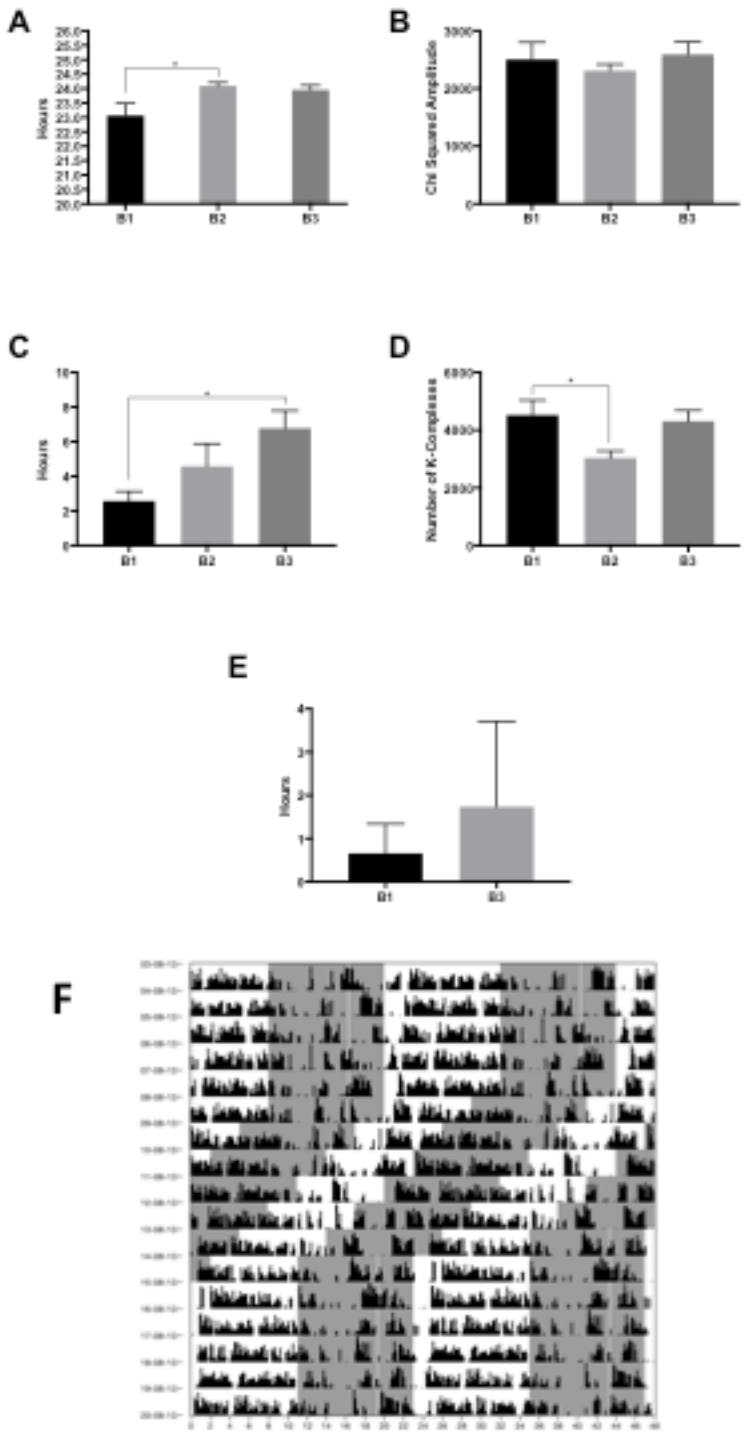

Figure 11. Spindles in male LE rats (n = 7).
A) Period length of rhythm. B) Amplitude of rhythm. C) Activity onset error. D) Light activity. E.) Phase of activity onset. F) Actogram-like representation of spindles. Normalized representative actogram-like representation from one animal. Shaded area indicates periods in which the lights were off. Error bars represent standard error of the mean and * indicates a *p* value of less than .05.

Table 4. Summary of the effects of a T21 light dark cycle on sleep in male LE rats. For comparisons sake, the running wheel data from Chapter 1 for these animals is also included.

Circadian	Running-Wheel	Total Sleep	Rem	SWS	K-complexes	Spindles
Period	No Change	No Change	No Change	No Change	No Change	Elongated During Shift
Amplitude	No Change	No Change	No Change	No Change	No Change	No Change
Onset Activity Error	No Change	Increased Post Shift	No Change	Increased Post Shift	No Change	Increased Post Shift
Light Phase of Process	Decreased During & After Shift	Decreased During Shift	Decreased During shift	Decreased During Shift	Decreased During and After Shift	Decreased During Shift
Phase	Significantly Advanced After Shift	No Change	No Change	No Change	No Change	No Change

Discussion

The T21 affected electrophysiological correlates of sleep in the male LE rats. For most aspects of sleep there were no change in period length, rhythm amplitude, or phase of entrainment. Similar to the running wheel activity, the amount of sleep and associated components that occurred during the light phase of the cycle decreased during the shift and this decrement was actually maintained for K-complexes. Similarly the activity onsets were less precise post shift for total sleep, SWS sleep, and spindles.

Some rhythms are more prone to rhythm splitting – presence of a free-running rhythm and another light entrained rhythm – than others. In rats, a symmetrical T22 cycle splits sleep, slow wave sleep, activity, and core body

temperature, but REM sleep has one free-running rhythm (Cambras et al., 2007). While we did not find evidence of rhythm splitting, interestingly, the onset of SWS sleep was less precise after the shift, whereas the onset of REM sleep remained precise.

As with the activity rhythms, all aspects of sleep also showed signs of circadian misalignment with K-complexes even remaining depressed during the light phase of the cycle after the shift. Except for K-complexes all other aspects of sleep during the light phase of the cycle increased when a normal L12:D12 cycle was reintroduced. While sleep was uncoupled from the external light dark cycle, there was no change in the total amount of sleep and its associated components. This is consistent with other studies in mice and rats, in which phase shifting the light-dark cycle (Altimus et al., 2008; Castanon-Cervantes et al., 2010; Loh et al., 2010; Sei et al., 1992; Van Dycke et al., 2015), or restricting feeding to the light phase of the cycle (Loh et al., 2015) desynchronizes the sleep cycle, but generally does not change the total amount of sleep, or sleep stages (Castanon-Cervantes et al., 2010; Loh et al., 2010; Sei et al., 1992).

To our knowledge no study has extended the findings of sleep misalignment to K-complexes and sleep spindles in the rat, which are thought to be important for memory consolidation (Diekelmann & Born, 2010). These processes seemed particularly susceptible to the T21 cycle. First spindles were the only process in which the period changed during the shift. Furthermore after the shift the onset of spindle activity was more variable and K-complexes were still misaligned from the light dark cycle despite it being a normal 12:12.

Interestingly, dark phase running wheel activity also did not significantly increase after the T21 light dark cycle, suggesting that activity and K-complexes/spindles take more time to re-entrain than sleep and its associated stages. Generally though, sleep was more affected by the T21 than the activity rhythm. This does not preclude the possibility that sleep misalignment/disruption is involved in the memory impairment elicited by circadian misalignment. The fact that spindles and K-complexes were most affected by T21 light dark cycle in particular enticing, as these processes are thought to be integral for memory consolidation, which coincidently is thought to be affected by circadian misalignment.

[6]Experiment 4.2. Stress

Introduction

Stress is influenced by circadian rhythms with the primary stress hormones (glucocorticoids) being expressed in a circadian manner (Chrousos, 1998; Dickmeis, 2009; Zelinski et al., 2014). Elevated glucocorticoids can impair hippocampal dependent learning and memory (Shors, 2006). Our group demonstrated that hamsters with age-induced dampening of baseline and corticosterone rhythms had poorer hippocampal dependent memory than age-matched controls with better corticosterone rhythms (Cain, Karatsoreos, et al., 2004).

Although correlative, these data open the possibility that the memory impairment induced by circadian rhythm disruption involves an altered stress response (Deibel, Hong, et al., 2014; Zelinski et al., 2014). However, in rodent circadian misalignment models the story is inconclusive and seems to depend on

[6] This experiment was reported in Deibel and colleagues (Deibel, Hong, et al., 2014).

experimental methodology (Deibel, Hong, et al., 2014). While most studies with circadian manipulations do not find increases in baseline corticosterone (Castanon-Cervantes et al., 2010; Kort & Weijma, 1982; Logan et al., 2012; Loh et al., 2010; Sei et al., 2003), it can be elevated at specific times during some circadian misalignment manipulations (Gibson et al., 2010), or potentiated when these animals are exposed to stressors (Loh et al., 2010). As our use of the T21 schedule is inherently different from many of the other methods, it was crucial to determine if glucocorticoid levels during our rigorous paradigm are elevated.

Methods

Animals

Sixteen three-month old female Long Evans rats that were obtained from Charles River Colony (Laval, Quebec) were used for all experiments. Rats were pair-housed in clear Plexiglass cages with beta- cob bedding. Wheels were not used so that the effects of the T21 cycle and mini-strokes were not mitigated in any way. During a 3-week acclimation period, water and Purina Rat chow were available ad-libitum. The housing rooms were maintained on a 12:12 light-dark cycle, with an ambient temperature and humidity of 21◦C and 35%, respectively. Rats were weighed weekly throughout the acclimation period.

T21 Exposure

After the acclimation period, one group of rats (shifted: n=8) underwent chronic T21 exposure that was interleaved with partial re-entrainment periods (see Table 5). As before the animals received six days of T21 exposure, but then there were 10 days with a normal 12:12 light-dark cycle. This sequence was repeated four times for a total of 64 days. The other group of rats (control: n = 8) remained on a

12:12 LD cycle for 64 days. The rats were weighed every three days at the same time of day (ZT2).

Table 5. T21 and blood extraction schedules.
Table appeared in (Deibel, Hong, et al., 2014).

Day		Day	
1	Lights off at 13:30	33	Lights off at 22:30
2	Lights off at 10:30	34	Lights off at 19:30
3	Lights off at 7:30	35	Lights off at 16:30
4	Lights off at 4:30	36	Lights off at 13:30
5	Lights off at 01:30	37	Lights off at 10:30
6	Lights off at 22:30	38	Lights off at 07:30
7[a]–16	Re-entrainment-lights off at 19:30	39[a]–48	Re-entrainment-lights off at 04:30
17	Lights off at 16:30	49	Lights off at 1:30
18	Lights off at 13:30	50	Lights off at 22:30
19	Lights off at 10:30	51	Lights off at 19:30
20	Lights off at 07:30	52	Lights off at 16:30
21	Lights off at 04:30	53	Lights off at 13:30
22	Lights off at 01:30	54	Lights off at 10:30
23[a]–32	Re-entrainment-lights off at 01:30	55[a]–64	Re-entrainment-lights off at 7:30

[a] Blood sampled at ZT01 on the first day of re-entrainment after each phase of photoperiod shifting.

Blood Extraction and Corticosterone Measurement

The rats were anesthetized with Isoflurane and the tail was wiped with alcohol. A butterfly style 26 guage needle and tubing was flushed with heparin and then blood was extracted from the tail vein and collected in 1ml syringe. For corticosterone analyses, blood samples were taken at ZT01 on the first day of entrainment following each of the four cycles of photoperiod shifting. The blood samples were centrifuged for 10 min at 5000 revolutions per minute, with a temperature of 4 ◦ C. The plasma was then extracted and frozen at −20 ◦ C. The samples were analyzed in duplicate with an Abcam corticosterone enzyme-linked immunosorbent assay (ELISA) kit. The samples were disrupted across three plates, in such a way that an entire phase for both groups was conducted in the same plate (except for one control sample and one photoperiod shifted sample in phase three).

Discriminative Fear Conditioning to Context

Training commenced four days after the cessation of the rotating T21 schedule (day 68). As described previously (Antoniadis & McDonald, 1999, 2000; Ferbinteanu, Holsinger, & McDonald, 1999; McDonald, Lo, King, Wasiak, & Hong, 2007), discriminative fear conditioning to context (DFCTC) evaluates the rat's ability to associate cues and locations with a mild foot-shock. The apparatus is comprised of two different chambers that were connected via an alley-way. The chambers differ in color (black vs. white), shape (triangle vs. square), and odor (Amyl-acetate vs. Vick's Vapor Rub). Amyl-acetate was used for the odor in the black triangle context, which measured 61 cm × 61 cm with a depth of 30 cm. Vick's Vapor Rub was used for the odor in the white square context, which measured 41 cm × 41 cm with a depth of 20 cm. On each training day, depending on the context in question, a drop of the odorant was applied to a cotton ball, which was then placed in an aerated plastic pill bottle that was attached to one of the walls in the chamber. For pre-exposure and the preference test, a gray alley (16.5 cm long × 11 cm wide × 11 cm high) provided access to both of the contexts. The entire apparatus was placed on Plexiglass table that was elevated 100 cm above the floor. Finally, a mirror (91 cm long × 61 cm wide) inclined on a 45◦ angle, provided visual access to the experimenter and allowed the trials to be recorded via a video camera that was placed 60 cm in front of the mirror.

During pre-exposure, each rat was placed in the alleyway and given 10 min to explore both chambers. An animal was considered to be in a chamber or alley when both its forepaws were in the area of question. For the training trials, the animal was confined to the desired context by inserting a door that blocked access to the alley. For each of the eight context exposures (four exposures in each context and one exposure per training day), the rats spent 5 min in the context and a 0.6 mA shock

was either given (paired context) or not given (unpaired context). For each group, exposure to contexts was counterbalanced so that half of the rats experienced the paired context on odd days (1, 3, 5, 7) and the unpaired context on even days (2, 4, 6, 8). The remaining rats experienced the unpaired con- text on odd days and the paired context on even days. The location of the paired context was also counterbalanced so that half of the rats received the shock in the triangle chamber and the remaining rats received the shock in the square chamber. The paired sessions also occurred in a different training room to decrease generalization. The shifted animals were tested at ZT 09 and the control animals were tested at ZT 05. The day after training, the test sessions began, in which the rats were placed in the paired or unpaired contexts for 5 min and the amount of time spent freezing was recorded. The rats received two exposures in each context and for each rat only one context exposure was conducted per day. The context exposure order was counterbalanced so that half of the rats were exposed to the paired context first, whereas the remaining rats were exposed to the unpaired context first. Normal rats typically spend more time freezing in the paired context during the testing sessions.

The day after the test trials, the rats were given a 10-min preference test that allowed them access to both of the contexts. The amount of time in each context was recorded. Normal rats usually spend more time in the unpaired context during the preference test.

Statistics

IBM SPSS Statistics 21 (IBM, Armonk, New York) was used to conduct the statistical analyses and GraphPad Prism software (GraphPad, La Jolla, CA) was

used to create the graphs. All analyses were two-tailed, with statistical significance set at p = 0.05.

In each of the four phases of fear conditioning a 2 (context) × 2 (group) mixed model ANOVA, with Context as the within factor and Group as the between factor, was used to assess freezing behavior or dwell time in the two different contexts. During each of the four phases of fear conditioning, planned Fisher's least significant difference comparisons were also conducted to evaluate performance between and within the groups in the paired and unpaired contexts.

For several reasons, a one-way ANOVA was used to analyze corticosterone expression in each of the four phases of photoperiod shifting. First, as previously mentioned, an entire phase for both groups was conducted on the same plate and multiple plates were ran. Second, the comparison between the groups at each level of phase was also planned a priori. To control for family-wise error that might have resulted from running multiple one-way ANOVAs, alpha was reduced to .005.

Results

Corticosterone

As can be seen in Figure 12, corticosterone only differed between the groups in phases three ($F(1, 14) = 12.199$, $p = .004$) and four ($F(1, 14) = 21.169$, $p < .001$). The average intra-assay coefficient of variation for all of the samples was 3.43%.

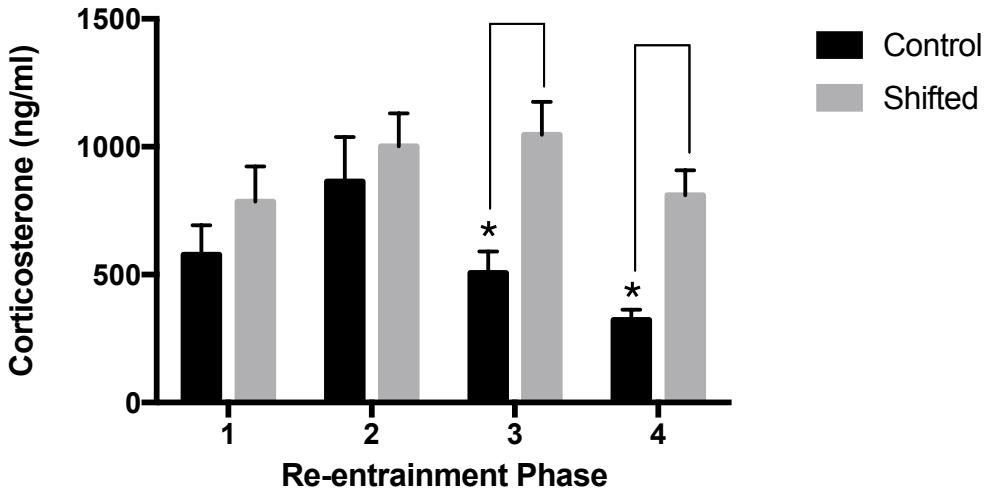

Figure 12. Corticosterone (ng/ml) values during chronic T21 exposure.
Figure reproduced from (Deibel, Hong, et al., 2014). Samples were taken on the first day of each 10 day re-entrainment period. So re-entrainment phase 1 refers to the day after six days of T21 exposure. The shifted (n = 8) rats had elevated corticosterone compared to the control animals (n = 8) during phase three (p = .004) and four (p = < .001). The * indicates a significant effect and error bars represent standard error of the mean.

Discriminative fear conditioning to context

Pre-exposure

During pre-exposure there was no effect of context, nor was there a context×group interaction. Similarly neither the control group ($t(14) = 0.175$, $p = .864$), nor the photoperiod shifted group ($t(14)=0.048$, $p=.962$) had a preference for either context.

Freezing

During the first fear-conditioning test, although the rats spent more time freezing in the context that was paired with the shock during training, there was not a main effect of Context. Nor, was there a context × group interaction. The average amount of freezing time in the paired and unpaired contexts did not differ between the groups. Also, neither the control group, nor the photoperiod shifted group froze more in one of the contexts.

During the second fear-conditioning test, as can be seen in Figure 13A, there was a main effect of context ($F(1, 14) = 6.817$, $p = .021$), however there was no context × group interaction.. Although the freezing time for the control group did not differ in the two contexts, the photoperiod shifted animals froze more in the paired context compared to the unpaired context ($t(14) = 2.350$, $p = .034$).

Preference test

As can be seen in Figure 13B, during the preference test, although the rats spent more time in the unpaired context, this was not significant. There also was no context × group interaction. Similarly neither the control group, nor the photoperiod shifted group demonstrated a preference for either context.

Weight Gain

As is shown in Figure 13C, the percent change in weight during fear conditioning indicated that the photoperiod shifted animals gained significantly more weight than the control animals ($t(9.99) = -2.64$, $p = .024$).

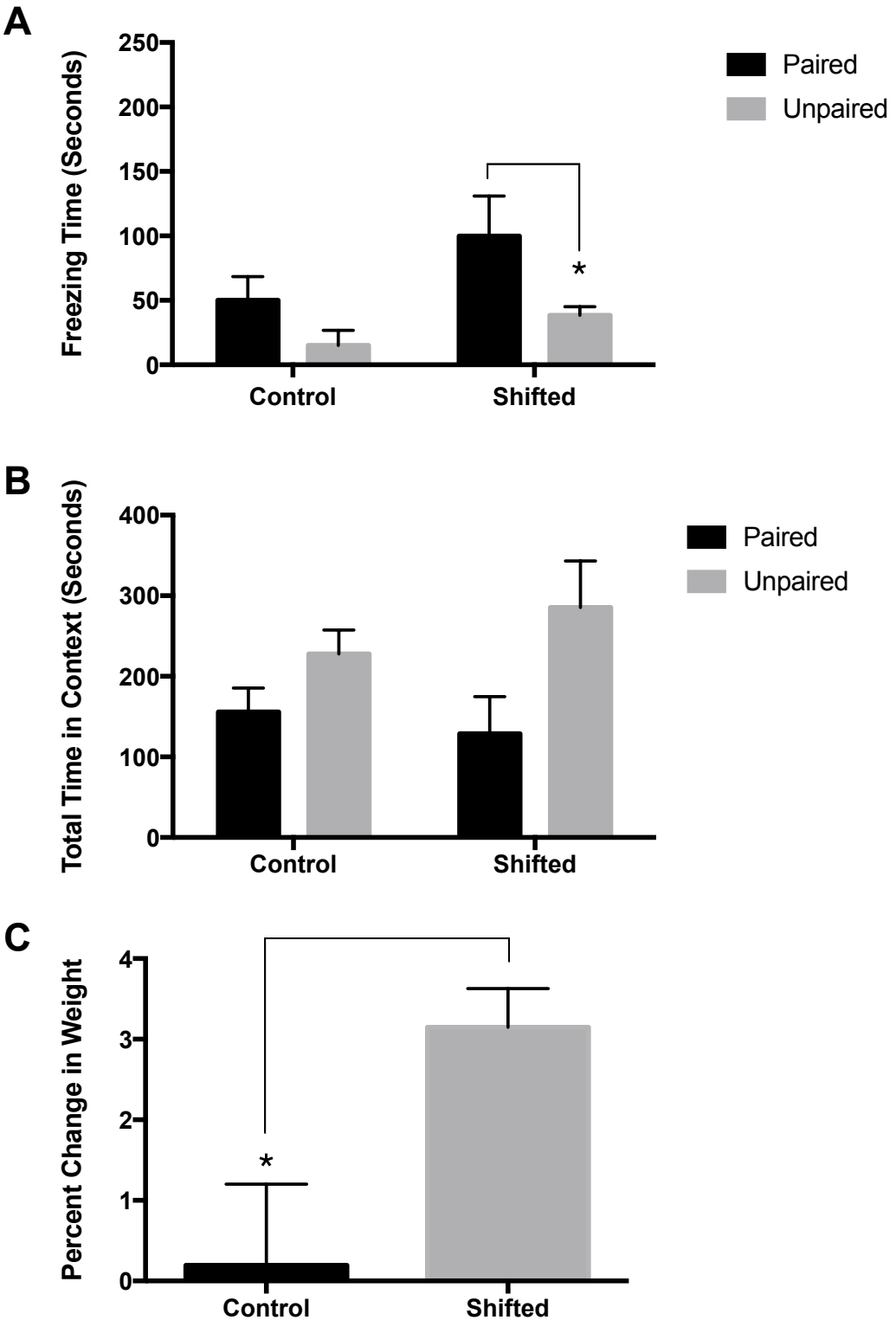

Figure 13. Discriminative fear conditioning to context data in female LE rats.

Figure and caption reproduced from (Deibel, Hong, et al., 2014). (A) Freezing time (seconds) during the second retention test in the paired and unpaired context. Only the photoperiod shifted animals spent significantly more time freezing in the context paired with the shock ($p = .034$). (B) Dwell time (seconds) in the paired and unpaired contexts during the preference test. Neither group displayed a preference for the unpaired context. (C) Average percent change in weight during fear conditioning. Without ingesting more food the photoperiod shifted rats gained significantly more weight than the control rats during the 15 days of fear conditioning ($p = .024$). Error bars represent standard error of the mean. The * indicates a significant effect and error bars represent standard error of the mean.

Discussion

One explanation for the cognitive deficits observed in animals with circadian misalignment is that manipulations of the light dark cycle are stressful. Corticosterone was only increased in the shifted animals towards the end of our 64day rotating T21 paradigm. This effect qualitatively appears to be a result of reduced corticosterone values for the control group during phases three and four, rather than the shifted group having increased corticosterone values during these phases. The data suggest that the control animals, eventually habituated to the stress elicited by blood sampling, while the shifted animals did not.

However, corticosterone was not elevated after six days of T21 exposure. This likely precludes the possibility that it is the sole culprit involved in the memory impairment induced by our paradigm. However, we were only assessing whether or not baseline corticosterone was elevated. While animal models of circadian rhythm disruption don't typically find elevated baseline corticosterone (Castanon-Cervantes et al., 2010; Kort & Weijma, 1982; Logan et al., 2012; Loh et al., 2010; Sei et al., 2003), it is extremely likely that as with activity and sleep, the corticosterone rhythm was misaligned with the T21 cycle. This fits with the finding that simulated shift work can phase advance the acrophase of the corticosterone rhythm (Barclay et al., 2012).

It is also possibly that the stress response in animals that were experiencing circadian misalignment would be potentiated during a stressful event. Several aspects of these data suggest that might be the case. First, the data qualitatively suggest that the control animals habituated to the stress of blood sampling in re-entrainment phases three and four, whereas the shifted animals did not. This is similar to the finding that hamsters exposed to normal lighting conditions appeared to habituate to blood sampling, whereas hamsters exposed to a jet-lag paradigm actually had a potentiated stress response (Gibson et al., 2010). Second, the fact that the shifted animals had enhanced fear conditioning memory also suggests that the stress response might have potentiated when exposed to the stresses of behavioural testing. In rodents, without causing increased baseline corticosterone, restraint stress (R. L. Wright, Lightner, Harman, Meijer, & Conrad, 2006), and photoperiod shifting (Loh et al., 2010) can cause elevated corticosterone during behavioral testing and concomitant memory impairments. Increased weight gain by the shifted animals during the 15 days of fear conditioning training and testing without ingesting more food also suggests that there could have been an altered stress response. Mild or moderate stressors paired with high caloric diets such as the one the rats had access to in the present study can induce weight gain (Michel, Levin, & Dunn-Meynell, 2003; Torres & Nowson, 2007).

One caveat of the present study was that ZT was used for blood sampling and memory testing. As a result of the T21 the shifted animals' circadian time was likely different from ZT. Therefore the relative sampling time and memory testing time for the shifted and control animals may not have been consistent. However, when taken together, the fact that there was increased corticosterone, weight gain during fear

conditioning, and a potentiated fear response suggest that these animals were experiencing stress.

In summary, while increased baseline corticosterone is likely not responsible for the memory deficit that occurs when learning during circadian misalignment, it is possible there is an increased stress response when exposed to a stressful event, such as behavioural testing. Whether or not a potentiated stress response impairs or facilitates memory depends on many factors. Unfortunately, the contention surrounding stress and circadian misalignment remains with a myriad of variables, such as strain, sex, diet, experience etc. interacting in each specific case.

[7]Experiment 4.3. Hippocampal Morphology

Introduction

Another possible explanation for the memory impairment induced by circadian perturbations is that circadian disruption is causing cell death in the hippocampus. Chronic jet-lag was associated with temporal lobe atrophy and spatial memory impairments in humans (Cho, 2001). Similar to impaired LTP, this would also be a downstream player that could be influenced by factors like stress, oxidative stress, and an altered epigenome (Deibel & McDonald, 2017; Deibel et al., 2015; Smarr et al., 2014; Zelinski et al., 2014). For example, chronically elevated cortisol elicited by chronic trans-meridian travel by airline staff was thought to be responsible for temporal lobe atrophy and the concomitant cognitive impairments (Cho, 2001). With this in mind, chronic exposure to a T20 schedule reduced dendritic length and complexity in the medial prefrontal cortex of mice (Karatsoreos et al.,

[7] This dataset appeared in Gidyk and colleagues (Gidyk et al., 2015).

2011). In the hippocampus, chronic dim light at night reduces dendritic density in the CA1 region of hamsters (Bedrosian, Galan, Vaughn, Weil, & Nelson, 2013). In terms of hippocampal integrity, we have only looked at cholinergic tone and obvious signs of structural damage in rats exposed to six days of a T21 cycle (Craig et al., 2009). While these animals did not show signs of hippocampal dysfunction in these measures (Craig et al., 2009), it remains to be seen if T21 exposure increases neuron degeneration or reduces hippocampal volume.

Another possibility is that circadian rhythm misalignment makes the hippocampus more susceptible to damaging agents (Craig et al., 2009; Deibel & McDonald, 2017; Deibel et al., 2015; Gidyk, Deibel, Hong, & McDonald, 2015; McDonald, 2002; McDonald, Craig, & Hong, 2010). Circadian rhythm misalignment has been linked as a possible risk factor for the cognitive decline that occurs both during natural aging and neurodegenerative diseases such as Alzheimer's disease (Coogan et al., 2013; Craig et al., 2009; Gidyk et al., 2015). Six days of T21 exposure did not exacerbate the effects of cholinergic depletion on the brain or behaviour (Craig et al., 2009). However, this does not discount the possibility that circadian rhythm misalignment could make the brain susceptible to other damaging agents, as the co-factor theory of Alzheimer's disease suggests that it pathology depends on the specific risk factor combination (Gidyk et al., 2015; McDonald et al., 2010).

The present study investigated the effects of circadian rhythm misalignment and mini-strokes in isolation and in tandem on hippocampal integrity.

Methods

Animals

Thirty-six three-month old male LE rats obtained from Charles River Colony (Laval, Quebec) were used. The rats were pair-housed in clear Plexiglass cages with beta-cob bedding. Wheels were not used so that the effects of the T21 cycle and mini-strokes were not mitigated in any way. During a 3-week acclimation period, water and Purina Rat chow were available ad libitum. The housing rooms were maintained on a 12:12 light-dark cycle, with an ambient temperature and humidity of 21◦C and 35%, respectively. The rats were divided into four groups (described below) of nine animals each.

Experimental Groups

sham

These animals were anesthetized with inhaled isoflurane and then received incisions and sutures. Post-surgery the animals were given buprenorphine and Metacam

shift

These animals received six days of T21 exposure as described in the previous chapters (T21 with 57% light was used).

stroke

As described previously (McDonald, Craig, & Hong, 2008), animals received mini-strokes in the hippocampus. The animals were anesthetized with inhaled isoflurane, incised, burr holes were drilled, and then 0.5µl of 6 pmol endothelin-1 (ET-1; Sigman) was injected into the hippocampus via 30 gauge cannulae into two sites bilaterally (AP -4.1, -5.3; ML ± 3.0, ± 5.2; DV -3.7, -7).

shift/stroke

These animals received six days of T21 exposure and then ET-1 infusions.

Histology

Twenty-two days after these manipulations, the rats were perfused transcardially with phosphate buffer solution and 4% buffered paraformaldehyde. Sliced sections (40 μm) were either stained with Cresyl violet for volume analysis, or 0.0004% Fluorojade-B (FJ; Millipore) for identification of damaged neurons (McDonald et al., 2008). Whole hippocampal volumes were measured using the Cavalieri method via StereoInvestigator (Microbrightfield, Williston, VT). Every sixth section was counted with an average of 14 sections assessed for each animal. If sections were missing from a series (on average 1 section per animal), stereo investigators correction for missing sections was used. For the FJ analyses, ImageJ was used to create a threshold that selected the FJ fluorescent signal in the whole hippocampus and then the area of this selected region was quantified in each of three representative sections per animal (−2.56, −3.8, and −4.8mm from bregma respectively). All histology was conducted blind to treatment group.

Statistics

Some animals were removed from the analyses because of poor tissue quality (see Figure 14 caption). One-way ANOVAs were used to determine if volumes or amount of FJ staining differed among the groups. For each analysis a planned contrast comparing the shift-stroke to the other three groups was conducted. A natural log transformation was performed for the FJ data as Levene's test was violated.

Results

As depicted in Figure 14, hippocampal volume (Figure 14 A & C; $F(3, 27) = 6.058$, $p = 0.003$) and the amount of FJ staining (Figure 14 B & D; $F(3, 22) = 8.918$, $p < 0.001$) differed among the groups. The shift/stroke group had decreased hippocampal volume ($p = .001$) and increased FJ staining ($p = .001$) when compared to the other groups.

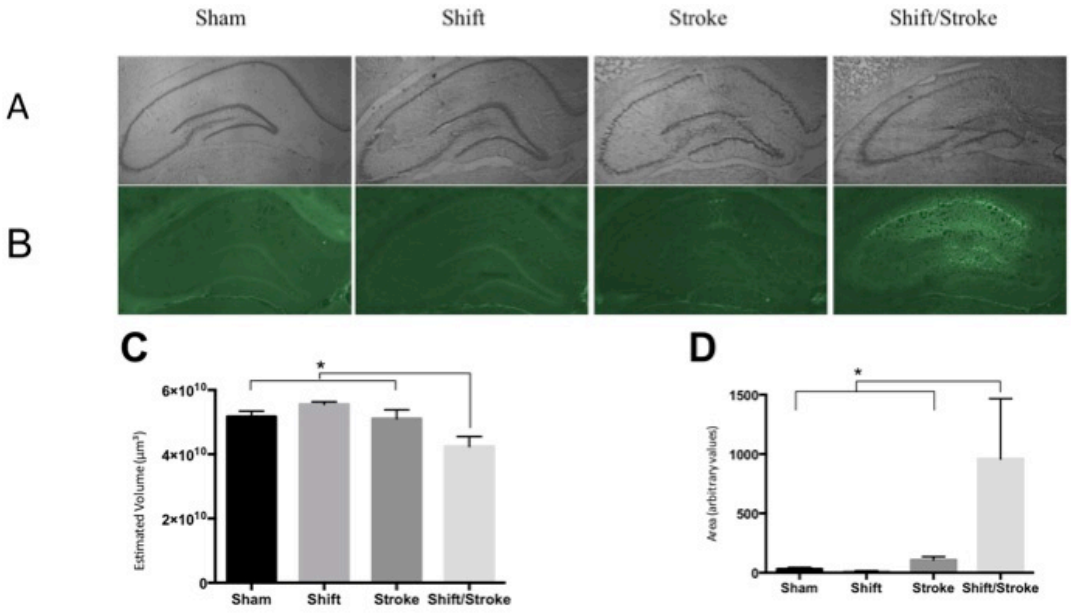

Figure 14. Hippocampal Integrity in male LE rats with T21 exposure.
Figure from Gidyk and Colleagues (Gidyk et al., 2015) (A) Representative images used for the volume analyses: sham ($n = 8$); shift ($n = 9$); stroke ($n = 8$); shift/stroke ($n = 6$). (B) Representative images used for the FJ analyses: sham ($n = 5$); shift ($n = 7$); stroke ($n = 8$); shift/stroke ($n = 6$). (C) The shift/stroke had smaller volumes when compared to the other groups ($p = 0.001$). (D) The shift/stroke group had a greater area of damaged neurons (FJ labeled cells) when compared to the other groups ($p = 0.001$). Error bars represent standard error of the mean and * represents a significant effect less than $p = .05$.

Discussion

These data suggest that six days of T21 exposure does not cause cell death in the hippocampus as evidenced by an increased number of degenerating neurons or reduced hippocampal volume. However, hippocampal mini-strokes immediately after

this T21 exposure did exacerbate both of these measures. These data suggest that while T21 exposure is not itself a damaging factor, it makes the brain more susceptible to other damaging factors. In terms of the co-factor theory of Alzheimer's disease it would be a passive rather than an active factor (McDonald, 2002). This could possible explain why the T21 exposure paired with cholinergic depletions did not lead to exacerbated hippocampal pathology because cholinergic depletions are also thought of as passive factor (Deibel et al., 2016; McDonald, 2002). Thus in accordance with the co-factor theory it fits that T21 exposure would have more of an effect on pathology when paired with an active factor like stroke than a passive factor such as cholinergic depletions.

These data indicate that the memory impairment induced by circadian misalignment is not due to hippocampal cell death. However, when paired with other brain insults it can have a compound effect that results in hippocampal cell death and atrophy. Thus, in humans it doesn't preclude the possibility that circadian rhythm disruption is contributing to hippocampal atrophy and the concomitant memory impairment when combined with other risk factors such as obesity, stroke, aging, stress, etc.

General Discussion

While many questions still remain regarding how circadian misalignment/disruption can induce memory impairments, the data presented in this chapter do rule out several possibilities. Notably that six days of the T21 cycle does not cause sleep deprivation, nor does it increase baseline corticosterone. However, this does not discount the fact that sleep and corticosterone rhythms that are out of phase with the light dark cycle could be contributing to hippocampal

dysfunction. Another conclusion from this chapter, is that circadian misalignment on its own does not cause cell death or hippocampal atrophy. Taken together, these data support the notion that the memory impairment is a result of altered hippocampal plasticity. As presented in Deibel and colleagues (Deibel & McDonald, 2017; Deibel et al., 2015), circadian rhythm disruption might affect hippocampal plasticity crucial for memory consolidation.

Hippocampal clock genes are likely involved in the influence of circadian rhythms on memory. As mentioned previously clock genes and molecules involved in plasticity such as such as cAMP, Ca2 stimulated adenylyl cyclases, Ras, and MAPK, display circadian oscillations in the hippocampus (Eckel-Mahan et al., 2008; Feillet et al., 2008; Jilg et al., 2010; Phan et al., 2011; Rawashdeh et al., 2014; Wakamatsu et al., 2001; Wang et al., 2009). Interestingly a recent, study extended these findings to other molecules involved in memory that oscillate in the hippocampus: *Arc, Bdnf*, *CBP*, and *P300* (Peixoto et al., 2015).

Clock genes have been directly linked to the molecules involved in the hippocampal signal transduction pathway as decreased amounts, and abolition of circadian oscillations of phosphorylated CREB are found in *Per2* (Wang et al., 2009) and *Per1* (Rawashdeh et al., 2014) mutant mice, respectively. Similarly, oscillations of MAPK and cAMP are abolished in *Bmal1* mutant mice (Wardlaw et al., 2014). Not surprisingly, mutant mice with deletions of various clock genes such as *Per1-/-* (Rawashdeh et al., 2014), *Per2-/-* (Wang et al., 2009), and *Bmal1-/-* (Wardlaw et al., 2014) have impaired LTP. Mice with mutations of the core clock genes are also impaired in a variety of learning and memory tasks thought to rely on the hippocampus (*Per1−/−*: (Jilg et al., 2010; Rawashdeh et al., 2014); *Cry1−/−*

Cry2−/−: (Van der Zee et al., 2008a); *Per2−/−:* (Wang et al., 2009); *Bmal1-/-*: (Kondratova, Dubrovsky, Antoch, & Kondratov, 2010a; Wardlaw et al., 2014); *Clk*: (Kondratova et al., 2010a).

There is a central caveat in virtually of all of the studies mentioned above linking clock genes to these hippocampal signal transduction pathways linked to plasticity. As transgenic methods are used to alter circadian rhythms, it is not known if the effects on hippocampal plasticity molecules are a result of global circadian rhythm arrhythmicity at the level of the SCN and/or altered hippocampal oscillations. Clock gene and plasticity molecule oscillations in the hippocampus are for the most part dependent on an intact SCN (Lamont, Robinson, Stewart, & Amir, 2005; Phan et al., 2011). With this is mind; one would assume that SCN lesions impair hippocampal dependent memory. Indeed SCN lesions impair retention of contextual fear conditioning and MWT in mice (Phan et al., 2011). However, other studies in rats and hamsters fail to find an effect of SCN lesions in various types of memory tasks (Fernandez et al., 2014; Mistlberger, De Groot, Bossert, & Marchant, 1996b; Stephan & Kovacevic, 1978). These data suggests that the hippocampus might be able to maintain some degree of rhythmicity despite the abolition of SCN afferents. This claim is supported by the finding that *Per2* chronically maintains oscillations in tissue culture (Wang et al., 2009).

One possibility is that circadian misalignment is affecting SCN oscillations and then this is having downstream effects. The SCN is often resistant to perturbations though, with manipulations that affect molecular rhythms in peripheral oscillators having no effect on the SCN. For example, in an interesting recent paper that described experiments that induced circadian rhythm

misalignment in mice by restricted feeding during the daytime, the authors observed memory impairments, a major phase shift in hippocampal but not SCN clock gene oscillations, impaired LTP, and reductions in the total amount of CREB (Loh et al., 2015). Amazingly activity and sleep rhythms misaligned with the light dark cycle but SCN oscillations were normal (Loh et al., 2015). This fits with the finding that diurnal Nile grass rats with different chronotypes had indistinguishable SCN oscillations, despite molecular oscillations in peripheral oscillators and activity in these animals with early and late chronotypes being in antiphase (Ramanathan, Stowie, Smale, & Nunez, 2008). It is likely that in our T21 paradigm the SCN is unaffected. With this in mind, even though in our paradigm activity and sleep are misaligned during T21 exposure it is likely that SCN oscillations are unaffected; this is supported by the findings from Chapter 1, demonstrating that period length does not change during T21 exposure.

Rather it appears that the hippocampal oscillator is affected. This could be evidenced by the abolishment of rhythmic oscillations. More likely the phase of the rhythms are altered. This is what occurred with circadian misalignment that was induced by restricted feeding. The phase of rhythms were drastically changed in the hippocampus and liver, while it did not change in the SCN (Loh et al., 2015). This suggests that the problem might lie in the phase relationship between the hippocampus and the SCN.

Some very surprising recent results speak to this theory. First, Ralph and colleagues (Ralph et al., 2013) recently suggested that there might be a context-entrainable oscillator. Contextual memory that was dependent on a time of day drifted to a new time of day without input (training) to the theorized contextual

oscillator. Remarkably this drift was dependent on the SCN; meaning that the temporally gated memory was maintained over time only when the SCN was lesioned (Ralph et al., 2013). This finding fits with an idea proposed by Eckel-Mahan and Storm (Eckel-Mahan & Storm, 2009) that the hippocampal clock might be entrained by learning. In the case of the Ralph data, my interpretation is that as days progressed without training, the relationship between the SCN and contextual oscillator were no longer synchronized as they were during encoding. Without a zeitgeber for the contextual oscillator, the SCN attempted to entrain it, which gradually pushed the contextual oscillator so that the memory was now at a new time.

Another fascinating finding also speaks to the fact that the issue lies with the crosstalk between oscillators. Fernandez and colleagues (Fernandez et al., 2014), have recently investigated the role of the SCN in circadian rhythm induced memory impairments. In their hamster paradigm a phase advance, followed by a phase delay the next night produces circadian arrhythmicity and impairments in novel object recognition (Fernandez et al., 2014; Ruby et al., 2008). While SCN lesions did not impair novel object recognition, remarkably SCN lesions rescued the memory impairment elicited by the phase shifts (Fernandez et al., 2014). In accordance with this group's previous finding that a y-aminobutyric acid type A (GABA) antagonist also rescues the same memory impairment, they are suggesting that the SCN has an inhibitory effect on memory during certain phases of the circadian cycle and this inhibitory effect is constitutive under circadian rhythm disruption (Fernandez et al., 2014; Ruby et al., 2008).

I feel this interpretation is unlikely as there are a myriad of possible circadian outputs in addition to GABA that could be involved so the influence of circadian rhythms on memory is probably not exclusively inhibitory. In conjunction with the findings from Ralph and colleagues (Ralph et al., 2013), an alternative explanation, is that the impairment is a result of misalignment between the SCN and a contextual oscillator that disappears when SCN inputs are abolished. Under circadian misalignment their memory impairment could be an example of the SCN trying to entrain the "memory oscillator". Without the SCN the integrity of the phase of the "memory oscillator" is unaffected. The Ralph and Fernandez data hint that maybe the "memory oscillator" is semi-autonomous. This is supported by long-term persistence of *Per2* oscillations in tissue cultures (Wang et al., 2009). Amazingly, a phase change in hippocampal oscillations like those induced by circadian misalignment, can cause the *Per2* rhythms in culture to decay faster (Loh et al., 2015).

Although there is no evidence to directly support this claim, it is expected that our paradigm is going to disrupt the synchrony or phase of the hippocampus clock with that of the SCN. It remains to be seen how this desynchrony between the SCN and the hippocampal clock specifically influences plasticity. It is likely that mistimed excretions of melatonin, corticosterone, and GABA elicited by circadian misalignment are responsible for the deysnchrony between the SCN and hippocampus. This is bolstered by the fact that the SCN influences excretion of these molecules and the hippocampus is rife with receptors for these molecules (Antle & Silver, 2005; Deibel & McDonald, 2017; Zelinski et al., 2014).

In summary, according to my analysis it seems that circadian misalignment among rhythms or with rhythms and zeitgebers is more plausible and potentially more detrimental than the abolition of rhythmicity at the level of the master or slave oscillator.

Chapter 5

Final Thoughts

Conclusions

Four main conclusions can be gleaned from this thesis: 1) Six days of T21 light-dark cycle causes circadian misalignment in activity and sleep rhythms. 2) Effects of six days of T21 light-dark cycle vary depending on the sex and strain of rat. 3) Six days of T21 exposure has long lasting effects on circadian entrainment and hippocampal functioning. 4) Hippocampal dysfunction elicited by six days of T21 exposure is not solely due to sleep deprivation, increased baseline stress response, or hippocampal cell death.

Implications

There are several notary findings from this thesis that might change the way the interaction between circadian rhythms and memory are thought about.

How Much is Too Much?

We and to our knowledge others did not anticipate that relatively brief periods of circadian misalignment might have subtle long-term deleterious effects on circadian entrainment and hippocampal-dependent memory. As previously mentioned, the effects of chronic shiftwork on memory can last for years (Rouch et al., 2005), so it is not inconceivable that a T21 cycle can have long lasting impacts on rhythms and memory in rats. However, we thought that the amount of exposure time to circadian misalignment for these problems to develop was much greater than the six days used here (Zelinski et al., 2014). This leads us perhaps to the most crucial question in this story, which is how much exposure to circadian rhythm

disruption is too much? This answer ultimately involves an infinite number of variables, however, we hope that the finding observed here will serve as a preliminary finding that will ignite investigations into lengths of exposure and how that affects length of symptom presentation.

Can Misaligned Sleep Affect Memory Consolidation

Another finding from this thesis that we hope will inspire future discovery is the notion that aspects of SWS that are involved in memory consolidation might be particularly susceptible to circadian misalignment. As touched on earlier, we have always posited that the memory impairment that occurs during normal and abnormal aging is partially due to deteriorating circadian rhythmicity (Antoniadis, Ko, Ralph, & McDonald, 2000; Deibel & McDonald, 2017; Deibel et al., 2015). As SWS sleep and some of its components such as K-complexes and spindles are reduced in aging (Crowley et al., 2002; Diekelmann & Born, 2010), we thought this warranted further investigation in experimentally induced circadian rhythm disruption. For the first time to our knowledge, we demonstrated that K-complexes and sleep spindles not only show signs of circadian misalignment, but also that this misalignment is slightly more severe and long lasting than in most of the other aspects of sleep.

While there was not a reduction in the amount of these processes, the temporal distribution was altered. In other words these processes were occurring at non-optimal times. The interval between learning and sleep can affect consolidation of that information (Diekelmann & Born, 2010). In our animals, it is possible that the interval between learning and SWS sleep is different from in entrained conditions as less sleep is occurring when in typically should (light phase of cycle). It

is possible that this could effect memory consolidation even in the absence of sleep deprivation. Along the line of altered temporal dynamics, it is possible that the synchrony between the hippocampus and cortex is altered. The finding that sleep spindles were particularly affected by the T21 cycle suggests that this hypothesis deserves further exploration. In summary, sleep that is misaligned with the environment and subsequently learning could affect consolidation of that information.

Animal Models are Not Perfect

We as animal researchers, and as I did earlier in thesis, sometimes jump at the chance to criticize epidemiological studies for self-selection bias and the like. Obviously, we can often limit and control extraneous variables to a much higher degree in animal research. We can also use manipulations and assessments that are not possible in humans. However, just like anything else, animal research has some crucial flaws.

Ultimately a rat is not a rat. I came to this realization the hard way early on during my scientific career. My first assignment during my Masters program was to assist with an ongoing learning and memory project in which the location of food reward varied depending on the time of day (C M Thorpe et al., 2012). This started out as a simple replication with the goal of adding an additional measurement, however after several cohorts of animals and six months later we could not replicate the behaviour. A further look at the methods enlightened us to the fact that the previous study had used Sprague Dawley rats and we had been using Long Evans rats. We had overlooked this detail because we thought it impossible that this was the missing link, as Long Evans rats are superior spatial learners. We got Sprague

Dawley rats and much to our amazement they acquired the task just fine (C M Thorpe et al., 2012) and as reported previously.

Unfortunately, my experience is much more pervasive than we would all like to hope. In our lab alone there several key examples. Again with timing, only some rat strains display the time-stamping of memory phenomenon (Cain, Ko, et al., 2004; McDonald et al., 2002). A recent PhD graduate in our lab Robin J. Keeley, specially investigated the effects of adolescent Δ9-tetrahydrocannabinol (THC) exposure on learning and memory, with rat strain and sex as potential mediators for these effects. She found that not only were the effects of THC on the brain and behaviour dependent on rat strain and sex, behaviour and brain morphology varied across rat strains in control animals (Keeley, 2014). Finally, perhaps even more amazing/disconcerting is that a LE rat from Charles River behaved differently than a LE rat bred in our facility (Keeley, 2014).

The point of this polemic is not to terrify, nor lambast animal research, but rather to encourage slight trepidation when interpreting the overarching implications from any animal research. Chapters 2 and 3 from this thesis, along with the finding that the effects of circadian misalignment are worse for male rats (Zelinski et al., 2014, 2013), and the strain differences regarding timing in rats (Cain, Ko, et al., 2004; McDonald et al., 2002; C M Thorpe et al., 2012) all suggest that perhaps this issue might be very pertinent for circadian rhythm research. We encourage the field to embrace this issue with the hope that delving into why different rat strains or sexes are affected differently might reveal ways to protect more vulnerable populations like the aged. For example, somewhat unexpectedly, analysis of the genetics that contributes to the variation in free running period

among inbred mouse strain (Schwartz, 1990), or the discovery of the tau hamster were paramount to our understating of circadian rhythm generation (Ralph & Menaker, 1988). It is my hope that the field does not lose sight of the importance of this type of basic research.

Future Directions

To Be or Not to Be Entrained, That is the Question

When I first started learning about circadian rhythm disruption in the context of memory, I assumed that there was a positive correlation with circadian rhythm changes and cognition. Recently, with more knowledge and the integration of some really interesting recent studies into my schema, I realize that the story is more complex. As talked about in the general discussion of Chapter 4, the synchrony of the "memory clock" and the SCN is key rather than the synchrony of the SCN with the environment. For example, in Ralph and colleagues time-stamping drift paper, arrhythmicity at the level of the SCN actually prevented the time-of-day memory from extinguishing (Ralph et al., 2013). Amazingly in this case circadian rhythm disruption as evidenced by abolition of the activity rhythm via SCN lesions actually facilitates the retention of a time-of-day memory. This suggests that it is not circadian rhythm disruption at the level of the SCN that is affecting memory but rather circadian rhythm disruption between oscillators. Findings that SCN lesions do not impair memory that involves time-of-day support this notion (Cain & Ralph, 2009; Fernandez et al., 2014; Ko et al., 2003; Mistlberger et al., 1996b). On the flip side hippocampal lesions can affect free-running activity rhythms (Mistlberger & Mumby, 1992). But hippocampal lesions do not impair behavioral anticipation of a single meal that occurs at the same time each day (Mistlberger & Mumby, 1992), nor does it impair circadian time-place-learning (Cole et al., 2016). While SCN lesions in

mice impaired MWT performance (Phan et al., 2011), hippocampal memory was intact in SCN ablated hamsters (Fernandez et al., 2014). I predict that hippocampal dysfunction would take time to develop and would not be initially seen until there was proper time for the "memory clock" to unravel. To our knowledge hippocampal dependent memory in SCN ablated rats has not been investigated, nor has this incubation theory in any species.

Fernandez and colleagues (Fernandez et al., 2014), take this story a step further. They have demonstrated in the past that a phase advance one day followed by a phase delay the next day abolishes circadian rhythmicity indefinitely and induces hippocampal dependent memory impairments that appear to be due to elevated GABA (Fernandez et al., 2014; Ruby et al., 2008). These hamsters have a completely abolished activity rhythm so it is assumed that there is dysfunction at the level of the SCN. Remarkably lesioning the SCN in these hamsters rescues hippocampal dependent memory. They interpret the following: "The present study demonstrates that chronic arrhythmia per se does not cause memory impairments in animals. Rather, an arrhythmic SCN is necessary to realize these deficits …". The mechanism for this effect was hypothesized to be elevated GABAergic tone in the hippocampus due to SCN dysrhythmia. If this interpretation were true then they should find that SCN lesions produce impairments in hippocampal dependent memory tasks. It is possible however that the arrhythmia produced by SCN lesions differs from that induced by phase shifts. The authors did not report or mention whether activity rhythms were different in SCN lesioned compared to phase shifted animals.

I think that the "memory clock" is in the hippocampus and is semi/fully autonomous. With SCN lesions hippocampal dependent memory is fine because the clock continues to tick and be entrained with it's own zeitgeber, which as first suggested by Eckel-Mahan (Eckel-Mahan & Storm, 2009) and shown by Ralph (Ralph et al., 2013) is likely behavioural training. The SCN can entrain this clock, but is likely a weak zeitgeber that takes over in the absence of the preferred zeitgeber: training (Ralph et al., 2013). It remains to be seen if this hypothesized clock can continue to tick chronically without SCN input.

Another interesting point regarding Ruby's paradigm is that they are getting memory impairments with the abolishment of the activity rhythm. We get memory consolidation failures despite the activity rhythm continuing to be expressed in a circadian manner. This again suggests that the memory impairment is likely due to a misalignment of the "memory clock". This misalignment could take several forms: misalignment from the SCN and or misalignment from Zeitgebers. An interesting possibility is that under circadian misalignment the SCN tries very hard to entrain the "memory clock" and this pull from the SCN is what causes the memory impairment. With the Ruby and Colleagues (Ruby et al., 2008) data in mind, maybe a GABA antagonist rescued memory because the SCN was trying to entrain the hippocampus. Thus, maybe the GABA antagonist releases the hold of the SCN on the hippocampus and memory is fine again. Melatonin is another likely candidate for the SCN to try to entrain the "memory clock". A mistimed melatonin rhythm has been suggested to elicit hippocampal dysfunction (Deibel & McDonald, 2017; Deibel et al., 2015; Jenwitheesuk, Nopparat, Mukda, Wongchitrat, & Govitrapong, 2014).

Clock gene analyses in the SCN and hippocampus under conditions of circadian misalignment are needed to investigate this theory. While analysis of gene activity in post-mortem tissue would shed light on this matter, *in vivo* imaging of clock genes in these regions would be ideal because it would be easier to investigate how these clocks interacted with zeitgebers over time. Another interesting idea is that it if the issue is desynchrony between the "memory clock" and the SCN or environment, possibly manipulating zetigebers like training or food could bring these clocks back together.

In summary, in terms of memory, misalignment between oscillators might be more important than abolishment of central rhythmicity. Therefore, in reference to the title for this section, memory should be fine if an animal is able to entrain to a T cycle. Seems like an obvious answer, however, I first thought that more changes in the activity rhythm under a T cycle would have more of an effect on the hippocampus.

The Future is Epigenetic

A common trait inherent to both circadian rhythms and memory is that these are incredibly plastic processes that require frequent input from the environment. Epigenetics provides an avenue for the environment to influence the brain and behaviour by manipulating gene expression without changing the genome. I will briefly talk about how epigenetics might be involved in the memory impairment elicited by circadian rhythm disruption. This section will summarize the ideas and material presented in (Deibel & McDonald, 2017; Deibel et al., 2015).

Epigenetic modifications influence gene expression by affecting chromatin state by changing the arrangement of histones, or by blocking DNA in promoter

regions (Goldberg, Allis, & Bernstein, 2007). Epigenetic modifications, are generally tissue specific and can be transient, long lasting, and even heritable (Feil & Fraga, 2012; Gallou-kabani & Junien, 2007; Goldberg et al., 2007; Ledón-Rettig, Richards, & Martin, 2012).

Epigenetic modifications are involved in and can be necessary for both circadian rhythms and memory (Masri & Sassone-Corsi, 2010; Penner, Roth, Barnes, & Sweatt, 2010; Rudenko & Tsai, 2014). Circadian entrainment and learning/memory require epigenetic modifications in the SCN and hippocampus respectively (Azzi et al., 2014; Chwang, O'Riordan, Levenson, & Sweatt, 2006; Dagnas & Mons, 2013; Levenson et al., 2004; Miller & Sweatt, 2007; Rudenko & Tsai, 2014). Interestingly, epigenetic histone modifiers oscillate in the SCN (Qureshi & Mehler, 2014) and hippocampus (Rawashdeh et al., 2014).

"Our group has recently assessed the effects of our circadian rhythm disruption paradigms on large-scale miRNA and mRNA expression in mammary gland tissue from female Sprague Dawley rats. Both acute and chronic paradigms induced changes in many miRNAs involved in cancer and circadian rhythms that were dependent on the zeitgeber (ZT) time of tissue extraction (Kochan et al., 2015). Remarkably, there were still changes in miRNA two weeks after the cessation of the chronic paradigm, an effect that was absent in the acutely shifted animals (Kochan et al., 2015). In terms of mRNA expression, genes involved in DNA repair were down regulated two weeks after the chronic paradigm in the animals that were sacrificed at ZT19 (Kochan et al., 2016). As a whole these data suggest that environmental circadian misalignment similar to that elicited by shift work induces immediate and/or long lasting changes in miRNA and mRNA expression in the mammary gland

tissue of rats. To our knowledge these are the first studies to conduct large-scale miRNA and mRNA assessments in mammary gland tissue of rats exposed to circadian rhythm disruption." (Deibel & McDonald, 2017).

Sirtuin1 (SIRT1), a metabolic sensor involved in the regulation of cellular energy, is thought to be key player as it is involved in the core circadian clock, and is oscillatory in both the SCN and hippocampus (Asher et al., 2008; H. C. Chang & Guarente, 2013; Nakahata et al., 2008; Rawashdeh et al., 2014). Mutant mice with deletions of SIRT1 have impaired circadian rhythms, memory, and LTP (H. C. Chang & Guarente, 2013; Gao et al., 2010; Michán et al., 2010). A recent study implicated that SIRT1 might be a link between rhythms and memory as learning induced epigenetic changes and hippocampal oscillations of SIRT1 and other histone modifiers are abolished in *Per1* mutant mice (Rawashdeh et al., 2014).

The effects of circadian misalignment on SIRT1 expression in the SCN and hippocampus are unknown. Inadvertently, several studies have spoken to this by demonstrating that hippocampal SIRT1 and concomitant memory impairments are elicited by sleep deprivation (H. M. Chang, Wu, & Lan, 2009) and high fat diet (Heyward et al., 2012), both of which coincidently can disrupt circadian rhythms (Antle & Mistlberger, 2000; Deboer, Détári, & Meijer, 2007; Kohsaka et al., 2007). Findings that SIRT1 decreases with aging and can partially rescue rhythms when artificially increased further implicate it as a key player in this story (H. C. Chang & Guarente, 2013; Quintas, de Solis, Díez, Carrascosa, & Bogónez, 2012). Perhaps not coincidently, SIRT1 also has neuroprotective properties involved in the defense of AD (Brunet & Berger, 2014; Jenwitheesuk et al., 2014; Kim et al., 2007; Qin et al.,

2008). Possibly aging/circadian rhythm disruption reduces hippocampal SIRT1 expression making it more susceptible to damaging agents like stroke.

Epigenetics is a particularly attractive mechanism for the memory impairment induced by circadian rhythm disruption because these affects can often be reversed by environmental manipulations. Calorie restriction promotes healthy and SIRT1 is thought to be involved as it is increased in the SCN and hippocampus of aged calorie restricted rodents (Chen et al., 2008; Quintas et al., 2012; Satoh et al., 2013). Some have suggested that the improved longevity elicited by calorie restriction is partially a result of improved circadian rhythmicity (Froy & Miskin, 2007, 2010; Gutman, Genzer, Chapnik, Miskin, & Froy, 2011). Interestingly, melatonin which is attenuated by circadian misalignment, can partially rescue hippocampal SIRT1 expression and SCN clock gene expression when applied exogenously (H. M. Chang et al., 2009; Korkmaz, Sanchez-Barcelo, Tan, & Reiter, 2009; Mattam & Jagota, 2014; Schwimmer et al., 2014). It would be particularly interesting to see how calorie restriction and exogenous melatonin would influence memory in circadian misaligned animals.

Summary

In closing, circadian misalignment between clocks or a specific clock and it's most salient zeitgeber is likely causing the memory impairment elicited by circadian rhythm disruption. Studies investigating the synchrony among clocks rather than the master clock and the environment are needed. We hope others delve into this story with the hope that the harmful impacts of circadian rhythm disruption on memory can be mitigated. Epigenetics might serve, as an avenue to entrain clocks with each other, which we hypothesize will restore memory.

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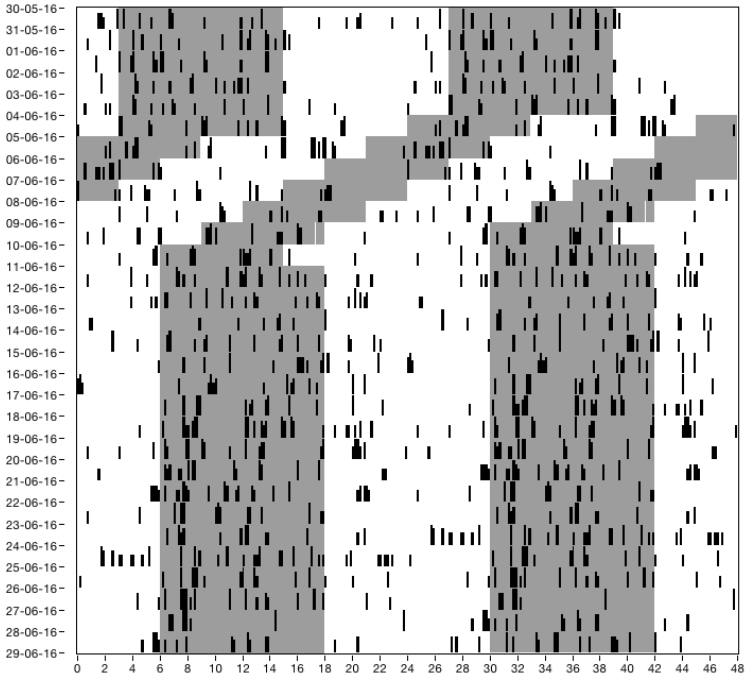
Appendices

Chapter 2

FBN male rats



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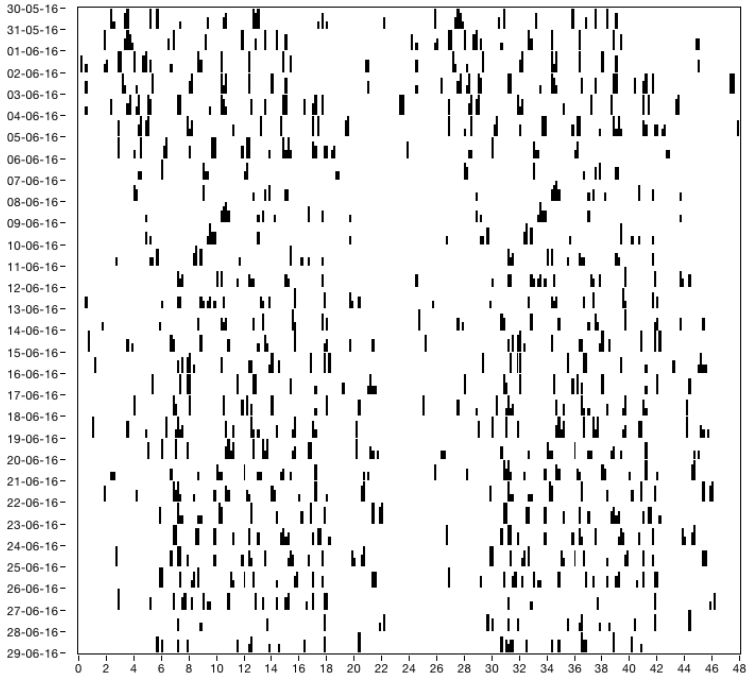

Rat2

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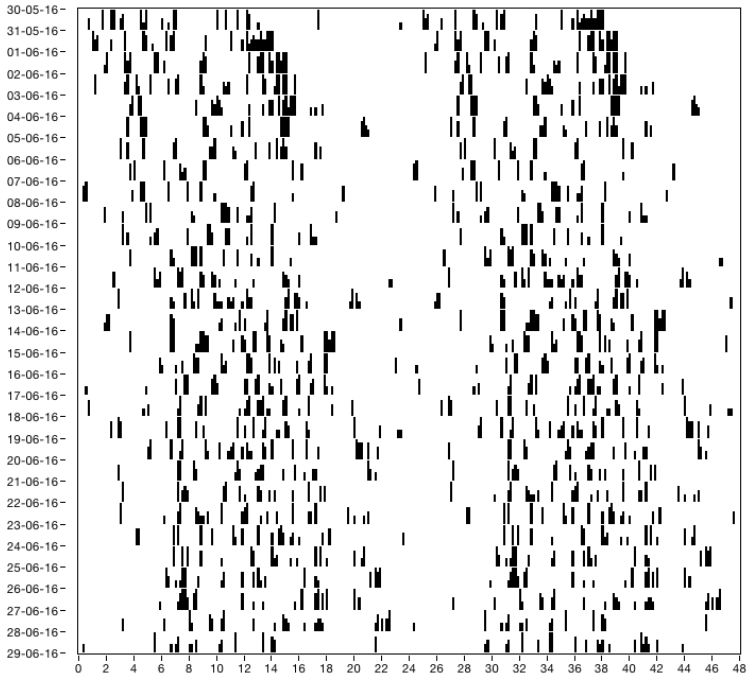
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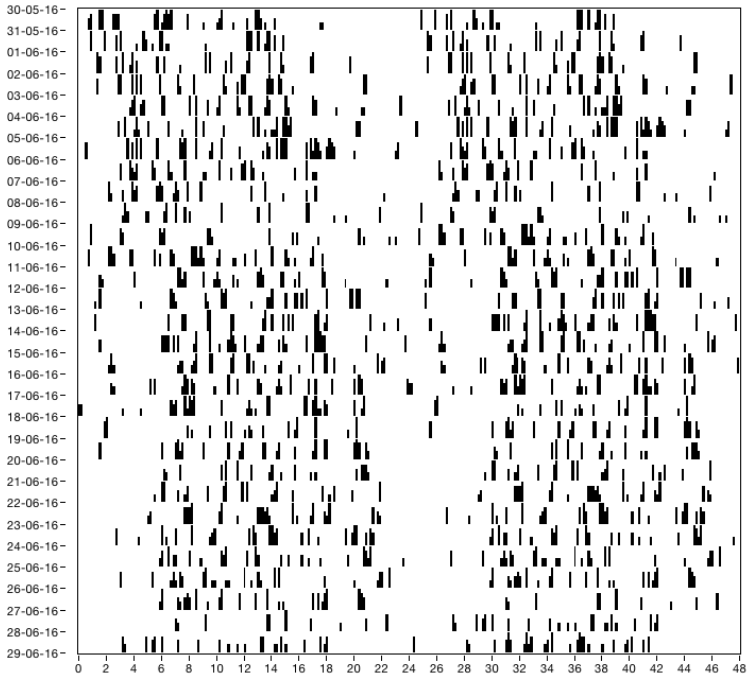
Rat3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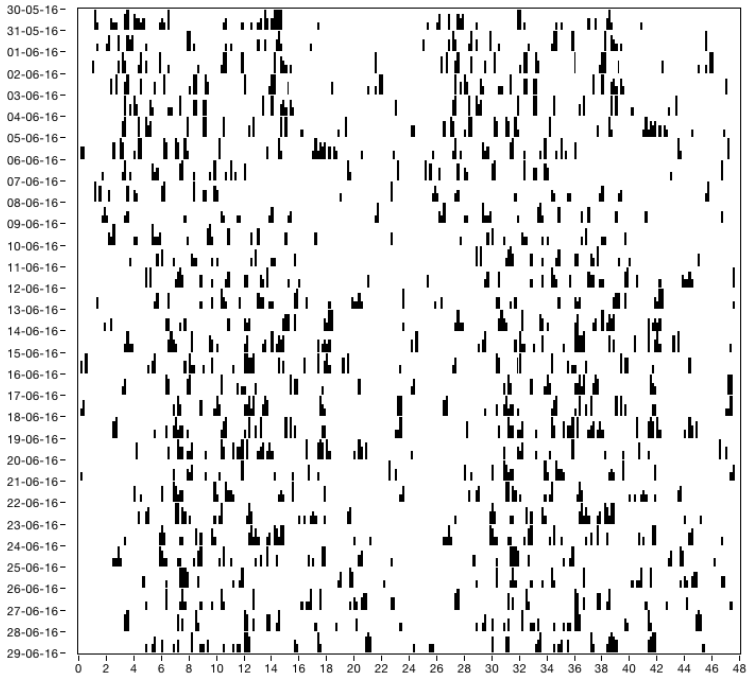
Rat4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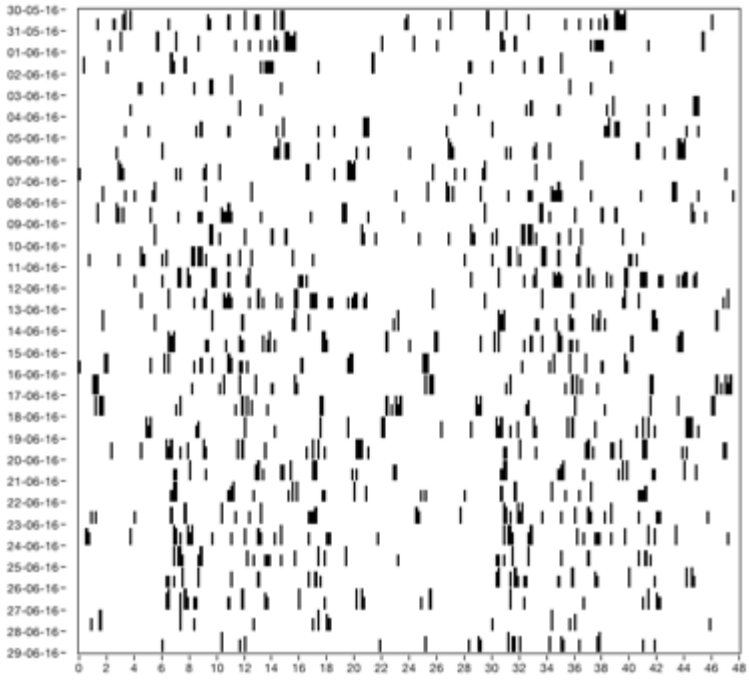
Rat5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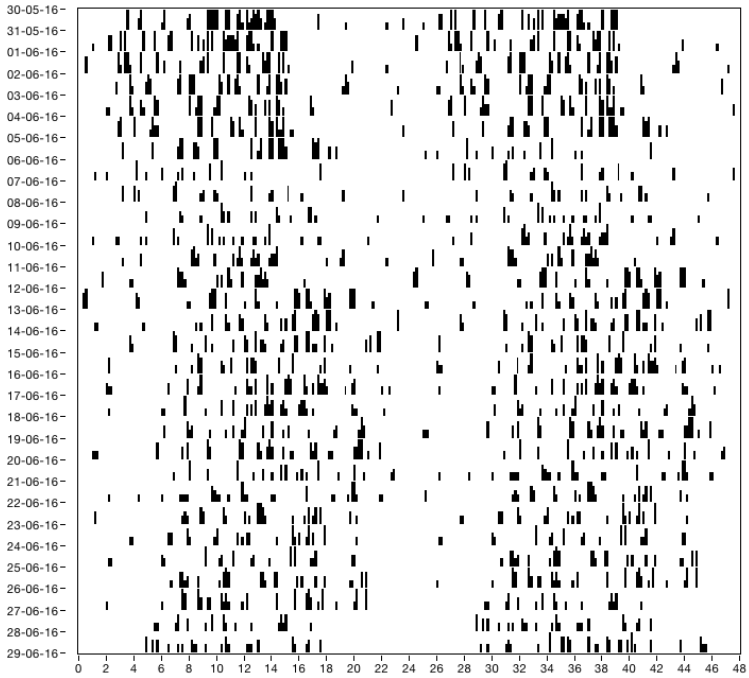
Rat6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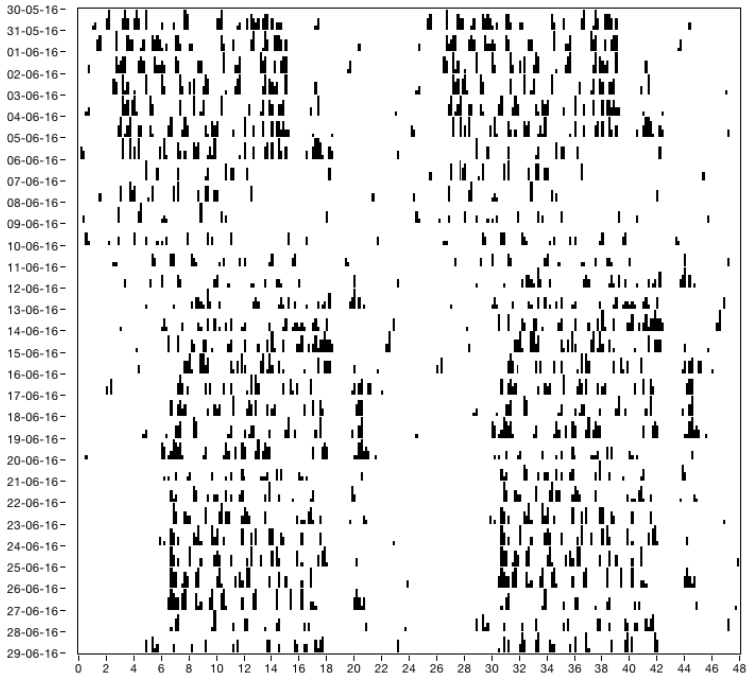
Rat7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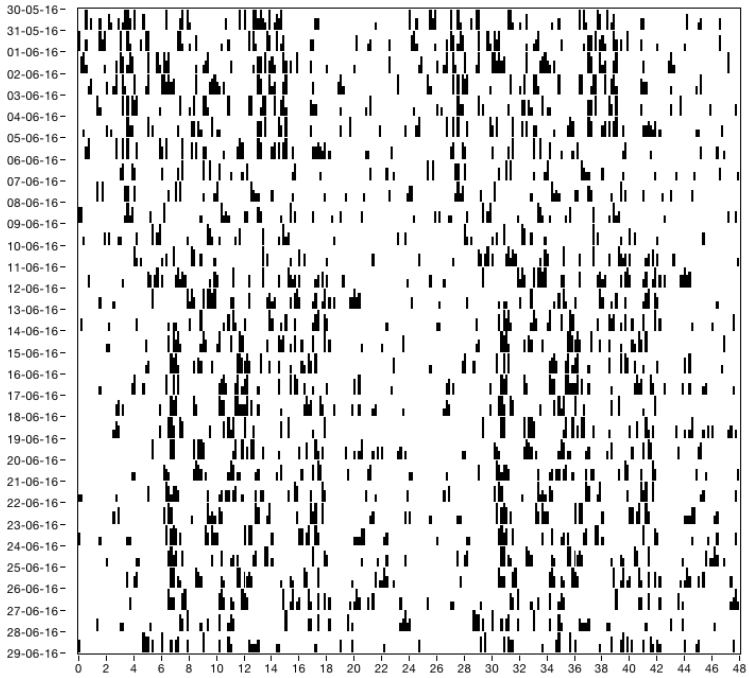
Rat8



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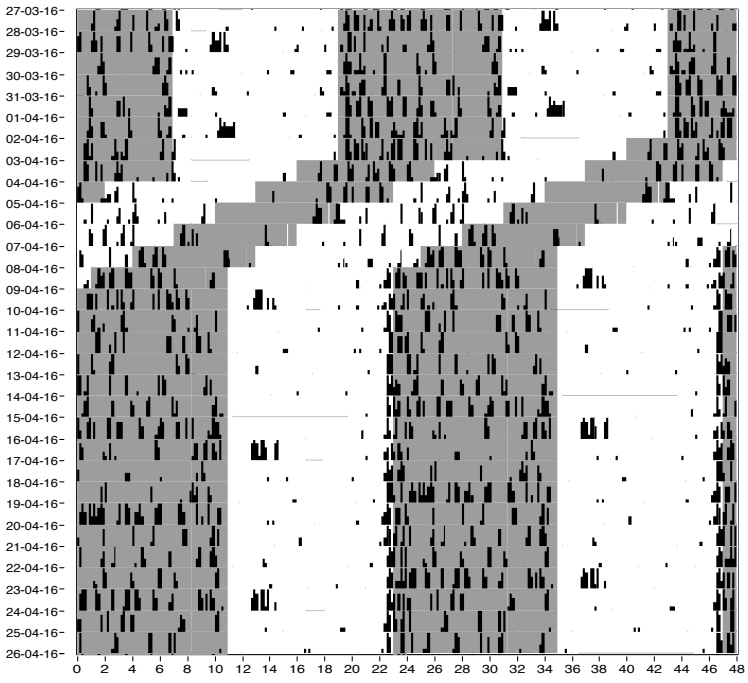
FBN Females



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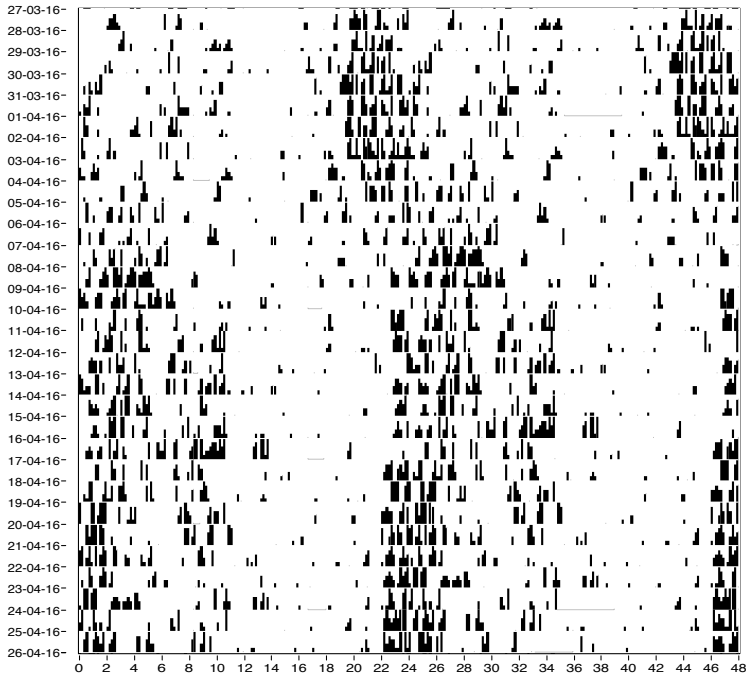
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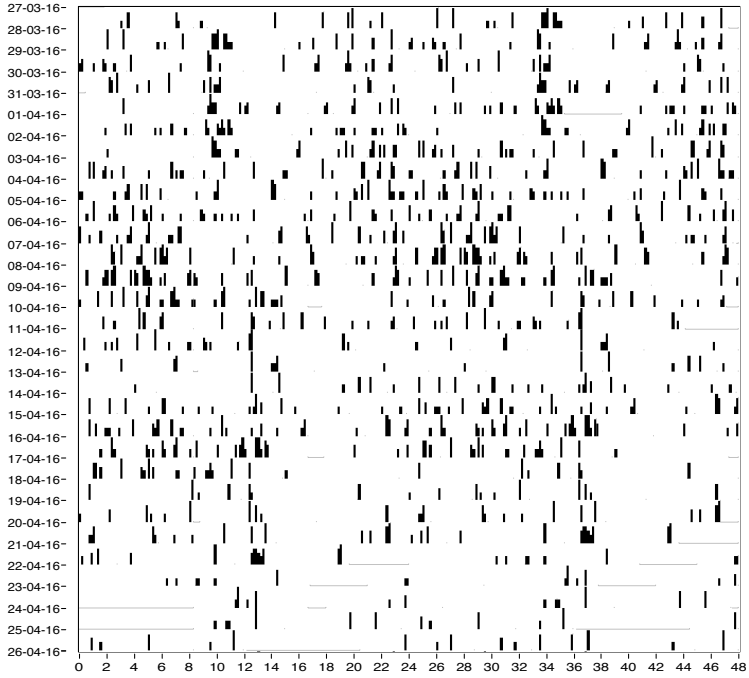
Rat2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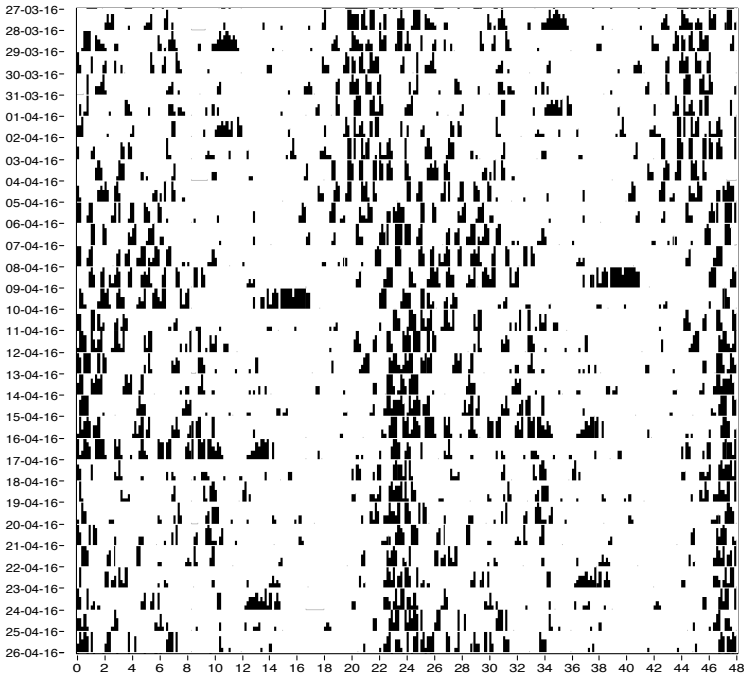
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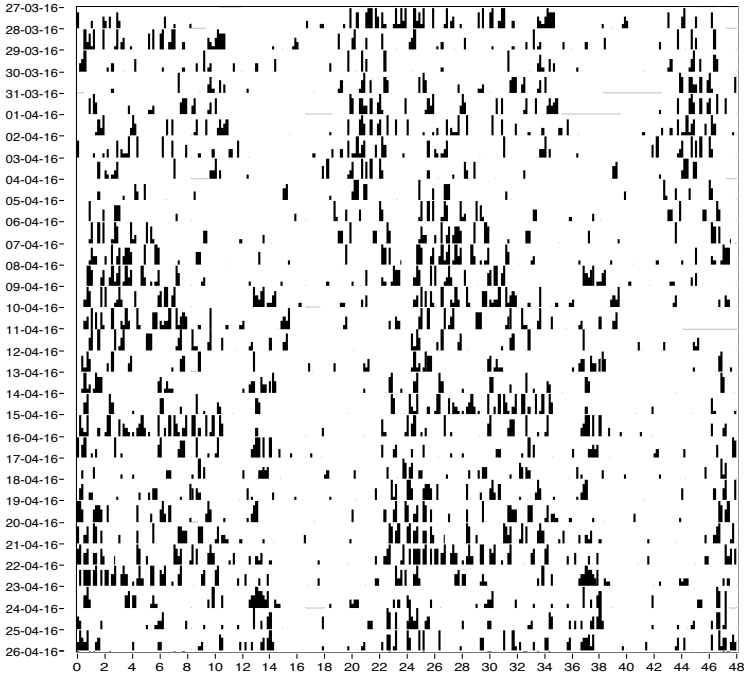
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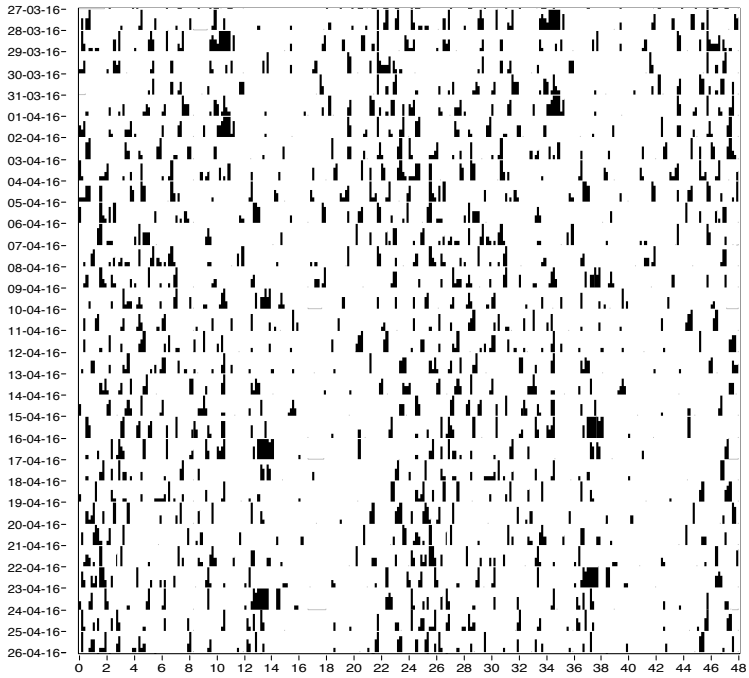
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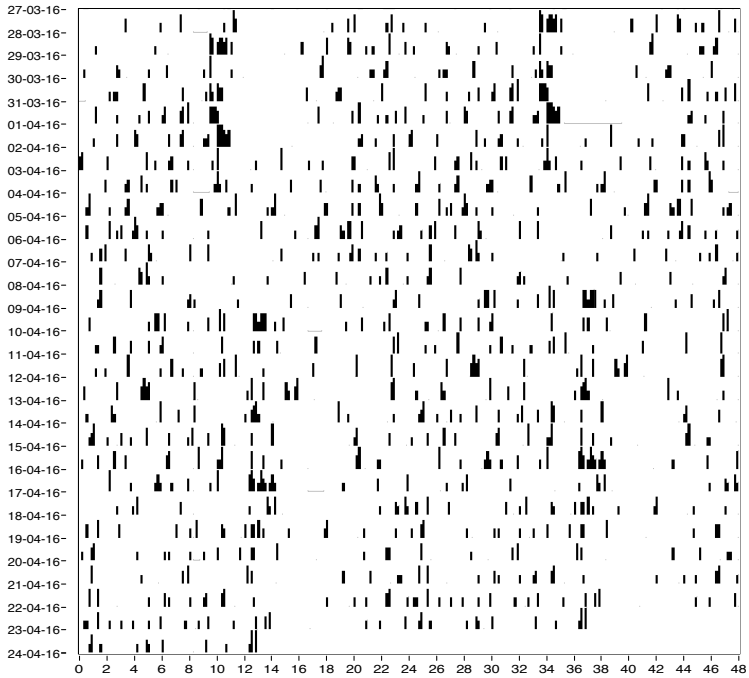
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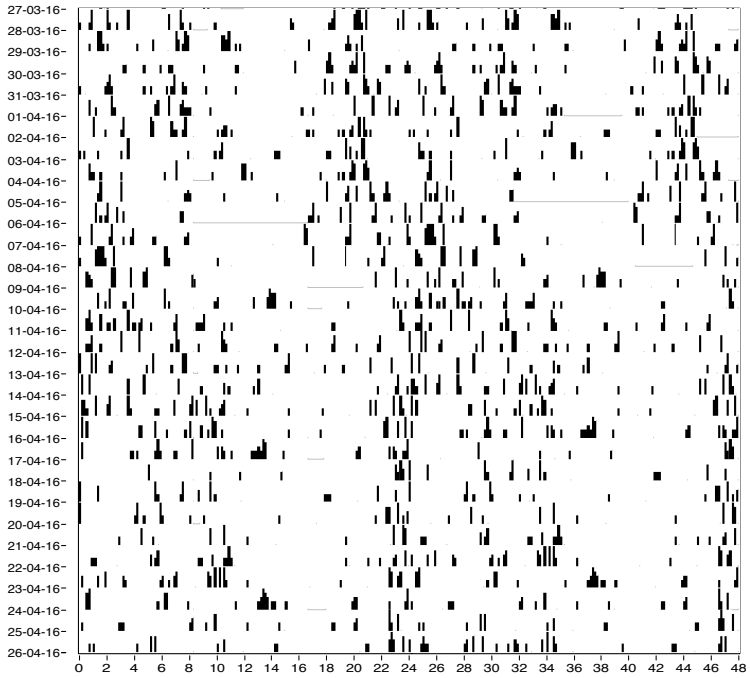
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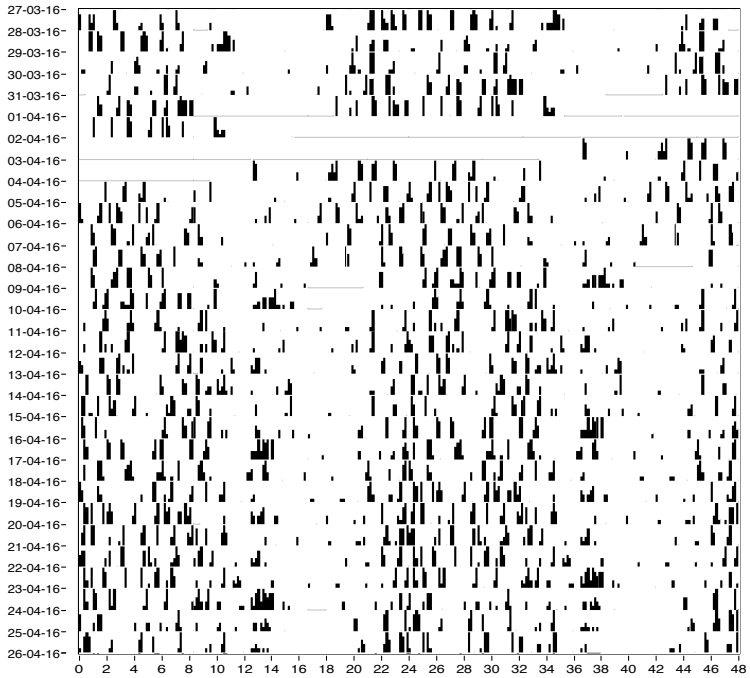
Rat8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Rat9

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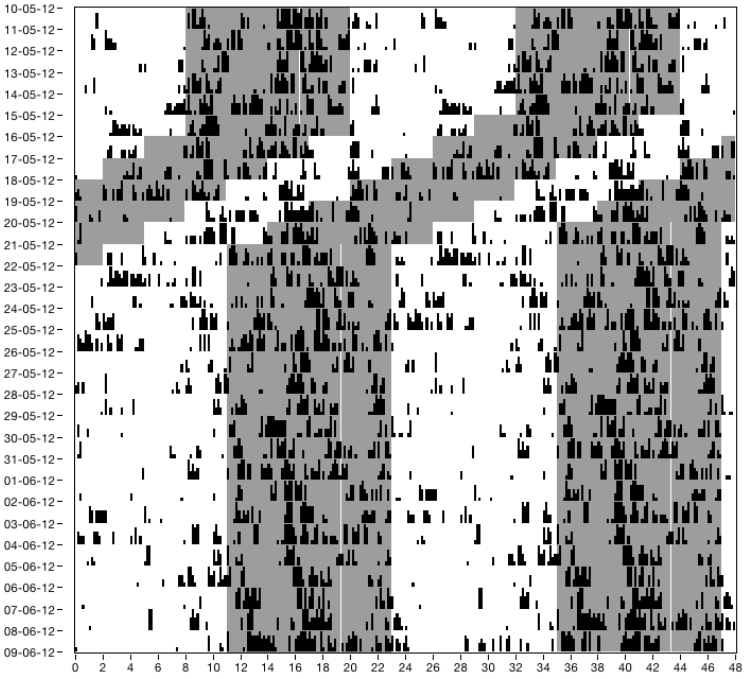

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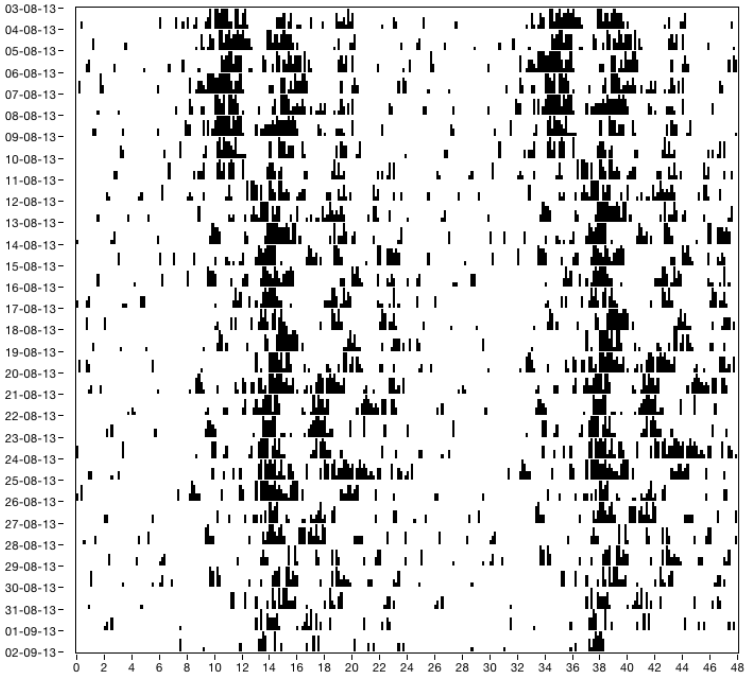
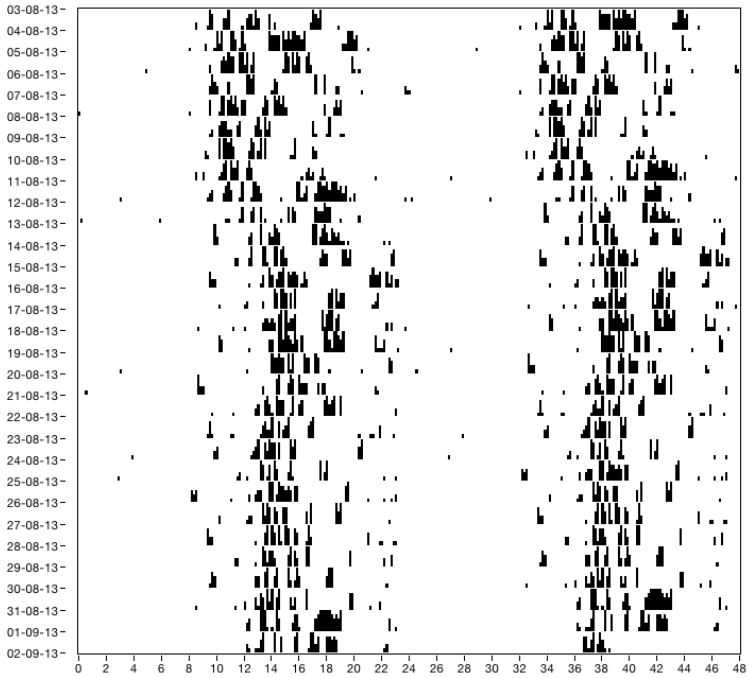


Rat2

[10] Rat1 appears in text above.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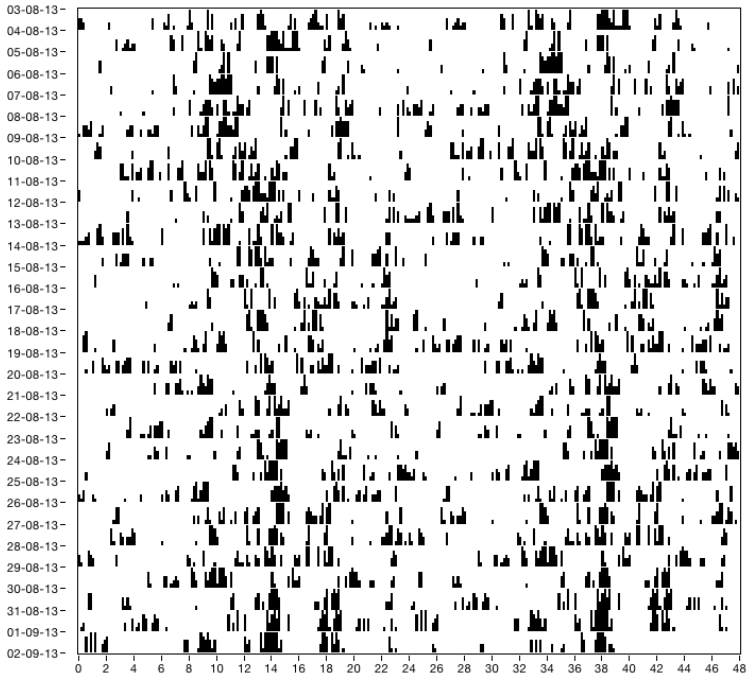
Rat3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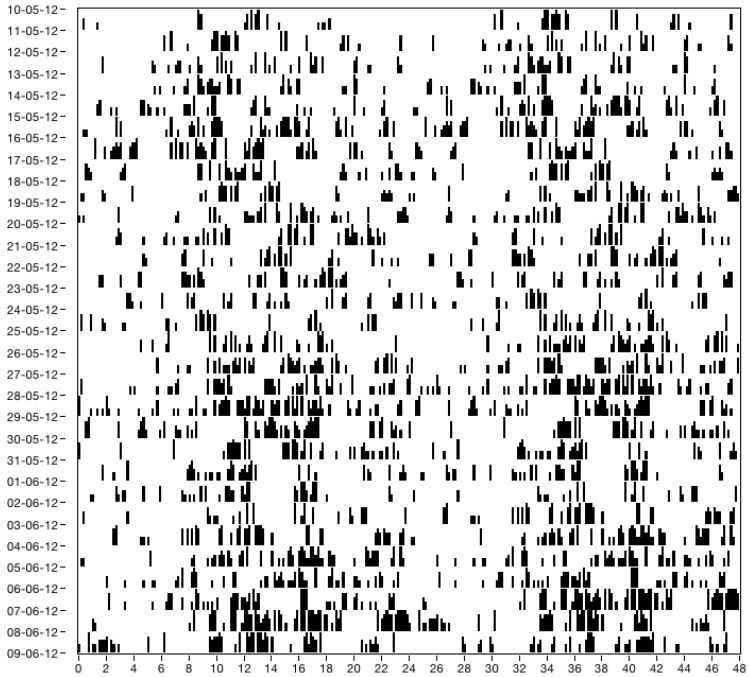
Rat4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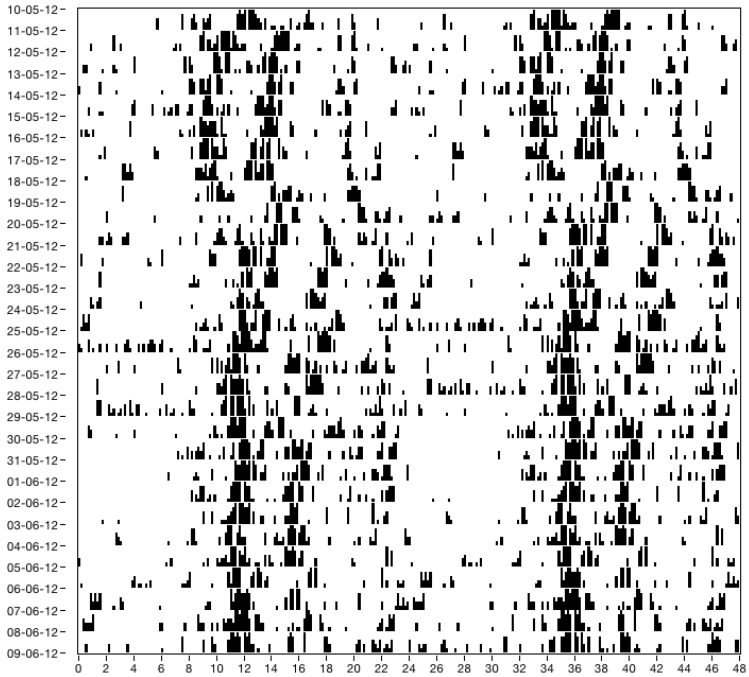
Rat5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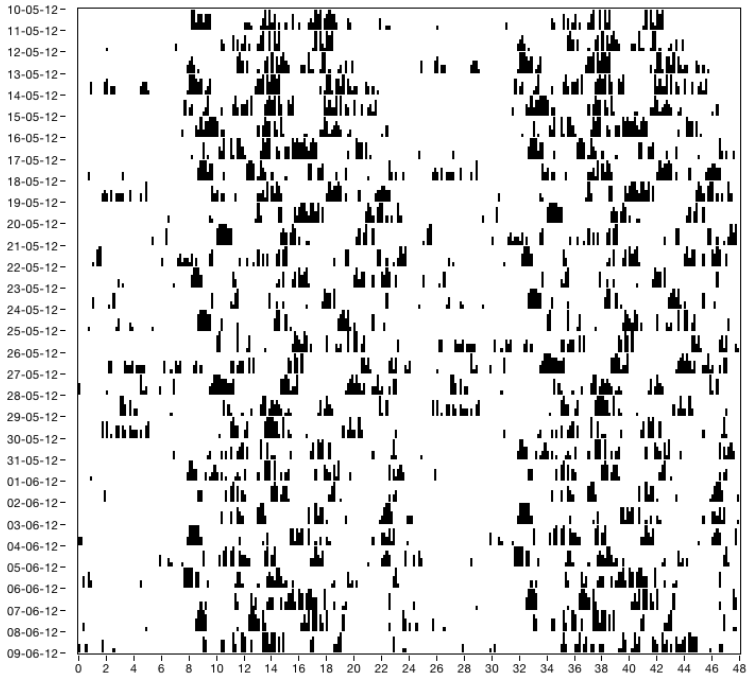
Rat6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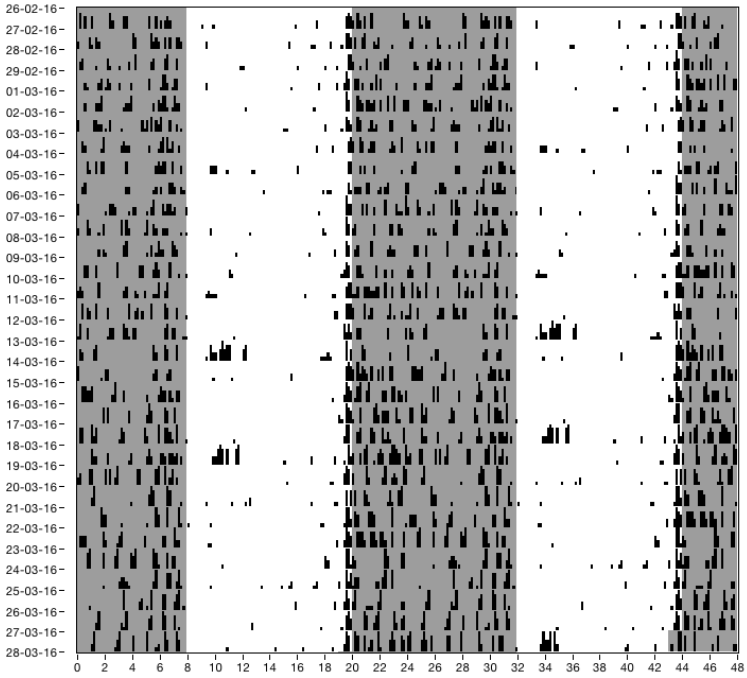
Rat7

Controls



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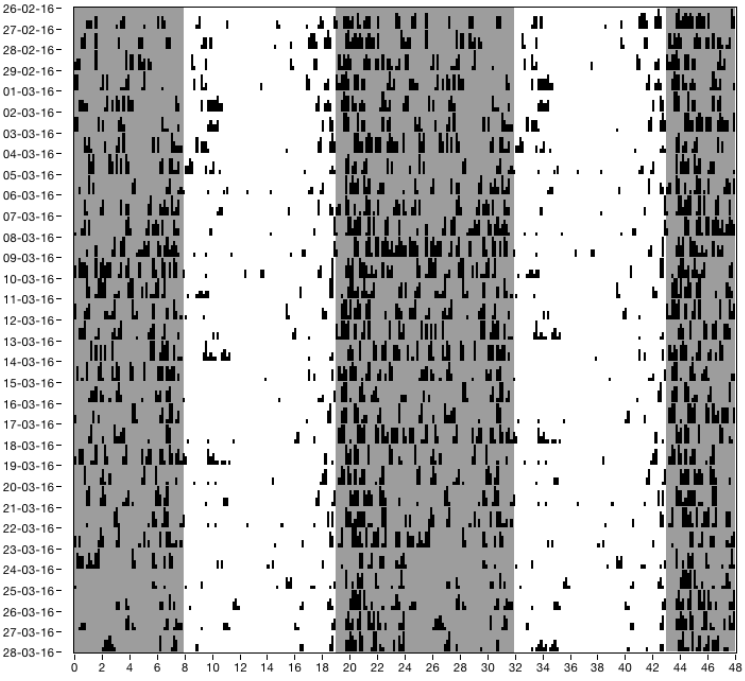

Rat2

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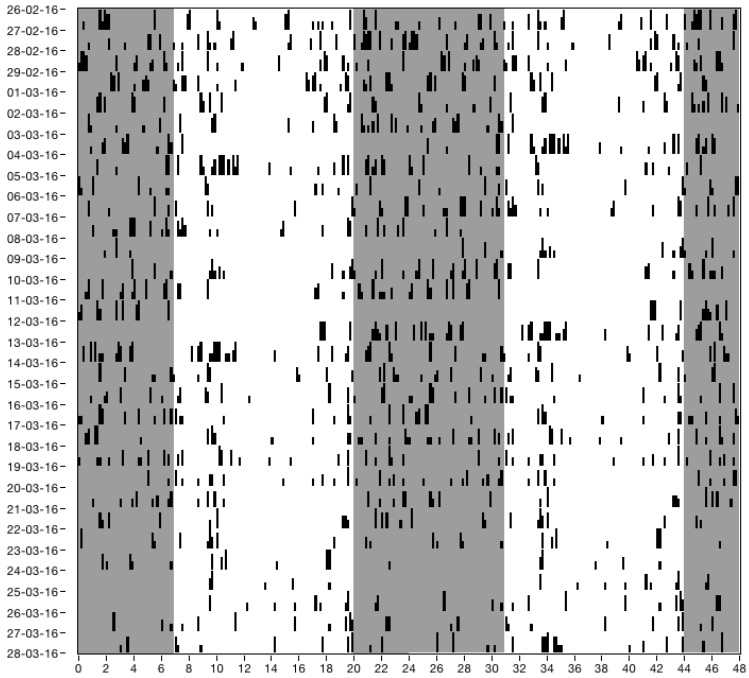
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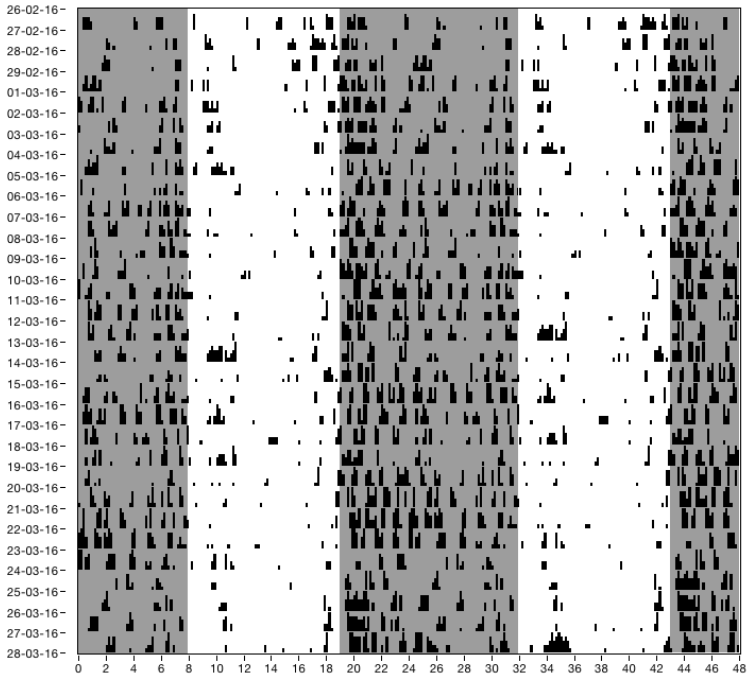
Rat3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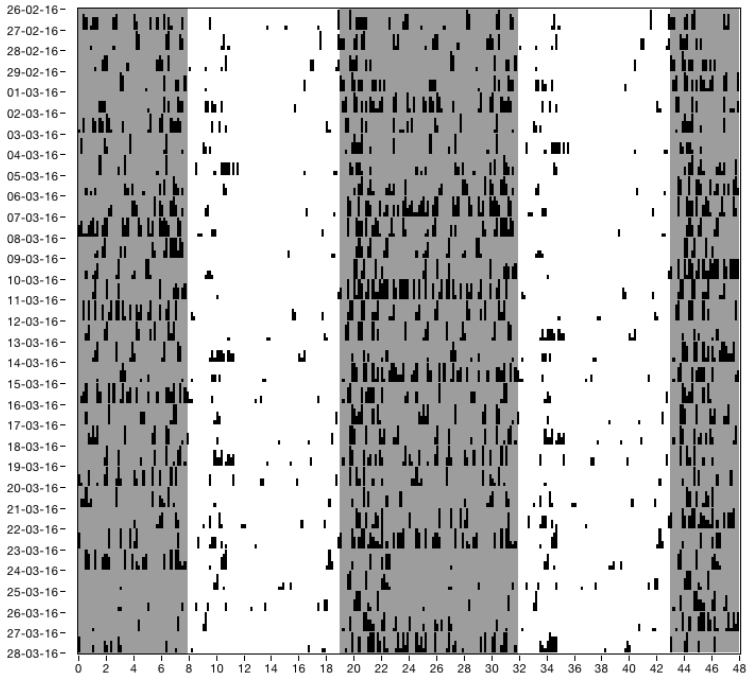
Rat4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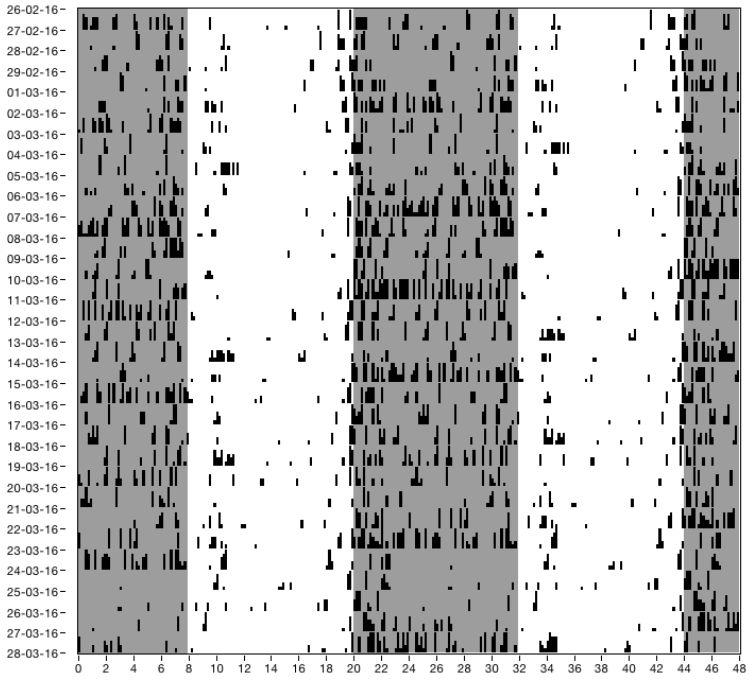
Rat5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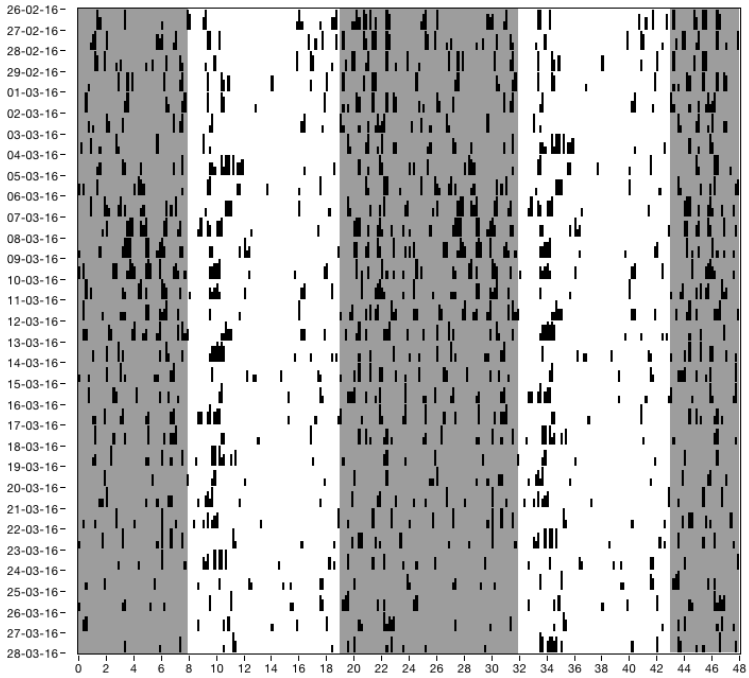
Rat6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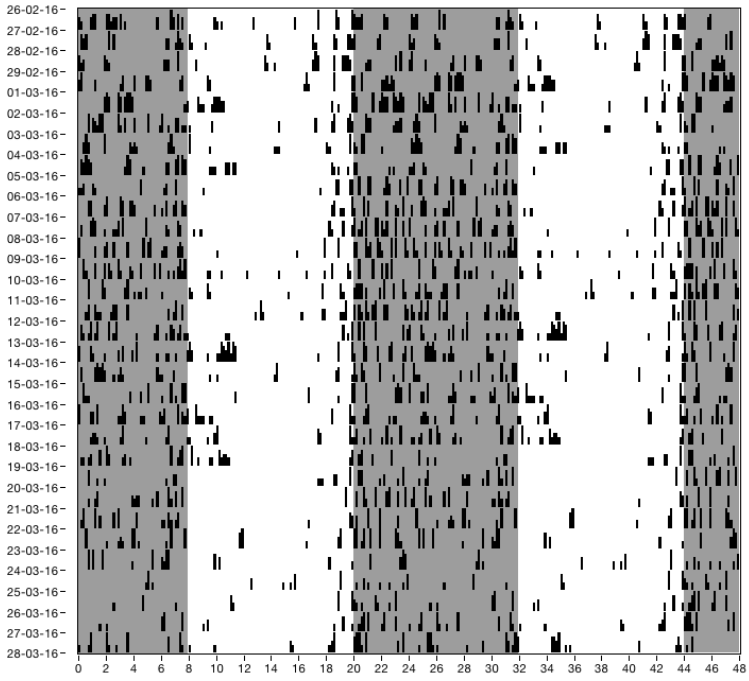
Rat7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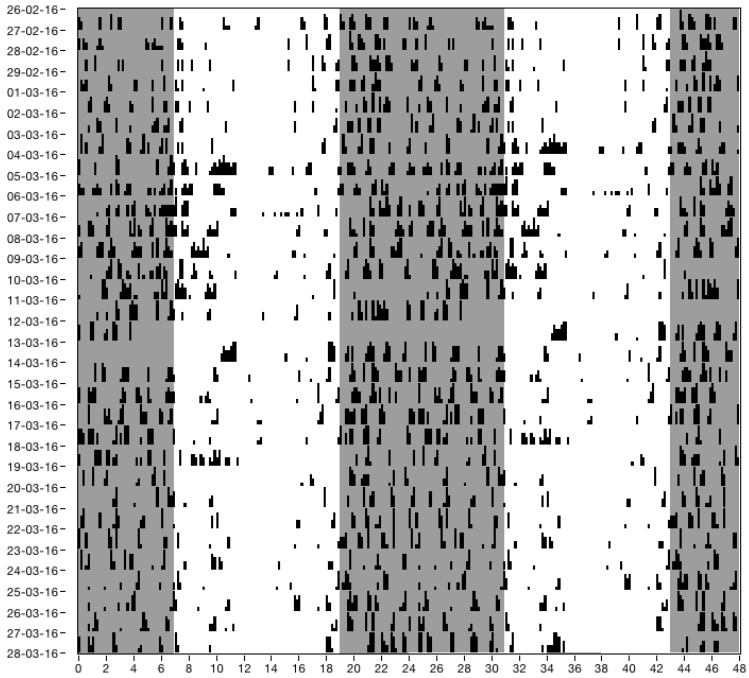
Rat8



Rat9

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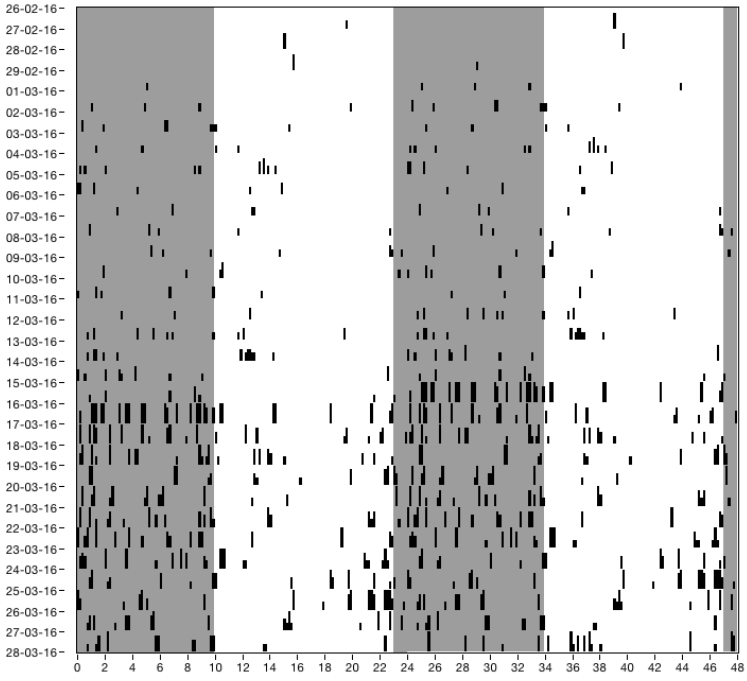
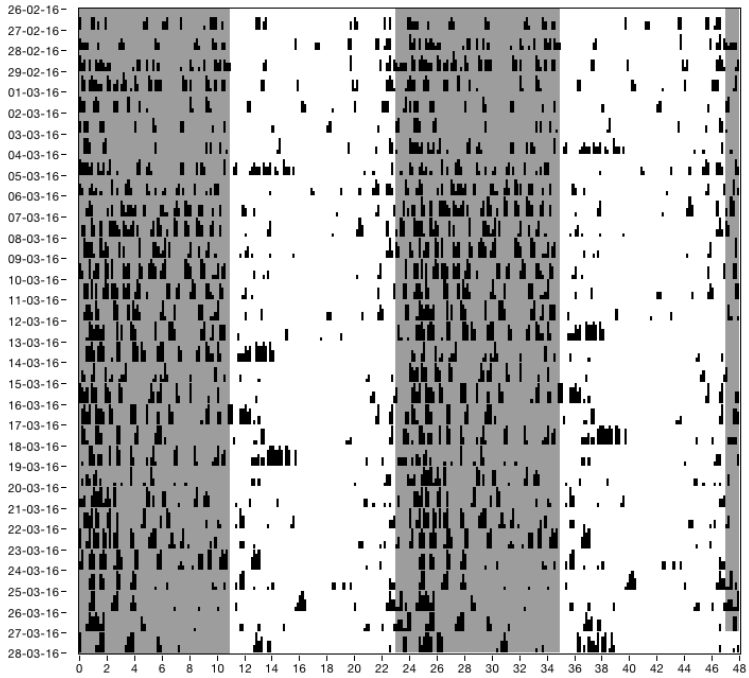


Rat10



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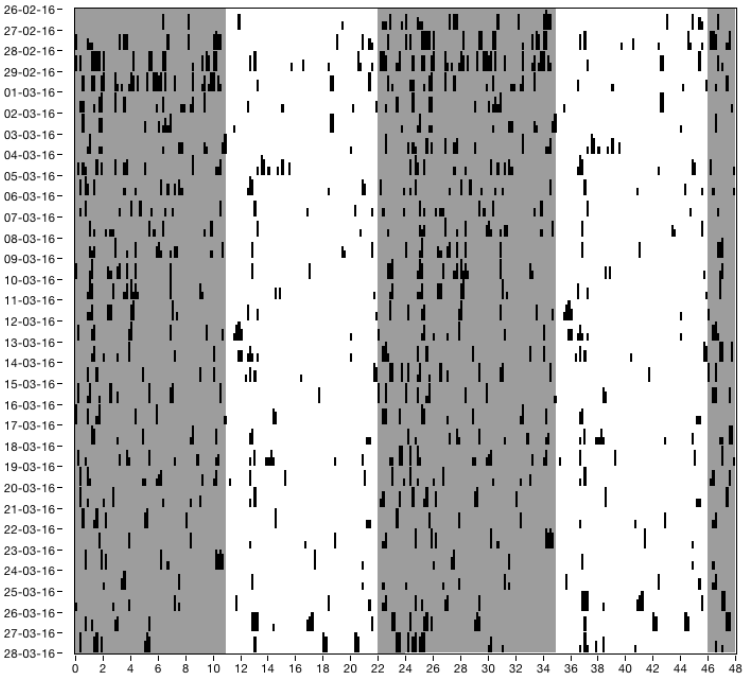

Rat12

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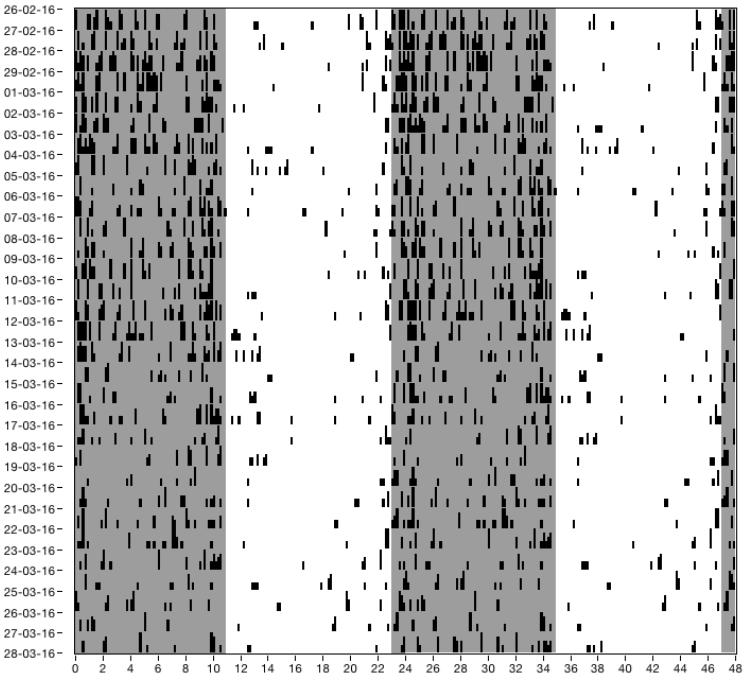
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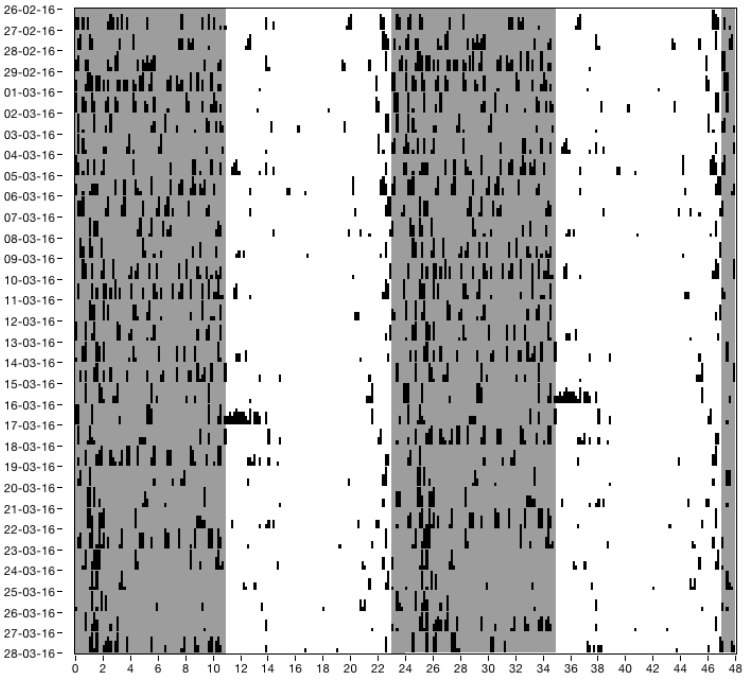
Rat13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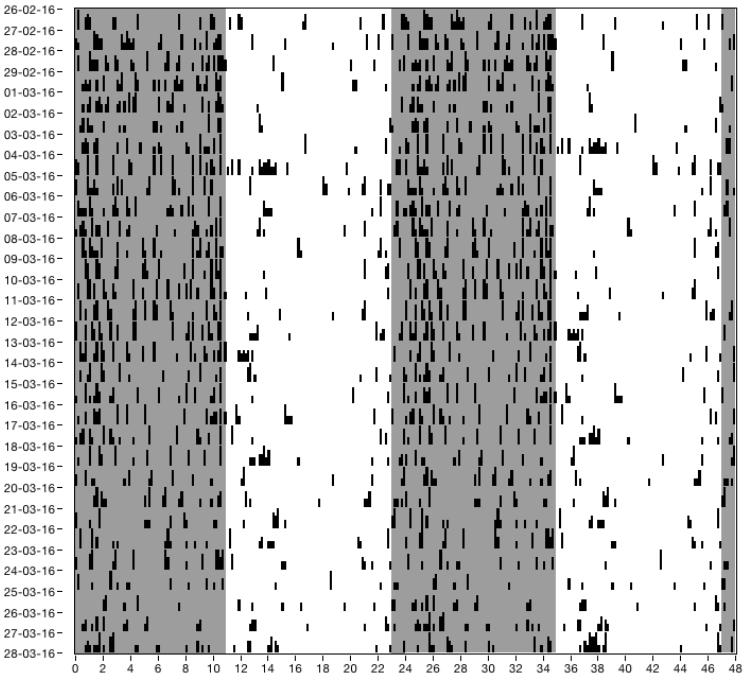
Rat14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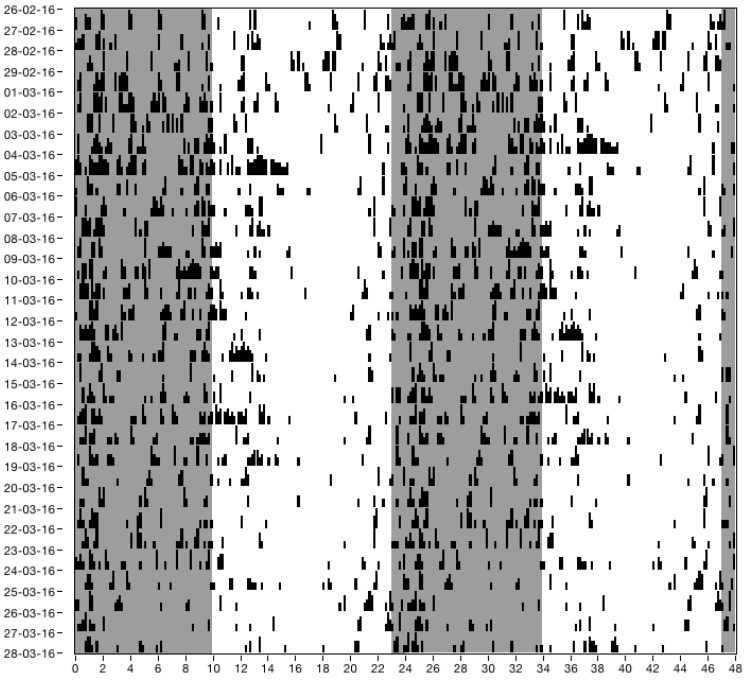
Rat15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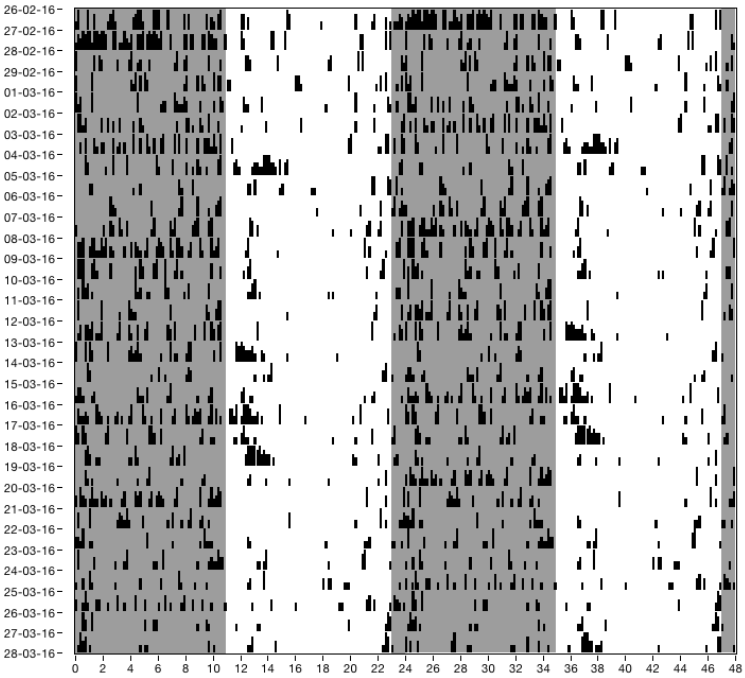
Rat16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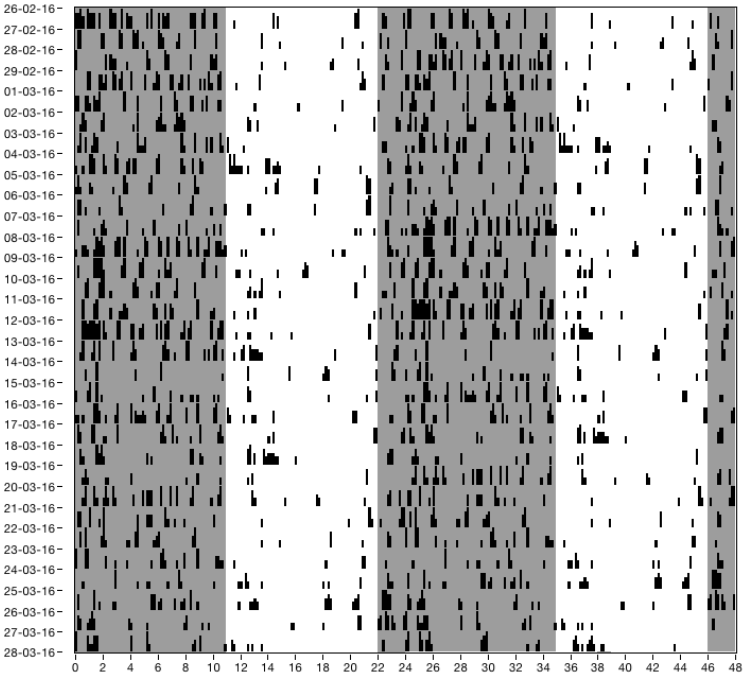
Rat17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Rat18