The Interplay between the Circadian and Stress Systems: How does Early Environmental Impoverishment Impact Circadian Phenotype and Clock Gene Expression?

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Abstract

The Interplay between the Circadian and Stress-Systems

How does Early Environmental Impoverishment Impact Circadian Phenotype and Clock Gene Expression?

Miranda Benoit

The circadian system and the stress system each impact an organism’s ability to adapt to environmental challenges (Weibel, Maccari, Van Reeth, 2002). Within the field of circadian studies, much research has been conducted examining the way in which stressors affect clock gene regulation and circadian phenotype. Despite this, very few studies, if any, have looked at the role of early social and environmental impoverishment on circadian phenotype and clock gene expression. As a result, the present study explored whether there was a difference in circadian phenotype and clock gene expression between two groups of rats, one of which had experienced typical early social and physical enrichment, and the other that had been raised in a socially and physically impoverished environment. It was hypothesized that animals that had been raised in an impoverished environment would have a less robust circadian rhythm and be more prone to negative perturbations to both the circadian and stress systems following an acute stressor. Though there was a trend towards significant differences between treatment groups for wheel running behaviours in experiment 1, the only treatment effects found were those of PER1 expression in the amygdala and hippocampus. In experiment 2 a novelty-induced locomotor test was administered to see if the treatment had been effective, and there was a significant main effect of treatment condition. Furthermore, a significant difference between treatment conditions was seen again in the hippocampus. Beyond these treatment effects, there were multiple significant main effects of both sex and ZT for nearly all tests carried out in both experiments.
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How does Early Environmental Impoverishment Impact Circadian Phenotype and Clock Gene Expression?

Animals must have a way in which to regulate the hormones and cues within their body in order to interact with changes in their external environment. This leads to animals showing cyclical patterns of behaviour and physiological processes, which cycle roughly every 24 hours (Verwey, Dhir, & Amir, 2016). Altogether, these cycles are referred to as circadian rhythms. More specifically, these circadian rhythms are regulated by environmental cues such as light, activity status, and accessibility to food, allowing animals to adapt by developing patterns of behaviour that anticipate cyclical events (ex: availability of food around a certain time each day). In other words, the circadian system’s role is to match behaviour and bodily processes with relatively consistent environmental cues (Chousos, 2009). In mammals, these circadian rhythms are regulated in a hierarchical fashion, with the main regulatory clock being found within the suprachiasmatic nucleus (SCN) of the hypothalamus.

However, recent research has found that subordinate clocks are present in other areas of the brain and other parts of the body, including the adrenal glands. In relation to the adrenal circadian clock, adrenal glucocorticoids are of importance as they serve multiple purposes including helping to regulate the stress response (Oster et al., 2016). Whereas the circadian system is involved in helping animals to adapt to predictable, cyclical stimuli, the stress system is involved in adaptation to unpredictable stimuli. Furthermore, glucocorticoids follow a strong circadian rhythm, and changes to this rhythm can lead to desynchronization between the master clocks and peripheral and extra-SCN clocks, which has the potential to lead to adverse health and behavioural effects (Oster et al., 2016; Segall & Amir, 2010; Segall, Walker, Stewart, & Amir, 2006).
Researchers have been interested in the effects of external stressors on the body for decades (Farrell, Simpson, Carlson, Englund, & Sung, 2016; Marten & Landers, 1970). Stress, in this instance, is defined as the body’s physiological and behavioural response to stimuli, called a stressor, that results in feelings of agitation, strain, pressure, etc. (APA Dictionary). Moreover, high levels of stress can result in a myriad of negative physical health outcomes (Oster et al., 2016). For instance, rats who experience chronic stress have been shown to exhibit increased anxiety-like behaviours, have increased corticosterone levels, and there is some evidence that DNA methylation is affected, leading to long lasting alterations in gene expression (Niknazar, Nahavandi, Peyvandi, Peyvandi, Akhtari, & Karimi, 2016).

Research with both humans and rodents has shown that the circadian rhythm of cortisol cannot adapt quickly to changes brought about by external stressors such as changes in the light-dark cycle, feeding cycles, and jet lag or shift work (Oster et al., 2016). This inability to quickly adapt can lead to desynchronization between the SCN and peripheral adrenal clock, causing either overproduction or deficiency in levels of glucocorticoids. This, in turn, can increase the risk of experiencing some pathologies. Disturbances to the circadian system are often discussed in terms of the sleep-wake cycle and, therefore, sleep disorders. However, such disturbances to the glucocorticoid rhythm have also been shown to impact memory and learning, and to increase the risk of other psychopathologies (Bartlang, Oster, & Helfrich-Förster, 2015; Oster et al., 2016).

**Circadian Dysregulation and Psychopathology**

It has been shown that stress induced pathologies are often comorbid with disturbed circadian rhythms (Bartlang, Oster, & Helfrich-Förster, 2015). One study found that there appears to be a link between circadian dysregulation and increased risk of suicidality in patients
who were diagnosed with PTSD based on the DSM-5 criteria. In this study, 65 participants were asked to complete self-report measures of rhythmicity (sensitivity to seasons, changes in weather, etc.) and vegetative functions (sleep schedule, eating habits, sexual functions, and menstrual cycle). This method was used as it had been shown in the past to be associated with risk of suicidality in patients with schizophrenia, manic-depressive disorder, borderline personality disorder, and panic disorder (Dell’Osso et al., 2014). Results showed that there was a significant association between self-report measures of circadian dysregulation, and increased risk of suicidality. More specifically, all sub-sections (rhythmicity, sleep, appetite and weight, sexual function, and physical symptoms) of the self-report measure were associated with greater likelihood of suicide ideation. However, only dysregulation in appetite and weight were found to be associated with increased risk of suicide attempts (Dell’Osso et al., 2014).

It is also relevant to note that disruptions of the hypothalamic-pituitary-adrenal (HPA) axis are often seen in individuals with stress-related disorders (Christiansen et al., 2012). The HPA axis, consisting of the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary gland, and the adrenal gland. This is of importance as the circadian rhythm of glucocorticoids is controlled not only from the peripheral clock within the adrenal tissue, but also by the HPA axis through means of corticotropin-releasing factor. This plays a vital role in the stress response by leading to the downstream production of glucocorticoids from the adrenal cortex (Smith et al., 2006). In healthy individuals, glucocorticoid levels show a circadian rhythm, whereby levels peak around waking time, and drop to their lowest around late afternoon (although subjective, depending on whether the individual is a diurnal or nocturnal animal) (Christiansen et al., 2012). However, it is common in individuals with stress and anxiety related
psychopathologies to have circadian rhythms of glucocorticoids that deviate from this norm, with peak elevations occurring at the wrong time of day (Christiansen et al., 2012).

Furthermore, previous research has demonstrated that there are compelling time-related interactions between the stress and circadian systems. For example, mice will show varied response to an acute stressor as a function of time of day. Particularly, male mice who were exposed to a psychosocial stress (social defeat) showed changes in their activity levels that were dependent on which social defeat paradigm they were exposed to (Bartlang, Oster, & Helfrich-Förster, 2015). There were three groups of wild-type mice: those who experienced social defeat during the dark/active phase (SDD), those who experienced it during the light/inactive phase (SDL) and the control group who did not experience social defeat. The authors hypothesized that the control group would show a typical pattern of activity, with 3 large spurts of activity. The first would be a short one that followed lights out, the second would be within the first half of the night, and third would occur soon before the lights were turned back on (Bartlang, Oster, & Helfrich-Förster, 2015).

However, it was expected that the activity patterns of the SDD and SDL mice would differ. Results of the study supported this hypothesis. The SDD mice showed increased activity during the first half of the dark phase (ZT12 – ZT18), during which they were exposed to social defeat. This was then followed by a decrease in locomotor activity for the remainder of the dark/active phase (ZT18-Z0). On the other hand, SDL mice showed increased activity during the first half of the light/inactive phase (ZT0-ZT6) in response to the stressor, followed by significantly decreased behaviour in the rest of the light/inactive phase and throughout the entire dark/active phase (ZT6-ZT0). These same results were seen with Per1/2 mutant mice as well,
suggesting that they are a direct response to the stressor itself, as opposed to a dysregulation of the internal clock system (Bartlang, Oster, & Helfrich-Förster, 2015).

Additionally, research suggests that the impact of stress will vary depending not only on the time of day for an organism, but also on the point of the lifespan of that organism (Farrell, Simpson, Carlson, Englund, & Sung, 2017). One study that supports this statement was conducted by Jauregui Huerta et al. (2015), who exposed 4 different groups of adult rats to chronic variable stress. These 4 groups were: a control group (standard laboratory conditions), an early-life stress group (exposed to environmental noise from post-natal days 21-35), a group who had previously been exposed to the early life stress protocol and were now being exposed to a chronic variable stress as adults, and the last group who were exposed to a chronic variable stress only as adults.

Results of this study showed that chronic variable stress overall lead to negative changes in hippocampal proliferation and special learning in rats (Jauregui Huerta et al., 2015). Notably, it was also shown that these negative effects may be lessened in rats who were exposed to early life stress in the form of environmental noise. This is important as it provides further evidence that early life experiences can lead to long lasting changes in the brain and behaviour.

**Early Life Experiences and Resilience to Later Life Stressors**

Cohen et al., (2006) defined early life stress as exposure to moderate and/or severe external stressors during an individual’s childhood or adolescent years. These stressors can occur prenatally (ex: maternal drug use), postnatally or during adolescence (ex: poverty, family conflict). Ample research has supported the hypothesis that early life experiences can have an impact on the way in which an organism reacts to stress later in development. Those who have experienced early life stress tend to have higher prevalence rates for various mental disorders
including anxiety, depression, and PTSD, as well as sleep disorders (Carr et al., 2013). It has been shown that rats who experience early enrichment showed less anxiety-like behaviours as adults as compared to rats reared in a typical or impoverished environment. One study examined this by rearing 4 different groups of rats: a control group reared in standard laboratory conditions, and three groups who experienced varying lengths of time in early enrichment (EE) (Baldini et al., 2013).

Of the three EE groups, the first had pregnant mothers placed in an enriched environment one week before the birth of the pups. After their birth, the pups stayed in the enriched environment for 12 days. The second and third groups differed only in that they stayed in the enriched environment for 45 days and 60 days after birth, respectively. Results of this study showed that when required to run on an elevated plus maze as adults, the rats who had experienced early enrichment spent significantly more time in the open arms of the maze and entered these open arms more frequently. This was in comparison to the control group. This is taken as a sign of decreased anxiety-like behaviours.

Interestingly, there was no significant difference in the decrease of anxiety-like behaviours between the rats who had experienced either 12, 45, or 60 days of early enrichment (Baldini et al., 2013). This may suggest that prenatal enrichment was enough to alter the pup’s subsequent anxiety response as adults, or perhaps somewhere under 12 days was the minimum amount of time to see long-term effects of early enrichment. Given these findings, it is probable that early enrichment leads to changes within the circadian system and, therefore, in brain regions that play a role in its regulation.
The Role of Clock Genes in Stress and Anxiety-like Behaviours

Considering that early life experiences can impact an individual’s capacity to deal with stressors much later in life, genetic and physiological changes must be occurring during these early life experiences. Following an acute stressor, there is increased interplay between mineralocorticoid and glucocorticoid receptors (MR and GR), and glucocorticoid response elements (GREs) regulating the expression of glucocorticoid dependant genes. While an acute release of glucocorticoids experienced after a stressor is advantageous, chronic stress or trauma can increase the risk of stress-related psychopathologies (Mifsud & Reul, 2016).

Within the hippocampus it has been shown that, following the use of a forced swim test as an acute stressor, the MR’s and GR’s bind transiently to GREs within certain glucocorticoid target genes, including per1, thereby upregulating gene expression (Mifsud & Reul, 2016). This upregulation of gene expression, if occurring during an early developmental period, could lead to significant behavioural changes for the animal as an adult. More specifically, the Per1 gene contains a glucocorticoid response element which, upon exposure to corticosterone, regulates gene transcription. Forty-five minutes to one hour after the onset of an acute stressor, increased PER1 protein expression has been found paraventricular nucleus and dorsomedial hypothalamus of rats (Al-Safadi et al., 2014).

The clock protein PER1 is of importance in relation to glucocorticoids as it has been shown that stressors that lead to the release of glucocorticoids also impact the expression of PER1 in extra-SCN and peripheral clocks, but not in the SCN itself (Al-Safadi, Branchaud, Rutherford, & Amir, 2015; Takahashi et al., 2001; Yamamoto et al., 2005). One study found that, following chronic social stress, mice showed significantly lowered levels of MR expression, which was likely the result of epigenetic changes in gene expression. MR’s have been shown to
be critically involved in maintaining homeostasis; they regulate ion transportation and impact muscle cell’s membrane excitability, among other functions (Gomez-Sanchez & Gomez-Sanchez, 2014).

Moreover, if the mice underwent the social stress during adolescence, this led to behavioral changes that persisted into adulthood. Based on these findings, researchers concluded that if the animals were exposed to chronic stressors during early development, there were long-lasting effects on both the physiology and behaviours of said animals. These effects appear to increase the animals’ vulnerability to stress-related disorders later in development (Sterlemann et al., 2008).

**Sex Differences: Clock Genes, Early Impoverishment, and the Estrous Cycle**

An individual’s behavioural and physiological response to stress is partially dependent upon their sex. One reason for this may be that the SCN communicates with extra-SCN clocks through the release of corticosterone (Chun et al., 2018). Given that female rats show higher levels of stress-induced plasma corticosterone than males this may be a partial explanation as to why females show higher prevalence rates of mood disorders than males (Babb, Masini, Day, & Campeau, 2014). Additionally, these sex differences are largely a result of the differential effects of gonadal hormones on the HPA axis.

The HPA and HPG (hypothalamic–pituitary–gonadal) axes communicate with one another so that the HPA axis can act in such a way as to make an organism’s response to environmental stressors fitting of their current reproductive status. As an example, female rats having differing responses to stress across the estrous cycle, as well as having a more robust HPA response in general as compared to male rats. This is due to circulating levels of estradiol
which elevates stress hormone (ex: corticosterone) levels during and after stressors (Oyola & Handa, 2017).

In female rats, there are cyclical variations in both estradiol and progesterone which, together, characterize the estrous cycle (Oyola & Handa, 2017). There are four stages of the cycle: proestrus, estrus, metestrus, and diestrus. These stages can be discerned from one another by changes in patterns of circulating hormones, as well as by conducting vaginal cytology (Cora, Kooistra, & Travlos, 2015). Progesterone peaks during proestrus, and vaginal cytology yields predominantly nucleated cornified cells. This correlates with higher stress hormone levels in females during proestrus as compared to estrus, when progesterone and estradiol levels are lower and cytology yields predominantly cornified epithelial cells (Cora, Kooistra, & Travlos, 2015; Viau & Meaney, 1991.) Due to these hormonal differences, anxiety-like behaviour in response to stressors is higher during proestrus (Lovick, 2012). These hormonal and behavioural differences are important to note, as they highlight the importance of monitoring estrous phase when using intact females in experiments.

Males do not show the same cyclical fluctuations in gonadal hormones that are seen in females as a result of the estrous cycle. However, males experience a change in the HPA axis’ response to stress during puberty, with testosterone levels increasing shortly beforehand, and then remaining rather stable (Oyola & Handa, 2017). Interestingly, despite similarities in both corticosterone and adrenocorticotropic hormone levels following an acute stressor between pre-pubertal and adult males, pre-pubertal males take approximately 30 minutes more to return to baseline levels than adult males (Romeo et al., 2006; Romeo, Lee, & McEwen, 2004). Altogether, research has shown that the increase in testosterone levels that occurs during the
pubertal period in males is required for healthy adulthood responses of the HPA axis (Oyola & Handa, 2017).

Research has also shown that HPA axis activity can be altered in a sex dependent manner during pre-pubescence. Studies using maternal separation in combination with either food restriction or restraint stress have shown that there are sex-dependent and enduring alterations in HPA axis activity. Early life stress leads to changes in the estrous cycle and HPA axis feedback of female rats, whereas no significant changes in HPA axis feedback were seen in male rats (Mourlon et al., 2011). Despite ample findings that suggest that the estrous cycle impacts stress-induced corticosterone, few lab studies have included female rats; this is likely due to the added difficulty of accounting for fluctuations in gonadal hormones in female rats.

Although gonadal hormones can help to beneficially regulate an organism’s stress response, unwanted communication between the HPG and HPA axis can result in irregularities of the stress response. In humans, such irregularities are frequently seen as symptoms of various psychopathologies (Oyola & Handa, 2017).

Conclusion

Even with the extensive number of publications pertaining to early impoverishment and stress, few studies include discussions of how the circadian system is affected. Understanding the ways in which the circadian and stress systems are interrelated with one another could lead to a more comprehensive accumulation of research concerning the way in which humans react to stress. Moreover, a better understanding of the interplay between these two systems could lead to more effective and targeted treatments for sleep irregularities that are so often present for those with stress and anxiety-type disorders.
Although this study focussed on wild-type rats, as opposed to a clinical population, it will have significance in guiding future research in the field of stress and circadian disorders. The bulk of previous research includes samples from clinical populations. Though important, this previous research does not help further the understanding of the ways in which the average individual will be impacted by stressful events, nor does it help researchers determine which factors lead to these individuals becoming resilient to said stressors. Acquiring data that allows researchers to understand the typical way in which animals are impacted by stress will also grant researchers the ability to notice changes between typically developing and clinical populations with more ease.

Based on the above studies, and the finding that early life experiences can shape health and behaviour later in life, this study aimed to achieve two major goals. Firstly, to clarify whether typical early social and environmental enrichment, versus early impoverishment, had an influence on overall circadian phenotype, the ability to effectively re-entrain to various light cycles, and of resilience to later life stressors. Of particular interest was each group’s levels of anxiety-like behaviours in response to acute, artificial stressors following early enrichment or impoverishment. The second was to determine if early enrichment influences the functioning of the circadian master clock, the SCN (suprachiasmatic nucleus), or other important areas such as the amygdala and hippocampus by impacting clock protein expression. The bulk of previous research has shown that stress has an impact on the circadian phenotype and clock protein expression (Al-Safadi et al., 2014; Al-Safadi et al., 2015; Koch, Leinweber, Drengberg, Blaum, & Oster, 2017; Logan, Edgar, Gilman, Hoffman, Zhu, & McClung, 2015). However, there is little research within this area on the ways in which early enrichment may mitigate these
circadian disruptions that are caused by external stressors.

The present study was separated into two experiments, each of which aimed to contribute further to research regarding the effects of stress on the circadian system; specifically, by investigating the way in which typical early social and environmental enrichment versus early impoverishment can alter the impacts that stress has on the circadian system. This was accomplished by rearing rat pups in either a typical environment (control) or an impoverished environment (treatment). The first study was conducted with all male rats. The reasoning behind this decision was that there are sex differences in response to social isolation in rats, with female rats being more negatively impacted than males (Ahern, Goodell, Adams, & Bland, 2016; Weintraub, Singaravelu, & Bhatnagar, 2010)

Following two weeks in either a control or impoverished rearing environment, the rats were moved to single-housing, sound attenuated cages, equipped with running wheels. At this point, rats were given 1 week to entrain to a 12:12 Light/Dark (LD) cycle. Rats were then exposed to a -6 hour phase advance for three weeks, followed by a +10 hour phase delay for another three weeks. After this point, the LD cycles ended, and rats were exposed to 15 days in constant darkness (DD), and then remained in constant light (LL) until the completion of the experiment. During the constant light, rats underwent behavioural measures of stress and anxiety, and samples of tail blood were collected to measure corticosterone levels. Finally, the rats were sacrificed and levels of PER1 were examined using immunohistochemistry. The light cycles of the second experiment differed in that the rats were placed back in a 12h-12h LD cycle with lights on from 08:00-20:00 following constant light, and underwent behavioural measures of stress in LD, and not LL, lighting.
Methods

Experiment 1

Animals and Housing

All experiments were carried out in accordance with the Concordia University Ethics Committee’s guidelines for the care of laboratory animals. Twelve male Wistar rats were obtained from Charles River Laboratory, St-Constant, QC, one day post weaning. The rats were housed in the Animal Care Facility of Concordia University for two weeks. Six rats were randomly assigned to the control group, where they were group-housed with three rats per cage. They had shredded paper for nesting material, as well as nyla-bones and plastic tunnels in their cages. The other six rats were assigned to the treatment group, an impoverished environmental paradigm. These rats were individually caged in opaque plastic cages, with no extra physical enrichment.

After two weeks in either the control or impoverished environment, at which point the rats all weighed approximately 200 grams, the animals were moved and housed individually in a circadian laboratory. Rats were individually housed in sound-attenuated, ventilated chambers. Each chamber was equipped with a computer-controlled light system (ie: VitalView). Running wheel rotations were recorded with VitalView and analyzed and plotted into actograms using ActiView. Food and water were provided ad libitum.

Experimental Procedure

Rats were kept in a 12:12 LD cycle with lights on at 08:00 h for one week, to allow for entrainment. Following this, the rats experienced a phase advance of 6 hours for 21 days (12:12 LD with lights on at 02:00h), followed by a phase delay of 10 hours for 21 days (12:12 LD with lights on at 12:00 h). Upon completion of a phase advance and phase delay, the rats were placed...
in constant darkness (DD) for 15 days. The rats were then given 10 days to re-entrain to a typical 12:12 LD cycle, with lights on at 08:00 h, after which they were placed in constant light (LL) for 6 weeks. At the end of the 6 weeks of constant light, the rats underwent a forced swim test and had blood collected from their tail veins to measure corticosterone levels.

**Forced Swim Test**

Rats were transported to the testing room 30 minutes before the onset of the forced swim test to allow for them to acclimate to the testing environment. Two glass cylinders, placed next to one another with a plain white divider between them, were filled with tap water at a temperature of 23 ± 1°C. Each rat was placed in a water filled cylinder for 5 minutes, and their behaviour was video recorded. Following the 5-minute test, animals were dried off and placed in clean, dry cages with absorbent pads. After 30 minutes in the drying cage, each rat underwent a tail clipping procedure to collect blood for corticosterone measures. Half of the rats were tested at ZT1 during the inactive phase while the other half were tested at ZT13 during the active phase.

**Tail Vein Blood Collection**

Rats were restrained under a box, allowing easy handling of the animals during the procedure. The animals tail remained outside of the box, allowing access for a small incision to be made at the tip of the tail. This allowed for the collection of blood into a heparinized tube. The blood was immediately transferred into an Eppendorf tube and placed on ice and the incision was cleaned and covered with FastKlot. Once all blood samples were collected, they were centrifuged at 10,000RPM for 10 minutes, at 4°C. After being centrifuged, the serum was collected using a pipette and transferred to a separate Eppendorf tube. This serum was then stored at -80°C until an ELISA was run according to the manufacturer’s instructions (Corticosterone ELISA Kit 900-097; Enzo Life Sciences, Inc., Farmingdale, NY, USA).
Perfusion and Tissue Preparation

Rats were anesthetized using an intraperitoneal injection of euthanol. The rats who underwent the forced swim test at ZT1 were sacrificed and perfused 24 hours later, also at ZT1. Rats who underwent the forced swim test at ZT13 were perfused 24 hours later, at ZT13. They were perfused with saline (0.9% NaCl, 300mL) and finally with paraformaldehyde (4% in a 1M phosphate buffer, 300mL). Brains were kept in paraformaldehyde for 24 hours. Brains were then sliced into 30 μm thick coronal slices using a vibratome (Vibratome, St-Louis, MO). The slices were rinsed in TBS (3 washes, 10 minutes each) to remove any remaining paraformaldehyde before being immersed in Watson’s Cryoprotectant for storage at -20ºC.

Immunohistochemistry

PER1

Immunohistochemistry was performed using established protocols (Al-Safadi et al., 2014; Amir, Lamont, Robinson, & Stewart, 2004). Brain slices were moved from Watson’s Cryoprotectant into a cold 0.9% Trizma buffered saline (TBS) and rinsed (6 rinses, 10 minutes each). Using a fine-bristled brush, slices were transferred to a quenching solution (3% H2O2 in TBS) and left for 30 minutes at room temperature before being moved to a TBS rinse (3x10 minutes). Sections were then moved into a pre-blocking solution (0.3% Triton TBS, 5% milk powder, 7% normal goat serum in TBS) for 1 hour at 4ºC. Immediately after, slices were moved into the primary solution (1:10 dilution of Novus Biologicals rabbit anti-Per1 to TBS, in pre-blocking solution) and placed on a rotator at 4ºC for 48 hours. The brain slices were then rinsed with cold TBS (3x10 minutes), after which they were placed into the secondary solution (biotinylated anti-rabbit IgG in pre-blocking solution) and for 1 hour on a rotator at 4ºC. Upon completion of the secondary incubation, the slices were again rinsed in cold TBS (3x10 minutes)
and then moved into an avidin/biotinylated peroxidase (Vectastain Elite ABC Kit; Vector Labs) tertiary solution and placed back onto the rotator at 4°C for 2 hours. Following the tertiary phase, the slices were rinsed with cold TBS (3x10 minutes), followed by one 50mM Tris -HCl rinse (10 minutes). Immediately following this, slices were placed in a DAB solution for 10 minutes at room temperature, and then moved into a NiCl₂ solution for 10 minutes at room temperature. Following this, the slices were rinsed again in cold TBS (3x10 minutes). The following day they were mounted onto gel-coated slides. Once the slices had dried onto the slides they were dehydrated using alcohol baths of increasing concentrations. Following this, slides were immersed in Citrisolv (Fischer Scientific, Houston, TX) for at least 30 minutes to remove any contaminants. Finally, the slides were coverslipped with Permount (Fischer Scientific).

**Experiment 2**

**Animals and Housing**

Twelve male Wistar rats and twelve female Wistar rats were obtained from Charles River Laboratory, St-Constant, QC, one day post weaning. The rats were housed in the Animal Care Facility of Concordia University for two weeks. Twelve rats (six males, six females) were randomly assigned to the control group, where they were group-housed with three rats per cage. They had shredded paper for nesting material, as well as nyla-bones and plastic tunnels in their cages. The other twelve rats (six males, six females) were assigned to an impoverished environmental paradigm. These rats were individually caged in opaque plastic cages, with no extra physical enrichment. Food and water were provided ad libitum.

**Experimental Procedure**

Rats were kept in a 12:12 LD cycle with lights on at 08:00 h for one week, to allow for entrainment. Following this, the rats experienced a phase advance of 6 hours for 21 days (12:12
LD with lights on at 02:00h), followed by a phase delay of 10 hours for 21 days (12:12 LD with lights on at 12:00 h). Upon completion of a phase advance and phase delay, the rats were placed in constant darkness (DD) for 15 days. The rats were then given 10 days to re-entrain to a typical 12:12 LD cycle, with lights on at 08:00 h, after which they were placed in constant light (LL) for 4 weeks. At the end of the 4 weeks of constant light, the rats were given 10 days to re-entrain to a typical 12:12LD cycle. Following this final re-entrainment, the rats underwent a forced swim test and had blood collected from their tail veins to measure corticosterone levels.

**Novelty-Induced Locomotor Test**

Rats were transported to the testing room 30 minutes before the onset of the test to allow for them to acclimate to the testing environment. Locomotor activity was recorded in one of four locomotor chambers. Each of the four chambers was a clear acrylic chamber measuring 26x26 inch, sitting inside a TruScan activity monitor. Each chamber was equipped with an infrared emitter. Each time the rat broke the infrared beam, information was recorded using TruScan 2.01 on a personal computer. Activity was measured in 5-minute bins, for a total testing time of 30 minutes. Fluorescent lights in the ceiling, as well as lamps were used to illuminate the testing room.

**Vaginal Lavage**

A single male rat was place in the same room as the female rats for approximately 2 weeks before starting the vaginal lavages. Approximately 0.1-0.2 mL of phosphate-buffered saline was drawn into a pipette tip. The tip was then placed on the exterior of the vaginal orifice with, at most, 1-2 mm being inserted in the vaginal orifice. The saline was then flushed into the vagina 2-3 times, or until the fluid appeared cloudy. After the lavage, the fluid was placed in a small, thin layer upon a glass slide. After air drying, the slides were stained by being immersed
in cresyl violet for 1 minute, followed by 2 one-minute baths in distilled water. Slides were then left to air dry once again and, once dry were coverslipped using glycerol.

Forced Swim Test Procedure

The same forced swim test protocol was used in both Experiment 1 and Experiment 2, except for the addition of a plexiglass extender added to the cylinders in experiment 2. This allowed the cylinders to hold more water, ensuring the larger rats were not able to easily touch the bottom of the cylinders with their tails.

Tail Vein Blood Collection

The same blood collection method was used as in Experiment 1.

Perfusions and Tissue Preparation

The same perfusion and tissue preparation protocols were used as in Experiment 1.

Immunohistochemistry

PER1

The same immunohistochemistry protocol was used as in Experiment 1.
Data Analysis

Experiment 1

Behaviour

Double-plotted actograms were generated using VitalView. Actograms were visually analyzed using ActiView to establish the rats’ entrainment to multiple 12:12 LD light cycles as well as various other circadian parameters, as previously described (Verwey, Robinson, & Amir, 2013).

Forced Swim Test

Four forced swim test behaviours were analyzed (climbing, swimming, immobility, and latency to immobility) for the 5-minute time period that the rats were in the water, as previously described (Porsolt, Brossard, Hautbois, & Roux, 2011; Yankelevitch-Yahav, Franko, Huly, & Doron, 2015).

These four behaviours were examined using a t-test with treatment condition (control vs impoverished) as the independent variables, where the alpha level was set at 0.05.

Serum Corticosterone Levels

An ELISA was run according to the manufacturer’s instructions (Corticosterone ELISA Kit 900-097; Enzo Life Sciences, Inc., Farmingdale, NY, USA). Every sample was assayed in duplicate; values were gathered by averaging the values between wells that contained the same sample.

A mixed-ANOVA with treatment condition (control vs. impoverished) as the between-subject factor and timing or corticosterone collection (baseline, post-stressor, and pre-perfusion) as the within-subject factor was run where the alpha level was set at 0.05. Any significant results
were further analyzed using post-hoc tests to determine where the differences were between the factors.

**Brain**

Five brain areas (SCN, bed nucleus of the stria terminalis (BNST), amygdala, hippocampus, and striatum) were examined under a light microscope and images were captured using a Sony XC-77 video camera (Sony, Tokyo, Japan), a Scion LG-3 frame grabber (Scion Corporation, Frederick, MD, USA) and image SXM software (v1.6, S D Barrett; http://www.ImageSXM.org.uk). Bilateral images of each brain area were photographed within a 400x400µm template at 20x magnification. Cells immunopositive for PER1 were counted using ImageJ software using the captured images. For analysis, the mean number of PER1 immunoreactive cells per region was calculated for each animal from the counts of six images showing the highest number of labeled nuclei as previously described (Al-Safadi et al., 2014).

These five areas were examined using a 2-way ANOVA with treatment condition (control vs impoverished) and sex (male vs female) as independent variables, where the alpha level was set at 0.05. Any significant results were further analyzed using post-hoc tests to determine where the differences were between the factors.

**Experiment 2**

**Behaviour**

The same behavioural data analysis protocols were used in both Experiment 1 and Experiment 2.

**Forced Swim Test**

The same four behaviours as described in experiment 1 were analyzed. These four behaviours were examined using a 2-way ANOVA with treatment condition (control vs
impoverished) and sex (male vs female) as independent variables, where the alpha level was set at 0.05. Any significant results were further analyzed using post-hoc tests to determine where the differences were between the factors.

**Brain**

The same brain imaging and analysis protocols were used in both Experiment 1 and Experiment 2.

**Estrous Cycle**

Each female underwent multiple vaginal lavages, which were then imaged at 10x magnification to identify cell density and type and, therefore, current phase of the estrous cycle. Samples were taken on consecutive days so that images could be compared to both the previous and following days images, allowing a more accurate identification of estrous phase.

**Novelty-induced Locomotor Activity**

At 5 months of age, locomotor activity in response to a novel environment was measured for 30 minutes during the light phase. Each rat was placed into a locomotor testing chamber and total distance travelled during the testing session was recorded. To ascertain activity levels, rats were placed in clear acrylic chambers measuring 26x26 inch. Total distance traveled was measured in centimetres. Novelty-induced locomotor activity (total distance travelled for one 30-minute session in the locomotor chamber) was analyzed by a 2-way (sex × treatment condition) ANOVA. Any significant results were further analyzed using post-hoc tests to determine where the differences were between the factors.
Results

Experiment 1

Behaviour

Rats were entrained to a 12:12 LD schedule for one week before beginning to analyze changes in their wheel running activity in various light cycles. Proper entrainment was defined as wheel running behaviour that began at the onset of the dark phase of the light cycle. Furthermore, wheel running behaviour must have occurred predominantly within this dark phase.

All animals were entrained by the end of the week, with a minimum of 80% of wheel running behaviour occurring during the dark phase, and less than 20% occurring during the light phase. Control group rats showed 82.19-93.06 percent (M = 88.59, SEM = 1.75) of their wheel running behaviour during the dark phase and impoverished rats showed 83.44-97.52 percent (M = 94.16, SEM = 2.23) of their wheel running behaviour during the dark phase. The differences were not statistically different (p = 0.07) (See figure 1, from day 0 to day 10).

Constant Darkness

Animals were placed in constant darkness for 15 days with constant access to a running wheel. All animals displayed typical free-running rhythms in constant darkness, as is shown by the diagonal pattern of delay of daily activity onset. The free-running rhythm in constant darkness is marginally longer than 24 hours. Control group rats had tau values between 24.29-24.67 (M = 24.50, SEM = 0.05) and impoverished group rats had tau values between 24.41-24.74 (M = 24.54, SEM = 0.05). These differences were not statistically significant (p = 0.63). Both the control and impoverished rats showed daily cycles of locomotor activity with approximately 12 hours of activity followed by 12 hours of rest. (See figures 1, from day 55 to day 70).
Figure 1. Representative double-plotted actograms of rat wheel-running behaviour from one impoverished (left) and one control (right) rat. The bar (top) indicates the ZT of the day. The y axis (left) indicates the number of days during the experiment. Rats were first entrained to a 12:12 LD (8-20) cycle for 7 days and then the LD cycle was advanced by 6 hours for 21 days. Next, the light cycle was delayed 10 hours for 21 days. Then the animals were placed in constant darkness for 15 days and, afterwards, re-entrained to 12:12 LD (8-20) for 10 days. After 10 days in the 12:12 LD (8-20) cycle, the rats were placed in constant light for 42 days, which is shown on figure 2. Grey areas indicate dark periods. Vertical black marks indicate periods of activity of a minimum of 10 wheel-revolutions per 10-minute bin.
**Constant Light**

After 10 days in a 12:12 LD (8-20) cycle animals were placed in constant light for 42 days with constant access to running wheels. Qp values and days to arrhythmicity were examined. Qp values indicate the strength of periodicity for a specific period length. The control ($M = 433.35$, $SEM = 68.49$) and impoverished ($M = 267.95$, $SEM = 5.52$) group rats did not have significantly different Qp values in constant light. The robustness of the free-running period in constant light was not significantly different between treatment conditions, $t(5) = 2.41, p = 0.06$, $d = 1.39$. All animals eventually displayed arrhythmicity in constant light, as is shown by the lack of pattern in the daily activity onset and offset. Control group rats became arrhythmic within 22-48 days ($M = 34.50$, $SEM = 4.39$) following the onset of constant light and impoverished group rats became arrhythmic within 9-32 days ($M = 23.33$, $SEM = 3.42$). The differences were not statistically significant, $t(10) = 2.01, p = 0.07, d = 1.16$. (See figure 2).
Figure 2. Representative double-plotted actograms of rat wheel-running behaviour from one control (left) and one impoverished (right) rat. The bar (top) indicates the ZT of the day. The y axis (left) indicates the number of days during the experiment. Vertical black marks indicate periods of activity of a minimum of 10 wheel-revolutions per 10-minute bin. These actograms represent the time during which animals were in constant light.
**Re-entrainment**

Re-entrainment was defined as wheel running behaviour that began at the onset of the dark phase and that occurred predominantly within the dark phase. Both control and impoverished rats re-entrained to multiple 12:12 LD cycles. There was no significant difference between treatment groups in number of days to re-entrainment during a phase advance (LD 12:12 (2-14)), $t(10) = 2.22, p = 0.66$. Control animals ($M = 6.17, SEM = 0.17$) took 6-7 days to re-entrain and impoverished animals ($M = 6.33, SEM = 0.33$) took 6-8 days to re-entrain.

There was no significant difference in number of days to re-entrain during a phase delay (LD 12:12 (12-24)), $t(10) = 2.23, p = 1.00$. Both control ($M = 3.5, SEM = 0.22$) and impoverished ($M = 3.5, SEM = 0.22$) animals took 3-4 days. There was no significant difference in number of days to re-entrain to the second LD 12:12 (8-20), $t(10) = 2.23, p = 0.60$. Both control ($M = 2.33, SEM = 0.21$) and impoverished ($M = 2.5, SEM = 0.22$) animals took 2-3 days.

**Phase Angle of Re-entrainment**

Phase angle of entrainment is defined as the relationship between the timing of an endogenous circadian phase and the timing of an external time cue (ex: light) (Emens, Yuhas, Rough, Kochar, Perters, & Lewy, 2009; Pittendrigh & Daan, 1976). Phase angle was measured by selecting a time point at which exposure to an external time cue occurred (lights off), and comparing it to the onset of wheel running activity. Therefore, phase angle of entrainment indicated the number of hours between lights off and onset of wheel running activity. There was a significant difference between treatment conditions in phase angle of entrainment during the final LD 12:12 (8-20) cycle, following constant dark, $t(10) = 2.42, p = 0.036$. Control animals ($M = 2.23, SEM = 0.12$) had significantly longer intervals between light onset and activity onset than impoverished animals ($M = 1.59, SEM = 0.23$).
There was no significant difference between treatment conditions in phase angle of entrainment during the initial LD 12:12 (8-20) cycle, $t(10) = 0.42, p = 0.967$. Control ($M = 2.57, SEM = 0.36$) and impoverished animals ($M = 2.59, SEM = 0.25$) did not differ in their phase angles of entrainment. There was no significant difference between treatment conditions during a phase advance (LD 12:12 (2-14)), $t(10) = 1.15, p = 0.275$. Control ($M = 3.22, SEM = 0.38$) and impoverished animals ($M = 2.67, SEM = 0.29$) did not differ in their phase angles of entrainment. There was no significant difference between treatment conditions during a phase delay (LD 12:12 (12-24)), $t(10) = 2.07, p = 0.066$. Control ($M = 4.39, SEM = 0.43$) and impoverished animals ($M = 3.39, SEM = 0.22$) did not differ in their phase angles of entrainment (See figure 3).

Figure 3. Phase angle of entrainment for various LD 12:12 light cycles (a) Phase angle of entrainment during the first LD 12:12 (8-20) cycle by both treatment groups (b) Phase angle of entrainment during the phase advance LD 12:12 (2-14) cycle by both treatment groups (c) Phase angle of entrainment during the phase delay LD 12:12 (12-24) cycle by both treatment groups (d) Phase angle of entrainment during the LD 12:12 (8-20) cycle that followed constant darkness by both treatment groups. There was a significant main effect of treatment condition for this light cycle ($p = 0.036$).
**Forced Swim Test**

Each rat was placed in a water filled cylinder for a single, 5-minute test and their behaviour was video recorded. Following the 5-minute test, animals were dried off and placed in clean, dry cages with absorbent pads. Videos were analyzed for four behaviours: swimming, climbing, immobility, and latency to immobility. A two-factor independent-measures ANOVA showed a significant main effect of ZT as a function of total time spent swimming (sec/300 sec), $F(1, 8) = 14.96, p = 0.005, \eta^2_p = .652$. Rats spent significantly more time swimming at ZT 13 ($M = 93.72, SEM = 19.03$) than at ZT 1 ($M = 10.40, SEM = 3.57$). The main effect of treatment condition was not significant; there was no significant difference in the amount of time spent swimming between the control ($M = 52.52, SEM = 22.15$) and impoverished ($M = 51.60, SEM = 24.05$) groups $F(1,8) = 0.002, p = 0.967, \eta^2_p = .000$. There was no significant interaction effect, $F(1,8) = 0.075, p = 0.791, \eta^2_p = .009$.

There was a significant main effect of ZT as a function of total time spent climbing (sec/300 sec), $F(1, 8) = 11.68, p = 0.009, \eta^2_p = .593$. Rats spent significantly more time climbing at ZT 1 ($M = 210.08, SEM = 16.61$) than at ZT 13 ($M = 119.00, SEM = 23.39$). The main effect of treatment condition was not significant; there was no significant difference in the amount of time spent swimming between the control ($M = 140.80, SEM = 24.37$) and impoverished ($M = 188.28, SEM = 28.86$) groups, $F(1,8) = 3.17, p = 0.113, \eta^2_p = .284$. There was no significant interaction effect, $F(1,8) = .409, p = 0.540, \eta^2_p = .049$.

There was no significant main effect of ZT as a function of total time spent immobile (sec/300 sec), $F(1,8) = 0.12, p = 0.747, \eta^2_p = .014$. There was also no significant effect of treatment condition, $F(1,8) = .401, p = 0.080, \eta^2_p = .334$. There was no significant interaction effect, $F(1,8) = 0.23, p = 0.645, \eta^2_p = .028$. There was no significant main effect of ZT as a
function of latency to immobility, $F(1,8) = 0.18, p = 0.681, \eta_p^2 = .022$. There was also no significant effect of treatment condition, $F(1,8) = 0.34, p = 0.577, \eta_p^2 = .041$. There was no significant interaction effect, $F(1,8) = 0.09, p = 0.774, \eta_p^2 = .011$. (See figure 4).

Figure 4. Forced swim test behaviours. (a) Total time spent engaging in climbing behaviour, in seconds, during forced swim test by both treatment groups (Out of 300 seconds total). There was a significant main effect of ZT for climbing behaviour (b) Total time spent engaging in swimming behaviour, in seconds, during forced swim test by both treatment groups (Out of 300 seconds total). There was a significant main effect of ZT for swimming behaviour (c) Total time spent engaging in immobility behaviour, in seconds, during forced swim test by both treatment groups (Out of 300 seconds total). (d) Latency to immobility behaviour, measured as time (seconds) until the first bout of immobility.
**Serum Corticosterone Levels**

Thirty minutes following the forced swim test, rats were restrained and had blood samples taken through a small incision on the tail. Blood samples were collected and centrifuged, then the serum transferred to Eppendorf tubes. These were stored at -80°C until an ELISA to examine corticosterone levels was run. A 2x3 mixed factorial ANOVA was conducted on the influence of two independent variables, treatment condition and timing of blood collection, on serum levels of corticosterone. Treatment condition included two levels, control and impoverished, and time of blood collection included three levels (baseline, post-stressor, and pre-perfusion).

There was a significant main effect of time, $F(1.47, 11.77) = 15.65, p = .001$. Pairwise comparisons for the main effect of time indicates a significant difference between baseline levels of corticosterone and post-stressor serum levels of corticosterone ($p = .014$), and between post-stressor and pre-perfusion serum levels of corticosterone ($p = .008$). There was no significant main effect of treatment condition, $F(1, 8) = 0, .025, p > .05$. There was no significant interaction between treatment condition and time, $F(1.47, 11.77), p > .05$. (See Figure 5).

![Serum Corticosterone Levels](image)

Figure 5. Serum corticosterone levels for impoverished and control rats at three time points: baseline - before any behavioural tests while animals were in a 12:12 LD (8-20) cycle, post-forced swim test – within 30 minutes following the animals being tested in the forced swim test, perfusion – immediately before animals were perfused.
**Brain**

Brains were collected 24 hours after the forced swim tests. For animals who underwent the forced swim test at ZT 1, brains were collected at ZT 1 the following day. The same procedure was used for those animals tested at ZT 13. A two-factor independent-measures ANOVA showed a significant main effect of ZT as a function of total PER1 expression in the striatum, $F(1, 8) = 8.34, p = 0.020, \eta_p^2 = .51$. Brain slices collected at ZT 13 (M = 147.94, SEM = 5.29) showed significantly higher PER1 expression in the striatum than those at ZT 1 (M = 124.11, SEM = 6.59). The main effect of treatment condition was not significant, $F(1, 8) = 2.40, p = .160, \eta_p^2 = .230$. There was no significant interaction effect, $F(1, 8) = .080, p = .785, \eta_p^2 = .010$.

A significant main effect of treatment condition was found when examining PER1 expression in the Cornu Ammonis and dentate gyrus subareas of the hippocampus, $F(1, 8) = 8.203, p = .021, \eta_p^2 = .506$. Impoverished rats (M = 176.16, SEM = 9.32) showed significantly higher PER1 expression in the hippocampus than control rats (M = 153.67, SEM = 7.12). The main effect of ZT time was not significant, $F(1, 8) = .029, p = .869, \eta_p^2 = .004$. There was a significant interaction of treatment condition*zt, $F(1, 8) = 14.26, p = .005, \eta_p^2 = .641$. The effects of ZT on PER1 levels in the hippocampus were significant for impoverished animals, $F(1, 8) = 6.50, p = .034$. Among impoverished animals, PER1 expression was significantly higher at ZT 13 (M = 190.33, SEM = 15.18) than at ZT 1 (M = 162.00, SEM = 1.76). It was also significant for control animals, $F(1, 8) = 7.79, p = .024$. Specifically, among control animals, PER1 levels were higher at ZT 1 (M =169.17, SEM = 3.60) then they were at ZT 13 (M = 138.17, SEM = 0.48).
A significant main effect of treatment condition was found when examining PER1 expression in the basolateral amygdala, $F(1, 8) = 7.30$, $p = .027$, $\eta_p^2 = .477$. Impoverished rats ($M = 127.39$, SEM = 4.00) showed significantly higher PER1 expression in the amygdala than control rats ($M = 115.97$, SEM = 5.32). The main effect of ZT time was not significant, $F(1, 8) = .27$, $p = .618$, $\eta_p^2 = .033$. There was a significant interaction of treatment condition*zt, $F(1, 8) = 16.56$, $p = .004$, $\eta_p^2 = .674$. The effects of ZT on PER1 levels in the basolateral amygdala were significant for impoverished animals, $F(1, 8) = 16.30$, $p = .036$. Among impoverished animals, PER1 expression was significantly higher at ZT 13 ($M = 134.89$, SEM = 1.81) than at ZT 1 ($M = 119.89$, SEM = 4.57). It was also significant for control animals, $F(1, 8) = 10.53$, $p = .012$. Specifically, among control animals, PER1 levels were higher at ZT 1 ($M = 125.67$, SEM = 6.31) then they were at ZT 13 ($M = 106.28$, SEM = 2.74). No significant effects or interactions were found in the BNST or the SCN. (See Figure 6)
Figure 6. Representative images of immunolabeling for PER1 in control and impoverished rats. (a) High-magnification images of the striatum for both treatment conditions and ZT points. (b) There was a significant main effect of ZT in the striatum with significantly higher PER1 expression at ZT 13 than ZT 1 ($p = 0.020$), as visible in the bar graph. (c) High-magnification images of the amygdala for both treatment conditions and ZT points. (d) There was a significant main effect of treatment in the amygdala with impoverished showing significantly higher PER1 expression ($p = 0.027$), as visible in the bar graph. (e) High-magnification images of the hippocampus for both treatment conditions and ZT points. (f) There was a significant main effect of treatment in the hippocampus with impoverished rats showing significantly higher PER1 expression ($p = 0.021$), as visible in the bar graph.
Experiment 2

Behaviour

Rats were entrained to a 12:12 LD schedule for one week before beginning to analyze changes in their wheel running activity in various light cycles. Proper entrainment was defined as wheel running behaviour that began at the onset of the dark phase of the light cycle. Furthermore, wheel running behaviour must have occurred predominantly within this dark phase. (See figure 7, from day 0 to day 7)

All animals were entrained by the end of the week, with a minimum of 85% of running their wheel running behaviour occurring during the dark phase, and less than 15% occurring during the light phase. A two-factor independent-measures ANOVA showed no significant main effect of sex, $F(1,19) = 2.51$, $p = 0.13$, $\eta^2 = .117$. There was no significant main effect of treatment condition, $F(1,19) = .002$, $p = 0.96$, $\eta^2 = .000$. There was no significant interaction of sex and treatment condition, $F(1,19) = 3.75$, $p = 0.07$, $\eta^2 = .165$.

Constant Darkness

Animals were placed in constant darkness for 15 days with constant access to a running wheel. All animals displayed typical free-running rhythms in constant darkness, as is shown by the diagonal pattern of delay of daily activity onset. Control group rats had tau values between 24.03-24.51 and impoverished group rats had tau values between 24.12-24.62. These differences were not statistically significant ($p = 0.77$). The main effect for sex was not significant ($p = 0.75$). The interaction between sex and treatment condition was also not significant ($p = 0.48$) Both the control and impoverished rats showed daily cycles of locomotor activity with approximately 12 hours of activity followed by 12 hours of rest. (See figure 7, from day 52 to day 66).
**Constant Light**

After 10 days in a 12:12 LD (8-20) cycle animals were placed in constant light for 28 days with constant access to running wheels. Qp values, as well as days to arrhythmicity were statistically examined. Qp values indicate the strength of periodicity for a specific period length. There was a significant difference in Qp value as a function of sex, $F(1,19) = 12.39, p = .002, \eta^2_p = .39$. Female rats ($M = 501.82, SD = 186.34$) had higher Qp values than male rats ($M = 320.46, SD = 62.34$). In other words, females had a more robust free-running rhythm before becoming arrhythmic than male rats. The main effect of treatment condition was not significant $F(1,19) = 2.91, p = .11, \eta^2_p = .13$). There was no significant difference in Qp values between rats who had been raised in a control environment ($M = 369.19, SD = 108.18$) versus those who had been raised in impoverished environments ($M = 448.66, SD = 203.41$). The interaction between sex and treatment condition was also not significant, $F(1,19) = 1.99, p = .18, \eta^2_p = .10$.

All animals eventually displayed arrhythmicity in constant light, as is shown by the lack of pattern in the daily activity onset and offset. Control group rats became arrhythmic within 7-27 days following the onset of constant light and impoverished group rats became arrhythmic within 14-26 days. The differences were not statistically significant, $F(1,19) = 0.44, p = 0.51, \eta^2_p = .02$. There was no main effect of sex, $F(1,19) = 0.17, p = 0.68, \eta^2_p = .01$. The interaction effect between sex and treatment condition was also not significant, $F(1,19) = 0.01, p = 0.92, \eta^2_p = .001$. (See figures 7, from day 77 to day 104).
Figure 7. Representative double-plotted actograms of rat wheel-running. The environmental bar (top) indicates the ZT of the day. The y axis (left) indicates the number of days during the experiment. Rats were first entrained to a 12:12 LD (8-20) cycle for 7 days and then the LD cycle was advanced by 6 hours for 21 days. Next, the light cycle was delayed 10 hours for 21 days. Then the rats were placed in constant darkness for 15 days and, afterwards, re-entrained to 12:12 LD (8-20) for 10 days. After 10 days in the 12:12 LD (8-20) cycle, the rats were placed in constant light for 28 days. Finally, the rats were re-entrained once again to a 12:12 LD (8-20) light cycle. Grey areas indicate dark periods. Vertical black marks indicate periods of activity of a minimum of 10 wheel-revolutions per 10-minute bin.
**Re-entrainment**

Re-entrainment was defined as wheel running behaviour that began at, or shortly after, the onset of the dark phase of the light cycle. Furthermore, this wheel running behaviour must have occurred predominantly within the dark phase. The control and impoverished rats of both sexes successfully re-entrained to multiple 12:12 LD cycles (LD 2-14, LD 12-24, and LD 8-20). For the LD 12:12 (2-14) cycle a two-factor independent-measures ANOVA showed a significant main effect of sex of days to re-entrainment, $F(1,19) = 8.19, p = 0.01, \eta_p^2 = .301$. There was no significant main effect of treatment condition, $F(1,19) = .93, p = 0.35, \eta_p^2 = .047$. There was no significant interaction of sex and treatment condition, $F(1,19) = 0.66, p = .43, \eta_p^2 = .033$. No other significant main effects or interaction effects were found for the LD 12:12 (12:24) or the second LD 12:12 (8-20) light cycles.

**Phase Angle of Re-entrainment**

Phase angle was measured by selecting a time point at which exposure to an external time cue occurred (lights off), and comparing it to the onset of wheel running activity. Therefore, phase angle of entrainment indicated the number of hours between lights off and onset of wheel running activity. For the initial LD 12:12 (8-20) cycle a two-factor independent-measures ANOVA showed a significant main effect of sex as a function of phase angle of entrainment, $F(1,19) = 20.27, p = 0.013, \eta_p^2 = .284$. Males ($M = 2.40, SEM = 0.44$) had significantly longer intervals between lights off and activity onset than females ($M = 0.50, SEM = 0.48$). In other words, males took significantly longer than females to begin wheel running after lights off. There was no significant main effect of treatment condition, $F(1,19) = .04, p = 0.835, \eta_p^2 = .002$. There was no significant interaction of sex and treatment condition, $F(1,19) = 0.17, p = .684, \eta_p^2 = .009$. 
For the second LD 12:12 (8-20) cycle, following constant dark, there was a significant main effect of treatment condition as a function of phase angle of entrainment, F(1,19) = 8.46, p = 0.009, $\eta^2_p = .308$. Impoverished rats ($M = -1.99$, $SEM = 0.75$) had significantly longer intervals between lights off and activity onset than control rats ($M = 1.13$, $SEM = 0.68$). Impoverished rats began wheel running before lights went off, whereas control rats began running after lights went off. There was no significant main effect of sex, F(1,19) = .65, p = 0.431, $\eta^2_p = .033$. There was no significant interaction of sex*treatment condition, F(1,19) = .01, p = .937, $\eta^2_p = .000$.

There was no significant main effect of sex, F(1,19) = .001, p = 0.975, $\eta^2_p = .000$. or treatment condition F(1,19) = 1.31, p = 0.267, $\eta^2_p = .064$. for the phase advance (LD 12:12 2-14). There was no significant main effect of sex, F(1,19) = .14, p = 0.711, $\eta^2_p = .007$. or treatment condition F(1,19) = .12, p = 0.729, $\eta^2_p = .006$. for the phase delay (LD 12:12 12-24). There were no significant interaction effects of sex*treatment condition for the phase advance, F(1,19) = 1.73, p = .204, $\eta^2_p = .084$ or the phase delay, F(1,19) = .14, p = .711, $\eta^2_p = .007$ (See figure 8).
Figure 8. Phase angle of entrainment for various LD 12:12 light cycles (a) Total phase angle of entrainment during the first LD 12:12 (8-20) cycle. There was a significant main effect of sex for this light cycle ($p = 0.013$) (b) Total phase angle of entrainment during the phase advance LD 12:12 (2-14) cycle (c) Total phase angle of entrainment during the phase delay LD 12:12 (12-24) cycle (d) Total phase angle of entrainment during the second LD 12:12 (8-20) cycle. There was a significant main effect of treatment condition for this light cycle ($p = 0.009$).
**Estrous Cycle**

Each female underwent vaginal lavages on consecutive days. The lavages were then imaged at 10x magnification to identify cell density and type and, therefore, current phase of the estrous cycle. All female rats had regular estrous cycles while vaginal lavage was being conducted; this was defined as a cycle that lasted 4-5 days, and included all 4 stages – proestrus, estrus, metestrus and diestrus (See figure 9).

![Figure 9](image)

Figure 9. Vaginal smears from varying phases of the estrous cycle. Plate A shows the proestrus phase, characterized by nucleated epithelial cells. Plate B shows the estrus phase, characterized by cornified epithelial cells. Plate C shows metestrus phase, characterized by leukocytes, gathered around cornified epithelial cells. During metestrus it is also possible to see low numbers of nucleated epithelial cells. Plate D shows diestrus, characterized by a lowered cell density and predominantly leukocytes.
**Novelty-induced Locomotion**

Rats were placed in a locomotor chamber that measured 26x26 inches and total distance travelled (in centimetres) during the 30-minute testing session was recorded. There was a significant effect of treatment condition on novelty-induced locomotor activity, $F(1, 19) = 5.74, p = 0.03, \eta^2_p = .23$. Impoverished rats ($M = 3401.78, SEM = 168.66$) travelled significantly more distance than control rats ($M = 2696.13, SEM = 225.51$). There was no significant effect of sex, $F(1, 19) = 0.11, p = 0.75, \eta^2_p = .06$. The interaction between sex and treatment condition was not significant, $F(1, 19) = 0.01, p = 0.094, \eta^2_p = .00$. (See Figure 10).

The amount of time spent in the center of the box as a ratio over time spent in the margins of the box (center:margin) was also examined. This was examined as if it were an open-field parameter despite the disparity in size of the novelty-induced locomotor boxes as compared to a true open field. The novelty-induced locomotor boxes measured 26x26 inches, whereas an open-field suitable to test rats would need to have measurements of approximately 40x40 inches.

There was no significant main effect of treatment condition as a function of time spent in the center of the box as a ratio over time spent in the margins, $F(1, 19) = 1.79, p = 0.196, \eta^2_p = .09$. Impoverished rats ($M = 0.33, SEM = 0.09$) showed no significant difference in amount of time spent in the center of the box than control rats ($M = 0.48, SEM = 0.07$). There was no significant main effect of sex, $F(1, 19) = 1.39, p = 0.254, \eta^2_p = .07$. Female rats ($M = 0.47, SEM = 0.08$) showed no significant difference in amount of time spent in the center of the box than male rats ($M = 0.33, SEM = 0.06$). The interaction between sex and treatment condition was not significant, $F(1, 19) = 0.25, p = 0.626, \eta^2_p = .01$. 
Figure 10. Total distance travelled, in centimetres, during a 30-minute novelty-induced locomotor test. There was a significant main effect of treatment condition, with impoverished rats travelling significantly more distance than control rats ($p = 0.03$)
Forced Swim Test

Each rat was placed in a water filled cylinder for a single, 5-minute test and their behaviour was video recorded. Following the 5-minute test, animals were dried off and placed in clean, dry cages with absorbent pads. Videos were analyzed for four behaviours: swimming, climbing, immobility, and latency to immobility. A two-factor independent-measures ANOVA showed a significant main effect of sex as a function of total time spent swimming (sec/300 sec), $F(1, 15) = 4.70, p = 0.047, \eta^2_p = .24$. Male rats spent significantly more time swimming ($M = 14.31, SEM = 3.59$) than female rats ($M = 4.94, SEM = 1.86$). The main effect of treatment condition was not significant; there was no significant difference in the amount of time spent swimming between the control ($M = 20.83, SEM = 2.33$) and impoverished ($M = 22.18, SEM = 3.85$) groups $F(1,15) = 0.34, p = 0.57, \eta^2_p = .02$. The main effect of ZT was not significant, $F(1,15) = 0.32, p = 0.58, \eta^2_p = 0.2$. There were no significant interaction effects.

Significant main effects of sex were found when examining all other forced swim test behaviour (ie: climbing, immobility, and latency to immobility). There was a significant main effect of sex when examining climbing behaviour, $F(1, 15) = 12.65, p < 0.003, \eta^2_p = .46$. Male rats ($M = 203.89, SEM = 13.86$) spent significantly less time climbing than female rats ($M = 265.53, SEM = 5.43$). The main effect of treatment condition was not significant, $F(1,15) = 0.79, p = 0.39, \eta^2_p = .05$. The main effect of ZT was not significant, $F(1,15) = 0.49, p = 0.50, \eta^2_p = .03$. There were no significant interaction effects.

Significant main effects of sex were found when examining amount of time spent immobile, $F(1, 15) = 10.51, p < 0.005, \eta^2_p = .41$. Male rats ($M = 81.79, SEM = 12.79$) spent significantly more time immobile than female rats ($M = 29.52, SEM = 5.05$). The main effect of treatment condition was not significant, $F(1,15) = 0.65, p = 0.43, \eta^2_p = .04$. The main effect of
ZT was not significant, $F(1, 15) = 0.36$, $p = 0.56$, $\eta^2_p = 0.2$. There were no significant interaction effects.

Furthermore, a two-factor independent ANOVA examining latency to immobility showed a significant main effect of sex, $F(1, 15) = 10.01$, $p = .006$, $\eta^2_p = .40$. Male rats ($M = 85.42$, $SEM = 13.39$) had a significantly lower latency to immobility than female rats ($M = 143.18$, $SEM = 14.17$). The main effect of treatment condition was not significant, $F(1, 15) = 1.91$, $p = 0.19$, $\eta^2_p = .11$. The main effect of ZT was not significant, $F(1, 15) = 3.73$, $p = 0.07$, $\eta^2_p = 0.2$. There were no significant interaction effects. (See figure 11).
Figure 11. Forced swim test results of a 300 second, single forced swim test with no habituation day. (a) Total time spent swimming for male rats, and female rats in diestrus, out of 300 seconds total. Swimming was defined as diving, as well as active paddling behaviours in the middle of the vase. (b) Total time spent climbing for male rats, and female rats in diestrus, out of 300 seconds total. Climbing was defined as active behaviour including the movement of both the front and back paws along the sides of the vase. (c) Total time spent immobile for male rats, and female rats in diestrus, out of 300 seconds total. Immobility was defined as no movement, or only enough movement of the front paws to keep the head above water. (d) Latency to immobility for male rats, and female rats in diestrus, out of 300 seconds total. Latency to immobility was defined as time, in seconds, until the start of the first bout of immobility.
Serum Corticosterone Levels

30 minutes after the forced swim test, rats had blood samples taken through an incision on the tip of the tail. Once all blood samples were collected, they were centrifuged, the serum transferred to separate Eppendorf tubes, and then stored at -80°C until an ELISA was run to examine serum corticosterone levels. A mixed factorial ANOVA was conducted on the influence of four independent variables, treatment condition, ZT time, sex, and timing of blood collection on serum levels of corticosterone. Treatment condition included two levels, control and impoverished, ZT time included two levels, ZT1 and ZT 13, sex included two levels, male and female, and time of blood collection included three levels (baseline, post-stressor, and pre-perfusion). There was no significant main effect of treatment condition, F(1, 14) = 0.28, p = .61. There was a significant main effect of sex, F(1, 14) = 72.65, p = .000. Females (M = 50539.56, SEM = 16058.62) had significantly higher corticosterone levels than males (M =2647.92, SEM = 1518.46).

There was a significant main effect of time, F(2, 28) = 22.51, p = .000. Pairwise comparisons indicate a significant difference between baseline levels (M = 5927.71, SEM = 1494.56) and post-stressor serum levels of corticosterone (M =84246.19, SEM = 22667.47) (p = .000) and between post-stressor and pre-perfusion (M = 23172.90, SEM = 8545.03) serum levels of corticosterone (p = .001). There was a significant interaction between time of corticosterone collection and sex, F(2, 38) = 17.43, p = .000. The effect of sex on time was non-significant for males, F(2, 38) = .28, p = .760. The effect of sex on time was significant for females, F(2, 38) =40.56 , p = .000. Among the females, corticosterone levels were significantly higher post-stressor (M = 178099.33, SEM = 34852.01) than they were at baseline (M = 11895.67, SEM = 2230.92) (p = .001). Corticosterone levels were also significantly higher post-stressor (M =
178099.33, \( \text{SEM} = 34852.01 \) than they were pre-perfusion \( (M = 50539.56, \text{SEM} = 16058.62) \) (\( p = .009 \)). (See Figure 12).

Figure 12. Serum corticosterone results taken at three time-points: baseline (before any behavioural tests, while animals were in 12:12 LD (8-20) cycles), within 30 minutes following the forced swim test, and again immediately before perfusion. (a) serum corticosterone results collected at ZT 1 for control and impoverished rats. This graph includes both the male and female rats who had corticosterone measures leveled at ZT 1. (b) serum corticosterone results collected at ZT 1 for control and impoverished rats. This graph includes both the males and females who had corticosterone measures leveled at ZT 1. (c) serum corticosterone results collected for males, at both ZT 1 and ZT 13 time points. (d) serum corticosterone results collected for females, at both ZT 1 and ZT 13 time points. Note the difference in the scale of graph c. As mentioned previously, there was a significant main effect of sex (\( p = .000 \)) with males having significantly lower corticosterone levels than females.
**Brain**

Brains were taken 24 hours after the forced swim test, at either ZT1 or ZT13. A two-factor independent-measures ANOVA showed a significant main effect of sex as a function of total PER1 expression in the BNST, $F(1, 15) = 4.54, p = 0.050, \eta^2_p = .23$. Males ($M = 202.11$, $SEM = 10.03$) showed significantly higher PER1 expression in the BNST than females ($M = 175.61$, $SEM = 5.33$). The main effect of ZT was not significant, $F(1, 15) = .26, p = .615, \eta^2_p = .017$. Nor was the main effect of treatment condition, $F(1, 15) = 1.32, p = 0.269, \eta^2_p = 0.08$. There were no significant interaction effects.

A significant main effect of sex was found when examining PER1 expression in the basolateral amygdala, $F(1, 14) = 21.19, p = .000, \eta^2_p = .60$. Females ($M = 172.95$, $SEM = 5.89$) showed significantly higher PER1 expression than males ($M = 135.55$, $SEM = 4.29$). The main effect of ZT time was not significant, $F(1, 14) = .03, p = .86, \eta^2_p = .002$. Nor was the main effect of treatment condition, $F(1, 14) = 1.11, p = 0.31, \eta^2_p = .07$. No significant interaction effects were found. A significant main effect of treatment condition was found when examining PER1 expression in the Cornu Ammonis and dentate gyrus of the hippocampus, $F(1, 15) = 7.5, p = .015, \eta^2_p = .33$. Impoverished rats ($M = 193.40$, $SEM = 3.81$) showed significantly higher PER1 expression in the hippocampus than control rats ($M = 176.61$, $SEM = 5.16$). The main effect of ZT time was not significant, $F(1, 15) = .52, p = .48, \eta^2_p = .48$. There was no significant main effect of sex, $F(1, 15) = 1.11, p = 0.31, \eta^2_p = .07$. No significant interaction effects were found. No further significant differences in PER1 expression were found in the striatum, or SCN. (See figure 13).
Figure 13. Representative images of immunolabeling for PER1 in control and impoverished rats. (a) Low magnification images of the BNST. White squares indicated the areas examined under higher magnification. (b) High-magnification images of the BNST, for both treatment conditions, sexes and ZT points. (c) Note that there was a significant main effect of sex in the BNST with males showing significantly higher PER1 expression ($p = 0.050$), as seen in the bar graphs. In these graphs, dark blue bars represent control animals and light blue bars represent impoverished animals. (d) Low magnification images of the amygdala. White squares indicated the areas examined under higher magnification. (e) High-magnification images of the basolateral amygdala, both treatment conditions, sexes and ZT points. (f) Note that there was a significant main effect of sex in the basolateral amygdala with females showing significantly higher PER1 expression ($p = 0.000$), as shown in the bar graphs. (g) Low magnification images of the hippocampus. White squares indicated the areas examined under higher magnification. (h) High-magnification images of the hippocampus, for both treatment conditions, sexes and ZT points. (i) Note that there was a significant main effect of treatment in the amygdala with impoverished rats showing significantly higher PER1 expression ($p = 0.015$), as shown in the bar graphs.
Discussion

The present study was designed to analyze whether there was a difference in circadian phenotypes and clock gene expression between two different treatment groups, impoverished and control. The study also examined whether rats who had been exposed to typical early environmental and social enrichment as pups (control group) would show more resilience to acute stress later in development. This was in comparison to rats who had been exposed to early environmental and social impoverishment (impoverished group). It was hypothesized that control group rats would spend more time immobile during the forced swim test, travel less distance in the locomotor chambers, have a more robust circadian system, and re-entrain to various light cycles more efficiently.

It was also hypothesized based on previous literature that there would be increased PER1 expression following an acute stressor in the hippocampus, and suppressed PER1 expression in the BNST and amygdala (Al Safadi et al., 2014; Al Safadi et al., 2015; Misfud & Reul, 2016). An interesting consideration was that an increase of PER1 would likely not be seen in the SCN itself, but still be present in extra-SCN tissue; the SCN does not contain GRs, so if acute stress modifies clock gene expression through increased corticosterone levels, the SCN would not express higher levels of PER1 (Al Safadi, Branchaud, Rutherford, & Amir, 2015; Balsalobre et al., 2000). This finding was replicated many times, showing altered expression of PER1 in the hypothalamus and some peripheral tissues, but not the SCN (Al Safadi et al., 2015; Balsalobre et al., 2000).

Data showed that impoverishment modulates various behaviours, including novelty-induced locomotor behaviours and multiple forced swim test behaviours. There were also numerous sex differences found when analyzing the forced swim test. Males spent more time
engaging in swimming behaviour, and more time immobile than females. However, females spent more time climbing and had a higher latency to immobility than males.

Moreover, Experiment 1 corticosterone ELISA results validated the forced swim test protocol used as an acute stressor, showing a significant main effect of time ($p < .01$). The significant main effect of time showed that corticosterone levels were significantly higher in all rats following the forced swim test than during both the baseline corticosterone measurements and the pre-perfusion corticosterone measurements. Had the forced swim test not been an acute stressor, corticosterone measures taken following the forced-swim test likely would not have differed so significantly from the baseline and pre-perfusion corticosterone levels.

Although there was a significant main effect of time, there was no significant main effect treatment condition. The lack of significant main effect of treatment condition suggests that the stressor did not differentially affect the rats as a function of whether they were reared in impoverished versus control environments. Perhaps if an enriched treatment condition had been tested, there would have been a significant difference in corticosterone levels post-stressor between the impoverished and enriched group.

**Circadian Phenotypes and Behaviour**

In this portion, we discuss circadian locomotor phenotypes and behavioural results, for which the data was obtained before conducting any further immunohistochemical analyses. We then focus on results of the immunohistochemical analyses for the five brain regions of interest. Lastly, we discuss the overall results of the current study; particularly in the framework of recent literature on associations between clock gene expression, early life experience, and stress disorders.
Research has shown that impoverishment affects motor behaviour, with impoverished animals displaying higher levels of locomotor activity, and less exploration than enriched animals (Haupt & Schaefers, 2010). Novelty-induced locomotor data from the current experiment fits this finding, showing that impoverished animals travelled significantly more distance, in centimetres, during the 30-minute novelty-induced locomotor test than control animals. Social factors and developmental environment affect locomotion in a novel environment and have also been shown to impact locomotor response to cocaine (Zakharova, Starosciak, Wade, & Izenwasser, 2012).

These effects can be seen after animals have experienced only two weeks of either the enriched or impoverished housing conditions. In two studies that looked at the effects of amphetamines and nicotine, respectively, it was found that drug effects were dependent not only on type of drug, but also rearing environment. Adult rats who were reared in an enriched environment showed higher sensitivity to amphetamines, but lower sensitivity to nicotine than rats who were reared in an impoverished environment (Bowling & Bardo, 1994; Green, Cain, Thompson, & Bardo, 2003).

Moreover, one study found that adults who had previously experienced childhood trauma were more likely to experience mental health issues as adults; specifically, high rates of substance use disorders (SUD) were reported. The participants of this study were 112 individuals diagnosed with comorbid psychiatric and SUD (SUD group), and 112 matched controls with a diagnosis of a psychiatric disorder but no SUD (control group). All participants completed a Childhood Trauma Questionnaire – Short Form (CTQ-SF). Results showed that the SUD group scored significantly higher on the CTQ-SF for all five subscales (emotional, physical, and sexual abuse, emotional neglect, and physical neglect) than the control group, suggesting that early
impoverishment and abuse can lead to an increased risk of SUD in adulthood (Rasmussen, Arefjord, Winje, & Dovran, 2018).

This has important implications in terms of addictions. Individuals who have experienced childhood trauma and environmental impoverishment are more likely to develop substance use disorders than those who have not. In other words, the type of environment an individual was reared in may provide insight into the risk of developing substance use disorders later in life. This information can help to further develop addictions treatment programs, incorporating treatments that take into consideration past experience and their biological affects on the body.

Overall, the results of these two experiments seem to support previous literature in that impoverished and control rats show significant differences from one another on many behavioural parameters. Interestingly, constant light data from Experiment 1 showed a higher Qp, a value which indicates robustness of the free-running period in constant light, for control animals than impoverished animals. While these data were not statistically significant ($p = .06$) they are intriguing. Experiment 1 constant light data also showed a higher number of days for control rats to become arrhythmic as compared to the impoverished rats and, although this was not statistically significant ($p = .07$) it fits well with the higher Qp values of the control animals. These data together suggest that control animals have a more robust free-running rhythm.

Additionally, when examining circadian phenotypes and locomotor behaviours, it is important to consider sex. To limit complexity of the data analysis, all female rats in this experiment were in diestrus when undergoing the forced swim test and the novelty-induced locomotor test. Studies have shown that the forced swim test behaviours of both latency to
immobility and total time spent immobile are influenced by which phase of the estrous cycles the females are in (Gouveia et al., 2008).

During diestrus, females take longer to reach immobility, and spend less total time immobile as compared to when they are in other phases of the estrus cycle; this same pattern of behaviour is seen when comparing females in diestrus to males (Gouveia et al., 2008). The present study confirms these findings, comparing the forced swim test behaviours of diestrus females to those of males. Females spent significantly more time climbing, less time swimming, significantly less time immobile, and had a higher latency to immobility as compared to males.

Though commonly considered a marker of depressive-like behaviours, it has been argued that the forced swim test more accurately measures anxiety-like behaviours (Anyan & Amir, 2018). In humans, depression is highly comorbid with anxiety disorders. When interpreting the forced swim test traditionally, such that immobility indicates a depressive-like behaviour, this leads to an inconsistency between animal and human research; in animal research, animals high in depressive-like behaviours score low in anxiety-like behaviours (Anyan & Amir, 2018). Anyan & Amir (2018) suggest that this inconsistency is likely the result of a misinterpretation of the forced swim test behaviours. When interpreting climbing as an anxiety-like and escape oriented behaviour, and immobility as signifying lower anxiety, forced swim test results no longer conflict so strongly with human research.

**Serum Corticosterone Levels**

Upon analyzing serum corticosterone levels for experiment 1 it was found that there was a significant main effect of time, validating the use of the forced swim test as a stressor. Animals had significantly higher corticosterone levels following exposure to the forced swim test than
they had when baseline measures were taken. They also had lower corticosterone levels immediately before perfusion as compared to after the forced swim test.

An interesting observation is that, though the difference is not statistically significant, baseline levels of corticosterone are higher for the control group than they are for the impoverished group. This may be a result of HPA dampening in the impoverished animals. Hypocortisolism, or lowered levels of HPA axis responsiveness to stress, have been seen in approximately one-quarter of individuals with stress-related disorders such as PTSD (Fries, Hesse, Hellhammer, & Hellhammer, 2004; Heim, Ehlert, Hanker, Hellhammer, 1998; Heim, Ehlert, Hellhammer, 2000).

A model of hypocortisolism was proposed decades ago that theorized it was due to prolonged periods of stress, HPA axis hyperactivity, and high levels of glucocorticoids, after which the HPA axis response becomes dampened (Hellhammer & Wade, 1993). This theory has been further examined and is supported by studies that show many individuals with stress-related disorders experience the onset of fatigue, pain, and stress-sensitivity following intense periods of stress (Fries et al., 2004; Van Houdenhove & Egle, 2004). This same pattern of impoverished rats having lower baseline serum corticosterone levels than control rats was also seen in experiment 2 when examining the female rats, and all rats at ZT1.

**Implications of Desynchrony between the SCN and Extra-SCN clocks**

Desynchrony between the SCN and extra-SCN clocks has been shown to lead to a heightened risk for adverse health effects. For example, shift workers have been shown to be at higher risk for cardiovascular disease due to desynchrony between the hypothalamic and other peripheral circadian clocks (Young & Bray, 2007). Previous research has also established that polymorphisms in some circadian genes are associated with psychiatric illnesses (McClung,
Perturbations to the circadian system are a frequent feature in depression, bipolar disorder, and schizophrenia (Jagannath, Peirson, & Foster, 2013).

Studies have shown that mice with mutations in both mPer1 and mPer2 show heightened anxiety-like behaviours (Spencer et al., 2013; Zhang et al., 2011). In terms of anxiety disorders, both the circadian and stress system play an important role. Due to the intertwined nature of the HPA axis and the circadian system anxiety-like phenotypes may be a result of changes to systems other than the circadian system more so than in other psychiatric disorders (Koch et al., 2017; McClung, 2013).

This is illustrated by glucocorticoids abilities to stabilize rhythms in peripheral clocks in the presence of external perturbations as well as mediating changes in gene expression the limbic forebrain (Koch et al, 2017; Segall, Milet, Tronche, & Amir, 2009). The circadian system is more resilient to perturbations of the peripheral clocks when glucocorticoid levels are heightened, showing that glucocorticoids play a role in the coordination of these clocks (Koch et al., 2017).

Corticosterone levels and clock gene expression: Implications for stress disorders

As well as their role in peripheral clock coordination, glucocorticoids (GCs) also have an important function within the stress system. As a result of HPA activation, when an organism is faced with a stressor, increased levels of GCs are released from the adrenal glands. It seems that the HPA axis is regulated by both the circadian and stress systems, as well as having its own influence on these two systems (Koch et al, 2017). Furthermore, as previously stated, seeing that there is a reciprocal interaction between Perl and corticosterone, perturbations that affect one of these can lead to further dysregulation of the other. For example, corticosterone release
following an acute stressor results in increased PER1 protein expression in the paraventricular nucleus and hypothalamus (Al-Safadi et al., 2014).

**Limitation of the Study**

The lack of statistically significant results in this study may be due to several limitations. One limitation is the small sample size, which leads to low power studies. Effect sizes were calculated to mitigate this limitation. There were few similar studies published pertaining to early experience and circadian dysregulation, indicating the need for further research that include studies with larger sample sizes.

A limitation specific to the first experiment was the lack of a final 12:12 LD light cycle following a period of constant light. Animals’ behaviour during the forced swim test was examined while the animals were still in constant light conditions. The forced swim test was being used as an acute stressor, and measures of corticosterone were taken. The animals were also sacrificed 24 hours following the forced swim test to analyze PER1 levels in multiple brain regions. Given that PER1 expression is downregulated in constant light conditions, if the forced swim test dramatically lowered PER1 levels we may have been left unable to gather any useful results from an immunohistochemistry. This limitation was considered and in the second experiment animals were re-entrained to a 12:12 LD (8-20) light cycle before the forced swim test was carried out.

An important limitation of Experiment 2 was that both males and females were used, but females were not tested for novelty-induced locomotor behaviour or forced swim test behaviours in any estrous phase other than diestrus. While this simplifies data analysis, it also makes the study less generalizable. During the estrous cycle, levels of hormones such as estrogen and progesterone fluctuate (Haim, Shakhar, Rossene, Taylor, & Ben-Eliyahu, 2003; Sportnitz et al.,
Hormone levels during diestrus in rats are similar to those during the beginning of the follicular phase of the menstrual cycle in humans (Staley & Scharfman, 2005). By testing during only one phase of the estrous cycle, we get little insight into behaviour during other estrous phases that corresponded to phases in the menstrual cycle other than the follicular phase. Moreover, in terms of behavioural testing, animals were not run in any behavioural tests that provide direct measures of anxiety-like behaviours (ex: elevated plus maze, or open field test).

**Future Directions**

Future studies would benefit from an increased sample size. Additionally, given the close relationship between stress and anxiety, behavioural measures of anxiety would prove useful. This would allow a better understanding of whether impoverishment affected the stress system’s ability to regulate itself and react to acute stressors effectively, or if it led to an overall anxious phenotype but maintained an intact response to acute stress. A simple way to accomplish this would be to run the animals in both an open field test and an elevated plus maze before being exposed to the forced swim test.

Future studies would also benefit from examining the circadian phenotypes and stress responses of rats other than the wild-type animals used in these experiments. Given the role of glucocorticoids in both the stress and circadian systems, it would be interesting to look at *per1* knockouts as well as analyzing the effects of adrenalectomy and corticosterone injections.

The current study will be helpful in allowing a more comprehensive understanding of the way in which the circadian and stress systems interact. However, this field of study of stress and early environmental impoverishment may benefit from including more female animals as research subjects. There are differences in the prevalence of various stress and anxiety related
disorders between male and female humans, with females showing higher prevalence rates (McLean, Asnaani, Litz, & Hofmann, 2011; Pesce et al., 2016). Taking into consideration differences in socialization, brain differences, and susceptibility to adverse reactions to stress in both male and female animals would lead to a more comprehensive pool of literature that would be more representative of the human population.

While this study on its own will not lead to therapeutic changes for those with stress and circadian disorders, when taken into consideration with other literature within the field, research is one step closer to illuminating the potential underlying circadian and HPA mechanisms that result in various stress and anxiety disorders.
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