Investigating individual differences in circadian locomotor parameters, depression- and anxiety-like behaviors and comorbidity in healthy Lewis rats

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ABSTRACT

Investigating individual differences in circadian locomotor parameters, depression- and anxiety-like behaviors and comorbidity in healthy Lewis rats

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Disrupted circadian rhythms are a core feature of various psychiatric disorders, including, but not limited to depression and anxiety. At this point, it remains uncertain whether disrupted circadian rhythms are implicated in the pathophysiology of mental disorders or whether the disrupted circadian rhythms are simply a byproduct of the disorder. The objective of this thesis is to explore the relationship between circadian rhythms and mood. The first study examines individual differences in circadian locomotor behavior and mood-related behaviors in rats. The circadian phenotype of male Lewis rats was characterized by analyzing daily wheel running activity under multiple lighting conditions: standard 12h:12h LD conditions, constant dark, constant light, and rate of re-entrainment to a phase advance. Rats were then tested on a battery of behavioral tests: activity box, elevated plus maze (EPM), forced swim test (FST), and fear conditioning.

We found an interesting relationship between entrainment parameters and mood-related behaviors. Under 12h:12h LD conditions, percent of daily activity in the light phase and variability in activity onset were associated with latency to immobility in the FST. Variability in onset was also associated with anxiety-like behavior in the EPM. Rate of re-entrainment correlated with anxiety-like behaviors in the activity box and EPM. Our findings suggest that Lewis rats may be a suitable strain for studying the relationship between circadian rhythms and mood-related behaviors in rodents.

In human clinical populations, comorbidity rates between depression and anxiety are very high. Surprisingly, little attention has been given to the question of comorbidity in

animal models. To further examine the utility of Lewis rats for the study of mood-related behaviors we then assessed for comorbidity between depression- and anxiety-like behaviors. Interestingly, our findings indicate suggest that Lewis rats do not exhibit comorbid depression- and anxiety-like behaviors. While the absence of a comorbid relationship between depression- and anxiety-like behaviors may come as a surprise, we present an alternative interpretation of escape-directed behaviors in the FST.

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the way: Genevieve, Isis, Kristyn, Sarah, and of course Cam and Jonny back home, get the records spinning and fill the fridge boys because it's going to be a long night!

"A Healthy man is like a well functioning clock, and an ill man is like a clock that needs repairing"

René Descartes

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

List of Abbreviations

β-CCE: beta-carboline-3-carboxylic acid ethyl ester

ACTH: Adrenocorticotrophic hormone

BMAL1: Brain and muscle arnt-like protein-1

CLOCK: Circadian locomotor output cycles kaput

CMS: Chronic mild stress

CREB: cAMP response element binding protein

CRY: Cryptochrome

DD: Constant dark

DMH: Dorsal medial hypothalamus

dPVZ: Dorsal paraventricular zone

Drd1: Dopamine receptor D1

DSM: The Diagnostic and Statistical Manual for Mental Disorders

EPM: Elevated plus maze

FST: Forced swim test

HAB: High anxiety- and depression-like behavior

ipRGCs: Intrinsically photosensitive retinal ganglion cells

LHA: Lateral hypothalamic area

LD: Light-Dark

LL: Constant light

MPO: Medial preoptic area

NAc: Nucleus accumbens

OCD: Obsessive-compulsive disorder

PVH: Paraventricular nucleus of the hypothalamus

PER: Period

PACAP: pituitary adenylate cyclase activating polypeptide

SCN: Suprachiasmatic nucleus

SNP: Single nucleotide polymorphism

SSRI: Serotonin selective reuptake inhibitor

UCMS: Unpredictable chronic mild stress

vPVZ: Ventral paraventricular zone

VLPO: Ventrolateral preoptic area

VIP: Vasoactive intestinal polypeptide

ZT: Zeitgeber time

Chapter 1: General Introduction

Investigating individual differences in circadian locomotor parameters, depression- and anxiety-like behaviors and comorbidity in healthy Lewis rats

Mental illness is the number one cause of disability in Canada with an estimated one in five Canadians experiencing mental health issues, such as depression, anxiety and addiction (Canada, 2013; Lim, Jacobs, Ohinmaa, Schopflocher, & Dewa, 2008). The financial burden of mental health in Canada is over 50 billion dollars a year (Smetanin, 2011). Having a mental illness can be an impediment in acquiring a job and there are at least 500,000 Canadians who are unable to work due to some form of mental illness. Moreover, unemployment rates can reach as high as 90% for severe mental conditions such as schizophrenia (Marwaha & Johnson, 2004). Given that mental illness takes a tremendous toll on individual, their family, and society at large, it is important to identify risk factors and potential causes in order to reduce the burden not only in Canada but also globally.

Depression and anxiety are the most commonly diagnosed mental disorders. Although the underlying pathophysiology of these disorders is unknown, there is reason to believe that disrupted circadian rhythms may be involved. There are diverse circadian disruptions in depression and anxiety, including hormone/endocrine release, sleep/wake cycles and daily activity (Breslau, Roth, Rosenthal, & Andreski, 1996; Ford & Kamerow, 1989; Luik et al., 2015; Souetre

et al., 1989). The strong link between depression and anxiety disorders has been known for a long times, however, the link between circadian rhythms and mood has yet to be elucidated. The purpose of this dissertation is to examine the relationship between circadian rhythms and depression- and anxiety-like behaviours in rats. This chapter begins with an overview of circadian rhythms and the endogenous circadian system, followed by a review of circadian rhythm disruptions in human clinical populations and animal models of depression- and anxiety-like behaviour.

Circadian Rhythms

Circadian rhythms are daily patterns in physiology and behaviour. Virtually all living things exhibit circadian rhythms, from single cell bacteria to plants and humans. In mammals, circadian rhythms are coordinated and sustained by an endogenous master clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. Circadian rhythms are endogenous, however they must be synchronized to the environment through external time cues known as zeitgebers. The primary zeitgeber in mammals is light, but food, exercise, and temperature can also influence circadian rhythms (S. Hughes, Jagannath, Hankins, Foster, & Peirson, 2015). The circadian system likely evolved as a way promote survival as it allows an organism to predict reoccurring events in the environment, such as the availability of food or mates (Albrecht, 2012). On the other hand, when the circadian system is not properly synchronized to the

environment, it is associated with physical and mental health issues, to be elaborated upon further below (Stevens et al., 2007).

The Master Circadian Clock

At a cellular level, circadian rhythms are coordinated by the master clock located in the SCN. This master clock is comprised of approximately 10,000 cells bilaterally, all of which are cell autonomous, meaning they self-sustain rhythms in the absence of any peripheral inputs (e.g., constant conditions). In vitro the SCN continues to display circadian rhythms in electrical firing rate (Gillette et al., 1995), Ca2+ concentration levels (Welsh, Logothetis, Meister, & Reppert, 1995), action potentials (Inouye & Kawamura, 1979), and gene expression (Yamazaki et al., 2000). Although individual cells within the SCN continue to oscillate, they must be synchronized to one another to create the emergent circadian clock. Vasoactive intestinal polypeptide (VIP) and its receptor VPAC2 have been shown to be fundamental for cellular synchrony at the level the SCN (Maywood et al., 2006) because genetically removing VIP or VPAC2 in mice causes arrhythmicity or a blunting of circadian rhythms in body temperature, hormone release, locomotor activity and clock gene expression in the SCN (Harmar et al., 2002; Loh, Abad, Colwell, & Waschek, 2008; Schroeder, Loh, Jordan, Roos, & Colwell, 2011). Synchrony between cells in the SCN is important as brain regions downstream of the clock and various peripheral tissues rely on signals from the SCN to determine the phase of their rhythms.

There are several lines of evidence demonstrating that the SCN is the master circadian pacemaker. Lesioning the SCN abolishes rhythms in locomotor activity, body temperature, hormone secretion, sleep-wake cycles, eating and drinking behaviours, thus demonstrating the necessity of this region (Meyer-Bernstein et al., 1999; Stephan & Zucker, 1972; Tahara et al., 2012). Moreover grafting fetal SCN tissue onto a lesioned animal restores circadian locomotor rhythms but with the endogenous period of the donor (Ralph, Foster, Davis, & Menaker, 1990). These studies clearly demonstrate the fundamental nature of the SCN in generating circadian rhythms. It is also important to note that recovery of circadian locomotor activity is not dependent on the re-establishment of afferent and efferent connections between the SCN and surrounding brain regions because transplanting an SCN within a semipermeable capsule, which prevents any connections being made, restores circadian locomotor activity (Silver, LeSauter, Tresco, & Lehman, 1996). How the re-establishment of locomotor rhythms are restored under such conditions is uncertain (Dibner, Schibler, & Albrecht, 2010) however, it likely involves neurochemical signals to the subparaventricular zone (SPZ) and the dorsomedial nucleus (Saper, Lu, Chou, & Gooley, 2005). The SPZ is broken down into dorsal and ventral regions, each of which regulate different circadian parameters. Lesions to the ventral SPZ disrupts circadian locomotor output while lesions to the dorsal SPZ disrupts body temperature rhythms. The ventral SPZ has significant projections to the dorsomedial nucleus of the hypothalamus (DMH) (Saper, Lu, et al., 2005; Saper, Scammell & Lu, 2005). The DMH acts as a hub (see Figure 1.) for regulating a

host of circadian rhythms and lesions to this regions disrupts circadian rhythms in a host of circadian parameters, including sleep/wake cycles, corticosteroid release, feeding and locomotor behavior (Chou et al., 2003). In summary, the SCN is the master pacemaker, which regulates circadian rhythms in physiology and behavior through a network of brain regions and neurochemical signaling pathways.

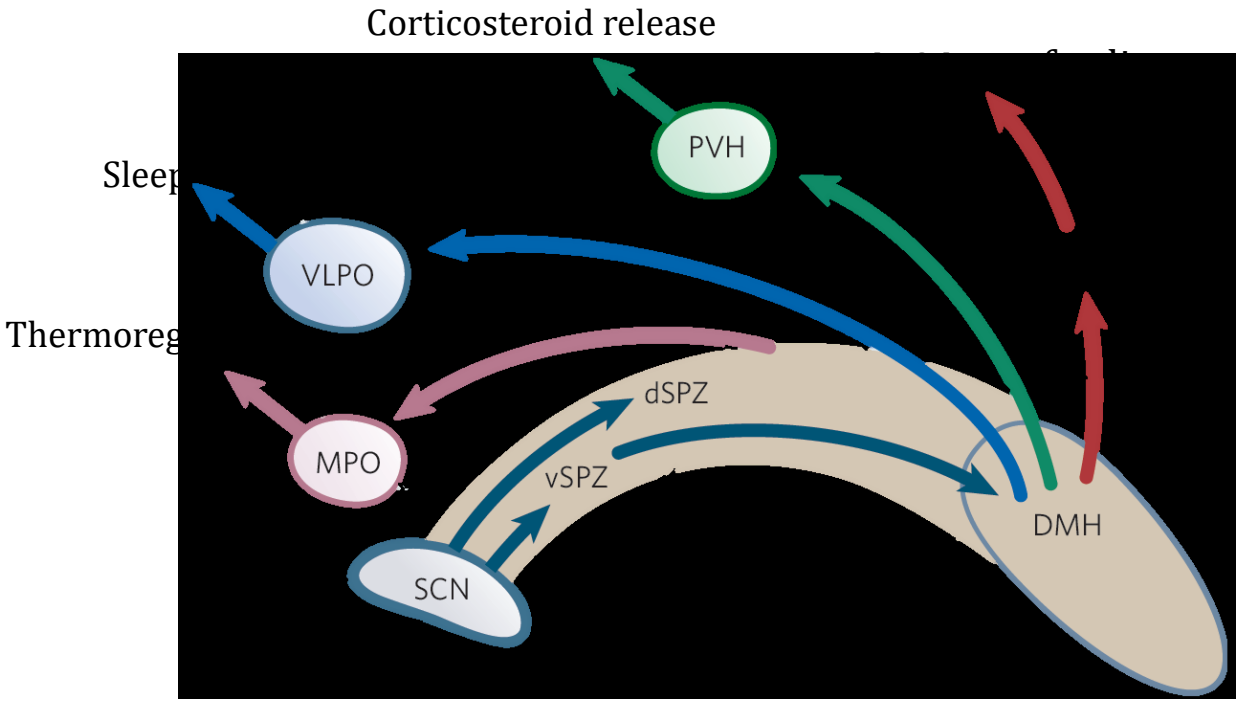

Figure 1. SCN coordination of circadian rhythms.

SCN: suprachiasmatic nucleus; vSPZ: ventral subparaventricular zone; dSPZ: dorsal subparaventricular zone; DMH: dorsomedial nucleus of the hypothalamus; LHA: lateral hypothalamic area; PVH: paraventricular nucleus; VLPO: ventrolateral preoptic area; MPO: medial preoptic area. Adapted from Saper, Scammell & Lu, 2005.

At the molecular level, circadian rhythms are generated by an autoregulatory transcriptional-translational feedback loop. The feedback loop begins with the transcription and translation of two core circadian genes: circadian locomotor output cycles kaput (*Clock*) and brain and muscle arnt-like protein-1 (*Bmal1*). The Clock and Bmal1 proteins form dimers that bind to the e-box promoter region of its target circadian genes Period (*Per*) and Cryptochromes (*Cry*). This constitutes the driving or positive arm of the feedback loop. The Per and Cry proteins begin to accumulate in the cell's cytoplasm where they form dimers and translocate back into the cell's nucleus inhibiting their own transcription, thus forming the negative arm of the feedback loop, see figure 1 (Reppert & Weaver, 2002). In addition to the core transcriptional-translation feedback loop just described there are additional loops and regulatory mechanisms involved in the molecular clock, interested readers are directed to (Ko & Takahashi, 2006). This process takes approximately 24h to complete one full cycle (Dickmeis, Weger, & Weger, 2013).

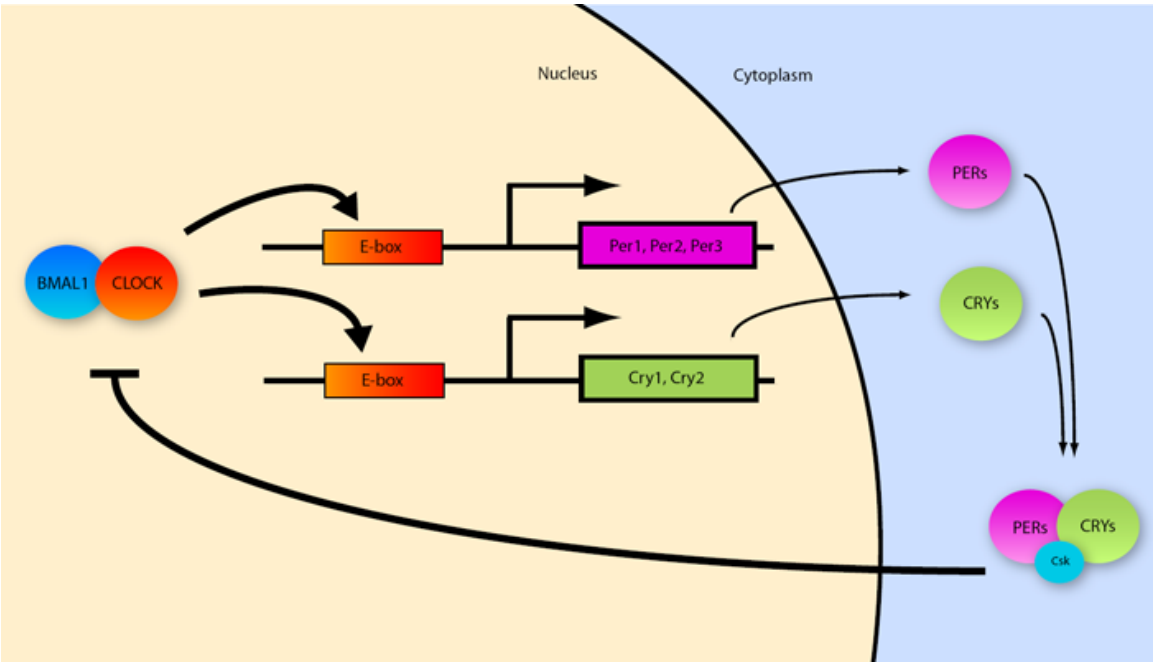

Figure 2. Transcriptional translational feedback loop.

Source: https://naef-lab.epfl.ch/files/content/sites/naeflab/files/shared/figures/figure1_guil.png

Synchronizing the Master Clock to the Environment

Various environmental cues can act as time cues for the circadian clock, however the earth's rotation on its axis and the 24h light/dark cycle that it produces is the primary zeitgeber. At dawn, light enters the eyes and this photic information is registered by specialized retinal photoreceptors (i.e., cones, rods and intrinsically photosensitive retinal ganglion cells) located in the back of the eye in the retina (Paul, Saafir, & Tosini, 2009). Photic information is then transmitted from the retina to the SCN through the retinohypothalamic tract in a process known as photic transduction. The terminals of the retinohypothalamic tract terminate in the ventral SCN releasing glutamate, pituitary adenylate cyclase activating polypeptide (PACAP) and substance P (Ebling, 1996;

Hannibal, 2006; Kim et al., 2001). Glutamate, PACAP and substance P reset the clock through activation of NMDA, AMPA/kainite and PACAP-specific receptor 1, causing an increase in intracellular concentrations of Ca2+ and cAMP. Phosphorylation of Ca2+/cAMP response element binding protein (CREB) (Ginty et al., 1993) leads to the expression of immediate early genes (Rusak, Robertson, Wisden, & Hunt, 1990) and the induction of clock genes Per1 and Per2 (Albrecht, Sun, Eichele, & Lee, 1997; Travnickova-Bendova, Cermakian, Reppert, & Sassone-Corsi, 2002).

Light is the primary zeitgeber and effectively resets the phase of the endogenous clock each day to remain entrained with the environment. Presenting light at different points in the circadian phase differentially affects the phase of locomotor output. The presentation of a light pulse to nocturnal rodents at the beginning of the dark phase causes a delay in locomotor output on the subsequent day (phase delay). On the other hand, presenting a light pulse at the end of the dark phase causes a phase advance (i.e., onset of behaviour is advanced on the subsequent day) (Pittendrigh & Daan, 1976). The circadian clock rapidly synchronizes to the minor time differences in sunrise and sunset, however, in the event that there is a large discrepancy from one cycle to the next (e.g., during shift work or travelling across time zones) the circadian system takes longer to readjust. When most of us travel across time zones we experience jetlag for a few days without any significant health concerns. However, individuals who chronically disrupt their circadian system through trans-meridian travel or

shiftwork are at greater risk for developing physical and mental health problems, which will be discussed next (DeCoursey, Walker, & Smith, 2000; Karatsoreos, 2014; Stevens et al., 2007).

Circadian Rhythms and Mental Health

Mental health is intimately linked to circadian rhythms. Most, if not all, mental disorders are associated with some form of disrupted circadian rhythm. The following section discusses circadian disruptions in depression and anxiety from two perspectives: human clinical studies and animal models of depression- and anxiety-like behaviors. Both depression and anxiety are associated with a host of circadian disruptions, however, what remains to be understood is whether disrupted circadian rhythms are implicated in the pathogenesis of these disorders or are an epiphenomenon.

Depression and Anxiety

According to The World Health Organization, depression is one of the leading causes of disability worldwide and by 2020 it is expected to be the second leading cause of disability (Organization, 2001). Given the increasing rates of depression, it is important to distinguish what differentiates clinically significant depression from feeling down or having the blues. In North America, clinicians use The Diagnostic and Statistical Manual for Mental Disorders (DSM) to diagnose depression and other mental disorders. Clinical depression, or Major Depression, is characterized by a persistently low mood, loss of interest, fatigue,

feelings of worthlessness and sometimes suicidal thoughts. It is normal for everyone to experience symptoms of depression at different points in their life but in order to be diagnosed with depression, the symptoms must be severe enough to interfere with daily life and last at least two weeks.

Just as most people will experience symptoms of depression at different points in their life, everyone also experiences fear. Having a fear response is normal when one's life is threatened or there is a real threat of danger; it serves an adaptive purpose when you avoid certain objects, places or animals that pose a threat. Anxiety on the other hand is when an individual experiences an exaggerated fear response in the absence of any legitimate threat. In other words, a healthy fear response occurs when one is faced with a threat whereas anxiety is the anticipation of threat. Anxiety disorders are a heterogeneous with diverse clinical presentations. In the most recent version of the DSM (American Psychiatric, American Psychiatric, & Force, 2013) the anxiety disorders section underwent some revisions and certain disorders were removed and given their own sections (e.g., post traumatic stress disorder and obsessive-compulsive disorder (OCD)). That being said, it is beyond the scope of this general introduction to discuss the rationale for the changes in diagnostic terminology and the debate that surrounds it, therefore these disorders will be discussed alongside DSM's canonical anxiety disorders.

Circadian Disturbances in Depression and Anxiety

Depression and anxiety disorders are associated with a host of alterations in circadian rhythms. One of the most common complaints is an alteration to the sleep/wake cycle, with upwards of 90% of depressed individuals (Tsuno, Besset, & Ritchie, 2005) and 70% of individuals with generalized anxiety disorder (Papadimitriou & Linkowski, 2005) reporting some form of sleep disturbance, including difficulty falling asleep, remaining asleep and/or early waking. The directional relationship between sleep disturbances and depression and anxiety is unclear. Some claim that there is a unidirectional relationship between insomnia and depression, where insomnia predicts future onset of depression (Johnson, Roth, & Breslau, 2006). Sleep disturbances do not appear to be a byproduct of depression or anxiety because disrupted sleep patterns tend to appear prior to the onset of the depression or anxiety disorder. One study found a spike in self-reported sleep disturbances in the weeks immediately preceding the onset of a depressive episode, suggesting that sleep disturbances may be prodromal feature (Perlis, Giles, Buysse, Tu, & Kupfer, 1997), with similar results have been reported for depression and anxiety disorders (Gillin, 1998). Two epidemiological studies found that in the absence of any pre-existing psychiatric condition, sleep disturbances are associated with increased risk of depression and anxiety disorders at one- and three-year follow-ups (Breslau et al., 1996; Ford & Kamerow, 1989). On the other hand, one study reported that symptoms of depression and anxiety at baseline were associated with new cases of insomnia at a 1-year follow up, suggesting that there may be a bidirectional

relationship between depression, anxiety and insomnia (Jansson-Frojmark & Lindblom, 2008). Disrupted sleep cycles are a prominent feature of depression and anxiety disorders and the disrupted sleep have been suggested to represent a prodromal phase in these disorders. If disrupted sleep cycles are a prodromal feature, closer monitoring of sleep could help identify individuals at risk for developing depression or an anxiety disorder (Gillin, 1998).

In addition to sleep disturbances, depression and anxiety disorders are also associated with a host of other circadian disruptions in physiology and behaviour. For instance, individuals with depression have elevated body temperature (Duncan, 1996), individuals with depression or anxiety have blunted activity rhythms and daily patterns are more fragmented compared to controls (Berle, Hauge, Oedegaard, Holsten, & Fasmer, 2010; Luik et al., 2015), and hormone and endocrine rhythms are disrupted (Monteleone, Catapano, Del Buono, & Maj, 1994; Souetre et al., 1989; Wingenfeld, Whooley, Neylan, Otte, & Cohen, 2015). In a now seminal study, Souêtre and colleagues (1989) collected blood samples every hour over a 25h period to compare circadian rhythms in plasma cortisol, norepinephrine, thyroid stimulating hormone and melatonin in healthy participants, individuals with depression and individuals with depression in remission. Compared to the other two groups, depressed individuals had elevated cortisol, lower levels of thyroid stimulating hormone during the night and blunted rhythms in melatonin. Similarly, in OCD, cortisol levels are elevated around the clock and there is a blunted rhythm in melatonin (Catapano,

Monteleone, Fuschino, Maj, & Kemali, 1992). More recently, urinary norepinephrine and cortisol levels were shown to correlate positively with depression and anxiety symptoms suggesting a synergistic relationship (J. W. Hughes, Watkins, Blumenthal, Kuhn, & Sherwood, 2004).

Taken together, depression and anxiety are associated with diverse circadian disruptions, including disrupted sleep/wake cycles, daily activity patterns, and hormone and endocrine rhythms. It is interesting to note that certain circadian parameters, such as sleep, become irregular in the time leading up to the onset of an episode. The disrupted rhythms then persist throughout the course of the episode but when in remission certain physiological rhythms resemble healthy. Although there is some evidence to suggest that circadian rhythms go awry prior to the onset of clinical symptoms, it is unclear why this is the case. There are two interpretations worth considering, either the disrupted circadian rhythms are causal in depression and anxiety or the disorders lead to disrupted circadian rhythms.

Clock Genes in Humans

To further understand the relationship between circadian rhythms and mood, some researchers are focusing on clock genes. Circadian rhythms are driven by a collection of core clock genes, which form a transcriptional-translational feedback loop. It was noted earlier that depression and anxiety are associated with disrupted behavioural and physiological rhythms, all of which are regulated

at least in part by the master clock, therefore a closer examination of the genes that comprise the molecular feedback loop is warranted.

Healthy individuals exhibit rhythmic expression of clock gene expression throughout the brain. One study, using postmortem tissue from healthy controls and depressed individuals found considerable discrepancies in the total gene expression across brain regions. Clock gene expression (including BMAL1, PER1-3) was lower in the dorsolateral prefrontal cortex, the anterior cingulate cortex, the hippocampus, the amygdala, the NAc, and cerebellum of depressed patients (J. Z. Li et al., 2013). In addition to attenuated expression, phase of clock gene rhythms were also altered indicating that there is desynchrony between brain regions and an uncoupling of the endogenous rhythms from the solar day.

The postmortem analysis suggests a role for internal desynchrony in depression. Currently, the best method for estimating circadian misalignment in living patients is to calculate dim light melatonin onset and the time of mid-sleep (Hasler, Buysse, Kupfer, & Germain, 2010). Melatonin peaks during the dark phase in healthy controls but is blunted in clinical populations (Souetre et al., 1989). In clinically depressed populations desynchrony is observed between dim light melatonin onset and core body temperature minimum, and dim light melatonin onset and sleep mid-point. Furthermore, this desynchrony is associated with the severity of depressive symptoms (Emens, Lewy, Kinzie, Arntz, & Rough, 2009;

Hasler et al., 2010). The internal desynchrony between melatonin and other circadian parameters may be due to aberrant cortisol rhythms. Individuals with severe depression have flatter diurnal rhythms in cortisol compared to mild or moderate depression, but also have lower cortisol at awakening and higher cortisol at night (Hsiao et al., 2010).

Buckley and Schatzberg (2010 (Buckley & Schatzberg, 2010)) found that by comparing the degree of uncoupling between the peak time of cortisol release and dim light melatonin onset they could distinguish healthy controls from those suffering from depression. It is unclear how the phase of cortisol and melatonin are related to one another, but the authors speculate that vasopressin receptors, which are dispersed throughout the SCN are implicated (Reghunandanan, Reghunandanan, & Marya, 1991). This is interesting argument because depression is associated with a reduction in the number of vasopressin-immunoreactive neurons in the SCN as well as a decrease in mRNA expression and amplitude (Zhou et al., 2001). Vasopressin V1a receptors in the SCN are important for regulating circadian clock genes as well as circadian locomotor activity (J. D. Li, Burton, Zhang, Hu, & Zhou, 2009), both of which are perturbed in depression (J. Z. Li et al., 2013; Luik et al., 2015), making vasopressin a likely candidate implicated in disrupted circadian rhythms in mood. One mechanism by which vasopressin could be effected in depression is through the increased basal levels of circulating stress hormones because glucocorticoids have an inhibitory

effect on vasopressin activity in the SCN (Liu, Unmehopa, Zhou, & Swaab, 2006).

Although there is accumulating evidence for disrupted phase relationships between physiological parameters in depression, relatively little is known about phase of clock genes in this population. One study found that individuals with a history of depression have increased levels of CLOCK, BMAL1 and PER1 mRNA in the morning compared to healthy controls (Gouin et al., 2010), whereas another study reported that CRY2 mRNA was significantly lower in the early afternoon (Lavebratt, Sjoholm, Soronen, et al., 2010). Unfortunately, neither of these studies collected data over a 24h period, which would have provided additional support for phase discrepancies in clock gene rhythms.

Lavebratt and colleagues (2010a) compared 115 single nucleotide polymorphisms (SNP) from 18 clock genes in depressed, healthy controls and mentally resilient participants. Participants were classified as mental resilient if they experienced one or more adverse life events in the 12-months preceding the study (e.g., experiencing a serious illness, being the victim of abuse or crime, homelessness, severe conflicts with spouse or others, and/or financial strain) but met the criteria for being in the control condition. They found an association between PER2 haplotypes (a combination of polymorphisms or DNA variations that are frequently inherited together) and risk of developing depression. Specifically, individuals classified as mentally resilient were more likely to have

the CTA haplotype than the depressed individuals who were more likely to have the CCG haplotype. In another study, Lavebratt and colleagues (2010b) found an association between CRY2 and seasonal depression in Swedish and Finnish populations (Lavebratt, Sjoholm, Partonen, Schalling, & Forsell, 2010; Lavebratt, Sjoholm, Soronen, et al., 2010). CRY2 has since been linked to dysthymic depression (a chronic and generally more mild form of depression) (Kovanen, Kaunisto, Donner, Saarikoski, & Partonen, 2013) and most recently with major depression (Kovanen, Donner, Kaunisto, & Partonen, 2017). Lastly, polymorphisms in CRY1 and NPAS2 (functionally equivalent to CLOCK) have been linked to unipolar depression (Soria et al., 2010). It is interesting to note that of the different clock genes, polymorphisms in CRY appear to have the strongest associations with depression. As stated above, CRY is one of the core clock genes involved in generating circadian rhythms in the master clock. CRY acts as a repressor of the molecular feedback loop, effectively shutting down the cycle, thus altering the endogenous period and an organism's capacity to transition between dark and light phases (Vitaterna et al., 1999; Ye et al., 2014).

Animal Models of Depression- and Anxiety-Like Behaviour

Due to some of the difficulties of measuring circadian rhythms in human clinical populations, animal models of depression- and anxiety-like behaviour provide a useful alternative to examine the relationship between circadian rhythms and mood. Numerous animal models have been developed over the years, but the most common models are based on brain lesions, chronic exposure to stress, or

genetic manipulations. When it comes to assessing the validity of an animal model, it is important that it meets certain criteria, including face validity, construct validity and predictive validity (Willner, 1984). Face validity refers to the similarity between the animal's phenotype and the symptom profile of the specific disorder. Construct validity implies that there are similar neurobiological mechanisms and sometimes this includes an independent assumption that there is also a similar underlying etiology (Geyer & Markou, 1995 In: (Bloom, Kupfer, & Neuropsychopharmacology, 1995). Predictive validity refers to the amelioration of clinical phenotype with appropriate treatments, for example, antidepressant or anxiolytic drugs. Although numerous animal models of depression and anxiety have been developed, the following section focuses on the most common paradigms and their effect on circadian rhythms.

The first animal model being discussed in based on the surgical ablation of the olfactory bulbs (i.e., the part of the brain that is necessary for processing odors). The olfactory bulbs are unique amongst brain tissues, because it is one of only a few tissues that maintain circadian rhythmicity in the absence of inputs from the SCN (Granados-Fuentes, Prolo, Abraham, & Herzog, 2004). Interestingly, olfactory bulbectomy leads to hyperactivity in a novel environment, decreased sexual behaviour, elevated levels of anxiety in the elevated plus maze (EPM), and deficits in associative learning (in the form of less freezing in contextual fear-conditioning) (Wang et al., 2007). Beyond, exhibiting strong face validity for an animal model of comorbid depression and anxiety, olfactory bulbectomy also

causes alterations in endocrine and neurotransmitter release, including a general elevation in cortisol (Marcilhac et al., 1997), increased cAMP in the SCN (Vagell, McGinnis, Possidente, Narasimhan, & Lumia, 1991) and reduced 5-HT turnover in the striatum, hippocampus, nucleus accumbens (NAc), frontal cortex, and the hypothalamus (Hellweg, Zueger, Fink, Hortnagl, & Gass, 2007), all brain regions implicated in depression (Lumia, Teicher, Salchli, Ayers, & Possidente, 1992). Olfactory bulbectomy also affects circadian locomotor parameters, including increased locomotor activity in LD (Marcilhac et al., 1997) and constant dark (DD), a lengthening of the free running period, delayed onset of running-wheel activity (Possidente, Lumia, McGinnis, Rapp, & McEldowney, 1996), and slower re-entrainment to phase advance and phase delays (Perret, Aujard, Seguy, & Schilling, 2003). It has yet to be determined why circadian locomotor parameters are affected by olfactory bulbectomy, but it is possible that the changes in 5-HT turnover in the hypothalamus is affecting cAMP through 5-HT receptors in the SCN (Baumgarten & Göthert, 2012) given that cAMP is important for regulating different aspects of circadian entrainment at the level of the SCN (O'Neill, Maywood, Chesham, Takahashi, & Hastings, 2008; O'Neill & Reddy, 2012). In support of this idea, treatment with Fluoxetine, a selective 5-HT reuptake inhibitor, reduces hyperactivity and shortens the free running period under DD (Possidente et al., 1996). Although the olfactory bulbectomy model of comorbid depression and anxiety has face and predictive validity it lacks construct validity.

Another approach to modeling mood-related behaviours in rodents with face, predictive and construct validity is the chronic mild stress (CMS) paradigm. In this paradigm, animals are exposed to various stressors (e.g., food or water deprivation, disrupting the light/dark cycle, tilting the cage, social isolation) typically for at least two weeks. Exposure to CMS leads to anhedonia-like behaviour, psychomotor retardation and decreased sexual activity (Gronli et al., 2005), fewer entries into the open arms of the EPM, and decreased swimming in the forced swim test (FST) (Logan et al., 2015). Exposure to CMS paradigm also disrupts circadian rhythms in physiology and locomotor parameters. These animals are less active under LD, DD and constant light (LL) (Gorka, Moryl, & Papp, 1996). They also exhibit fragmented locomotor rhythms with increased activity in the light phase relative to the dark (Logan et al., 2015). In respect to circadian physiological parameters, CMS is associated with higher melatonin secretion during the dark phase, a biphasic rhythm in cortisol peaking in the early light and early dark phases, and lower body temperature during the dark phase (Christiansen, Hojgaard, Wiborg, & Bouzinova, 2016).

Chronic mild stress also affects clock gene rhythms in the brain. Specifically, CMS attenuates the amplitude of Per2::luc in the SCN but increased in the amplitude in the NAc. Notably, the changes in amplitude in these two regions correlate with depression- and anxiety-like behaviours. Using real-time reverse transcriptase-polymerase chain reaction, it was shown that CMS alters the phase and amplitude of several other clock genes in the SCN and NAc. Stress

attenuated the amplitude of Per1 in the SCN and NAc, reduced the amplitude of Bmal1 in the NAc, and interestingly induced a circadian rhythm in Clock expression. Besides changes in amplitude, CMS also affects the phase of clock gene rhythms in the SCN and NAc. Clock and Bmal1 were delayed in the SCN, Per1 was advanced in the SCN and Bmal1 was delayed in the NAc (Logan et al., 2015).

Christiansen and colleagues (2016) have also characterized clock gene rhythms following CMS. They reported robust circadian rhythms in Per1, Per2 and Bmal1 in the SCN. Consistent with Logan et al (2015) Bmal1 was delayed in the SCN, whereas Per2 was phase advanced and Per1 was unaffected (Christiansen, Bouzinova, Fahrenkrug, & Wiborg, 2016). The fact that the phase and peak expression of Per1 did not differ between CMS and control groups suggests that Per1 is resilient against stressors, which is consistent with results from our lab (Al-Safadi et al., 2014). Taken together, these findings indicate that stress induces depression- and anxiety-like behaviours in rodents as well as alterations in circadian locomotor activity. It is feasible that the effect of CMS on mood, locomotor and physiological parameters is driven by the alterations in the molecular clock.

Animal models of depression and anxiety can help uncover the underlying pathogenesis of these disorders. Importantly, the olfactory bulbectomy and CMS paradigm both induce mood-related behaviours as well as alterations in circadian

rhythms. Changes in clock gene profiles in the master clock likely contribute to the disrupted circadian locomotor and physiological parameters, however clock genes in and of themselves may contribute to mood-related behaviours more directly through pleiotropic effects. For example, a mutation in the core circadian gene, *Clock*, produces a mania-like phenotype. These mutant mice exhibit less anxiety-like behaviour on the EPM and open field tests, swim more during the FST, and are more sensitive to the rewarding properties of cocaine (Easton, Arbuzova, & Turek, 2003; Roybal et al., 2007). Whereas a mutation in *Clock* produces a mania-like phenotype with decreased anxiety, *mPer1* and *mPer2* double mutant mice exhibit increased anxiety-like behaviours. Interestingly, a mutation in either *mPer1* or *mPer2* alone does not reliably induce anxiety-like behaviours (Spencer et al., 2013).

Phenotyping mice with different clock gene mutations and combinations thereof is informative, however a global mutation will affect circadian rhythms throughout the brain and the periphery, not only the SCN. This begs the question of whether changes in the SCN are necessary or sufficient for inducing mood-related phenotypes. Recently, Landgraf and colleagues (Landgraf, Long, Proulx, et al., 2016) showed that selectively knocking down Bmal1 in the SCN induces a depression- and anxiety-like phenotype. Not surprisingly, Bmal1 knockdown also led to an 80% reduction in Per2::luc amplitude and altered circadian locomotor output in the form of reduced locomotor amplitude and a lengthening of the free running period under DD. The PER genes appear to be specifically important in

the NAc because knocking down both *mPer1* and *mPer2* with selective RNA interference causes anxiety-like responses (Spencer et al., 2013). Knocking down *mPer1* or *mPer2* in isolation is associated with inconsistent impact on anxiety with more circumscribed anxiety suggesting that *Per* genes are in some way related to anxiety, which is supported by the finding that anxiolytics rapidly reduce *mPer1* mRNA in the cerebellum, whereas antipsychotics did not (Akiyama et al., 1999).

To conclude, it is important to note that there is a large degree of overlap between human clinical populations and animal models of depression- and anxiety-like behaviours. For instance, at a behavioural level, both human and animal models indicate that there is a reduction in overall activity in depression and anxiety, but there also appears to be a pattern of disorganized output. For example, clinical populations and animal models both report increased activity during the inactive phase as well as more variability in their behavioural patterns. From a physiological perspective hormone/endocrine rhythms are shifted and or blunted in human and animal models. There is also evidence for altered clock gene expression, amplitude and phase in both human and animal models. These findings indicate that depression and anxiety disorders are characterized by internal desynchrony and this may explain the ubiquity and heterogeneity of circadian disruptions. Post mortem analyses confirm that clock gene expression is blunted and out of phase in depressed individuals. In summary, animal

models not only corroborate human clinical studies but also provide a means to further explore potential underlying mechanisms.

Current Limitations

There is a clear link between disrupted circadian rhythms on the one hand and currently diagnosed individuals with depression and anxiety disorders on the other. There is some existing literature which suggests that circadian rhythms become overtly disrupted in the time leading up to the onset of an episode (Perlis et al., 1997). Two epidemiological studies were also reviewed above that show how sleep disturbances in otherwise healthy individuals can be used to predict future onset of depression and anxiety disorders at one or three year follow ups (Breslau et al., 1996; Ford & Kamerow, 1989). Apart from these few extant studies with sleep, there are no studies investigating any of the numerous other circadian disturbances that are so closely related to depression and anxiety in non-clinical subjects. Therefore, it is probable that other circadian parameters, such as behavioural patterns or physiological rhythms, could also predict the future onset of depression or anxiety in healthy populations.

Of the different circadian parameters that go awry in mental disorders, monitoring behavioural rhythms would be the easiest and least invasive approach. It was demonstrated above that human clinical population and animal models of depression- and anxiety-like behaviour are associated with numerous alteration in daily locomotor patterns. For example, daily rhythms are more fragmented,

there is increased activity in the inactive phase, and a general blunting of overall activity in both human and animal studies (Berle et al., 2010; Gorka et al., 1996; Landgraf, Long, Proulx, et al., 2016; Logan et al., 2015; Luik et al., 2015). Animal models also suggest that there are alterations in the free-running period under constant dark, that these animals take longer to re-entrain to phase advances and phase delays, and that they are potentially less responsive to the phase shifting effects of light (Griesauer et al., 2014; Perret et al., 2003). Taken together there is ample reason to suspect that differences in behavioural rhythms could reliably predict future onset of depression and anxiety as sleep disturbances.

While it is theoretically possible to monitor circadian locomotor parameters in healthy populations (e.g., using an activity monitoring watch see (Berle et al., 2010; Luik et al., 2015)) and assess symptoms of depression and anxiety at multi-year follow-ups, no such investigations have been conducted. The benefit to monitoring behavioural rhythms over physiological parameters is that it is less invasive and can be performed continuously. In summary, there is a large gap in the literature regarding the relationship between circadian parameters in healthy populations and symptoms of depression and anxiety. Filling this gap in the literature could potentially aid in the development of non-invasive and reliable predictors of future onset of mental conditions.

The Present Thesis

A properly functioning circadian system is fundamental for overall physical and mental health, whereas disrupting circadian rhythms is a risk factor for disease (Karatsoreos, 2014; Stevens et al., 2007). When it comes to investigating the relationship between circadian rhythms and mental health, most studies are performed after the onset of the illness, which means little is known about the state of the circadian system before the onset of the disorder. The present thesis was designed to address this question. The results of this project promote our understanding of the relationship between circadian rhythms and depression- and anxiety-like behaviours in healthy animals.

Objectives and rationale

Disrupted circadian rhythms in physiology and behaviour are a core feature of mood and anxiety disorders (Karatsoreos, 2014; Lamont, Legault-Coutu, Cermakian, & Boivin, 2007). There is strong support for a relationship between disrupted rhythms in the SCN and depression- and anxiety-like behaviours (Christiansen, Bouzinova, et al., 2016; Jiang et al., 2011; Landgraf, Long, Proulx, et al., 2016; Landgraf, Long, & Welsh, 2016; Logan et al., 2015). While there is a clear link between disrupted circadian parameters and mood-related behaviours in animal models of pathology, such links have not been investigated in healthy rats. This thesis attempts to fill part of the gap in the literature by determining whether individual differences in circadian locomotor parameters relate to depression- and anxiety-like behaviours. The rationale for focusing on circadian

locomotor parameters is twofold. First, there is a robust relationship between circadian locomotor output and depression and anxiety in both human and animal models. Second, monitoring circadian locomotor output can act as a proxy for stability and robustness of the circadian clock. The precision of circadian locomotor output is regulated by intercellular communication in the SCN (Herzog, Aton, Numano, Sakaki, & Tei, 2004) and individual differences in SCN neuron coupling correlates with individual differences in rate of re-entrainment to a phase advance and the phase of locomotor rhythms (Evans, Leise, Castanon-Cervantes, & Davidson, 2015).

The purpose of this thesis is to describe the relationship between individual differences in circadian locomotor parameters and mood-related behaviours in healthy rats. This thesis project is broken down into three parts. The first part of the thesis characterizes the circadian locomotor phenotype of male Lewis rats. To this end, rats were exposed to the following light conditions: standard 12h:12h LD, constant dark, simulated jet lag in for form of a 6h phase advance, and constant light. Numerous circadian parameters were calculated for each light condition.

The second part of the thesis investigates the relationship between circadian locomotor behaviour and depression- and anxiety-like behaviour. Individuals with depression and/or anxiety exhibit fragmented behavioural patterns, including increased activity during the night, reduced activity during the day, and greater

variability in behavioural rhythms across days (Luik et al., 2015). Based on these findings, we hypothesized that variable activity onset, activity during the light phase, and amplitude would be related to anxiety- and depression-like behaviours. Given that a longer rate of re-entrainment is associated with greater phase heterogeneity within the SCN (Evans et al., 2015), we also hypothesized that animals that take longer to adjust to simulated jetlag would exhibit more mood-related behaviours. The findings from this section indicate that individual differences in circadian locomotor activity predict anxiety and depression-like behaviours in male Lewis rats.

The final section of the thesis focuses on comorbidity of depression- and anxiety-like behaviours in rats. In humans there is a high degree of comorbidity between depression and anxiety disorders (Lamers et al., 2011), therefore we hypothesized strong positive correlations between depression- and anxiety-like responses. The findings from this part of the study have implications for translational research because Lewis rats display comorbidity in an analogous way to humans.

In conclusion, we demonstrate that individual differences in circadian locomotor parameters are associated with depression- and anxiety-like behaviours in healthy rats. The results from this project are important because it is the first time individual differences in circadian locomotor parameters have been related to mood-related behaviours in healthy rats. With a more comprehensive

understanding of the relationship between individual differences in circadian rhythms and mood there is potential to identify novel biomarkers that can be used to identify individuals at risk for developing depression and/or anxiety, the two most common psychiatric disorders.

CHAPTER 2

Individual differences in circadian locomotor parameters correlate with anxiety- and depression-like behavior

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Abstract

Disrupted circadian rhythms are a core feature of mood and anxiety disorders. Circadian rhythms are coordinated by a light-entrainable master clock located in the suprachiasmatic nucleus. Animal models of mood and anxiety disorders often exhibit blunted rhythms in locomotor activity and clock gene expression. Interestingly, the changes in circadian rhythms correlate with mood-related behaviours. Although animal models of depression and anxiety exhibit aberrant circadian rhythms in physiology and behavior, it is possible that the methodology being used to induce the behavioral phenotype (e.g., brain lesions, chronic stress, global gene deletion) affect behavior independently of circadian system.

This study investigates the relationship between individual differences in circadian locomotor parameters and mood-related behaviors in healthy rats. The circadian phenotype of male Lewis rats was characterized by analyzing wheel running behavior under standard 12h:12h LD conditions, constant dark, constant light, and rate of re-entrainment to a phase advance. Rats were then tested on a battery of behavioral tests: activity box, restricted feeding, elevated plus maze, forced swim test, and fear conditioning.

Under 12h:12h LD conditions, percent of daily activity in the light phase and variability in activity onset were associated with longer latency to immobility in the forced swim test. Variability in onset also correlated positively with anxiety-like behavior in the elevated plus maze. Rate of re-entrainment correlated positively with measures of anxiety in the activity box and elevated plus maze. Lastly, we

found that free running period under constant dark was associated with less anxiety-like behaviors in the activity box. Our results provide a previously uncharacterized relationship between circadian locomotor parameters and mood-related behaviors in healthy rats and provide a basis for future examination into circadian clock functioning and mood.

Individual differences in circadian locomotor parameters correlate with anxiety- and depression-like behavior

Disrupted circadian rhythms in physiology and behavior are a core feature of many psychiatric conditions including mood and anxiety disorders (Karatsoreos, 2014; Lamont et al., 2007). Aberrant sleep/wake cycles (e.g., insomnia, hypersomnia) are observed in various psychiatric conditions (Szelenberger & Soldatos, 2005). Concurrent to these changes in sleep/wake rhythms, circadian alterations in daily body temperature rhythms, daily activity patterns, and hormone release have also been reported (Berle et al., 2010; S. G. Jones & Benca, 2015). Because disruptions are observed in several different circadian rhythms, these findings suggest a fundamental connection between the master circadian clock and psychiatric illnesses.

Circadian rhythms in mammals are coordinated by an endogenous circadian clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. At the molecular level, rhythms in the SCN are driven by a collection of core clock genes that form a transcriptional-translational feedback loop, and go on to orchestrate rhythms in downstream brain regions and the periphery, which produce rhythms in behavior and physiology (Dardente & Cermakian, 2007; Kalsbeek et al., 2006). Several studies have found links between single nucleotide polymorphisms in core clock genes and mood disorders and schizophrenia (Kripke, Nievergelt, Joo, Shekhtman, & Kelsoe,

2009; Nievergelt et al., 2006; Patel et al., 2010; Pickard et al., 2009; Soria et al., 2010). Collectively, these findings point to some fundamental circadian mechanism acting at the core of certain affective disorders.

Animal models of depression and anxiety have advanced our understanding of this relationship between circadian rhythms and affective disorders (Cryan, Markou, & Lucki, 2002). For example, the unpredictable chronic mild stress (UCMS) paradigm induces a depression-like phenotype in rats that share similar symptoms to humans, such as reduced sexual activity and anhedonia (Gronli et al., 2005). Remarkably, in this same paradigm, the daily rhythm in the expression of the core clock protein Per2 is blunted in the SCN (Jiang et al., 2011), and the amplitude of this oscillation in clock gene expression correlates with depression-like behaviors (Logan et al., 2015). Moreover, mice selectively bred for high anxiety- and depression-like behaviors (HAB) also exhibit aberrant circadian rhythms in the form of fragmented locomotor activity under 12h:12h light-dark (LD) conditions, a longer free running period in constant dark (DD), and are less responsive to the phase shifting effects of light (Griesauer et al., 2014). Treating these HAB mice with the anti-depressant fluoxetine did not alter the free running period but normalized the fragmented rhythms in locomotor activity and light responsiveness (mood-related behaviors were not assessed after fluoxetine treatment) (Schaufler et al., 2016). Finally, it has also been shown that when expression of the core clock gene *Bmal1* is selectively knocked down in the SCN, a behavioral phenotype with increased depression- and anxiety-like behaviors

emerged (Landgraf, Long, Proulx, et al., 2016). Thus, there is some evidence to argue for a causal relationship between SCN-based circadian rhythms and mood-related behaviors.

While there is a clear link between disrupted circadian parameters and mood-related behaviors in animal models of pathology, such links have not been investigated in a healthy population. To fill this gap, several circadian parameters were characterized in a healthy group of rats. We looked at circadian locomotor activity under a 12h:12h LD cycle, during constant darkness, after a 6h phase advance (simulated “jet-lag”), and under constant light. The same rats were then tested on a battery of depression and anxiety-like tests in order to determine which circadian parameters are associated with depression- and anxiety-like behavior. We found several key associations between these circadian parameters and mood related behaviors in this otherwise healthy population of rats.

Materials and Methods

Animals and Housing

Twenty-four inbred male Lewis (LEW/Crl) rats weighing 145-188g upon arrival (Charles River, St-Constant Quebec) were used in this study. All efforts were made to minimize the number of animals used and limit the distress. All rats were individually housed in cages (9.5”x8”x16” deep) equipped with access to running wheels and had ad libitum access to rat chow and water (except for 10-

days of restricted feeding, described below). Animals were handled and weighed on weekly basis. At the time of handling, rats were also visually inspected for any health concerns. None of the rats appeared to have health concerns at any point during the study. Each cage was kept in a custom built sound-attenuated and light-sealed ventilated chamber (17.5”x27.5”x27.5” deep). Light cycles were computer-controlled, and programmed to a 12h:12h LD cycle. All procedures were carried out in accordance with the Canadian Council on Animal Care guidelines (http://www.ccac.ca) and were approved by the Animal Care Committee of Concordia University (Montreal, Quebec).

Lighting Schedules

Rats were entrained to a 12h:12h LD cycle for two weeks, and between each light schedule rats were given another two weeks of 12h:12h LD to re-entrain before subsequent manipulations. All rats underwent the same sequence of manipulations: two weeks of 12h:12h LD, two weeks of constant dark (dark-dark, DD; Fig 1A-B), two weeks of 12h:12h LD, a 6h phase advance (Fig 1C-D), two weeks of 12h:12h LD, two weeks of constant light (LL; Fig 1E-F), and finally were maintained on 12h:12h LD for the behavioral assays of mood and anxiety (Fig 1G-H). Under standard circadian notation, zeitgeber time (ZT) 0 denotes the time when environmental lights were turned on, and ZT 12 represents the time when the lights were turned off.

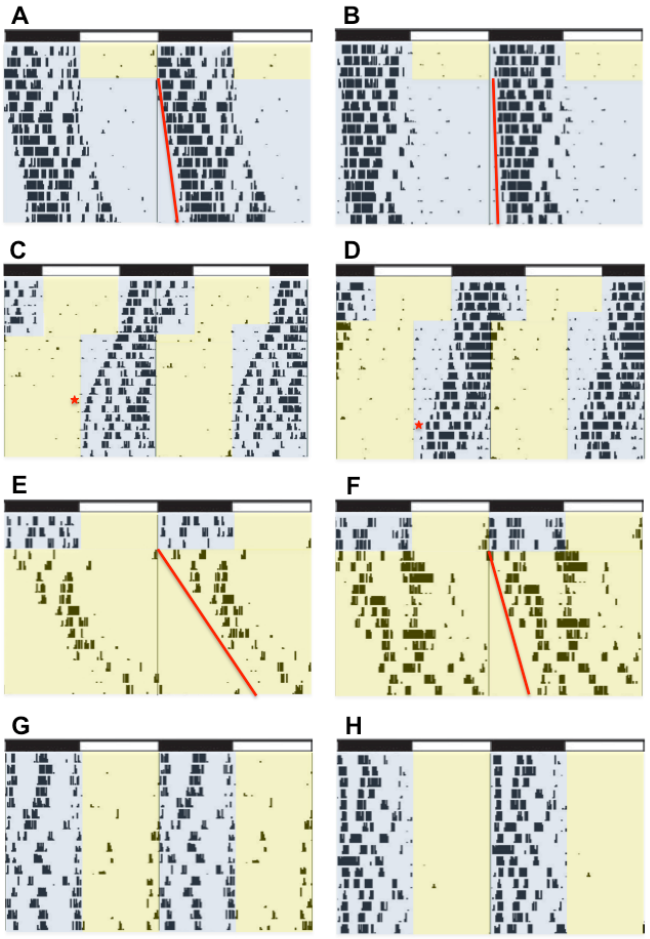

Fig 1. Representative actograms illustrating individual differences under each light each light condition.

(A-B) Last 3 days of baseline LD entrainment followed by constant dark (A τ=24.3, B τ=24.11.), (C-D) rate of re-entrainment to a 6h phase advance (C=7 days, D= 11 days), (E-F) 3 days of stable entrainment to 12h:12h LD followed by constant light (E τ=25.67., , F τ=24.73), and (G-H) entrainment to the final 12h:12h LD after completing all of the light schedules and immediately preceding the start of behavior testing. Fig 1G is representative of a rat with high activity in the light phase and H is a representative of a rat that remains inactive during the light phase.

Circadian Analysis of Locomotor Activity

Wheel running activity was recorded continuously and displayed in 10min bins, and analyzed using VitalView (VitalView software; Mini Mitter Co. Inc., Sunriver, OR). ClockLab (Actimetrics Software, Wilmette, IL) was used to calculate total wheel rotations in the dark, total wheel rotations in the light, activity onset and activity offset over the last 7 days of 12h:12h LD prior to behavior testing. Microsoft Excel (Mac 2011, version 14) was used to calculate percent of activity during the light, variability in activity onset and variability in activity offset using the coefficient of variation equation (Standard deviation/mean*100). Under constant darkness, the free running period was calculated using χ^2 over the last 10 days of this 2-week period. Total activity was also calculated for the last 10 days of constant darkness. The rate of re-entrainment to a 6h phase advance was calculated as the number of days required to shift the daily onset of activity to the same phase angle of entrainment observed under baseline. To verify this method, rate of re-entrainment was also calculated by visually inspecting the actograms (with three independent observers). A Cronbach's alpha of .833 indicates that the two methods are consistent with one another. The free running period and total activity under constant light were calculated in the same way as constant dark.

Behavioral Assays of Mood and Anxiety

Activity Box

Each rat was tested in a standard locomotor activity box during the light phase between ZT2-ZT4 (2-4h after environmental lights turned on). Testing was carried out under standard fluorescent lighting because a previous report did not find an effect of illumination on exploratory behaviors or center entries in an open field apparatus (N. Jones & King, 2001). Activity boxes (15”x16.5”x19.5”) with transparent Plexiglas walls and removable plastic tray floors were used, and were cleaned with 70% ethanol between sessions. Each locomotor box was housed in a sound-attenuated chamber and activity was recorded by computer via infrared beam breaks on two sensor rings that created a 16”x16” matrix. Rats were placed in one corner of the activity box and the TruScan Software (TruScan, Activity Monitoring System, Coulborn Instruments Whitehall, PA) collected number of ambulatory movements, total distance travelled (cm), distance travelled in the margins (cm), center time (s). For the purposes of this experiment, ambulatory moves and margin distance are being interpreted as high anxiety-like responses, whereas distance travelled and center time are low anxiety-like responses.

Elevated Plus-Maze

In order to test anxiety-like behavior, a standard elevated plus-maze (EPM) was used. The EPM is an ethologically valid assay for measuring anxiety-like behavior (Carobrez & Bertoglio, 2005). The maze consisted of two open arms (20”x4”), two closed arms (20”x4”, surrounded by 20.5” walls) and a center square measuring 4”x4”, and the apparatus was 20.5” off the floor. Animals were

tested between ZT2-4 under dim red light because it has previously been shown that although circadian phase and illumination do not affect anxiety-like behaviors on the EPM, testing in low light promotes activity (N. Jones & King, 2001). At the start of each trial, each rat was placed in the center square facing one of the open arms and was given 5 min to explore. The maze was cleaned between sessions with alcohol wipes and all trials were video recorded from a camera attached to the ceiling. Videos were scored for percent of open arm entries, time in open arm (s), time in closed arm (s), and time in the center square (s). Percent of open arm entries and time spent in the open arms are low anxiety-like behaviors, more time in closed arms reflects high anxiety-like behavior, and time spent in the center square is interpreted as 'decision-making' (Sestakova, Puzserova, Kluknavsky, & Bernatova, 2013).

Forced Swim Test

A standard depression-related test that is commonly used is the forced swim test (FST) (Porsolt, Brossard, Hautbois, & Roux, 2001), and was administered 3-5 days after the EPM. Testing was performed at the start of the active phase (ZT13-ZT14) under dim red light. Rats were tested under dim red light to prevent exposure to light during the active phase and because it is less stressful for the animals (Kelliher et al., 2000). In this test, glass cylinders (25.5"x10.75" diameter) were filled with tap water (25±2°C) to a depth of 14". The water was replaced between each test. Rats were habituated to the FST for 15 min on the first day and were tested 24h later for 5 min. Both the habituation and test day

were carried out under dim red light and video recorded. After the test, each rat was dried and placed back in its home cage. None of the animals drowned or struggled to survive during the FST beyond what is normal escape oriented behaviors, therefore no experimenter interventions were required. Latency to immobility (s) is being used as a measure of active coping, time spent immobile (s) is being interpreted as passive coping, swimming (s) and climbing are both being interpreted as active coping responses.

Fear Conditioning

In order to induce contextual fear conditioning, rats were placed in a novel environment (14”x10”x10”) equipped with a stainless steel grid floor connected to a shock source and scrambler (Med Associates Inc. Georgia, VT, U.S.A). On day 1 the rat was placed in the fear conditioning box, and after 3 min of habituation to this novel environment, an aversive foot shock (0.5 mA current for 1s) was delivered, and given again 1 min later. The rat was then removed from the conditioning chamber and returned to the home cage (~30s after last shock). Boxes were cleaned after every test. The next day, each rat was placed back in the same context and the contextual fear response was scored. All testing was conducted at ZT 2 and video recorded for the following conditioned fear responses: latency to freeze (s) and time spent freezing (s).

Data Screening and Statistical Analyses

All variables were initially screened for outliers. A z-score +/-3 was used as the cutoff and the next closest data point was used to replace the outlier value. All variables were assessed for normal distribution using D'Agostino's K-Squared test (Prism 6). Scatterplots were visually inspected for linearity, and bivariate correlations were conducted to examine co-linearity. After visual inspection of all relevant scatterplots it was determined that one variable was non-linear (percent of activity in the light). An inverse transformation was applied to produce a linear relationship. A Pearson r=1.00 between raw and transformed data indicates that the integrity of the data and the distribution were retained after applying this transformation. After data screening was complete, SPSS (version 21) was used to conduct bivariate correlations between behavioral assays and locomotor parameters.

The use of a correction for multiple comparisons is debated within the literature. On the one hand, there are some that argue that correction for multiple comparisons is unnecessary (Perneger, 1998; Rothman, 1990; Savitz & Olshan, 1995)but for others it comes down to the nature of the study. For instance, it is generally accepted that corrections for multiple comparisons should be used in confirmatory studies (Koch & Gansky, 1996; Sankoh, Huque, & Dubey, 1997). When it comes to exploratory studies, it seems that corrections for multiple comparisons are not advised (Bender & Lange, 2001). Given that this study was more of an exploratory analysis of the relationship between individual differences

in circadian locomotor parameters and performance on mood-related behaviors we decided against the restricting nature of Bonferroni or similar test. The use of a Bonferroni correction could have led to type II errors, which may lead future investigators to omit a particular circadian parameter when designing a confirmatory study based on the current findings.

Results

Individual Differences in Locomotor Parameters

All behavior testing was carried out during the inactive/light phase except for the FST, which was performed at the beginning of the active/dark phase. On the days where behavior testing was conducted during the light phase, all rats used the running wheel after being placed back in their home cages. This activity was brief and subsided within 1h; 48h post behavior testing all animals have returned to baseline activity in the light phase. See Fig. 2 for representative waveforms of daily activity rhythms under baseline conditions and Fig. 3 for scatter plots of individual variability in the primary circadian locomotor variables.

Correlations Between Locomotor Parameters and Behavior Testing

See Table 1 for complete bivariate correlation matrix between circadian locomotor parameters and mood-related behavior tests.

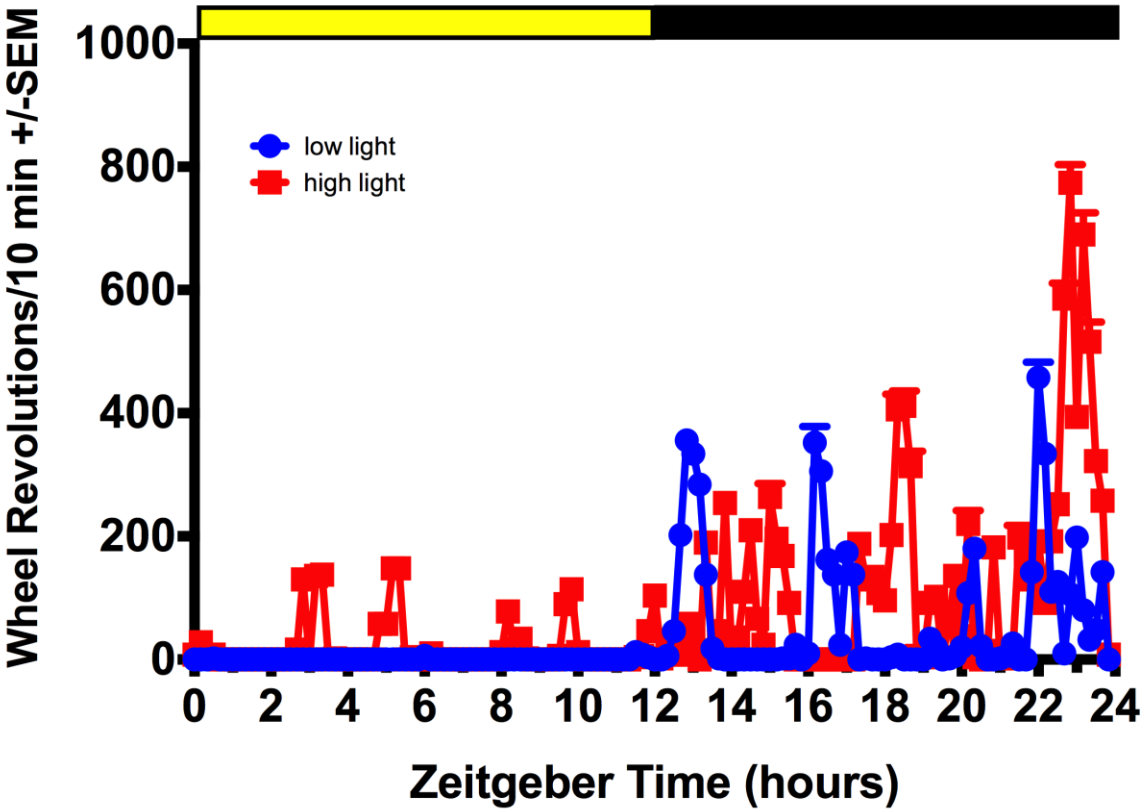

Fig 2. Activity patterns under 12h:12h LD.

Representative waveforms of individual variability in circadian locomotor output under 12h:12h LD.

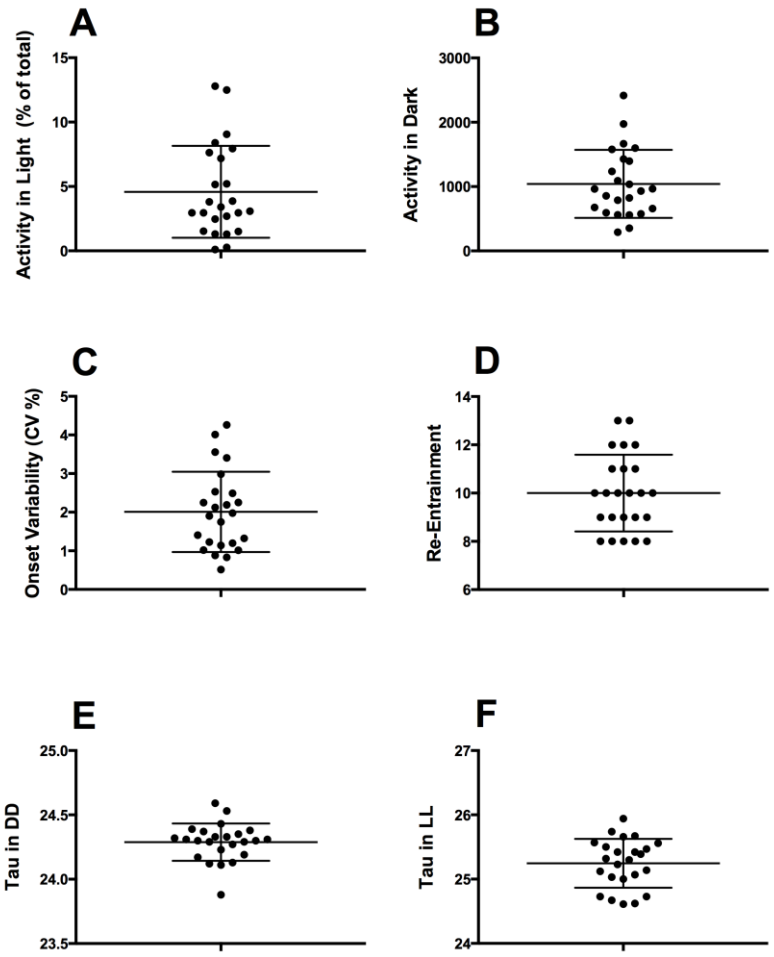

Fig 3. Scatter plot of individual differences in core circadian parameters.

(A) Individual variability in total activity in light phase (B) Individual variability in total activity in the dark phase (C) Individual variability in activity onset (D) Individual variability in rate of re-entrainment (E) Individual variability free running period in DD (F) Individual variability free running period in LL.

Table 1. Correlation Matrix: Circadian Locomotor Parameters by Behavior Tests

		Act. Box				EPM				FST					FC	
		Ambulatory Moves	Total Distance Travelled	Margin Distance	Center Time	% Open Arm Entries	Time in Open Arms (s)	Time in Closed Arms (s)	Center Time (s)	Latency to Immobility (s)	Immobility (s)	Swimming (s)	Climbing (s)	Dives	Latency to Freeze (s)	Freezing (s)
12:12 LD	Total Dark Activity	.056	.053	-.165	.260	.011	.249	-.306	.164	-.163	.301	-.194	-.306	-.130	-.004	-.101
	% Activity in Light	.147	-.273	-.015	-.242	-.274	-.309	.332	-.109	**.487****	**-.362***	.229	.341	-.135	-.035	.116
	Onset Variability	.087	-.038	.210	-.228	-.192	**-.372***	.115	**.415***	**.422***	-.272	.115	.246	.293	-.276	.138
	Offset Variability	.087	.059	.177	-.036	.193	.222	-.182	-.039	.162	-.050	.108	.032	.119	-.279	.085
Phase Advance	Re-Entrain	.232	-.136	**.454***	**-.521****	**-.461***	**-.394***	**.401***	-.098	.334	-.312	.132	.314	-.131	-.081	-.178
Constant Dark	Period in DD	.318	**-.466***	**-.510****	.015	.077	-.030	.006	.045	.021	.038	-.187	-.023	.039	.029	.187
	Activity: DD	.018	-.137	-.272	.164	-.088	-.093	.154	-.133	.044	.172	-.089	-.172	**-.438***	.134	.062
Constant Light	Period in LL	-.201	-.095	-.031	.084	-.008	.079	-.105	.074	-.112	.245	-.113	-.269	-.307	.298	-.197
	Activity: LL	.321	-.315	-.196	-.063	-.056	-.031	.174	-.292	.101	-.004	-.197	.029	-.193	-.243	.205

Re-Entrain: number of days to adjust to 6h phase advance; Activity DD, total activity across 24h averaged over last week; Activity LL, total activity across 24h averaged over last week; Act. Box, activity box; EPM, elevated plus maze; FST, forced swim test; FC, fear conditioning.

*p<.05, **p<.01

Total Activity Under 12h:12h LD

To examine the relationship between circadian locomotor activity under stable entrainment to a 12h:12h LD cycle we correlated activity in the dark phase and light phase with behavioral assays of mood and anxiety. Total activity in the dark phase did not correlate with any mood-related measures. To assess individual differences in the masking effect of light, percent of activity in the light phase was correlated with each behavioral assay (Fig 4). Percent of activity in the light phase correlates positively with latency to immobility in the FST (r=.487, p=.008. Fig 4E) and negatively with time spent immobile in the FST (r=.-362, p=.041) but not with any of the anxiety measures.

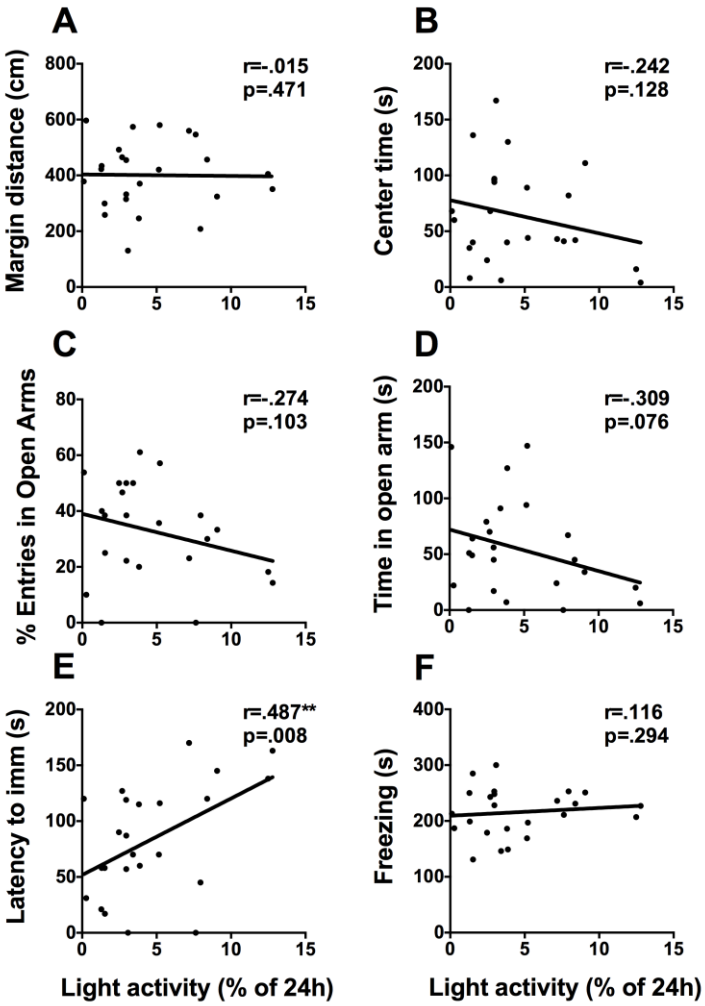

Fig 4. Activity in the light phase is positively correlated with latency to immobility in the FST.

Scatterplots representing the relationships between percent of activity in the light phase and (A) distance travelled in the margins of the activity box, (B) time spent in the center of the activity box, (C) percent of open arm entries, (D) time spent in the open arms of the EPM, (E) latency to immobility in the FST, and (F) time spent freezing on day 2 of contextual fear conditioning.

* p<.05, ** p<.01.

Variability in Activity Onset and Offset Under 12h:12h LD

Variability in activity onset and offset was calculated as a proxy for the precision of the SCN-based clock in each animal (Welsh, Engel, Richardson, & Dement, 1986). Scatterplots for variability in activity onset by core mood-related measures are shown in Fig 5. Variability in onset correlates positively with latency to immobility in the FST ($r=.422$, $p=.020$. Fig 5E), time spent in the center square of the EPM ($r=.415$, $p=.024$), and correlates negatively with time spent in the open arms of the EPM ($r=-.372$, $p=.040$. Fig 5D). Variability in activity offset was not associated with any of the behavioral assays, $p>.05$.

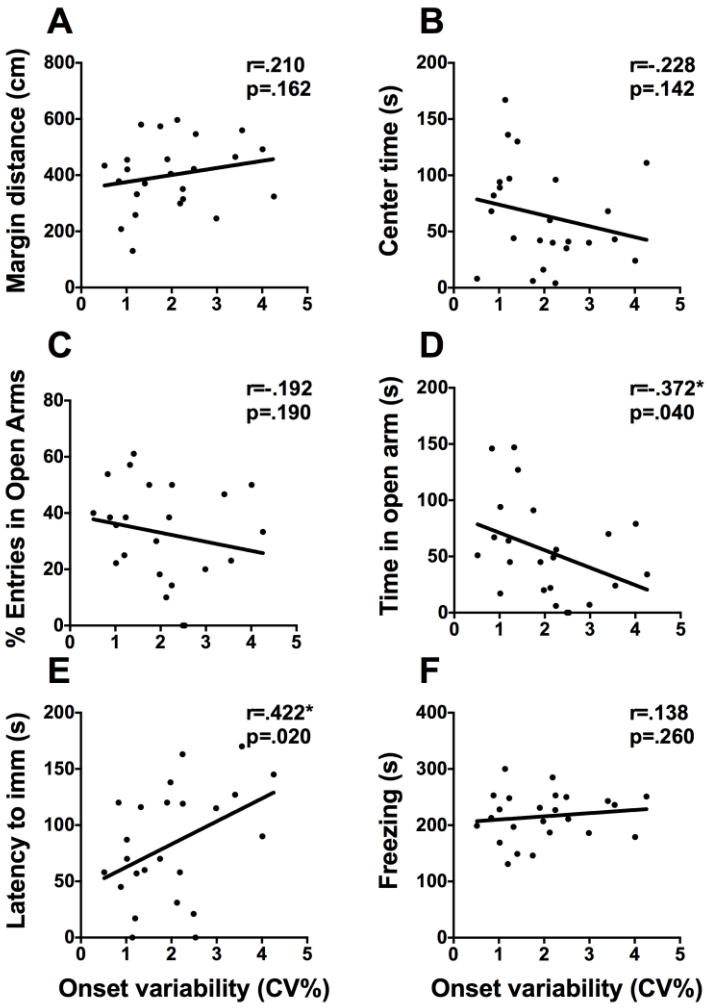

Fig 5. Variability in onset is associated with performance on the EPM and FST.

Scatterplots representing the relationships between variability in onset (CV) and (A) distance travelled in the margins of the activity box, (B) time spent in the center of the activity box, (C) percent of open arm entries, (D) time spent in the open arms of the EPM, (E) latency to immobility in the FST, and (F) time spent freezing on day 2 of contextual fear conditioning.

* p<.05, ** p<.01.

Rate of Re-Entrainment

The number of days required to re-entrain to a 6h advance of the light cycle, a marker of the adaptability of the circadian clock, is associated with performance in the activity box, and the EPM (Fig 6). Rate of re-entrainment correlates positively with distance traveled in the margins of the activity box (r=.454, p=.013. Fig 6A), negatively with time spent in the center of the activity box (r=-.521, p=.004. Fig 6B), percent of open arm entries (r=-.461, p=.013), and time spent in the open (r=-.394, p=.032. Fig 6D) and time spent in closed arms of the EPM (r=.401, p=.029). Re-entrainment is not statistically correlated with any measure from contextual fear conditioning or the FST, however there was a trend towards significance with latency to immobility in the FST (r=.334, p=.055, Fig 6E).

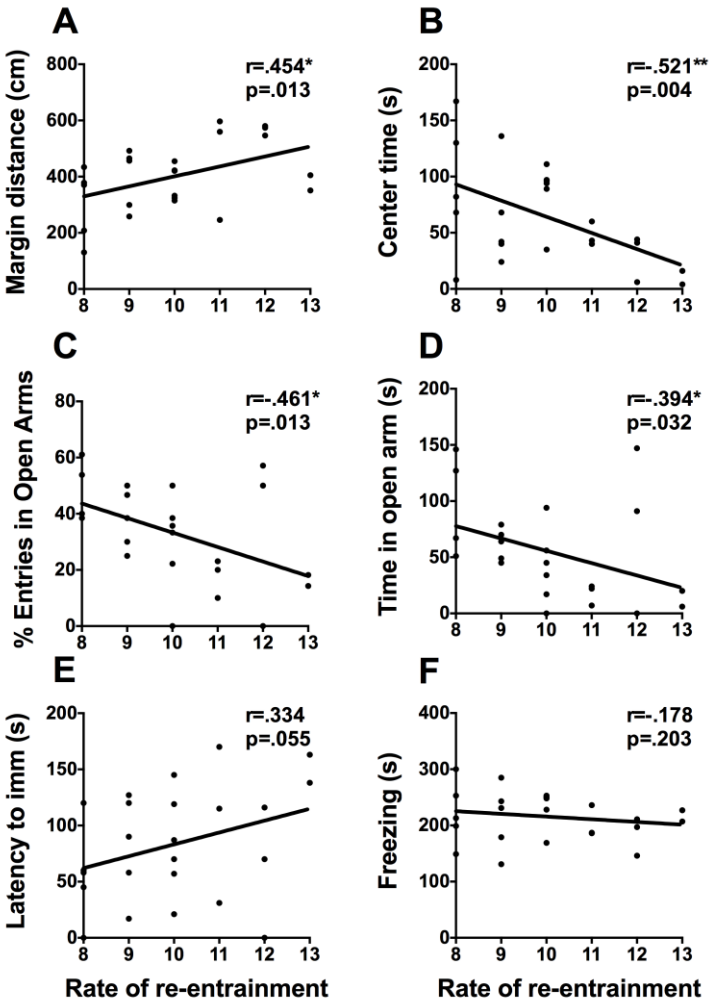

Fig 6. Rate of re-entrainment and mood-related behaviors

Scatterplots representing the relationships between rate of re-entrainment to a 6h phase advance and (A) distance travelled in the margins of the activity box, (B) time spent in the center of the activity box, (C) percent of open arm entries, (D) time spent in the open arms of the EPM, (E) latency to immobility in the FST, and (F) time spent freezing on day 2 of contextual fear conditioning.

* p<.05, ** p<.01.

Constant Dark

Free running period under DD correlates negatively with total distance travelled (r=-.466, p=.011) and margin distance in the activity box (r=-510, p=.005, Fig 7A) and correlates positively with closed arm entries (r=.479, p=.01). It does not correlate with any behaviors from the FST (Fig 7 D-E). Total activity in constant dark correlates negatively with diving in the FST (r=-.438, p=.016).

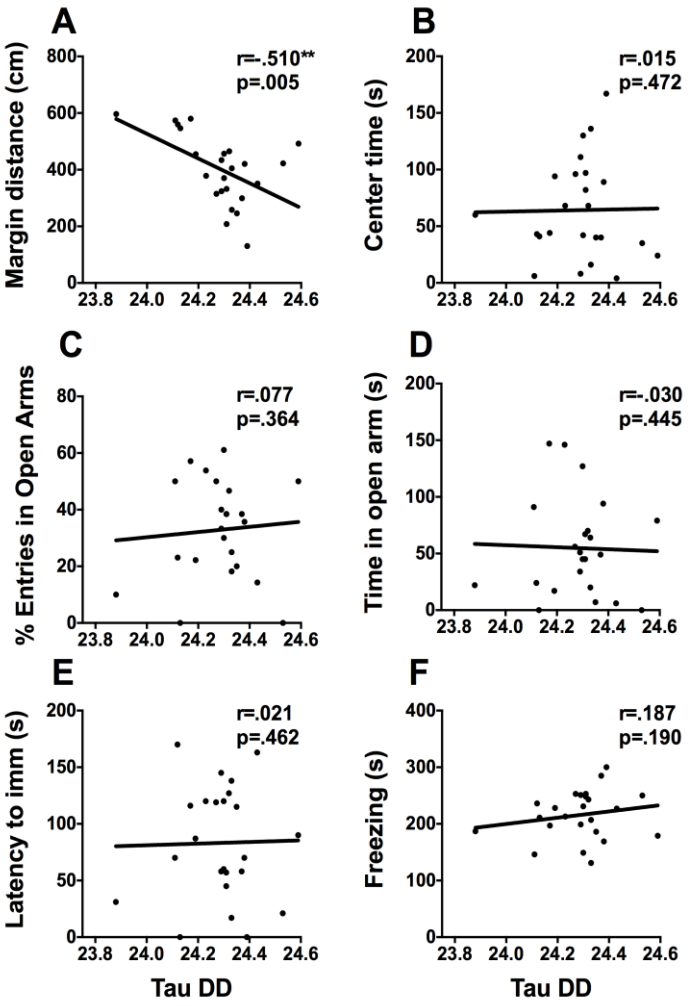

Fig 7. Free-running period in DD and mood-related behaviors.

Scatterplots representing the relationships between endogenous period (tau) in constant dark (DD) and (A) distance travelled in the margins of the activity box, (B) time spent in the center of the activity box, (C) percent of open arm entries, (D) time spent in the open arms of the EPM, (E) latency to immobility in the FST, and (F) time spent freezing on day 2 of contextual fear conditioning.

* p<.05, ** p<.01.

Constant Light

All animals had a measurable free running period under constant light. Neither free-running period nor total activity under LL correlated with any of mood-related measures (Fig 8).

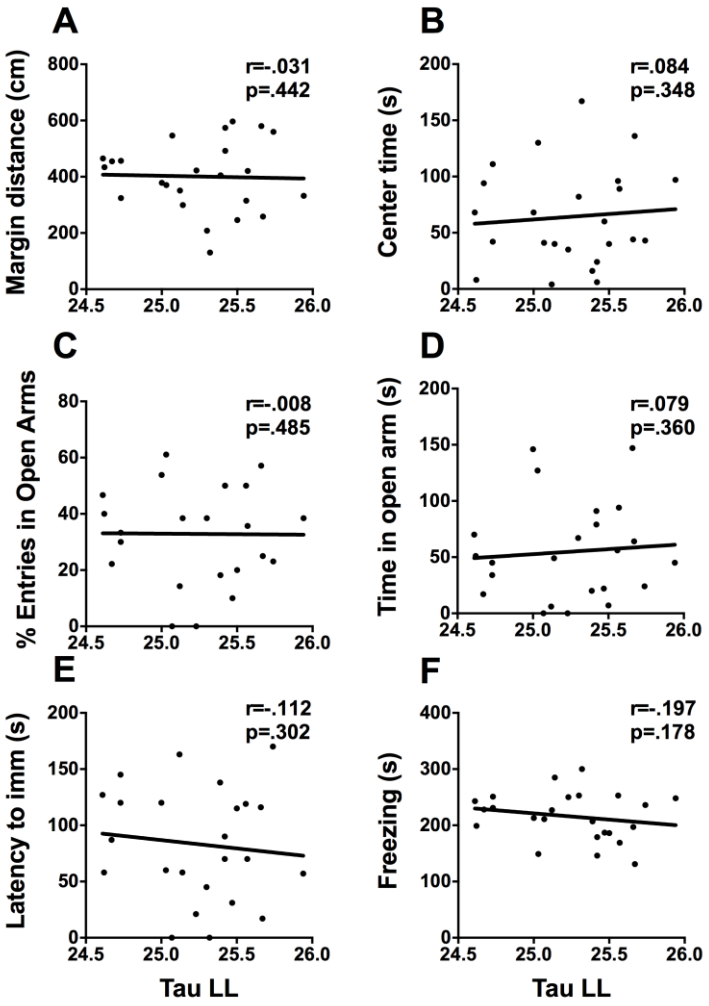

Fig 8. Free-running period in LL and mood-related behaviors.

Scatterplots representing the relationships between free running period (tau) in constant light and (A) distance travelled in the margins of the activity box, (B) time spent in the center of the activity box, (C) percent of open arm entries, (D) time spent in the open arms of the EPM, (E) latency to immobility in the FST, and (F) time spent freezing on day 2 of contextual fear conditioning.

* p<.05, ** p<.01.

Discussion

Disrupted circadian rhythms in physiology and behavior are a core feature of mood and anxiety disorders (Karatsoreos, 2014; Lamont et al., 2007). There is a complex and bidirectional relationship between disrupted circadian rhythms and mood. Numerous studies have shown that environmental (e.g., stress, shiftwork, transmeridian travel) and genetic factors (e.g., clock gene polymorphisms) influence circadian rhythms and are potential risk factors for the development of pathology (Jiang et al., 2011; McClung, 2007; Spencer et al., 2013; Stevens et al., 2007). While there is clearly a role for environmental and genetic factors in the pathogenesis of mood and anxiety disorders, there is also evidence to suggest that aberrant circadian rhythms can be a risk factor for depression and anxiety independently. In the present study we investigated individual differences in circadian locomotor activity and how they relate to depression- and anxiety-like behaviors in a healthy population of inbred Lewis rats. A major benefit of using this inbred strain is that we have previously carried out an extensive characterization of the daily expression clock gene rhythms in this strain (Harbour, Weigl, Robinson, & Amir, 2013) and they are commonly used for experiments on stress, mood, and anxiety (Dhabhar, McEwen, & Spencer, 1997; Goswami, Cascardi, Rodriguez-Sierra, Duvarci, & Pare, 2010; Hinojosa et al., 2006; Ramos et al., 2002). In the current project, we observed several important associations between individual differences in circadian parameters, based on

locomotor activity rhythms, and performance on common assays of depression- and anxiety-like behaviors.

Rats that were more active during the light phase in the home cage under 12h:12h LD, generally had a longer latency to immobility and spent less time immobile in the FST. The FST occurred across 2 days and rats transitioned from being highly active on day 1 to primarily immobile on day 2. Immobility measured on day 2 is frequently interpreted as a sign of 'despair', however, it has recently been argued that immobility reflects a switch from active to passive coping (Molendijk & de Kloet, 2015). Immobility in the FST may in fact be an adaptive response that promotes survival because rats that become immobile float, conserve energy and are less likely to sink (Nishimura, Tsuda, Oguchi, Ida, & Tanaka, 1988). As a result, the current findings suggest that activity during the light phase is associated with the persistence of active coping in the FST. Increased activity during the light phase could suggest that some rats are less well entrained or are less responsive to the masking effects of light under LD conditions. Therefore, this behavioral result could reflect more general changes in the light-responsivity or entrainment parameters of individual rats.

To evaluate whether entrainment parameters may be associated with behavioral assays of mood and anxiety we also assessed variability in the daily onset of locomotor activity. Similar to the amount of activity during the light phase, higher variability in activity onset was associated with longer latency to immobility in the

FST. Variability in onset was also associated with less exploratory behavior and more anxiety-like behaviors in the EPM. Greater variability in onset was associated with more time spent in the center square and less time in the open arms. The present findings demonstrate that variability in onset is associated with active coping in the FST and increased anxiety-like behavior in the EPM. Different factors can account for variability in the precision of activity rhythms, including precision of the SCN clock, the strength of the entraining stimulus as well as individual differences in sensitivity to the entraining stimuli (Aschoff, Daan, Figala, & Muller, 1972). Because our lighting was standardized, these behavioral results likely reflect individual differences in precision of the SCN clock or individual differences in light responsiveness.

To test the “strength” of entraining stimuli, we measured how long it took rats to adapt to simulated jet-lag in the form of a 6h phase advance of the light cycle. We found that the rate of re-entrainment was associated with anxiety-like behaviors. Rats that took longer to re-entrain spent less time in the center of the activity box but were more active in the margins. Both avoidance of the center square and increased activity in the margins are interpreted as increased anxiety-like response. We observed that rate of re-entrainment was also associated with more time spent in the closed arms of the EPM, a lower percentage of open arm entries, and less time spent in the open arms, which are also indicative of anxiety. It was recently demonstrated that individual differences in the rate of re-entrainment is associated with the degree of phase

heterogeneity between individual SCN neurons (Evans et al., 2015). These data suggest, that the phase heterogeneity or coherence of neuronal oscillations within the SCN could modulate anxiety-related behaviors.

To explore the relationship between locomotor activity under constant conditions and mood-related behaviors, animals were housed in DD and LL. Although some animal models of depression do exhibit alterations in free running period under DD (Martynhak, Pereira, de Souza, & Andreatini, 2015) we did not find any relationship between period in DD and the FST. We did however find an association with anxiety-like behaviours in the activity box and EPM. In respect to constant light we found that total activity in is associated with fewer arm changes in the EPM, suggesting that activity during constant light is associated with less exploratory behavior in the EPM.

We found a strong relationship between several circadian locomotor parameters and mood related behaviors in rats. In particular there is an association between activity during the light, under entrained conditions as well as LL, and anxiety and depression-like behaviors. We speculate that individual differences in the responsiveness to light could be mediating these responses. Consistent with this type of association, it has been shown that mice selectively bred for high anxiety- and depression-like behaviors are less responsive the phase shifting effects of light (Griesauer et al., 2014). An alternative explanation would be that light is directly influencing mood independently of the circadian system, as aberrant light

schedules have been shown to alter mood related behaviors in mice (LeGates et al., 2012). Bright light therapy is an effective treatment for individuals with either seasonal or non-seasonal depression (Wirz-Justice et al., 2005). The circadian phase of bright light therapy may be important for the antidepressant effects. Light therapy is frequently prescribed in the morning (Lewy, Lefler, Emens, & Bauer, 2006); however, evening treatments have also been shown to be effective (Wirz-Justice et al., 1993), suggesting that there may be some "non-circadian" pathways mediating these effects.

The current study contributes to our understanding of the relationship between circadian locomotor rhythms and mood-related behaviors. One of the limitations to this study is that all of the subjects were exposed to multiple lighting conditions prior to behavior testing. Exposure to different light cycles may produce aftereffects (Aschoff et al., 1972), but these are not usually observed after constant dark or constant light schedules. To minimize potential aftereffects, rats were given two weeks of 12h:12h LD between manipulations. It is possible that the circadian manipulations could have an effect on mood-related behavior. To rule this possibility out, future studies can include a control group that is single housed in 12:12 LD before undergoing the same behavioral tests. The choice to use an inbred rat strain allowed us to control for individual differences in locomotor and mood-related behavior due to genetic variability. Although individual differences in circadian locomotor parameters are determined in part by genetics, there are other variables that can affect an individual's rhythms,

including, social interactions, exercise, age and environmental lighting (Duffy, Kronauer, & Czeisler, 1996; Monk, Petrie, Hayes, & Kupfer, 1994; Mrosovsky, 1988; Valentinuzzi, Scarbrough, Takahashi, & Turek, 1997). To account for the influence of social interactions we single housed the rats, to control for the effect of age all rats were young, and environmental lighting was standardized between boxes. Our choice to minimize the number of extraneous variables influencing circadian rhythms, particularly the use of an inbred strain may limit the degree of inter-subject variability and thereby reduce the number of correlations we observed. The results from the current study reflect the relationship between circadian locomotor parameters and mood-related behaviors in healthy Lewis rats. It would be interesting for future replications to compare across strains to determine whether the associations observed between circadian parameters and mood-related behaviors are consistent across strains. Lastly, although rats were housed under 12h:12h LD conditions at multiple points during the study we chose to focus on the final 12h:12h LD cycle. It is possible that circadian parameters fluctuate over time, but this does not appear to be the case as we found a significant positive correlation between circadian locomotor activity between entrained conditions pre and post light manipulations. This suggests that individual differences in these circadian parameters are stable over time, which is consistent with previous findings (Evans et al., 2015). Therefore, we are confident that the results from this time point reflect the general relationship between circadian locomotor parameters and mood-related behaviors.

Conclusion

Disrupted circadian rhythms are a cardinal feature of many psychiatric conditions, and there is accumulating evidence to suggest that disrupted circadian rhythms are involved in the pathogenesis of some of these disorders. We demonstrate here that individual differences in circadian locomotor parameters are associated with depression- and anxiety-like behaviors in Lewis rats. With a more comprehensive understanding of the relationship between individual differences in circadian rhythms and mood there is potential to identify novel biomarkers that can be used to identify individuals at risk for developing certain psychiatric conditions.

CHAPTER 3

Examining comorbidity between anxiety- and depression-like behaviors in a healthy cohort of Lewis rats.

Jeffrey Anyan, Shimon Amir

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Funding acquisition: Shimon Amir.
Investigation: Jeffrey Anyan.
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Project administration: Shimon Amir.
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Supervision: Shimon Amir.
Validation: Shimon Amir.
Visualization: Jeffrey Anyan, Shimon Amir.
Writing ± original draft: Jeffrey Anyan.
Writing ± review & editing: Shimon Amir.

Abstract

There is a remarkably high degree of comorbidity between depression and anxiety in clinical populations, yet surprisingly the degree of comorbidity between depression- and anxiety-like behaviors in animal models has received little attention. The purpose of this study is to examine individual differences in depression- and anxiety-like behaviors in a healthy population of Lewis rats. A large battery of tests, including the activity box, elevated plus maze, forced swim test and fear conditioning, was used. We found that time spent in the center of the activity box was associated with more immobility in the FST, while center time was negatively associated with climbing. Time spent in the closed arms of the EPM was associated with less time swimming. We did not observe any relationships between fear conditioning and the FST. We also found that rats that exhibit high anxiety-like behaviors on one test tend to exhibit increased anxiety-like responses on other measures. This paper addresses some of the limitations in using animals to model psychopathology as well as the inherent difficulties of interpreting animal behavior as anxiety- or depression-like.

Examining comorbidity between anxiety- and depression-like behaviors in a healthy cohort of Lewis rats

Depression and anxiety are the most commonly diagnosed forms of mental illness. Depression alone affects approximately 350 million people and by 2030 it is expected to be the number one cause of disability worldwide (Health, 2012). Anxiety disorders affect approximately 1 in every 13 individuals, or ~7% of the general population (Baxter, Scott, Vos, & Whiteford, 2013). In addition to being the most prevalent disorders they are highly comorbid with one another, with some estimates being as high as 80% (Lamers et al., 2011), leading some researchers to suggest a shared genetic etiology (Gorwood, 2004), however elucidating the underlying mechanisms in these clinical populations has been difficult (Lamers et al., 2011).

To overcome some of the difficulties in working directly with clinical populations, animal models are frequently used to investigate the pathophysiology of depression and anxiety. There are various approaches of inducing a depression- or anxiety-like phenotype in rodents, including chronic stress, selective breeding, genetic manipulations (e.g., gene knockouts, RNA interference), and brain lesions (Farhan, Ikram, Kanwal, & Haleem, 2014; Griesauer et al., 2014; Holmes, Murphy, & Crawley, 2003; Kelly, Wrynn, & Leonard, 1997; Landgraf, Long, Proulx, et al., 2016; Spencer et al., 2013; Wang et al., 2007). Although these methods can produce mood-related phenotypes, they are not without their limitations. For instance, significant morphological changes manifest in the brain

following some of the aforementioned manipulations (Czeh, Fuchs, Wiborg, & Simon, 2016). Moreover, not all animals exposed to a particular manipulation will exhibit the expected phenotype. In other words, some animals are more resilient to these manipulations. By focusing strictly on responsive animals and comparing them to healthy controls, one loses valuable information regarding individual variability in susceptibility to a given disease state. Animal models also fail to recreate some fundamental characteristics of psychiatric disorders. For example, no animal model can mimic the episodic and recurrent nature of mental disorders. Existing animal models of depression and anxiety reproduce psychopathology in its acute phase where symptoms are at their peak. From a clinical perspective however, information regarding the premorbid state is equally valuable because it has the potential to inform researchers and clinicians alike on the prodromal characteristics of a disorder. Lastly, rodents cannot communicate their phenomenological state, therefore researchers must infer mood-related states based on behavioral observations, which is often ambiguous and open to different interpretations (this topic is explored further in the discussion).

An alternative and complementary approach to animal models of depression and anxiety is to characterize and study individual differences in healthy unselected animal populations (Pawlak, Ho, & Schwarting, 2008). Studying individual differences in psychopathology is a relatively underutilized approach and there are very few studies examining depression- and anxiety-like behaviors using this methodology. In one study, researchers used healthy Wistar rats and assessed

for low and high anxiety-like phenotypes based on time spent in the open arms of the elevated plus maze (EPM). They found that high anxiety rats spend significantly more time freezing when tested in a standard fear conditioning paradigm with an inescapable foot shock (Borta, Wohr, & Schwarting, 2006). Another study reported that high anxiety rats exhibit more anxiety-like behaviors in the object burying test, which was considered a defensive reaction to an aversive stimuli, and took longer to learn how to escape a foot shock in a two-way active avoidance test. Interestingly, no association was found between performance on the EPM and the forced swim test (FST) (Ho, Eichendorff, & Schwarting, 2002). The absence of a relationship between performance on the EPM and the FST is important because it suggests that animals with high trait anxiety do not exhibit concomitant depression-like behaviors. To date, only one study investigating comorbidity in healthy unselected rat strains observed a significant relationship between performance on the FST and the EPM. Estanislau and colleagues (2011) reported that low anxiety-like behavior in the EPM was associated with shorter latency to immobility and more time spent immobile in the FST. Latency to immobility and time spent immobile are traditionally interpreted as measures of depression- or despair-like behaviors. These results are surprising as they are in the opposite direction to what one would expect based on clinical populations where depression and anxiety have a very high comorbidity rate. Given the scarcity of studies on comorbidity in healthy rodent populations, more research is warranted.

There are considerable phenotypic differences between rodent strains (Ramos, Berton, Mormede, & Chaouloff, 1997; Trullas & Skolnick, 1993) therefore selecting an appropriate strain is important. Individual differences are determined by an individual's genetic makeup, the environment, and gene by environment interactions. In order to limit the number of variables that can influence an individual's phenotype, some researchers use inbred strains such as Lewis rats. Lewis rats are frequently used to study mood-related behaviors and are regarded as a good genetic model of anxiety (Hinojosa et al., 2006; Ramos, 2008; Ramos et al., 1997; Ramos et al., 2002). Compared to other strains Lewis rats have a higher ratio of at-risk to resilient phenotypes for developing a prominent anxiety-like phenotype (Cohen et al., 2006). The increased anxiety-response in this strain combined with individual differences in the rate of extinction also make them a useful animal model for studying the underlying causes of anxiety disorders such as posttraumatic stress disorder (Goswami et al., 2010). Another study found that individual differences in baseline anxiety-like behavior correlate with dendritic morphology in the prefrontal cortex, where high anxiety rats had smaller dendrites (Miller, Morrison, & McEwen, 2012). Most recently, we used Lewis rats to demonstrate that individual differences in circadian locomotor parameters are associated with anxiety- and depression-like behaviors (Anyan, 2017). Taken together Lewis rats are a good strain for studying individual differences in susceptibility to disease states.

At the present time there are very few studies examining comorbidity in healthy rats. The purpose of this study is to characterize individual differences in depression- and anxiety-like behaviors in Lewis rats and evaluate comorbidity. Rats were tested on a large battery of mood-related tests: the EPM, activity box, fear conditioning, and the FST. The results from this study contribute to our understanding of individual variability in healthy rat populations and comorbidity.

Methods

Animals and Housing

The study consisted of twenty-four male Lewis (LEW/Crl) rats weighing 145-188g upon arrival (Charles River, St-Constant Quebec). These rats were also used in a previous report where rats underwent a series of different light schedules including constant dark, constant light, and a 6h phase advance (Anyan, 2017). Rats were individually housed in plastic cages (9.5”x8”x16” deep) equipped with a running wheel. Food and water were available ad libitum throughout the experiment. Individual cages were maintained in a custom built, sound-attenuated, and light-sealed ventilated chamber (17.5”x27.5”x27.5” deep). Animals were housed under a standard 12h:12h LD cycle throughout behavioral testing. All procedures were carried out in accordance with the Canadian Council on Animal Care guidelines (http://www.ccac.ca) and were approved by the Animal Care Committee of Concordia University (Montreal, Quebec). All possible efforts were made to minimize animal suffering and the number of animals used.

Behavioral Assays of Mood and Anxiety

All rats underwent behavior testing in the same sequence: activity box, EPM, FST and finally fear conditioning. The behavioral assays were ordered from least to most stressful and there was a minimum of 4 days between tests.

Activity Box

The activity box is used as a measure of anxiety-like behavior. Testing was conducted during the light phase, 2-4h after lights on). We chose to test animals under standard fluorescent lighting because it has previously been shown that lighting does not affect exploratory behavior or the number of center entries (N. Jones & King, 2001). The activity box measured 15”x16.5”x19.5” with transparent Plexiglas walls and a removable plastic floor tray. The tray was removed between tests and cleaned with 70% ethanol. Activity boxes were maintained in a sound-attenuated chamber. Activity was automatically recorded by a computer registering infrared beam breaks on two sensor rings, creating a 16”x16” matrix. Rats were placed in one corner of the activity box and the TruScan Software (TruScan, Activity Monitoring System, Coulborn Instruments Whitehall, PA) calculated total number of movements, total distance travelled, number of entries into the center square and amount of time spent in the center over a 5 min testing period. Animals that show less exploratory behavior, make fewer center entries or spend less time in the center are interpreted as being more anxious.

Elevated Plus-Maze

The EPM is an ethologically valid assay for measuring anxiety-like behavior (Carobrez & Bertoglio, 2005). The EPM pits a rat's innate drive to explore against its desire to avoid open spaces thus providing a measure of active and passive avoidance (Montgomery, 1955; Sestakova et al., 2013). The EPM consists of two open arms (20"x4"), two closed arms (20"x4", surrounded by 20.5" walls) and a center square measuring 4"x4". The entire apparatus was raised 20.5" off the ground. At the start of each test, a rat was placed in the center square facing one of the open arms and given 5 minutes to explore the maze. The EPM was cleaned with alcohol wipes between each trial. Testing was completed under dim red light, 2-4h after lights on. We chose to conduct this test under dim red light because low lighting has been shown to promote activity in the EPM (N. Jones & King, 2001). Trials were recorded by a video camera fixed to the ceiling. Percent of open arm entries, time spent in the open arms (s), time spent in the closed arms (s), and time spent in the center square (s) were scored from the video recording. A higher percent of open arm entries and time spent in the open arms are interpreted as low anxiety-like responses whereas time spent in the closed arms is considered an anxiety-like response. Lastly, time spent in the center square is interpreted as 'decision-making' (Sestakova et al., 2013).

Forced Swim Test

The FST is the gold standard for measuring depression-like behaviors in rodents (Porsolt et al., 2001). In this study rats were habituated to the FST for 15 min on day 1 and then underwent a 5 min test 24h later. The FST was performed in glass cylinders (25.5”x10.75” diameter) filled with 14” of tap water at 25±2°C. The water was changed between each rat. Both the habituation and test day were carried out 1-3 h after the onset of the dark phase under dim red light and video recorded. After being removed from the FST, rats were dried with towels before being placed back in their home cages. The following behaviors were scored for day 2 only: latency to immobility (s) (the first time a rat becomes immobile for >3s), time spent immobile (s), swimming (s) and climbing (s). Swimming and climbing were differentiated based on specific movements: swimming was defined as smooth rhythmic movements where the forepaws are not breaking water, and climbing was defined by frantic escape oriented behaviors where the forepaws do break water. For the purposes of this study, time spent immobile is interpreted as passive coping, whereas, latency to immobility, swimming and climbing are being interpreted as active coping responses.

Fear Conditioning

Contextual fear conditioning was included here to complement the behavioral assays of anxiety. The activity box and EPM capture trait levels of anxiety, whereas contextual fear conditioning captures anxiety as a state (Ramos, 2008), therefore we wanted to assess both state and trait anxiety. Freezing behavior is

considered a state fear response because it is an acute response associated with a cue, in this case context previously paired with an aversive event (footshock). To test contextual fear conditioning, rats were placed in a clear Plexiglas box (14”x10”x10”) equipped with a stainless steel grid floor connected to a shock source and scrambler (Med Associates Inc. Georgia, VT, U.S.A). On day 1 of fear conditioning rats were placed in the shock chamber and after 3 min of habituation the rat received a mild foot shock (0.5 mA current) for 1s. One minute later a second foot shock of equal current and duration was administered. Thirty seconds after the second foot shock, the rat was removed from the fear conditioning chamber and returned to its home cage. The fear conditioning chamber was cleaned with an alcohol wipe after each trial. Twenty-four hours later the rat was placed back in the shock chamber to measure contextual fear. Both days of fear conditioning were performed two h after lights on. Day 2 was video recorded for latency to freeze (s) and time spent freezing (s).

Data Screening and Statistical Analyses

All variables were initially screened for outliers. A z-score +/-3 was used as the cutoff and the next closest data point was used to replace the outlier value. All variables were assessed for normal distribution using D’Agostino’s K-Squared test (Prism 6). Scatterplots were visually inspected for linearity, and bivariate correlations were conducted to examine co-linearity. After visual inspection of all relevant scatterplots it was determined that all variables were linear. After data screening was complete, SPSS (version 21) was used to conduct Pearson

bivariate correlations between each behavioral assay. We chose to use Pearson bivariate correlations over a more advanced statistical modeling technique because there was not enough power to use a multivariate technique.

Results

Activity Box

To examine the relationship between anxiety-like behavior in a novel environment and depression-like behaviors, behavioral parameters from the activity box were correlated with performance on the FST (see Table 1 for a correlation matrix). Total number of movements correlates positively with climbing behavior in the FST (r=.366, p=.039). Center time correlated positively with time spent immobile in the FST (r=.404, p=.025. Fig. 1A), meaning that animals that exhibit low anxiety-like behaviors in the activity box spend more time immobile in the FST. Center time correlated negatively with climbing (r=-.406, p=.025. Fig. 1C), indicating that climbing behavior is associated with increased anxiety-like behavior. Although it did not reach statistical significance, there was a trend towards a significant negative correlation between time spent in the center square and latency to immobility (r=-.342, p=.051. Fig.1D). Taken together, these findings suggest that low anxiety-like behavior in the activity box is associated with more immobility and less struggling in the FST.

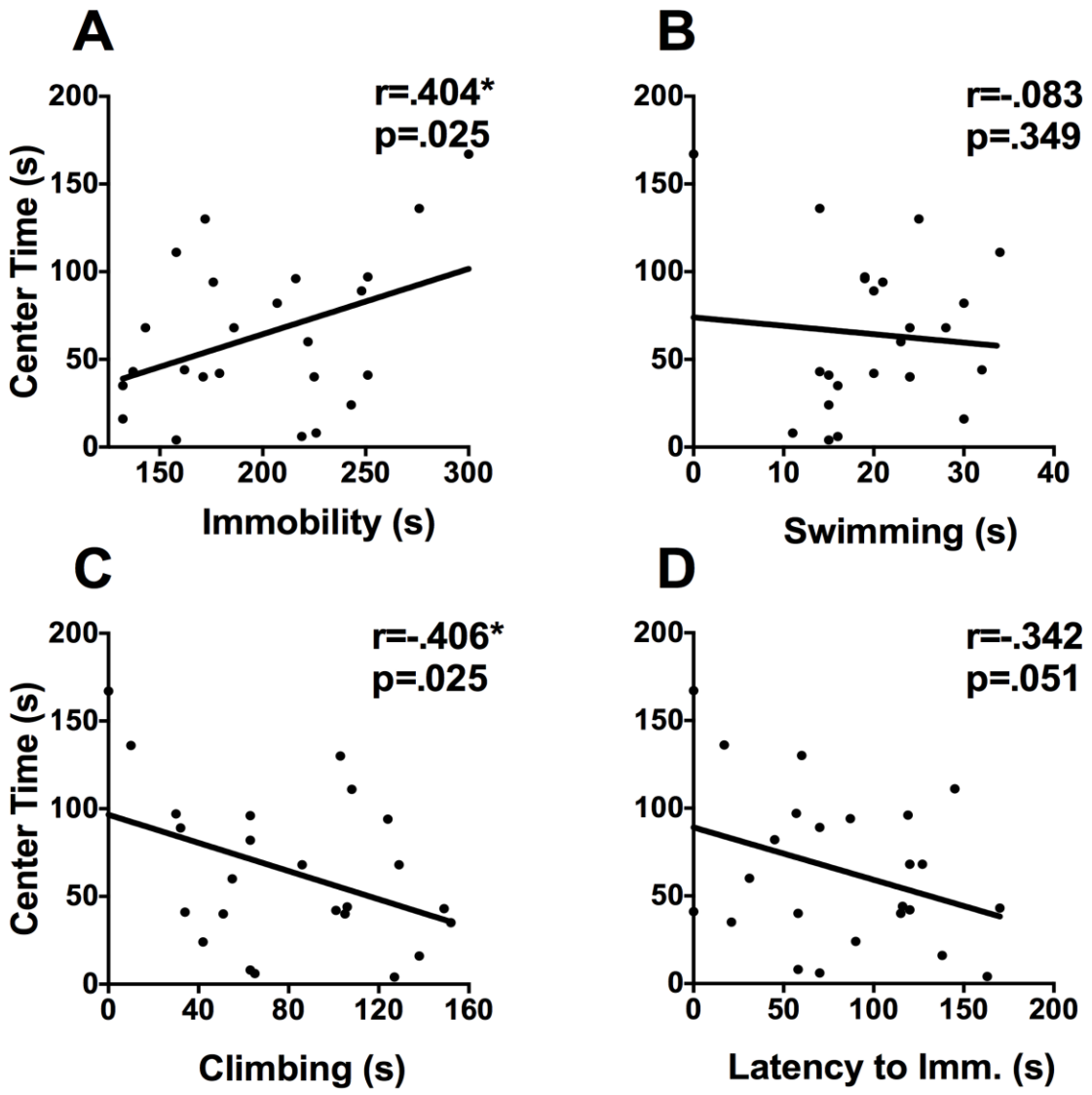

Fig 1. Relationship between time spent in the center square of the activity box and performance on the FST.

Scatterplots depicting the correlations between time spent in the center square of the activity box and (A) time spent immobile in the FST, (B) time spent swimming in the FST, (C) time spent climbing, and (D) latency to immobility in the FST.

* p<.05, ** p<.01.

Table 1. Correlation Matrix: Activity box by forced swim test

	Latency to imm.	Immobility	Swimming	Climbing	Dives	Total Movements	Distance travelled	Center entries	Center time
Latency to imm.	-								
Immobility	**-.687****	-							
Swimming	**.418***	**-.565****	-						
Climbing	**.661****	**-.984****	**.433***	-					
Dives	.219	-.042	.090	.004	-				
Total movements	.280	-.325	-.145	**.366***	-.035	-			
Distance travelled	-.073	.081	.143	-.078	.250	**-.488****	-		
Center entries	-.030	.064	.232	-.093	.263	**-.390***	**.776****	-	
Center time	-.342	**.404***	-.083	**-.406***	.056	**-.437***	**.483****	**.682****	-

Elevated Plus Maze

See Table 2 for a correlation matrix between EPM parameters and performance on the FST. Time spent in the closed arms correlates negatively with swimming (r=-.491, p=.009), meaning that rats with high anxiety-like responses in the EPM spend less time swimming. Time spent in the center square of the EPM correlated positively with swimming (r=.384, p=.035).

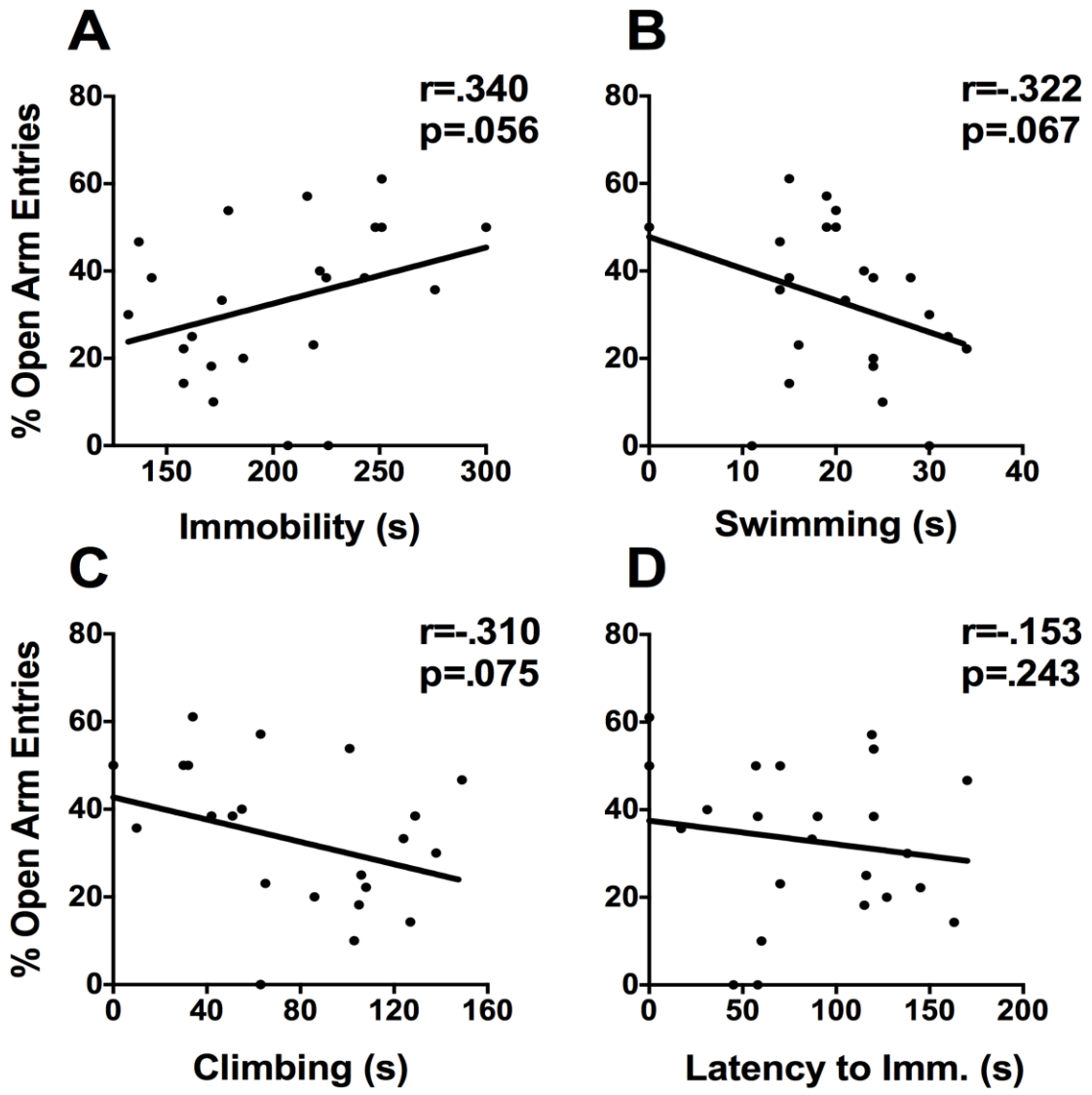

Fig 2. Relationship between percent of open arm entries in the EPM and performance on the FST.

Scatterplots depicting the correlations between percent of open arm entries in the EPM and (A) time spent immobile in the FST, (B) time spent swimming in the FST, (C) time spent climbing the FST, and (D) latency to immobility in the FST.

* p<.05, ** p<.01.

Table 2. Correlation Matrix: Elevated plus maze by forced swim test

	Latency to imm.	Immobility	Swimming	Climbing	Open arm time	Closed arm time	Center time	% Open arm entries
Latency to imm.	-							
Immobility	**-.687****	-						
Swimming	**.418***	**-.565****	-					
Climbing	**.661****	**-.984****	**.433***	-				
Open arm time	.041	.064	.329	-.139	-			
Closed arm time	-.157	-.059	**-.491****	.158	**-.859****	-		
Center time	.232	.011	**.384***	-.077	-.056	**-.463***	-	
% Open arm entries	-.153	.340	-.322	-.310	-.157	-.039	.347	-

Fear Conditioning

See Table 3 for a correlation matrix between fear conditioning and performance on the FST. None of the behavioral parameters from the FST correlated with any of the parameters from fear conditioning (Fig. 3A-D).

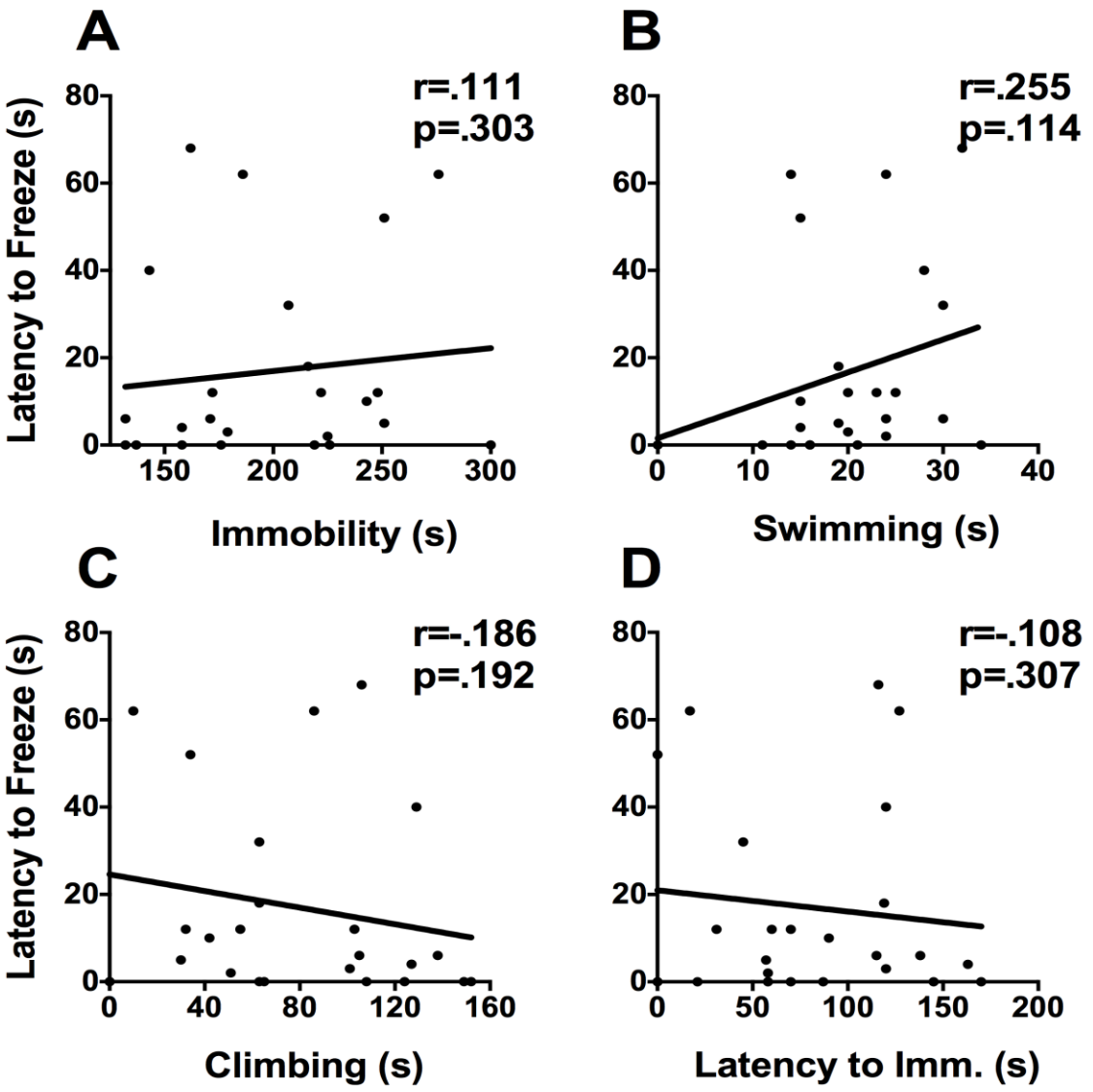

Fig 3. Relationship between latency to freeze during fear conditioning and performance on the FST.

Scatterplots depicting the correlations between latency to freeze and (A) time spent immobile in the FST, (B) time spent swimming in the FST, (C) time spent climbing the FST, and (D) latency to immobility in the FST.

* p<.05, ** p<.01.

Table 3. Correlation Matrix: Fear conditioning by forced swim test

	Latency to imm.	Immobility	Swimming	Climbing	Latency to freeze	Freezing
Latency to imm.	-					
Immobility	**-.687****	-				
Swimming	**.418***	**-.565****	-			
Climbing	**.661****	**-.984****	**.433***	-		
Latency to freeze	-.108	.111	.255	-.186	-	
Freezing	.070	-.056	-.071	.073	-.236	-

Correlations Between Measures of Anxiety

Previous reports indicate that different measures of anxiety-like behavior assess distinct aspects of anxiety (Ramos, 2008), therefore we wanted to assess the strength of the relationship between each of the anxiety-like parameters used in this study. Moreover, we wanted to examine the relationship between trait and state anxiety. Table 4 provides a complete correlation matrix for anxiety parameters. Total number of movements in the activity box correlates positively with time spent in the closed arms of the EPM ($r=.410$, $p=.026$). Distance travelled in the activity box is associated with less time spent in the closed arms of the EPM ($r=-.360$, $p=.046$) and a longer latency to freeze during fear conditioning ($r=.355$, $p=.044$). Taken together, low anxiety-like behaviors in the activity box and EPM are associated with low state anxiety during fear conditioning.

Table 4. Correlation Matrix: Anxiety tests

		Activity Box				Elevated Plus Maze				Fear Conditioning	
		Total movements	Distance travelled	Center entries	Center time	Open arm time	Closed arm time	Center time	% Open arm entries	Latency to freeze	Freezing
Activity Box	Total movements	-									
	Distance travelled	**-.488****	-								
	Center entries	**-.390***	**.776****	-							
	Center time	**-.437***	**.483****	**.682****	-						
Elevated Plus Maze	Open arm time	-.292	.287	.254	.260	-					
	Closed arm time	**.410***	**-.360***	**-.470***	**-.418***	**-.859****	-				
	Center time	-.292	.205	**.479***	**.370***	-.056	**-.463***	-			
	% Open arm entries	-.206	.267	.317	.205	-.157	-.039	.347	-		
Fear Conditioning	Latency to freeze	-.086	**.355***	.282	.149	**.428***	-.304	-.153	-.003	-	
	Freezing	-.050	-.190	.082	.142	**-.367***	.125	**.385***	.210	-.236	-

Animals that enter the center of the activity box and spend more time therein are interpreted as being less anxious. Center entries and center time both correlate negatively with time spent in the closed arms (r=-.470, p=.012 and r=-.418, p=.024, respectively) and correlates positively with time spent in the center square of the EPM (r=.479, p=.010 and r=.370, p=.041, respectively). Time spent in the open arms, a low anxiety-like behavior, have a longer latency to freeze (r=.428, p=.021) and less time spent freezing (r=-.367, p=.043) during fear conditioning. Lastly, we found a positive correlation between center time and time spent freezing (r=.385, p=.035).

Discussion

Depression and anxiety are complex mental disorders and despite extensive experimental and epidemiological studies, the underlying pathophysiology remains unclear. Although there is a high degree of comorbidity between anxiety and depression in clinical populations, much less is known about comorbidity between depression- and anxiety-like behaviors in healthy rats. This question is of particular interest to psychiatry and neuroscience considering that rodents are frequently used in translational research and in testing new pharmacological agents. The purpose of this study is to characterize individual differences in depression- and anxiety-like behaviors in a healthy cohort of rats and assess for comorbidity.

We found a number of associations between measures of anxiety-like behavior and performance on the FST. There are a limited number of studies examining comorbidity

between depression-like behaviors on the FST and anxiety-like behaviors on the on the EPM, but this is the first study to include a large battery of tests. We found that avoidance of the center square of the activity box, a sign of anxiety-like behavior, was associated with more time spent climbing in the FST. We also found that total number of movements in the activity box, interpreted here as an agitated behavioral response to novelty, was also associated with avoidance of the center square of the activity box. It is important to note that total number of movements is not related to distance travelled, which is a low anxiety-like behavior, because distance travelled was not related to climbing ($r=-.078$, $p>.05$). Taken together, these results suggest that climbing behavior in the FST is associated with anxiety-like behaviors in both the activity box and the EPM.

In contrast to climbing, we found that swimming behavior is associated with less anxiety-like behaviors. Swimming is distinct from climbing in that it is rhythmic and does not break water with the forepaws. We observed a positive correlation between swimming and time spent in the center square of the EPM, and a negative correlation between swimming and time spent in the closed arms. This suggests that swimming behavior is associated with increased decision making (Sestakova et al., 2013) in Lewis rats. The lack of comorbidity between 'depression-like' behaviors in the FST and anxiety-like behaviors in the EPM is consistent with past research (Ho et al., 2002). Lastly, we report that there is a significant relationship between immobility in the FST and center time in the activity box. This finding suggests that immobility in the FST is associated with less anxiety-like behavior in the activity box.

We investigated the relationship between different behavioral assays of anxiety-like behavior. We found that individual differences in trait levels of anxiety-like behavior are associated with conditioned fear response. Animals that are more exploratory in novel space, in the form of distance travelled in the activity box, had a longer latency to freeze during fear conditioning. We also found that animals that spend more time in the open arms of the elevated plus maze have a longer latency to freeze and spend less time freezing. Our findings are consistent with two previous studies (Borta et al., 2006; Walker, Hinwood, Masters, Deilenberg, & Day, 2008) demonstrating that individual differences in baseline trait anxiety are associated with differences in state anxiety during fear conditioning paradigm.

Socially isolating rats can lead to depression- and anxiety-like behaviors, equivalent to chronic mild stress paradigms (Brenes Sáenz, Villagra, & Fornaguera Trías, 2006). In addition to inducing mood-related behaviors, social isolation is also associated with altered hypothalamic pituitary adrenal axis functioning. Isolated rats exhibit higher basal levels of adrenocorticotropic hormone (ACTH), reduced brain derived neurotrophic factor mRNA in the hippocampus, as well as increased release of ACTH and corticosterone in response to stress (Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004; Zheng et al., 2006). Although the animals in the current study were socially isolated they also had free access to a running wheel. Voluntary exercise has previously been shown to reverse the molecular and behavioral changes following chronic mild stress in mouse and rat models (Huang et al., 2017; Zheng et al., 2006), therefore we believe that providing our rats with a running wheel mitigates the behavioral and physiological effects of social isolation.

This study contributes to our understanding of the relationship between depression- and anxiety-like behaviors in healthy rodents. At the present time there are very few studies examining comorbidity in healthy rats and the results are mixed (Estanislau et al., 2011; Ho et al., 2002). This study is unique in that it incorporates a large number of anxiety tests, including state and trait anxiety tests, as well as the FST. We found significant relationships between measures of anxiety-like behavior and performance on the FST. We report here that healthy male Lewis rats high in anxiety-like behaviors do not exhibit concomitant depression-like behaviors. These findings are similar to Estanislau et al., (2011) who reported that high anxiety-like behavior was inversely related to depression-like behavior in healthy male Wistar rats. These results are surprising given the degree of comorbidity between depression and anxiety in clinical populations (Lamers et al., 2011). It is possible that rats do not exhibit comorbidity in the same way as clinical populations, but on the other hand it is also possible that the interpretations of standard mood-related tests are lacking in construct validity. In other words, what is being interpreted as an ‘anxious’ or ‘depressed’ phenotype may not be appropriate. In line with this possibility, the FST as a behavioral assay for ‘despair’ has come into question. Not only is it being argued that the FST does not measure ‘despair’, but it is also being suggested that immobility is an adaptive response (Commons, Cholanians, Babb, & Ehlinger, 2017; Molendijk & de Kloet, 2015), in part because immobility conserves energy and likely promotes survival (Hawkins, Hicks, Phillips, & Moore, 1978). A recent review article postulates and summarizes literature that suggests that immobility is a learned response mediated through glucocorticoid dependent learning (Molendijk & de Kloet, 2015). If this argument is true, then the current findings and those of Estanislau

et al., (2011) do not refute the possibility of comorbidity in healthy rodent models. What it would mean however is that we need to pay careful attention to the way behavioral assays of mood are interpreted.

Conclusion

There is a high degree of comorbidity between depression and anxiety disorders in clinical populations, yet this remains an under-investigated area in animal research. We demonstrate here that individual differences in anxiety-like behaviors correlate with performance on the FST. Consistent with previous studies, anxiety-like behaviors do not correlate with immobility in the FST. Traditionally, immobility has been interpreted as a sign of despair; however, the phenomenological experiences of patients combined with the anomalous findings from the few extant animal studies that do exist, suggest that this interpretation is untenable. Rather, immobility is more likely an adaptive learned response and continued struggling on the test day may reflect less plasticity in the neural networks underlying anxiety. A better understanding of individual differences in mood-related behaviors and more sound interpretations of different behavior tests will improve translational research from animal models to human clinical populations and vice versa.

Chapter 4: General Discussion

Jeffrey Anyan, Shimon Amir

Part of this chapter was published in Neuropsychopharmacology

Author contributions:

Conceptualization: Jeffrey Anyan, Shimon Amir.

Writing ± original draft: Jeffrey Anyan.

Review & editing: Shimon Amir.

General Discussion

Disrupted circadian rhythms are a core feature of many psychiatric conditions, including depression and anxiety disorders. Although there is a strong association between disrupted circadian rhythms and mental health, the exact nature of this relationship has yet to be elucidated. In other words, are aberrant circadian rhythms a cause or consequence of the psychiatric disorder? The findings from this thesis contribute to our understanding of the interaction between circadian rhythms and mood. The first study examines individual differences in circadian locomotor parameters and their relationship to depression- and anxiety-like behaviors in Lewis rats.

Our results suggest that rate of re-entrainment to a 6h phase advance is the best predictor of depression- and anxiety-like behaviors in rats. We incorporated a 6h phase advance into the study as a way test the adaptability of the SCN. Rate of re-entrainment is associated with anxiety-like behaviors in the activity box and the EPM. Rats that take longer to re-entrain spend less time in the center of the activity box and spend more time in the margins. We also report that rate of re-entrainment is associated with more time spent in the closed arms of the EPM, a lower percentage of open arm entries, and less time spent in the open arms - all of which are indicative of anxiety-like responses.

There are two explanations that can account for individual differences in rate of re-entrainment. First, individual differences in rate of re-entrainment may be driven by individual the degree of phase heterogeneity in SCN cells. Evans et al., (2015) demonstrate that individual differences in the rate of re-entrainment to a phase advance are associated with the degree of synchrony/phase heterogeneity between individual

SCN neurons. Cell synchrony within the SCN is regulated by vasoactive intestinal polypeptide and its receptor VPAC2R (Harmar et al., 2002; Maywood et al., 2006). To further investigate whether degree of phase heterogeneity between SCN cells affects mood, future studies can selectively knock down vasoactive intestinal polypeptide in the SCN or by micro-infusions of VPAC2R antagonists. An alternative way to account for differences in rate of re-entrainment is individual differences in expression of dopamine receptor D1 (Drd1) expression in the SCN. It was recently shown that Drd1 knockout mice take significantly longer to entrain to either a phase advance or a phase delay and that selectively restoring Drd1 in the SCN restores rate of re-entrainment to wild-type rates (Grippo, Purohit, Zhang, Zweifel, & Güler). Future studies can examine the relationship between individual differences in Drd1 expression in the SCN with rate of re-entrainment and performance on mood-related tests to elucidate the role of Drd1 receptors in entrainment parameters and mood.

We also report an interesting relationship between circadian entrainment parameters and mood-related behaviors. Rats that are more active during the light phase of a standard 12h:12h LD cycle spend less time immobile and have a longer latency to immobility in the FST. We also report that variability in activity onset is associated with longer latency to immobility in the FST, more time spent in the center square of the EPM and less time spent in the open arms. Taken together, variability in onset is associated with increased escape-directed behaviors in the FST and increased anxiety-like behavior in the EPM. Different factors could account for individual differences in these entrainment parameters, including the precision of the SCN clock, the strength of the entraining stimulus or individual differences in sensitivity to the entraining stimuli

(Aschoff et al., 1972). Because the room lighting was standardized across rats, we believe that the increased variability in activity onset and increased activity in the light phase are mediated by individual differences in responsiveness to light.

Responsiveness to light has been linked to aberrant circadian rhythms and mood-related behaviors in rodent models. Mice selectively bred for high anxiety-like behaviors exhibit a number of aberrant circadian locomotor parameters, including a longer free running period in constant dark, more fragmented wheel-running rhythms in 12h:12h LD and constant dark conditions, and are less responsive to the phase shifting effects of light in constant dark (Griesauer et al., 2014). Similarly, individuals with seasonal depression also exhibit aberrant circadian rhythms and are less responsive to light (Hebert, Dumont, & Lachapelle, 2002). Seasonal affective disorder is a specific form of depression where depressive symptoms manifest during the winter months and predominantly occurs in northern climates where exposure to sunlight is reduced during winter months. Individuals with seasonal and non-seasonal forms of depression are often phase delayed compared to the environment, have blunted amplitude in daily activity patterns and have increased variability in daily acrophase (i.e., the peak time of a rhythm) (Berle et al., 2010; Luik et al., 2015; Teicher et al., 1997). Interestingly, when endogenous circadian rhythms are realigned with the environment through chronotherapies such as bright light therapy or melatonin treatment, depressive symptoms subside (Lewy et al., 2007). Based on these findings, we speculate that individual differences in responsiveness to light are influencing circadian entrainment parameters and mood-related behaviors through the SCN. Although individual differences in responsiveness to light can affect mood independently of the clock (i.e.,

via projections from ipRGCs to brain regions implicated in mood (LeGates et al., 2012)), given that the SCN regulates circadian rhythms in locomotor behavior, we believe that the current findings support an association between SCN functioning and mood-related behaviors. Future experiments can examine the role of individual differences in responsiveness to light by looking at how individual rats respond to phase shift effects of light under constant dark and negative masking during the active phase. Negative masking is when a light pulse (e.g., 2-3h) is presented during the active phase. Animals that are less responsive to light would be expected to have less suppression of running wheel behavior.

The results from the first study contribute to our understanding of the relationship between circadian rhythms and mood. We believe that these findings support the hypothesis that circadian rhythms are implicated in the etiology of certain psychiatric disorders. Our findings also contribute to the growing number of studies using Lewis rats to study individual differences in susceptibility to different disease states, including adjuvant-induced arthritis (Sajti et al., 2004) and post-traumatic stress disorder (Goswami et al., 2010). In order to further validate the utility of Lewis rats as a model for circadian disruption and mood-related behaviors we then wanted to examine whether Lewis rats exhibit a comorbid relationship between depression- and anxiety-like behaviors. This is an important question to address in animal models because in clinical populations depression and anxiety are highly comorbid, with some estimates as high as 80% (Lamers et al., 2011). Despite this high degree of comorbidity little attention has been given to the question of comorbidity between depression- and anxiety-like behaviors in animal models, particularly from the perspective of individual differences.

The purpose of the second study was to assess for comorbid-like relationships between depression- and anxiety-like behaviors using a large battery of mood-related assays. Interestingly, we did not observe a 'comorbid' relationship between anxiety-and depression-like behaviors; in fact we observed the opposite effect. Rats that exhibit more anxiety-like behaviors in the activity box tend to spend more time immobile in the FST. Moreover, rats that spend more time in the center of the activity box also spend significantly more time immobile in the FST. Consistent with these findings, Estanislau and colleagues (2011) reported an inverse relationship between performance on the FST and the EPM in Wistar rats. Based on these findings, there appears to be an inverse relationship between depression- and anxiety-like behaviors. This is surprising as it is diametrically opposed to what is seen in clinical populations. There are two possible explanations for the discrepancies between human and animal research: either, the underlying mechanisms driving depression and anxiety are distinct in humans and rodents, or there is a fundamental problem with how we are interpreting animal behavior in one or more of the behavioral assays. Of all the behavior tests used, the FST is the only test with a significant amount of controversy surrounding the validity of how it is interpreted.

There are a limited number of behavioral assays available to assess depression-like behaviors in rodents but by far the most common is the forced swim test (FST). Although the FST is considered the gold standard for studying depression-like behaviors there are strong reasons to question the interpretation that immobility represents 'despair' and escape-directed behaviors such as climbing represent the absence of a

depression-like phenotype. It has recently been proposed that immobility in the FST is an adaptive learned response and reflects a switch from active to passive coping strategies (de Kloet & Molendijk, 2016). While we agree with de Kloet and Molendijk (2016) that immobility is adaptive we disagree on the interpretation of active versus passive coping strategies. Instead we believe that escape-directed behaviors are driven by anxiety. We argue this perspective on the basis of comorbidity, gene targeting and pharmacological studies.

Depression rarely strikes alone. In clinical populations, depression is highly comorbid with anxiety disorders, with estimates as high as 80% (Lamers et al., 2011). Despite the high degree of comorbidity between depression and anxiety in clinical populations there are only a few studies examining individual differences in comorbidity between depression- and anxiety-like behaviors in rodents. One study found no relationship between performance on the FST and the elevated plus maze (EPM) (Ho et al., 2002), whereas another study reported an inverse relationship between performance on the FST and the EPM. In other words, animals that were high on measures of depression-like behaviors were low on measures of anxiety-like behaviors (Estanislau et al., 2011). More recently, we conducted a study looking at individual differences in depression- and anxiety-like behaviors where our results are more consistent with Estanislau and colleagues (2011). The inverse relationship between depression- and anxiety-like behaviors is surprising as it is diametrically opposed to human clinical populations. There are two possible explanations for the discrepancies between human and animal research: either, the underlying mechanisms driving depression and anxiety are distinct in humans and rodents, or we are misinterpreting animal behavior. We believe the

underlying mechanisms are conserved and therefore it is more likely due to interpretation error.

While comorbidity is the norm within clinical populations, much less is known about comorbidity in standard lab rodents. Different methodologies can be employed in the search for parallels between human comorbidity and animal models including gene knockouts (KO), selective breeding, and RNA interference. Although there are numerous animal models at one's disposal, we address two KO models: the serotonin transporter (5-HTT) KO and the serotonin 1A receptor (5-HT_{1A}) KO. These KO lines were chosen because the 5-HT system is implicated in both depression and anxiety disorders in clinical populations and that the most commonly used drugs to treat these disorders target the 5-HT system.

The precise mechanisms underlying the therapeutic effects of SSRIs have yet to be elucidated, however, it is known that SSRIs increase the amount of 5-HT available at the synaptic cleft through blocking the re-uptake of 5-HT at 5-HTT sites. Holmes and colleagues (2003) have done extensive work characterizing the phenotype of 5-HTT KO mice and demonstrated that they exhibit increased anxiety-like behaviors in the EPM, open field, and light-dark box, spend more time immobile in the FST, are more passive and less aggressive in the resident-intruder paradigm, but are also less active overall as measured by baseline activity in their homecage (see: (Holmes, Yang, Lesch, Crawley, & Murphy, 2003). Considering that 5-HTT KO exhibit increased anxiety-like behaviors across a host of behavioral tests and spend more time immobile in the FST one could argue that knocking out 5-HTT generates an animal model of comorbid depression- and

anxiety-like behavior. The fact that this KO model is generally less active is important to note because anxiety-like phenotypes are often based on activity levels, such as reduced exploratory activity in the open field and exploration of the EPM. As such, the general blunting of activity in 5-HTT KOs raises the question: can an overall reduction in activity account for the increased immobility observed in the FST as well as the anxiety-like phenotype? Given this caveat, researchers using KO models should pay close attention to overall activity levels to rule out this possibility.

In their work with the 5-HTT KOs, Holmes *et al.,* (2003) found that the anxiogenic phenotype is mediated through 5-HT_{1A} receptors. As with the 5-HTT gene, the 5-HT_{1A} receptor has been implicated in mood and anxiety in humans. 5-HT_{1A} receptor KO mice exhibit increased anxiety-like behaviors (Klemenhagen, Gordon, David, Hen, & Gross, 2006) as well as increased escape-directed behaviors in the FST (Freeman-Daniels, Beck, & Kirby, 2011), making 5-HT_{1A} KOs similar to 5-HTT KOs in respect to anxiety-like behaviors but opposite in respect depression-like behaviors in the FST. Both the 5-HTT and the 5-HT_{1A} receptor are implicated in depression and anxiety in humans, yet KO mouse lines exhibit different behavioral phenotypes. To explain these differences, we maintain that the 'depression-like' phenotype associated with 5-HTT KO is the result of an overall blunting of activity. In respect to the 5-HT_{1A} KO, we argue that the persistance of escape-directed behavior in the FST does not represent an antidepressant-like response, but is instead caused by the anxiogenic effect of knocking out 5-HT_{1A} receptors.

In addition to exhibiting an anxiety-like phenotype, 5-HT_{1A} KOs also exhibit deficits in hippocampal-dependent learning tasks including contextual fear conditioning (Klemenhagen et al., 2006). There are a number of convincing lines of evidence which support the position that immobility is a learned response (reviewed in: (de Kloet & Molendijk, 2016). In brief, if memory consolidation following the habituation phase is blocked with the protein synthesis inhibitor anisomycin, animals do not become immobile on the test day 24h later (De Pablo, Parra, Segovia, & Guillamon, 1989). Memory consolidation in the FST appears to be mediated by glucocorticoid signaling because adrenalectomy has the same effect on immobility as anisomycin. Moreover, adrenalectomized rats that receive an injection of corticosterone 15-min after the habituation trial become immobile on test day (Veldhuis, De Korte, & De Kloet, 1985).

The claim that the FST measures depression-like behaviors is based on evidence from preclinical trials that antidepressant compounds reduce immobility. It is consistently shown that an acute injection of a putative antidepressant reduces immobility in the FST (see de Kloet and Molendijk, 2016; Van der Meersch-Mougeot *et al.,* 1993). The fact that acute exposure to an antidepressant can produce an antidepressant-like response in rodents has been criticized because antidepressants require weeks of chronic use in clinical populations. Moreover, many patients do not respond to the first antidepressant prescribed and many patients are deemed treatment-resistant because they do not respond to two or more courses of antidepressant treatment. The issues of delayed onset and questionable efficacy are acknowledged in some animal behavior studies, however, the fact that antidepressants are associated with the induction or exacerbation of anxiety, particularly during the acute stage of treatment, has not been acknowledged.

The fact that antidepressants induce or exacerbate anxiety in clinical populations suggests that antidepressants would also have anxiogenic properties in rodents. This is particularly important given that protocols designed to assess antidepressants in rodents focus on acute exposure to the drug. Consistent with our anxiogenic hypothesis, Silva and colleagues (1999) demonstrated that an acute injection of fluoxetine induces anxiety-like behaviors in rodents. Interestingly, chronic exposure to fluoxetine had sustained anxiogenic properties (Silva, Alves, & Santarem, 1999). The lack of anxiolytic properties associated with fluoxetine is surprising given that SSRIs are the first line treatment for anxiety disorders.

There is evidence from both clinical reports and animal studies demonstrating that antidepressants induce anxiety. Therefore, we must consider whether the anxiogenic effects of antidepressants are being misinterpreted as antidepressant properties. If reduced immobility and increased escape-directed behaviors are driven by anxiety, it follows that anxiogenic compounds would have an anti-immobility effect in the FST. Nishimura and colleagues (1989) administered either beta-carboline-3-carboxylic acid ethyl ester (β-CCE; an inverse agonist at the benzodiazepine receptor site) or diazepam (an anxiolytic) to rats and tested their performance on the FST. They reported increased climbing and escape-directed behaviors in animals treated with β-CCE, whereas rats treated with diazepam spent significantly more time immobile (Nishimura, Ida, Tsuda, & Tanaka, 1989). Likewise, the alpha-2-receptor antagonist yohimbine, which exerts its anxiogenic properties through antagonism of alpha-2-receptors, has been shown to potentiate the anti-immobility effects of fluoxetine (Dhir & Kulkarni, 2007).

Furthermore, benzodiazepines have also been shown to reverse the anti-immobility effects of antidepressants (Van der Meersch-Mougeot et al., 1993). Interestingly the reversal of anti-immobility is specific to antidepressants because benzodiazepines do not affect caffeine-induced activity in the FST. As such, we believe that the anxiolytic is attenuating the anxiogenic effect of acute exposure to antidepressants. Within the clinical sphere, some physicians and psychiatrists prescribe an anxiolytic for the first weeks when commencing antidepressant treatment in order to manage the anxiety associated with the treatment and maintain adherence. One possible side effect of anxiolytics is sedation, thus one could argue that the decreased activity in the diazepam condition is driven by the sedative effect of the drug. Although possible, this is unlikely given that anxiolytics promote exploratory activity in the EPM. Therefore, any sedative effects associated with the anxiolytic likely do not account for the increase in immobility in the FST. In summary, there is converging evidence from pharmacological studies demonstrating that antidepressants can induce anxiety in both human and animal models. We argue that the antidepressant-induced anxiety is the driving force behind the increased escape-directed behaviors seen in the FST.

The FST is a widely used behavioral assay for depression-like behavior yet what immobility and escape-directed behaviors mean exactly remains unclear. Given the ubiquitous use of the FST to assess mood in gene KOs and other animal models of mood it is necessary to have a clear understanding of what the test represents and what the behaviors actually mean. Drawing upon comorbidity, gene KOs and pharmacological studies from both animal models and clinical samples we propose an alternative interpretation of the FST. To further our understanding of the FST, future

studies can continue to examine comorbidity between depression- and anxiety-like behaviors in healthy rodents. The study of individual differences as more of a top-down approach to understanding psychopathology will compliment what is learned from bottom-up approaches including gene KOs. While genetic models of mood and anxiety disorders provide insight into molecular pathways of a particular state, one must pay attention to confounding effects that can make inferring mood states more difficult. It is also important to focus on sex differences in mood-related behaviors. There are sex differences in the prevalence of depression and anxiety disorders in humans, which means future animal studies should include both male and female subjects. Finally, we believe that the understanding and interpretation of animal behavior would be improved by looking to clinical research. Currently there is a resurgence of interest in the therapeutic benefits of compounds such as lysergic acid diethylamide and psilocybin. These compounds were not developed for antidepressant or anxiolytic purposes but have therapeutic benefits, most likely mediated through their high affinity for 5-HT receptors. It will be interesting to see how these drugs affect performance on the FST and other measures of anxiety-like behavior. When designing experiments with these drugs it will be important to look to human clinical trials for the time course. For example, it will be important to administer the drug at least 2-3 days prior to behavior testing. In moving forward, it is important that the methodology used in animal studies mirrors human clinical trials as closely as possible. Utilizing similar methodologies, in respect to dosages and time course in particular will promote a better understand of the relationship between performance on the FST and anxiety-like behaviors.

Conclusion

Disrupted circadian rhythms are a cardinal feature of various psychiatric disorders, including depression and anxiety. There is accumulating evidence to suggest that disrupted circadian rhythms are implicated in the pathogenesis of these disorders. We are the first to report that individual differences in circadian locomotor parameters are associated with depression- and anxiety-like behaviors. These results have important implications in the assessment of individuals at risk of developing depression or anxiety disorders. With a more comprehensive understanding of the relationship between circadian rhythms and mood, there is potential to identify individuals at risk of developing a psychiatric condition. Many people today carry either a smart phone or a watch that can track the number of movements made in a day and at what time. This data has the potential to be translated into a diagnostic tool to identify individuals at risk of a psychiatric episode. When locomotor rhythms begin to go awry, therapeutic interventions can be implemented, including chronotherapies such as bright light therapy.

Much remains to be understood in the landscape of circadian rhythms and mental health. The findings herein contribute to our understanding of how outputs from the master clock located in the SCN relate to mood-like behaviors in healthy rodents. Robust and stable circadian rhythms are the reflection of a finely tuned clock. The end point of a stable circadian system is optimal health. Likewise, when circadian rhythms are disrupted, the overall health of the organisms tends to deteriorate, which is seen in

the form of both physical and mental health issues. By continuing to study the circadian system we will continue to learn more about how to optimize health and minimize disease.

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