The effects of circadian food entrainment on the dopamine system and behavioral measures of affect in the male rat.

Hanna N. Opiol

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Dr. Ian Ferguson	Chair
Dr. Florian Storch	External Examiner
Dr. Richard Courtemanche	External to Program
Dr. Andrew Chapman	Examiner
Dr. Uri Shalev	Examiner
Dr. Shimon Amir	Thesis Supervisor

Approved by: _____

Dr. A. Chapman, Graduate Program Director

August 15, 2019 _____

Dean A. Roy, Faculty of Arts and Science

ABSTRACT

The effects of circadian food entrainment on the dopamine system and behavioral measures of affect.

Hanna N. Opiol, Ph.D.

Concordia University, 2019

When feeding is time restricted, the brain and body shift their circadian rhythms to adjust to the feeding time, a process called food entrainment. Dopamine (DA) has been suggested to play a key role in the mechanism through which food entrainment develops. Since amphetamine (AMPH) sensitization is known to enhance DA, we tested whether it would enhance behavioral output of food entrainment, called food anticipatory activity (FAA). We did not find evidence for enhancement of FAA, however, restricted feeding (RF) did result in enhanced output when measuring behavioral cross-sensitization to an acute injection of AMPH. Further investigation found that sufficient exposure to RF was necessary, for the enhancement in DA function. Cross-sensitization remained present post-RF and was not related to body weight at the time of testing, however caloric restriction was necessary. Molecular measures of DA function were examined in the dorsal striatum (DS) during RF and/ or in the re-feeding phase at which time cross-sensitization to AMPH was present. Consistent with the results of behavioral cross-sensitization, immunohistochemistry staining of dopamine transporter (DAT) in the DS revealed higher density of DAT present in rats exposed to at least two weeks of RF. No differences were found in tyrosine hydroxylase (TH) or FOS protein staining of the DS between rats exposed to RF and controls. Since one of the primary mechanisms

through which AMPH acts is DAT, it is possible that the cross-sensitization in food-entrained rats may be due to an upregulation of DAT in DA fibers of the DS. Finally, since DA is known to affect mood, we examined how RF would affect behavioral measures of depression and anxiety in the rat. The results indicate that RF does not negatively affect mood and/or learning of an adaptive response to a stressor, as measured by the elevated plus maze and open-field. RF may also positively affective regulation of depression, since it results in increased swimming behavior in the forced swim test. Taken together, the findings support the role of DA in the DS as part of the food entrainment pathway and lends further support to the idea that food entrainment is a dietary regimen that does not negatively affect mental health, with possible implications for the treatment of depression.

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"There is neither happiness nor misery in the world; there is only the comparison of one state with another, nothing more. "

Alexandre Dumas

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CHAPTER 1

LIST OF ABBREVIATIONS

AMPH: Amphetamine

ANOVA: Analysis of variance

DA: Dopamine

DAT: Dopamine transporter

DS: Dorsal striatum

EPM: Elevated plus maze

FAA: Food anticipatory activity

FOS: FOS protein

FST: Forced swim test

OF: Open field test

LD: Light-dark

LL: Constant light

LS: Locomotor sensitization

NRF: Night restricted feeding

NVRF: Night variable restricted feeding

PER: Period protein

RF: Restricted feeding

RT: Restricted treat

SCN: Suprachiasmatic nucleus

TH: Tyrosine Hydroxylase

VRF: Variable restricted feeding

VRT: Variable restricted treat

VTA: Ventral tegmental area

ZT: Zeitgeber time

GENERAL INTRODUCTION

Circadian Rhythms, a hierarchical system of clocks

Our bodies are designed to function under a 24-h cycle. Each day the biological rhythms that drive our hormonal, behavioral and cognitive processes reset themselves to adapt to changes in the length and timing of daylight. Every cell in our body that contains a nucleus contains the genes necessary to keep track of time. In essence, cells in our body are able to act as clocks to regulate the timing of their own biological functions. Liver cells oscillate together and regulate the circadian response of insulin to sugar (Van Cauter et al., 1991; Damiola et al., 2000), while cells of the adrenal gland oscillate together to regulate the circadian production of the stress hormone cortisol (Damiola et al., 2000; Leliavski et al., 2015), and different brain cells oscillate according to their own schedule of protein expression (Hastings & Maywood, 2000). As a result, our physiology and cognition varies with time of day.

At the level of each cell, there is a small subset of genes, called clock genes, whose transcription / translation is driven by a negative feedback loop of ~ 24 hours. The negative feedback loop is what gives each cell the ability to act as an independent oscillator. However, each individual cell does not have the ability to act as a pacemaker by setting its own rhythm; it must receive physiological input from the brain or body that can determine differences in time of day. In mammals, a single brain region, the suprachiasmatic nucleus (SCN) acts as the pacemaker for clock gene oscillation. The SCN is a small bilateral cluster of ~20,000 cells located within the hypothalamus, which

receives electrical signals via the melanopsin photoreceptors in the retinohypothalamic tract extending from the eyes (Reppert & Weaver, 2002). It is the intensity of light from the sun that the SCN detects and synchronizes to, while simultaneously relaying the information to downstream clock genes in the brain and body (Warren et al., 2006; Liu et al., 2007). The SCN organizes the timing of circadian rhythms that further drive the subdivision of sleep/wake and feeding/fasting cycles.

The SCN is deemed the "master clock" as it is the only known tissue that is self-sustainable, in that it can continue to oscillate on its own when removed from all other inputs (Abe et al., 2002). The superiority of the SCN's function as the master clock is further observed when it is removed. SCN lesions result in most physiological, hormonal and behavioral rhythms becoming arrhythmic (Moore & Eichler, 1972; Stephan & Zucker, 1972; Yamamoto et al., 1987). Furthermore when an SCN is transplanted from a donor, rhythmicity is reinstated in both lesioned and genetically arrhythmic animals, with a new circadian periodicity specific to the donor SCN (Ralph et al., 1990; Sujino et al., 2003). Finally, when the SCN is lesioned, clock genes in peripheral tissues of the brain and body become arrhythmic or desynchronized (Sakamoto et al., 1998). Thus, the body's time-keeping system is a hierarchical multi-oscillator network composed of a multitude of downstream slave oscillators and a single master clock that sets the pace based on its own reference of local time. The SCN ensures that circadian rhythms stay synchronized both amongst each other and to the external world.

Maintaining clock function

Circadian rhythms can become misaligned or disrupted. Misalignment refers to the endogenous circadian rhythms that have been shifted out of phase from the local environment. A common example of circadian misalignment is "jet lag". When we travel across time zones clock gene expression becomes out of phase with the new location and must shift to adjust to the new light cycle. Jet lag can be unpleasant, however circadian misalignment is temporary and the negative effects can be minimized. Treatment for jet lag consists of controlled intake of synthetic melatonin and exposure to bright light before or after travel to the new time zone (Eastman & Burgess, 2009). Some studies report mixed or negative findings for the latter method. A likely reason for this is that studies do not always control for the effects of other stressors, which may or may not affect the circadian system, such as travel-associated fatigue related to length of travel, temperature changes, noise or low oxygenation on flight, as well as meal-timing and composition (Bin et al., 2018). Nevertheless, even without treatment, circadian misalignment is short lived since the body is adapted to resynchronize rhythms to changes in the environment. Circadian misalignment can however become a health concern when it is chronic, rendering the body inefficient at dealing with other stressors in the environment.

Circadian disruption refers to a chronic miscommunication between the environment and internal clocks that results in long-term negative health effects. Chronic miscommunication results in the impairment of critical functions that require tight inter-communication between endogenous oscillators. Circadian disruptors can be

endogenous or exogenous in origin, which disrupt the temporal organization of biological function and hierarchy (Erren & Reiter, 2009). Due to the hierarchical organization of time-keeping systems in our body, disruption at a single level of clock function can produce negative effects at multiple levels of gene function and health. Consistent with this hypothesis, circadian disruption has been linked to: impairments in metabolic function, hormonal rhythms, immune function, increased risk of cancer, negative effects on the gut-microbiome and mental health (Evans & Davidson, 2013; Haus & Smolensky, 2013; Bedrosian et al., 2016; Bedrosian & Nelson, 2017; Smolensky et al., 2016; Touitou et al., 2017; Reynolds et al., 2017). Many psychological disorders, in particular those that affect emotion regulation, also show circadian disruption between various hormonal rhythms (Evans & Davidson, 2013; Bedrosian & Nelson, 2017).

It is important to understand and implicate behavioral patterns that maintain circadian synchrony rather than oppose it. A common example of circadian disruption is in shift work. Shift-workers are often exposed to various schedules within a short period of time, whereby exposure to light and food intake is inconsistent. Both light and food intake have strong effects on the circadian system. Since the sleep/wake cycle is partly driven by circadian rhythms, behavioral patterns in light exposure and food intake can work against each other to result in disrupted sleep. Impairments in sleep feedback to produce additional negative health outcomes. In particular, cardiometabolic disorders are a frequent consequence of circadian disruption in shift workers (Reutrakul & Knutson, 2015).

Metabolic disorders resulting from circadian disruption have been linked to an inefficiency of glucose metabolism. Glucose metabolism is under the control of circadian rhythms in the liver, pancreas, muscle, adipose tissue and gut (Qian & Scheer, 2016). When glucose metabolism does not function efficiently it can result in obesity, type II diabetes and downstream effects on hormonal rhythms that further affect feeding habits and sleep (Jha, Challet & Kalsbeek, 2015). In light of our understanding of circadian function, a novel field of treatment has emerged called "chronotherapeutics" (Ballesta et al., 2017). The approach takes into consideration the time of biological functions, or implements changes in hormonal rhythms as to maximize the outcomes of medicine. For example, recent work has demonstrated that metabolic disturbances to the liver resulting from circadian disruption can be prevented by aligning feeding patterns with the circadian rhythm of two critical hormones, melatonin and cortisol (Baez-Ruiz et al., 2017). Nevertheless, chronotherapeutics is at its infancy and requires more research to help understand how internal clocks communicate amongst one another, what external factors pose a risk in their disruption, and what methods can be implemented to maintain and correct circadian disruption.

Feeding patterns

Clock genes in the body

Although clock genes outside the SCN cannot independently synchronize to the day-night, light-dark (LD) cycle, they are not solely dependent on signals from the SCN. Food intake can be a potent zeitgeber (time-giver) for clock genes. For some clock

genes, such as those in peripheral tissues and organs, the time of food intake is a more potent time cue than light. When feeding time occurs out of phase with the SCN, clock genes in the periphery will uncouple from the SCN to shift their rhythms inline with feeding. Clock genes in the liver appear to shift the fastest, followed by kidney, heart and pancreas (Damiola et al., 2000). It is suggested that digestive organs, such as the liver, are directly affected by feeding, whereas clock genes in other tissues, such as the lung, are shifted by secondary mechanisms, such as changes in hormonal signals that affect behavior and thereby breathing (Stokkan et al., 2001).

The idea that peripheral clocks are more sensitive to feeding than light is consistent with the idea that anticipation of feeding time is critical for optimal health, by reducing negative effects of food and maximizing the benefits. More specifically, food is a necessity, but it is also a stressor, as it disrupts many homeostatic systems (Woods et al., 1991). Food is most energy efficient when the physiology involved in its breakdown is prepared. For example, mice with liver-specific clock gene disruption develop hypoglycemia, as the lack of circadian rhythmicity in the liver leads to exaggerated glucose clearance (Lamia, Storch & Weitz, 2008). Conversely, digestion, of both nutrient content and indigestable substances, is more efficient when feeding time is anticipated (Cagampang & Bruce, 2012). Thereby, aligning peripheral clocks with feeding time helps to prepare the body with appropriate metabolic and hormonal responses in order to efficiently maintain energy homeostasis (Wood et al., 1998).

Clock genes in the brain

In the brain, clock genes are also sensitive to feeding, however each brain region varies in sensitivity to different feeding patterns and in which clock genes are affected (Feillet et al., 2008; Verwey & Amir 2009). Under ad libitum feeding conditions, the clock protein PERIOD (PER) 2 in the oval nucleus of the bed nucleus of the stria terminalis (BNSTov) and central nucleus of the amygdala (CEA), areas important for emotion and motivational processes, have been found to oscillate in phase with PER2 in the SCN (Amir & Stewart, 2009). Furthermore, these two regions are directly under the control of SCN driven glucocorticoid rhythms (Segall et al., 2006; Segall et al., 2009), but also affected by other hormonal rhythms such as the estrous cycle (Perrin et al., 2006) and thyroid hormones (Amir & Robinson, 2006), but not melatonin (Amir, Harbour & Robinson, 2006). In contrast, melatonin, a hormone important for sleep, affects the clock protein PER1 in the pituitary gland and striatum (Messager et al., 2001; Uz et al., 2003). Therefore, under free-feeding conditions each region of the brain utilizes it's own measure of clock gene rhythmicity to carry out brain-region specific functions. However, under conditions of scheduled feeding, clock genes in the brain respond differently depending on: caloric restriction, feeding time in respect to the light cycle, or elimination of the light cycle altogether.

The SCN remains largely unaffected by scheduled feeding, unless there is hypocaloric restriction or the light cycle is eliminated for prolonged periods (Feillet et al., 2008). It appears that PER1 and PER2 in the brain respond differently, with PER2 being more sensitive to phase shifts in more brain regions overall (Verwey & Amir, 2009). Daytime

feedings appear to produce larger shifts in the PER2 and in more brain regions than nighttime feedings (Verwey et al., 2008). Furthermore, scheduled feeding can promote beneficial resynchronization of clock genes in the brain when signals from the SCN have been disrupted (Lamont et al., 2005; Novakova et al., 2011), as well as resynchronizing clock genes rhythms in the SCN itself (Waddington Lamont et al., 2007). Therefore the neuronal network of clocks in the brain is complex and responds differently to different feeding circumstances. The importance of regular feeding schedules on clock genes in the brain emerges when we evaluate variable feedings, whereby time of feeding is chronically altered.

Variable feedings

Consuming food on a variable schedule, as in the case of many shift workers, can take a toll on the body. Effects of variable feeding can be seen both short term in sluggish digestion or insulin response, and long term as with the development of insulin resistance and obesity (Escobar et al., 2011; Zarrinpar et al., 2016). The effects of variable feeding on mental health have received less focus, however there is evidence to suggest that variable feedings negatively affect clock gene function in the brain and thereby the cognitive functions of those regions (Escobar et al., 2007; 2011). The extent of disruption is also dependent on the brain region and time of feeding (Verwey et al., 2012). In particular, variable feedings impair PER2 rhythms in the dorsal striatum (DS), a region important for emotional processing and behavioral output (Verwey et al., 2012). The daily rhythm of the hormone corticosterone is also blunted, indicating that biological functions relying on this hormone may be disrupted (Escobar et al., 2007; Verwey et al.,

2012). In contrast to scheduled feedings, variable feedings result in daily resetting of circadian phase in brain tissues to the time of previous feeding, and neuronal activation in the SCN is not inhibited (Escobar et al., 2007). Thereby, if feeding time is constantly changing the body is not able to adapt in preparation to those feedings; and signals from the SCN remain intact whether it is beneficial or disruptive to health.

Restricted feeding activates an SCN-independent clock mechanism

Restricted feeding (RF), a feeding schedule whereby food intake is time restricted to a few hours within a 24-h cycle, acts as an external time cue to which clock genes in the brain and body can synchronize (Stephan, 2002; Mistlberger, 2009). The potential for feeding to act as a time-keeping mechanism is related to three important aspects: meal size, nutrient content (Mistlberger & Rusak, 1987), and time spacing between meals (Mendoza et al., 2008; Luby et al., 2012). Although caloric restriction itself is not necessary to shift circadian rhythms, as entrainment can occur with a nutritious palatable snack and no caloric deficit (Mistlberger & Mistlberger 1987; Mendoza et al., 2005; Verwey et al., 2007; Hsu et al, 2010a), it does appear to affect the amplitude of circadian rhythms and their magnitude to shift rhythms. Palatable meals without restrictions to feeding do not have the same capacity to shift clock genes in the brain as RF (Verwey et al., 2007; Waddington Lamont et al., 2007). Behavioral rhythms are also weakly affected by palatable snacks compared to RF (Hsu et al., 2010a). Thereby some element of compartmentalized feeding/fasting has a strong potency for shifting circadian rhythms. It is currently uncertain through what mechanism clock genes are able to synchronize to feeding time, however studies have pointed out a few important clues.

The food-sensitive clock is not an hourglass mechanism that requires daily "winding up" (Mistlberger, 1994). In other words, food is not required every day in order for rhythms to remain cycling with feeding time. If food is withheld for a few days, behavioral rhythms will remain in sync with time of previous feeding. RF results in the gradual development of food anticipatory activity (FAA). FAA is defined as the increase in activity in the hours prior to previous feeding. In rats, over the course of several days to one week FAA will increase, while previously predominant activity at night is reduced. Similarly, in the case of a meal shift, FAA requires time to readjust. The gradual changes are also seen internally, albeit with differences between tissues in how quickly circadian rhythms adjust to a new feeding time; FAA develops in parallel to the internal reorganization of clock gene expression around feeding time (Damiola et al., 2000; Verwey & Amir, 2009). Thereby, the emergence of FAA and internal clock reorganization to a new feeding window is not sudden, and remains intact despite a daily positive reinforcement, both of which would be expected of an hour-glass mechanism.

A second clue is that RF affects clock function independent from the SCN. When the SCN is removed clock genes begin cycling out of tune with neighboring cells, resulting in internal disorganization in biological function of gene expression, hormonal communication, and the behavioural arrhythmicity of the sleep/wake rhythms (Moore & Eichler, 1972; Stephan & Zucker, 1972; Stephan & Nunez, 1977; Yamamoto et al., 1987). Yet, in the absence of the SCN, the food clock mechanism remains intact. The

application of RF results in the restoration of internal circadian rhythmicity to peripheral organs, the brain and behavioral output, despite the lack of a functioning SCN (Stephan 1979; Verwey & Amir, 2009).

Searching for the food entrainable oscillator

The mechanism by which RF shifts clock genes to feeding time is referred to as food entrainment, whereby entrainment refers to the phase of an internal rhythm synchronizing to the phase of an external rhythm (Mistlberger, 1994). Food entrainment is currently believed to be the result of multiple interconnected structures in the brain and body that communicate through many metabolic and hormonal signals. However, for many years, researchers hypothesized that just as the SCN controlled light entrainment, a single brain region would be found to control food entrainment. The hypothesized region was termed the food entrainable oscillator (FEO). To try to identify this region, lesion studies targeted candidate regions linked to gustatory and visceral inputs that modulate feeding, including the parabrachial nucleus (Davidson et al., 2000), ventromedial hypothalamus (Kreiger, 1980; Inouye, 1982; Saito and Shimazu, 1982; Mistlberger and Rechtschaffen 1984), the dorsal medial hypothalamus (Landry et al. 2006, 2007), as well as a number of other non-feeding related regions (Davidson, 2009). To date, no single brain region has been shown to fully abolish circadian food entrainment.

The idea that the FEO is (or requires) a hormonal signal entrained to food intake has also been previously suggested. Potential candidates included hormones involved in

metabolic breakdown of food such as insulin and glucagon (Davidson & Stephan, 1999; Davidson et al., 2003; Dailey et al., 2012), leptin (Elimam & Marcus, 2000), and ghrelin (Drazen et al., 2006) acting at various hypothalamic sites after food ingestion. The conclusion of prior studies was that feeding hormones are unnecessary for the FEO. Both hormonal knockout and receptor blockade studies demonstrate food entrainment, in at least one physiological measure, remains intact (see review Patton & Mistlberger, 2013). Global knockout models of clock genes also do not abolish FAA rhythms (Pitts et al., 2003; Iijima, et al., 2005; Pendergast et al., 2009; Storch and Weitz, 2009). As a result, it is currently believed that there are multiple pathways through which food entrainment can be attained and simply impairing one pathway will not bring down a network-wide FEO (Carniero and Araujo, 2009). The hypothesized model of the FEO as a network of interconnected brain structures would involve many brain regions processing feeding and/or time related information to which each region may be differentially sensitive to (Feillet et al., 2008; Verwey et al., 2007; Verwey and Amir, 2009, 2011; Pezuk et al., 2010). Current interest is focused on reward-processing regions of the brain and the role they play in influencing circadian rhythms through the dopamine (DA) neurotransmitter system.

Similar to how FAA develops with entrainment to RF, recent studies have shown that circadian rhythms in behavioral output can also be entrained to daily time-restricted rewarding events. For example, a daily scheduled palatable treat (Mistlberger and Rusak 1987; Angeles-Castellanos et al., 2008) or mating window (Landry et al., 2012) and daily injections of DA-activating drugs (Kosobud, 1998; Gallardo, Darvas, Oviatt,

Chang, Michalik et al., 2014) all result in the development of anticipatory activity prior to the time of the scheduled event. These findings suggest that the time-keeping of salient events may be controlled by clock genes in reward-related brain regions. It has been suggested that the entrainment of behavioral rhythms to external salient events could result through the effect of daily surges of DA rhythms acting on clock genes in motor output regions of the brain. Evidence for the latter hypothesis comes from studies demonstrating that DA receptor activation regulates both clock genes in the DS (Imbesi et al., 2009; Sahar et al., 2010; Hood et al., 2010; Frederick et al., 2014) as well as circadian locomotor output (Hood et al. 2010; Smit et al., 2013; Blum et al., 2015). Therefore, the question arises whether food entrainment controls clock genes and FAA through DA signaling in the DS.

The working hypothesis: rhythmic DA as an entrainment factor for the FEO

Entrainment of the clock protein PERIOD 2 (PER2) by DA in the DS is one of the most conclusive studies to show DA-controlled clock gene expression (Hood et al., 2010). Hood et al. demonstrated that the clock protein PER2 in the DS requires the fluctuation in circadian rhythmicity of DA to this region in order to maintain its own circadian gene expression. If the circadian rhythm of DA in this region is blunted, PER2 is no longer rhythmic. Moreover, the evidence that fluctuations of DA are required for driving a clock gene, and not vice versa, is sufficient grounds to assume that factors from the external environment that affect extracellular DA levels (such as food or reward-related stimuli) may be acting as input signals, which could then entrain parts of the brain responsible for food entrainment.

Since DA in the striatum is involved in both motivation and motor output, it has been difficult to test its role in food entrainment by suppressing DA function or genetically knocking it down. Motivational impairments that are the result of low DA levels result in insufficient food intake during a single period of time to properly implement RF, while maintaining a healthy body weight. DA suppression also results in impaired motor movement, such that food entrainment cannot be measured by FAA when animals are unable (or unwilling) to move around. However, even then if the animal would not show FAA, a neurochemical "memory" of circadian phase may still develop and remain intact (Mistlberger, 1994), ready to resurface when locomotor function is restored (Mistlberger and Mumby, 1992; Gallardo et al., 2014; Caine et al., 2017).

Recent advances in technology have made use of vector viruses to selectively restore the gene expression of DA to a specific brain region in mice that lack the gene to make DA (DA knock out mice). This technology has allowed for a closer inspection of DA's role in different brain regions, such as the role of reward processing in incentive salience, goal-directed learning and motivation pertaining to feeding. DA deficient mice that lack feeding behavior and the capacity for food-related learning regain these functions if DA is selectively restored into the DS, but not if DA is restored to the nucleus accumbens or ventral tegmental area (Szczypak et al., 2001; Hnasko et al., 2006). DA in the DS is a reasonable candidate for a brain location that could be entrained by DA release, such as to a bout of feeding when food is restricted. The question remains how this process would take place. It is possible that food could

regulate rhythms of DA, perhaps in the DS, which may be necessary for food entrainment. If so, then RF may provide a simple tool for regulating disrupted circadian rhythms through its effects on DA, such as in the case of mood disorders whereby circadian rhythms and DA function are both affected.

Circadian rhythms and mood disorders

Bipolar disorder, major depressive disorder and seasonal affective disorder are mood disorders that consistently show impairments in the sleep/wake cycle. Impairments to the sleep/wake cycle in depressed and bipolar patients are paralleled by disruptions in circadian rhythms of hormone and neurotransmitter function (Atkins et al 1974; Kripke et al., 1978; Souetre et al., 1989). The chronological development between sleep and mood disorders remains unknown (Salgado-Delgado et al., 2011). In other words, is it the development of a mood disorder that affects the sleep/wake cycle, which leads to further impairments of ones circadian rhythms? Or do environmental factors that impair sleep lead to disrupted rhythms that promote the development of a mood disorder? Currently, there is much focus on the later hypothesis. It is believed that clock genes, both SCN and peripheral, are altered by chronic environmental disruptions, which further lead to disruption of the sleep/wake cycle and downstream clock-controlled gene expression (Bunney and Bunney, 2000; Reppert & Weaver, 2001). The disruption in circadian function such as with night shift work, chronic jet-lag and lifestyle that promotes exposure to light at night all appear to promote mood disorders in individuals who are genetically predisposed to their development (McClung, 2013; Verwey, Al-Safadi & Amir, 2015).

The idea that clock genes are disrupted in mood disorders is supported by evidence that shows therapy treatments targeting sleep and the function of the SCN lead to beneficial outcomes. Total sleep deprivation and bright light therapy are both known to reset circadian rhythms, including the rhythm of the SCN (Czeisler et al., 1987; 1989; Wirz-Justice, 2006; McClung, 2011; Bunney & Bunney, 2013). Mood stabilizers and antidepressants on the market are believed to derive at least part of their therapeutic effects through correction of clock function in patients (Atkins et al., 1974; Kafka, 1983; Souetre et al., 1989). It is evident that correcting circadian disruption can benefit treatment outcomes of mood disorders. It is less clear to researchers how circadian function is related in the developmental trajectory of mood disorders. What roles do genetics and environmental disruptors of the SCN play in the development of mood disorders? The animal literature appears to suggest that both genetics and environment can disrupt circadian rhythms that result in alterations to affective behavior, and human studies are revealing similar findings.

In North America, prevalence rates of depression are higher during the winter months for populations at higher latitudes (Rosen et al., 1990; Patten et al., 2017), suggesting that less sunlight during the winter may reduce the ability of the SCN to maintain internal synchrony. However, the same has not been shown to be true of Northern European countries (Mersch et al., 1999). Therefore, socio-cultural factors may contribute to protection against season-induced depression at higher latitudes, or alternatively contribute to their development. In support of the latter hypothesis is the

finding that descendants of Icelandic emigrants living in Northern Canada have lower prevalence rates of seasonal affective disorder (Magnusson & Axelsson, 1993). It is possible that cultural practices, such as those related to dietary feeding patterns, may serve a protective function against SCN rhythm disruption under low light conditions. However, more research is needed to evaluate the effects of socio-cultural factors on clock function and its role in the development of mood disorders.

Genetic abnormalities that affect clock genes in humans have been shown to increase the risk of developing a mood disorder. Familial advanced phase sleep syndrome, whereby sleep onset and wake occur much earlier than in the general population shows comorbidity with depression (Xu et al., 2005). In contrast, delayed sleep phase syndrome, whereby patients fall asleep and awake at later hours, commonly show a psychological profile of nervousness, neurosis and anxiety (Shirayama et al., 2003). In college students, depressive-like symptoms have been shown to be more common among "evening chronotypes" or people that are predisposed to a preference for working in the evening (Chelminski et al., 1999). Therefore, even a tendency towards promoting circadian misalignment with the day/night cycle can lead to negative affects. Together, genetic predispositions or having a strong chronotype and socio-cultural factors may exacerbate the development of mood disorders.

Night work and rotating shiftwork have been associated with circadian disruption and mental health outcomes (Haus & Smolensky, 2006;). In a 2-year study of night shift nurses, a decrease in symptoms of depression and anxiety where observed when

nightshifts were changed to daytime (Thun et al., 2014). Although nurses tend to work longer hours, shift work in general has shown to play a role in the development of mood disorders over time (Scott, 2000). Some argue that it is the presence of light at night that impairs the signal from the SCN in promoting strong consolidation of the biological sleep/wake cycle (Ikeno & Yan, 2016). However, others believe that implementing proper sleep and feeding "hygiene" by reversing the rest/activity cycle can combat negative health outcomes of shift work by maintaining circadian rhythmicity through the food clock rather than the SCN (Moran-Ramos et al., 2016; Zarrinpar, Chaix & Panda, 2016; Reynolds et al., 2017). Ultimately, it is the communication between these two clocks on circadian output that would bring the best understanding for treatment and prevention of psychiatric illness that is either the result of, or exacerbated by, circadian disruption (Herzog et al., 2017).

The role of DA in mood disorders: past and future perspectives

The etiology of mood disorders remains unknown. The interplay of neurotransmitter systems in the brain is believed to be an integral role in mental disorders. DA, serotonin, and norepinephrine are known to regulate mood. The hypothesis that these neurotransmitters are out of balance in mood disorders has been widely accepted in the past (Schildkraut, 1965). As a result, the use of antidepressants targeting catecholamine function has become the gold standard in the pharmacological treatment of mood disorders. However, there has been much debate regarding this hypothesis with the development of brain neuroimaging techniques, whose findings are highly variable and inconsistent regarding the role of catecholamine function in the

pathophysiology of mood disorders (Nikolaus et al., 2009; Phillips et al., 2015). Furthermore, a large confound in these studies is the use of medication. It is known that patients show large variability in how they respond to the type of antidepressant taken, with many patients not responding to treatment (McIntyre et al., 2014). This has led to the speculation that inconsistency between study results regarding catecholamine function in a mood disorder are not reflective of the mood disorder itself, but rather the individual differences in how a patient's brain responds to medication. To resolve this issue, studies have aimed to identify which genetic and/or environmental factors may predict response rates to antidepressants (Kim et al., 2000; 2006; Biernacka et al., 2013; Hashimoto, 2015; Phillips et al., 2015), though little advancement has been made.

One possibility for the lack of advancement in our understanding of catecholamine function in depression may be related to the oversimplification of neurotransmitter function. Bioavailability in neurotransmitter function is an oversimplification of how the brain works. Neurotransmission is a dynamic and complex system that varies with time of day (Wirz-Justice, 1987; Sleipness et al., 2007). Furthermore, circadian rhythms of neurotransmitter function occur at many levels of brain function. DA, glutamate and GABA oscillate in neurotransmitter synthesis, receptor levels, synaptic release, and enzymes related to their breakdown. For example, extracellular DA cycles with a different circadian phase in the striatum compared to the nucleus accumbens (Castaneda et al., 2004). DA receptor binding in whole brain tissue has been shown to differ with time of day and season, despite being maintained in a constant LD cycle

(Wirz-Justice, 1987). Other proteins critical to DA function, such as tyrosine hydroxylase (TH) and dopamine transporter (DAT) also differ with time of day, brain region, and rely on signals from the SCN (Sleipness et al., 2007). As a result, the ability of environmental factors in shifting DA rhythms differs with brain region and time of day.

Whereas the circadian rhythm of DA function in many brain regions can be affected by signals from the SCN (Sleipness et al., 2007), only in the striatum is the extracellular rhythm of DA directly altered by environmental changes to light (Castandeda et al., 2004). Likewise, RF has been shown to alter the temporal rhythm of extracellular DA in the striatum, such that it reorganizes itself around the feeding window during a regular LD cycle (Liu et al., 2012). Therefore, both timing of light and food have the ability to change DA rhythms in the striatum, and RF can override signals from the SCN. What this means in terms of our mental health needs further investigation. However, understanding the importance of circadian rhythms in the brain has led some researchers to question whether it is the desynchrony between rhythms, and in particular DA, that disrupts the communication of neurotransmitter systems important to the pathophysiology of mood disorders (McClung, 2013; Verwey et al., 2016).

Increasingly more studies are demonstrating the role of DA in regulating circadian rhythms through environmental factors (Korshunov et al., 2017). Furthermore these influences are bidirectional whereby circadian rhythms can feed back to alter DA circuits (Verwey, Dhir & Amir, 2016). The development of a Clock gene mutant mouse, where the function of the clock gene *Clock* is impaired specifically in the ventral tegmental area

(VTA) has shown phenotypic behavior resembling depression and mania (Mukherjee et al., 2010). The VTA is a region rich in DA neurons, which has led to the speculation that circadian clock gene function in DA neurons is important in the development of mood disorders. Given that DA rhythmicity can control clock function we hypothesize that DA is also involved in the circadian food entrainment pathway. If so, food entrainment could regulate mood under normal conditions or prevent alterations in affective behavior due to circadian disruption.

Current thesis

The study objectives of the current thesis can be organized into three areas of interest. Firstly, the idea that DA sensitization is involved in food entrainment was evaluated through its effects on behavior. Both FAA and cross-sensitization to amphetamine (AMPH) were evaluated as measures of DA enhancement during food entrainment. Secondly, measures of DA function in the DS were measured in rats exposed to RF, namely cFOS protein (FOS), TH and DAT. Lastly, the idea that RF may promote mental health was investigated using animal models of anxiety and depression.

CHAPTER 1

Exploring the role of locomotor sensitization in the circadian food entrainment pathway

Hanna Opiol, Nuria de Zavalia, Tara Delorme, Pavel Solis, Spencer Rutherford, Uri Shalev, Shimon Amir

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Author Contributions:

Conceptualization: Hanna Opiol, Shimon Amir.
Data curation: Hanna Opiol, Shimon Amir.
Formal analysis: Hanna Opiol.
Funding acquisition: Shimon Amir.
Investigation: Hanna Opiol, Nuria de Zavalia, Tara Delorme, Pavel Solis, Spencer Rutherford.
Methodology: Hanna Opiol, Shimon Amir.
Project administration: Shimon Amir.
Resources: Shimon Amir, Uri Shalev.
Supervision: Shimon Amir.
Validation: Shimon Amir, Uri Shalev.
Visualization: Hanna Opiol, Shimon Amir.
Writing – original draft: Hanna Opiol.
Writing – review & editing: Shimon Amir, Uri Shalev, Nuria de Zavalia.

Abstract

Food entrainment is the internal mechanism whereby the phase and period of circadian clock genes comes under the control of daily scheduled food availability. Food entrainment allows the body to efficiently realign the internal timing of behavioral and physiological functions such that they anticipate food intake. Food entrainment can occur with or without caloric restriction, as seen with daily schedules of restricted feeding (RF) or restricted treat (RT) that restrict food or treat intake to a single feeding time. However, the extent of clock gene control is more pronounced with caloric restriction, highlighting the role of energy balance in regulating clock genes. Recent studies have implicated dopamine (DA) to be involved in food entrainment and caloric restriction is known to affect dopaminergic pathways to enhance locomotor activity. Since food entrainment results in the development of a distinct behavioral component, called food anticipatory activity (FAA), we examined the role of locomotor sensitization (LS) in food entrainment by 1) observing whether amphetamine (AMPH) sensitization results in enhanced locomotor output of FAA and 2) measuring LS of circadian and non-circadian feeding paradigms to an acute injection of AMPH (AMPH cross-sensitization). Unexpectedly, AMPH sensitization did not show enhancement of FAA. On the contrary, LS did develop with sufficient exposure to RF. LS was present after 2 weeks of RF, but not after 1, 3 or 7 days into RF. When food was returned and rats regain their original body weight at 10–15 days post-RF, LS remained present. LS did not develop to RT, nor to feedings of a non-circadian schedule, e.g. variable restricted feeding (VRF) or variable RT (VRT). Further, when RF was timed to the dark period, LS was observed only when tested at night; RF timed to the light period resulted in LS that was present

during day and night. Taken together our results show that LS develops with food entrainment to RF, an effect that is dependent on the chronicity and circadian phase of RF but independent of body weight. Given that LS involves reorganization of DA-regulated motor circuitry, our work provides indirect support for the role of DA in the food entrainment pathway of RF. The findings also suggest differences in neuronal pathways involved in LS from AMPH sensitization and LS from RF.

Introduction

Circadian locomotor activity, predominantly under the control of the light/dark cycle, can be reorganized to adapt to daily time-restricted events of biological importance. Timekeeping mechanisms in the brain and/or body can anticipate a circadian event by shifting endogenous clock gene rhythms and behavioral locomotor output. Daily time-restricted access to a palatable treat (Mistlberger & Rusak, 1987; Angeles-Castellanos et al., 2008) or mating window (Hsu et al., 2010b; Landry, Opiol, Marchant, Pavlovski, Mear, et al., 2012) has been shown to result in anticipatory behavioral activity prior to the scheduled event. Similarly, daily-timed injections of rewarding or DA-activating drugs show anticipatory activity preceding injection (Kosobud et al., 1998; Gallardo et al., 2014). The most notable example of circadian reorganization to a time-restricted event is the implementation of circadian RF.

RF requires two components: 1) that complete caloric intake be restricted to a specific feeding window, usually a few hours in length and 2) that the timing of feeding does not vary extensively between each circadian cycle. The effects of RF can be seen as soon as the following day, including shifted clock gene expression, neuroendocrine signaling and the reorganization of circadian behavior (Patton & Mistlberger, 2013). The biological changes that arise from circadian scheduled feeding are referred to as food entrainment. The mechanism of food entrainment is unknown, however one of the defining behavioral features is the development of FAA prior to feeding time. Feeding-related behavior, including FAA, has been related to DA function in motor regions of the brain (Gallardo et al., 2014; Palmiter, 2008). Taken together, it is speculated that daily

time-restricted events affecting DA function may alter components of the circadian clock, resulting in a mechanism by which organisms are able to anticipate the time of their occurrence.

One possibility is that clock genes within particular brain regions, such as those driving locomotor output, may be involved in the timekeeping of salient events by sensing changes to the circadian rhythmicity of DA. For example, stimuli that act on DA in a circadian fashion, when the organism deems the stimuli sufficiently salient (e.g. food under food-restricted conditions), may act as an input signal for clock genes regulating diurnal activity. DA has been shown to shift and regulate circadian locomotor output and influence clock genes in brain regions important for motor output (Gallardo et al., 2014; Imbesi et al., 2009; Sahar, et al., 2010; Hood et al., 2010; Smit et al., 2013; Blum et al., 2014; Frederick et al., 2015; Iijima et al., 2005). Of particular interest is the clock gene Per2, whose protein expression is under the control of DA in the dorsal striatum (Hood et al., 2010). The role of Per2 in food entrainment remains undetermined (Chaix & Panda, 2016), however mice with a mutation for Per2 show significant impairment of FAA (Feillet et al., 2006), suggesting it is important for locomotor output of food entrainment. Interestingly, time has previously been shown to act as a conditioned stimulus for the locomotor expression of AMPH sensitization (Arvanitogiannis et al., 2000), and clock genes appear to play a critical role in the expression of behavioral sensitization (Abarca et al., 2002; Liu et al., 2005; Liu et al., 2007; McClung et al., 2005). Therefore, time-keeping mechanisms through which food entrainment

reorganizes behavioral rhythms could involve neuroplastic changes to motor circuits that are also important for LS to reward-related stimuli (McClung & Nestler, 1998).

The term LS refers to the enhancement in behavior due to the (repeated) application of a stimulus (Stewart & Badiani, 1993). LS occurs as a result of long-term neurochemical changes in the brain, which produce hypersensitivity to neurotransmitter systems, including the DA system (Vanderschuren & Pierce, 2010; Robinson & Berridge, 1993). One of the defining factors of LS is that the effects last beyond the cessation of whatever stimuli initiated the locomotor changes, and is often enhanced during the time of withdrawal or period of "incubation" (Liu, Wang, Jiang, Wan, Zhou et al., 2007). Therefore, LS can be thought of as the composite of two critical stages: its development and its expression (Kalivas, Sorg, & Hooks, 1993; Pierce & Kalivas, 1997). Development involves neuroplastic changes that occur during the application of a repeated stimulus and during the incubation period after stimuli cessation. The expression of LS involves the persistence of the neuronal changes that occurred during development. Furthermore, when different stimuli act on the same neuronal networks involved in LS, often times cross-sensitization can be observed, such that LS resulting from one stimulus produces a hyper-response to the second stimulus (Avena & Hoebel, 2003). A recent study found that the presence of a locked running wheel resulted in the enhancement of FAA in mice with previous running wheel exposure, while mice naive to a running wheel showed no change in FAA (Flôres et al., 2016). The authors hypothesized that FAA, like running wheel activity, involves reward-signaling mechanisms that can further be enhanced by rewarding stimuli.

In the first part of our study we tested whether behavioral properties of food entrainment (FAA) could be augmented by enhancing DA activity through repeated AMPH exposure (AMPH sensitization). Unexpectedly, we found that there were no additive effects of AMPH sensitization on FAA, both when testing between and within group designs. We wondered whether our control groups, which were also subjected to RF, could have developed LS as a result of caloric restriction (Carr KD et al, 2003; Carr, 2007; Cabib et al., 2000). The threshold-lowering effects of rewarding electrical brain stimulation by drugs of abuse that act on DA, such as AMPH, can be potentiated with caloric restriction, leading to the speculation that caloric restriction produces additive effects through the DA system (De Vaca & Carr, 1998; De Vaca, Krahne, & Carr, 2004. However, AMPH sensitization (repeated exposure) does not result in the augmentation of electrical brain self-stimulation (De Vaca, Krahne, & Carr, 2004), suggesting that sensitization of drug reward in the rewarding electrical brain stimulation paradigm and LS might be mediated by different neural mechanisms. Given the previous findings, we hypothesized that either AMPH sensitization does not act through the same neuronal networks as food entrainment, and therefore we should not expect to see additive effects in FAA. Or else, a second possibility might be that control groups that were subjected to RF, also developed LS, which would then result in a ceiling effect of FAA between groups. We further investigated the latter option.

The second part of our study evaluated whether RF could result in LS. We tested various circadian and non-circadian feeding schedules on its effects of cross-

sensitization to AMPH. We found that the most important aspect of feeding-induced LS was the combination of a circadian feeding schedule and caloric restriction. Body weight appeared to be irrelevant for RF-induced LS. Previous work has shown that the effects of caloric restriction on the sensitization of drug reward are related to weight loss. The augmentation of drug reward by caloric restriction was reversed after one week of returning rats to free access feeding (Carr, 2002) and during longer periods of food restriction, the reversal of food-induced behavioral sensitization paralleled the return of body weight to baseline (De Vaca, Krahne, & Carr, 2004; Carr & Wonlinsky, 1993; Abrahamsen, Berman, & Carr, 1995). In our study rats were returned to free access feeding prior to testing, whereby they regained pre-RF body weight. Our findings suggest that it is the duration of RF and/or a period of incubation that is necessary for the behavioral expression of LS. These observations are consistent with the idea that endogenous mechanisms regulating food entrainment include LS.

Methods and Materials

Subjects

One hundred fifty-eight male Wistar rats (Charles River, St. Constant) weighing between 125–200 grams at the beginning of experiment were individually housed in running wheel cages placed inside individual sound attenuating chambers. A 12:12 light/dark cycle was maintained for 2 weeks before beginning each experiment. Light intensity inside the cage measured ~300 lux. Rats had free access to tap water and rodent lab chow, except when subjected to scheduled feeding. Injections of AMPH or saline were administered to rats via intraperitoneal route. All experimental procedures

were conducted under the guidelines of the Canadian Council of Animal Care and approved by the animal care ethics committee at Concordia University, Quebec.

Feeding schedules

Food (lab chow) was removed 22–24 hours prior to starting timed-restricted feedings with caloric restriction. As depicted in Fig 1, RF rats were fed during a daily 2-3h feeding window corresponding to Zeitgeber time (ZT) 5–8, where ZT 0 is lights on. Placing RF in the middle of the light period was done by convention to allow for a low background of activity from which FAA can be easily identified. Night RF (NRF) rats were fed during a daily 2-3h feeding window from ZT 17–20. To evaluate the effects of caloric restriction without food entrainment, variable feeding schedules were applied. Variable RF (VRF) and night VRF (NVRF) rats were fed at variable times each day between ZT 0–12 and ZT 12–24, respectively. Note that the hours for VRF were restricted to daytime as opposed to 24h-VRF due to a previous report of PER2 disruption in the dorsal striatum as a result of daytime-VRF but not 24h-VRF (Verwey & Amir, 2012). To evaluate the effects of food entrainment without caloric restriction, we allowed rats daily access to a restricted treat (RT) and variable RT (VRT). RT and VRT consisted of daily scheduled unlimited access to Oreo cookies (Cadbury, Mondelez-International) between ZT 5–7, and for 2 hours at variable times between ZT 0–12, respectively. RT and VRT had free access to lab chow at all times.

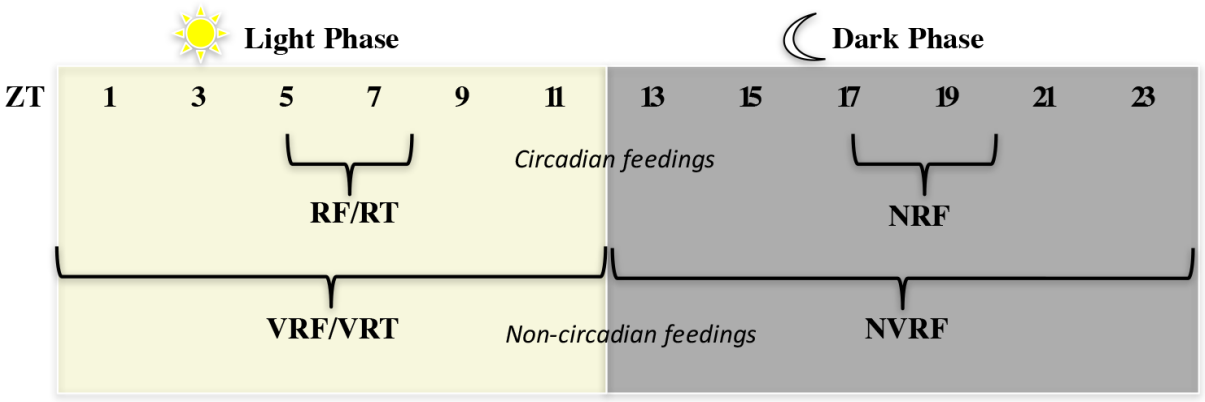


Fig 1. Schematic of daily feeding schedules. Circadian feedings occurred daily during a 2-3h feeding window during the lights on phase (RF/RT) or lights off phase (NRF). Non-circadian feedings occurred daily during a variable 2h-feeding window, restricted to the lights on phase (VRF and VRT) or lights off phase (NVRF).

Exp 1–3 protocols: AMPH sensitization effects on FAA

In experiment 1, rats were divided into 2 groups (*n* = 6) and given 5 x 1.5 mg/kg AMPH or saline every 48 hours during the lights on period ZT 4–6, followed by 7 days of incubation (no injection). Rats were then subjected to 12 days RF to evaluate FAA between groups. In experiment 2, rats were subjected to RF for 2 weeks before AMPH sensitization (RF-before) followed by a 1-week break. Next rats were divided into 2 groups (*n* = 6) and given injections of AMPH, 3 mg/kg daily for 6 days (AMPH group) (Avena & Hoebel, 2003), or no injection (control group). Controls were not handled to minimize stress-induced sensitization that can occur from repeated saline injections (Paulson & Robinson, 1996). AMPH injections occurred between ZT 14–16, 2-4h into the dark period to mimic the natural rhythm of DA in the dorsal striatum (Hood et al, 2010). A period of incubation (10 days) followed AMPH sensitization and rats were subjected to RF a second time (RF-after) for 2 weeks. Rats were again returned to free access feeding for 1 week and subjected to an AMPH challenge test to see whether differences in LS between AMPH pretreated rats and controls was present. Since non-handled controls used in this protocol had not previously been subjected to injections, a habituation day was done on the day prior to the AMPH challenge. On habituation day both groups received an injection of saline and open-field activity was measured. On AMPH challenge day both groups received a low dose of AMPH (.5 mg/kg) and open-field activity was measured again. Challenge injections occurred between ZT 2–8.
In experiment 3, rats were divided into 2 groups (*n* = 6) and given 5 x 1.5 mg/kg AMPH or saline every 72 hours, during ZT 14–16, followed by 14 days incubation (Vezina, 1996). Rats were then subjected to 5 weeks of RT. To observe whether there were any

masking effects of light on FAA, week 5 of RT was conducted in constant darkness. Following the cessation of RT, rats underwent a 1-week break and were subjected to an AMPH challenge. For the challenge test, both groups received a low dose of AMPH between ZT 2–8 and open-field activity was measured. See Fig 2 for a timeline of AMPH sensitization protocols in relation to feeding schedule used in experiments 1–3, to investigate the effects of AMPH sensitization on FAA.

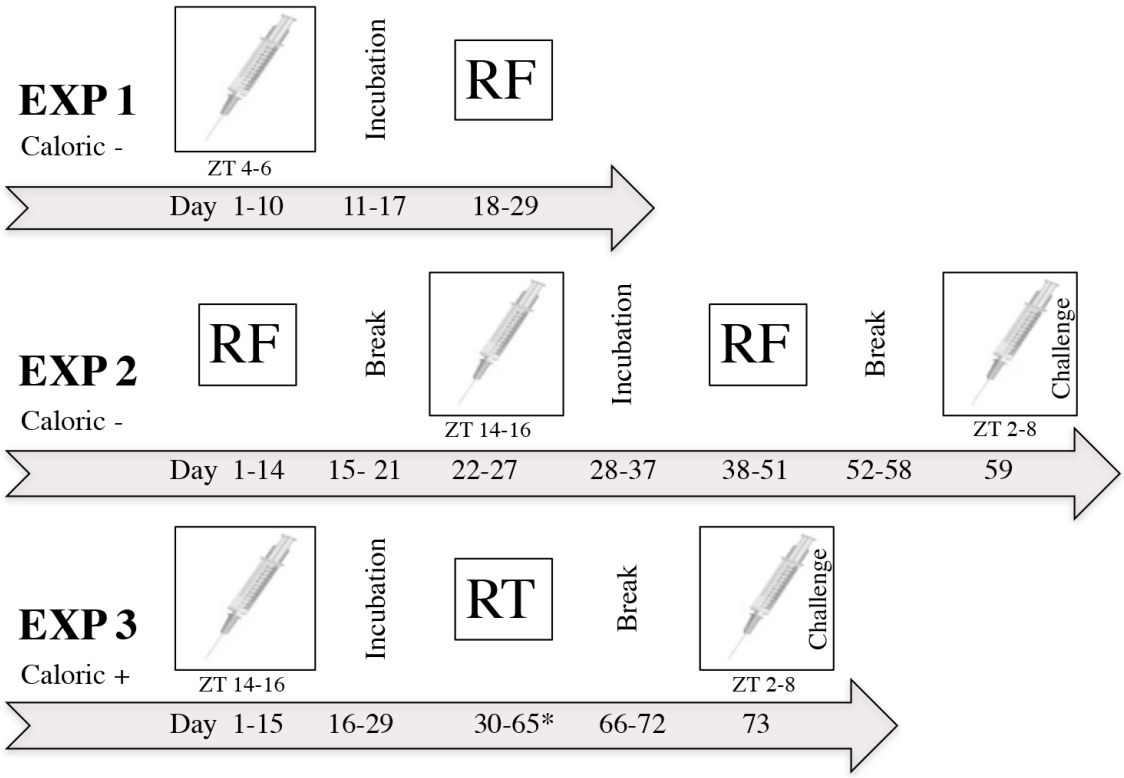


Fig 2. Exps 1–3: protocols for evaluating effects of AMPH sensitization on FAA. Experiments 1 and 3, use a between groups design to evaluate FAA in AMPH pretreated rats and controls, and experiment 2 uses a within group design to evaluate FAA before and after AMPH sensitization. Caloric restriction (-) or surplus (+) indicates the use of RF or RT, respectively, in each experiment. Timelines for AMPH sensitization and scheduled feeding are described below. Exp 1: rats were subjected to AMPH sensitization followed by an incubation period and RF. Exp 2: rats were placed on RF before AMPH sensitization (RF-before), followed by a break, and then divided into 2 groups: non-handled control or AMPH for AMPH sensitization, followed again by a break (incubation) and RF (RF-after). Rats were next returned to free access feeding and then challenged with a low dose of AMPH to see whether differences in LS were present between AMPH-pretreated rats and controls subjected to RF. The day prior to the AMPH challenge (day 58), a habituation run using a saline injection was conducted. Exp 3: rats were divided into 2 groups and subjected to AMPH sensitization followed by an incubation period prior to being placed on RT for 5 weeks. *During week 5 (days 59–65) the lights remained off (DD). As in experiment 2, following a break rats were challenged with AMPH to see whether differences in LS were present between AMPH pretreated rats and controls subjected to circadian feeding with no caloric restriction (RT). Both AMPH challenge tests (Exp 2 and 3) occurred during the lights on phase between ZT 2–8, consistent with time of previous FAA expression. Each AMPH sensitization protocol was selected based on the criteria that a prior study verified it to result in an increase of extracellular DA in the striatum and/or enhanced locomotor activity.

Exp 4–9 protocols: Feeding cross-sensitization to AMPH

In experiments 4–8, rats were divided into groups determined by feeding schedule (RF, VRF, RT, VRT, NRF or NVRF). Control (CTRL) groups had no changes to diet. Feeding schedules were followed by 10–15 days of free access (ad libitum, AL) feeding, which served as time for rats to regain body weight and as an incubation period. After the incubation period all rats were administered a low dose of AMPH (.5 mg/kg) to test for cross-sensitization of locomotor activity in an open-field box. Groups were assigned as follows. Exp 4: rats were divided into 2 groups, CTRL or RF (n = 6), that were further subdivided to include a saline injection subgroup for the cross-sensitization test. Exp 5: rats were divided into 3 groups: CTRL, RF or VRF (n = 4). Exp 6: rats were divided into 3 groups: CTRL, RT, and VRT (n = 4). Exp 7–8: rats were divided into 5 groups: CTRL, RF, VRF, NRF and NVRF (n = 5). Injections for the cross-sensitization test occurred during the light phase (ZT 2–8), with the exception of experiment 8, where injections occurred during the dark phase (ZT 14–17). In experiment 9, rats were divided into 7 groups (RF1, RF3, RF7, RF14, AL5, AL10 and CTRL), RF1, RF3, RF7 and RF14 were subjected to RF for 1, 3, 7 and 14 days, respectively, and tested for cross-sensitization on the last day of RF. AL5 and AL10 were subjected to 14 days RF and tested for cross-sensitization at 5 or 10 days post-RF. CTRL remained on free access feeding and were evenly divided across days of AMPH cross-sensitization tests. Injections for the cross-sensitization test occurred during the light phase (ZT 2–8). See Fig 3 for a detailed timeline of cross-sensitization protocols in experiments 4–9.

Feeding schedule and cross-sensitization to AMPH

EXP	Feeding schedule (groups)	Cal (+/-)	Feeding (days)	Return to AL (days)	Amph test (day)	Injection time (ZT)
4	RF	-	1-14	15-25	25	2-8 (light phase)
5	RF, VRF	-	1-14	15-25	25	2-8
6	RT, VRT	+	1-21	22-32	32	2-8
7	RF, VRF, NRF, NVRF	-	1-14	15-30	25-30	2-8
8	RF, VRF, NRF, NVRF	-	1-14	15-30	25-30	14-17 (dark phase)
9	RF1, RF3, RF7 RF14, AL5, AL10	-	1, 1-3, 1-7 1-14	n/a n/a, 14-20, 14-25	1, 3, 7 14, 20, 25	2-8

Fig 3. Exps 4–9: protocol timelines for testing feeding schedule cross-sensitization to AMPH. In experiments 4–8, different feeding schedules were tested for cross-sensitization to AMPH 10–15 days after returning to free access feeding (ad libitum, AL). In experiment 9, cross-sensitization of RF was tested on days 1, 3, 7 or 14 of RF (RF1, RF3, RF7, RF14) and at 5 or 10 into ad libitum feeding following 2 weeks of RF (AL5, AL10). The use of caloric (cal) restriction or surplus by experimental design are indicated with—or +, respectively. CTRL rats had no changes to diet and were tested on the same day as feeding groups. Experiment 4 also included a saline injection subgroup as a second control group on AMPH test day. All injections occurred between ZT 2–8, the time of day corresponding to FAA in RF rats, with the exception of experiment 8 where injections occurred between ZT 14–17, corresponding to FAA for NRF rats. Feeding group abbreviations: RF = restricted feeding, VRF = variable RF, RT = restricted treat, VRT = variable RT, NRF = night RF, NVRF = night VRF, CTRL = control.

Data collection and analysis

Running wheel activity was collected from the home cage of each rat using VitalView software (Mini Mitter, Bend, OR) and recorded in 10 min bins. The data was used to graph activity as group averages during different stages of each experiment. FAA ratios were calculated using the sum running activity 3h prior to feeding (ZT 2–5) divided by the sum nocturnal running activity (ZT 12–24).

Open-field data was collected using Tru Scan 2.0 activity monitoring system (Coulbourn Instruments, Whitehall, PA). Three or four rats were tested simultaneously within the same room using separate boxes (39 x 42 x 50 cm) with plexiglass transparent walls placed inside sound attenuation boxes constructed from insulation board. Total distance covered (cm) was collected in 5 min bins during a period of 1.5 h (30 min habituation, followed by 60 min post-injection). Distance-travelled ratios were later calculated as a measure of LS by summing distance travelled post-injection divided by total distance travelled.

Raw body weight scores were recorded for each group across stages of the experiment. To check whether rats had regained their pre-scheduled feeding (pre-RF/RT) body weight by test day, the percent pre-RF/RT weight was calculated (using weight on test day and pre-RF/RT weight).

Statistical analysis

Two-way repeated measures ANOVA was used to evaluate FAA ratios, with day or week of RF as the within subject time factor and AMPH sensitization versus control as the between subject group factor. Unpaired t-tests, one-way ANOVA or two-way ANOVA (in the case that injection was a factor itself) were used to evaluate open-field behavior by comparing the group means of distance-travelled ratios or total distance travelled post-injection. Post-hoc analyses, indicated below, were applied when the test yielded significance. Rats with ineffective injections were excluded from analysis.

Results

Exp 1–3: AMPH-pretreated rats do not show enhancement of FAA to RF or RT

Experiments 1–3 examined whether behavioral response of FAA is enhanced as a result of AMPH sensitization, since AMPH sensitization enhances DA activity in the dorsal striatum (Paulson & Robinson, 1995) and the dorsal striatum has been implicated in food entrainment (Gallardo et al., 2014). We hypothesized that if food entrainment acts through the same DA signaling pathway as AMPH sensitization, then there may be additive effects in the form of increased FAA in AMPH-pretreated rats subjected to a circadian feeding schedule.

In experiment 1, AMPH-pretreated rats and saline controls were placed on RF to see whether there would be any differences in FAA as measured by FAA ratios. Fig 4A shows FAA development for rats pretreated with saline or AMPH. Both groups

increased FAA from day 1 to 12 as expected, $F_{(11, 110)} = 11.99$, $p < 0.01$, but there were no differences between groups $F_{(1, 10)} = 0.48$, $p = 0.51$ and there was no interaction between time and group, $F_{(11, 110)} = 0.84$, $p = 0.60$ (Fig 4B). FAA ratios averaged across total RF time also did not differ significantly, $t_{10} = 1.47$, $p = 0.17$ (Fig 4C).

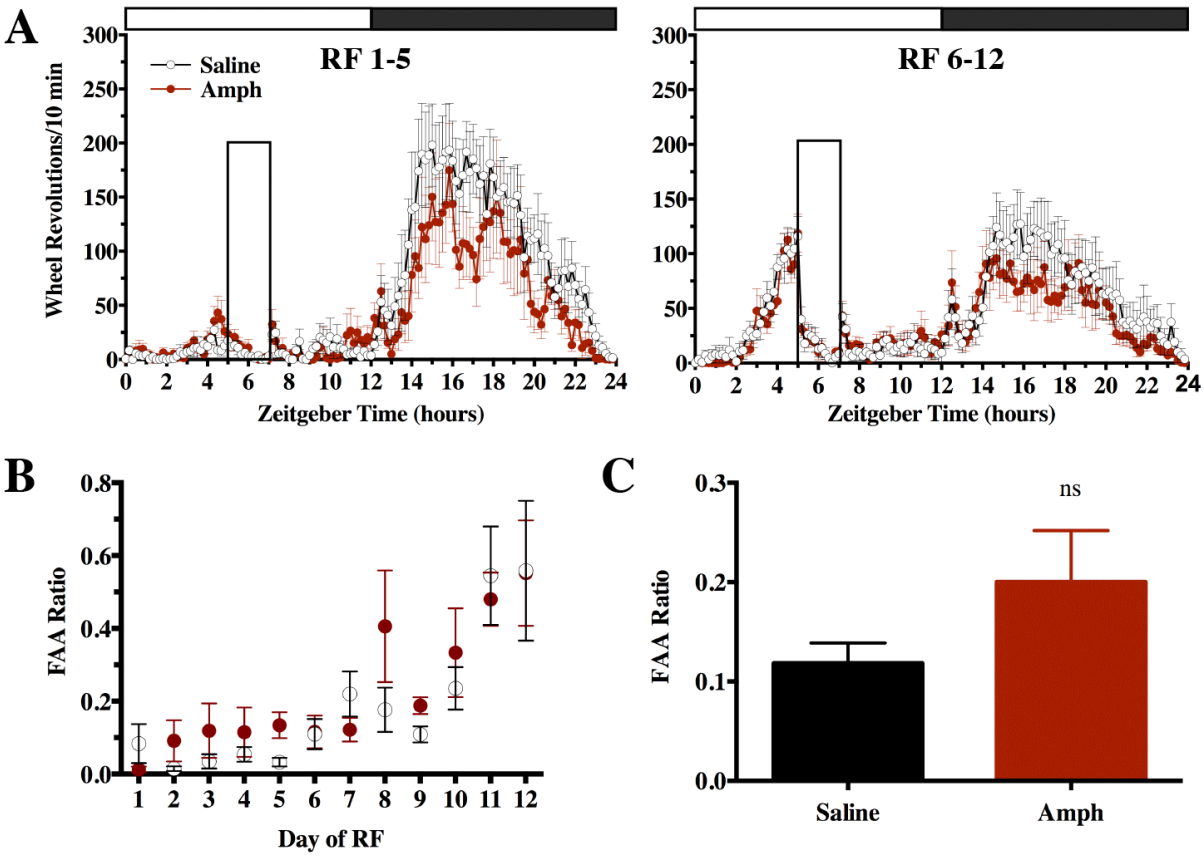


Fig 4. Exp 1: measuring FAA to RF in rats previously exposed to AMPH or saline injections, between group design. Home-cage activity expressed in wheel revolutions /10 min +/- SEM for AMPH and saline pretreated rats, during RF days 1–5 and 6–12, closed box indicates feeding window (**A**). FAA ratios +/- SEM shown as group averages for each day of RF (**B**) and for the total 12 days of RF (**C**), not significant, *ns*.

In experiment 2, rats were tested for within group differences in FAA before and after AMPH treatment (Fig 5A). Fig 5B shows no differences in FAA when comparing RF-before to RF-after in rats injected with AMPH and non-handled controls as measured by FAA ratios; time factor $F_{(1,10)} = 1.14$, $p = 0.31$, group factor $F_{(1,10)} = 0.52$, $p = 0.49$ and no interaction $F_{(1,10)} = 0.002$, $p = 0.96$. Unexpectedly, at the end of the experiment when all rats underwent an AMPH challenge procedure, there was no difference in distance-travelled ratios between groups, both on habituation day and on the following AMPH challenge day ($t_{10} = 0.94$, $p = 0.37$ and $t_{10} = 0.32$, $p = 0.76$, respectively). The results suggested that RF could have resulted in locomotor sensitization of the control group (Fig 5C).

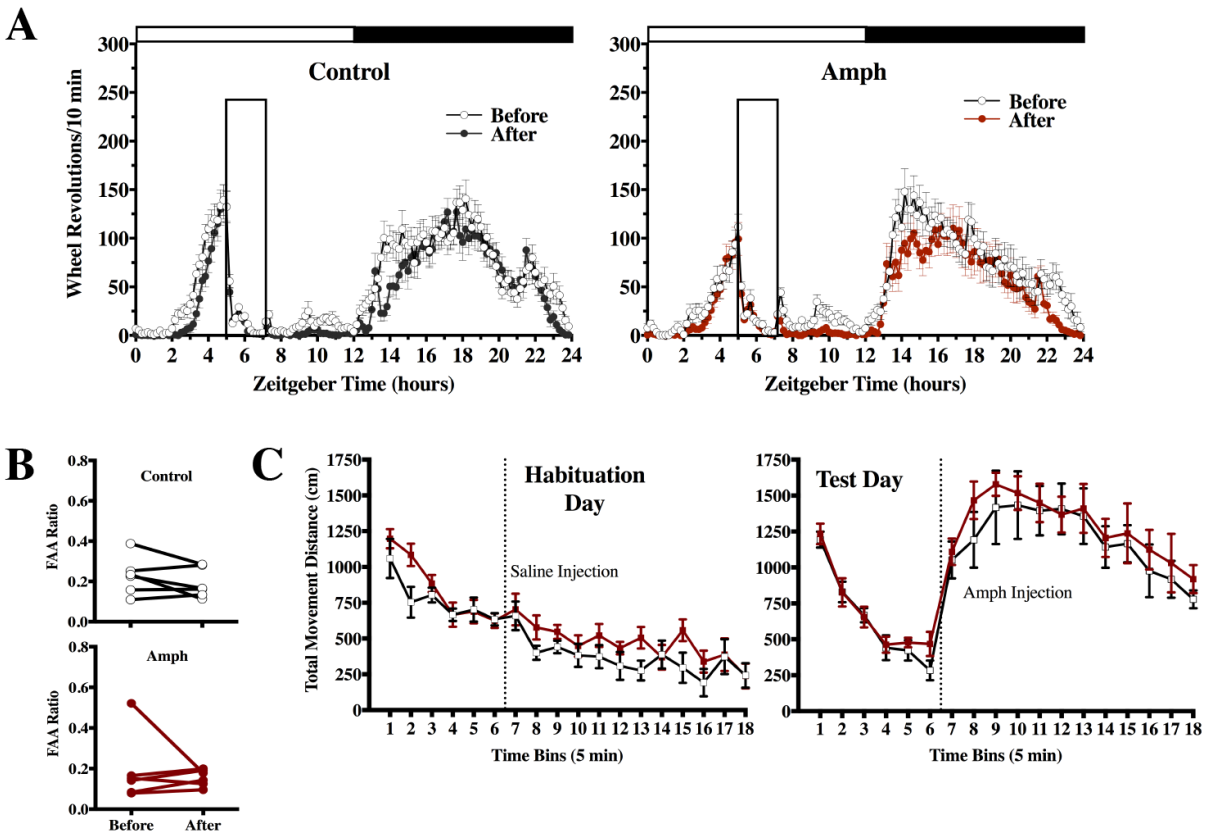


Fig 5. Exp 2: measuring FAA to RF before and after AMPH sensitization compared to non-handled controls, within group design. Home-cage activity expressed in wheel revolutions /10 min +/- SEM before and after AMPH sensitization for AMPH-pretreated and non-handled controls, closed box indicates feeding window (**A**). FAA ratios +/- SEM expressed as individual rat FAA ratios during RF-before and RF-after (**B**). AMPH-pretreated rats and non-handled controls injected with saline on habituation day and AMPH on test day, with open-field activity measured as total movement distance +/-SEM in 5 min bins (**C**).

In experiment 3, we tested whether rats fed a daily RT, without caloric restriction, would show enhancement of FAA when pretreated with AMPH compared to saline controls. RT is known to induce FAA prior to a daily scheduled treat, although this activity is not as robust as FAA in rats with caloric restriction and takes longer to develop (Mistlberger & Rusak, 1987; Angeles-Castellanos, Salgado-Delgado, Rodriguez, Buijs, Escobar, 2008). Even by prolonging the feeding schedule of RT and placing rats into constant darkness to check for any masking effects of light on activity during the final week, we found no difference in FAA between rats pretreated with saline or AMPH as measured by FAA ratios, $F_{(1, 10)} = 0.85$, $p = 0.38$. Both groups increased FAA from week 1 to week 5, $F_{(4, 40)} = 4.40$, $p < 0.01$, with no significant interaction between time and group $F_{(4, 40)} = 0.73$, $p = 0.58$ (Fig 6A and 6B). At the end of the experiment, an AMPH challenge was conducted, which appeared to show that AMPH-pretreated rats were more active than controls (Fig 6C), although these distance-travelled ratios were not significantly different $t_9 = 0.31$, $p = 0.76$, and neither were scores summing total activity post-injection, $t_9 = 1.69$, $p = 0.13$.

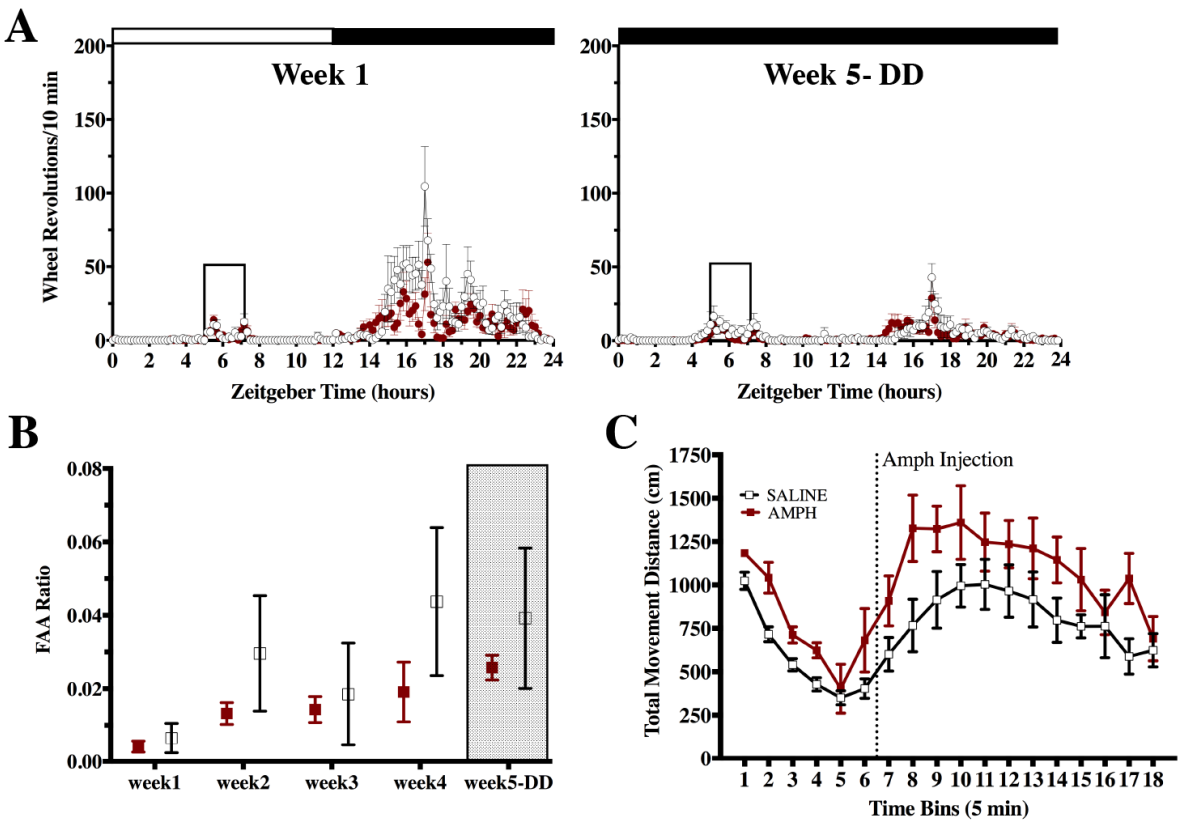


Fig 6. Exp 3: measuring FAA to a RT in rats previously exposed to AMPH or saline injections, between group design. Home-cage activity of AMPH and saline pretreated rats subjected to RT during week 1 and week 5 (constant darkness, DD), expressed in wheel revolutions /10 min +/- SEM, closed box indicates feeding window (**A**). FAA ratios +/- SEM for each week of RT (**B**). Open-field activity measured as total movement distance +/- SEM in 5 min bins for AMPH and saline pretreated rats injected with AMPH (**C**).

Exp 4–9: Crossover LS of circadian feeding to AMPH depends on caloric restriction, time of day and continuity of circadian RF

Experiments 4–9 tested the hypothesis that RF itself resulted in LS. We suspected that the reason experiments 1–3 failed to show an enhancement in FAA after AMPH treatment was that RF resulted in LS of the control group, which could then produce a ceiling effect in FAA between groups. We reasoned that if circadian RF resulted in sensitization of motor output, then possibly it was through the same mechanism as AMPH sensitization and therefore crossover sensitization should be possible from RF to an acute injection of AMPH. To test this hypothesis we placed rats under various feeding paradigms, returned them to AL feeding to restore body weight and then administered a low-dose of AMPH to measure locomotor activity in an open-field test. Feeding schedules varied, with the intent of testing the importance of caloric versus non-caloric restriction, circadian versus non-circadian feeding times, and feeding windows restricted to the day versus night. Because experiments 4–8 tested for cross-sensitization after a period of incubation (10–15 days post-RF), in experiment 9 we tested cross-sensitization during RF after 1, 3, 7 and 14 days of RF as well as 5 and 10 days post-RF.

In experiment 4 we tested whether RF resulted in cross-sensitization to AMPH 10 days post-RF. On test day, feeding groups were divided and administered an acute injection of AMPH or saline. A significant difference in distance travelled scores was found between injection treatment groups $F_{(1, 8)} = 15.35$, $p < .01$, but not between feeding groups, $F_{(1, 8)} = 0.08$, $p = 0.78$, and there was no interaction, $F_{(1, 8)} = 0.17$, $p= 0.69$.

Sidak's multiple comparison found differences between RF-AMPH and RF-saline, but not between CTRL-AMPH and CTRL-saline (Fig 7). In experiment 5, we tested whether cross-sensitization to AMPH was present in rats fed a VRF schedule. On test day differences in open-field activity between groups was detected, $F_{(2, 8)} = 4.62$, $p < 0.05$. Dunnett's post hoc test ($\alpha < .05$) revealed RF to be significantly higher than CTRL, but not VRF compared to CTRL (Fig 7). In experiment 6, we tested whether cross-sensitization to AMPH was present in rats subjected to RT or VRT. On test day there were no statistically significant differences in open-field activity between RT and VRT compared to CTRL, $F_{(2, 9)} = 1.91$, $p = 0.20$ (Fig 7). In experiment 7, we tested whether cross-sensitization to AMPH was present in rats subjected to RF, VRF, NRF and NVRF and found that there was a significant increase in open-field activity between groups, $F_{(4, 18)} = 2.98$, $p = 0.05$. Dunnett's multiple comparison ($\alpha < .1$) revealed RF to be significantly higher than CTRL. NVRF, although it appeared to have a high response to AMPH, was not statistically significantly different than CTRL (Fig 7). Upon inspection of individual rats' responses for NVRF, we found high variability that was evenly divided; 2 rats had high responses and 2 rats had a response similar to CTRL. In experiment 8, we tested whether cross-sensitization was present in rats subjected to RF, VRF, NRF and NVRF when tested during the lights off phase. On test day there was a significant difference in open-field activity between groups, $F_{(4, 16)} = 3.35$, $p = 0.04$. Dunnett's multiple comparison ($\alpha < .1$) revealed differences for RF and NRF compared to CTRL (Fig 7). In experiment 9, we tested whether cross-sensitization to AMPH was present in rats during day 1, 3, 7 or 14 of RF (RF1, RF3, RF7, RF14) and 5 or 10 days post 2 weeks RF (AL5, AL10) compared to CTRL rats. A significant difference in open-field

activity was detected between groups, $F_{(6, 37)} = 7.607$, $p < 0.01$. Dunnett's revealed significant differences for RF14 and AL5 compared to CTRL (Fig 7).

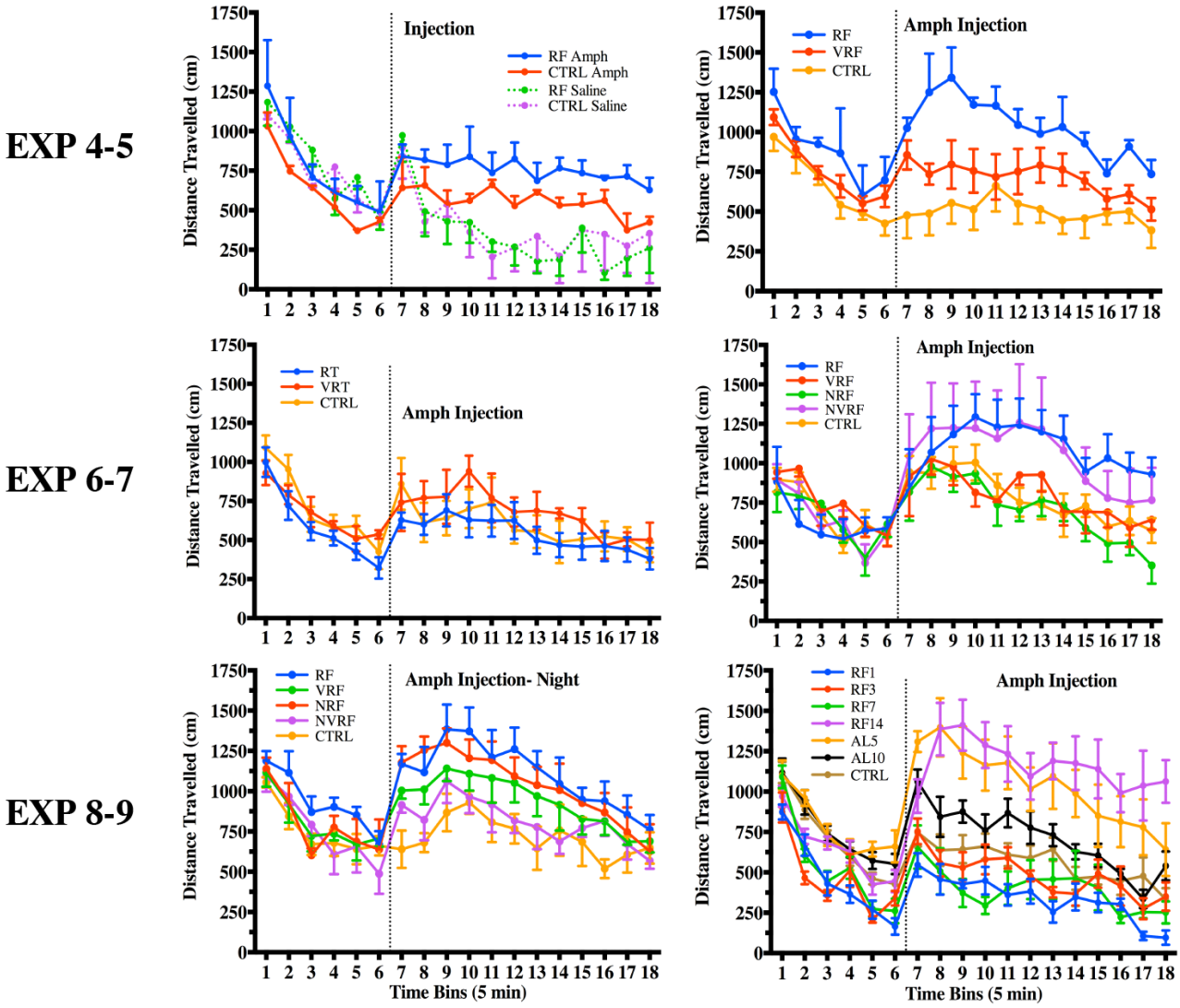


Fig 7. Feeding schedule cross-sensitization to AMPH test.
Distance travelled to an acute injection of AMPH, plotted in 5-min time bins +/- SEM for feeding groups in experiments 4–9, respectively. Dotted line indicates injection time.

Percent of pre-RF/RT body weight on cross-sensitization test day

Rats typically regained 100% pre-RF/RT body weight by test day, indicating RF cross-sensitization to AMPH does not run in parallel to the return of body weight (S1 Fig).

Discussion

Restricting caloric intake is known to enhance behavioral response to drugs of abuse in rats. We thought to investigate whether the opposite could be true of circadian RF, namely whether enhancing LS through repeated AMPH exposure would enhance FAA to RF. The latter hypothesis was of particular interest since AMPH sensitization is known to act through the DA system and food entrainment is believed to involve DA. Therefore, if both AMPH sensitization and food entrainment act through the same DA neuronal networks, then we should see additive effects on FAA. We found no evidence for AMPH sensitization enhancing FAA. This outcome was not completely explained by our second hypothesis that RF resulted in LS of control groups and therefore a ceiling effect in FAA, when compared to AMPH-pretreated rats subjected to RF.

With the second part of our study we verified that RF results in LS as measured by RF cross-sensitization to an acute injection of AMPH. LS from RF was found to require more than one week for behavioral expression, since an acute injection of AMPH on days 1, 3, and 7 of RF did not result in cross-sensitization. The latter finding was unexpected, as we had expected LS to be present as soon as caloric restriction was implemented. Therefore, if LS does not occur during the initial stages of food entrainment, some alternative explanation needs to be offered for why FAA among control groups did not differ from AMPH pretreated rats during the first week of RF in

experiments 1–3. One explanation may be related to the high variability and instability of FAA during the initial stages of food entrainment, typically seen in the first week of RF. Although a more plausible explanation is that AMPH sensitization and food entrainment have partly different neuronal mechanisms that do not overlap to produce additive effects on downstream locomotor output. In the intracranial self-stimulation paradigm it has been shown that caloric restriction increases AMPH reward, while AMPH sensitization does not increase AMPH reward (De Vaca, Krahne, & Carr, 2004). Our results may likewise be attributed to the inability of AMPH sensitization to produce additive effects on FAA.

The effectiveness of the chosen AMPH sensitization protocols to our experimental design may also be questioned. It is possible that AMPH sensitization protocols were not ideal for home-cage testing. Placing animals into a novel environment following AMPH injection facilitates sensitization (Badiani & Robinson, 2004) something that we did not do in the current protocols; however, the doses used for each protocol were high enough for sensitization to occur regardless of environmental conditions (Browman, Badiani, & Robinson, 1998). Different doses of AMPH and diurnal time of administration during sensitization and incubation have also been shown to produce differences in behavioral sensitization (Gaytan, Swann, & Dafny, 1998a, Gaytan, Swann, & Dafny, 1998b) and these factors were taken into account when selecting AMPH sensitization protocols. Additionally, AMPH withdrawal is known to result in an initial decrease in DA levels in the dorsal striatum (Paulson & Robinson, 1996), which is believed to contribute to the sensitization process. Since we did not measure DA levels directly, we cannot

confirm whether DA levels in AMPH-pretreated rats were low at initiation of RF, and perhaps this was why we did not observe FAA enhancement.

Given that DA is involved in food entrainment (Gallardo et al., 2014; Smit, et al., 2013; Liu et al., 2012; Opiol et al., 2015), a second reason why FAA may not have increased in rats repeatedly exposed to AMPH is that the neuroplasticity of DA through which AMPH achieves behavioral sensitization is different from the changes in DA that occur with RF. For example, AMPH sensitization relates more to dopaminergic changes involving the D1 receptor, rather than D2 (Vezina, 1996; Vezina & Stewart, 1989; Fletcher et al., 2005). Circadian links to DA appear to work closely with D2 receptor function, e.g. clock gene expression in the dorsal striatum (Hood et al., 2010) and FAA is shifted by treatment with D2 receptor agonists, but not D1 agonists (Smit et al., 2013) or D1 antagonists (Mistlberger & Mumby, 1992). Future experiments should test whether there are additive effects on FAA with psychostimulants that achieve behavioral sensitization through the DA D2 receptor (Beyer & Steketee, 2002; Song et al., 2014).

Since rats also show FAA to a RT, we examined whether a circadian feeding schedule without caloric restriction would show differences in FAA after being pretreated with AMPH. No differences in FAA were observed in AMPH-pretreated rats compared to controls while on a RT schedule, despite maintaining the schedule for an extended time and placing rats into constant darkness to unmask potentially hidden locomotor activity. Additionally, both RT and VRT schedules did not result in crossover sensitization to an acute injection of AMPH, indicating that some element of negative energy balance may

be necessary for feeding-related LS. Circadian feeding schedules without caloric restriction do not alter forebrain and hypothalamic oscillators to the same extent as RF (Verwey et al., 2007; Verwey et al., 2008). Caloric restriction is an important aspect contributing to the clock function of food entrainment and likewise is necessary for circadian feeding-induced LS.

As seen in experiments 4–8, cross-sensitization to AMPH in groups subjected to circadian RF was present when tested 10–15 days post-feeding. Experiment 9 showed that RF did not result in LS during the first week of RF. Similar findings have been demonstrated with acute food deprivation, and its failure to enhance brain stimulation reward (Fulton, Woodside, & Shizgal, 2000). FAA also requires about a week before it reaches its maximum and stable amplitude in daily locomotor activity, suggesting that LS may be related to the enhancement (or reorganization) of locomotor activity during the first week of RF. One limitation of our study is that we cannot rule out the possibility that behavioral expression of LS may have been detected earlier had we used a higher dose of AMPH. Whether the expression of LS can be present during the first week of RF with a higher AMPH dose, or whether brain neuroplastic changes that underlie LS require more than a week to manifest as behavioral enhancement remains to be determined. It does not appear that LS is related to the amount of daily running wheel activity between groups. Both RF and VRF groups have similar levels of 24-h running activity, which are divided equally between the night and day (S2 Fig). LS, if affected by locomotor activity, may be related to the way it is reorganized across the 24-h cycle during the period of RF rather than the total amount of activity itself.

LS in RF but not VRF may be explained by RF's ability to maintain shifted clock gene expression. Recent studies have implicated transcription factors that comprise the molecular circadian clock to changes of neural plasticity (McClung & Nestler, 2008). By maintaining the same feeding time, RF may result in more consolidated neuroplastic changes important for LS, whereas VRF, which must readjust clock gene expression on a daily basis (Escobar et al., 2007), may not. It is important to note that even though changes to clock gene expression by VRF may not be to the same extent as RF, VRF has been shown to dampen rhythms of PER2 in several brain regions, including the dorsal striatum (Verwey & Amir, 2011), which may be relevant to the development or maintenance of RF LS. Finally, since we did not test whether LS is present in VRF groups during the VRF period, we cannot say that caloric restriction during VRF would not result in cross-sensitization; only that LS is maintained 10–15 days post-RF but not post-VRF. Therefore, LS may persist longer after cessation of circadian RF than non-circadian caloric restriction.

It is uncertain why cross-sensitization to AMPH was present in some NVRF rats when tested during the day, as this response was not present when NVRF were tested at night. In contrast, rats fed a circadian feeding schedule at night (NRF) showed cross-sensitization only when tested during the night. Unpredictable schedules involving non-caloric/ non-circadian food rewards have also been shown to result in cross-sensitization to a low dose of AMPH (Singer, Scott-Railton, & Vezina, 2012). Both in the latter and present study the effects of cross-sensitization were unrelated to body weight

loss. Similarly, Avena & Hoebel evaluated various feeding schedules comprised of restricted access to sugar and/or lab chow and came to the hypothesis that cyclic feeding affects DA signaling to produce sensitization independently of body weight changes (Avena & Hoebel, 2003).

Our results show that the combination of circadian feeding and caloric restriction result in LS as measured by an acute injection of AMPH. VRF, RT and VRT do not result in LS and NVRF shows high within-group variability. Interestingly, NRF only resulted in cross-sensitization when AMPH was administered during the night, while daytime RF resulted in cross-sensitization irrespective of the time of day. The latter may not be surprising, given that daytime RF results in the uncoupling of circadian oscillators from the SCN, such that both brain and peripheral oscillators can oscillate in anti-phase compared to oscillations under free-feeding conditions (Verwey & Amir, 2009). The amount of time it takes for non-SCN oscillators to resynchronize to the SCN, once animals are returned to free-feeding conditions, has not been extensively studied; however it is reasonable to hypothesize that brain regions important for both LS and/or food entrainment may remain coupled to the timing of feeding for some extended time post-RF. Unexpectedly, LS was not present during the first week of RF, but was present at 2 weeks into RF and 10–15 days post-RF. Body weight per se did not appear to play a role in these results since rats typically regained pre-RF body weight by test day. These results signify that food entrainment can play a role in experimental outcomes when chronic food restriction is used to study drug addiction. Moreover, LS resulting

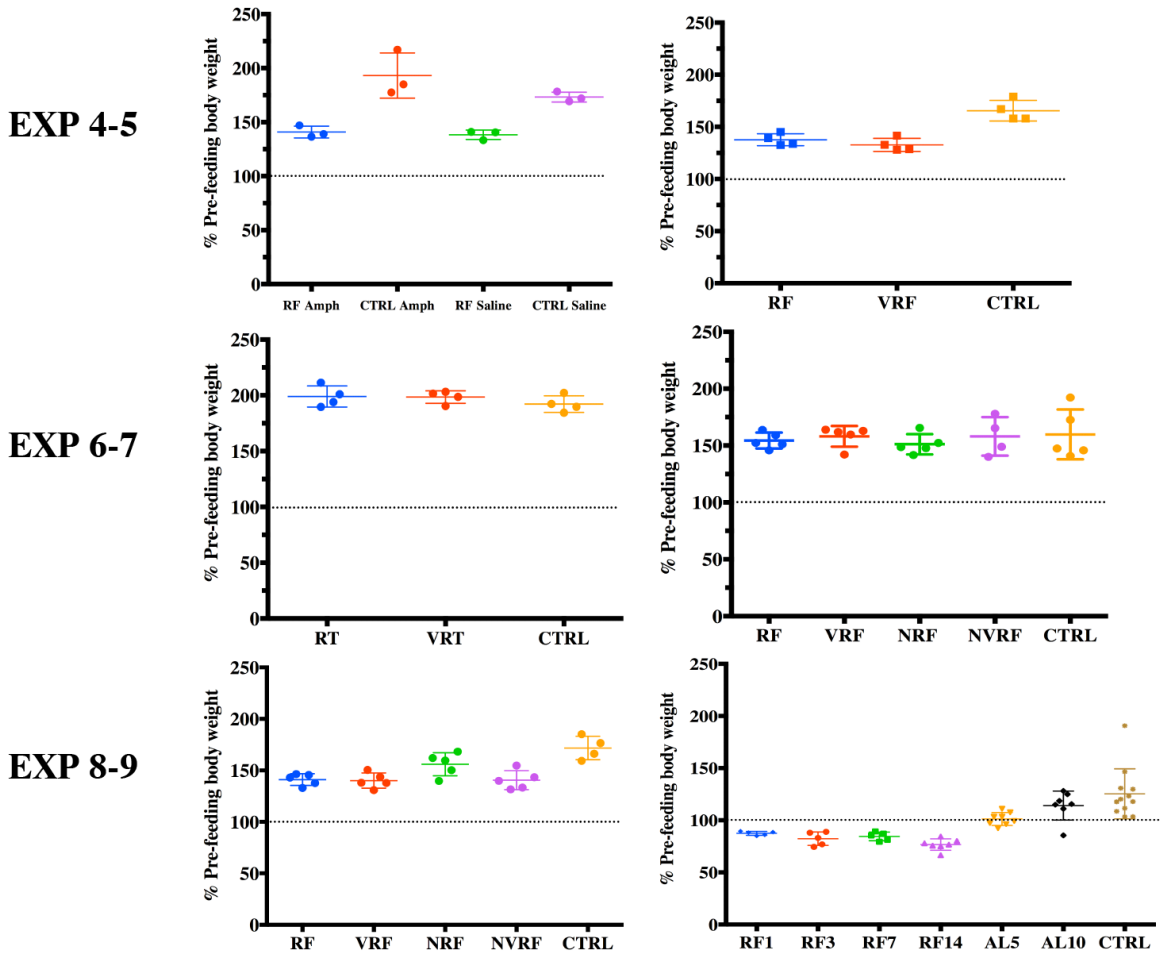
from circadian RF appears to be a distinguishing feature of food entrainment, and further supports the role of DA in the food entrainment pathway.

Acknowledgments

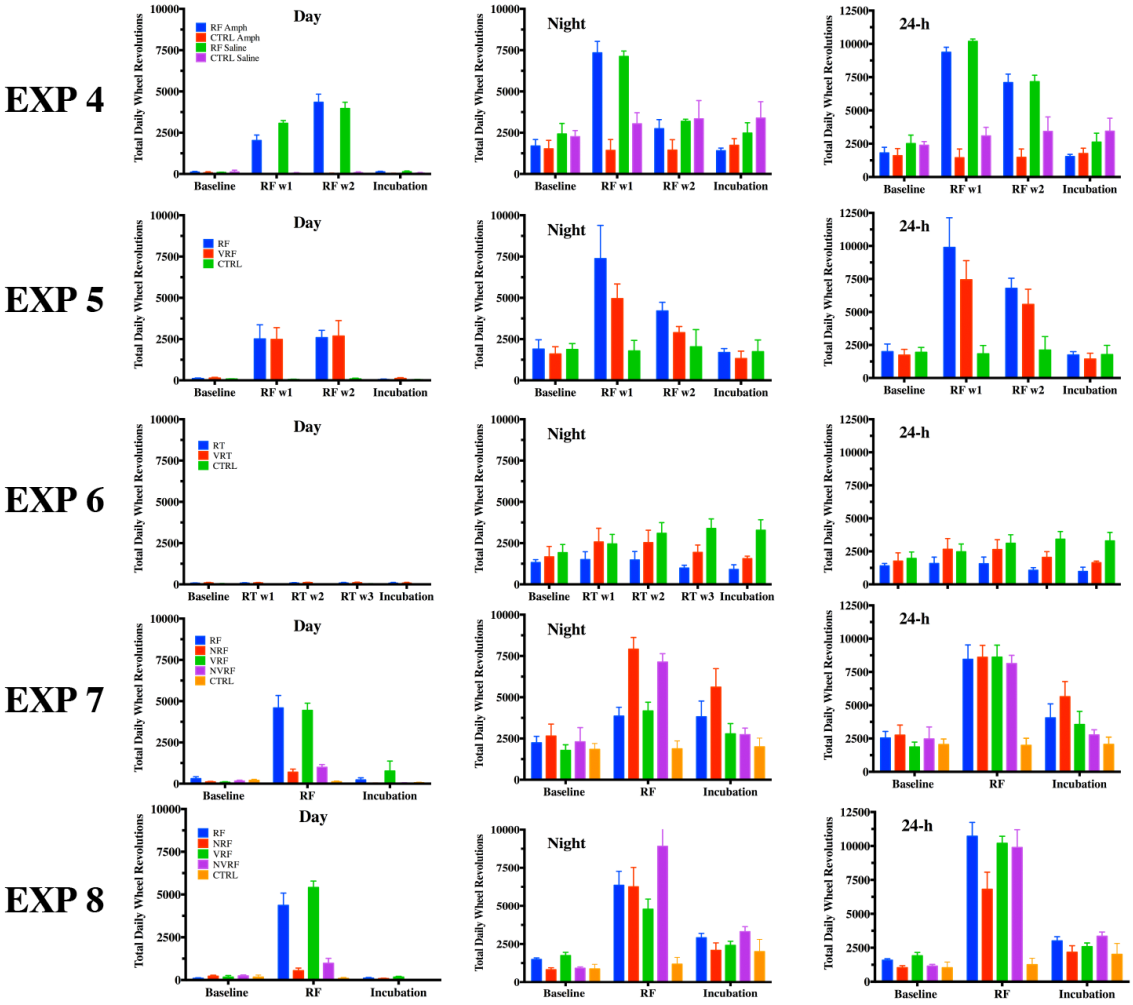
This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada, Les Fonds de la recherche en santé Québec, and the Canadian Institutes for Health Research.

Supplementary information

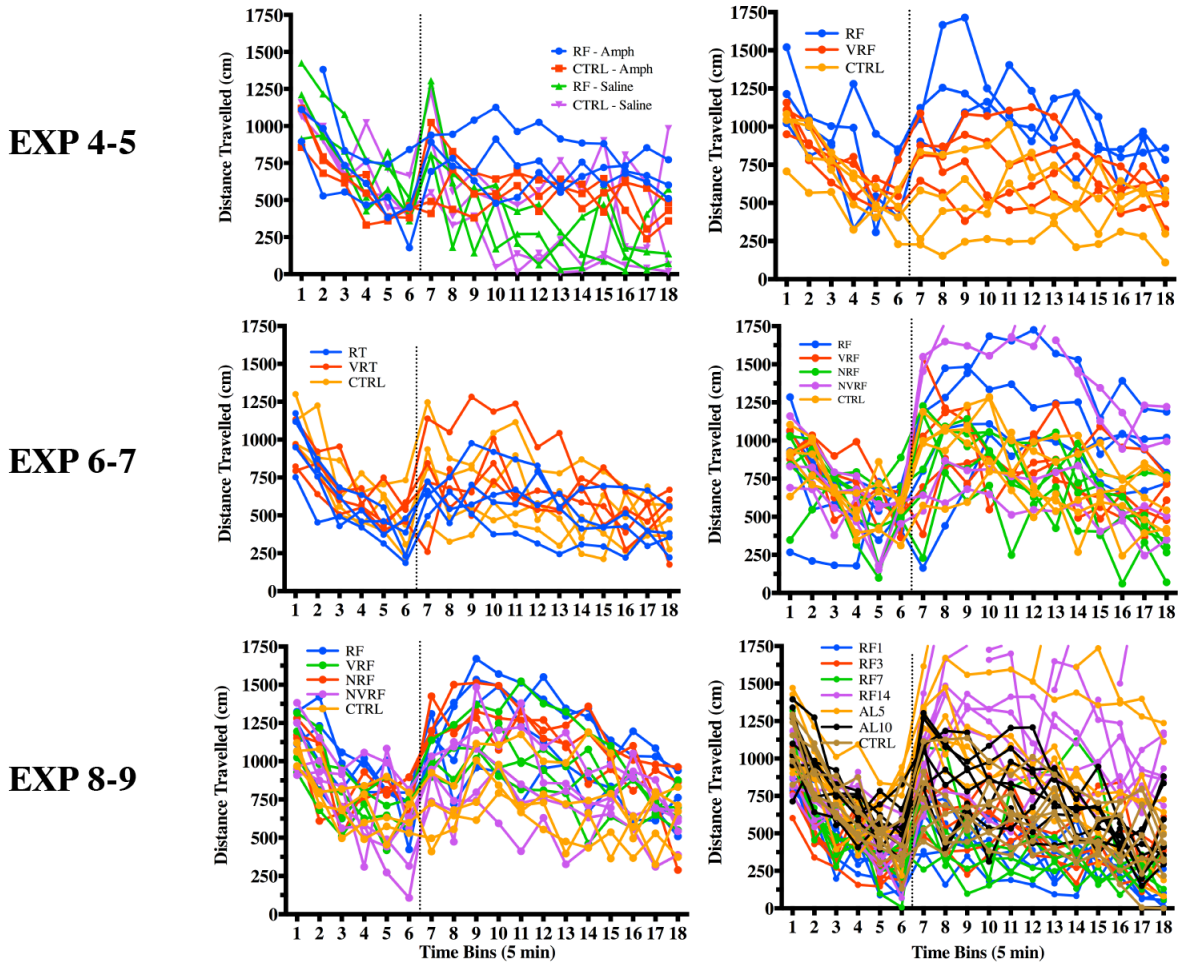
S1 Fig. Percent pre-RF/RT body weight +/- SD for individual rats within each group for experiments 4–9, respectively.



S2 Fig. Running wheel activity by stage of experiment.



S3 Fig. Feeding schedule cross-sensitization to AMPH test: Individual rats.



CHAPTER 2

Molecular measures of dopamine function in the dorsal striatum resulting from circadian restricted feeding

Hanna Opiol, Tara Delorme, Nick Van Oirschot, Pavel Solis,
Nuria de Zavalia, Shimon Amir

Author Contributions:

Conceptualization: Hanna Opiol, Shimon Amir.
Data curation: Hanna Opiol, Shimon Amir.
Formal analysis: Hanna Opiol.
Funding acquisition: Shimon Amir.
Investigation: Hanna Opiol, Tara Delorme, Nick Van Oirschot, Pavel Solis.
Methodology: Hanna Opiol, Shimon Amir.
Project administration: Shimon Amir.
Resources: Shimon Amir, Uri Shalev.
Supervision: Shimon Amir.
Validation: Shimon Amir, Nuria de Zavalia.
Visualization: Hanna Opiol, Shimon Amir.
Writing – original draft: Hanna Opiol.
Writing – review & editing: Shimon Amir.

Abstract

Placing rats on restricted feeding (RF) for 2 weeks results in behavioral cross-sensitization to amphetamine (AMPH) as measured by increased activity in an open-field test. Cross-sensitization to AMPH and the absence of this phenotype in the first week of restricted feeding are indicators that neuroplastic changes in dopamine (DA) function develop with sufficient time exposure to RF. To investigate which dopaminergic mechanism may be responsible for the enhanced response to AMPH, three different measures of DA function from the dorsal striatum (DS) were evaluated in rats exposed to RF. In the first experiment, rats were subjected to 2 weeks of 3h-RF followed by 10 days re-feeding. Tissue was analyzed for FOS expression and found no differences compared to controls. In the second experiment, rats were subjected to 4 weeks of 5h-RF and tissue was analyzed for tyrosine hydroxylase (TH), revealing no differences from controls. In the third experiment, rats were subjected to 1, 3, 7, or 14 days of 3h-RF, and 5 or 10 days of re-feeding, and tissue was stained for dopamine transporter (DAT). Optical density of DAT in the DS was significantly higher in rats exposed to RF for 14 days, including groups that had been re-fed. The increase in DAT is consistent with the timeline by which behavioral cross-sensitization to AMPH is manifest.

Introduction

Dopamine (DA) in the dorsal striatum (DS) is currently believed to play a key role in the circadian food entrainment pathway (Liu et al., 2012; Smit et al., 2013; Gallardo et al., 2014; De Lartigue et al., 2018; LeSauter et al., 2018). Previous work from our lab is consistent with this hypothesis, whereby food entrainment results in an enhanced locomotor response (cross-sensitization) to amphetamine (AMPH). AMPH is known to exert its effects through the DA system. The enhanced locomotor effects of AMPH have also been shown to increase over time, whereby levels of DA in the DS increase in response to AMPH the longer the period of incubation (Paulson & Robinson, 1995). Similarly, RF cross-sensitization to AMPH manifests itself only if there is sufficient time exposure to RF (Opiol et al., 2017). In particular, 14 days of RF, but not 1, 3 or 7 days, is required for cross-sensitization. Food entrainment also requires time in order for circadian rhythms driving behavioral output to readjust with feeding time. We hypothesize that food entrainment is resulting from neuroplastic changes in the DS, which are reflected in the enhanced behavioral response to AMPH. Our intent for this study was to determine what type of neurosplastic changes are occurring that could explain the enhanced DA function resulting from food entrainment.

To further investigate the role of the DS in RF cross-sensitization to AMPH, we analyzed three molecular measures of this brain region following RF and an acute injection of AMPH. Firstly, we checked to see the levels of FOS activation. FOS is a protein product of the immediate early gene *c-FOS*, and serves as an indirect marker of neuronal activity. Nikulina et al. (2004) found that cross-sensitization to AMPH resulting

from repeated social defeat stress showed elevated levels of FOS in the DS. Likewise, we hypothesized that food entrainment would result in elevated FOS in the DS. Secondly, a western blot analysis of tyrosine hydroxylase (TH) was conducted. TH is a marker for dopaminergic neuronal activity, since it is the rate-limiting enzyme needed for DA synthesis. It is possible that food entrainment results in increased DA turnover in the DS. Increased TH activity has been shown to elevate the response to acute AMPH (Vecchio et al., 2017). Finally, we evaluated DAT density at various time-points during RF and post-RF to see whether DAT function may contribute to the enhanced response to AMPH. DAT is known to be one of the three main mechanisms through which AMPH produces its behavioral effects (Calipari & Ferris, 2013).

Methods and Materials

Animals and Housing

Eighty-four male Wistar rats (150-175 g at arrival) purchased from Charles River (St. Constant, QC, Canada) were single housed in clear plexiglass containers (9.5"x8"x16") with a running wheel and mesh flooring (experiments 1 and 3) or corncob bedding and frizzy paper (experiment 2). Rats were maintained in a 12:12 LD cycle and had 10-14 days to habituate to their home environment prior to beginning RF, whereby there was free access to tap water and lab chow. Rats were handled every other day prior to beginning feedings and once a week thereafter. All experimental procedures were conducted under the guidelines of the Canadian Council of Animal Care and approved by the animal care ethics committee at Concordia University, Quebec.

Experimental procedure

Exp 1: Rats were divided into two groups: CTRL and RF. CTRL had no changes to diet. RF was subjected to 2 weeks of time-restricted feeding, whereby food was restricted to a 3h-feeding window at Zeitgeber time (ZT) 5-8, where ZT 0 is lights on. Free access to food was provided for 10 days post-RF for rats to regain body weight. All rats were administered a low dose of AMPH (.5 mg/kg i.p.) between ZT 5-7. Animals were perfused ~90 minutes after injection and the DS was analyzed for FOS (Nikulina et al., 2004).

Exp 2: Rats were divided into two groups: CTRL and RF. CTRL had no changes to diet. RF was subjected to 3-4 weeks of daily 5-h time-restricted feeding, from ZT 2-7. 5-h feedings did not result in significant body weight loss compared to CTRL. On the last day of RF, rats were allowed at least 1 h of food prior to AMPH injection. Thirty minutes after injection rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.) and brains flash frozen in isopentane, placed on dry ice and stored in -80°C for analysis of TH.

Exp 3: Rats were divided into 7 feeding groups: RF1, RF3, RF7, RF14, AL5, Al10 and CTRL. RF1-14 were subjected to 1, 3, 7 and 14 days of 3h RF, respectively. AL5 and AL10 were subjected to 14 days RF followed by 5 or 10 days re-feeding, respectively. CTRL had no changes to feeding. All rats were injected with AMPH on the final day of RF or re-feeding and tissue was collected for DAT analysis, as in experiment 1.

Immunohistochemistry

Following the acute injection of AMPH, rats were anesthetized with sodium pentobarbital (100 mg/kg) and perfused intracardially with 300 ml of cold 0.9% saline, followed by 300 ml of cold 4% paraformaldehyde. Brains were removed and post-fixed for 24 h in 4% paraformaldehyde and stored at 4°C overnight, then sliced to 50 µm serial coronal sections on a vibratome in phosphate buffered saline, and stored at −20°C in Watson's Cryoprotectant. Immunohistochemistry for FOS (1:100,000 - Calbiochem Gibbstown, NJ, USA) was as described in Al-Safadi et al. (2014). Staining for DAT was first prepared with a quenching phase of 30% H2O2 for 30 min, followed by a pre-block containing Triton TBS and 5% normal goat serum for 1 h. The primary was a monoclonal antibody raised in rat (1:1500; Millipore) in Triton TBS containing 2% NGS incubated for 40 h at 4°C, followed by an anti-rat IgG secondary (1:500 - Millipore) for 1 h at 4°C, and an avidin-biotin peroxidase solution for 2 h at 4°C (Vectastain Elite ABC kit; Vector Laboratories). Sections were then rinsed in a 0.5% 3,3-diaminobenzidine (DAB) solution, and IR nuclei were visualized with a solution containing 0.5% DAB, 0.1% H2O2, and 8% NiCl2.

Microscopy

Stained sections were mounted onto gel-coated slides and dehydrated in a series of alcohols and Citrisolv (Fisher), then coverslipped. The sections were examined under a light microscope (Leica, DMR) and images were captured with a Sony XC-77 video camera, Scion LG-3 frame grabber with a 400 x 400 µM template, and Image J 1.52a software (http://imagej.nih.gov/ij, W. Rasband). The mean number of FOS and DAT

immunoreactive (IR) cells per region was then calculated for each animal from the counts of six unilateral images showing the highest number of labeled nuclei, using ImageJ freeware (http://imagej.nih.gov). Images were calibrated for optical density measures using a 21-step tablet with optical density range of 0.05-3.05, as provided by Image J (https://imagej.nih.gov/ij/docs/examples/calibration/).

Western blot

Sleipness et al. (2007) found that TH is higher in the DS at ZT 20 compared to ZT 4, which is consistent with the idea that DA levels peak during the mid-dark phase when rats are most active (Hood et al., 2010). Since DA levels realign with the time of feeding (Liu et al., 2012), we hypothesized that TH, a rate-limiting enzyme of DA, would peak in parallel with DA for RF rats. We expected that collecting brains during RF at feeding time ~ZT 5 would yield high levels of TH, while control brains collected at this time would reflect low levels. Brains were sliced using the cryostat (~ 0.1 mm) and bilateral punches (1.5 mm diameter) were taken from the DS of 3-4 slices/ brain.

For protein quantification, tissue punches were homogenized using a sonicator with 150 μL of lysis buffer (3 x 5 sec) cooling in between on ice. Samples were then frozen and thawed (3x) using liquid nitrogen and a heat block (37°C) and centrifuged for 30 min at 4°C. Using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), a standard curve was generated (R^2= 0.9214) and protein concentrations for each sample were interpolated using optical density. Running and stacking gel preparations were made the day before running the western, as indicated

in Mahmood & Yang (2012). Tissue homogenates were thawed, centrifuged (30 sec) and the loading buffer was prepared, containing 20 μg of protein sample, 4.5 μL loading dye, 0.5 μL of 10% Beta-mercaptoethanol and distilled water for a total volume of 25 μL. Loading buffer was loaded onto prepared gels and subjected to electrophoresis. Proteins were transferred to nitrocellulose membrane for 60 min. Following transfer, the membrane was blocked in 5% non-fat dry milk/10mM Tris, 150 mM NaCl, 0.1% Tween-20 (TBST) with shaking and incubated overnight at 4°C with anti-TH (1:1000, Chemicon). The following day, the membrane was rinsed in TTBS (3 x 10 min) and placed into the secondary antibody (1:2500) in 5% non-fat dry milk/TTBS on a rotator for 1 h at room temperature. Finally the membrane was rinsed in TBST (2 x 10 min), TBS (2 x 10 min) and developed for 5 min using the enhanced chemiluminescence (ECL) substrate kit (PerkinElmer, Inc.).

Luminescence from the blots was detected using the Amersham Imager 600 (GE Healthcare). Analysis of the bands was performed using Image J 1.52a. Raw densities were normalized according to the following steps: A relative value was calculated for each sample's actin optical density value by dividing it by the largest actin value in the control group. TH adjusted values were calculated by dividing TH optical density by its corresponding relative value. Lastly, TH adjusted values were normalized by dividing each value by the average in the control group.

Statistical analysis

Differences between groups were analyzed using an unpaired t-test or a one-way ANOVA, with $\alpha = 0.05$. Post-hoc testing was applied if ANOVA detected significance.

Results

RF cross-sensitization to AMPH is not reflected in FOS or TH

The DS is an important brain region for driving locomotor activity; therefore we hypothesized that the enhanced locomotor response from RF cross-sensitization to AMPH would be reflected in the neuronal activation of this region. Unexpectedly, there was no difference in FOS between CTRL and RF after an acute injection of AMPH, $t_{18} = 0.33$, $p = 0.75$ (Fig 1). Next, we evaluated TH levels from the DS to see if this indirect measure of DA correlated with RF cross-sensitization to AMPH. Again, there were no differences in TH between CTRL and RF, $t_9 = 0.36$, $p = 0.73$ (Fig 2).

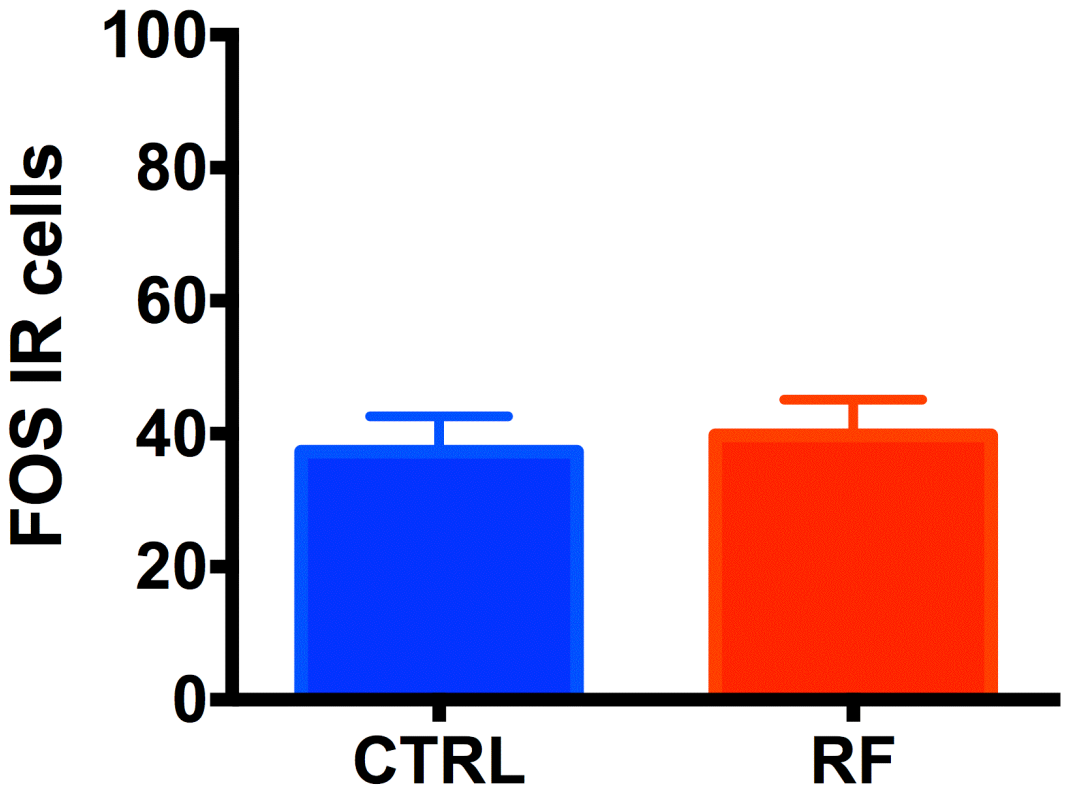


Fig 1. No difference in number of FOS immunoreactive cells (IRC) in the DS following acute AMPH 10 days post RF, compared to controls. Means +/-sem are shown, *n* = 10 per group.

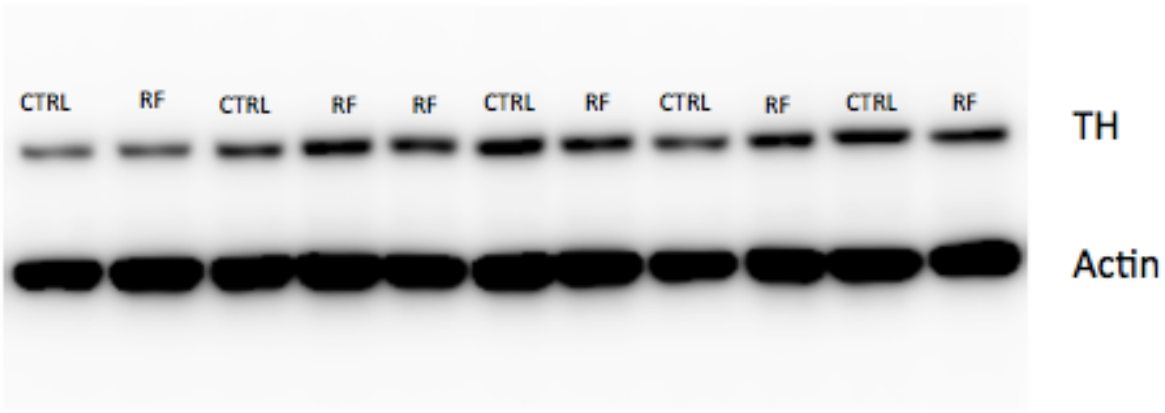


Fig 2. Western blot of TH protein levels in the DS of rats subjected to 4 weeks RF, relative to CTRL. *n* = 5-6 per group.

DAT levels reflect the timeline of cross-sensitization to AMPH

AMPH is known to act through DAT in exerting its effects on locomotor activity, therefore we intended to measure whether DAT density could explain the timeline of AMPH cross-sensitization seen in RF. DAT optical density scores for rats subjected to 1, 3, 7, or 14 days of RF and after 5 or 10 days of re-feeding following AMPH were analyzed using ANOVA. A significant difference was detected $F_{(6,23)} = 6.41$, $p = 0.0004$. Dunnett's multiple comparison found differences for RF14, AL5 and AL10 compared to CTRL.

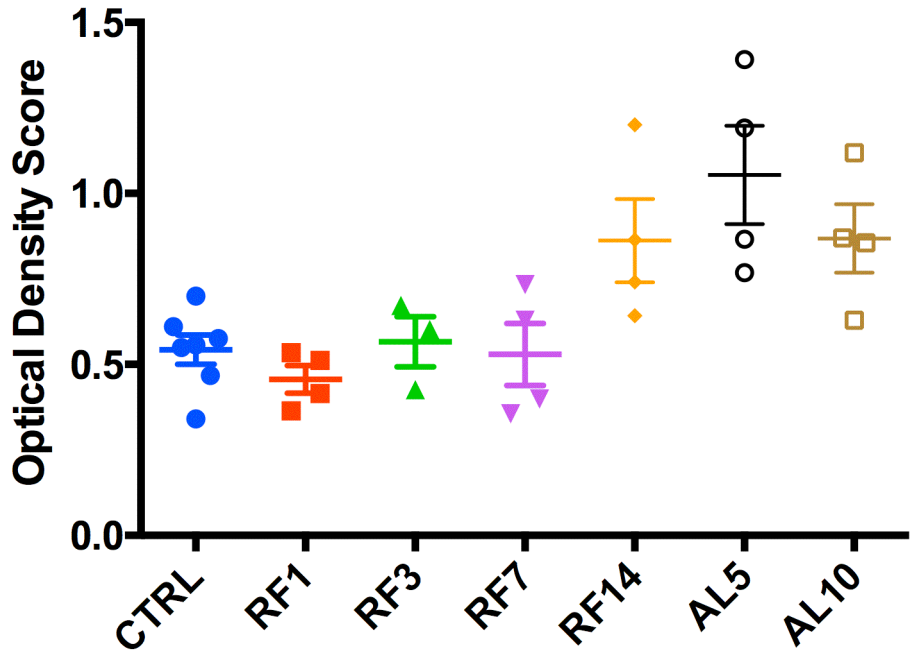


Fig 3. DAT optical density scores for the DS in rats subjected to RF for 1, 3, 7, or 14 days RF and 5 or 10 days post-RF. Means +/- sem are shown, *n* = 4 per group.

Discussion

In this study we evaluated three measures of neuronal activity in the DS, which could explain RF cross-sensitization to AMPH. In experiment 1 and 2, it was shown that FOS and TH expression in the DS did not differ between feeding groups. The lack of difference in FOS expression indicates that the acute effect of AMPH on the DS, intracellularly, does not differ as a result of RF, and therefore does not explain the behavioral differences in cross-sensitization. TH protein levels in the DS also showed no difference as a result of RF, which may indicate that RF does not affect DA synthesis. Similarly, chronic caloric restriction resulted in no changes to total TH (Pan et al., 2006). However, the latter study also reported that there were differences in TH activation (phosphorylation at Ser-40) in the DS as a result of acute AMPH, but not under basal conditions (no AMPH). Since TH phosphorylation at serine residues is unaffected by AMPH treatment itself (Janenaite et al., 2017), it supports the idea that TH activation by AMPH is due to chronic caloric restriction. TH phosphorylation at Ser40 results in an increase in TH activity and DA synthesis (Dunkley et al., 2004), which could explain the effects of AMPH cross-sensitization. An alternative hypothesis may be that RF results in changes to DA regulation at the synapse, such as through DAT, which would enhance homeostatic feedback by increasing TH phosphorylation when blocked by AMPH.

DAT is the main mechanism through which AMPH produces its effects on striatal DA and behavioral output (Sulzer et al., 2005; Calipari & Ferris, 2013). Genetic depletion of DAT results in a two-fold increase of DA synthesis (Gainetdinov et al., 1998), and

increased TH phosphorylation (Ser-40) in the DS (Salvatore et al., 2016). Mice with overexpression of DAT show elevated response to acute AMPH, without a hyperactive phenotype during baseline testing (Salahpour et al., 2008). Thereby, our final experiment tested whether RF might change DA metabolism due to elevated DAT. Additionally, we evaluated whether the differences in DAT developed over time, as seen with behavioral cross-sensitization. To our surprise, DAT levels were significantly higher at 14 days into RF, as well as, 5 and 10 days after food was returned compared to CTRL, consistent with the timeline of RF behavioral cross-sensitization. Therefore, DAT may explain the locomotor sensitization effects of RF, although it is not necessary for food entrainment. DAT knockout mice develop normal food anticipatory activity (FAA) (Enriquez, 2018).

Enhanced DAT function may be an important component of the behavioral sensitization that develops with food entrainment. In humans, during a weight-loss program, the time of day when the largest meal is consumed has been shown to differentially affect DAT binding in the striatum (Versteeg et al., 2017). It is hypothesized that Insulin may play a critical role in regulating striatal DA release and uptake through DAT. In particular insulin has been shown to indirectly result in striatal DA release, through insulin receptors located on striatal cholinergic interneurons (Patel et al., 2018). Furthermore, the effects of insulin on striatal DA uptake via DAT are the result of chronic food restriction rather than acute insulin exposure. In contrast, rats that are hypoinsulenemic have decreased DAT surface area and impaired response to AMPH due to insufficient DA release into the synapse (Williams et al., 2007). It is unknown how food entrainment

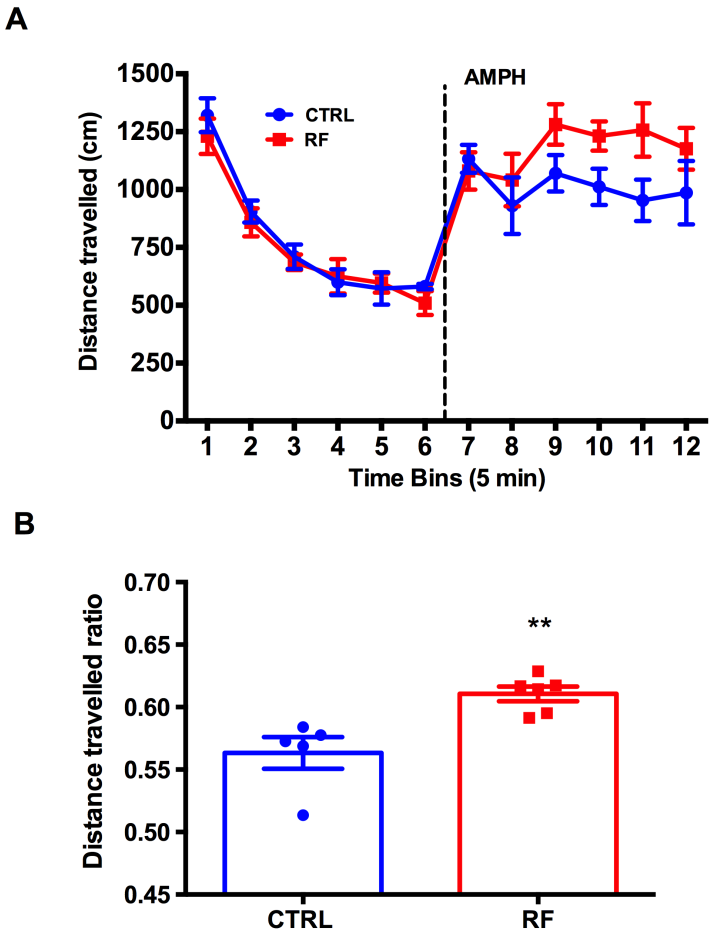
affects insulin receptors on cholinergic interneurons, however we do know that food entrainment shifts the hormonal rhythm of insulin to anticipate feeding time (Diaz-Munoz et al., 2000), which could then regulate striatal DAT expression. We hypothesize that RF cross-sensitization reflect changes to DAT function that occur with prolonged changes to insulin levels. It has been suggested that insulin's effects on enhanced striatal DA function could be regulating motivation and mood (Stouffer et al., 2015). Future work should evaluate the effects of RF for the potential role it may have in regulating mood or in preventing the development of mood disorders under conditions of circadian disruption.

Acknowledgments

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada, Les Fonds de la recherche en santé Québec, and the Canadian Institutes for Health Research.

Supplementary information

S1 Fig. Cross-sensitization to AMPH is present using 5h RF. (A). Distance travelled (cm) +/-sem across 30 minutes of habituation to the open-field, followed by 30 minutes post-AMPH injection. (B). Distance travelled ratio +/-sem is significantly different between CTRL and RF. ** Significance $p < 0.01$. The AMPH test was intended to verify that cross-sensitization was present in rats using a 5h RF schedule, which did not result in significant weight loss. Previously, cross-sensitization to AMPH was confirmed using 3h RF (Opiol et al., 2017).



CHAPTER 3

The effects of circadian restricted feeding on behavioral measures of anxiety and depression in the male rat

Hanna Opiol & Shimon Amir

Author Contributions:

Conceptualization: Hanna Opiol, Shimon Amir.
Data curation: Hanna Opiol, Shimon Amir.
Formal analysis: Hanna Opiol.
Funding acquisition: Shimon Amir.
Investigation: Hanna Opiol.
Methodology: Hanna Opiol, Shimon Amir.
Project administration: Shimon Amir.
Resources: Shimon Amir
Supervision: Shimon Amir.
Validation: Shimon Amir.
Visualization: Hanna Opiol, Shimon Amir.
Writing – original draft: Hanna Opiol.
Writing – review & editing: Shimon Amir.

Abstract

Circadian restricted feeding (RF) is known to have many health benefits in relation to metabolic function. However, there is very little research to evaluate how RF could affect mental health. Previous work in our lab showed that RF results in the enhancement of dopamine (DA) function as measured by cross-sensitization to amphetamine (AMPH). Current understanding of the DA system implies that it is critical for emotion, motivation, and is believed to underlie mood disorders. In the present study we evaluate the effects of RF on measures of depression and anxiety in the male rat. Two rat strains are evaluated in their behavioral response to the forced swim test (FST) 10 days post-RF, a time previously shown to result in cross-sensitization to AMPH. No significant differences were detected in either strain. To test whether RF may have some preventative effects on the development of mood disorders, rats were tested in the FST, elevated plus maze (EPM) and open-field (OF) over the course of RF during constant light conditions. RF increased swimming behavior in the FST and some measures of activity in the EPM and OF, indicating possible antidepressant and/or anxiolytic effects. More work is needed to further investigate the effects of RF on mental health.

Introduction

The study of RF on circadian rhythms has traditionally confined feeding to a single 2-3 hour feeding window. Recent studies have shown that larger feeding windows of 12 hours can also harness circadian rhythms and provide health benefits. The health benefits are attributed to appropriate circadian function rather than calorie restriction or the type of diet eaten. High-fat diets with no caloric restriction, known to result in diet-induced obesity, can have their detrimental effects on metabolic disease prevented or reversed by simply restricting the time of food consumption (Hatori et al., 2012; Chaix et al., 2014; Manoogian & Panda, 2017; Zarrinpar et al., 2014). In rodents, maintenance of appropriate circadian function through RF results in improved cardiac health and sleep, reduced inflammation, reduced hepatic glucose production, increased metabolism and innate immune function (Froy, 2011; Gill et al., 2015; Cisse et al., 2018). In humans, overnight fasting of >12 hours is correlated with reduced breast cancer incidence (Marinac et al., 2015a; Marinac et al., 2015b), improved sleep and elevated daytime alertness (Gill & Panda, 2015), enhanced immune function in young and older men (Gasmi et al., 2018), as well as improved blood pressure, insulin sensitivity and oxidative stress in prediabetic men (Sutton et al., 2018). The maintenance of circadian rhythms by RF reveal numerous benefits to metabolic function, however research has yet to evaluate if the benefits of RF are extended to mental health. RF shifts clock gene rhythms in the brain, including regions important for learning and memory, motivation and reward processing (Verwey & Amir, 2009). If circadian disruption or misalignment of these brain regions is a contributing factor in affective disorders (Salgado-Delgado et

al., 2011; Li et al., 2013; Vadnie & McClung, 2017; Bedrosian, 2017; Lyall et al., 2018), then RF may serve a preventative or therapeutic function to mental health.

From animal studies it is known that RF can resynchronize biological rhythms under conditions of circadian disruption, whether the disruption originates from removing the suprachiasmatic nucleus (SCN) (Stephan et al., 1979; Hara et al., 2001), clock gene mutation (Storch & Weitz, 2009) or external factors that indirectly affect clock function (Lamont et al., 2005; Novakova et al., 2011; Oike et al., 2015; Baez-Ruiz et al., 2018). In the case of chronic constant light (LL)-induced disruption on the SCN, it is the individual cells in the SCN that become unsynchronized from each other that cause circadian arrhythmicity (Ohta, Yamazaki & McMahon, 2005). LL does not compromise the ability of the SCN to generate rhythms and thereby RF can rescue circadian function by resynchronizing cells in the SCN. However, since RF can also correct circadian rhythms in the absence of the SCN and clock gene function, it is evident that a second independant clock mechanism exists and can be controlled by RF. The molecular mechanisms through which this food entrainable oscillator (FEO) functions have yet to be determined. Recent work suggests an important role for dopamine (DA) motor circuits (Smit et al., 2013; Gallardo et al., 2014; De Lartigue & McDougle, 2018). Previous work in our lab supported the idea that RF results in sensitization of DA function (Opiol et al., 2017). Therefore, in addition to correcting circadian disruption, RF may elevate levels of DA, a neurotransmitter critical for driving motivation and affective processes. We hypothesized that since DA is implicated in the regulation of affective

disorders and since the pharmacological treatments often target the DA system, RF may serve a non-pharmacological preventative or treatment option for regulating mood.

The current study sought to evaluate the effects of RF on mood by observing their effects in animal models of anxiety and depression. Firstly, we tested the effects of RF on the forced swim test (FST). The FST is the gold standard for measuring antidepressant efficacy. Since there are strain differences in the FST, we tested two popular rat strains known to behave differently (Bogdanova et al., 2013). Initial testing was done 10 days post-RF since previous work showed cross-sensitization to AMPH at this time point. Secondly, we tested how RF could affect behavior in animal measures associated with anxiety-like behavior, the elevated plus maze (EPM) and open-field (OF). Lastly, we tested how rats would behave under these behavioral tests when subjected to short-term chronic LL. If LL did disrupt emotional processing, perhaps RF could prevent its development by maintaining circadian rhythmicity.

Methods and Materials

Animals and Housing

Forty male Wistar rats and 10 male Long Evans (150-175g at arrival) purchased from Charles River (St. Constant, QC, Canada) were single housed in clear plexiglass containers (9.5"x8"x16") with corncob bedding and frizzy paper. Rats had free access to tap water and rodent lab chow, except when subjected to RF. Rats were handled every other day at the end of the light phase/ beginning of the dark phase prior to beginning RF, and 1-3 times weekly to measure body weights during RF. All experimental

procedures were conducted under the guidelines of the Canadian Council of Animal Care (http://www.ccac.ca) and approved by the animal care ethics committee at Concordia University, Quebec.

Feeding and light schedules

Rats were introduced to a 12:12 light/dark cycle prior to starting experimental conditions and were maintained under this light schedule throughout the experiment unless undergoing LL. Half the rats in experiment 3 were subjected to LL following habituation until the final day of testing. Light intensity inside each homecage ranged from 90 to 130 lux. For a visual representation of feeding and light schedules see fig 1.

In experiment 1 and 2, rats were subjected to 3h RF in the middle of the light phase, ZT5-8 (where ZT0 is lights on) for 14 days, followed by 10-14 days of ad libitum feeding to regain body weight. In experiment 3, rats were subjected to a 5h RF, ZT 2-7. The longer feeding window intended to reduce weight loss over the course of RF. Similarly, LL is known to increase body weight (Fonken et al., 2010; 2013), therefore body weight was monitored and the FST was conducted prior to significant changes in weight, ~3 weeks into LL. Rats were given at least 1 hour to eat prior to testing. The intention was not to test rats in a fasting state, however, still within the hours that food-entrained rhythms were driving arousal.

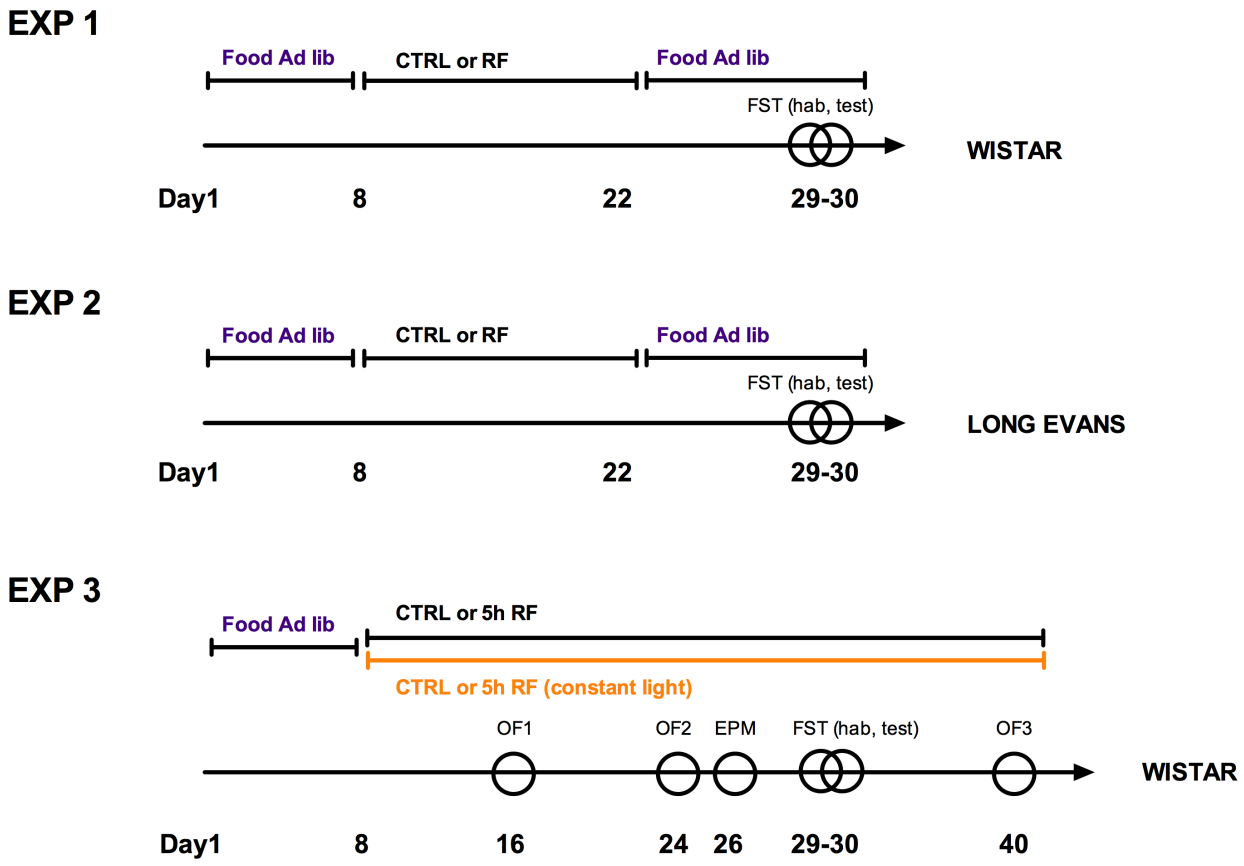


Fig 1. A schematic of experiments 1-3. All rats first underwent 7 days of habituation to home cage environment prior to feeding or lighting changes. In experiments 1 and 2, rats were divided into a feeding group for 14 days that included 3h of restricted feeding (RF) or no changes to feeding (CTRL) followed by 10 days of re-feeding to regain body weight prior to the FST. In experiment 3, rats were subdivided into 4 groups: CTRL, RF CTRL-LL, or RF-LL. The latter two groups were kept in LL from day 8 until the end of the experiment. Since body weight can affect behavior in the FST, RF was extended to 5h to avoid weight loss and LL did not exceed 3 weeks to avoid weight gain. Multiple open-field tests (OF1-3) and the elevated plus maze (EPM) were conducted during experiment 3.

Forced swim test

The modified forced swim test (FST) was conducted as described by Cryan, Valentino, & Lucki (2005). Briefly, the test consists of two days: habituation day and test day. On habituation day, the rat is placed into a cylinder of water where it cannot escape. After 10 minutes the rat is removed from the water, dried with a towel, and placed back into its home cage. Twenty-four hours later the test is repeated for 5 min. Fresh water is provided for each trial and the water temperature must be between 23-25 degrees Celsius. Each trial is recorded using a standard video camera.

The modified FST uses a water depth of 30-35 cm, compared to the original Porsolt (1977) protocol that uses 15-18 cm. By increasing the water depth the modified FST prevents rats from balancing on their tails and using this posture as a behavioral adaptation. Pharmacological and lesion studies have shown that by using the modified FST the active behaviors of climbing versus swimming can be differentiated as being under the control of separate neurotransmitter systems. More specifically, antidepressants acting on the catecholamine system selectively increase climbing behavior and serotonergic agents selectively increase swimming (Slattery & Cryan, 2012).

Testing was done during the light phase ZT4-6 with the lights on in the testing room, to stay consistent with the timing of the light schedule in their home environment. As noted by Kelliher et al. (2000) there is more escape-oriented behavior when testing during the diurnal phase. Our rational was to conduct the FST during the peak time for anxiety-like

response, as some have argued the FST may be more appropriate for measuring anxiety-like behavior than depression-like behavior (Anyan & Amir, 2018).

Elevated plus maze

To test for anxiety-like behavior, a standard elevated plus-maze (EPM) test was conducted. The EPM is the most common method used by researchers to evaluate behavior in a novel environment, which simultaneously evokes curiosity and fear and creates an approach-avoidance conflict that is beneficial to detecting an anxiety-like state (Carobrez, Kincheski, & Bertoglio, 2015). The maze consisted of two open arms (50 x 10 cm), two closed arms (50 x 10 x 52 cm), and was elevated 52 cm off the floor. Testing was conducted in the light phase between ZT 4-6, under similar lighting conditions to home environment. Rats undergoing LL were tested at the same time as control counterparts. It has previously been shown that circadian phase and illumination do not affect anxiety-like behaviors (Jones & King, 2001). Each trial began by placing the rat in the center of the EPM facing an open arm, and left to explore for 5 minutes. The maze was cleaned using sanitizer wipes prior to the next rat. Activity was recorded using a camera placed above the EPM.

Open-field

In experiment 3, rats were subjected to the open-field at three time points throughout the experiment to monitor if there would be changes in behavior under RF, LL, or RF-LL compared to controls. The open-field is traditionally used as a measure of stress-response to a novel enviornment, in which thigmotaxis, among other measures, is

evaluated (Prut & Belzung, 2003). Thigmotaxis refers to anxiety-like behavior that rodents show by maintaining close proximity to the wall of the OF, which then diminishes within a few minutes. In particular we were interested in whether LL resulted in an increased stress response over time that could be seen with repeated OF testing, and if so, whether RF-LL would show a reduced stress-response or anxiolytic-like behavior in parallel to their CTRL-LL counterparts. Testing was done over a 30-minute period. Rats were placed in a sound-attenuated plexiglass open-field box (39 x 42 x 50 cm) facing the left corner and allowed to explore for 30 minutes. Three to four rats were tested simultaneously within the same room, in separate boxes. Dim lighting (~5-10 lux) was used in the room to promote explorative behavior. Boxes were cleaned out using 70% ethanol after each session.

Data collection and analysis

Video recordings of the FST were scored for climbing, swimming or immobility using a time-sampling technique described by Slattery & Cryan (2012). The technique labels the predominant behavior for each 5-sec bin out of the 300-second test. Climbing is defined as frantic/vertical behavior directed at the wall. Swimming is defined as horizontal behavior not directed at the wall. Immobility is defined as floating or minimum movement to maintain head above water. Although the modified FST does not recommend scoring for time to immobility, we decided to evaluate this measure for both habituation and test days in experiment 3 to see a within subject comparison of this learned response. Time to immobility was scored as the first 5-sec bin in which predominant behavior was defined as floating.

Video recordings of the EPM were scored for total time in the center, total time spent in the closed arms, total time spent in the open arms, latency to enter open arm, the number of open arm entries and the number of transitions between arms. These behavioral scores for the EPM were chosen to evaluate any differences between groups in anxiety-like behavior, as compared to the control group. If the rat fell off the arm he was disqualified from analysis unless there was sufficient data for the measure of interest.

Open-field data was collected using Tru Scan 2.0 activity monitoring system (Coulbourn Instruments, Whitehall, PA). Total distance travelled, total distance in the center, total time in the center, total time moving and total entries into the center was collected in 5-min bins during a period of 30 min during OF1-3 for each rat. To measure anxiolytic behavior, a ratio of total distance in the center/ total distance travelled was calculated.

Statistical analysis

Unpaired t-tests or one-way ANOVA were used to evaluate the FST and EPM for experiments 1-3 and body weight for experiments 1 and 2. Two-way repeated measures ANOVA was used in experiment 3 to evaluate each OF test over 30 minutes (six 5-min bins), time to immobility comparing habituation to test day, and body weight over the course of RF. Post-hoc analyses were applied when the test yielded significance.

Results

Exp 1-2: No differences in the FST 10 days post-RF

As shown in Fig 2A, there were no significant differences between RF and CTRL in immobility $t_{11} = 0.72$, $p = 0.49$, swimming $t_{11} = 0.52$, $p = 0.61$, or climbing $t_{11} = 1.31$, $p = 0.21$ for male Wistar 10 days post-RF. Similarly, shown in Fig 2B, there were no differences in immobility $t_8 = 1.347$, $p = 0.22$, swimming $t_8 = 0.8998$, $p = 0.40$, or climbing $t_8 = 0.1286$, $p = 0.90$ for male Long Evans 10 days post-RF.

According to Slattery & Cryan (2012) controls should have a score of 25-30 for total immobility on test day. An analysis of mean scores for immobility found Wistar controls to have a mean +/- SD score of 25.33 +/- 8.98, which appears to be within the appropriate range as stated above. Long Evans controls had a mean +/- SD score of 18.60 +/- 7.83, which is lower than indicated by the above protocol, and is likely due to strain differences (Bogdanova et al., 2013).

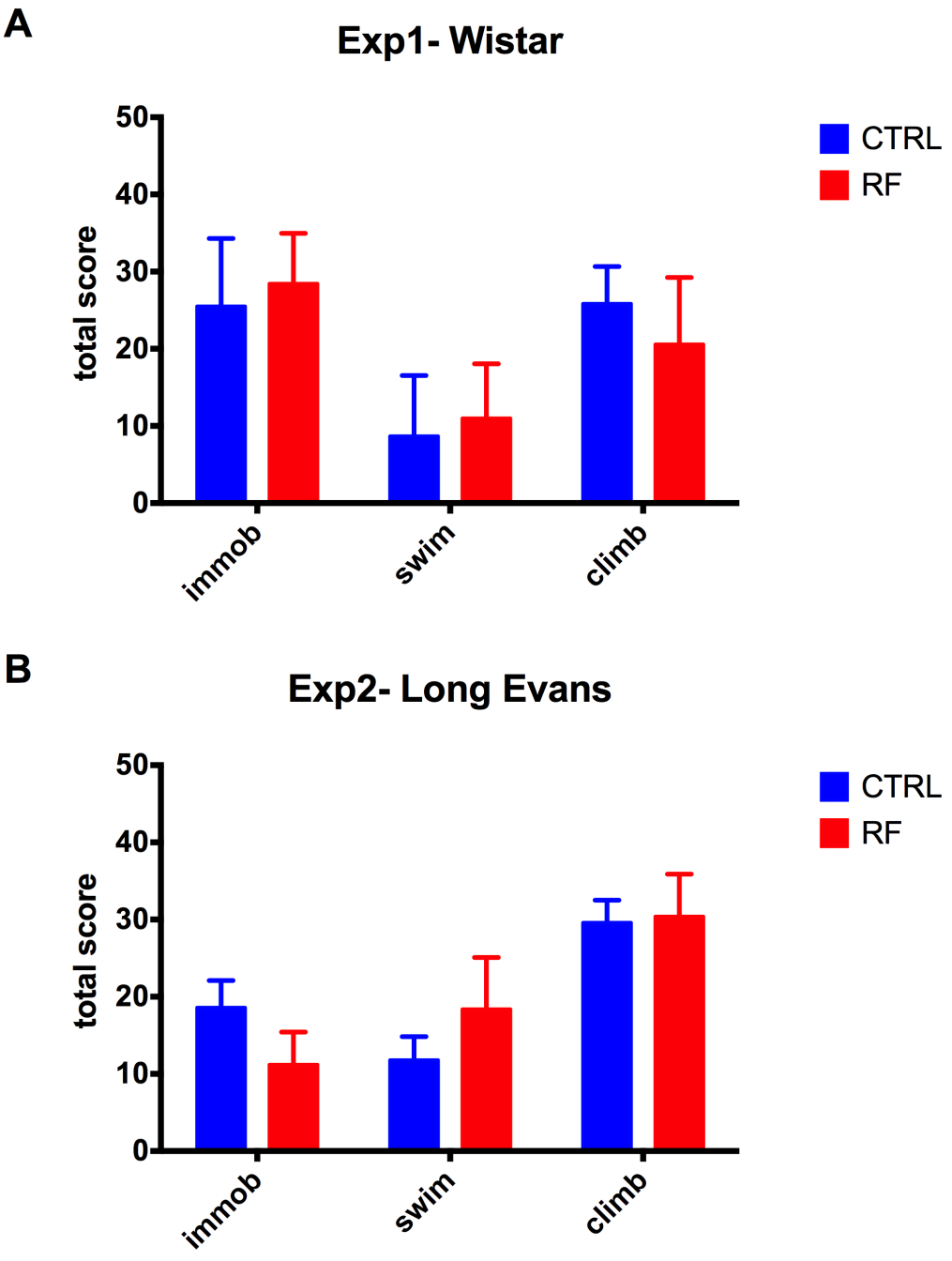


Fig 2. FST scores for immobility, swimming and climbing behavior 10 days post-RF. (A). Total score means +/- sem for Wistar rats in experiment 1, *n* = 6 or 7 per group. **(B).** Total score means +/- sem for Long Evans rats in experiment 2, *n* = 5 per group.

Exp 3: Swimming increases in the FST for rats under RF, but not RF-LL

As shown in Fig 2, ANOVA revealed a difference in swimming behavior $F_{(3, 21)}$ = 3.674, p = 0.029. Dunnett's post hoc test found a difference between RF, but not RF-LL or CTRL-LL, compared to CTRL. There were no significant differences between groups in immobility $F_{(3, 21)}$ = 1.313, p = 0.30 or climbing $F_{(3, 21)}$ = 0.142, p = 0.93.

An analysis of mean scores for immobility found CTRL to have a mean +/- SD of 32.8 +/- 13.37, which appears to be within the appropriate range for the modified FST (Slattery & Cryan, 2012). CTRL-LL and RF-LL had similar immobility scores to the CTRL group: 31.33 +/- 18.75 and 30.14 +/- 5.58 respectively. RF had a lower mean score for immobility at 20.29 +/- 10.78.

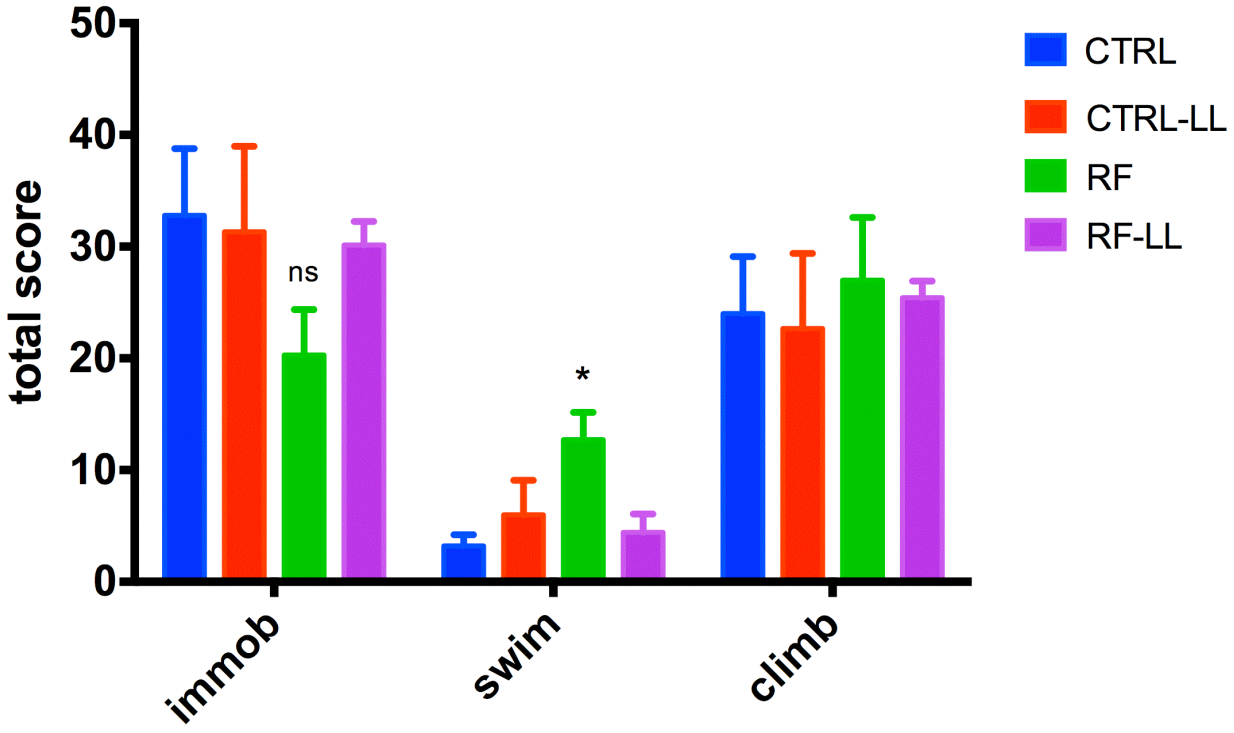


Fig 3. FST scores for immobility, swimming, and climbing behavior during 5h RF. Total score means +/- sem for CTRL, CTRL-LL, RF and RF-LL in experiment 3. *n* = 5-7 per group. * Significantly different from CTRL, $p < .05$, *ns*, not significant.

Exp 3: No differences in time to immobility on test day

Figure 4 shows time to immobility in the FST on habituation day and test day had no significant effect of group $F_{(3, 21)} = 0.8376$, $p = 0.49$, however there was an effect of time $F_{(1, 21)} = 4.545$, $p = 0.05$, and an interaction effect $F_{(3, 21)} = 3.536$, $p = 0.03$. Bonferroni revealed RF-LL to have a significantly reduced time to immobility from habituation to test day.

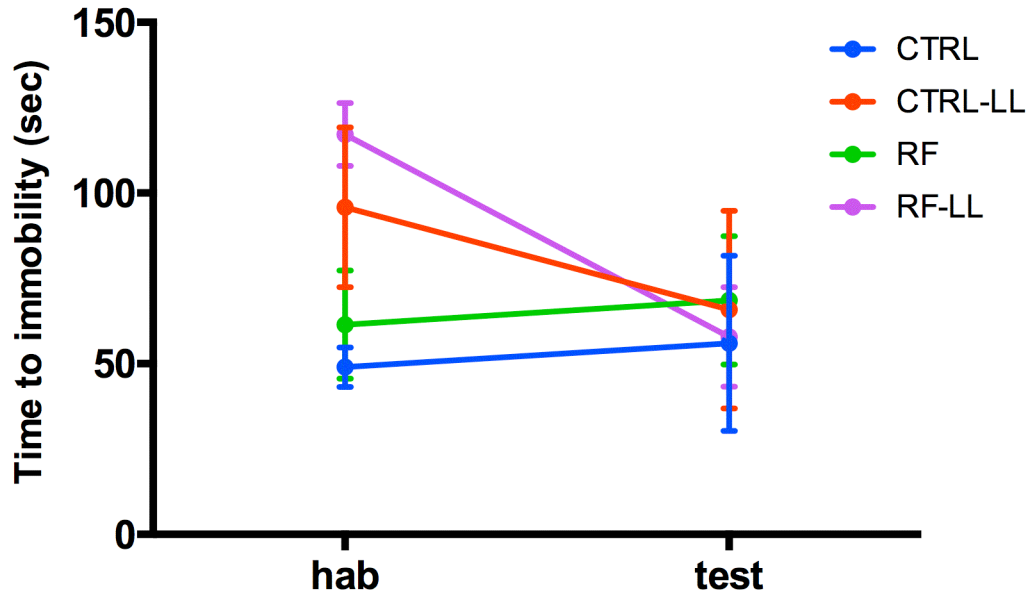


Fig 4. Time to immobility in the FST. Mean time to immobility +/- sem on habituation and test days of the FST for CTRL, CTRL-LL, RF and RF-LL. *n* = 5-7 per group.

Exp 3: EPM and OF during RF and/or LL

To evaluate anxiety-like behavior, the EPM was conducted after >2 weeks of RF and/ or LL. Fig 5A shows a difference between groups for time spent in the center, $F_{(3, 19)} = 15.59$, $p < 0.001$. Dunnett's post hoc test (α =.05) revealed the difference to be for CTRL-LL compared to CTRL. Fig 5B-E shows no differences between groups for time spent in the open arm $F_{(3, 19)} = 1.163$, $p = 0.35$, latency to enter the open arm $F_{(3, 19)} = 1.534$, $p = 0.24$, number of entries into the open arm $F_{(3, 19)} = 1.617$, $p = 0.22$, and number of transitions between arms $F_{(3, 19)} = 0.6050$, $p = 0.62$, respectively. Fig 5F shows a difference in the ratio of total open arm entries/ total transition between arms $F_{(3, 19)} = 3.037$, $p = 0.05$.

To evaluate how rats adapted to the OF over the course of LL and whether RF served any preventative function, CTRL and RF fed rats were tested after 1, 2 and 4 weeks of LL. Statistical analysis found the group factor to be significantly different for time in the center, with significant changes over time for every measure. Post-hoc testing revealed that the CTRL group did not significantly change over time for any measure, and that the group difference was between CTRL and CTRL-LL for time in the center at OF3. See Table 1 for two-way ANOVA (Group x Time), with post-hoc analysis shown in Fig 6A-E.

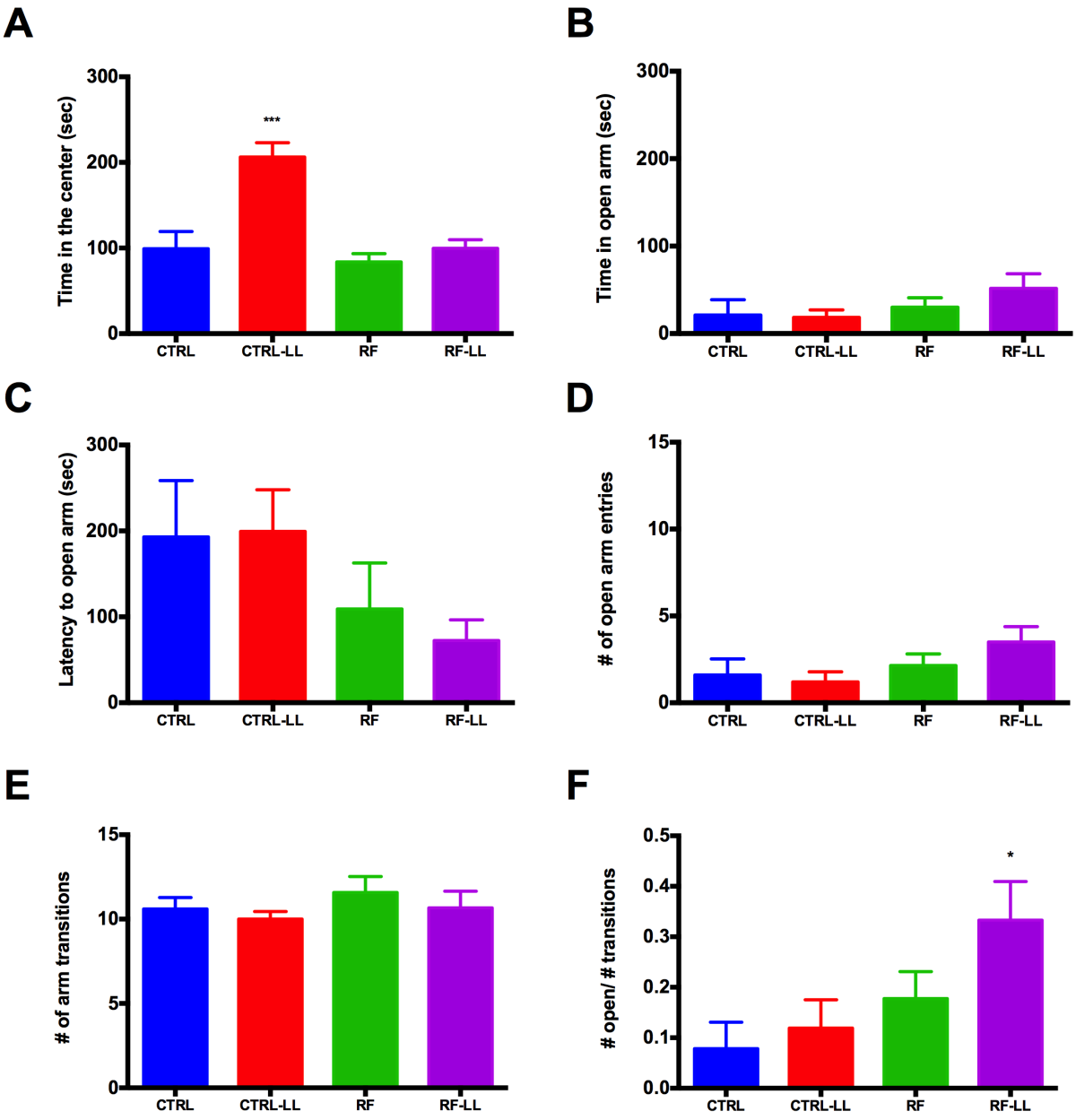


Fig 5. Activity in the EPM after 2 weeks RF and/or LL. Various measures evaluating anxiety-like activity in the EPM for CTRL, CTRL-LL, RF and RF-LL. Means +/-sem are shown for (**A**). Time in the center (sec), (**B**). Time in the open arm (sec), (**C**) Latency to open arm (sec), (**D**). Number of open arm entries (sec), (**E**). Number of transitions between arms and (**F**). Number of open arm entries/ number transitions between arms. *n* = 5- 7 per group. Significant difference * $p < .05$, ***$p < .001$.

Table 1.
Two way ANOVA, OF activity measures across time exposure to LL and/or RF, OF1-3 corresponding to 1, 2 and 4 weeks in LL and/or RF

OF activity (5min)	Group	TIme in LL (OF1-3)	Group x time in LL
Total distance	F (3, 21) = 0.3708 P = 0.7748 ns	F (2, 42) = 5.406 P = 0.0081 **	F (6, 42) = 0.8571 P = 0.5340 ns
Center distance	F (3, 21) = 2.077 P = 0.1338 ns	F (2, 42) = 5.424 P = 0.0080 **	F (6, 42) = 0.6684 P = 0.6755 ns
Time moving	F (3, 21) = 1.957 P = 0.1514 ns	F (2, 42) = 7.389 P = 0.0018 **	F (6, 42) = 1.305 P = 0.2762 ns
Time in center	F (3, 21) = 3.718 P = 0.0274 *	F (2, 42) = 12.62 P < 0.0001 ****	F (6, 42) = 1.009 P = 0.4323 ns
Center/total distance ratio	F (3, 21) = 1.279 P = 0.3073 ns	F (2, 42) = 16.57 P < 0.0001 ****	F (6, 42) = 1.360 P = 0.2531 ns
Entries into center	F (3, 21) = 1.243 P = 0.3192 ns	F (2, 42) = 2.244 P = 0.1186 ns	F (6, 42) = 1.852 P = 0.1120 ns

ns, not significant

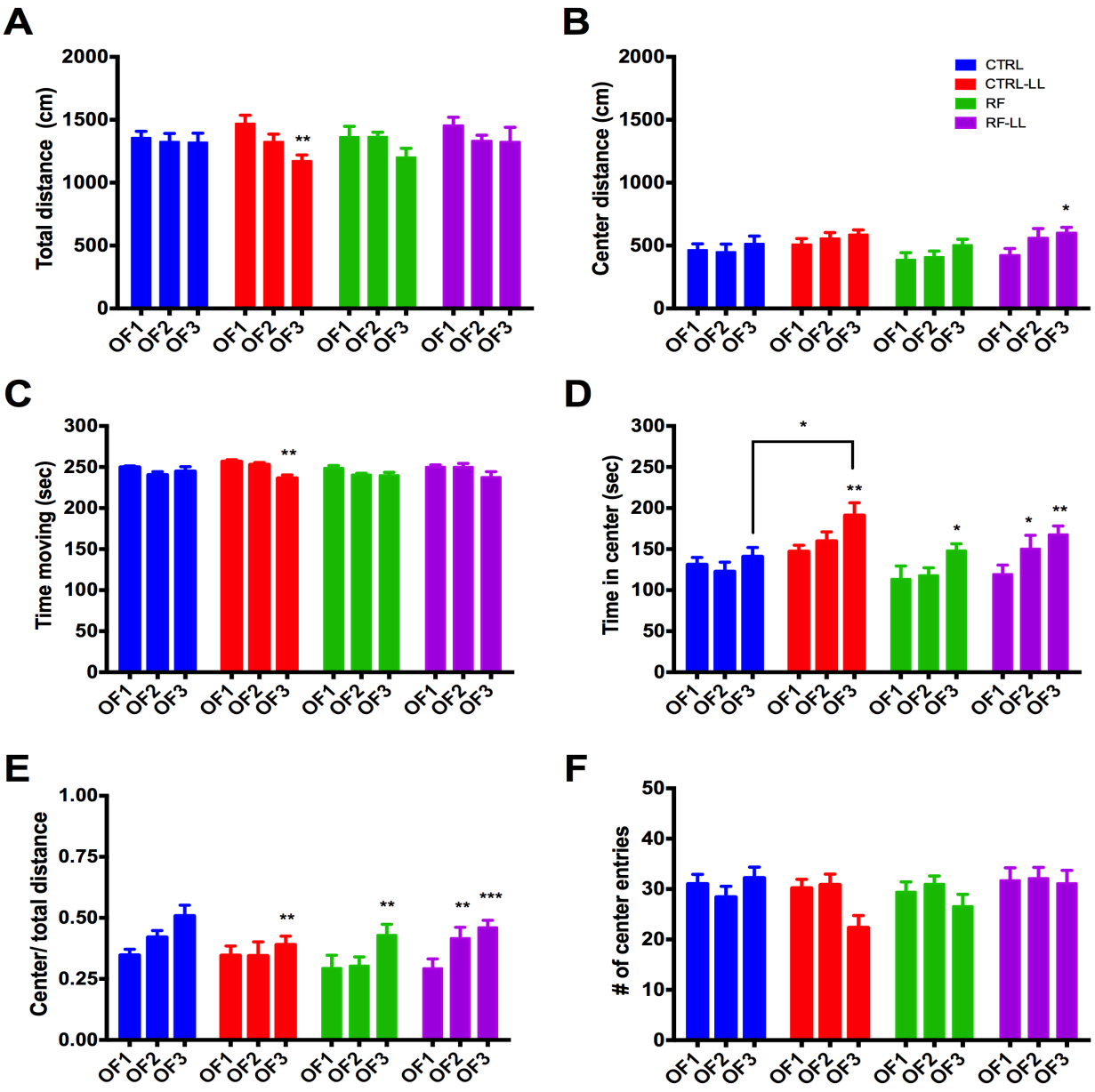


Fig 6. Activity in OF 1-3, corresponding to 1, 2 and 4 weeks into LL and/or RF. Means +/-sem are shown for (**A**). Total distance (cm), (**B**). Center distance (cm), (**C**). Time moving (sec), (**D**). Time in center (sec), (**E**). Center/ total distance ratio, and (**F**). Number of center entries. *n* = 6 or 7 per group. Significant differences between groups compared to CTRL. Significant differences within groups over time, compared to OF1. * $p < .05$, ** $p < .01$, *** $p < .001$.

No differences in body weight during FST

Experimental designs for RF took into consideration that body weight can affect behavior in the FST (Bogdanova et al., 2013). In experiments 1 and 2, the FST was conducted 10 days post-RF. Previous work in our lab found that 10 days post-RF is the earliest time at which body weight of RF is no longer significantly different from controls and cross-sensitization to AMPH is still present. Body weight of RF compared to CTRL in exp1 and 2 do did not significantly differ during the FST, t_{20}= 1.76, p= 0.01. In experiment 3, RF groups were subjected to a 5h-feeding window intended to maintain similar body weight to controls throughout the experiment. From time of arrival to the final day of RF there was a significant increase in weight among groups $F_{(6, 120)}$ = 2179, $p < 0.10$, with an interaction between time and group $F_{(18, 120)} = 4.49$, $p < 0.01$, however there was no difference in body weight between groups $F_{(3, 20)} = 2.97$, p = 0.058. Post-hoc analysis found one significant difference between groups RF and CTRL-LL on day 16 of RF, which was not present at day 20 of RF.

Discussion

Overall, our findings revealed no negative effects of RF for measures of depression and anxiety-like behavior in the male rat. Behavior in the FST showed no differences at 10 days post-RF for both Wistar and Long Evans rats. Furthermore, when testing rats during a 5h RF window, which did not result in significant weight loss, it was found that RF increased swimming behavior. Although there are conflicting views on the interpretation of the FST, swimming behavior is not attributed to anxiety or depression-like behavior, as is the case with climbing and immobility (De Kloet & Molendijk, 2016;

Commons et al., 2017; Anyan & Amir, 2018). In particular, increased swimming could reflect elevated activity in the serotonin system (Cryan, Valentino & Lucki, 2005), which is a targeted system in pharmacological treatments for depression. Finally, time to immobility for rats on RF did not differ from the control group on both habituation day and test day. RF-LL took significantly longer to become immobile on habituation day relative to test day, indicating an elevated response to acute stress over other groups, however this difference was not present on test day. Unexpectedly, we did not find differences in the FST as a result of LL. Previous studies evaluating the effects of light at night (LAN) on FST behavior in mice (3 weeks LL) and hamsters (8 weeks LL) have noted depressive-like phenotypes as indicated by increased immobility (Fonken et al., 2009; Bedrosian et al., 2011). It is important to note that in these studies the FST is a one-day test, which does not reflect a learned response, but rather an acute response to stress.

Caloric restriction in rodents has previously been shown to have anxiolytic effects in EPM and OF (Lutter et al., 2008; Dietze et al., 2016), as has circadian RF in the EPM (Inoue et al., 2004). Interestingly, in the latter study they determined that the anxiolytic effects reflect the same timeline as RF's effects on cross-sensitization to AMPH (Opiol et al., 2017). In the current study we did not find significant differences in the EPM for RF compared to CTRL, however there appeared to be a trend toward anxiolytic behavior for both RF groups. CTRL-LL spent more time in the center of the EPM, which may reflect a delay in decision-making strategy (Sestakova et al., 2013) as a result of LL. If so, RF may have prevented the delay in decision-making, since RF-LL did not

significantly differ from CTRL. RF-LL also displayed more entries into the open arm in proportion to total arm transitions, indicating less anxiety. Three weeks of LL has previously been shown to result in increased time in the open arm (Ma et al., 2007; Fonken et al., 2013), a finding we did not confirm.

In the OF there was a significant difference between CTRL and CTRL-LL for time spent in the center after >4 weeks in LL. One interpretation is that LL resulted in an anxiolytic effect. However, it is more likely that the increased time spent in the center may be a maladaptive effect of LL. Examining the within group changes over the course of LL, CTRL-LL was the only group that significantly reduced its activity in total distance travelled and decreased time spent moving from OF1-3. Distance travelled in the center for CTRL-LL did not change, although time spent in the center significantly increased from OF1-3. In contrast, RF-LL also increased time spent in the center from OF1-3, however this was (appropriately) paralleled by an increase in total distance travelled in the center. Thereby, it appears that the increase in time spent in the center for CTRL-LL is not spent moving or "exploring" and may reflect a delay in decision-making as in the case of the EPM. Evaluating the behavior as a ratio of distance travelled in the center relative to total distance, it appears that over time both RF and LL result in an increased amount of exploratory activity. Albeit the reason that CTRL-LL has an elevated ratio score at OF3 is due to a significant decrease in total distance travelled rather than increased distance travelled in the center.

In contrast to our findings, studies that tested acutely fasting (24-48h) and calorie restricted (25-80% body weight) rats using similar methodology, including testing at the same time of day and after rats had fed, found more center activity on the first trial in the OF relative to controls (Heiderstandt, 2000; Levay et al., 2007; Dietze et al., 2016). Perhaps weight loss or caloric restriction may be a contributing factor to exploratory activity or overall increased activity in the OF. In our case, RF did not result in significant weight loss and OF activity did not differ relative to controls at any time point.

A limitation of this study is that rats were not monitored for circadian rhythms in locomotor activity or body temperature, which are indicators of internal circadian rhythmicity. Therefore, we cannot ensure that there was sufficient circadian disruption. Tapia-Osorio et al. (2013) evaluated the effects of 6 weeks LL on OF activity and found elevated levels of grooming stereotypy. We found no differences in stereotypic behavior (not shown), thereby ~4 weeks LL may not have been enough time for circadian disruption. Future investigation should evaluate whether RF prevents negative effects of chronic stress models of depression, social-defeat and repeated light cycle changes that simulate jet-lag or shiftwork schedules to better understand whether RF could prevent any disruptions to stress-coping strategies, which may translate to better outcomes in human mental health.

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GENERAL DISCUSSION

The work presented in this thesis supports the idea that DA is involved in the circadian food entrainment pathway. We show that food entrainment results in changes to the DA system, which are paralleled by an enhanced locomotor response (cross-sensitization) to AMPH. One, 3 and 7 days of RF are insufficient to produce cross-sensitization. Cross-sensitization is present at 2 weeks of RF and persists for at least 10-14 days following the return of ad libitum food. Counter to what previous studies have indicated in regards to food restriction and reward-related cross-sensitization to AMPH (Carr, 2002; De Vaca, Krahne, & Carr, 2004), the effects of circadian RF on cross-sensitization are unrelated to body weight at the time of testing. Our work finds that cross-sensitization by RF is present after body weight is regained post-RF, as well as, when a larger feeding window is used that does not result in significant weight loss. However, the element of negative energy balance is necessary, as the presence of a daily-restricted treat does not result in cross-sensitization.

Interestingly, we did not find an enhancement of FAA from AMPH sensitization. Three different AMPH sensitization protocols were used to evaluate the question of whether increasing DA sensitivity via AMPH sensitization would likewise show an increase in locomotor output prior to feeding time. Our speculation is that FAA may have reached a ceiling effect, and thereby does not imply that DA is not involved in food entrainment. The ceiling effect may be the result of the instrumentation used to measure FAA or perhaps, more likely, it may reflect an upper limit of locomotor output. The latter is supported by the finding that RF itself results in the enhancement of locomotor response to AMPH.

To investigate the role of the DS in RF cross-sensitization to AMPH, three measures of DA function were evaluated. Unexpectedly, we did not find differences in FOS expression in the DS, which has been reported in cross-sensitization to AMPH resulting from repeated social defeat stress (Nikulina et al. 2004). DA in the DS has been shown to be critical in the expression of FAA (Gallardo et al., 2014) and the resetting of clock rhythms (Hood et al., 2010), thereby we hypothesized this region might show increased neuronal activation that would explain the effect of RF on cross-sensitization to AMPH. In measuring total TH in the DS, we also did not find differences that would explain RF cross-sensitization. However, it is possible that had we measured phosphorylated TH we might have found differences, as is suggested by one study evaluating chronic food restriction (Pan et al., 2006).

Ironically, we found that the expression of DAT increased with time exposure to RF. Although DAT is one of the main mechanisms through which AMPH produces its effects, it has also been shown that DAT is not necessary for the development of FAA (Enriquez, 2018). One explanation for our finding is that DAT in the DS acts as a critical regulator of DA clearance in the synapse during FAA, since DA and its metabolites increases prior to the feeding window (Liu et al., 2010). In the absence of DAT, a compensatory mechanism for regulating DA would be required to take its place, such as through enzymatic degradation, astrocytes, or a secondary neuronal uptake mechanism, such as norepinephrine transporter (Jennings & Rusakov, 2016; Sulzer, Cragg & Rice, 2016). The compensatory mechanism is likely not through the DA D2 autoreceptor, as DAT knockout mice do not show elevated levels of D2 when placed on

RF and still display FAA (Enriquez, 2018).

One interesting hypothesis for the role of DA in the DS in driving circadian activity is that it is responsible for setting the pace (De Lartigue & McDougle, 2018). This hypothesis is largely supported from work conducted in Dr. Kai-Florian Storch's lab. Storch's lab has demonstrated that dopaminergic neurons oscillate with ultradian rhythms, which can be observed in activity patterns when dissociated from the SCN. When circadian clock function is disrupted through SCN lesion or genetic manipulation, locomotor activity will occur in shortened bouts of ~ 4h (Blum et al., 2014). Conversely, when SCN lesioned mice have DAT function genetically disrupted, such that DA accumulates in the synapse, the 4h ultradian rhythms are lengthened to 12 hours. It is argued, rather convincingly, through strong correlational data between DA levels, period-manipulation and the resulting locomotor output, that extracellular DA levels act as a period determinant. Thereby, given the ongoing work showing that DA release in the DS occurs in food restricted individuals during initial food intake, as an immediate orosensory response and as a delayed post-ingestive response (Small, Jones-Gotman & Dagher, 2003; Thanarajah et al., 2018) and given its necessity for conditioned learning (Voorn et al., 2004; De Lartigue & McDougle, 2018), time-restricted food intake through DA in the DS could act as the period determinant in food entrainment.

We hypothesize that elevated DAT with time exposure to RF is likely a homeostatic regulatory response to manage the clearance of DA in the synapse. A new feeding time would trigger an elevated DA response in the DS that would initially result from food

intake, and over time could develop into a conditioned, anticipatory, response of DA that could drive circadian food entrained rhythms, such as FAA. AMPH cross-sensitization seen with RF likely reflects this new diurnal rhythm of DAT to feeding time, as DAT in the DS is a primary mechanism through which AMPH produces its locomotor effects (Sulzer et al., 2005; Calipari & Ferris, 2013). On a side note, DAT appears to play a critical role in driving the diurnal cycle of extracellular DA in the DS, since mice with disrupted DAT function do not show a rhythm (Hood et al., 2010; Gallardo et al., 2014). It is possible that DAT may likewise be important for maintaining DA rhythms in the absence of a food reinforcement on DA, such as with the maintenance of FAA when meals are omitted. Food entrained rhythms are also hypothesized to compete with input from the SCN, when the feeding window is out of phase with the LD cycle, either through inhibition of the SCN or elevated activation of arousal circuits (Acosta-Galvin et al., 2011; Datollo et al., 2016). Therefore, food entrainment could result from a new rhythm in DA regulation, one that occurs with a stronger DA tone driving circadian periodicity, rather than an ultradian rhythm that is overshadowed by or integrated together with input from the SCN.

Though there has been much research conducted towards understanding the underlying mechanisms of food entrainment, equally important is the research that has shown the beneficial health outcomes of food entrainment. Although we cannot answer how exactly the benefits arise, we can evaluate the practicality of its implementation. As mentioned in chapter 3, there are many physiological benefits to RF; however, less is known about how RF affects mental health. Affective disorders often stem from or result in the disruption of

circadian rhythms, whether that is through chronic stress, inability to attain sleep, a traumatic event, or any number of negative lifestyle habits that result in changes to food intake or exposure to light at night. Given that DA is involved both in the locomotor sensitization of RF and in the pharmacological treatment of affective disorders, RF may serve a therapeutic function. The ability for RF to maintain and strengthen circadian rhythms through dopaminergic brain circuits may serve a preventative or non-pharmacological treatment option for depression and/or anxiety.

To investigate how RF may affect emotional processing, we evaluated behavioral tests for depression and anxiety in the male rat. We found that RF did not result in any negative effects in the FST, a common measure of depression-like behavior. Firstly, we found no behavioral differences in the FST at 10 days post-RF, a time previously shown to display cross-sensitization to AMPH. This finding was confirmed in two rat strains known to behave differently in the FST. Next, we tested rats during RF, by using a longer 5h RF schedule that did not result in significant weight loss, but which did yield cross-sensitization to AMPH. In this scenario, RF resulted in increased swimming behavior. Increased swimming is indicative of elevated serotonergic activity (Slattery & Cryan, 2012). Although there are conflicting viewpoints on the behavioral interpretations of the FST, the gray area lies with the definitions for climbing and immobility in regards to which one reflects adaptive or maladaptive behavior- anxiety or depression (De Kloet & Molendijk, 2016; Commons et al., 2017; Anyan & Amir, 2018). In either case, swimming behavior is not argued as maladaptive. Therefore, the effects of RF on measures of depression or anxiety-like behavior in the FST do not suggest a negative outcome on affect, and perhaps reflect an

increase in adaptive response to a stressor.

We also investigated whether LL, a known disruptor of circadian rhythms, would have negative effects in behavioral measures of the FST, EPM or OF, of which the latter two are measures of anxiety-like behaviour. We postulated, that whatever negative effects LL may precipitate, RF might serve a preventative function against LL. To our surprise, LL itself did not result in any significant negative effects in the FST. Perhaps 3- 4 weeks LL was insufficient to disrupt circadian rhythms in rats; however previous work in mice reported depressive-like behavior after 3 weeks LL (Fonken et al., 2009). In the EPM, LL may have resulted in deficits to decision-making strategy (Sestakova et al., 2013), to which RF may have prevented this deficit. RF appeared to show a trend towards more activity in the open-arms, a sign of reduced anxiety, also previously reported with caloric restriction (Inoue et al., 2004). Finally, we evaluated OF activity at three time points during LL and/or RF exposure. We found only one group difference, for time spent in the center in the CTRL-LL group during the final week of LL. After evaluating within group activity, we believe that the time spent in the center reflects a maladaptive behavior. The time spent in the center was not indicative of active behavior, and therefore, similar to the EPM, may reflect a deficit in decision-making strategy. Taking the latter interpretation, we believe that RF prevented the development of the maladaptive effect of LL.

The ability of food entrainment to harness circadian rhythms is an appealing idea for health practitioners. It is a relatively simple, non-pharmaceutical intervention, through which internal rhythmicity can be resynchronized at multiple levels of physiology. Both rhythms in

the body and brain respond to RF under conditions of disrupted communication from the SCN. Given that artificial lighting, shift work, jet-lag from travel or social events are increasingly a part of our culture, the environment is more likely to impair circadian rhythms and weaker at reinstating them. Our work suggests that RF does not have negative effects on mental health, and even positive effects, depending on one's interpretation. Our work also suggests a regulatory effect of RF through the DA system, an area known to be involved in many psychological disorders. Due to the limitations that animal research has on the evaluation of mental health, future work on RF will need to be conducted in humans. Future considerations should acknowledge that RF might require an extended timeframe for the reorganization of circadian rhythms and DA function to positively contribute to mental health. We hypothesize that any negative effects RF may have on mental health would likely be temporary, as the animal literature is decidedly in agreement in regards to the long-term benefits of RF at multiple levels of physiology.

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