Chapter 7

General Discussion
The circadian system generates internal time in an organism and synchronizes it to external time, a property that permits anticipation and preparation so that physiological and behavioral processes occur with maximal efficiency. In mammals, the most important signal to reset the master oscillator, located in the SCN, is the light-dark cycle. Although other signals such as food and temperature are able to influence peripheral oscillators, the SCN does not seem to be affected by them. The main aim of this thesis was to evaluate whether chronic social defeat stressor exposure to glucocorticoid stress hormones can perturb the master clock and/or peripheral oscillators.

1. Effects of social defeat stress

We found that in male mice repeated social defeat stress on 10 consecutive days results in a strong suppression of locomotor activity (Chapters 3 and 4), which confirms earlier findings in both mice (Wells et al., 2017) and rats (Meerlo et al., 1997, Meerlo et al., 2002). Activity levels from defeated animals returned to the levels of control animals after a few days of recovery. The strong suppression of activity indicates that one way or another the mice were severely impacted by defeat, which therefore seems to represent a good model to study effects of stress on circadian function.

Although the overall level of locomotor activity was suppressed by social defeat stress, the period and phase of the rhythm as calculated from successive activity onsets did not differ between defeated animals and control mice. These results are in agreement with earlier findings on effects of social defeat stress in rats (Meerlo et al., 1997, Meerlo and Daan, 1998). In our new experiments the mice were subjected to defeat repeatedly for 10 days to assess of this chronic intermittent stress potentially has small cumulative effects that perhaps went unnoticed in the earlier studies in rats that were subjected to defeat stress only once or twice. This was not the case. Even chronic stress did not affect period and phase of the activity rhythm.

In chapters 4 and 5, we used a different approach to assess the effects of stress on the molecular circadian clock in the SCN directly. We used knock-in mice that have the *Luciferase* gene fused to the *Per2* gene, which allowed us to analyze PER2::LUC protein expression, by recording the bioluminescence in tissue cultures (Yoo et al., 2004). The mice were exposed to 10 days of social defeat stress and subsequently the SCN was cultured to assess the circadian rhythm in PER2::LUC expression in vitro. As we hypothesized, the rhythm of PER2::LUC expression was not affected in the SCN, neither by repeated social stress prior to the collection of SCN tissue (Chapter 4) nor by the stress hormone corticosterone added to the recording medium directly (Chapter 5). Again, these findings clearly support the general notion that the master clock in the SCN is well protected against any perturbing influence of stress.

In contrast, our studies showed that the circadian oscillator in the liver was affected by both prior social defeat stress (Chapter 4) and direct exposure to glucocorticoids in vitro.
The phase of PER2::LUC expression was delayed, both by social defeat and corticosterone treatment at ZT11 on isolated liver tissue. Given that the effects of prior social defeat stress and direct exposure to glucocorticoids on the liver rhythm were at least qualitatively similar, it is likely that the effects of stress are mediated by glucocorticoid hormones. This might also explain why stress appears to have little effect on the master clock in the SCN since, unlike most other tissues and brain regions, the adult SCN does not contain glucocorticoid receptors (Balsalobre et al., 2000, Rosenfeld et al., 1988).

Tahara and colleagues (2015) had already demonstrated that restraint stress for 3 consecutive days was able to phase shift the peak of PER2::LUC expression and RNA expression of other clock genes in different tissues, but not in the SCN of mice under LD. In the same study, social defeat and injection of dexamethasone for 3 days were also able to phase shift PER2 expression in peripheral tissues (liver, kidney and submandibular gland). Balsalobre and colleagues (2000) had previously reported that injections with the synthetic glucocorticoid dexamethasone were able to phase shift clock gene transcription in peripheral tissues but not in the SCN. Moreover, our in vitro measurements demonstrate that the effects of glucocorticoids may be directly mediated by binding to glucocorticoid receptor in the liver tissue itself (chapter 5).

In chapter 6, we have described a study to assess whether social defeat stress in mice would result in depressive-like behavior. This study was based on the hypothesis that circadian dysfunction might contribute to disease processes, including the development of psychiatric disorders such as depression. While the earlier chapters clearly demonstrate a very strong defeat stress-induced suppression of general home cage activity, which might be taken as an indicator of depression in itself, the more specific read outs of anxiety and depression in chapter 6 were not affected. This unexpected finding is in contrast with our earlier work in rats, in which the behavioral changes following social defeat stress have been presented as a model of depression (Koolhaas et al., 1997). Based on our mixed findings in mice, it is possible that defeat stress did not truly result in a depression-like state or that the behavioral read outs in chapter 6 were not sufficiently sensitive for mice. The behavioral assessment in chapter 6 started 4 days after the last defeat, when the suppression of home cage shown in chapter 3 had largely recovered and were no longer different from control. Perhaps in our model of social defeat stress in this particular strain of mice, behavioral changes are mostly acute and normalize within a few days after the last stressor. It is not excluded that stronger and more persistent stress-induced behavioral changes may occur under slightly different conditions and/or in mice with different traits. For example, one factor of importance that may have attenuated behavioral changes in chapter 6 is the age of the mice. Golden and colleagues (2011) have suggested that depressive-like behavior is best induced in younger mice. Since we were interested in observing the effects of stress in adulthood, we exposed our mice to defeat after 3 months of age, when the mouse brain is considered to be fully developed (Hammelrath et al., 2016). Future experiments are
required to assess depression-like behavior in more detail and also its potential relationship with changes in circadian function.

In summary, our studies in mice show that severe, uncontrollable social defeat stress does not appear to affect the master clock in the SCN, but it does phase shift the peripheral clock in the liver, an effect that may be mediated by glucocorticoid stress hormones.

2. Role of CORT in stress effects on circadian function

Stress is also associated with an activation of the sympathoadrenal system, resulting in the rapid release of the catecholamines noradrenaline and adrenaline. Tahara and colleagues (2015) also tested the effects of adrenaline and noradrenaline in vivo injections and reported that these hormones can also phase shift PER2 peak in the liver, kidney and submandibular gland. Therefore, it is still a question whether effects of stress on the liver clock are mediated by glucocorticoids, catecholamines, or both. Based on our own findings, one logical follow-up study to address this particular issue would be to inhibit the release of corticosterone during each successive social defeat stress by administration of metyrapone, a reversible inhibitor of an enzyme involved in cortisol and corticosterone synthesis. Then, if the phase shifts of the liver clock are no longer observed, one could conclude that corticosterone is necessary for the stress effects on this peripheral oscillator.

Alternatively, to test if effects of stress on peripheral clocks are mediated by catecholamines, a follow-up study could be the administration of catecholamine receptors blockers during the 10-days social defeat stress. Carvedilol, for example, is a β1, β2 and α1 adrenergic receptor blocker used to treat hypertension and congestive heart failure, and it is known to reduce the amplitude of heart rate (Valentina et al., 2015). Another approach could be the inhibition of dopamine beta-hydroxylase, the enzyme that converts dopamine into noradrenaline, during the days of social defeat exposure by administration of nepicastat, although no studies on the circadian effects of this drug were found.

It is possible that some effects of social defeat stress might be mediated by changes in body temperature (Pittendrigh, 1981). Especially circadian rhythms in peripheral tissues are sensitive to temperature changes (Buhr et al. 2010). Like most stressors, defeat is associated with an acute and strong increase in body temperature (Koolhaas et al. 1997). Moreover, defeat stress in particular has been reported to cause rather long-lasting elevations of body temperature, mainly during the circadian resting phase (e.g., Meerlo et al., 1996, Meerlo et al., 1997). However, increased body temperature as well as increased activity caused by cage change stimulation for 3 days does not seem to shift the peak phase of PER2 expression in the liver, kidney and submandibular gland (Tahara et al., 2015). Therefore, stress-induced temperature increases do not seem to be the most plausible explanation for the shift in the liver clock following social defeat.
3. Changes in PER2 rhythm and liver function

The liver plays an important role in the regulation of glucose homeostasis, together with other tissues such as the pancreas. The circadian system in turn provides the rhythmicity in baseline glucose levels in synchrony with the environment and in anticipation to regular periodic events, such as feeding during the active phase and fasting during the inactive phase (Reinke and Asher, 2016). Several hepatic enzymes participating in lipid biosynthesis and catabolism are expressed in a daily manner (e.g. cytochrome P450s, HMGCoA reductase, Lipin), (Panda, 2016) and the liver oscillator appears to buffer excessive circadian fluctuations of glucose levels in the blood caused by behavioral rhythms. Mice with liver-specific deletion of the core gene Bmal1, showed hypoglycemia during the fasting phase, exaggerated glucose clearance, and loss of the circadian expression rhythm of glucose regulatory genes in the liver (Lamia et al., 2008).

Mice with a Per2 knockout are heavier than wild type mice during the pre-adolescence and adolescence phase of life. After this, their growth rate slows down, and they become lighter than the wild types in adulthood. It was shown that Per2 knockout mice have the same daily food intake as wild type mice, but around 50% reduced triglycerides plasma levels (Grimaldi et al., 2010). In the same study, the authors demonstrated that PER2 controls lipid metabolism by repressing peroxisome proliferation activated receptor γ2 (PPARγ2), an important regulator of lipid metabolism and adipocyte differentiation. These findings together demonstrate that the circadian system and specific clock genes play an important role in metabolic regulation. Moreover, altered circadian function and changes in clock gene expression may in the long run sensitize individuals for metabolic disorders such as diabetes and obesity (Knutsson and Kempe, 2014, Tucker et al., 2012). Our own findings on social defeat-induced phase shifts of the liver clock may imply that stress could be one of the factors leading to disturbed circadian regulation of metabolic function and ultimately metabolic disorders. However, it remains difficult to study the relationship of phase shifts caused by stress with metabolic diseases, since the available techniques to track, for example, PER2 expression involve in vitro culture or anesthetizing the animals. Nevertheless, it is an interesting issue, because individuals under chronic stress often have clinical manifestations of metabolic syndrome (Nicolaides et al., 2014).

4. Oscillators in other brain tissues

As discussed above, circadian disruption between peripheral oscillators, such as the liver, might implicate in metabolic issues. Another important question to address is, whether stress might affect oscillators or clocks that exist in the brain, outside the SCN. This may be particularly important in relation to psychiatric disorders such as depression.

Indeed, brain regions outside the hypothalamus have also been reported to present daily oscillations in expression of clock genes. A study with immunohistochemistry to assess PER2 expression in rats in basal conditions, under a 12:12 LD cycle, showed that 18 forebrain
areas important for motivated and appetitive behavior displayed PER2 rhythms with brain region specific phases (Harbour et al., 2013). PER2::LUC rhythm in CA1, CA2, CA3 and DG of the hippocampus was also confirmed in isolated mouse hippocampus (Wang et al., 2009). Other areas involved in regulation of mood (cortex area 1, lateral habenula, periaqueductal grey and ventral tegmental area) were also reported to exhibit PER2::LUC circadian rhythm with more than two peaks in isolated cultures (Landgraf et al., 2016).

Two forebrain nuclei involved in fear and stress-related behavior, the bed nucleus of the stria terminalis and the central nucleus of the amygdala also present circadian PER2 expression rhythm and it is dependent on adrenal gland integrity (Amir et al., 2004). Furthermore, corticosterone replacement in adrenalectomized rats in drinking water, but not via constant-release pellets, can restore the circadian expression of PER2 in these areas. The results show the importance of corticosterone rhythm to maintain the rhythm in these nuclei. Other two stress sensitive structures, the hippocampus and cortex may also be sensitive to variations in corticosterone, since 3 days of restraint stress phase-advanced Per1 and Per2 expression in these areas (Tahara et al., 2015).

These studies suggest that chronic stress, by disrupting glucocorticoid rhythm, may affect brain areas important in emotional processing and consequently play a role in the development of mood disorders. Chronic high levels of glucocorticoids have been observed in depressed patients (Aihara et al. 2007, Bauer et al. 2003) and particularly for major depression, circadian desynchronization is associated with more severe symptoms, as observed in depressed patients who show desynchronization between the sleep/wake cycle and the SCN (Emens et al., 2009). Since it is very difficult to establish the relationship of cause and consequence between stress, circadian disruption and psychiatric disorders in humans, we attempted to untangle these associations by using the social defeat model in mice. Although the defeated animals did not show depressive-like behaviors (Chapter 6), we showed that stress can cause an internal desynchronization, at least between the SCN and liver, and that glucocorticoids might be the mechanism.

5. Future prospects and conclusion

There are still many questions to be answered about the mechanisms and consequences of chronic stress on circadian rhythms.

Future studies that could follow are mentioned in item 2: inhibition of glucocorticoids by metyrapone, and perhaps by adrenalectomizing the animals, and inhibition of catecholamines production or blocking their action, to determine if those stress hormones are essential for the phase-shift observed in the liver oscillator.

Another line to further investigate are the consequences of the disruption between master and peripheral oscillators caused by stress. However, since glucocorticoids are involved not only in timing information for peripheral oscillators, but in metabolic and physiological processes, it might be difficult to differentiate the effects of rhythm disturbance
and effects caused by elevation of glucocorticoids per se.

From our studies, we can conclude that chronic social stress does not seem to affect the SCN, but it does affect the peripheral oscillator in the liver and CORT is a viable candidate for mediating this effect (Figure 1).

Figure 1. Conclusions on chronic stress effects on circadian rhythms. The master clock in the SCN is synchronized by the photic information, but it is not affected by stress stimuli. On the other hand, the peripheral clock in the liver is affected by chronic stress and one pathway is by corticosterone signal. Although other experiments are necessary to assess whether catecholamines can also affect the liver or other peripheral oscillators, the phase shift caused by chronic stress could result in internal desynchronization among different tissues.
References


