"Circadian rhythms in Djungarian hamsters (*Phodopus sungorus*) with an attenuated ability to synchronize"

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ADDITIONAL NOTE ON THIS ISSUE

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SUMMARY

A number of Djungarian hamsters (*Phodopus sungorus*, Pallas 1773) bred at the Zoology Institute of the University of Halle show aberrations in their daily patterns of locomotor activity, leading to a distinction between three different circadian phenotypes. Wild type (WT) hamsters display robust nocturnal rhythms of locomotor activity according to the ambient light/dark (LD) conditions, i.e. the activity onset is stably coupled to "light-off" and the activity offset is stably coupled to "light-on". In contrast to this behavior, the activity onset is continuously delayed in hamsters designated as DAO (delayed activity onset) phenotype. Since the activity offset remains coupled to "light-on", the activity period (α) in those hamsters becomes compressed up to a critical value of 3:02 ± 0:12 h. Exceeding the critical value leads to free-running activity rhythms for a certain time period, despite animals being kept in a LD photocycle. Finally, the rhythm breaks down and hamsters show arrhythmic activity patterns, hence characterized as the arrhythmic (AR) phenotype. Preliminary results revealed that hamsters of the DAO phenotype are characterized by a diminished ability to synchronize with its periodic zeitgeber (i.e. the LD cycle) and the aim of the thesis was therefore to identify the origin and underlying mechanism of this phenomenon.

Investigations of further markers of the circadian pacemaker beside the locomotor activity rhythm, more precisely the circadian body temperature and 6-sulfatoxymelatonin rhythm, have revealed that all three markers of the circadian clock, which is the suprachiasmatic nucleus (SCN) in mammals, show similar patterns according to the rhythmic phenotype (Chapters IV + V). Thus, the signal coding for the rhythmic phenotype must arise from the SCN.

Experiments have been conducted to evaluate intrinsic properties of the circadian system in DAO and WT hamsters as well as its interaction with the exogenous zeitgeber. Though general features of the free-running rhythms in DAO and WT hamsters were similar when animals were kept under constant darkness, the free-running period (tau) was significantly longer in the DAO phenotype (Chapter II). However, the longer tau in DAO hamsters cannot be taken as only reason for the delayed activity onset. Particularly, the resetting mechanism of the circadian clock as a function of the LD-zeitgeber became the focus of interest, as these should compensate the daily deviation from the 24-h day caused by tau > 24 h. Clearly, the interaction of light with the pacemaker, and in particular the non-parametric effect of light, has been identified as an important clue to the underlying mechanism of the DAO phenomenon, which has been demonstrated by investigation of phase responses following

brief light pulses in the early and late subjective night (Chapter II). Phase advances of the activity onset and offset following brief light pulses in the late subjective night were significantly smaller in DAO hamsters, despite the longer tau. As a consequence, the overall phase response for the activity onset in DAO animals is insufficient to compensate the long tau, thus leading to its delay (Chapter II). This was confirmed by a phase response curve (PRC Aschoff type VI) constructed when animals were kept under a LD cycle (Chapter III).

A diminished sensitivity to light has been proposed as a possible reason for the reduced phase response and with that the delayed activity onset in DAO hamsters. Particularly, the different reactions of DAO and WT hamsters to low constant light emphasize that the sensitivity to light is altered in DAO animals (Chapter II). Furthermore, the results of the pineal melatonin investigation support the assumption that the sensitivity to light is reduced in DAO hamsters, as the melatonin decline at the beginning of light phase was slightly less than in WT hamsters (Chapter V). Since the amount of Fos immuno-reactive cells, however, was similar between DAO and WT hamsters, functionality of light reception and afferent signal transduction seems not to be compromised in DAO hamsters (Chapter VI).

In summary, the results point to differences in key characteristics of the circadian system between DAO and WT hamsters, namely the free-running period on the one hand and the interaction of the LD cycle with the circadian system, which is altered in DAO hamsters, on the other. On the basis of the studies on the body temperature and 6-sulfatoxymelatonin rhythms, as well as c-Fos expression in the SCN following photic stimulation, it was possible to localize the SCN itself as the origin of the phenomenon. Therefore, the interplay between free-running period and resetting of the circadian pacemaker by photic cues, particularly by phase advances, seems to be the crucial factor determining the circadian phenotype in Djungarian hamsters of our breeding stock. One reason seems to be a reduced sensitivity to light in DAO hamsters. However, since the perception and transduction of the photic signal to the SCN does not seem to be constrained, downstream processes within the SCN that use light information to reset the circadian pacemaker have to be taken into account as a possible origin of the signal coding for the DAO phenomenon. In AR hamsters, the SCN produces no circadian signal and this is evidence in favor of the hypothesis that the mechanism for rhythm generation is defective in these animals. Thus, hamsters of the WT, DAO and AR phenotype provide an excellent model to study the underlying molecular mechanisms of photic entrainment with special regard to light-induced resetting of the circadian pacemaker and the two-oscillator theory of entrainment.

Key words:
Circadian rhythms; Djungarian hamsters; Delayed activity onset; Free-running period; Disturbed photic entrainment; Body temperature rhythm; Melatonin rhythm; C-Fos expression; SCN; Arrhythmic hamsters

ZUSAMMENFASSUNG

In Dsungarischen Hamstern (*Phodopus sungorus*, Pallas 1773) aus der Zucht des Instituts für Biologie/Zoologie der Martin-Luther-Universität Halle wurde eine bestimmte Anzahl von Hamstern beobachtet, welche Auffälligkeiten in ihrem Tagesmuster der lokomotorischen Aktivität aufwiesen. Anhand der beobachteten Muster kann zwischen drei circadianen Phänotypen unterschieden werden: der Wildtyp (WT) zeigt robuste Rhythmen nächtlicher Aktivität entsprechend der Licht/Dunkel-Bedingungen unter denen die Tiere gehalten werden. Das heißt, der Aktivitätsbeginn ist stabil an „Licht-aus" und das Aktivitätsende stabil an „Licht-an" gekoppelt. Bei Hamstern des DAO (delayed activity onset)-Phänotyps hingegen ist der Aktivitätsbeginn kontinuierlich verzögert. Da das Aktivitätsende weiterhin stabil an „Licht-an" gekoppelt ist, verkürzt sich die Aktivitätszeit zunehmend bis ein kritischer Wert von 3:02 ± 0:12 h erreicht wird. Ein Unterschreiten der kritischen Aktivitätsdauer führt zu freilaufenden Aktivitätsrhythmen und letztlich zum Zusammenbruch des Rhythmus. Die Hamster zeigen dann nur noch arhythmische Muster und werden dementsprechend als AR-Phänotyp bezeichnet. Erste Untersuchungen konnten zeigen, dass DAO-Hamster eine verminderte Synchronisationsfähigkeit gegenüber ihrem Hauptzeitgeber, dem Licht/Dunkel-Wechsel, aufweisen. Ziel der Promotionsarbeit war nun zum einen die Lokalisation des Ursprungs des DAO-Phänomens und zum anderen erste zugrunde liegende Mechanismen, welche zur kontinuierlichen Verzögerung des Aktivitätsbeginnes in DAO-Hamstern beitragen, zu identifizieren.

Die Untersuchung weiterer Marker des circadianen Schrittmachers neben dem Aktivitätsrhythmus, speziell dem Körpertemperatur- und Sulfatoxymelatonin-Rhythmus, haben gezeigt, dass alle drei Marker der circadianen Uhr, welche der Suprachiasmatische Nukleus (SCN) bei Säugern ist, ähnliche Muster entsprechend des circadianen Phänotyps zeigen (Kapitel IV + V). Anhand dieser Ergebnisse kann man ableiten, dass der SCN selbst die rhythmischen Signale für den jeweiligen Phänotyp generiert.

Weiterhin wurden Experimente durchgeführt, um sowohl Eigenschaften des circadianen Systems als auch seiner Interaktion mit dem Hauptzeitgeber in DAO- und WT-Hamstern zu untersuchen. Obwohl sich beide Phänotypen im Freilaufverhalten unter konstanten Bedingungen (Dauerdunkel) nicht wesentlich voneinander unterschieden, war die Spontanperiode (Tau) in DAO-Hamstern signifikant länger (Kapitel II). Trotzdem kann die längere Spontanperiode nicht allein ursächlich für den verzögerten Aktivitätsbeginn sein, da speziell die Rückstellungsmechanismen des SCN in Abhängigkeit vom Licht/Dunkel-

Zeitgeber die durch die lange Periodendauer hervorgerufene tägliche Abweichung von 24 Stunden kompensieren sollten. Die Ergebnisse der Versuche aus Kapitel II haben jedoch gezeigt, dass die Interaktion des circadianen Schrittmachers mit Licht, speziell den nicht-parametrischen Effekten von Licht, wesentlich an dem Zustandekommen des verzögerten Aktivitätsbeginnes in DAO-Hamstern beteiligt ist. Dies wurde durch kurze Lichtpulse in der frühen und späten subjektiven Nacht (Aktivitätsphase) ermittelt (Kapitel II). So konnte gezeigt werden, dass trotz der längeren Spontanperiode die phasenvorverlagernden Effekte von Licht in der späten subjektiven Nacht in DAO-Hamstern signifikant geringer war als im WT Phänotyp. In der Konsequenz führt dies dazu, dass speziell die Phasenantwort des Aktivitätsbeginnes in DAO-Hamstern nicht mehr ausreicht, die lange Spontanperiode zu kompensieren und dies letztlich zur kontinuierlichen Verzögerung des Aktivitätsbeginnes führt (Kapitel II). Eine Bestätigung dieser Hypothese lieferten die Ergebnisse einer Phasenantwortkurve nach dem Aschoff Typ VI-Protokoll (Kapitel III).

Eine verminderte Lichtsensitivität des circadianen Systems in DAO-Hamstern wurde als Ursache für die geringere Phasenantwort und damit der Verzögerung des Aktivitätsbeginnes in Hamstern des DAO-Phänotyps postuliert. Grund der Annahme waren die unterschiedliche Reaktionen des DAO- und WT-Phänotyps im Dauerlicht von geringer Intensität (Kapitel II) und das leicht verzögerte Ende der Melatoninsynthese im Pineal von DAO- Hamstern in der Lichtphase (Kapitel V). Den Ergebnissen der c-Fos Untersuchung zufolge beruht diese verringerte Sensitivität aber nicht auf einer beeinträchtigten Perzeption von Lichtsignalen in der Retina sowie deren Weiterleitung zum SCN, sonder eher auf eine Weiterverarbeitung dieser Signale im SCN selbst (Chapter VI).

Zusammenfassend kann also festgehalten werden, dass sich DAO- und WT Hamstern in zwei Komponenten des circadianen Systems unterscheiden, die wesentlich an der Synchronisation circadianer Rhythmen beteiligt sind, nämlich der Spontanperiode und der Interaktion des LD-Wechsels mit dem circadianen Schrittmacher. Anhand der Untersuchungen zur Tages-rhythmik von Körpertemperatur und Sulfatoxymelatonin sowie der c-Fos Expression im SCN konnte der circadiane Schrittmacher als Ursprungsort des DAO-Phänomens lokalisiert werden. Damit scheint das Zusammenspiel zwischen Periodenlänge und entsprechender Phasenantwort des SCN durch Lichtreize, speziell der Phasenvorverlagerung, ein entscheidender Faktor für das Zustandekommen des DAO-Phänotyps in unserer Zuchtlinie. Ursache dafür scheint eine verminderte Lichtempfindlichkeit des circadianen Systems zu sein, die aber nicht auf einer fehlerhaften Lichtperzeption und –transduktion zum SCN, sondern möglicherweise auf nachgeschalteten Prozessen im SCN selbst beruht, welche die

Lichtinformationen zur Einstellung des circadianen Schrittmachers nutzen und zum Auftreten des fehlerhaften Signals in DAO-Hamstern führt. In arhythmischen Hamstern hingegen scheint der SCN kein rhythmisches Signal mehr zu produzieren, was ein Hinweis darauf ist, dass die Mechanismen der Rhythmusgenerierung in diesen Tieren defekt sind. Damit stellen die Hamster aller drei Phänotypen ein exzellentes Modell dar, um die zugrunde liegenden Mechanismen der photischen Synchronisation und der Zwei-Oszillatoren-Theorie im SCN auf molekularer Ebene weiter zu untersuchen.

Stichwörter:
Circadiane Rhythmen; Dsungarische Hamster; Verzögerter Aktivitätsbeginn; Spontanperiode; Gestörte photische Synchronisation; Körpertemperaturrhythmus; Melatoninrhythmus; C-Fos Expression; SCN; Arhythmische Hamster

CHAPTER I

GENERAL INTRODUCTION

The adaptation to temporal changes of the geophysical environment is one of the most striking challenges which almost all living organisms are confronted with. Natural selection led to the evolution of biological clocks to match predictable changes of the environment caused by the Earth's rotation and planetary movements. This enables organisms to anticipate rather than passively respond to periodic environmental variations and guaranties optimal timing of metabolism, physiology and behavior (Paranjpe and Sharma, 2005; Sharma, 2003a). The most ubiquitous biological rhythm is the circadian rhythm, which has been found in a variety of organisms including bacteria, insects, mammals and plants (Sharma, 2003a). Circadian rhythms are self-sustained and persist with a period close to 24 h in the absence of cyclic environmental changes (Aschoff, 1965a). This property reflects the existence of an endogenous circadian pacemaker or clock, which is believed to have evolved from the selection pressure of environmental periodicities caused by changes of the solar day (Sharma and Chandrashekaran, 2005). Since the inherent period (tau) of the circadian pacemaker deviates from the exact 24-h environment, it has to be corrected or synchronized by environmental cues, so-called zeitgebers (Aschoff, 1960). The daily light/dark (LD) cycle is the major zeitgeber to which the circadian system entrains (Roenneberg and Foster, 1997; Sharma and Chandrashekaran, 2005). In addition to the photic zeitgeber, non-photic zeitgebers can entrain circadian clocks. Though being temperature compensated, it has been shown that circadian clocks entrain to temperature cycles (Aschoff and Tokura, 1986; Liu et al., 1998; Rajaratnam and Redman, 1998). Also, food availability, behavioral feedback and social cues can act as non-photic zeitgebers for the circadian system, whereas it is necessary to note that the influence varies greatly between non-mammal and mammal species (Challet and Mendoza, 2010; Mrosovsky, 1988; Reebs and Mrosovsky, 1989; Sharma and Chandrashekaran, 2005).

Entrainment is achieved by resetting mechanisms of the circadian clock to establish a stable phase relationship (phase angle) between the endogenous circadian pacemaker and the entraining stimulus (Johnson et al., 2003). This is an inevitable prerequisite for proper adaptation to external periodic alterations not only on a daily, but also on a seasonal basis (Goldman, 2001). It is believed that circadian clocks have an adaptive significance in order to gain fitness advantages particularly under natural conditions: firstly, to provide an internal

temporal order to coordinate various metabolic processes and, secondly, to provide the right time for daily and seasonal physiological and behavioral events (Sharma, 2003a). Empirical evidence, however, is admittedly limited. The persistence of circadian clocks in animals permanently living in constant environments provides a good basis for the intrinsic adaptive hypothesis (Schöttner et al., 2006; Sheeba et al., 2002; Trajano and MennaBarreto, 1996). This is supported by the fact that the expression of genes controlling metabolism is regulated by the circadian clockwork itself (Hatanaka et al., 2010). Nevertheless, clear evidences concerning an increase of fitness is still awaited. Empirical evidence in favor of a fitness advantage come from circadian resonance studies, indicating that a similar frequency of a zeitgeber cycle and the intrinsic period of an organism contribute to longevity (von Saint Paul and Aschoff, 1978) or reproductive fitness (Ouyang et al., 1998). Studies under natural conditions point to an increase in individual fitness in animals with intact pacemakers compared to animals with destroyed clocks, by enabling the intact animals to avoid the risk of predation (DeCoursey et al., 2000), thus supporting the hypothesis of an extrinsic adaptive advantage. Also, the adaptation to annual cycles, which is essential for seasonal species and increases the individual fitness, is mediated by the circadian system and may even represent a strong selection pressure for accurate daily timing (Hut and Beersma, 2011). On the other hand, the adaptive value was questioned when animals with disturbed daily rhythms were investigated under laboratory conditions, revealing that they did not necessarily lack benefits conducive to survival when compared to the wild type (Ruby et al., 1998; Vitaterna et al., 1994).

In this context, investigations on Djungarian hamsters (*Phodopus sungorus*, Pallas 1773) became the focus of interest when it was shown that a certain number of animals posses specific characteristics which seem incompatible with stable entrainment of the circadian system. The Djungarian hamster is a nocturnal and highly seasonal species that has been used mainly in laboratory studies to investigate photoperiodic time measurement associated with seasonal adaptation (Figala et al., 1973; Hoffmann, 1982; Milette and Turek, 1986; Palchykova et al., 2003; Puchalski and Lynch, 1994; Scherbarth et al., 2007; Steinlechner and Heldmaier, 1982). The significance of Djungarian hamsters as research animals to investigate seasonal rhythmicity arises from the natural environment they inhabit, one that requires precise timing of daily and seasonal events for survival. They naturally occur in Steppes and semi-deserts of central and eastern Kazakhstan and south-western Siberia, regions that are characterized by extreme changes in environmental conditions, particularly in ambient temperature, food and water availability (Feoktistova, 2008). Thus, it is expected that a robust

and properly functioning circadian system will be found in hamsters of this species. Hence, it was a surprising result in studies of Puchalski and co-workes (1986, 1988) when they found that animals in their breeding stock did not react to changes of the photoperiod. These animals, so-called non-responders, did not decompress the activity time nor undergo gonadal regression when transferred from a long-day to short-day photoperiod (Puchalski and Lynch, 1986, 1988). Though non-responsiveness may have fitness benefits by lengthening the time of reproduction, it arises from a failure in keeping up a stable phase angle of entrainment under short-day conditions. As a consequence, the correct signal of day length cannot be transmitted to the effector systems (Gorman and Zucker, 1997; Prendergast et al., 2001). These results focused investigations on daily rhythms in Djungarian hamsters and revealed further peculiarities in the circadian system in this species. A number of hamsters failed to re-entrain to phase shifts of the photocycle and showed freerunning or arrhythmic patterns instead (Ruby et al., 1996). Arrhytmicity in a large fraction of Djungarian hamsters was also induced when they were exposed to two successive light pulses during the scotophase. The authors of that study concluded that the Djungarian hamster is a species with a labile circadian pacemaker (Steinlechner et al., 2002), which is a rather unexpected outcome. This assumption, however, was supported by comparative investigations on circadian activity rhythms in hamsters of all three species of the genus *Phodopus* at the Zoology Institute in Halle. It was shown that Djungarian hamsters are characterized by a considerably higher instability of circadian activity rhythms under laboratory conditions compared to the other two species (Weinert et al., 2009). Moreover, a number of Djungarian hamsters showed aberrations in their daily patterns of locomotor activity, leading to a distinction between three different circadian phenotypes (Weinert and Schöttner, 2007). Wild type (WT) hamsters display robust activity rhythms according to the ambient LD conditions, i.e. the activity onset is stably coupled to "light-off" and the activity offset is stably coupled to "light-on". In contrast to this behavior, the activity onset is continuously delayed in hamsters designated as DAO (delayed activity onset) phenotype. Since the activity offset remains coupled to "light-on", the activity period (α) in those hamsters becomes compressed up to a critical value of 3:02 ± 0:12 h. Exceeding the critical value leads to free-running activity rhythms for a certain time period, despite animals being kept in a LD photocycle. Finally, the rhythm breaks down and hamsters show arrhythmic activity patterns, hence characterized as the arrhythmic (AR) phenotype. In some instances, DAO hamsters become arrhythmic immediately, presumably having passed the critical value of α (Weinert and Schöttner, 2007). The distinctive phenotypical characteristics in connection with specific properties of the circadian system in the DAO hamsters clearly

distinguish it from other phenomenon like the non-responders and offer a unique opportunity to gain new insights into the mechanism regarding generation and synchronization of circadian rhythms (Weinert and Schöttner, 2007). By a specific breeding program, it was possible to establish a breeding colony of DAO hamsters at the institute of Halle, and so to gain a sufficient number of animals to allow investigations of this phenomenon. First experiments revealed that hamsters of the DAO phenotype have a significant longer tau under constant darkness (DD) compared to WT hamsters. Also, preliminary results pointed to a diminished phase response to photic stimulation in the late subjective night in DAO hamsters (Weinert and Schöttner, 2007). Thus, two key properties of the circadian system associated with entrainment to a periodic environment (i.e. to establish a stable phase relation) seemed to be involved. Their importance becomes clear when considering the concepts that have been proposed to explain the mechanisms of photic entrainment: the discrete (non-parametric) and the continuous (parametric) model. The first concept proposes a phasic response of the pacemaker to light. Adjustment of the rhythm is achieved by instantaneous phase shifts following photic stimulation around the transients from light to dark and vice versa, either by phase delays or phase advances, and compensate for the difference between the period of the endogenous pacemaker and the entraining zeitgeber cycle (Pittendrigh, 1981; Pittendrigh and Daan, 1976a). According to this model, entrainment is a function of the rhythm's period and shape of the phase response curve (PRC), by which the magnitude and direction of phase shifts that depend on the circadian phase are produced (Aschoff, 1965b; Pittendrigh, 1981; Pittendrigh and Daan, 1976a). During the subjective day (i.e. the resting period), brief light pulses have no marked effect on the rhythm's phase. During the subjective night (i.e. the activity period), however, light pulses phase delay the activity rhythm in the first half while they will phase advance it during second half. On the other hand, the concept of parametric or tonic entrainment proceeds from the assumption of a continuous change in the pacemaker's velocity in response to light (Daan and Aschoff, 2001). This concept proposes changes in light intensity cause phase specific accelerations or decelerations of the pacemaker, thereby adjusting its intrinsic period to that of the environmental zeitgeber cycle. Phase-dependent changes of the angular velocity of the pacemaker can be depicted by so-called velocity response curves (VRCs), estimated from the PRC. Thus, the shape of the VRC is similar to that of the PRC, whereby the delay and advance region corresponds to the region when the clock slows down or speeds up, respectively. In fact, both processes are involved in the synchronization of circadian rhythms and depend on all three key properties of the circadian system to gain maximum stability: the period, the VRC and the PRC (Beersma et al., 1999;

Sharma, 2003b; Taylor et al., 2010). Therefore, parametric and non-parametric effects of light on daily activity rhythms in DAO and WT hamsters were investigated in order to characterize general properties and the functionality of the circadian system in both phenotypes (Chapter II). The focus was on the investigation of tau under constant lighting conditions of different intensities, and on the phase and period responses of the circadian pacemaker following light pulses in the early and late subjective night. These investigations were also designated to study intrinsic properties of the pacemaker in DAO and WT hamsters, particularly in connection with the two oscillator model for activity rhythms proposed by Pittendrigh and Daan (1976b). In brief, two mutually coupled oscillators with different responses to light drive activity rhythms, one of which is decelerated by light and tracks dusk (evening oscillator, E) whereas the other that is accelerated by light and tracks dawn (morning oscillator, M). Though evidence in favor of this model came from behavioral, electrophysiological and molecular studies (Daan et al., 2001; Jagota et al., 2000; Pittendrigh, 1981; Pittendrigh and Daan, 1976b), the overall validity of the concept is still under debate (Helfrich-Forster, 2009). The investigations may therefore contribute to a better understanding of the pacemaker structure in DAO and WT hamsters and will help to establish a "model" organism to study this specific issue in more detail.

The properties of the pacemaker (tau, PRC) as well as the properties of the zeitgeber cycle (period, zeitgeber strength, LD ratio) define the phase angle between the biological rhythm and the entraining stimulus, which is a key determinant of entrainment (Pittendrigh and Daan, 1976a). The time taken to re-establish a stable phase relationship following changes of this angle will shed light on the general capability of the circadian system to entrain to the corresponding zeitgeber cycle (Aschoff et al., 1975). For this purpose, experiments have been conducted to study re-entrainment behavior following phase shifts of the LD cycle; the aim has been to evaluate possible consequences of the DAO phenomenon concerning the adjustment of the circadian rhythm to environmental changes (Chapter III). In this context, a PRC according to the Aschoff type VI protocol (Aschoff, 1965b), i.e. when animals were kept under a LD cycle, was constructed to enhance the understanding of the resetting processes associated with entrainment of the circadian system and to get insights into the underlying mechanism of the DAO phenomenon. However, since these experiments will help to determine properties of the circadian system in DAO and WT hamsters by a more mechanistic approach, they will not necessarily identify the exact origin of the attenuated ability to synchronize, since this can be located in varying elements involved in circadian organization.

Generally, the circadian system comprises three fundamental components: a component upstream of the pacemaker and mediating the entraining signal, the circadian pacemaker itself and a downstream component, to convey the output signals of the pacemaker to the corresponding effector systems (Moore, 1996). In mammals, the site of the master circadian clock is the suprachiasmatic nucleus (SCN), a bilateral structure located in the anterior hypothalamus dorsal to the optic chiasm (Reuss, 1996). The SCN consists of approximately 20000 neurons, many of which act as single cell oscillators of different function that produce circadian rhythms on the basis of molecular transcriptional-translational feedback loops (Reppert and Weaver, 2002). The clockwork consists of a core negative feedback loop by which the positively acting heterodimeric transcriptional factor Clock/Bmal1 enhances the expression of *Period* (*per1*, *per2*) and *Cryptochrome* (*cry1*, *cry2*) genes during the circadian day (Bunger et al., 2000; Gekakis et al., 1998; Hogenesch et al., 1998; King et al., 1997). The proteins produced in turn form negatively acting Per/Cry dimers and repress their own transcription several hours later by inhibiting the activity of Clock/Bmal1 (Lee et al., 2001). Subsequent Per/Cry degradation during the circadian night leads to the reactivation of *per* and *cry* gene expression (Busino et al., 2007; Reischl et al., 2007; Shirogane et al., 2005), thereby starting a new circadian cycle. A second feedback loop involves the orphan nuclear receptors Rora and Rev-erbα (Preitner et al., 2002), whose expression is also regulated by Clock/Bmal1. Whereas Rora activates *bmal1* transcription, it is repressed by Rev-erbα, thereby contributing to robustness and precision of the clock (Welsh et al., 2010). Beside the expression of genes involved in the core clock mechanism described above, other (downstream) genes are regulated directly and indirectly by the circadian clock (Lowrey and Takahashi, 2004). Although these clock-controlled genes (CCGs) have no critical relevance for the function of the core clockwork, they are important in regulating metabolism and physiology of the cell and are involved in various output pathways (Hatanaka et al., 2010; Panda and Hogenesch, 2004; Panda et al., 2002; Ueda et al., 2002).

Neurons inside the SCN are not uniform and give rise to clusters of cellular and functional heterogeneity (Antle and Silver, 2005; Lee et al., 2003; Van Esseveldt et al., 2000). Two main subdivisions have been distinguished based on neuropeptide expression, afferent signal transduction and gene expression: the ventrolateral “core” region and the dorsomedial “shell” region (Morin, 2007). In the most general sense, the SCN is composed of a non-rhythmic, retino-recipient core region that expresses genes following photic stimulation and relays photic information to the intrinsically rhythmic, but light-non-responsive shell subdivision which, in turn, transmits efferent signals to targets downstream from the SCN. The role of the

SCN core is crucial for two matters, the regulation of photoresponsiveness to photic stimulation of the clock and maintaining coupling within SCN neurons, particularly of the SCN shell (Welsh et al., 2010; Yan et al., 2007). According to a model proposed by Antle and co-workers (2003), a SCN oscillatory network comprises the core region coordinating rhythmicity of independent oscillators in the shell. In turn, synchronized shell oscillators provide feedback signals to regulate the activity of core cells in terms of adjusting their sensitivity to light (Antle et al., 2003).

The SCN perceives photic information through photoreceptors in the retina. Photosensitive retinal ganglion cells, which express the photopigment melanopsin, send their projections via the monosynaptic retinohypothalamic tract (RHT) to the SCN (Rollag et al., 2003). Glutamate, aspartate and pituitary adenylate cyclase-activating protein (PACAP) are the major neurotransmitters by which the photic signal is conveyed directly from the RHT to the SCN (Chen et al., 1999; Ebling, 1996; Fahrenkrug, 2006; Hannibal, 2002). Indirect photic information is relayed from RHT projections via the intergeniculate leaflet (IGL) and geniculohypothalamic tract (GHT) to the SCN, mediated by neuropeptide Y (NPY) and gamma-aminobutyric acid (GABA) (Moore and Card, 1994). Both direct and indirect photic signals seem to be relevant to fine-tune the reaction of the SCN to light (Dibner et al., 2010; Van Esseveldt et al., 2000).

As described above, photic stimulation is the most significant signal for synchronizing the circadian clock and is mainly mediated by the RHT innervations passing to the SCN core. Neurotransmitter release by RHT axons at synaptic contacts with SCN core neurons triggers a number of signal transduction cascades, leading finally to gene expression (e.g. immediate early gene *c-fos* and clock genes *per1* and *per2*) (Golombek and Rosenstein, 2010; Welsh et al., 2010). Beside *per* genes, which mediate the phase shifting effects of light, special importance is attached also to immediate early genes (IEGs). The induction of these transcriptional factors provides an internal reaction of the neuronal cell by mediating short-term external signals like photic stimulation to long term responses (Sheng and Greenberg, 1990). On particular, *c-fos* has been recognized as a good marker for estimating photo-responsiveness and light sensitivity of the circadian clock (Caputto and Guido, 2000). As early experiments upon the DAO hamsters revealed that the interaction of the LD cycle with the SCN might involve such a phenomenon (Weinert and Schöttner, 2007), the expression of Fos-protein in the SCN following photic stimulation was examined in DAO and WT hamsters (Chapter VI). This investigation will allow conclusions regarding whether the attenuated ability to synchronize in DAO hamsters might be located in the upstream component of the

circadian system to be drawn. More precisely, it will help assess whether the reception and transmission of photic signals to the SCN may be impaired, thus leading to insufficient zeitgeber strength of the photic signal to entrain properly the circadian system in DAO hamsters. The experiment will also provide insights into the spatio-temporal pattern of Fos expression and thereby will give the first information about the functionality of the SCN in DAO hamsters.

However, in order to evaluate the functionality of the SCN as a possible origin of the DAO phenomenon, it is absolutely essential to assure that the signal coding for the phenotype does, indeed, arise from the circadian pacemaker and is not a consequence of modulation at effector sites downstream from the SCN. Therefore, an investigation of further markers of the SCN output in addition to locomotor activity is crucial, since the efferent signal relay pathway, particularly for activity rhythms, is rather complex and may provide targets for subsequent alteration. The SCN output is mediated primarily by neuronal and to a lesser extent by humoral signals. The hypothalamus, beside the thalamus and basal forebrain, is the main target of SCN efferents. Axons of the SCN densely innervate the subparaventricular zone (SPZ) and as well as the dorsomedial nucleus of the hypothalamus (DMH), the preoptic area (PAO) and the arcuate nuclues (ARC). In the thalamus, the paraventricular nucleus (PVN) and the IGL have been identified as targets of SCN efferent pathways (Dibner et al., 2010; Kriegsfeld et al., 2004; Leak and Moore, 2001; Saper et al., 2005). GABA, glutamate and AVP are the major neurotransmitters by which the signal is conveyed at synaptic contacts of SCN target sites (Dibner et al., 2010; Kalsbeek et al., 2010). In recent years, very marked process has been made in the identification of individual SCN target sites with their corresponding rhythms in physiology and behavior. For example, it was found that different rhythms can be controlled directly by the SCN (e.g. melatonin), or can be regulated by complex systems of one (e.g. body temperature) or two (e.g. activity and feeding rhythm) synaptic relays from the SCN (Saper et al., 2005). To exclude the possibility that the origin of the DAO phenomenon is located downstream from the SCN, both locomotor activity and body temperature rhythms of WT, DAO and AR hamsters were studied by means of implanted E-mitters. Investigation of both rhythms by this method allows long-term investigations over many cycles to examine whether both patterns correspond to each other (Chapter IV). Overt body temperature rhythms were therefore purified from the effect of activity as the purified rhythm is a reliable estimate of the endogenous rhythm. Additionally, the process of purification allows estimation of the thermoregulatory efficiency, thereby providing insights into whether the DAO phenomenon may have consequences for the

animals' physiology. Pineal melatonin was measured at three different times (in DAO, WT and AR hamsters) as an additional marker of the SCN output, since the signal for melatonin production is directly relayed from the SCN. Furthermore, 24-h profiles of urinary 6-sulfatoxymelatonin, the metabolic end-product of melatonin, were compiled to enable comparisons with the corresponding activity rhythm of each type of animal (Chapter V).

As described above, the main goals of the thesis are to identify the origin and underlying mechanisms of the phenomenon observed in DAO hamsters. The thesis is structured in five chapters according to the date of publication.

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CHAPTER II

EFFECTS OF LIGHT ON THE CIRCADIAN ACTIVITY RHYTHM OF DJUNGARIAN HAMSTERS (*Phodopus sungorus*) WITH DELAYED ACTIVITY ONSET

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Chronobiology International 27, 95-110, (2010)

Abstract:

A number of Djungarian hamsters (Phodopus sungorus) of our institute show activity patterns that seem incompatible with proper adjustment to a periodic environment. The activity onset of those animals is continuously delayed, whereas the activity offset is stably coupled to "lights-on", leading to compression of activity time. A series of experiments was conducted to evaluate the possible causes of the deteriorated ability of DAO (delayed activity onset) hamsters to synchronize. Thus, we investigated the properties of the endogenous circadian rhythm plus parametric and non-parametric light effects on hamsters of DAO and Wild type (WT) phenotypes. Free-running rhythms were studied in constant darkness (DD) or constant light (LL) of different intensities (1, 10, 100 lux). To investigate photic phase responses, hamsters were kept in DD and exposed to light pulses (100 lux, 15 min), at circadian time (CT) CT14 and CT22. Differences were verified statistically by ANOVA. Light intensity exerted significant effect on the free-running period (τ). In DD, τ was significantly longer in DAO than WT hamsters. With increasing light intensity, τ lengthened in both phenotypes, though not at a similar rate. In 10 and 100 lux LL, however, τ did not differ between the two phenotypes. The robustness of the circadian activity rhythm was highest in DD and decreased in LL. No differences between phenotypes were noted. The percentage of arrhythmic animals was low in DD, but remarkably high in LL, and always higher in WT hamsters. The total amount of activity/day was highest in DD; DAO hamsters were less active than WT hamsters under each lighting condition. Light pulses induced phase delays when applied at CT14 and phase advances at CT22, with advances being stronger than delays. Also at CT14, the response of the activity onset was stronger than the activity offset. The opposite was observed

at CT22. At CT14, the phase response did not differ between the phenotypes. However, at CT22 the phase advance was significantly weaker in DAO than WT hamsters despite their longer τ. The results provide further evidence that the distinct activity pattern of DAO hamsters is due to an altered interaction between the circadian clock and photic zeitgeber.

Keywords:
Djungarian hamster; Circadian activity rhythm; Photic zeitgeber; Freerunning period; Phase response

CHAPTER III

RE-ENTRAINMENT BEHAVIOR OF DJUNGARIAN HAMSTERS (*Phodopus sungorus*) WITH DIFFERENT RHYTHMIC PHENOTYPE FOLLOWING LIGHT-DARK SHIFTS

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Chronobiology International 28, 58-69, (2011)

Abstract:

Djungarian hamsters bred at the authors' institute reveal two distinct circadian phenotypes, the wild-type (WT) and DAO type. The latter is characterized by a delayed activity-onset, probably due to a deficient mechanism for photic entrainment. Experiments with zeitgeber shifts have been performed to gain further insight into the mechanisms underlying this phenomenon. Advancing and delaying phase shifts were produced by a single lengthening or shortening of the dark (D) or light (L) time by 6 h. Motor activity was recorded by passive infrared motion detectors. All WT hamsters re-entrained following various zeitgeber shifts and nearly always in the same direction as the zeitgeber shift. On the other hand, a considerable proportion of the DAO animals failed to re-entrain and showed, instead, diurnal, arrhythmic, or free-running activity patterns. All but one of those hamsters that re-entrained did so by delaying their activity rhythm independently of the direction of the LD shift. Resynchronization occurred faster following a delayed than an advanced shift and also after changes of D rather than L. WT animals tended to reentrain faster, particularly following a zeitgeber advance (where DAO hamsters re-entrained by an 18-h phase delay instead of a 6-h phase advance). However, the difference between phenotypes was statistically significant only with a shortening of L. To better understand re-entrainment behavior, Type VI phase-response curves (PRCs) were constructed. To do this, both WT and DAO animals were kept under LD conditions, and light pulses (15 min, 100 lux) were applied at different times of the dark span. In WT animals, activity-offset always showed phase advances, whereas activity-onset was phase delayed by light pulses applied during the first half of the dark time and not affected by light pulses applied during the second half. When the light pulse was given at the beginning of D, activity-onset responded more strongly, but light pulses given later in D produced significant changes only in activity-offset. In accord with the delayed activity-onset

in DAO hamsters, no or only very weak phase-responses were observed when light pulses were given during the first hours of D. However, the second part of the PRCs was similar to that of WT hamsters, even though it was compressed to an interval of only a few hours and the shifts were smaller. Due to these differences, the first light-on or light-off following an LD shift fell into different phases of the PRC and thus caused different re-entrainment behavior. The results show that it is not only steady-state entrainment that is compromised in DAO hamsters but also their re-entrainment behavior following zeitgeber shifts.

Keywords:
Circadian activity rhythm, Delayed activity-onset, Djungarian hamster, Re-entrainment, Type VI phase response curve, Zeitgeber shift, Light–Dark shift

CHAPTER IV

THE CIRCADIAN BODY TEMPERATURE RHYTHM OF DJUNGARIAN HAMSTERS (*Phodopus sungorus*) REVEALING DIFFERENT CIRCADIAN PHENOTYPES

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Physiology & Behavior 103, 352-358, (2011)

Abstract:

Djungarian hamsters (Phodopus sungorus) of our breeding stock show three rhythmic phenotypes: wild type (WT) animals which start their activity shortly after “lights-off” and are active until “lights-on”; delayed activity onset (DAO) hamsters whose activity onset is delayed after “lights-off” but activity offset coincides with “lights-on”; and arrhythmic hamsters (AR) that are episodically active throughout the 24-h day. The main aim of the present study was to investigate whether the observed phenotypic differences are caused by an altered output from the suprachiasmatic nuclei (SCN). As a marker of the circadian clock, the body temperature rhythm purified from masking effects due to motor activity was used. Hamsters were kept singly under standardized laboratory conditions (L:D=14:10 h, T: 22 °C±2 °C, food and water ad libitum). Body temperature and motor activity were monitored by means of implanted G2-E-Mitters and the VitalView® System (MiniMitter). Each phenotype showed distinctive rhythms of overt activity and body temperature, these two rhythms being very similar for each phenotype. Correcting body temperatures for the effects of activity produced purified temperature rhythms which retained profiles that were distinctive for the phenotype. These results show that the body temperature rhythm is not simply a consequence of the activity pattern but is caused by the endogenous circadian system. The purification method also allowed estimation of thermoregulatory efficiency using the gradients as a measure for the sensitivity of body temperature to activity changes. In WT and DAO hamsters, the gradients were low during activity period and showed two peaks. The first one occurred after “lights-on”, the second one preceded the activity onset. In AR hamsters, the gradients did not reveal circadian changes. The results provide good evidence that the different phenotypes result from differences in the circadian clock. In AR hamsters, the SCN

do not produce an obvious circadian signal. With regard to DAO hamsters, it remains to be investigated whether the clockwork itself or the afferent entraining pathways are abnormal in comparison with the WT hamsters.

Keywords:
Djungarian hamster, Circadian rhythm, Body temperature, Motor activity, Arrhythmic activity patterns, Unmasking

CHAPTER V

THE DAILY MELATONIN PATTERN IN DJUNGARIAN HAMSTERS DEPENDS ON THE CIRCADIAN PHENOTYPE

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Abstract:

Djungarian hamsters (Phodopus sungorus) bred at the Institute of Halle reveal three different circadian phenotypes. The wild type (WT) shows normal locomotor activity patterns, whereas in hamsters of the DAO (delayed activity onset) type, the activity onset is continuously delayed. Since the activity offset in those hamsters remains coupled to "light-on," the activity time becomes compressed. Hamsters of the AR (arrhythmic) type are episodically active throughout the 24 h. Previous studies showed that a disturbed interaction of the circadian system with the light-dark (LD) cycle contributes to the phenomenon observed in DAO hamsters. To gain better insight into the underlying mechanisms, the authors investigated the daily melatonin rhythm, as it is a reliable marker of the circadian clock. Hamsters were kept individually under standardized laboratory conditions (LD 14:10, T = 22°C ± 2°C, food and water ad libitum). WT, DAO (with exactly 5 h delay of activity onset), and AR hamsters were used for pineal melatonin and urinary 6-sulfatoxymelatonin (aMT6s) measurement. Pineal melatonin content was determined at 3 time points: 4 h after "light-off" [D + 4], 1 h before "light-on" [L − 1], and 1 h after "light-on" [L + 1]). The 24-h profile of melatonin secretion was investigated by transferring the animals to metabolic cages for 27 h to collect urine at 3-h intervals for aMT6s analysis. WT hamsters showed high pineal melatonin content during the dark time (D + 4, L − 1), which significantly decreased at the beginning of the light period (L + 1). In contrast, DAO hamsters displayed low melatonin levels during the part of the dark period when animals were still resting (D + 4). At the end of the dark period (L − 1), melatonin content increased significantly and declined again when light was switched on (L + 1). AR hamsters showed low melatonin levels, comparable to daytime values, at all 3 time

points. The results were confirmed by aMT6s data. WT hamsters showed a marked circadian pattern of aMT6s excretion. The concentration started to increase 3 h after “light-off” and reached daytime values 5 h after “light-on.” In DAO hamsters, in contrast, aMT6s excretion started about 6 h later and reached significantly lower levels compared to WT hamsters. In AR animals, aMT6s excretion was low at all times. The results clearly indicate the rhythm of melatonin secretion in DAO hamsters is delayed in accord with their delayed activity onset, whereas AR hamsters display no melatonin rhythm at all. Since the regulatory pathways for the rhythms of locomotor activity and melatonin synthesis (which are downstream from the suprachiasmatic nucleus [SCN]) are different but obviously convey the same signal, we conclude that the origin of the phenomenon observed in DAO hamsters must be located upstream of the SCN, or in the SCN itself.

Keywords:
Arrhythmic activity pattern, Circadian rhythm, Djungarian hamster, Daily melatonin rhythm, Disturbed photic entrainment

CHAPTER VI

C-FOS EXPRESSION IN THE SCN OF DJUNGARIAN HAMSTERS WITH A DELAYED ACTIVITY ONSET FOLLOWING PHOTIC STIMULATION

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in preparation

Abstract

C-Fos expression in the suprachiasmatic nucleus (SCN) following photic stimulation was investigated in Djungarian hamsters (*Phodopus sungorus*) of two different circadian phenotypes. Wild type (WT) hamsters display robust daily patterns of locomotor activity according to the light/dark conditions. Hamsters of the DAO (delayed activity onset) phenotype, however, progressively delay the activity onset, whereas activity offset remains coupled to "light-on", which leads to a compression of the activity time. Although the exact reason for the delayed activity onset is not yet clarified, it is connected with a disturbed interaction between the light/dark cycle and the circadian clock. The aim of the study was to test the link between photoreception and the circadian system in hamsters of both phenotypes, to get further insight in the underlying mechanism of the DAO phenomenon. Animals were kept individually under standard laboratory conditions (LD14:10, T = 22 ± 2°C, food and water *ad lib.*). Depending on the phenotype and the compression of the activity time in DAO hamsters, animals were divided into three groups: WT (wild type animals), DAO2 (DAO hamsters with 2-h delays in activity onset) and DAO6 (hamsters with 6-h delays in activity onset). Animals were exposed to light pulses (100 lx, 15 min) at different time points during the dark period and expression of Fos protein was analyzed by immuno-histochemical assays. Almost no Fos-immunoreactive cells were found in DAO6 hamsters during the dark period when animals were still resting (2 h before activity onset). During the activity time, however, elevated Fos expression following light pulses was observed, indicating that the photosensitive phase in DAO hamsters is restricted and compressed to the actual activity time. This was confirmed by the results from DAO2 and WT animals as well as from controls (kept in the dark). The results provide evidence that the photosensitivity of the circadian system

does not differ between WT and DAO hamsters and this lead us to conclude that downstream processes within the SCN that enable light information to reset the circadian pacemaker might offer an explanation for the DAO phenomenon.

ABBREVIATIONS

III V	3rd ventricle
AR	arrhythmic hamster
CET	Central European Time
CWFS	cold water fish gelatine
DAB	3, 3'-diaminobenzidine
DAO	hamster with delayed activity onset
DC	dark control
EtOH	ethanol
Fos-ir	Fos-immunoreactivity
h	hours
HALO(x)	x hours after light offset
H_2O_2	hydrogen peroxide
IU	international unit
LP	light pulse
lx	lux
min	minutes
ml	milliliter
µm	micrometer
NaN_3	sodium azide
OC	optic chiasm
PBS	phosphate-buffered saline
PEG	polyethylene glycol embedding
PLP	periodate-lysine-paraformaldehyde
SAV-POD	streptavidin-perioxidase
SCN	suprachiasmatic nucleus
SEM	standard error of the mean
TBI	tribromoimidazole
TBS	tris-buffered saline
TW20	Tween-20
WT	Wild-type hamster

INTRODUCTION

Daily rhythms of physiology, metabolism and behavior are ubiquitous features in almost all living organisms. These rhythms are generated by a pacemaker, or biological clock, which is the suprachiasmatic nucleus (SCN) in mammals, a bilateral structure located in the anterior hypothalamus dorsal to the optic chiasm (Reuss, 1996). The SCN generates rhythms by gene expression that changes under the influence of positive and negative transcriptional-translational feedback loops (Takahashi et al., 2008). Since the inherent period of these rhythms deviates from the external 24-h day, it needs to be reset, or synchronized, by so-called zeitgebers (Aschoff, 1960). Such resetting will guarantee the optimal timing of physiology and behavior according to the ambient lighting conditions. The most prominent zeitgeber to which the SCN entrains is the light/dark cycle (Sharma and Chandrashekaran, 2005). The SCN receives a direct photic input from retinal ganglion cells via the retinohypothalamic tract (RHT) (Abrahamson and Moore, 2001; Pickard, 1982). RHT projections mainly innervate the ventrolateral or core region of the SCN (Bryant et al., 2000). Cells of this sub-region show weak or even no rhythmic oscillation but rather express genes (e.g. immediate early genes and clock genes) following light stimulation (Guido et al., 1999a; Hamada et al., 2001; Schwartz et al., 1994; Yan et al., 1999). Furthermore, core neurons rapidly adjust their phase to a new LD-cycle (Albus et al., 2005; Nagano et al., 2003). Responsiveness or sensitivity to light in the core SCN is restricted to the subjective night which is, in turn, time-gated by the circadian clock itself (Hamada et al., 2003). By contrast, sparse RHT projections pass to the dorsomedial or shell region of the SCN, which is characterized by intrinsically rhythmic cells and only slow phase adjustment to a new LD-cycle (Davidson et al., 2009; Hamada et al., 2001; Moore et al., 2002; Schwartz et al., 2000). It is believed that the retino-recipient core region relays photic information to the shell region for readjustment of its phase, which then provides the appropriate output signal to the corresponding target sites (Albus et al., 2005; Antle and Silver, 2005). However, it is important to note that SCN targets also acquire direct light information from the SCN core region, supporting the idea that integration of phase and rhythmic information may occur at the level of the target site (Kriegsfeld et al., 2004).

In Djungarian hamsters (Phodopus sungorus) bred at the Zoology Institute of the University of Halle, we have observed three rhythmic phenotypes on the basis of circadian locomotor activity, body temperature and melatonin rhythms (Schöttner et al., 2011b; Schöttner et al., 2011c; Weinert and Schöttner, 2007). Wild type (WT) hamsters display stable rhythms appropriate to the light/dark conditions. Hamsters of the DAO (delayed activity onset)

phenotype, however, are characterized by a continuous delay of the activity onset even though activity offset remains coupled to "light on". Thus, the activity time becomes compressed up to a critical duration of approximately 3 h. Beyond this critical value, animals start to free-run despite the presence of a light/dark cycle, and this ultimately leads to a collapse of the rhythm. The animals then display arrhythmic patterns and are therefore designated as of the AR phenotype. The exact reason for the DAO phenomenon is not yet clarified, but it is connected to a disturbed interaction of the light/dark cycle with the SCN (Schöttner and Weinert, 2010). Analysis of phase responses following light pulses in the dark phase (when animals were kept under a light/dark cycle) revealed that WT hamsters significantly phase shifted their activity rhythm during the entire activity time, which corresponds to the dark period. By contrast, DAO hamsters reacted by phase shifts during their actual activity time but not during that part of the dark phase when they were still resting (Schöttner et al., 2011a). This is a strong indication that the light-sensitive phase of the SCN is compressed according to the observed activity pattern which, in turn, would point to a malfunction of the SCN. Therefore, investigations of c-Fos expression in the SCN, which is a reliable marker of light responsiveness of the circadian clock (Caputto and Guido, 2000; Kornhauser et al., 1990; Vuillez et al., 1996), were carried out following light stimulation in WT and DAO hamsters. Animals of both phenotypes were exposed to light pulses at different times of the dark phase. Furthermore, DAO hamsters with different stages of activity (alpha) compression were studied, particularly animals with 2 h (DAO2) and 6 h (DAO6) delays of activity onset (Fig. 1). Pronounced expression of c-Fos in the SCN following light stimulation 2 h after activity onset is expected (Kornhauser et al., 1990). This is 4 h after "light-off" (HALO4) in DAO2 and 8 h after "light-off" (HALO8) in DAO6 animals. Light pulses given at HALO8 in DAO2 and HALO4 in DAO6 serve as control within the DAO hamsters. This last case is particularly important since the light pulse falls in the dark phase when the hamsters were still resting. C-Fos expression following light pulses applied in WT hamsters at HALO2, HALO4 and HALO8 serve as standards in these experiments. From the results, it will be possible to get more insights into the process of photic synchronization in hamsters of DAO and WT phenotype with specific regard to photosensitivity of the circadian pacemaker.

MATERIAL & METHODS

Animals

Adult male and female Djungarian hamsters (*Phodopus sungorus*, Pallas 1773) of the WT and DAO phenotype were used. Animals were derived from two breeding lines which differed in

the proportion of WT to DAO offspring. One line yields almost exclusively WT offspring by pairing the WT hamsters that were most unrelated genetically. By contrast, the second line yields a high percentage of DAO offspring when unrelated DAO animals are paired. DAO hamsters with exactly 2 h and 6 h delay in activity onset were selected. For this purpose, locomotor activity of DAO animals was registered under standard housing conditions. Due to the progressive compression of alpha it was possible to select the animals when activity onset was delayed by 2 h or 6 h.

Standard housing condition:

Animals were kept individually in windowless air-conditioned rooms in standard plastic cages (Macrolon® type II) provided with wood shavings as nesting material. Animal bedding (Allspan®, The Netherlands) was renewed once every two weeks. Room temperature was 22 ± 2 °C, and relative humidity varied between 60 and 65 %. The light/dark condition was 14:10 h, with light switched on from 04:00–18:00 h Central European Time and with a light intensity between 80–100 lx during the light period and 0 lx in the dark period. Food pellets (breeding diet Altromin® 7014, maintenance diet Altromin® 7024, relation 1:2; Altromin GmbH, Lage, Germany) and water were provided *ad libitum*.

The experimental procedures were conducted in compliance with the German law for animal protection.

Experimental protocol:

Hamsters of both phenotypes (23.55 ± 0.96 weeks old, mean ± SEM) were kept under standard housing conditions for a minimum of 2 weeks to get stable activity patterns. Animals were divided into 3 groups dependent on their activity pattern, wild type hamsters (WT), hamsters with delays of activity onset of 2 h (DAO2) or 6 h (DAO6). At different time-points, as illustrated in Fig. 1, five hamsters from each group were exposed to light pulses (15 min, 100 lx) and three hamsters of each group were used as dark controls. Hamsters exposed to light pulses (LP) as well as the dark control animals (DC) were transferred from the standard housing to the experimental room at the appropriate times for treatment with light (or darkness, if controls). Thereafter, the animals were transferred back to the standard housing. One h after the beginning of the treatment (LP, DC), hamsters were perfused as described below.

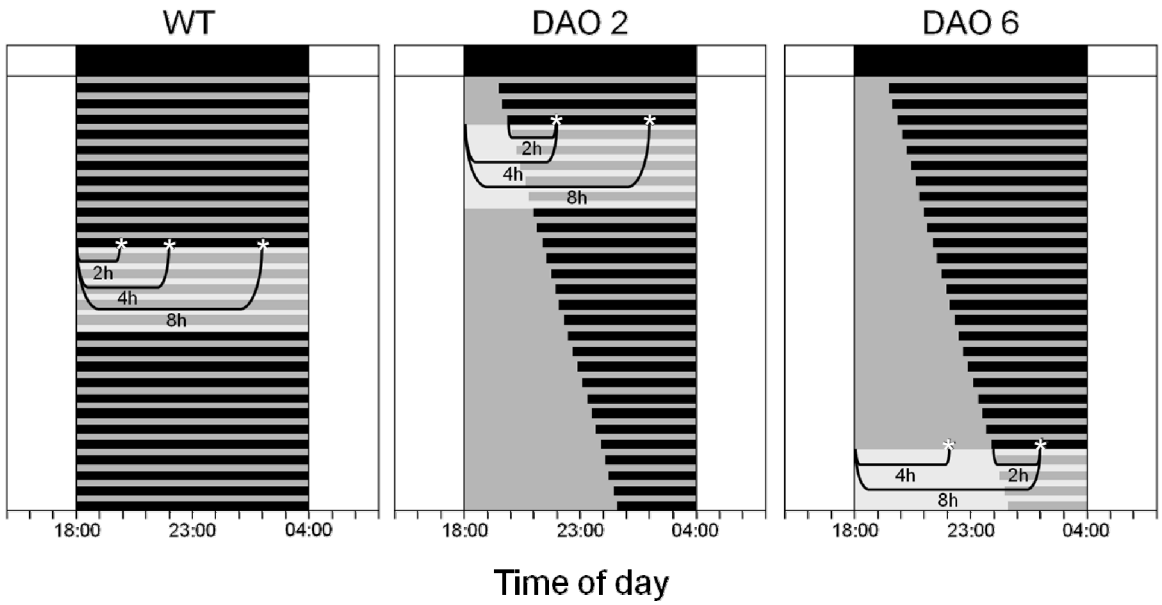

FIGURE 1: Illustration of the experimental design of the Fos-ir investigation in the SCN of Djungarian hamsters following light pulses during the dark period. Schematic actograms are shown for hamsters of each rhythmic phenotype. The time points when animals were exposed to light pulses (100lx, 15min) as well as the corresponding dark controls are indicated by asterisks. WT hamsters were exposed to light pulses 2 (HALO2), 4 (HALO4) and 8 h (HALO8) after "light-off" and served as standard in the experiment. DAO hamsters of both groups received light pulses at HALO4 and HALO8. The white/black bar on top together with the grey background displays the light/dark condition. The black lines below symbolize the activity pattern of the specific rhythmic phenotype.

Perfusion and embedding

Animals were deeply anesthetized by exposure to an atmosphere of isoflurane (Isofluran Baxter AG, Volketswil, Switzerland), heparinized (0.2 ml, 25000 IU/ ml, Heparin-Rotexmedica, Rotexmedica GmbH, Trittau, Germany) and perfused transcardially by 100 ml cold PBS (40 ml/ min) followed by 200 ml cold Periodate-Lysine-Paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) (20 ml/ min) using an infusion pump (KDS 200, KD Scientific Inc., Holliston, MA, USA). Brains were then removed and post-fixed in the same PLP fixative for 6 h at 4 °C. Subsequently, brains were washed in PBS (1 x 30 min, 1x overnight) and dehydrated sequentially by ethanol (EtOH 70 %, 2 x 1 h), 2-ethoxyethanol (3 x 1 h) and butanol (1 x 1 h). They were then stored in butanol and send to Strasbourg for PEG-embedding (Klosen et al., 1993) and immuno-histochemistry.

Sectioning and immuno-histochemistry

Vibratome transverse sections throughout the SCN (12 µm) were mounted on slides, treated with blocking buffer (dry skimmed milk in TBS-TW20 and 0.02 % NaN_3) and incubated with

anti-c-Fos antiserum (1:500 in TBS-TW20 and 0.2 % CWFS) overnight. The sections were washed with TBS-TW20 (3 x 10 min) the next day and incubated with biotinylated secondary donkey anti-rabbit antibody (Jackson, 1:2000, 1 h). Thereafter, sections were washed (TBS-TW20, 3 x 10 min), treated with streptavidin-perioxidase (SAV-POD, Roche, 2 h) and washed again (TBS-TW20, 3 x 10 min). Peroxidase detection occurs by treating sections with TBI (50 mM Tris and 10 mM imidazole, pH 7.6, 10 min) followed by incubation in DAB-solution (1/100 DAB in TBI and 3 % H_2O_2) for approximately 15 min. Thereafter, sections were rinsed using TBS (4 x 5 min) and dehydrated sequentially (EtOH 70 %, 1 x 2 min; EtOH 95 %, 1 x 2 min; EtOH 100 %, 2 x 2 min; Toluene, 2 x 10 min).

Data analysis

Sections of the rostro-caudal level of the SCN were taken to estimate c-Fos expression by semi-quantitative visual analysis. Sections from the median SCN (first part of the caudal half) have been taken for illustration (for details see result section).

RESULTS

In hamsters of the DAO and WT phenotypes, practically no c-Fos expression was observed in the DC animals at any time (Figs. 2 – 4, right panels). In a few instances, hamsters of both phenotypes showed Fos-ir cells (approximately 10 – 15 in number) in 2 – 3 sections of the caudal part of the SCN (e.g. Fig. 2E), this being independent of time and treatment received. WT hamsters displayed numerous well stained Fos-ir cells mostly in the ventral-caudal part of the SCN, when animals were exposed to light pulses at HALO2 (Fig. 2A). Light pulses at HALO4 and HALO8 led to c-Fos expression in the same part of the SCN (Fig. 2B) but also in the rostral part of the SCN. Additionally, Fos-ir cells were present in the dorsal part of the caudal half of the SCN and this was more pronounced at HALO8 (Fig. 2C). The results are summarized in Tab. 1.

TABLE 1: Semi-quantitative analysis of Fos expression in the SCN of WT hamsters at different times during the dark period

Phenotype	WT											
HALO (h)	2				4				8			
Hours after activity onset (h)	2				4				8			
Rostro-caudal region	R		C		R		C		R		C	
Ventro-dorsal region	V	D	V	D	V	D	V	D	V	D	V	D
Light pulse	-	-	+	-	+	-	+	+	+	-	+	+
Dark control	-	-	-	-	-	-	-	-	-	-	-	-

R … rostral part of the SCN; C … caudal part of the SCN; V … ventral part of the SCN; D … dorsal part of the SCN; - … Fos absent; + … Fos weakly present; + … Fos present

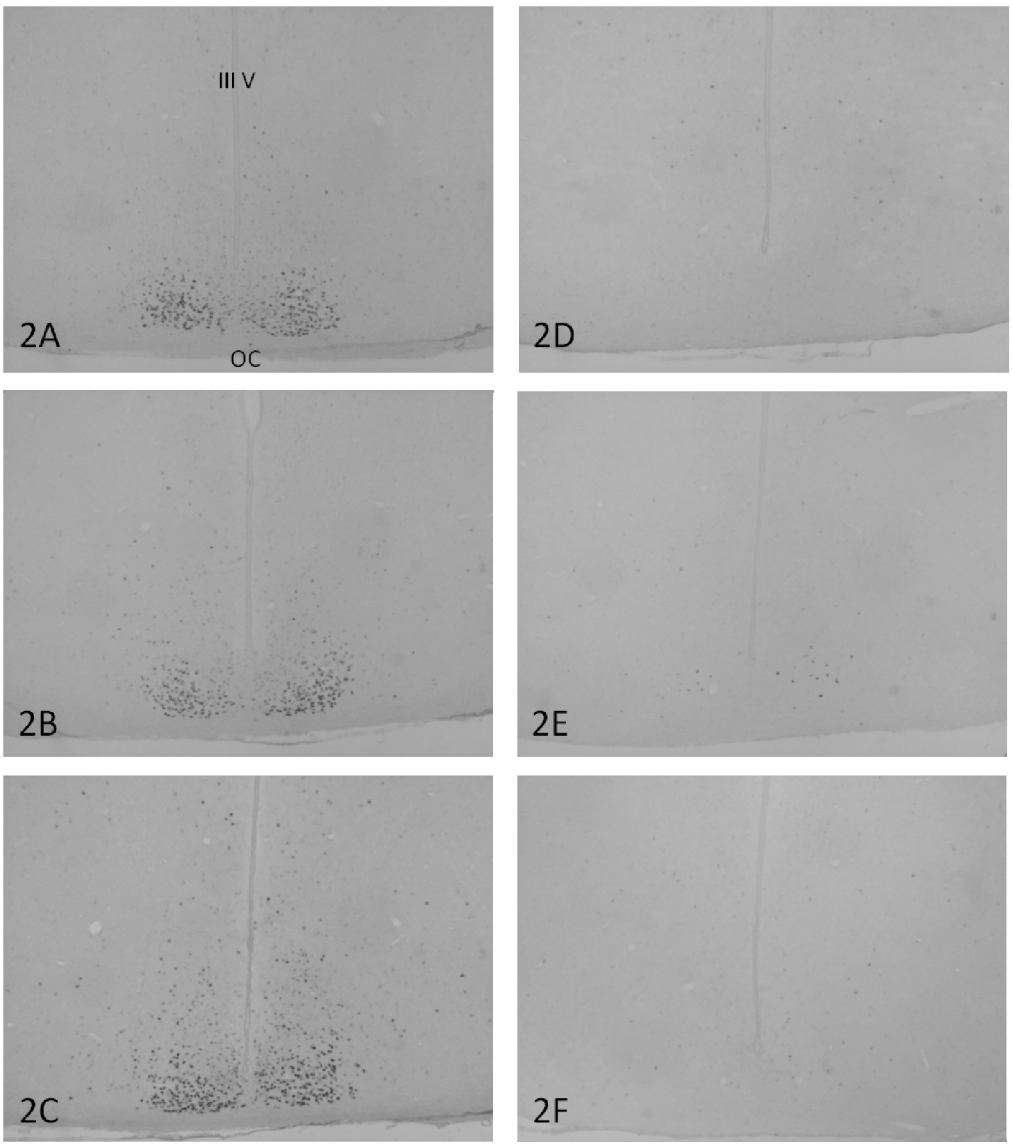

FIGURE 2: Image of the c-Fos expression in the SCN of WT hamsters following light pulses (left side) and the corresponding dark controls (right side) 2 (A+D), 4 (B+E) and 8 h (C+F) after "light-off", which coincided with the activity onset. Sections from the median SCN are depicted. Fos-ir cells were distinct in the ventral region of the SCN following light pulses 2 h and 4 h after "light-off" (A+B). Light pulses 8 h after "light-off" induced Fos-ir in cells of the dorsal SCN beyond to the expression in the ventral part (C). Almost no Fos-ir cells were found in the dark controls (D-F). III V … 3. ventricle; OC … optic chiasm

C-Fos expression was observed in DAO2 animals following light pulses at HALO4 and HALO8. Fos-ir cells were present in the ventral-caudal part of the SCN similar to the pattern observed in WT hamsters at HALO2 (Fig. 3A). At HALO8, the pattern of Fos-ir cell expression resembled the observations made in WT-HALO8 animals (Fig. 3B). The most striking result was found in DAO6 hamsters in which very few or even no Fos-ir cells were found in the SCN when they were exposed to light pulses at HALO4 (i.e. when animals were still resting) (Fig. 4A). However, Fos-ir cells were distinct in the SCN of DAO6 hamsters following light stimulation during the activity period at HALO8 (Fig. 4B). Fos-ir cells were

clearly distributed at the ventral–caudal part and to lesser extent in the dorsal part of the SCN. Summarized results are depicted in Tab. 2. No marked differences between the phenotypes were observed by visual analysis regarding the amount of Fos-ir cells when exposed to light pulses 2 h after activity onset.

TABLE 2: Semi-quantitative analysis of Fos expression in the SCN of DAO hamsters with 2 and 6 h delay of the activity onset at different times during the dark period

Phenotype	DAO2								DAO6							
HALO (h)	4				8				4				8			
Hours after activity onset (h)	2				6				-2				2			
Rostro-caudal region	R		C		R		C		R		C		R		C	
Ventro-dorsal region	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D
Light pulse	-	-	+	-	+	-	+	+	-	-	-	-	+	-	+	+
Dark control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

R … rostral part of the SCN; C … caudal part of the SCN; V … ventral part of the SCN; D … dorsal part of the SCN; - … Fos absent; + … Fos weakly present; + … Fos present

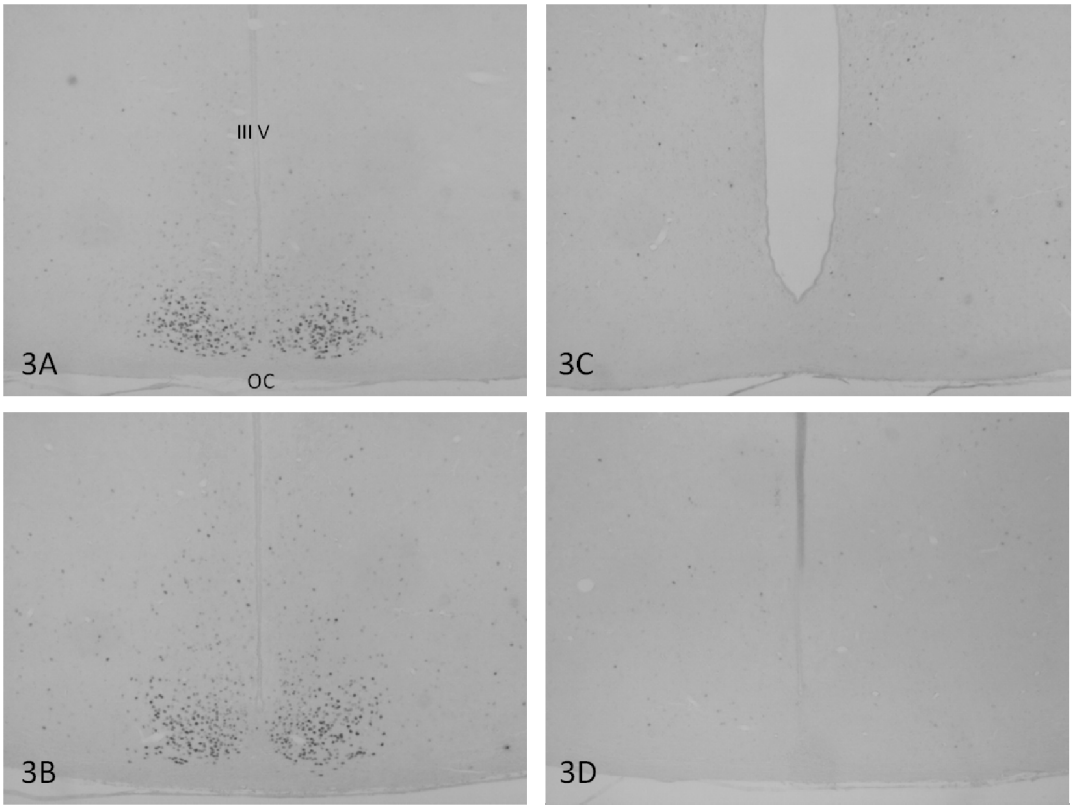

Figure 3: Image of the c-Fos expression in the SCN of DAO2 hamsters following light pulses (left side) and the corresponding dark controls (right side) 4 (A+C) and 8 h (B+D) after "light-off". Sections from the median SCN are depicted. Fos-ir cells were distinct in the ventral region of the SCN following light pulses 4 h after "light-off" (i.e. 2 h after the activity onset) (A). Light pulses 8 h after "light-off" induce Fos-ir in cells of the ventral and dorsal SCN (B). Almost no Fos-ir cells were found in the dark controls (C+D). III V … 3. ventricle; OC … optic chiasm

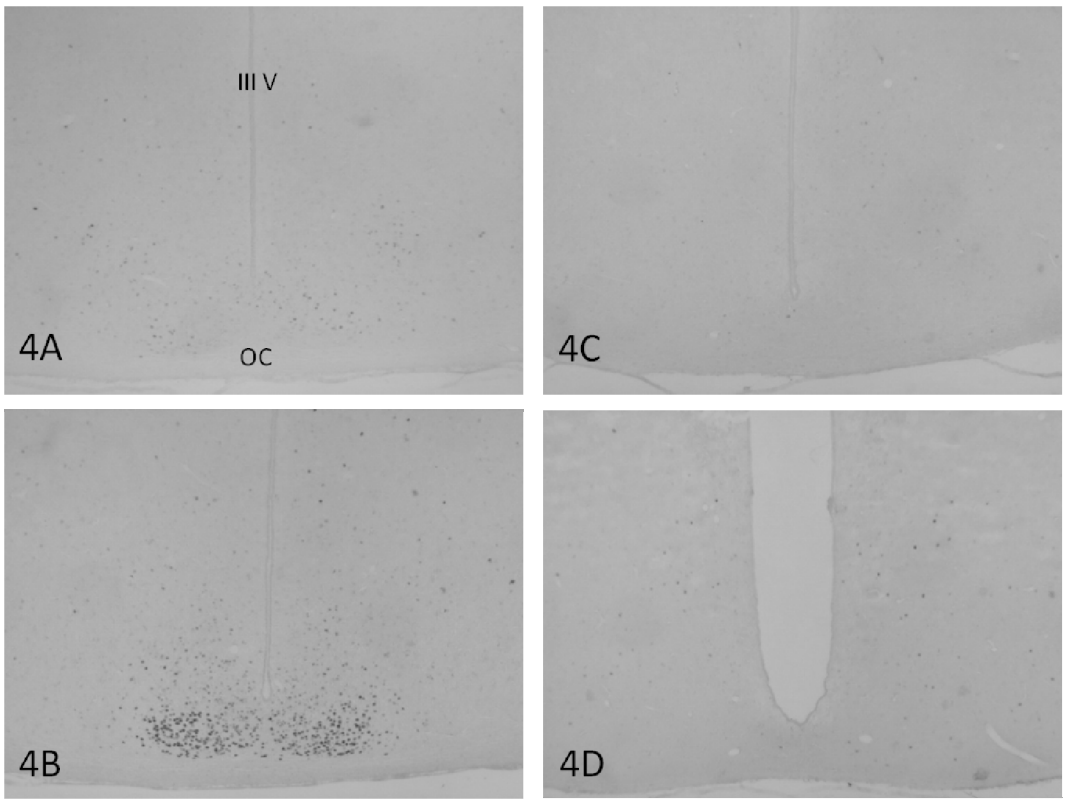

Figure 4: Image of the c-Fos expression in the SCN of DAO6 hamsters following light pulses (left side) and the corresponding dark controls (right side) 4 (A+C) and 8 h (B+D) after "light-off". Sections from the median SCN are depicted. Very few Fos-ir cells were distinct in the SCN following light pulses 4 h after "light-off" (i.e. 2 h before the activity onset) (A). Light pulses 8 h after "light-off" (i.e. 2 h after the activity onset) induce Fos-ir in cells of the ventral and and to a little extent in the dorsal SCN (B). Almost no Fos-ir cells were found in the dark controls (C+D). III V … 3. ventricle; OC … optic chiasm

DISCUSSION

C-Fos expression in the SCN has been widely used as a molecular marker to investigate the mechanism of light-responsive signalling associated with photic entrainment of the circadian system (Caputto and Guido, 2000; Guido et al., 1999a; Guido et al., 1999b; Kornhauser et al., 1996; Kornhauser et al., 1990). Fos immuno-reactivity following light pulses is limited to retino-recipient SCN neurons and can be induced only during the phase when the SCN is sensitive to light. This phase corresponds to the subjective night, when light pulses can phase shift the circadian system (Caputto and Guido, 2000). The results in WT hamsters of the recent study agree with those predictions. C-Fos labelling was observed in sections of the SCN at all three time points of photic stimulation. This is in accordance with a phase response curve of WT hamsters kept under light/dark conditions (Schöttner et al., 2011a). Animals of this phenotype showed significant phase shifts following light pulses at various time points throughout the dark period which corresponded to times of activity. We conclude that the

underlying mechanism of photic synchronization of the circadian system in WT hamsters is functioning properly, which guarantees a stable daily output from the SCN (Schöttner et al., 2011b; Schöttner et al., 2011c) and appropriate adaptation to changes of the photoperiod (Schöttner et al., submitted). In DAO hamsters by contrast, the light-sensitive phase of the SCN is limited to the actual activity phase only. Sparse Fos-ir cells were present in SCN sections of DAO6 hamsters when exposed to light pulses two hours before activity onset (HALO4); by contrast, c-Fos expression was clearly present in the same group of animals exposed to light two hours after activity onset (HALO8). This result also agrees with a phase response curve of DAO hamsters compiled in an earlier study (Schöttner et al., 2011a) in which DAO hamsters with 5 h delay of their activity onset and kept under light/dark (14:10 h) conditions showed significant phase shifts only during the remaining 5 h when they were active. In the present study, clear Fos expression in the SCN of DAO2 hamsters at HALO4 (i.e. 2 h after onset of activity) confirms the hypothesis that the phase of sensitivity to light of the SCN is coupled to the activity time in hamsters of the DAO phenotype. Though the appearance of Fos-ir cells in the SCN of DAO6 hamsters at HALO4 was slightly more pronounced than in the dark controls, it was considerably less than in sections of DAO6 hamsters exposed to light at HALO8. Elevated levels of Fos protein following photic stimulation compared to dark controls have been observed in rats 2 h before activity onset (Sumova et al., 1995a; Sumova et al., 1995b). However, it is important to note that these values were significantly less compared to levels measured following light pulses in the activity period. Also, small phase shifts, though not significantly different from zero, were observed in DAO hamsters during the dark time when animals were still resting (Schöttner et al., 2011a). These results indicate that the transition between the light-insensitive to the light-sensitive phase of the SCN is coupled to the beginning of the subjective night in DAO hamsters as well as in WT animals. Confirmation that it is, indeed, a light-sensitive phase that can be distinguished from a light-insensitive phase is obtained by the results of the dark controls. The results of the dark controls validate the view that Fos expression is not a consequence of non-photic stimulation (i.e. transfer of the cage during the experiment), as was observed in rats after various kind of manipulation (Edelstein and Amir, 1995).

Several studies have shown that the spatio-temporal distribution of Fos-ir cells in the SCN following photic stimulation changes throughout the subjective night. Whereas Fos expression is observed in the ventral-caudal part of the SCN in the early night, additional Fos-ir cells are distributed in the dorsal and rostral portion of the SCN in the mid and late night (Chambille, 1998; Chambille et al., 1993; Guido et al., 1999b; Rea, 1992; Teclemariam-Mesbah et al.,

1995). In the present study, the spatio-temporal pattern of Fos expression in the SCN of WT and DAO2 hamsters following light stimulation in the early and late subjective night was similar to the data described in the literature. DAO6 hamsters show c-Fos expression in the ventral-caudal part of the SCN comparable to that observed in WT and DAO2 in the early subjective night. However, Fos-ir cells are also present in the dorsal region of the SCN, though less distinct than was found in WT and DAO2 animals at HALO8. This is likely to be an effect of the short activity time. Since the light pulse at HALO8 falls in the middle of the active phase in DAO6 hamsters it seems plausible that cells in both the dorsal and rostral parts of the SCN will be stimulated. This result can be interpreted to indicate that the spatio-temporal profile of expression of c-Fos in DAO hamsters is similar to that in WT but compressed as it is dependent on the actual activity time. This is supported by the phase response curves of WT and DAO hamsters. Although the general shape of the curves is similar in both phenotypes, the proportion of areas where phase shifts can be induced by light pulses is limited to the actual activity time in DAO hamsters (Schöttner et al., 2011a). However, further evidence relevant to this concept requires investigation of the entire spatio-temporal expression profile of c-Fos in the SCN of DAO6 hamsters, and this can be achieved only by a higher resolution of light pulses covering the early and late phase of activity.

Nevertheless, the results of the recent study, coupled with the phase response curves to light pulses lead us to the conclusion that the rhythmic output (i.e. locomotor activity, body temperature, melatonin) coincides with the intrinsic state of the SCN, particularly in the DAO hamsters (Schöttner et al., 2011a; Schöttner et al., 2011b; Schöttner et al., 2011c). The underlying reason for the DAO phenomenon is not yet clear, but it is obviously connected with a disturbed interaction between the light/dark cycle and the SCN. This mainly concerns the non-parametric effects of light (Schöttner et al., submitted; Schöttner and Weinert, 2010). A previous study pointed to a lower sensitivity of the circadian system to light in DAO hamsters compared to the WT (Schöttner et al., 2011b). The results of the present study, however, show that the direct perception of light in the SCN is not diminished in DAO hamsters, since the level of c-Fos expression following photic stimulation was comparable to that of the WT. It is important to note that this conclusion is based on visual analysis of the SCN sections; quantitative analysis of Fos-ir cells is required before definitive conclusions can be drawn.

As mentioned above, the intrinsic state of the SCN coincides with its output, leading to the suggestion that the origin of the DAO phenomenon is located in the SCN. Since the results of the recent study indicate that light perception and transmission to the SCN are not different

between WT and DAO hamsters. Downstream processes within the SCN that use light information to reset the circadian pacemaker have to be taken into account as a possible origin of the DAO phenomenon. Accordingly, it becomes necessary to investigate expression of clock genes in the SCN - namely, the period genes - under various lighting conditions and following photic stimulation to test this hypothesis. It is known that *per1* and *per2* gene expression in the SCN differs in a temporally- and spatially-dependent manner (Antle and Silver, 2005; Johnston et al., 2005; Yan et al., 2007). Phase-shifting light pulses that induce phase delays are associated with *per2* gene expression whereas light pulses that induce phase advances are associated with *per1* gene expression (Albrecht et al., 2001; Miyake et al., 2000). Additionally, special attention should be devoted to the core and shell regions of the SCN since gene expression differs between them (Hamada et al., 2004; Hamada et al., 2001). With this information, it will be possible to get further insights into the mechanism underlying the attenuated ability of photic synchronization in DAO hamsters.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CHAPTER VII

GENERAL DISCUSSION

The main goal of the thesis was to identify the origin and underlying mechanism of the DAO phenomenon observed in Djungarian hamsters of the breeding colony at the Zoology Institute of the University of Halle.

Animals of the DAO phenotype are characterized by a progressive delay of activity onset relative to "light-off", whereas the activity offset remains coupled to "light-on", which leads to a continuous compression of the activity time (alpha). Once alpha falls below a critical value of approximately 3 h, the rhythm breaks down immediately or does so after a short period of free-running (Weinert and Schöttner, 2007). Continuous compression of alpha, however, is not simply the consequence of a free-running rhythm under a LD cycle whose activity is terminated by a masking effect of light at "light-on". The activity offset is in fact entrained, which has been demonstrated by animals released into constant darkness (Chapter II). The rhythm (i.e. onset and offset of activity) resumes free-running from the same phase as determined by the zeitgeber cycle before. Also, results from hamsters exposed to phase shifts of the LD-cycle (Chapter III) or to a symmetric extension of the dark time (Schöttner et al., submitted) accord with this concept. The activity offset re-entrained to a new zeitgeber cycle by phase delays over several cycles and not by an immediate adjustment of the phase. Despite the "free-running" characteristic of the activity onset, it underlies a certain influence of the zeitgeber cycle, since tau measured under LD conditions differs when compared to conditions of constant darkness (Chapter II, unpublished data). Thus, onset of activity is not completely uncoupled from a circadian regulation but rather characterized by a diminished ability to become synchronized to "light-off".

An important issue was to clarify whether the origin of the DAO phenomenon is located in the pacemaker itself, or whether it is caused by processes downstream of the SCN. Thus, beside locomotor activity, additional markers of the circadian clock, namely body temperature and melatonin, have been investigated in WT, DAO and AR hamsters. The results of those studies revealed that all three markers of the circadian clock show similar patterns according to the rhythmic phenotype (Chapters IV + V). Since the overt patterns are the same, even though the regulatory pathways for daily rhythms of activity, body temperature and melatonin downstream of the SCN are different (Saper et al., 2005), it is verified that the signal coding for the specific phenotype must arise from the SCN itself. Further evidence in favor of this

hypothesis come from the results of the Fos investigation in the SCN of DAO and WT hamsters, which reflects the functional state of the SCN regarding its sensitivity to light (Chapter VI). It was shown that light-induced expression of c-Fos in SCN neurons correspond to the pattern of locomotor activity of the respective phenotype, i.e. the light sensitive-phase is compressed in DAO hamsters according to their compressed activity time. Thus, the SCN provides the basis for the generation of a rhythmic signal coding for DAO and WT patterns, and which might even be abolished in the AR phenotype. In studies conducted by others, arrhythmic hamsters also lack 24-h patterns of body temperature, urinary aMT6s excretion, and clock-gene expression but it is important to note that the arrhythmicity was induced in different ways in these studies (Grone et al., 2011; Ruby et al., 1996; Steinlechner et al., 2002). It is assumed that arrhythmicity is a consequence of a collision of the circadian pacemaker oscillators by compressing alpha, which was achieved by exposing hamsters to two consecutive light pulses, may driving both oscillators towards each other until they collide (Steinlechner et al., 2002). Since arrhythmicity in hamsters of our breeding line most likely originates from DAO animals have passed the critical compression of alpha, they provide an excellent model to investigate the underlying mechanisms of this phenomenon at SCN level. AR hamsters are of also of interest as they behave functionally like SCN-lesioned animals. Evidence in favor of this viewpoint came from electrophysiological studies (Margraf et al., 1992). Investigations on extra-SCN oscillators may provide useful insights whether this phenomenon is restricted to the SCN only or whether peripheral clocks will be affected too. However, this was not examined in detail since the focus of the thesis was on the exploration of the DAO phenomenon.

Investigations have been conducted to evaluate intrinsic properties of the circadian system in DAO and WT hamsters as well as its interaction with the exogenous zeitgeber. Though general features of the free-running rhythms in DAO and WT hamsters were similar when animals were kept under constant darkness concerning after-effects, decompression of alpha and rhythm stability, tau was significantly longer in the DAO phenotype (Chapter II). Tau is determined by the self-sustained circadian oscillation of clock genes in SCN neurons that are regulated by positive and negative transcriptional-translational feedback loops (Reppert and Weaver, 2002). However, it is important to note that posttranscriptional modification of clock gene mRNA (Kojima et al., 2011) and posttranslational modification of clock proteins, predominately the phosphorylation by casein kinases, greatly influence the dynamics of dimerization, translocation and degradation, thereby influencing the clock's speed (Bellet and Sassone-Corsi, 2010; Gallego and Virshup, 2007; Lee et al., 2001; Takahashi et al., 2008).

Thus, it is tempting to speculate that the differences in tau between DAO and WT hamsters may be associated with an alteration of the fine-tuning mechanism of the molecular clockwork. Such an alteration has been identified as reason for the tau-mutant in the Golden hamster, though differences in tau between mutant and wild-type were much larger as compared to our DAO and WT phenotype (Lowrey et al., 2000). However, investigations on the molecular clockwork, particularly the temporal mRNA and protein expression of clock genes in the SCN, are necessary to draw further conclusions about this issue.

As described above, hamsters shared similarities concerning their free-running behavior under constant darkness, except for the longer tau in DAO hamsters. However, whether the longer tau in DAO hamsters can be taken as only reason for the delayed activity onset was questioned. Particularly, the resetting mechanism of the circadian clock as a function of the LD-zeitgeber became the focus of interest, as these should compensate the daily deviation from the 24-h day caused by tau > 24 h.

Clearly, the interaction of light with the pacemaker has been identified as an important clue to the underlying mechanism of the DAO phenomenon (Chapter II). Thus, it was questioned whether parametric and/or non-parametric effects of light contribute to the delayed activity onset. Evidence in favor of the hypothesis that parametric light effects may not play a central role in the entrainment of the circadian system in Djungarian hamsters came from animals kept under semi-natural conditions. These animals displayed activity at the ground surface exclusively during the dark time. Thus they may perceive light only when animals start to become active too early or stop activity too late, respectively (Weinert et al., 2009). This is in accordance with the current view that nocturnal burrowers would benefit less from parametric light effects than from non-parametric ones that entrain the pacemaker (Daan, 2000). Experiments with DAO and WT hamsters kept under skeleton photoperiods have been conducted in the laboratory to minimize the parametric light effect on the circadian pacemaker. These investigations also demonstrated that the influence of parametric light effects is small, if present at all, under LD conditions. In brief, DAO and WT hamsters were kept under standard LD conditions and subsequently exposed to skeleton photoperiods, i.e. brief light pulses at each of the former times of “light-off” and “light-on”. No changes in the activity patterns have been observed, either in WT or DAO animals (unpublished results). Most strikingly, the rate of the daily delay of the activity onset in DAO hamsters remained almost unaltered, which is a further indication that parametric light effects may only marginally contribute to the DAO phenomenon. Thus, non-parametric light effects on the circadian pacemaker in DAO animals must be a source of the diminished ability to

synchronize, which has been demonstrated by investigation of phase responses in the early and late subjective night (Chapter II). In particular, phase advances of the activity onset and offset following brief light pulses in the late subjective night were significantly smaller in DAO hamsters, whereas phase delays evoked in the early subjective night were similar between phenotypes. As a consequence, the overall phase response for the activity onset in DAO animals is insufficient to compensate the long tau, thus leading to its delay (Chapter II). This was confirmed by a PRC (Aschoff type VI) constructed when animals were kept under a LD cycle (Chapter III). Though the shape of the PRC was similar between phenotypes, it was compressed according to alpha in DAO hamsters, and the magnitude was smaller when compared to the WT. Whether phase shifts following light stimulation are generally reduced in DAO hamsters or whether this phenomenon is phase specific is an important question, the answer to which will enhance the identification of the underlying causes for the DAO phenomenon. The results of phase responses elicited under a LD cycle are in support of the former assumption, whereas the results, so far of phase shifts under conditions of constant darkness tend rather to support the latter hypothesis. However, as the methods by which the results have been obtained are different, direct comparison of the two outcomes is difficult. For instance, a change in the shape of the PRC as a function of tau, as it was observed in mice and Djungarian hamsters (Puchalski and Lynch, 1992; Schwartz and Zimmerman, 1990), may be a possible reason for the differences observed between DAO and WT hamsters when investigated under constant darkness. Such differences may not be present in synchronized animals tested under LD conditions. The construction of a complete PRC (Aschoff type I) of both phenotypes under constant darkness, which is currently in progress, may help to clarify this issue. On the other hand, it has been questioned whether the smaller magnitude of the Aschoff type VI PRC is a consequence of the short alpha in DAO hamsters, as it was found in Syrian and Djungarian hamsters investigated in constant darkness (Evans et al., 2004; Puchalski and Lynch, 1991). Our results disagree with the assumption that the phase response is a function of alpha; WT hamsters displayed no larger phase responses when kept under a LD8:16 compared to a LD14:10 light regimen (Schöttner et al., submitted). Also, phase responses following light pulses two hours before light onset were almost the same in DAO hamsters, irrespective of whether the activity onset was delayed by 2 or 6 h, but smaller compared to WT hamsters (unpublished results). Thus, the results provide good evidence for the hypothesis that a reduced phasic response to light is an inherent property in DAO hamsters, and that this contributes to the delay of activity onset.

The extension of the dead zone detected by the type VI PRC in DAO hamsters has been confirmed by the investigation of Fos expression in the SCN following photic stimulation (Chapter III and VI). According to this result, only the effect of "light-on" should have a considerable influence on synchronizing the clock under a LD photocycle, since "light-off" falls in the dead zone of the PRC in DAO hamsters. This has been verified in experiments, in which animals were first kept under skeleton photoperiods. Subsequently, either the evening or the morning pulse was deactivated, so animals received only one brief light pulse during the 24-h cycle. In the case of the morning pulse remaining, almost no changes were observed in the activity patterns. When only the evening pulse was retained, hamsters free-ran with tau > 24 h until activity offset was once again "captured" by the light pulse (unpublished results). Similar results have been observed in WT hamsters. According to those findings, entrainment is predominantly a function of a tau > 24 h and the phase-advancing effects of "light-on" in the morning upon hamsters of both phenotypes. In particular, since the phase-advancing effects of "light-on" seem to be insufficient to compensate the long tau, activity onset in DAO hamsters is continuously delayed.

The investigation of the underlying mechanism of the reduced phasic effect of light can undoubtedly contribute to a better understanding of the DAO phenomenon. A diminished sensitivity to light has been proposed as a possible reason for the delayed activity onset in DAO hamsters. Particularly, the different reactions of DAO and WT hamsters to low constant light emphasize that the sensitivity to light is altered in DAO animals (Chapter II). Whereas WT hamsters increase tau under constant light of 1 lx intensity, no such reaction was observed in DAO animals. However, higher light intensities (10 and 100 lx) led to similar reactions in both phenotypes. Preliminary results on investigations of zeitgeber strength also point to a lower sensitivity to effects of light on the circadian pacemaker in DAO hamsters. The rhythm was phase delayed when animals were kept under 1 lx daytime illumination compared to 100 lx whereas it remained unaffected in WT animals (unpublished data). Furthermore, the results of the pineal melatonin investigation support the assumption that the sensitivity to light is reduced in DAO hamsters, as the melatonin decline at the beginning of light phase was slightly less than in WT hamsters (Chapter V). Therefore, the question arises whether a reduced reception of light in the retina or an altered signal transduction by the afferent pathways to the SCN is the origin of a decreased sensitivity to light and thus the diminished ability to synchronize in DAO hamsters. Since the amount of Fos-ir cells was similar between DAO and WT hamsters, functionality of light reception and afferent signal transduction seems not to be compromised in DAO hamsters (Chapter VI). Thus, the

"communication" of the photic signal within the SCN may differ between the phenotypes. An intensity-dependent increase in *c-fos* expression, in combination with an increase of phase shifting effects of light has been demonstrated by others (Kornhauser et al., 1990). This is further evidence for the above hypothesis, since Fos expression seems to be similar between the phenotypes but phase shifts were smaller in DAO hamsters exposed to light pulses under LD conditions (see above). However, more specific analysis is required to investigate this hypothesis. Thus, the zeitgeber strength of light should be analyzed in more detail in future experiments, particularly by investigating phase-shifting effects of light of different intensities at the same time as Fos expression in the SCN in DAO and WT hamsters.

Investigations on the molecular clockwork of the SCN in hamsters of both phenotypes are also necessary, precisely because photic signal transduction within the SCN seems to be compromised in DAO hamsters. Particularly, *per1* and *per2* gene expression is of great interest, since these genes mediate the phase-shifting effects of light. Light-induced *per1* and *per2* expression initially begins in the SCN core and then spreads into the SCN shell. Moreover, the pattern of *per1* and *per2* expression is dependent on the time of light exposure: *per1*- and *per2*-induced expression in the SCN core followed by *per2* expression in the shell is associated with delaying light pulses in the early night. Advancing light pulses in the late night, on the other side, induce only *per1* expression in the core and, later, in the shell (Antle and Silver, 2005; Hamada et al., 2004; Yan et al., 2007; Yan and Okamura, 2002; Yan and Silver, 2002). Since the interaction of light with the pacemaker seems to be one of the determining factors for the DAO phenomenon, investigation of light-induced *per1* and *per2* messenger RNA expression is necessary to evaluate the effect of light on the circadian clockwork at a molecular level. Investigation will enhance understanding of the relationship between gene expression and behavior in the particular case of the DAO hamsters and enable gaining insight into the underlying mechanisms of the DAO phenomenon. Thus, expression profiles of both genes over one circadian cycle should be examined by in-situ hybridization using two approaches. First, free running rhythms under constant darkness in animals of both phenotypes and with activity durations of approximately 12 h should be investigated. This will enable the amplitude of oscillation and peak time, as well as the levels of *per1* and *per2* expression, to be examined. Such an investigation is important to evaluate the possibility that the DAO phenomenon induced by a malfunction in gene expression. Evidence in favor of such a hypothesis has come from transgenic rats, where constitutive (over)expression of *per1* led to impaired molecular and behavioral rhythms (Numano et al., 2006). In the second approach, animals of both phenotypes should be tested under LD conditions similar to the

protocol used for the corresponding PRC (Aschoff type VI), to evaluate the temporal expression profile of *per1* and *per2* under entraining conditions. Both approaches will allow detailed analysis of the effect of light on the expression of the two genes and will help to discriminate whether light-induced *per* gene expression is disturbed in DAO hamsters. Dependent on the results, the investigation of expression of Per proteins by immuno-histochemical methods might be conducted.

The investigation of compartment-specific gene expression in the SCN (i.e. core and shell region) is also of great interest. This concerns particularly the population of retinorecipient, calbindin-containing cells of the SCN core found in hamsters, which express *c-fos* as well as *per1* and *per2* in a phase-dependent manner following photic stimulation (Hamada et al., 2001; Silver et al., 1996). This subregion has been identified as temporarily "gating" light responsiveness (Hamada et al., 2003) and maintaining rhythmicity of the circadian clock (Kriegsfeld et al., 2004). Investigating the time course of photic-induced *per1* and *per2* expression in the SCN at the beginning and end of the subjective night, similar to a method proposed by Yan and colleagues (Yan et al., 1999), will contribute to a better understanding of the pacemaker function in DAO and WT hamsters. More importantly, such an investigation can provide insights whether the compartment-specific communication between SCN neurons may be disturbed in general or in a phase-dependent manner, i.e. the light pulses which produce phase advances (Golombek et al., 2004; Golombek and Rosenstein, 2010).

Additionally, the spatio-temporal expression profile of *per* genes on the rostral-caudal plane of the SCN should be examined, as structural heterogeneity of gene expression is associated with coding for day length (Hazlerigg et al., 2005; Naito et al., 2008). Systematic investigations under long, intermediate and short photoperiods have revealed a functional relationship between oscillating cell groups in the rostral SCN with the activity onset (evening) oscillator and in the caudal SCN with the activity offset (morning) oscillator, respectively (Inagaki et al., 2007). Investigation of the expression profiles will therefore help to determine whether a possible deficiency of light-induced gene expression is coupled to a designated region of the SCN and will also contribute to a better understanding of the clocks function regarding the two-oscillator theory.

In a model proposed by Daan and colleagues (2003), entrainment is a function of mutually coupled evening and morning components of the circadian pacemaker, both of which shown alternating delay and advance zones over the course of one cycle as predicted from the PRC. According to this model, if $tau_E < 24$ h and $tau_M > 24$ h delays should dominate over advances in the evening component (E, activity onset) whereas advances should dominate over delays

in the morning component (M, activity offset) if tau_E < 24 h and tau_M > 24 h. Also, coupling strength between both components has an important function (Daan et al., 2003). When this model is applied to DAO and WT hamsters, its implications are: since tau_E and tau_M in hamsters of both phenotypes are longer than 24 h (Chapter II), advances should always dominate over delays in the corresponding PRCs for both E and M. Moreover, this effect should be even stronger in DAO hamsters. Our results support this assumption for WT hamsters, when taking the resulting net phase response at CT14 and CT22 as reference point as a first approximation. By contrast, in DAO hamsters and contrary to the predictions from this model, the delay to advance (D/A) ratio seems to be larger compared to the WT due to smaller phase advances in the late subjective night, but still sufficient to compensate the long tau for the activity offset. This is not the case for the activity onset, the delay portion predominating over the advance portion, thus leading to its continuous delay (Chapter II).

The data indicate that the DAO phenomenon cannot be attributed to a deficiency of the single E or M component only. Both components show a reduced phase advancing effect of light, which is, however, insufficient to compensate tau of the E component only (Chapter II). Weak mutual coupling of the E and M components, as demonstrated by the different taus of the activity onset and offset as well as by the different magnitude (Chapter II) and direction (Chapter III) of phase shifts following light pulses, may facilitate the phenomenon. However, weak mutual coupling is not the explanation for the phenomenon since both phenotypes are characterized by weak oscillator coupling. Thus, the underlying mechanisms of the different abilities between E and M to synchronize still remain unclear and further experiments investigating the molecular basis are necessary to draw further conclusions. Temporal differences in mRNA oscillation of *per1* and *per2* as well as *cry1* and *cry2* have been taken as evidence in support of the hypothesis that the morning oscillator comprises the Per1/Cry1 and the evening oscillator the Per2/Cry2 heterodimeric loops (Daan et al., 2001). Several studies have supported this assumption, whereas others have failed to confirm the theoretical predictions connected with this hypothesis, leading to alternative suggestions (Hastings, 2001). More recent studies, however, indicate that the theory of a simple 2-oscillator model has to be reconsidered in favor of a distributed network model of individual neurons throughout the SCN that might be designated as morning or evening oscillators based on their response to the photoperiod (Helfrich-Forster, 2009). Therefore, investigations on the spatio-temporal expression of clock genes in the SCN, as mentioned above, will enhance understanding of the functionality and interaction of both oscillators in DAO and WT

hamsters. Also, the occurrence of arrhythmic activity patterns in the AR phenotype may be explained by such investigations.

Summarizing the results discussed above, it can be stated that a long free-running period coupled with a disturbed non-parametric effect of light on the circadian pacemaker itself leads to a diminished ability to synchronize in hamsters of the DAO phenotype. Studies on shifts of the zeitgeber cycle have demonstrated that this phenomenon has serious consequences for the re-entrainment behavior in DAO hamsters (Chapter III). Direction and duration of resynchronization were influenced, particularly when the LD cycle was phase advanced. DAO hamsters did not adjust their rhythm according to the direction of the shift of the LD cycle, but phase delayed instead, thus lengthening the time of re-entrainment when compared to WT hamsters. Additionally, a considerable amount of animals started free-running or became arrhythmic following such a shift of the LD cycle. The reason for the altered re-entrainment behavior was revealed by the PRC (according to the Aschoff type VI protocol) that was characterized by small phase shifts and an extended dead zone compared to WT hamsters, as mentioned above; these results fully account for re-entrainment behavior under LD conditions.

Another consequence of the disturbed interaction of the LD zeitgeber with the circadian pacemaker in DAO hamsters concerns seasonal adaptation. DAO hamsters did not react to changes of the photoperiod (from long-day to short day conditions by symmetrically lengthening of the dark time), either by activity decompression or body weight loss, gonadal regression or fur coloration (Schöttner et al., submitted). However, when hamsters were kept under constant darkness, alpha decompressed as a result of different taus of activity onset and offset and these hamsters then displayed the same adaptation responses as observed in WT hamsters kept under short-day conditions. Thus, the general endogenous mechanisms of short day adaptation are present in DAO hamsters, but this process is prevented rather than mediated by the effect of light (Schöttner et al., submitted). This hypothesis was supported when it was revealed that non-responsiveness in DAO hamsters is not simply an effect of the long tau or a consequence of strong coupling between evening and morning oscillator of the circadian system, as proposed for “non-responders” in the studies of other researchers (Gorman et al., 1997). WT were able to react to short photoperiods and display even longer taus than that observed in “non-responders” by others (Puchalski and Lynch, 1988). On the other hand, the coupling strength between morning and evening oscillator has been characterized as considerably lower in hamsters of both phenotypes (see above). The construction of a PRC (Aschoff type VI) under short photoperiods clearly revealed that the

underlying mechanism for the inability to respond to the photoperiod was connected with diminished phase responses in DAO hamsters (Schöttner et al., submitted).

Whether the DAO phenomenon may have adverse effects for the animals' fitness under constant LD conditions is difficult to assess based on the first results gained by body temperature investigations. Studies on the thermoregulatory efficiency gave the initial evidence that DAO hamsters are not negatively affected in this when compared to WT animals (Chapter IV). Even non-responsiveness to short photoperiods, as observed in DAO hamsters, is obviously no disadvantage to survive winter conditions, as revealed by own results under natural light and temperature conditions (unpublished results). It remains unknown generally, whether such phenomenon as observed in DAO hamsters as a result of a diminished interaction of the photic zeitgeber with the circadian pacemaker will be found in animals living freely in the field, since other, non-photic zeitgebers like temporal availability of food and water, daily fluctuations of environmental temperature, intra- and inter-specific interactions or behavioral feedback, all influence circadian rhythmicity (Aschoff and Tokura, 1986; Challet and Mendoza, 2010; Liu et al., 1998; Mrosovsky, 1988; Rajaratnam and Redman, 1998; Reebs and Mrosovsky, 1989; Sharma and Chandrashekaran, 2005). It is assumed that non-photic zeitgebers may have a complementary role in the synchronization of the circadian system, particularly in case of entrainment disruptions (Golombek and Rosenstein, 2010). First results on investigations of time-restricted feeding schedules, temperature cycles and social synchronization revealed minor effects on the activity rhythm in DAO hamsters. The continuous delay of the activity onset was weakened or even disappeared, but alpha never decompressed (unpublished data). Access to running wheels led to different reactions in DAO hamsters, as it induced decompression of alpha in a number of animals whereas others remained unaffected, independently of the level of running wheel activity (Weinert and Schöttner, 2007). The exact reason for the inconsistent result, however, remains still unclear. It was proposed that the effect of running wheel-induced activity shortens tau (Mrosovsky, 1999), so that phase-shifting effects of light may became sufficient to synchronize the circadian activity rhythm accurately. Studies in mice, on the other hand, revealed that spontaneous running-wheel activity did not increase phase shifting effects of light in the early and late subjective night (Mistlberger and Holmes, 2000). Therefore, it might be excluded as a possible explanation for the decompression of alpha in DAO hamsters, though it needs to be verified in our hamsters in future studies, particularly in that of the DAO phenotype. Thus, the overt rhythm of clock-controlled activity may provide modulatory feedback effects on the circadian system (Mrosovsky, 1996, 1999), and this will be sufficient

to initiate a "re-adjustment" of the pacemaker in some DAO hamsters with access to a running wheel. However, this hypothesis is speculative since the underlying mechanisms are still poorly understood. The SCN receives non-photic stimuli from the dorsal raphe nucleus (DRN) via neuropeptide Y (NPY)-containing neurons of the IGL, leading to the suggestion that the IGL integrates photic and non-photic information which is then transmitted to the SCN (Dibner et al., 2010; Janik et al., 1995; Meyer-Bernstein and Morin, 1996). Non-photic stimulation of the SCN is also provided by a serotonergic (5HT) projection emanating from the median raphe nucleus (MRN) (Leander et al., 1998; Meyer-Bernstein and Morin, 1996). However, though non-photic stimulation has its largest effects on the clock during the subjective day (Golombek and Rosenstein, 2010), this does not preclude the possibility that behavioral feedback mediated by NPY and serotonergic stimulation in the SCN may enhance initiation of resetting the activity onset in DAO hamsters (Marchant et al., 1997), which has to be investigated in future studies.

The outcomes of the present studies have enabled new insights into the underlying mechanisms giving rise to the phenomenon of a DAO phenotype in Djungarian hamsters of a breeding colony at the Zoology Institute of the University of Halle to be gained. The results point to differences in key characteristics of the circadian system between DAO and WT hamsters, namely the free-running period on the one hand and the interaction of the LD cycle with the circadian system, which is altered in DAO hamsters, on the other. Also, on the basis of the studies on the body temperature and melatonin rhythms, as well as c-Fos expression in the SCN following photic stimulation, it was possible to localize the SCN itself as the origin of the phenomenon. Therefore, the interplay between free-running period and resetting of the circadian pacemaker by photic cues, particularly by phase advances, seems to be the crucial factor determining the circadian phenotype in Djungarian hamsters of our breeding stock. One reason seems to be a reduced sensitivity to light in DAO hamsters. However, since the perception and transduction of the photic signal to the SCN does not seem to be constrained, downstream processes within the SCN that use light information to reset the circadian pacemaker have to be taken into account as a possible origin of the DAO phenomenon. In AR hamsters, the SCN produces no circadian signal and this is evidence in favor of the hypothesis that the mechanism for rhythm generation is defective in these animals. Thus, hamsters of the WT, DAO and AR phenotype provide an excellent model to study the underlying molecular mechanisms of photic entrainment with special regard to light-induced resetting of the circadian pacemaker and the two-oscillator theory of entrainment.

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APPENDIX

Curriculum Vitae

Personal information		Konrad Schöttner
	Birth	March 3rd 1981 in Dresden
	Nationality	German
	Languages	English, French

School education

09/1987 – 07/1992	82. Polytechnische Oberschule Dresden
08/1992 – 07/1997	Gymnasium Klotzsche, Dresden
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Academic education

from 10/2000	Study of Biology (Diploma) at the Martin-Luther-University Halle-Wittenberg (MLU)
from 10/2003	Advanced Studies in Zoology, Behavior, Chronobiology
2/2005 – 7/2005	Study Semester abroad: Institute of Zoology & Entomology, University of Pretoria, South Africa
from 8/2006	Diploma thesis: "Characterization of circadian activity rhythms in hamsters of the genus *Phodopus*"
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Research visits

6/2007 – 8/2007	Biological Station of the Russian Academy of Sciences Moscow, Chernogolovka, Russia
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Eigenständigkeitserklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig und ohne fremde Hilfe nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht zu haben. Ich erkläre weiterhin, dass ich mich noch nicht um den Doktorgrad beworben habe und diese Arbeit weder der Naturwissenschaftlichen Fakultät I – Biowissenschaften der Martin-Luther Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion vorgelegt wurde.

Halle, den 13.12.2011

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