Effects of stress on circadian organization

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Appendix I

English Summary

English Summary

Circadian rhythms are physiological, mental, and behavioral changes that are repeated over a roughly 24-hour cycle. This is one of the most striking features shared by all life forms on our planet. In mammals, almost every tissue or organ harbors circadian clocks. The one that resides in the neurons of the suprachiasmatic nucleus of the hypothalamus (SCN) is often referred to as the master clock or central clock, because it synchronizes circadian clocks in peripheral tissues and extra-SCN brain areas. Ambient light resets the SCN and synchronizes the master clock with the light-dark cycle in the external environment. The SCN in turn uses hormonal or neuronal signals to entrain the peripheral clocks, thereby keeping the whole endogenous mammalian clock system in pace with the external geophysical time.

A disruption of the circadian system can have adverse effects on performance, well-being, and health. Many stress-related disorders, such as psychiatric disorders, metabolic and cardiovascular diseases are often associated with abnormalities in sleep/wake cycles, body temperature patterns, and circulating hormone levels. This has motivated researchers to explore whether the circadian system is affected by stress, and whether stress-induced changes in circadian organization contributes to the development of stress-related disorders.

Previous studies including our own suggest that stress or stress hormones have little effect on the master clock in the SCN but can shift peripheral clocks (Meerlo et al., 2002; Ota et al., 2021). One explanation for this might be the fact that receptors for glucocorticoid stress hormones are abundant in most tissues but absent in the SCN (Rosenfeld et al., 1988; Balsalobre et al., 2000; Tahara et al., 2015). In order to further understand the effects of stress on peripheral clocks, in this thesis, we assessed (1) how social defeat stress affects the peripheral clocks, (2) what mechanisms are involved, and (3) if melatonin as an anti-stress hormone can counteract such effects.

In Chapter 2, we reviewed the current knowledge on the entrainment, properties, and functions of peripheral clocks. Independent circadian rhythms in mammalian peripheral tissues have been noticed in the 1960-70s of last century in various organs, including adrenal glands and liver. The first rhythmically expressed circadian genes outside of SCN were described by Balsalobre and colleagues in Ueli Schibler's lab in 1998. Now it is accepted that circadian clocks reside not only in the SCN but in almost all tissues or organs throughout the body.

The relationship between SCN and peripheral clocks is generally described in the "orchestra" model in which SCN behaves as a conductor and peripheral clocks as orchestra members. Daily light resets SCN, which in turn synchronizes the circadian clocks in peripheral tissues through a wide variety of indirect (behavioral rhythms, feeding, and temperature cycles) and more direct (autonomous nervous system, hormones) pathways.

In spite of the similar molecular makeup, the lasting times of self-sustained rhythms of in vitro cultures, the entraining cues, and some of the working mechanisms are different between SCN and the peripheral clock. In the last 10 years, the application of tissue-specific manipulations (up- and down-regulation) of gene expression technologies such as Cre-loxP, CRISPR, and RNAi, combining viral transgene delivery method, facilitate the understanding of the role of circadian clocks in local physiology. We reviewed the current knowledge of the effects of local tissue clock gene manipulation on the cardiovascular system, immune system, adrenals, liver, and hippocampus. Most of the studies focus on the complete knockout of certain clock genes, which is more like studying the function of clock genes themselves than examining what the rhythm changes of circadian clocks result in local physiology. Also, how the clock in one tissue affects another, or the physiological importance of phase alignment between circadian clocks in different tissues is not clear. Therefore, at the end of this review, we propose two important issues for future studies: (1) differentiate the "clock effect" from "non-clock effect" of the clock genes, (2) assess the communication of peripheral clocks, and how the integrity of the peripheral clock system affects local or systemic physiology.

In Chapter 3, we assessed whether peripheral clocks are differently affected by acute and chronic stress and whether this is time-of-day dependent and tissue specific. Adult male mice were subjected to acute (single) or chronic (10-day) social defeat stress either in the early or the late dark phase. One hour after the last stressor, mice were sacrificed and peripheral tissues (liver, lung, white adipose tissue, kidney) were collected for ex vivo assessment of PER2 rhythms. We found that chronic social defeat stress caused a phase delay of several hours in the rhythm of PER2 expression in the lung and kidney, but only when stress occurred in the late dark phase, not in the early dark phase. This suggests the effect of social defeat stress on peripheral clocks is dependent on the time of the day. PER2 rhythms in only the kidney and lung but not the liver and white adipose tissue were significantly affected by social defeat stress. This suggests the effect of social defeat stress on peripheral clocks is tissue-specific. In addition, the phase delays in the lung and kidneys after social defeat stress in the

late dark phase were much stronger after 10 days of repeated social defeat stress than after a single stress exposure (lung) or even non-significant after a single defeat (kidney). This suggests the shifts in peripheral clocks might be the consequence of a cumulative chronic stress effect. Overall, the findings in Chapter 3 suggest that chronic social defeat stress shifts peripheral circadian clocks in mice in a tissue-specific and time-of-day dependent fashion.

Stressors might affect the peripheral clock system by activating the hypothalamic-pituitary-adrenal (HPA) axis and stimulating the release of glucocorticoids from the adrenal cortex. However, no studies so far directly tested the role of endogenous GC in stress-induced phase shifts of peripheral clocks in vivo. Therefore, in Chapter 4 we examined the effect of social defeat stress on peripheral clocks in mice that were adrenalectomized to remove the main source of glucocorticoids. We found that the PER2 rhythm in the kidney cortex was phase delayed by over 4h following 5 daily social defeat stressors, and this delay was largely reversed by the removal of the adrenal glands. In the lung, PER2 rhythms displayed strong phase delays of ~8h after both stress and ADX, which might imply that both high peak levels of CORT and a lack of CORT affect the phase of the peripheral clock in this tissue. The liver clock was not responding to any of the GC manipulations, whether by stress, ADX, or additive CORT. Together the findings suggest that at least in the kidney, the induction of GC is clearly required in stress-induced phase shifts of peripheral clocks. The role of GC signaling in the lung is more complex. On the one hand, ADX itself can phase delay the PER2 rhythms in the lung which is comparable as seen after social defeat stress. On the other hand, stress did not further shift the delayed lung clock in ADX animals, suggesting a high possibility that GC is needed for stress to reset the peripheral clocks.

Melatonin is thought to be a molecule that the central pacemaker (SCN) uses to entrain the peripheral clocks, as well as an anti-stress hormone that interferes with hypothalamic–pituitary–adrenal (HPA) axis activity and glucocorticoid receptor (GR) translocation to the nucleus. In Chapter 5 we therefore assessed whether a rhythm in melatonin could prevent or reduce effects of stress on circadian organization. Unexpectedly, we did not see significant effects of rhythmic melatonin (peak at early dark phase) on PER2 rhythms in any tissues (pituitary, lung, kidney) examined in this experiment, nor did it affect the phase shifts of PER2 rhythms caused by repeated stress. In additional in vitro experiments, we add melatonin directly to cultured tissues, alone or in combination with dexamethasone. Again, neither the PER2 rhythms from naïve tissue nor from the dexamethasone-treated tissue were affected by melatonin.

Thus, results in Chapter 5 do not support the idea that melatonin could act as an anti-stress hormone in alleviating the stress effects on peripheral clocks. Also, it challenges the wide-accepted opinion that melatonin is the classic synchronizer used by SCN to entrain the peripheral clocks.

In conclusion, our studies suggest that the chronic social defeat stress phase-shifts peripheral circadian clocks in male mice in a tissue-specific and time-of-day dependent fashion and a cumulative manner. These stress effects appear to be mediated at least partly by activation of the HPA axis and release of glucocorticoids (corticosterone). Melatonin does not affect the peripheral clocks, nor does it reduce the effects of stress or stress hormones on peripheral clocks no matter *in vivo* or *in vitro.*

Appendix II

Nederlandse Samenvatting

Nederlandse samenvatting

Circadiane ritmes zijn fysiologische, mentale en gedragsmatige veranderingen die zich herhalen gedurende een cyclus van ongeveer 24 uur. Dit is een van de meest opmerkelijke kenmerken die voorkomen in alle levensvormen op onze planeet. Bijna alle weefsels en organen van zoogdieren hebben een circadiane klok. De klok in de neuronen van de suprachiasmatische nucleus (SCN) van de hypothalamus wordt vaak beschreven als de hoofdklok of centrale klok, die ritmiek in perifere klokken en hersengebieden buiten de SCN synchroniseert. Licht kan de SCN resetten en de SCN synchroniseren met de licht-donker cyclus van de externe omgeving. De SCN gebruikt hormonale en neuronale signalen om perifere klokken te entraineren. Hierbij wordt het hele interne klok systeem van zoogdieren gelijkgezet met de externe tijd.

Een verstoring van het circadiane systeem kan nadelige gevolgen hebben voor prestaties, welzijn en gezondheid. Veel stress-gerelateerde stoornissen, zoals psychiatrische stoornissen, metabole ziekten en hart- en vaat ziekten worden vaak geassocieerd met afwijkingen in slaap/waak ritmes, lichaamstemperatuur patronen en circulerende hormoon levels. Dit heeft wetenschappers gemotiveerd om onderzoek te doen naar het verband tussen het circadiane systeem en stress, en of veranderingen in het circadiane systeem die veroorzaakt worden door stress bijdragen aan de ontwikkeling van stress gerelateerde stoornissen. Voorafgaande studies suggereren dat stress en bijbehorende stress hormonen weinig effect hebben op de hoofdklok in de SCN maar dat het ervoor kan zorgen dat perifere klokken in fase verschuiven (Meerlo et al., 2002; Ota et al., 2021). Een verklaring hiervoor zou kunnen zijn dat de meeste weefsels receptoren bevatten voor glucocorticoïde stress hormonen maar deze receptoren zijn niet aanwezig in de SCN (Rosenfeld et al., 1988; Balsalobre et al., 2000; Tahara et al., 2015). Om de effecten van stress op perifere klokken verder in kaart te brengen hebben we in dit proefschrift onderzocht (1) hoe acute en chronische sociale stress perifere klokken kan beïnvloeden, (2) welke mechanismen hierbij betrokken zijn en (3) of melatonine als een anti-stress hormoon dergelijke effecten kan tegengaan.

In hoofdstuk 2 hebben we de huidige kennis over de eigenschappen en functies van perifere klokken en hoe deze gesynchroniseerd worden beschreven.

In de jaren 1960-70 van de afgelopen eeuw zijn voor het eerst onafhankelijke circadiane ritmes in perifere weefsels van zoogdieren beschreven in verschillende

organen zoals de bijnieren en de lever. De eerste ritmische circadiane genen buiten de SCN zijn beschreven in 1998 door Balsalobre en zijn collega's in het lab van Ueli Schibler. Tegenwoordig is duidelijk dat circadiane klokken voorkomen in bijna alle weefsels en organen van het lichaam.

Het verband tussen de SCN klok en perifere klokken wordt vaak beschreven als een 'orkest', waarbij de SCN functioneert als dirigent en de perifere klokken de orkestleden zijn. Licht kan de SCN resetten om vervolgens de circadiane klokken in perifere weefsels te synchroniseren via een breed scala aan directe (autonoom zenuwstelsel, hormonen) en indirecte (gedrags-, voedings- en temperatuurcycli) routes.

Ondanks dat de SCN en de perifere klokken eenzelfde soort moleculaire klok bevatten verschillen ze in hoe lang ze een zelfvoorzienend ritme kunnen onderhouden in *in vitro* cultures, en ook de signalen die voor synchronisatie zorgen zijn verschillend. In de afgelopen 10 jaar zijn er veel technieken ontwikkeld (zoals Cre-loxP, CRISPR, and RNAi) om weefselspecifieke manipulaties van genexpressie (omhoog of omlaag) uit te voeren. Deze technieken hebben eraan bijgedragen om de rol van circadiane klokken in lokale fysiologie beter te begrijpen. We hebben in hoofdstuk 2 de huidige kennis over de effecten van klok gen manipulaties in verschillende weefsels beschreven, alsmede de effecten hiervan op het hart- en vaat systeem, het afweersysteem, de bijnieren, lever en hippocampus. De meeste onderzoeken richten zich op het volledig uitschakelen van bepaalde klokgenen. Hierdoor kan de functie van een klokgen onderzocht worden, maar niet per se of het ritme van circadiane klokken en de lokale fysiologie hierdoor ook veranderen. Daarnaast is het niet duidelijk hoe de klok in een bepaald weefsel effect kan hebben op de klokken in ander weefsels. Ook het fysiologische belang van de fase koppeling tussen circadiane klokken in verschillende weefsels is niet duidelijk. Daarom beschrijven we aan het eind van dit hoofdstuk twee belangrijke suggesties voor toekomstige studies: (1) maak onderscheid tussen het 'klok effect' en het 'niet-klok effect' van klok genen, (2) stel de communicatie tussen perifere klokken vast en onderzoek hoe de integriteit van het perifere klok systeem de lokale of systemische fysiologie kan beïnvloeden.

In hoofdstuk 3 hebben we gekeken of perifere klokken verschillend beïnvloed worden door acute- en chronische stres en of dit effect weefselspecifiek en afhankelijk van de tijd van de dag is. Volwassen mannelijke muizen werden onderworpen aan een acute

(eenmalig) of een chronische (10 dagen) sociale stress vroeg of laat in de donker fase. De sociale stress bestond uit het plaatsen van de experimentele muizen in de kooi van een dominante soortgenoot, resulterend in een sociaal conflict en uiteindelijk een sociale nederlaag. Een uur na het laatste sociale conflict werden muizen opgeofferd en werden perifere weefsels (lever, long, wit vetweefsel) verzameld om vervolgens ex vivo PER2 ritmes te bekijken. We hebben ontdekt dat chronische sociale stress een fase verschuiving (vertraging) veroorzaakt van enkele uren in het PER2 ritme van zowel de longen als de nieren, maar alleen als de stress plaats vond aan het einde van de donker fase en niet aan het begin van de donker fase. Dit suggereert dat de effecten van sociale stress op perifere klokken tijdsafhankelijk is. PER2 ritmes in de nieren en de longen, maar niet in de lever en het witte vetweefsel zijn significant veranderd door sociale stress. Dit suggereert dat het effect van sociale stress op perifere klokken weefselspecifiek is. Daarnaast zijn de fase verschuivingen in de longen en nieren na sociale stress aan het eind van de donker fase veel groter na 10 dagen herhaalde sociale stres dan na een eenmalige sociale nederlaag (nier). Dit suggereert dat de verschuiving in perifere klokken het gevolg is van een geleidelijke opstapeling van chronische stress. In het algemeen laten de bevindingen in hoofdstuk 3 zien dat chronische sociale stress in muizen tot een verschuiving van perifere circadiane klokken leidt op een weefselspecifieke en tijdsafhankelijke wijze.

Stress factoren zouden een effect kunnen hebben op perifere klokken door activatie van de hypothalamus-hypofyse-bijnier-as en de daaruit voortkomende afgifte van glucocorticoïden uit de bijnierschors. Echter, tot dusver zijn er geen onderzoeken gedaan die de directe rol van endogene glucocorticoïden op de fase verschuiving van perifere klokken in vivo hebben onderzocht. Daarom hebben we in hoofdstuk 4 experimenteel onderzocht wat het effect van een sociale nederlaag op perifere klokken is in muizen waarvan de bijnieren verwijderd waren waardoor de belangrijkste bron van glucocorticoïde productie afwezig was. We vonden dat het PER2 ritme in de nierschors 4 uur verschoven (vertraagd) was na 5 dagen van sociale stress, maar deze verschuiving trad niet op in de muizen waarvan de bijnieren waren verwijderd. In de longen liet het PER2 ritme een sterke fase verschuiving (vertraging) van ongeveer 8 uur zien, niet alleen in de muizen die aan stress blootgesteld waren maar ook in controle muizen zonder bijnieren. Dit zou kunnen betekenen dat zowel hoge piekwaarden van corticosteron na stress als een gebrek aan corticosteron na verwijdering van bijnieren de fase van de perifere long klok kan verschuiven. De lever klok reageerde niet op de glucocorticoïden manipulaties, niet door geïnduceerde stress, nog door het verwijderen van de bijnieren. Tesamen suggereren deze bevindingen dat tenminste in de nier de verhoging van glucocorticoïden nodig is om de door stress-

geïnduceerde fase verschuiving van perifere klokken plaats te laten vinden. De rol van glucocorticoïden in de long is complexer. Aan de ene kant, kan het verwijderen van de bijnieren voor een fase verschuiving van het PER2 ritme leiden in de long, wat vergelijkbaar is met het effect van een sociale nederlaag. Aan de andere kant zorgt stress er niet voor dat de klok in de long verder verschuift in deze dieren. Dit suggereert dat glucocorticoïden waarschijnlijk nodig zijn om perifere klokken te resetten door middel van stress.

Melatonine wordt beschouwd als een molecuul dat de centrale pacemaker (SCN) gebruikt om perifere klokken te entraineren. Daarnaast wordt melatonine ook beschouwd als een anti-stres hormoon dat kan interfereren met de hypothalamus-hypofyse-bijnier-as en met de verplaatsing van glucocorticoïde receptors naar de celkern. In hoofdstuk 5 hebben we daarom onderzocht of een ritme in melatonine effecten van stress op het circadiane systeem kan verminderen of voorkomen. Het was onverwacht dat we geen significante effecten van het melatonine ritme (met een piek vroeg in de donker fase) op PER2 ritmes in verschillende weefsels (hypofyse, long, nier) zagen in dit experiment. Ook had het melatonine ritme geen invloed op de stress-geïnduceerde fase verschuiving van de PER2 ritmes in de weefsels. Vervolgens hebben we nog een *in vitro* experiment uitgevoerd waarin we melatonine of dexamethason toevoegden aan verschillende weefsel cultures. Ook hier was er geen effect van melatonine op de PER2 ritmes. De resultaten in hoofdstuk 5 ondersteunen dus niet de hypothese dat melatonine als een anti-stress hormoon werkt doormiddel van het verminderen van stress-geïnduceerde effecten op perifere klokken. Daarnaast stelt dit het algemeen aanvaarde standpunt ter discussie dat melatonine door de SCN wordt gebruikt om perifere klokken te entraineren.

Concluderend suggereren onze studies dat chronisch sociale stress kan leiden tot fase verschuivingen van perifere circadiane klokken in mannelijke muizen op een weefsel-specifieke en tijdsafhankelijke manier. De effecten van stress lijken deels gemedieerd te worden door activering van de hypothalamus-hypofyse-bijnier-as en door afgifte van glucocorticoïden (corticosteron). Melatonine heeft geen effect of perifere klokken en het zorgt niet voor een vermindering van de effecten van stres of stress hormonen op perifere klokken zowel *in vivo* als *in vitro*.

Appendix III

Curriculum Vitae

Curriculum Vitae

I was born on 15th April 1988 in Puyang, China. I love reading, thinking and am always fascinated by human brains. I obtained my bachelor's degree in clinic medicine in 2010 at Lanzhou University. In the same year, I joined the Neuroscience Institute of Lanzhou University to study sleep and wake mechanisms, and obtained my master's degree three years later (2013). During this period, I assessed the effect of a novel neuropeptide (neuropeptide S) on an anesthetics-induced sleep state, supervised by Prof. Yiping Hou. In July 2013, I started my career as a human anatomy teacher at Hunan Norman University, Changsha, China. In November 2018. I was accepted in the laboratory of Prof. Roelof A. Hut, co-supervised by Prof. Peter Meerlo, at the Groningen Institute for Evolutionary Life Sciences (GELIFES), the University of Groningen, the Netherlands. During my Ph.D., I examined how social defeat stress or stress hormones affected the circadian system in mice. All my work conducted during my Ph.D. are present in this thesis.

Employment history
July 2013 -Nov 2018
Lecturer, School of Medicine at Hunan Normal University, Changsha, China
Sep 2017- Dec 2017
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Education
Nov 2018- March 2023
Ph.D. University of Groningen, Groningen, the Netherlands
Sep 2010 - June 2013
Master of Medicine, Lanzhou University, Lanzhou, China
June 2009 - March 2010
Intern, the first hospital of Lanzhou University, Lanzhou, China
Sep 2005- June 2010
Bachelor of Clinical Medicine, Lanzhou University, Lanzhou, China

Publications

1. **Kong X**, Luxwolda M, Hut RA, Meerlo P. Adrenalectomy prevents the effects of social defeat stress on PER2 rhythms in some peripheral tissues in male mice. Hormones and Behavior 150 (2023) 105326
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3. Ota SM, **Kong X,** Hut R, Suchecki D, and Meerlo P The impact of stress and stress hormones on endogenous clocks and circadian rhythms. Front Neuroendocrinol, 2021; 63:100931

In submitting

1. **Xiangpan Kong**, Peter Meerlo, Roelof A. Hut. Melatonin does not prevent the social-defeat-stress induced phase shifts of peripheral clocks.
2. **Xiangpan Kong**, Roelof A. Hut. Peter Meerlo. The entrainment, properties and functions of peripheral clocks.

Appendix IV

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I especially thank CSC and Hunan Normal University, without whom I cannot start my Ph.D. study in the Netherlands. And actually, it was because of your sponsorship that I could visit UCLA in the US in 2017, which was an eye-opening and life-changing experience and made me set up my mind to start a Ph.D. life and pursue neuroscience academia. All the stories related to my Ph.D. life would not happen without that journey.

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