# WHAT IF DAYS WERE SHORTER?

# THE BEHAVIORAL EFFECTS OF A 22-HOUR PERIOD

# ON WISTAR RATS

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BOĞAZİÇİ UNIVERSITY

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# DECLARATION OF ORIGINALITY

I, Aybeniz Ece Çetin, certify that

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Date 24.05.2019

ABSTRACT

What If Days Were Shorter?

The Behavioral Effects of a 22-Hour Period in Wistar Rats

Light is a significant pacemaker for circadian, that is daily or ~24-h, rhythms that greatly influence mood and various physiological functions. As this connection is well known in psychology, there are many studies focusing on the seasonal affective disorder (SAD) and its connection to the light period (i.e. daytime) of the day. Briefly, when the light period in a circadian cycle is shortened, animals tend to be more vulnerable to mood alterations. Despite the effects of shortened light or dark periods are well-known in mood and mood disorders, there is no study investigating the effect of shortened days (i.e. < 24-h) on mood or locomotor activity.

To this end, 27 naïve female Wistar rats (2-month-old, 203±6 g) were placed into two identical cabinets: the experimental group was exposed to shortened periods (22-h, 11:11 L:D cycle), whereas the controls were kept in a standard period (24-h, 12:12 L:D cycle). Two months later, both groups were tested in a classical rodent depression paradigm (behavioral despair), the Forced Swim Test (FST) as well as an anxiety-like behavior and locomotor activity test, the Open Field Test (OFT). The 22-h group showed no behavioral despair in the FST and no anxiety-like behavior in the OFT, and higher locomotor activity compared to the 24-h controls. These results indicate that moderately shorter, that is 22-hour, days can produce an antidepressant and anxiolytic effect in Wistar rats.

# ÖZET

## Günler Daha Kısa Olsa Ne Olurdu?

## 22 Saatlik Periyodun Wistar Sıçanları Üzerindeki Davranışsal Etkileri

Işık, ruh halini ve çeşitli fizyolojik fonksiyonları modüle eden sirkadiyen ritimler (yaklaşık 24 saatlik/günlük) için önemli bir düzenleyicidir. Bu bağlantı psikolojide iyi bilindiğinden, ışığın mevsimsel duygudurum bozukluğuyla (SAD) ve günün aydınlık dönemiyle olan ilişkisine odaklanan birçok çalışma vardır. Kısaca, sirkadiyen bir döngüdeki aydınlık süre kısaldığında, deney hayvanları ruhsal değişikliklere karşı daha savunmasız olma eğilimindedir. Kısaltılmış aydınlık veya karanlık dönemlerin etkilerinin duygudurum ve duygudurum bozukluklarında iyi bilinmesine rağmen, kısaltılmış günlerin (toplam 24 saatten daha kısa) ruh hali veya lokomotor aktivite üzerindeki etkisini araştıran bir çalışma yoktur.

Bu amaçla, daha önce herhangi bir deneyde kullanılmamış (naif) 27 dişi Wistar sıçanı (2 aylık, 203 ± 6 g) iki özdeş kabine yerleştirildi: Deney grubu kısaltılmış güne (22 saat, 11:11 A:K döngüsü) maruz kalırken kontrol grubu standart gün yaşamaya devam etti (24 saat, 12:12 A:K döngüsü). İki ay sonra, her iki grup da klasik bir kemirgen depresyonu (davranışsal umutsuzluk) paradigması olan Zorunlu Yüzme Testi (ZYT) ile bir kaygı benzeri davranış ve lokomotor aktivite testi olan Açık Alan Testi'ne (AAT) tabi tutuldu. 22 saat grubu ZYT'de davranışsal umutsuzluk, OFT'de anksiyete benzeri bir davranış göstermedi ve AAT'de belirgin olarak daha yüksek lokomotor aktivite sergiledi. Bu sonuçlar kısaltılmış 22 saatlik günlerin Wistar sıçanları üzerinde antidepresan ve anksiolitik bir etkisi olduğuna işaret etmektedir.

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*To all women in science...*

TABLE OF CONTENTS

LIST OF FIGURES

# ABBREVIATIONS

BD: Bipolar Disorder

CR: Circadian Rhythm

EPM: Elevated Plus Maze

FST: Forced Swimming Test

GHT: Geniculohypothalamic tract

GLU: Glutamate

JLD: Jet Lag Disorder

L:D: Light:Dark cycle

L:L: Light:Light cycle

MDD: Major Depressive Disorder

OFT: Open Field Test

PER: Period (Genes)

RHT: Retinohypothalamic tract

SAD: Seasonal Affective Disorder

SCN: The Suprachiasmatic Nucleus

SSRI: Selective serotonin reuptake inhibitor

VIP: Vasoactive intestinal polypeptide

# CHAPTER 1

# INTRODUCTION

The mammalian body appears like a perfect machine in terms of its precise timing on both physiologic and behavioral processes such as hormone release and sleep-wake cycles, adjustment of body temperature, and various other metabolic and locomotor activity. Having such noteworthy rhythmicity helps an individual to adapt to exogenous changing factors (Aschoff & Pohl, 1978). It is now known that almost all organs and types of tissue possess their own clock cells (e.g. retina, liver; Reppert & Weaver, 2002). Yet, even though they have their own rhythms, they work in a synchronized pattern modulated by a *central clock* (Herzog, 2007; Golombek & Rosenstein, 2010).

The idea of a central clock has flourished since the early 1970s when the suprachiasmatic nucleus (SCN) of the hypothalamus has been identified as the master pacemaker of the body (Rosenwasser & Turek, 2005; Golombek & Rosenstein, 2010; Turek, 2016). Before the discovery of this master pacemaker, in the second half of the 1950s, the transatlantic travels became common and as a result, pilots and passengers experienced the Jet- Lag Disorder (JLD; for a broader explanation please see Chapter 1.2). These travels were basically causing an inconsistency between outer (destination's) clock and the internal clock which resulted in psychosomatic problems risking flight safety. Therefore, curiosity over the internal central clock increased and the following studies lead to the discovery of the master pacemaker, the SCN.

Two pioneering experiments were conducted in 1972 and the SCN of the hypothalamus was shown as the internal clock in rats (Moore & Eichler, 1972;

Stephan & Zucker, 1972). Both studies showed that the SCN is responsible for the production of internal rhythmic events. Moore and Eichler focused on the exact location of the SCN by lesioning firstly the optic tract and then ablating the SCN bilaterally (1972). Both research groups argued that the retinohypothalamic tract (RHT; for a broader explanation please see Chapter 1.1.2) and especially the SCN or near neuronal groups mediate circadian rhythms (CR) and sleep-wake cycles (Moore & Eichler, 1972; Stephan & Zucker, 1972).

## 1.1 How a maestro works: Structural organization of the SCN

### 1.1.1 Location and structure

The SCN, named after its location, is one of the eleven nuclei of the hypothalamus. It is positioned in the anterior hypothalamus, just dorsal to the optic chiasm and bilateral to the 3rd ventricle (Figure 1).

The hypothalamus is the key brain region for providing homeostasis ("similar standing" in Latin) and as it is one of the nuclei, the SCN the regulates CR (the term circadian also comes from Latin, where *circa* means "around" and *diēm* denotes "day"; hence it means "around the day") such as the sleep-wake cycle, body temperature, hormone release, gene expressions, and so on.

The SCN has approximately in total 20.000 neurons in humans and some of these neurons has its own individual endogenous rhythm (Welsh, Logothetis, Meister, & Reppert, 1995; Honma, Nakamura, Shirakawa, & Honma, 2004). However, all neurons in the SCN work in synchrony thanks to the non-self-sustained oscillatory neurons (Gu, Tang, Rohling, & Yang, 2016). Therefore, even in the absence of external stimulus (e.g. under constant darkness), it has its own intrinsic

rhythmicity. Czeisler et al. (1999) have measured this free-running rhythm of the SCN to be approximately 24 hours (>24-h).

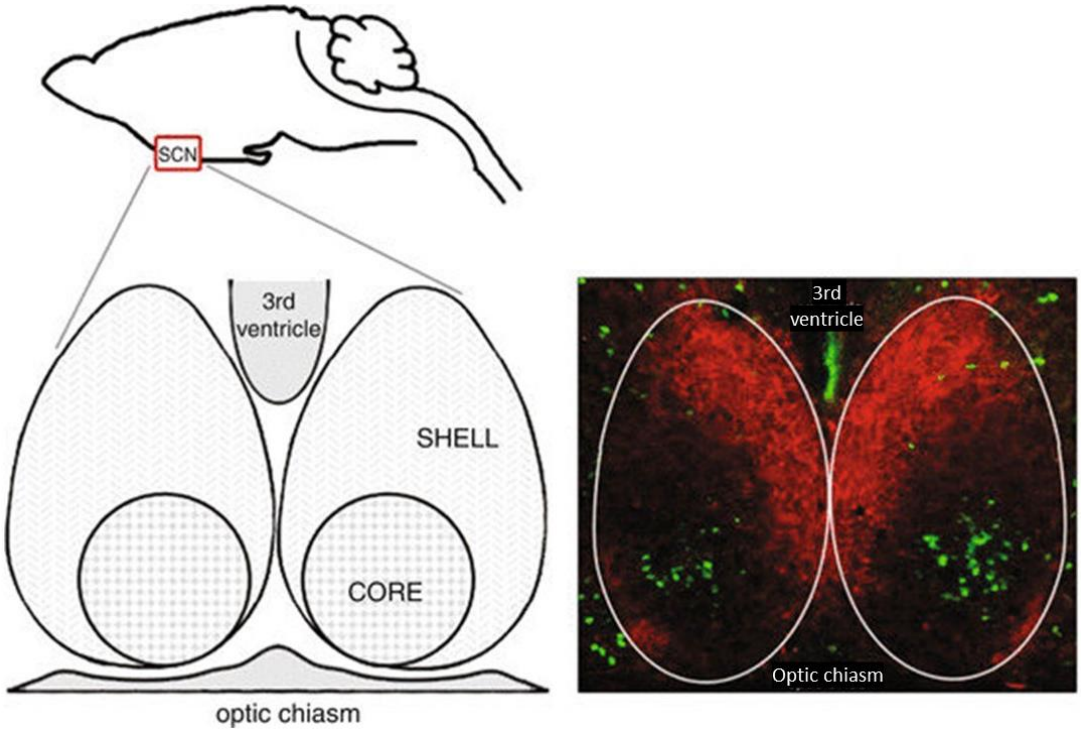


Fig. 1 Structure of the SCN
Note: The SCN consists of two nuclei on either side of the 3rd ventricle. In the photomicrograph, both the core (green staining, calbindin containing cells are dyed) and the shell (red staining, AVP cells) of the SCN is revealed clearly (modified from Silver & Rainbow, 2013)

The SCN is functionally divided into two parts: the core and the shell (Figure 1). The ventrolateral SCN (the core) is the major hypothalamic region for receiving photic input. It expresses the so called Period genes per1 and per2 with the innervation by the retinohypothalamic tract (RHT) (e.g. photic stimulation) and this expression ends with a circadian oscillation in the dorsomedial SCN (the shell) (Antle & Silver, 2005; Golombek & Rosenstein, 2010).

Consequently, the master pacemaker, the SCN, works in a complex way. This complex functioning serves to maintain a synchronized rhythmicity throughout the body. To this extent, the SCN has survival value; it receives various inputs (e.g. light,

temperature, etc.) from the external world and uses these to regulate other (sub)cycles (e.g. gene expressions, sleep-wake cycles) underlying different bodily functions. Despite to its relatively small size, it is a very powerful nucleus to control whole body's adaptation to the external world.

### 1.1.2 Afferent inputs and efferent projections

The SCN as the master pacemaker has its own free-running rhythm. However, another duty of the SCN is to preserve adaptation of the body, so it receives input essentially from the external world. This is by means of two main afferent projections: (1) the geniculohypothalamic tract (GHT) and (2) the RHT. The major input for the SCN is the light/visual projection. While the monosynaptic RHT is the main projection to the SCN (Johnson, Moore, & Morin, 1988; Golombek & Rosenstein, 2010), the GHT has a marginal role in the entrainment of the SCN and it mostly accompanies the RHT as the secondary projection by taking direct visual input from the optic tracts (Johnson et al., 1988).

The most important intra-SCN neurotransmitter is the vasoactive intestinal polypeptide (VIP). The VIP takes part in the preservation of the current rhythm and resetting the clock. The VIP is observed in the efferent projections, too. Another important chemical messenger is melatonin, released in the pineal gland, has an inhibitory role on SCN activity throughout the night (Reghunandanan & Reghunandanan, 2006).

In addition to mentioned neurotransmitters, the SCN has other ways to project the rest of the cells in the body. It is known that there are other oscillators in the rest of the brain and in peripheral organs (Reppert & Weaver, 2002; Stratmann & Schibler, 2006). Because there is a hierarchical relationship between the SCN and the

other cells in the peripheral organs imitating the activity of the SCN, they are called slave oscillators (Figure 2; Reppert & Weaver, 2002).

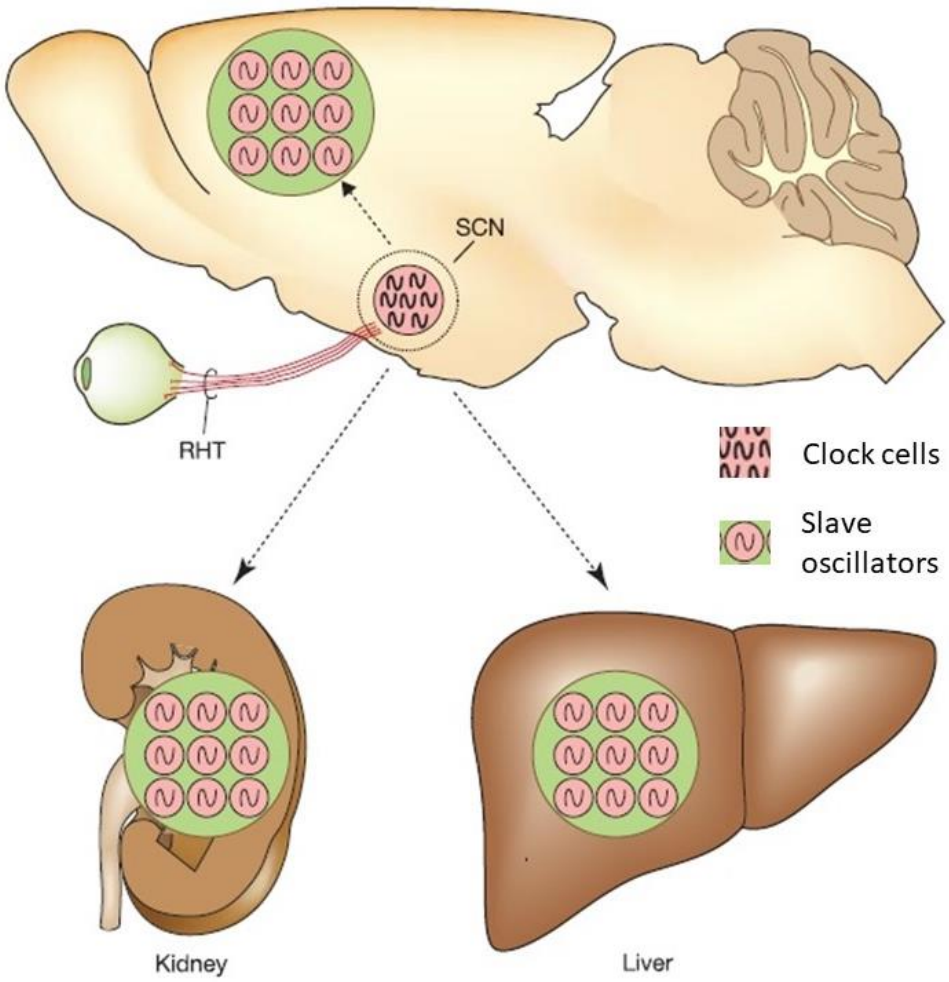


Fig. 2 Examples of slave oscillators
Note:Clock cells of the SCN orchestrates slave oscillators in the cortex, kidney, liver, and other peripheral organs (modified from Reppert & Weaver, 2002)

In addition to its tissue/organ-specific genetic regulation, the SCN has also been investigated for its metabolic and behavioral regulation (Sack et al., 2007; Takahashi, Hong, Ko, & McDearmon, 2008). These studies, which are out of the scope of this thesis, showed the substantial role of this tiny hypothalamic nucleus in various sleep disorders and types of cancer (Lowrey & Takahashi, 2004; Sack et al., 2007; Takahashi et al., 2008).

In order to orchestrate all these mechanisms, the SCN requires sufficient and versatile input. While it has its own intrinsic rhythm, it requires input from the external world, which is necessary for survival. There are many environmental cues such as light, temperature, sound, availability of food and social cues for the body to set the CR/biological clock. However, today it is very well known that the most effective one is light (Rosenwasser & Turek, 2005; Golombek & Rosenstein, 2010).

## 1.2 Light: the integral Zeitgeber

It is well-known that the SCN is entrained most powerfully by light which has direct and indirect effects on mood and behavior (LeGates, Fernandez & Hattar, 2014). Just as we set our wrist watches, light entrains the SCN. It regulates mood and behavior indirectly by modulating the CR (through the SCN) or by directly through projections from intrinsically photosensitive retinal ganglion cells (ipRGCs; LeGates et al., 2014; Fernandez et al., 2018).

If the SCN is not entrained by external cues it has a free-running rhythm. The word “entrainment” is chosen on purpose: light sets the biological clock, as I mentioned above, so it also can change the phase of it. The timing of the rhythms of bodily functions is refers to the phase of the biological clock. The phase change may occur in one of two ways; either a phase advance (setting the clock earlier) or a phase delay (setting the clock later).

Phase shifts are observed commonly in the JLD mentioned in the introduction. The JLD is the result of a trans-meridian flight and affects almost everyone. For example, a plane from Istanbul to San Francisco departures at 1 pm and lands at 4:30 pm. The flight duration is 13:30 hours yet according to destination’s clock only 3 hours passed compared to home country’s clock. Even if

the SCN tolerates such rapid travel and is set up immediately, the peripheral clocks mentioned above fail to synchronize instantly with the SCN and they keep the earlier rhythm until its totally entrained by the destination's clock (Sack et al., 2007; Takahashi et al., 2008). This is why not only sleep disturbances, but also gastrointestinal problems and low cognitive performance is observed during the JLD (Vosko, Colwell, & Avidan, 2010).

The JLD is one of the common disorders that is directly related to the CR. At first, they can be treated (e.g. via melanopsin administration, light exposure) yet in the long term it causes even more severe diseases (Vosko et al., 2010). That is to say, keeping the rhythm of the body regular is essential to avoid both psychological and physiological disorders.

As is seen in the example of the JLD, regular CR is maintained by environmental cues and the most effective cue is light. The power of light and the idea of entrainment was first mentioned by Jürgen Aschoff in 1965. In this seminal article, Aschoff states that circadian period is entrained to "the period of the earth's rotation by means of periodic factors in the environment, called Zeitgebers" ("time giver" in German; Aschoff, 1965, p. 1427). It is possible to observe physiological alterations either in short or long-term by altering only the light-period of the day.

### 1.3 Pharmacological agents are non-photic cues for internal clock

The SCN is mostly described as an interface between the outer world and bodily functions (Millar, 2004). However, as it is affected by Zeitgebers, there are several studies revealing the effects of pharmacological agents (e.g. caffeine, lithium, fluoxetine, and so on) on clock genes, and accordingly the CRs.

Caffeine is an easy-to-access agent in daily life; especially tea and coffee are the most known sources of it. Phase shifting effect of caffeine is recognized in several species (bacteria, insects, other mammalians). Both in vivo and in vitro studies in mice showed that caffeine causes a phase-delay and lengthens the CR (Oike, Kobori, Suzuki, & Ishida, 2011) when it is administered *ad libitum*. The same effect was observed in humans, showing that caffeine causes a 40-min phase-delay (Burke et al., 2015).

Another example is lithium, a multi-faceted, complex pharmacological agent with differential effects on norepinephrine and serotonin, and a common choice for the Bipolar Disorder (BD) treatment causes phase delays in the first administration, shortens active hours of hamsters and stabilize the CRs (Klemfuss & Kripke, 1995). Lithium changes the phase by not only delaying the it, but also by extending/lengthening the whole period. In patients who voluntarily lived in isolated huts (and therefore not exposed to Zeitgebers), it was shown that while treating depressive symptoms, lithium also extended their periods (Johnsson, Engelmann, Pflug, & Klemke, 1983).

Classical antidepressants such as the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and sertraline also resynchronize CRs in depressive patients (Mendoza, Revel, Pevet, & Challet, 2007; Nomura, Castanon-Cervantes, Davidson, & Fukuhara, 2008). These are frequently used for the treatment of the Seasonal Affective Disorder (SAD; Westrin & Lam, 2007; for a broader explanation see chapter 1.4).

## 1.4 One of the most common psychological disorders: the SAD

Phase changing effects of pharmacological agents point to a reciprocal relationship between the CR and affective disorders. For instance, the SAD mainly results from a substantial reduction of the light phase (e.g. change in the CR) during winter, and it comes with negatively changed mood and diminished cognitive performance. On the other hand, because affective disorders cause changes in the CR, pharmacological agents used in the treatment of these disorders also affect the CR (e.g. phase delay, phase lengthening etc.; McClung, 2007).

A well-known phenomenon affected by daylight is the SAD, defined as the "recurrent depressive episodes that occur annually" (Rosenthal et al., 1984, p. 72) and often observed, especially during fall and winter. This why it is also known as "winter blues". According to the so-called phase shift hypothesis, SAD is the result of an unordinary phase delay of the internal clock which is in charge with synchronizing itself to the external cues (Lewy, Sack, Miller, & Hoban, 1987).

There are numerous studies on how to cure the SAD symptoms and some of the methods are as follows: regulating sleep-wake times and temperature of the room, increasing indoor lighting and not avoiding social contact (Leahy, 2017). All these suggestions are related to the aforementioned Zeitgebers. Social cues, for instance, constitute a well-defined clock for all members of the society who have to wake up at a certain time to go to work, eat at defined time periods and go to bed at night. However, as a clinical intervention or even as prevention, it seems that light-box therapy (e.g. light exposure for 20 – 30 mins every day, in front of a UV-filtered light-box) is the most preferred end effective way against the SAD (Leahy, 2017).

From this point of view, the SAD clearly shows the severe effects of a significant alteration in the CR as well as the power of light as a pacemaker stimulus.

As light manipulation is strong enough to treat, or even cure, certain affective disorders, lack of sufficient lighting has substantial negative effects on mood.

## 1.5 Shortening the light phase of a 24-h photoperiod has negative effects on mood

Many studies, inspired by SAD, have experimented with different photoperiods in experimental animals to understand the disease and to determine what functions are affected in the body. Among the known effects of the shortened light period are depression-like behavior in the FST (Einat, Kronfel-Schor, & Eilam, 2006; Otsuka et al., 2014; Ben-Hamo, Tal, Paz-Cohen, Kronfeld-Schor, & Einat, 2016), higher anxiety scores in the Elevated Plus Maze (EPM; Ben-Hamo et al., 2016; Xu et al., 2016) and anhedonia in the Sucrose Preference Test (Ben-Hamo et al., 2016). However, the experimental manipulation of these studies consisted of shortening of the light period and accordingly increasing the dark period of the 24-hour day. Thus, although there is a plethora of information about the proportional changes of darkness and light within 24 hours (e.g. 10:14, 16:8 L:D cycles), there are few studies on the replacement of the whole 24 hours (e.g. a total day of 8 hours, shortening the day to 4-h light and 4-h dark).

One of the classical studies utilizing a significantly shorter day (8 hours) suggested that the 4:4 L:D cycle caused a significant decrease in locomotor activity scores (Park, Cheon, Son, Cho, & Kim, 2012). The same study also stated that their paradigm (4:4 L:D) was beyond the limits of internal clock mechanism and this is why the SCN cannot adapt to these extreme cycles. From this point of view, CR/internal clock has a biologically-set limit in terms of the shortened day.

## 1.6 Light as treatment

Light has the power of changing mood. Deficit or irregular phases (e.g. because of transatlantic flights) of light affect the mood negatively. Yet, this power can also be used in the reverse direction, to improve the affective state. As it has an ameliorative effect on SAD, different types of light manipulation may alleviate depressive symptoms. Furthermore, light therapy may alleviate depressive symptoms in patients with Major Depressive Disorder (MDD; Kripke, 1997; Lieverse et al., 2011; Li & Li, 2018).

Not only chronic manipulations of light therapy, but also acute applications have been utilized as positive mood modulators. For instance, when rats were exposed to a 12-h light period (e.g. they did not live 12:12 L:D day as usual, yet they lived a single 12:12 L:L day) they did not show behavioral despair in the Forced Swim Test (FST; Yılmaz, Aksoy, & Canbeyli, 2004).

The timing of the light stimulation is a fundamental feature of its antidepressant effects. For example, the antidepressant effect of light is observed in patients diagnosed with SAD, only when the light is delivered early in the morning (Lewy et al., 1987; Lewy, Sack, Singer, Whate, & Hoban, 1988).

The main positive result of the timing of the light comes with phase advances. When subjects are presented with light late at night, they experience phase advances (Rusak & Groos, 1982; Eastman, Young, Fogg, Liu, & Meaden, 1988; Vitaterna, Takahashi, & Turek, 2001). This is why both the SAD and MDD treatments has an ameliorative effect. Light exposure during night time has a positive effect on mood not only in clinical populations, but also in subjects with no diagnosis of a mood disorder. For instance, a 30-min photic stimulation in the second half of the dark phase has an ameliorative effect on rats (Schulz, Aksoy & Canbeyli, 2008).

In addition, a 10-min blue light exposure in the second half of the night is more effective than the blue light exposure at the first half of the night (İyilikçi, Aydın & Canbeyli, 2009).

Furthermore, the wavelength and intensity of light also matter when it comes to finding the most beneficial manipulation. Low-intensity short wavelength (i.e. blue light) has a strong ameliorative effect on depressive symptoms (Meesters, Dekker, Schlagen, Bos, & Ruiter, 2011). However, long wavelengths (e.g. red light) do not replicate this antidepressant effect (İyilikçi et al., 2009).

All in all, duration and timing of light matters when it comes to its effect on behavior both in human and animal models. It is important to understand the limits of the SCN in order to assess the psychological problems stemming from unfamiliar light exposure (e.g. during winter) and the ameliorative effect of the light (e.g. light-box therapy). There are two main concerns to assess mood-changing effect of light, and the interface between light and mammalian body, the SCN: First, as Park et al. (2012) mentioned a 4:4 L:D cycle is beyond limits of the SCN, so there must be a moderately-shortened-day-manipulation. Second, effect of duration of the dark period is as important as the light period, so the duration of both phases should be kept equal to eliminate the effect of a longer dark period as it is seen in the SAD experiments.

## 1.7 Present study

As mentioned above, there are several studies utilizing shorter or longer light phases in 24-h periods. However, there is no specific longitudinal study on possible outcomes of a shortened whole day (e.g. 11:11 L:D cycle). To investigate the effects

of a moderately shortened day that can be tolerated by the SCN, in the present study, adult female Wistar rats were subjected to 22-h days (11:11 L:D cycle) for 2 months.

To assess behavioral despair, the experimental (i.e. 22-h day) animals were tested in the FST. Their locomotor activity was recorded in the Open Field Test (OFT) in comparison to the 24-h control group. In this study, animals were tested in the OFT right after the Day 2 (test phase) of the FST.

I hypothesized to observe less behavioral despair and anxiety in the 22-h group, as the 22-h group experience repetitive phase-advances. As explained above, phase advances were shown to underlie the antidepressant effect observed as a result of early morning light manipulation in patients with SAD (Lewy et al., 1988). Furthermore, comorbidity of depression (behavioral despair in rats) and anxiety is observed in half of the patients (Zimmerman & Chelminski, 2003). I therefore postulate to find a difference in the anxiety scores of 22-h (lower anxiety) and 24-h groups (higher anxiety), which will be measured by their location/area preferences in the OFT.

Locomotor activity is recognized for its period-length changing and phase-shift aspects. Free-running period of blinded rats who have free access to the running wheel is shortened (Yamada, Shimoda, Ohi, Takahashi, & Takahashi, 1988). Locomotor activity is a non-photic cue that strongly influence the CR, especially through a phase-advances (Mrosovsky, 1996). It is known that benzodiazepine (an anxiolytic pharmacological agent) leads to phase-advances in CR and increase locomotor activity (Turek & Losee-Olson, 1986; Wickland & Turek, 1991). For this reason, I hypothesize that in this study, rats in the 22-h treatment group will show higher locomotor activity than the 24-h control group in the OFT.

# CHAPTER 2

# METHODS

## 2.1 Subjects

Twenty-seven naïve female Wistar rats (2-month-old, 203±6 g) were used in the experiment. They were kept under standard conditions before the manipulation, 12:12 L:D cycle, 22±1ºC with food and water were given ad libitum. All procedures were approved by the Institutional Ethics Committee for the Local Use of Animals in Experiments.

## 2.2 Experimental manipulation

While 13 rats lived a shortened photoperiod (11:11 L:D cycle) in a custom-made cabinet with several shelves (experimental group), 14 rats lived usual 24 hours (12:12 L:D cycle) in another identical designed cabinet (control group). All animals were given food and water *ad libitum* and housed in cages in groups of 3 or 4, at 22±1ºC. A series of 12V LED strip lights were used for luminance (35 lx at bedding level). Airflow in the cabinets was maintained by standard aquarium air pumps. With a constant white noise (70 dB), potential external auditory Zeitgebers were blocked.

In both cabinets, rats were constantly recorded with night-vision cameras. They lived 66 (according to the 24-h based clock) days in total in these custom-made cabinets. On Day 65, they were taken to the FST habituation/acclimation session, and on Day 66 they were first taken to the FST test session and then to the OFT. At the end of the OFT, they were placed back to the vivarium (Figure 3).

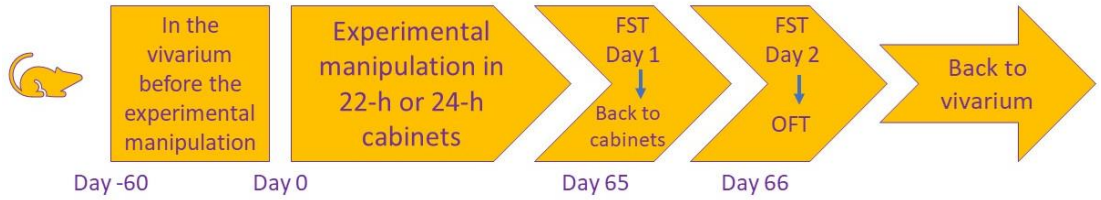

Fig. 3 Timetable of the experiments

## 2.3 Behavioral tests

### 2.3.1 The Forced Swim Test (FST)

The FST is an animal depression model that induces behavioral despair (Porsolt, Anton, Blavet, & Jalfre, 1978). It is a widely used behavioral rodent paradigm and the gold standard to test antidepressants in animals (Borsini & Meli, 1988; Cryan, Markou, & Lucki, 2002; Cryan, Valentino, & Lucki 2005; Slattery & Cryan, 2012). Rodents are placed in a cylinder-shaped, narrow pool filled with water and their behavior (e.g. immobility, swimming and struggling) is scored. While mice are tested in a single day/session, rats are tested in the FST for two consecutive days: in the first day, rats swim for 15 mins, and in the second day 24 hours later, they swim for 5 mins. The first 5 mins of the FST Day 1 and the Day 2 performance can be compared to assess whether the rat is experiencing behavioral despair (an animal model of depression). Increased immobility in Day 2 of the FST implies behavioral despair of the animal (Yankelevitch-Yahav, Franko, Huly, & Doron, 2015).

We used the standard FST protocol in our design following our altered rhythm manipulation in a Plexiglas cylinder shaped FST pool (height: 45 cm, diameter: 30 cm, water depth: 25 cm; Figure 4).

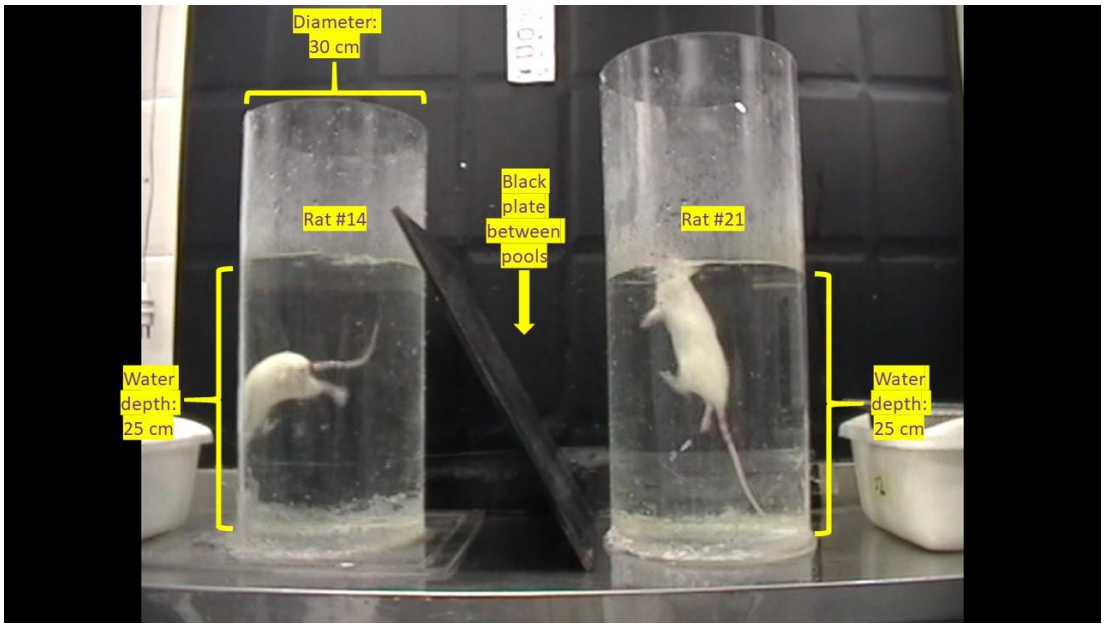


Fig. 4 Example FST setting
Note: An example of an FST trial. In each trial, 2 animals were tested. Pools and water qualities were identical. In this screenshot, Rat #14 (left) is diving and Rat #21 (right) is immobile; it does not move hind paws and moves only forelimbs to breathe by keeping her head over the surface of the water

To keep water level same and temperature at 22ºC for all animals, water was refreshed after each trial. In each trial, 2 rats were taken to 2 different FST pools separated by a black panel (Figure 4). Trials were recorded by a video camera (HDR-CX240 Handycam with Exmor® R CMOS sensor, SONY, CA). FST Pools were set in front of a black wall to have clear shootings.

Rats were tested in the FST according to their specific hour corresponding to the middle of the light onset (for 11:11 L:D group it was between ZT4 and ZT8 for 12:12 L:D group it was between ZT4,5 and ZT8,5). After the FST session, rats were taken to the individual cages filled with a paper towel to prevent hypothermia for 25±5 mins. Rats underwent the OFT after which they taken to these drying cages.

The rats were considered “immobile” when they were not swimming (i.e. diving, leaning toward the walls of the pool or floating; Figure 5). Immobility, taken as the main measure of behavioral despair, was measured for the first 5 minutes of

both FST days. Scoring was done offline (after the experiments) by two laboratory members via video recordings by using identical chronometers (ZSD808 – Water resistant chronometers).

### 2.3.2 The Open Field Test (OFT).

The OFT is a locomotor activity and anxiety-like behavior measure for rodents (Seibenhener & Wooten, 2015). The “open field" of the OFT is an 80x80cm dark area surrounded by 30-cm-high dark walls (Figure 5). In the OFT, overall locomotor activity, fecal boli counts, and location preferences (especially standing next to the walls vs. the center of the maze) were measured. The whole session was captured on camera with the animal tracking software EthoVision (Noldus, VA, USA).

After rats dried properly, they were introduced to the OFT. Rats were observed in the OFT for 10 minutes. At the end of each trial, rats were returned to the home cages and the maze was cleaned with ethanol (70%) to remove the odor from the prior test. The behavior of the animals was encoded using a chronometer. Both the location preferences and locomotor activity were measured in seconds.

The OFT was illuminated uniformly from 4 spots from the ceiling, which results in the periphery (near the walls) to be slightly darker. The rodents have an innate urge to seek darkness when they are anxious and they avoid the center of the OFT, which is more illuminated (Figure 5; Simon, Dupuis, & Costentin, 1994; Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002). When anxious, they often display thigmotaxis: approach to the slightly darker periphery and move in one direction while touching the walls (Simon et al.,1994; Seibenhener & Wooten, 2015).

Another way to measure anxiety is counting fecal boli. The number of fecal boli increases when the rats are anxious and it is usually negatively correlated with

locomotor activity (Carola et al., 2002). At the end of each trial, rats were taken to their home cages and fecal boli were counted.

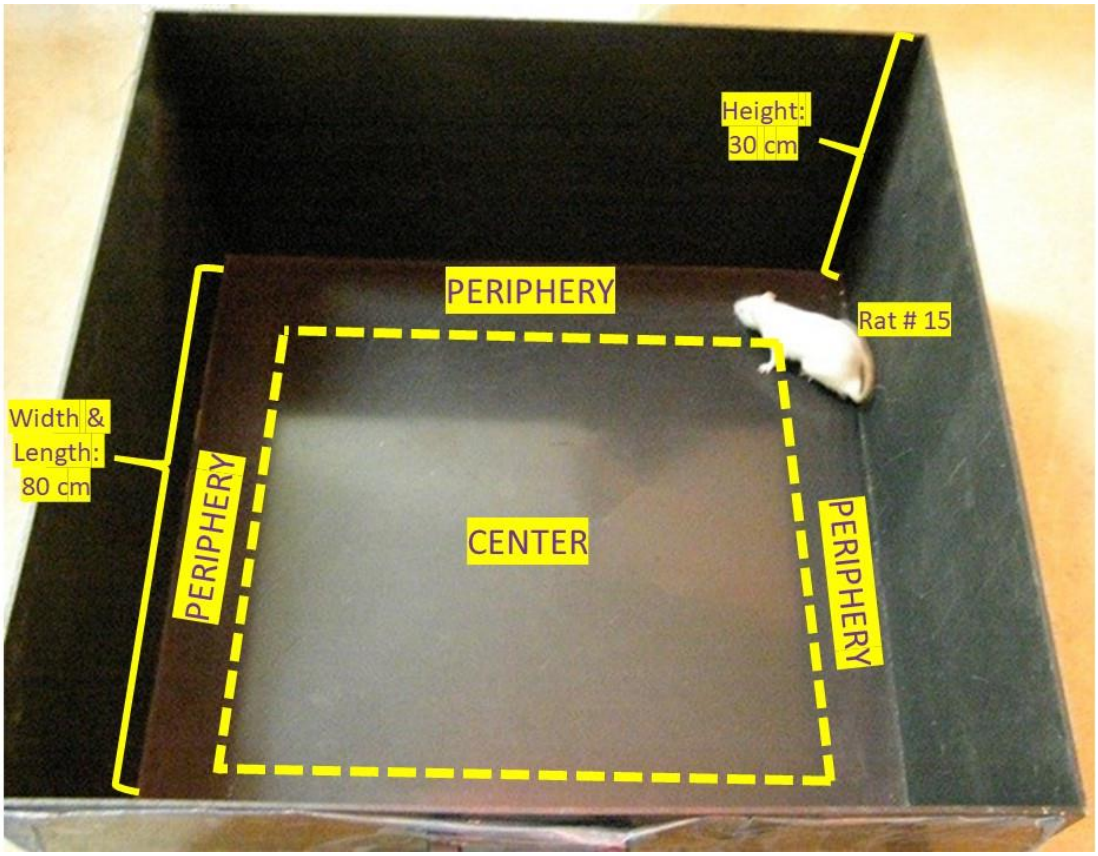


Fig. 5 Example OFT setting
Note: An OFT trial example. Rats were introduced into the maze at the center towards the same side/facing the same wall in each trial. The OFT is virtually split into two areas (yellow lines): the center and the periphery. In this screenshot, Rat #15 is in the periphery. If more than half of the body was in the center, but not a paw or the head, it would be scored as being in the center.

# CHAPTER 3

# RESULTS

## 3.1 Statistical analyses

Statistical analyses were carried out by using the Statistical Package for the Social Sciences (SPSS) 24.0, Excel (Microsoft Office 2016) and MATLAB (MathWorks). Data were also analyzed with the Student's t-test. Additionally, a two-way 2 (group) x 2 (FST day) mixed ANOVA was used to assess any significant main effect and interaction between the experimental groups and experiment days. Two animals from the 24-h control group had to be removed because the shooting of the FST session was not recorded properly.

## 3.2 The FST (Behavioral Despair Scores)

A 2x2 mixed ANOVA (between factor: experimental group, within factor: FST days) was conducted to assess the main effect of manipulation. There was a non-significant main effect of the FST day (habituation vs. test days), $F(1,23) = 1.55$, $p = .23$ and group (22-h vs. 24-h), $F(1,23) = 3.360$, $p = .08$. Additionally, to see the differences on test day, a Student's t-test was conducted. There was no difference in the mean raw scores of test day (Day 2) with the given sample size, $t(23) = -1.66$, $p = 0.1$. However, the data implied a clear trend toward an effect of the experimental group (Figure 6).

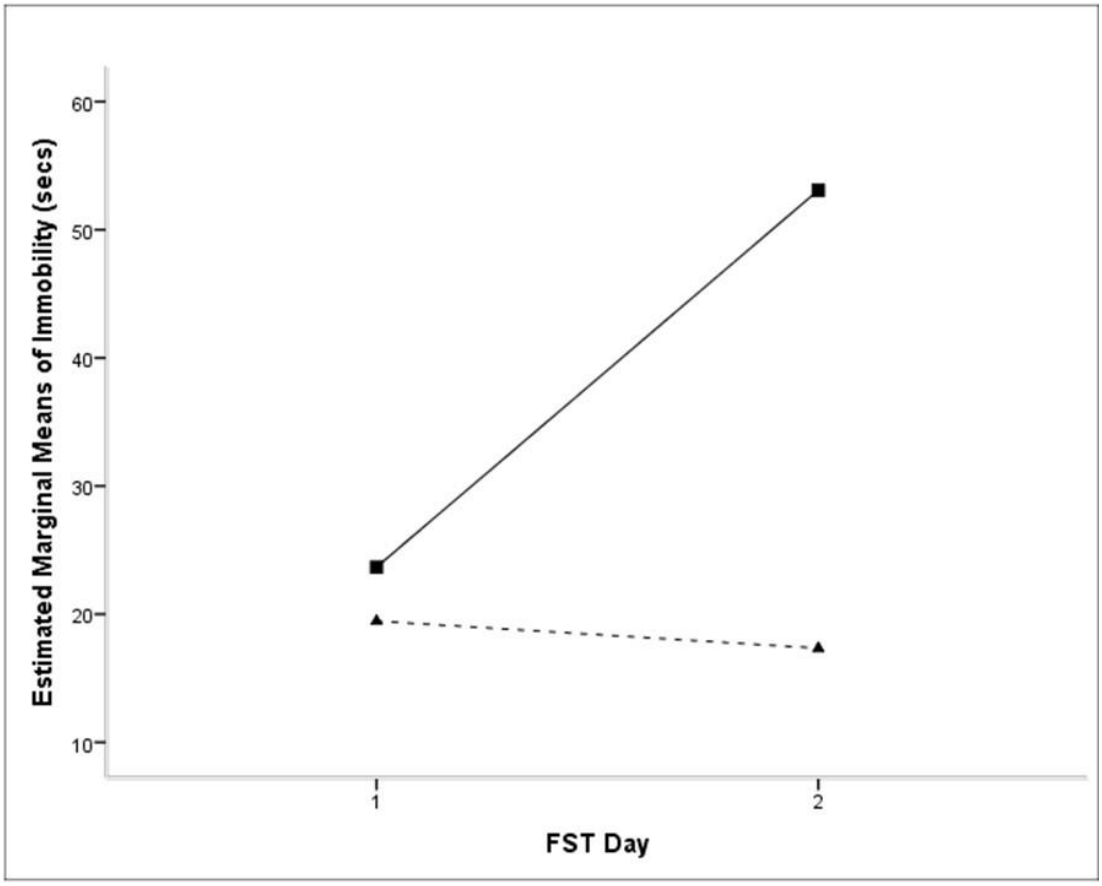


Fig. 6 Immobility change between 22-h and 24-h groups
Note: Straight line is for the 24-h control group and the dotted line is for the 22-h treatment group. Immobility change between 22-h and 24-h groups. In the analysis of the raw data, there was a no significant effect of experimental group or FST Day yet the data shows a certain trend especially for the main effect of the 22-h experimental group ($F(1,23) = 3.360$, $p = .08$)

Thus, immobility scores were analyzed with another commonly used method. In this method, total time (5 mins = 300 secs) is split into 5-sec intervals, and the behavior of the rat is scored if it is predominantly immobile (Slattery & Cryan, 2012; Yankelevitch-Yahav et al., 2015). An example presented in Table 1 below.

Table 1. Behavioral Despair Scoring

| 5-sec intervals for 20 secs, duration of immobility is given as x/5 | | | | |
|---|---|---|---|---|
| 2,4/5 | 2,5/5 | 1/5 | 4/5 | Total score for 20 secs |
| 0 | 1 | 0 | 1 | 2 |

According to Table 1, in the first 5 seconds, the rat is not mobile for 2,4 seconds – this is a 0-point. During the second 5 seconds, it is immobile half of the time – so it is a 1-point. This scoring was made for the whole 300-second test and the total number of points was taken as a behavioral despair score of the animal. In this way, animals' continuous immobility score was obtained with no error and momentarily stops (e.g. stops lasting in milliseconds for resting, breathing or headshaking is disregarded).

When these scores were compared, there was a significant difference between 22-h ($M$ = 1.15, $SD$ = 1.14) and 24-h ($M$ = 14.75, $SD$ = 20.16) groups, $t(23) = -2.43$, p = .01. The 22-h group had significantly lower immobility scores, which indicates higher behavioral despair of the 24-h group. Additionally, while the 22-h group has no significant difference between Day 1 and Day 2 ($t(12) = .30$, $p > .05$), the 24-h group significantly differed in its scores of Day 1 and Day 2 ($t(11) = -2.26$, $p = .04$; Figure 7).

The same procedure was applied to check coping strategies (whether the rats were actively swimming or struggling). A 2x2 mixed ANOVA (between factor: experimental group, within factor: FST days) was conducted on both swimming and struggling scores for this purpose.

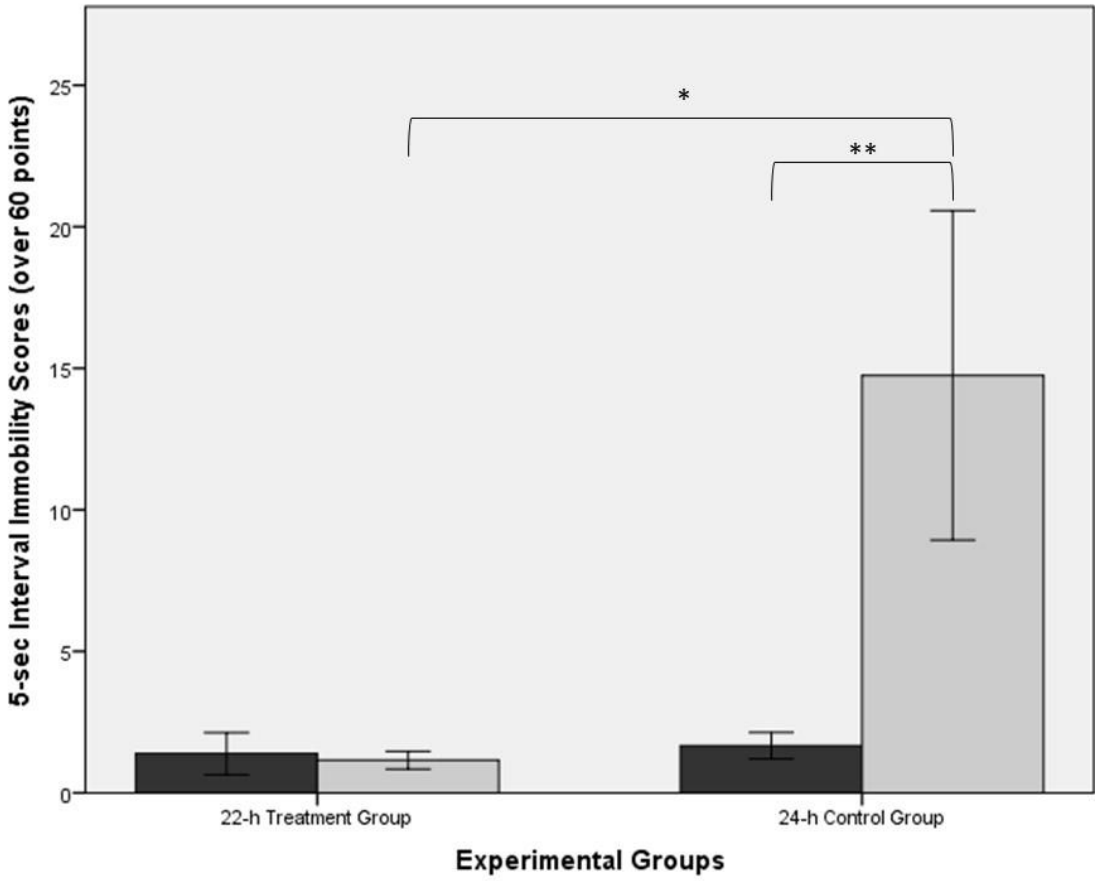


Fig. 7 Comparison of behavioral despair scores in the FST
Note: Dark bars are for the FST Day 1 (habituation day) and the light bars are for FST Day 2 (test day). *Statistically significant difference between test day scores of 22-h and 24-h groups. **24-h group showed significantly higher immobility at Day 2 compared to Day 1. Bars denote standard error of the mean (SEM)

For swimming scores there was a significant interaction between the experimental groups and FST day, $F(1, 23) = 654.50$, $p < .001$ (Figure 8). This indicates that swimming scores of the experimental groups were differed in FST Day 1 and Day 2. While the 22-h treatment group swam significantly more in the FST test day (Day 2) ($t(11) = -4.91$, $p < .001$), the 24-h control group showed no significant difference between FST Days ($t(11) = -4.91$, $p = .72$; Figure 9). For struggling scores there were no significant interaction, $F(1,23) = .747$, $p > .05$ (Figure 10).

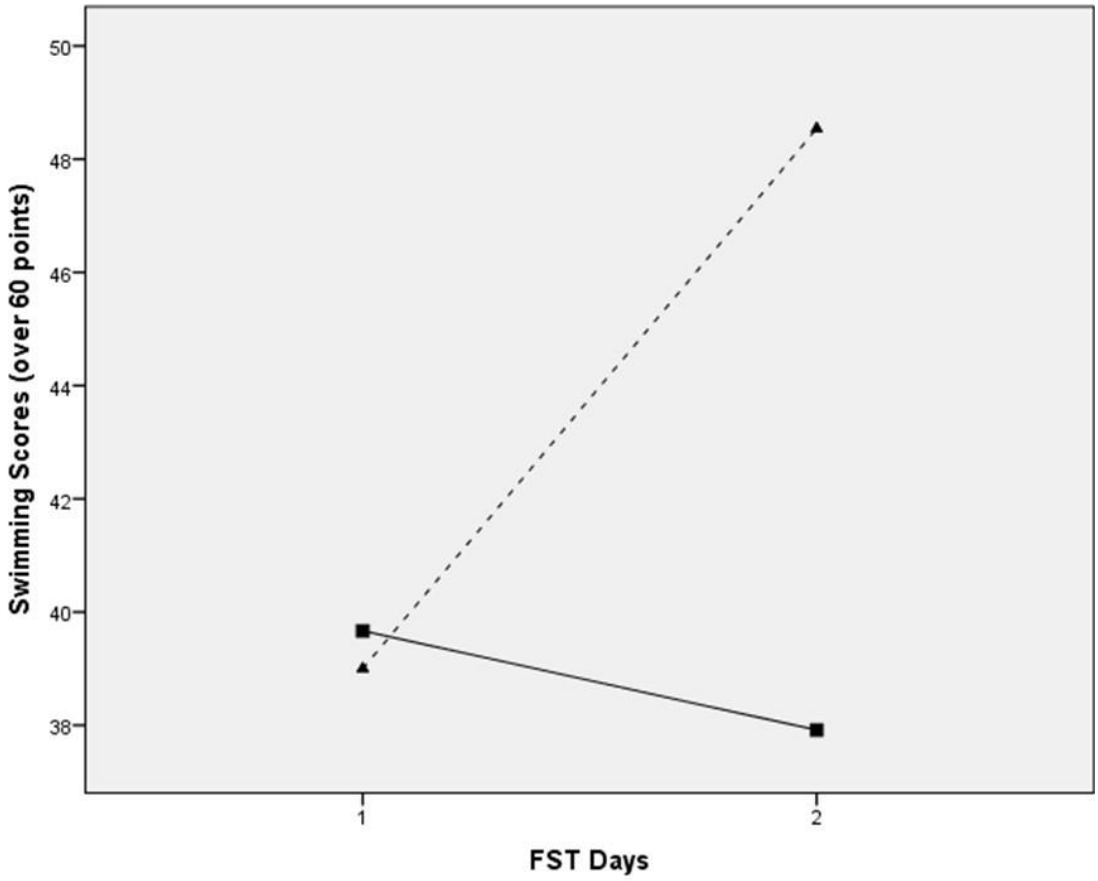


Fig. 8 Swimming change between 22-h and 24-h groups
Note: Straight line is for the 24-h control group and the dotted line is for the 22-h treatment group. There was a significant interaction between the groups and FST day, $F(1, 23) = 654.50$, $p < .001$. While the 24-h group's swimming scores decreased in Day 2, the 22-h group showed more swimming than they did on Day 1

## 3.3 The OFT (anxiety-like behavior and locomotor activity scores)

### 3.3.1 Locomotor Activity

An independent samples t-test was conducted to compare mean locomotor activity scores between groups. There was a significant difference between the 22-h ($M$ = 78.15, $SD$ = 29.18) and 24-h ($M$ = 30.86, $SD$ = 12.91) groups, $t(25) = 5.52$, $p < 0.001$ (Figure 11). The control group was significantly less mobile during the OFT.

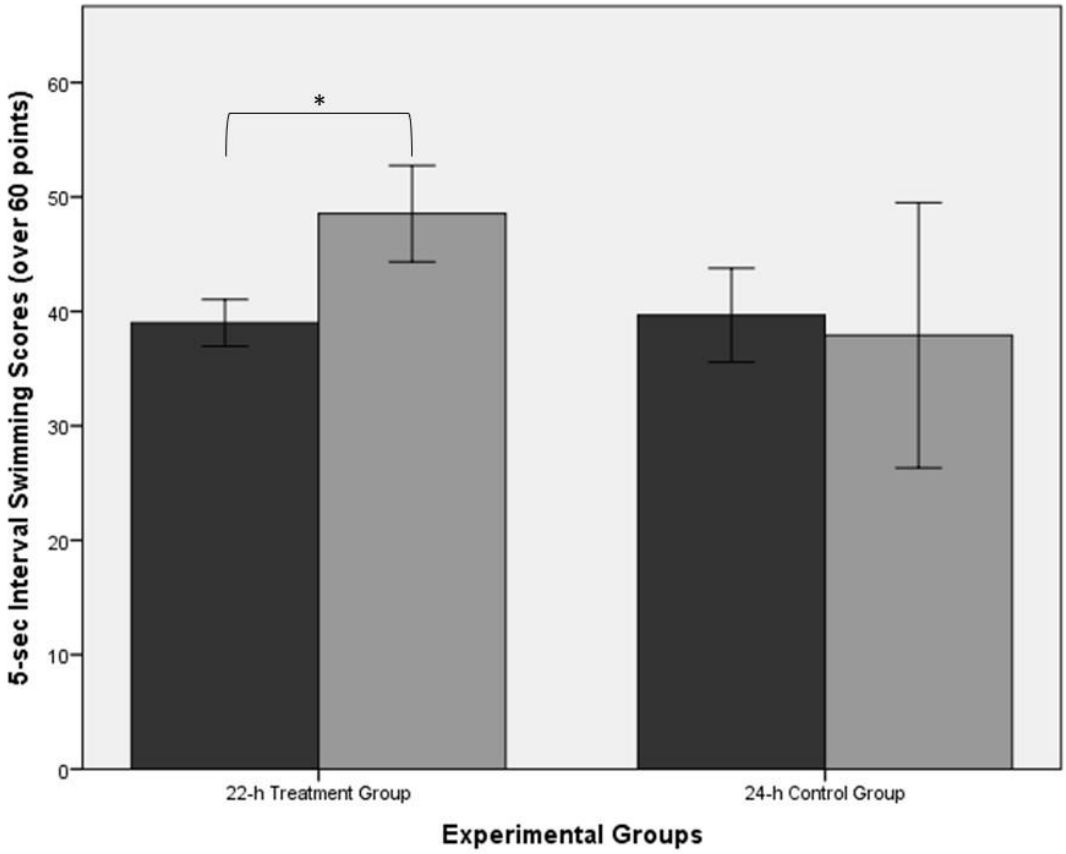


Fig. 9 Comparison of swimming scores in the FST
Note: Dark bars are for the FST Day 1 (habituation day) and the light bars are for FST Day 2 (test day). In Day 1 swimming scores did not differ between 22-h and 24-h groups. Also, the 24-h group showed no statistically significant difference between Day 1 and Day 2. *Statistically significant difference between Day1 and Day 2 swimming scores of the 22-h group. Bars denote standard error of the mean (SEM)

### 3.3.2 Anxiety-like behavior

The 22-h group was significantly more mobile in the OFT. Accordingly, the difference between fecal boli counts was statistically significant ($t(25) = -1.72$, $p = 0.049$). The 24-h group ($M = 2.21$, $SD = 1.89$) had higher fecal boli counts than the 22-h group ($M = 1$, $SD = 1.78$; Figure 12). There was no statistically significant difference between 22-h and 24-h groups on location preference, $t(25) = -.235$, $p > .05$.

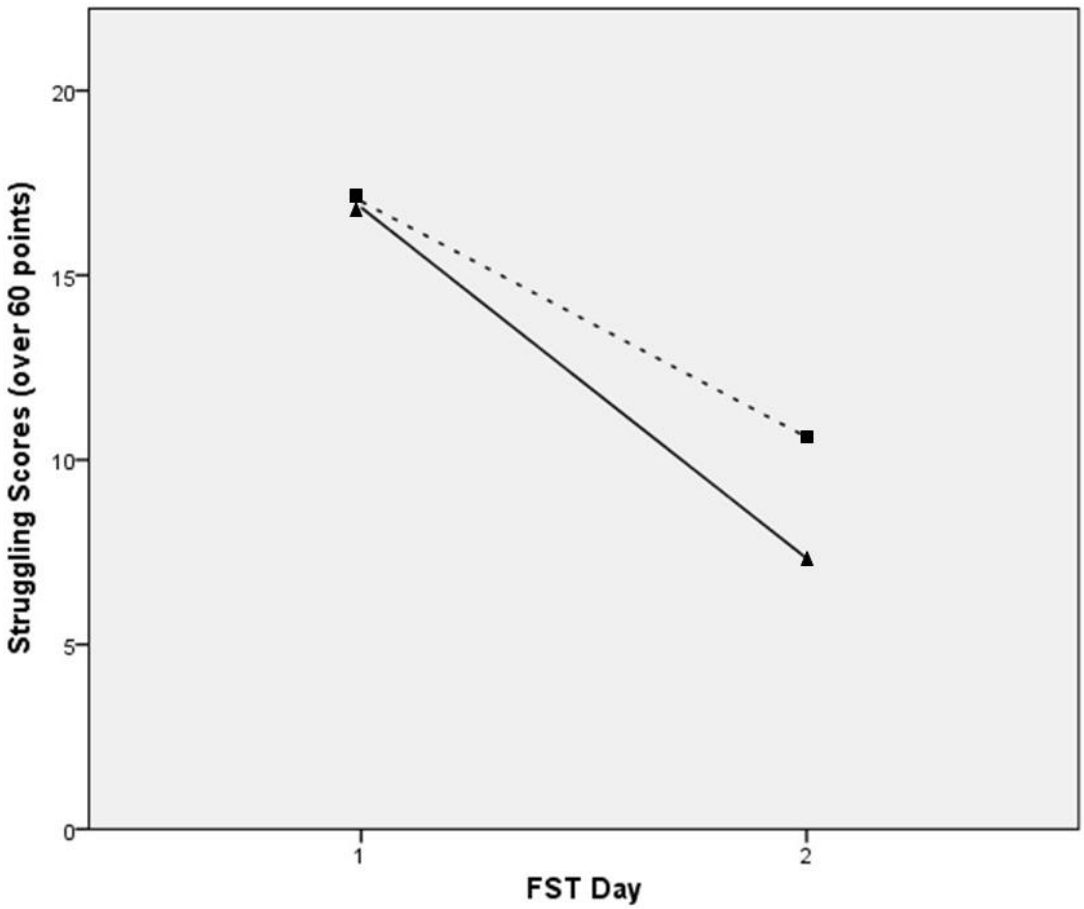


Fig. 10 Struggling change between 22-h and 24-h groups
Note: Straight line is for the 24-h control group and the dotted line is for the 22-h treatment group. There was no significant interaction between the groups and FST day, $F(1,23) = .747$, $p > .05$

3.4 Relationship between the FST and the OFT

Another important issue with this experiment was the relationship between two behavioral tests. To assess the relationship between the FST and the OFT, Spearman's correlation test was conducted. Correlations between FST immobility scores for both Day 1 and Day 2, OFT locomotor activity, OFT fecal boli counts, and OFT location preference was assessed (Table 2).

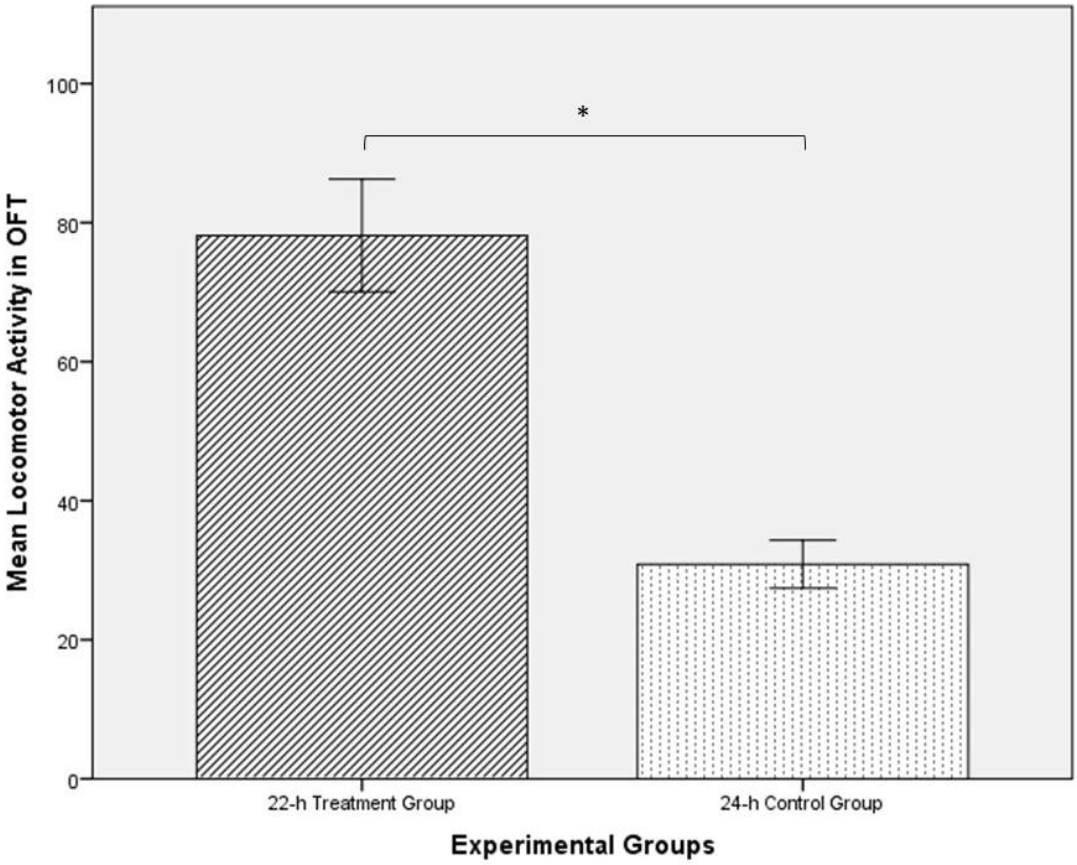


Fig. 11 Comparison of locomotor activity in the OFT
Note: The lined bar denotes 22-h treatment group and the dotted bar denotes 24-h control group. *There was a statistically significant difference between locomotor activity of 22-h and 24-h groups. The 22-h group was significantly more mobile during the OFT. Bars implies standard error of the mean (SEM)

### 3.4.1 FST immobility scores and locomotor activity in the OFT

The 22-h treatment group showed no correlation between FST immobility scores and locomotor activity in the OFT. However, while there was no significant correlation between FST Day 1 immobility scores and locomotor activity in the 24-h control group, there was a significant positive correlation between FST Day 2 immobility scores and locomotor activity ($r$ = .525, $p$ = .04; Table 2).

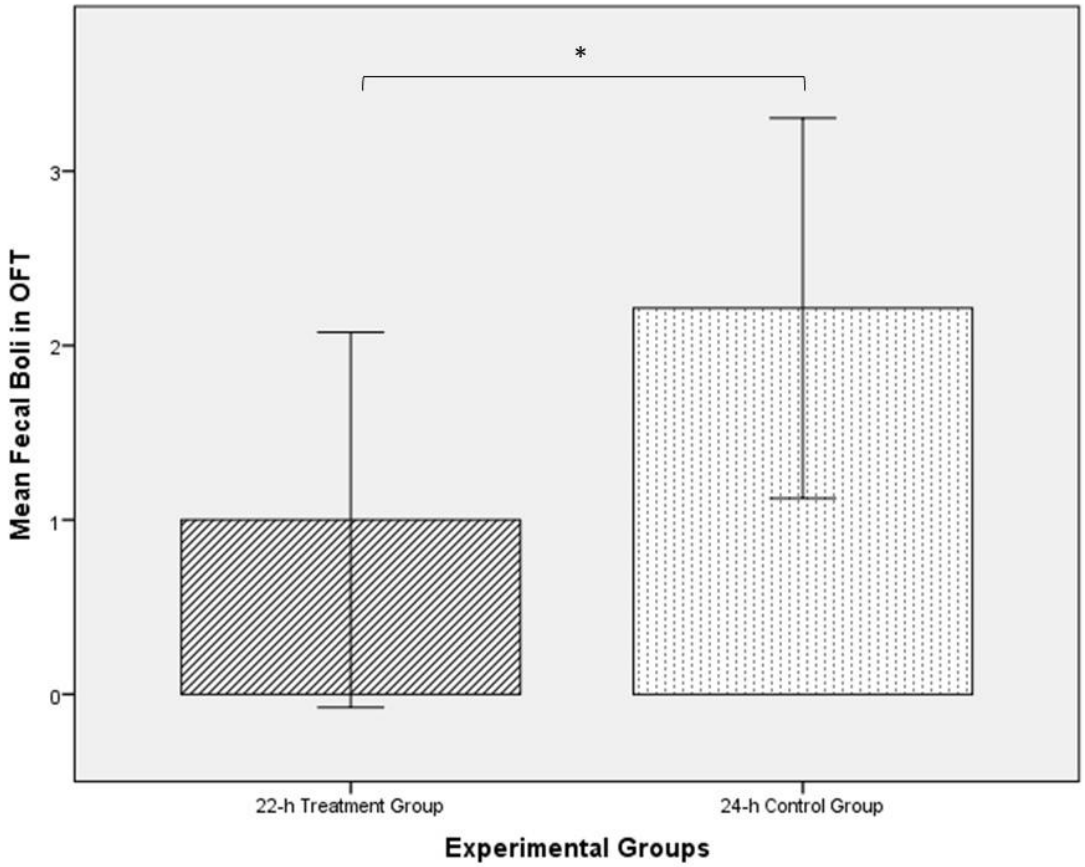


Fig. 12 Comparison of fecal boli counts in the OFT
Note: The lined bar denotes the 22-h treatment group and the dotted bar denotes the 24-h control group. Fecal boli counts were significantly different between the 22-h and 24-h groups. As an indication of anxiety, fecal boli counts were higher in the 24-h control group

### 3.4.2 FST immobility scores and fecal boli counts in the OFT

The 22-h treatment group showed no correlation between FST immobility scores and fecal boli counts in the OFT. However, while there was no significant correlation between FST Day 1 immobility scores and fecal boli counts in the 24-h control group, there was a significant positive correlation between FST Day 2 immobility scores and fecal boli counts ($r$ = .616, $p$ = .016; Table 2).

Table 2. Correlation Table

| | | | FST Day 1 (in secs) | FST Day 2 (in secs) | OFT Center Preference | OFT Locomotor Activity | Fecal Boli Count |
|---|---|---|---|---|---|---|---|
| 22-h Group | FST Day 1 (in secs) | Cor. Coef. | 1 | .039 | -.108 | -.251 | -.027 |
| | | p | . | .450 | .363 | .204 | .465 |
| | FST Day 2 (in secs) | Cor. Coef. | .039 | 1 | .512* | .138 | .014 |
| | | p | .450 | . | .037 | .327 | .482 |
| | OFT Center Preference | Cor. Coef. | -.108 | .512* | 1 | -.163 | .603* |
| | | p | .363 | .037 | . | .298 | .015 |
| | OFT Locomotor Activity | Cor. Coef. | -.251 | .138 | -.163 | 1 | -.358 |
| | | p | .204 | .327 | .298 | . | .115 |
| | Fecal Boli Count | Cor. Coef. | -.027 | .014 | .603* | -.358 | 1 |
| | | p | .465 | .482 | .015 | .115 | . |
| 24-h Group | FST Day 1 (in secs) | Cor. Coef. | 1 | .028 | -.696** | -.373 | .110 |
| | | p | . | .465 | .003 | .094 | .355 |
| | FST Day 2 (in secs) | Cor. Coef. | .028 | 1 | .204 | .525* | .616* |
| | | p | .465 | . | .263 | .040 | .016 |
| | OFT Center Preference | Cor. Coef. | -.696** | .204 | 1 | .309 | .041 |
| | | p | .003 | .263 | . | .141 | .445 |
| | OFT Locomotor Activity | Cor. Coef. | -.373 | .525* | .309 | 1 | .418 |
| | | p | .094 | .040 | .141 | . | .068 |
| | Fecal Boli Count | Cor. Coef. | .110 | .616* | .041 | .418 | 1 |
| | | p | .355 | .016 | .445 | .068 | . |

Correlation coefficient is Spearman's rho (ρ). * $p < .001$, ** $p < .05$

### 3.4.3 FST immobility scores and location preference in the OFT

The 22-h treatment group showed no correlation between FST Day 1 immobility scores and location preference in the OFT yet there was a significant positive correlation between FST Day 2 immobility scores and OFT center preference ($r$ = .512, $p$ = .037; Table 3). Contrarily, while there was a significant negative correlation between FST Day 1 immobility scores and center preference in the OFT in the 24-h control group ($r$ = -.696, $p$ = .015), there was a no significant correlation between FST Day 2 immobility scores and location preference (Table 2).

### 3.4.4 Fecal boli counts and location preference in the OFT

The 22-h treatment group showed a significant positive correlation between fecal boli counts and center preference in the OFT ($r$ = .603, $p$ = .015). However, in the 24-h control group there was a no significant correlation between fecal boli counts and location preference (Table 2).

### 3.4.5 Locomotor activity, fecal boli counts and location preference in the OFT

For both groups there were statistically significant correlations neither between locomotor activity and fecal boli counts, nor between locomotor activity and location preference in the OFT (Table 2).

# CHAPTER 4

# DISCUSSION

The aim of this study was to investigate the behavioral effects of a day shortened-to-22-hour. Because it is thought that the SCN has a limit in terms of conforming shortened days (Park et al., 2012), a relatively modest, 22-h-long-day cycle was chosen. In the present study, it was shown that a 22-h day cycle has significant effects on behavioral despair, anxiety and locomotor activity. While increasing locomotor activity, the 22-h day application had ameliorative effects in anxiety as well as behavioral despair.

## 4.1 Ameliorative effect of 22-h day

Other than shortening the day, underlying reason for the ameliorative effect of 22-h day might be the phase advances experienced by the 22-h animals. The 22-h group has chronically experienced phase advances during the experiment as compared to the 24-h control group. Also, the 24-h control group experienced a phase advance as humans do every day. This phase advance is the natural result of the free-running rhythm of the SCN which is a few minutes longer than 24-h (Czeisler et al. 1999).

Some antidepressants and anxiolytics (e.g., agomelatine, benzodiazepine) are known to cause phase advances in circadian rhythms (Turek & Losee-Olson, 1986; Wickland & Turek, 1991; Germain & Kupfer, 2008). Reciprocally, as shown in both rat and human studies, experiencing regular phase advance has an ameliorative effect on depressive symptoms (Wehr, Wirz-Justice, Goodwin, Duncan, & Gillin, 1979; Germain & Kupfer, 2008; Schulz et al., 2008; İyilikçi et al., 2009).

Light-box therapy is another example showing the antidepressant effect of phase advances. In fact, it is a common treatment for the symptoms of the SAD. Besides, the most effective improvement in mood occurs when patients diagnosed with SAD are exposed to “morning light”, which causes phase advances in patients’ CR (Lewy et al., 1988).

In this study, mentioned chronic manipulation might have led to an antidepressant effect and protected the rats in the 22-h group in the FST from behavioral despair. Additionally, there was a significant positive correlation between FST Day 2 and center preference in OFT. This finding shows that while 22-h day cycles decrease behavioral despair, they do not produce the same ameliorative effect on anxiety.

### 4.2 Anxiety-like behavior in 24-h group

Anxiety in rodents is commonly assessed using the Elevated Plus Maze (EPM) or the OFT. In the EPM, there are two open and two closed arms, and the time spent in the closed arms (dark ones) denotes anxiety-like behavior.

Although the EPM and the OFT are different tests in terms of their design/structure, the periphery preference in the OFT also relies on the innate urge of the anxious rodent (Seibenhener & Wooten, 2015) . Independent of darkness, periphery preference is related to thigmotaxis — the urge to move towards one direction while touching the walls of the maze. Thigmotaxis is a common indicator of anxiety in rodent models (Simon et al., 1994). In the present study, 22-h and 24-h groups did not differ in terms of their center or periphery preferences. As a result, there was no difference in anxiety-like behavior between 22-h and 24-h groups, which shows that experiencing 22-h days has no anxiolytic effect.

However, another measure in the current study, fecal boli counts, is also accepted as a powerful sign of anxiety in rodents (Hall, 1934; Ramos, 2008). Defecation is an action of the autonomic nervous system and it is an inevitable process in situations of fear, anxiety and strong excitement. In the present study, the 24-h control group displayed higher rates of defecation during the OFT. Even if the location preference did not differ between groups, this finding indicates that the 22-h days may actually have an anxiolytic effect. Accordingly, not only the depressive symptoms, but also anxiety-like behavior was triggered in the 24-h group and shortening the days to 22-h protected rats from both behavioral despair and anxiety.

The general expectation in OFT is that fecal boli counts and location preference mostly verifies each other (Ramos, 2008). For example, if fecal boli count is higher in an experimental group, it is expected to find that the group also spends more time in the periphery. In this study, location preference (center vs. periphery) check for anxiety failed to support the aforementioned findings.

Furthermore, the significant positive correlation between center preference and fecal boli counts in the 22-h group might be an indication of different types of anxiety. Because there is more than one stressful factor in the OFT (e.g. separation stress, agoraphobia, and so on; Gould, Dao, & Kovacsics, 2009), rats in the 22-h group might be affected by one of these factors while the other factors are not effective that much.

Additionally, there was a significant negative correlation between FST Day 1 immobility scores and OFT center preference, and significant positive correlation between FST Day 2 immobility and fecal boli counts of 24-h group, while there was no correlation in 22-h group. This reflects the expected comorbidity of high level of anxiety and behavioral despair.

### 4.3 Locomotor activity was lower in the depressive group

In the present study, shortening of the circadian period caused a substantial increase in locomotor activity, implying a reciprocal relationship between locomotor activity and the length of the period.

There was a significant positive correlation between FST Day 2 immobility and OFT locomotor activity score, showing that the less mobility an animal displays during the FST, it becomes more mobile in the OFT. This correlation implies that the difference in immobility scores in the FST (indicator of behavioral despair) between 22-h and 24-h groups does not originate from the increase in the locomotor activity. Instead, the mobility in the FST did not alter locomotor activity in the OFT.

It should be noted that significantly lower immobility in the open field is considered as anxiety-like behavior (Carola et al., 2002; Ramos, 2008). In the present experiment, the depressive group, that is the 24-h control animals, had lower locomotion in the OFT. This may be taken as further evidence that the antidepressant effect of 22-h days has remained during the OFT, which was run and completed 25±5 minutes after the FST.

### 4.4 General discussion

In the present study, rats showing higher behavioral despair and anxiety had lower locomotor activity scores in line with the literature (LaPerriere et al., 1994; Solberg, Horton, & Turek, 1999). As a result of 22-h days, locomotor activity was increased, and animals were protected from behavioral despair and anxiety.

In a different perspective, 22-h day might have caused an overall increase in locomotor activity as it was observed in the OFT and therefore caused low immobility of the 22-h group in the FST. However, the correlation analyses between

FST immobility scores and locomotor activity in the OFT rule out this possibility. First, there were no significant correlation between FST immobility scores and OFT locomotor activity in the 22-h group. Second, there was a positive correlation between FST Day 2 immobility and OFT locomotor activity scores in 24-h group. Which means even though they were immobile in the FST they were active during the OFT. However, they were showing still significantly less locomotor activity in the OFT and more immobility in FST Day 2 than 22-h treatment group.

In addition, the essential finding here is the significantly increased immobility of the 24-h group, and it should be noted that the difference in baseline mobility (in FST Day 1) of the 22-h and 24-h groups was not different. If there was an overall increase in locomotor activity of 22-h treatment group, we would also observe a significant difference in the habituation day of the FST (Day 1) between experimental groups.

The general idea of the FST is that a naïve rat performs lower mobility in the test day (Day 2) because of a learned helplessness-like behavior. It is an inescapable stress inducer to be in a deep, water-filled pool, which makes animals to either stop swimming (e.g. immobility) or trying to escape actively (e.g. swimming or struggling). If learned helplessness/behavioral despair remains at FST Day 2, a rat does not struggle or swim but rather shows high immobility during the test. If mobility score does not change in the test day (Day 2), this means that the animal does not experience behavioral despair.

In this study, the 22-h group was mostly swimming or struggling, in other words actively coping with the inescapable stress, during the FST while 24-h experiencing control animals stayed mostly immobile and showed less active coping. This difference implies once more that 22-h animals did not experience behavioral

despair and their lower immobility scores were not due to a general increase in locomotor activity.

It is important to note that changing the living space/cages of the animals for the study may have biased the results for both groups. However, unlike other period studies utilizing such manipulations lasting less than a month,(Einat et al., 2006; Flaisher-Grinberg, Gampetro, Kronfeld-Schor, & Einat, 2011; Park et al., 2012; Otsuka et al., 2014), in this experiment the manipulation lasted for 2 months, which gave the animals sufficient time for habituation.

Besides, unlike the SAD experiments, dark and light periods were kept equal in this experiment. Shortening 1 hour from light and 1 hour from dark phases of the day could be a more easy-to-adapt alteration.

It is known that being exposed to an inescapable shock, as in the FST, lengthens the circadian period (Stewart, Rosenwasser, Hauser, Volpicelli, & Adler, 1990). In this study, by shortening the period (keeping light and dark onsets equal) rats were protected from behavioral despair in the FST. For this reason, the present study indicates that there is a reciprocal relationship between period length and mood.

As a result, it can be concluded that shortening days to 22-h cycles increases locomotor activity of the rats and 22-h days protect rats from behavioral despair as well as certain types of anxiety.

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