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„Circadian abnormalities in the Cav1.4 IT mouse model for congenital stationary night blindness 2"

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Contents

1 Introduction

1.1 Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels (VGCCs) are responsible for Ca^{2+} influx in response to action potentials. Hence, Ca^{2+} serves as a second messenger of electrical signaling, activating various cellular events such as contraction, secretion, integration of synaptic input in neurons, synaptic transmission and regulation of gene expression (Catterall, 2011). Reuter et al. first described Ca^{2+} currents in myocytes, and since then it has become clear that there are multiple types of Ca^{2+} currents which can be defined by their physiological and pharmacological properties (Table 1) (Reuter, 1979).

Table 1. Nomenclature and function of Ca^{2+} channel types

Ca^{2+} current type	α1 Subunits	Specific blocker	Principal physiological functions	Primary tissues
L	$Ca_V1.1$	DHPs	Excitation-contraction coupling in skeletal muscle, regulation of transcription	skeletal muscle
	$Ca_V1.2$	DHPs	Excitation-contraction coupling in cardiac and smooth muscle, endocrine secretion, neuronal Ca^{2+} transients in cell bodies and dendrites, regulation of enzyme activity, regulation of transcription	heart, smooth muscle, brain, heart, pituitary, adrenal
	$Ca_V1.3$	DHPs	Endocrine secretion, cardiac pacemaking, neuronal Ca^{2+} transient in cell bodies and dendrites, auditory transduction	brain, pancreas, kidney, ovary, cochlea
	$Ca_V1.4$	DHPs	Visual transduction	retina
N	$Ca_V2.1$	ω-CTx-GVIA	Neurotransmitter release, Dendritic Ca^{2+} transients	brain, cochlea, pituitary
P/Q	$Ca_V2.2$	ω-Agatoxin	Neurotransmitter release, Dendritic Ca^{2+} transients	brain, nervous system
R	$Ca_V2.3$	SNX-482	Neurotransmitter release, Dendritic Ca^{2+} transients	brain, cochlea, retina, heart, pituitary
T	$Ca_V3.1$	None	Neurotransmitter release, Dendritic Ca^{2+} transients	brain, nervous system
	$Ca_V3.2$		Pacemaking and repetitive firing	brain, heart, kidney, liver
	$Ca_V3.3$		Pacemaking and repetitive firing	brain

Note. DHP = dihydropyridine; ω-CTx-GVIA = ω-conotoxin GVIA from the cone snail *Conus geographus*; SNX-482 = synthetic version of a peptide toxin from the tarantula *Hysterocrates gigas* (adopted from *Ertel et al., 2000* and *Catterall, 2011*)

Curtis and Catterall purified the first Ca^{2+} channels from skeletal-muscle transverse tubules. Further analysis led to the discovery of three subunits: α_1, β and γ (Curtis and Catterall, 1984). In-depth biochemical investigations revealed an additional $\alpha_2\delta$ subunit (Takahashi et al., 1987). The α_1 subunit builds the pore-forming complex and consists of four repeated domains (I – IV), each containing six transmembrane segments (S1 – S6). Additionally, the α_1-subunit contains a membrane-associated loop between transmembrane segments S5 and S6 (Catterall, 2000). The segments S1-S4 form the voltage sensor, whereas segments S5 and S6 build the conduction pore. Hence, a central conduction pore is surrounded by four voltage sensors (Bezanilla, 2008). Thus, Ca^{2+} channels open upon membrane depolarization.

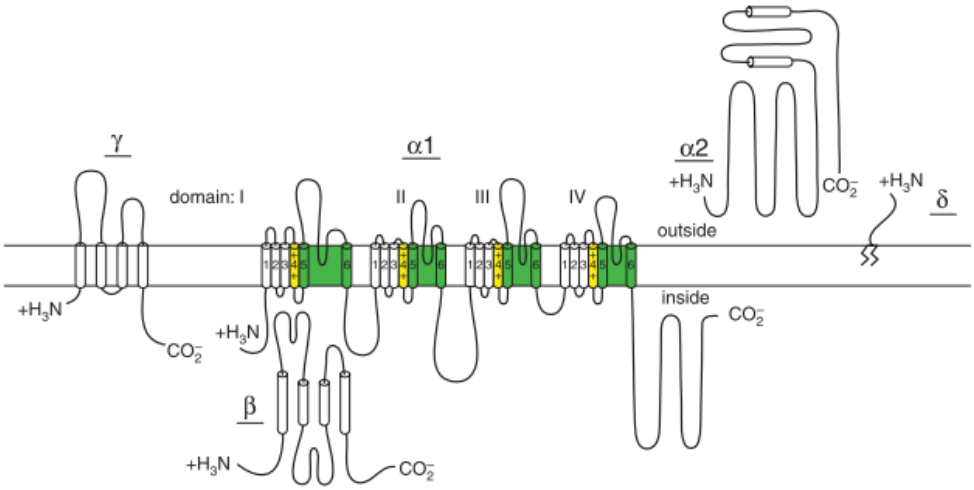

***Figure 1*. Subunit structure of Ca^{2+} channels.** Expression of the α_1-subunit would be sufficient to produce functional Ca^{2+} channels. However, the expression of the other subunits is important for expression levels and gating properties (Perez-Reyes et al., 1989) (Image source: *Catterall, 2000)*

Different α_1 subunits define the different types of Ca^{2+} currents. Currently, ten distinct Ca^{2+}-channel α_1 subunits are defined. A nomenclature divides them into three functionally and structurally related families: Ca_V1, Ca_V2 and Ca_V3 (Ertel et al., 2000). The Ca_V1 family is responsible for the conduction of L-type calcium currents, which is necessary for muscle contraction, gene transcription and endocrine secretion. These channels are primarily regulated by second-messenger-activated protein phosphorylation pathways. Channels of the Ca_V2 channel family conduct N-type, R-type and P/Q type Ca^{2+} currents. They are primarily important for rapid synaptic transmission. Interactions with G-proteins

and SNARE proteins regulate these types of Ca^{2+} channels. Additionally, protein phosphorylation plays a role in their regulation. The Ca_V3 family conducts T-type Ca^{2+} currents. Compared to other Ca^{2+} channels these channels activate and inactivate more rapidly, and also activate at more negative membrane potential (Catterall, 2000, Catterall et al., 2005). They are therefore important in repetitively firing tissues such as the sinoatrial node (SAN) (Mangoni et al., 2006), or in relaying neurons of the thalamus, where they drive sleep spindles and control sleep (Lee et al., 2004). Generally, L- and T-type currents are found in a wide range of cell types, while R-, Q-, P- and N-type Ca^{2+} currents are predominantly recorded in neurons (Catterall, 2011). To this end, due to the different structures and patterns of regulation, the distinct Ca^{2+} channel families provide a flexible range of Ca^{2+} entry pathways in return for alterations in membrane potential (Catterall, 2000).

1.1.1 L-type calcium channels

The Ca_V1 Ca^{2+} channel family is called L-type Ca^{2+} channels (LTCCs) because they lead to a long-lasting inward current during depolarization. Such channels additionally show high voltage of activation and slow voltage-dependent inactivation and are very sensitive to Ca^{2+} antagonist drugs such as dihydropyridines, benzothiazepines and phenylalkylamines (Catterall, 2011). The four distinct members of LTCCs are $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$. The genes coding for the respective channel proteins are CACNA1S ($Ca_V1.1$), CACNA1C ($Ca_V1.2$), CACNA1D ($Ca_V1.3$) and CACNA1F ($Ca_V1.4$.) (Bech-Hansen et al., 1998, Catterall et al., 2005, Zamponi et al., 2015).

$Ca_V1.1$ channels are primarily restricted to skeletal muscles, where they are expressed within the membranes of the T-tubule system. They physically contact ryanodine-sensitive Ca^{2+} release channels (RyRs) in the sarcoplasmic reticulum. This leads to a rapid release of Ca^{2+} and subsequently results in muscle contraction (Tanabe et al., 1987).

As depicted in figure 2, $Ca_V1.2$ and $Ca_V1.3$ channels are expressed in virtually all excitable cells, including endocrine cells (Marcantoni et al., 2010), the brain (Dragicevic et al., 2014, Chan et al., 2007) and the heart (Mangoni et al., 2003). They contribute to the following physiological processes:

i. *$Ca_V1.2$ and $Ca_V1.3$ channels in the heart.* In cardiomyocytes contraction is mainly triggered by $Ca_V1.2$ channels, whereas $Ca_V1.3$ channels are the predominant isoform in the SAN and atrioventricular node (AVN). There, $Ca_V1.3$ channels are essential for normal pacemaker function, since loss-of-function mutations lead to bradyarrhythmia (Baig et al., 2011).

ii. *$Ca_V1.2$ and $Ca_V1.3$ channels in the brain.* In the brain, $Ca_V1.2$ and $Ca_V1.3$ channels modulate neuronal firing and induce Ca^{2+} pathways involved in gene-expression control. Subsequently, they play a role in learning and memory, drug addiction, and neuronal development (Striessnig et al., 2014). The $Ca_V1.2$ isoform is mainly responsible for hippocampal function, where it is required for spatial memory formation (White et al., 2008). Additionally, both isoforms contribute to other types of memory such as fear memory and drug-taking behaviors associated with memory (Moosmang et al., 2005, Busquet et al., 2008). Furthermore, deficiencies in $Ca_V1.2$ and $Ca_V1.3$ may provoke anxiety- and depression-like behavior (Busquet et al., 2010, Lee et al., 2012). Further, these isoforms contribute to the vulnerability of substantia nigra dopaminergic neurons to cell death in Parkinson's disease (Surmeier et al., 2011). In addition, $Ca_V1.3$ are important for maintaining normal synaptic connectivity (Zamponi et al., 2015).

iii. *$Ca_V1.2$ and $Ca_V1.3$ channels in the endocrine cells.* Although $Ca_V1.2$ and $Ca_V1.3$ channels are expressed in many endocrine cells, they are mainly present in pancreatic island cells, aldosterone-producing cells in the adrenal cortex, and adrenal chromaffin cells (Zamponi et al., 2015). In the pancreas $Ca_V1.2$ channels therefore control insulin secretion, whereas $Ca_V1.3$ channels are required for β-cell proliferation (Sinnegger-Brauns et al., 2004, Namkung et al., 2001). Both isoforms are additionally coupled to catecholamine secretion during long depolarizing stimuli

(Marcantoni et al., 2010). Furthermore, $Ca_V1.3$ channels are important for aldosterone secretion.

iv. *$Ca_V1.2$ and $Ca_V1.3$ channels in auditory and vestibular hair cells.* $Ca_V1.3$ channels are essential for hearing, since $Ca_V1.3$ deficiency leads to deafness (Baig et al., 2011).

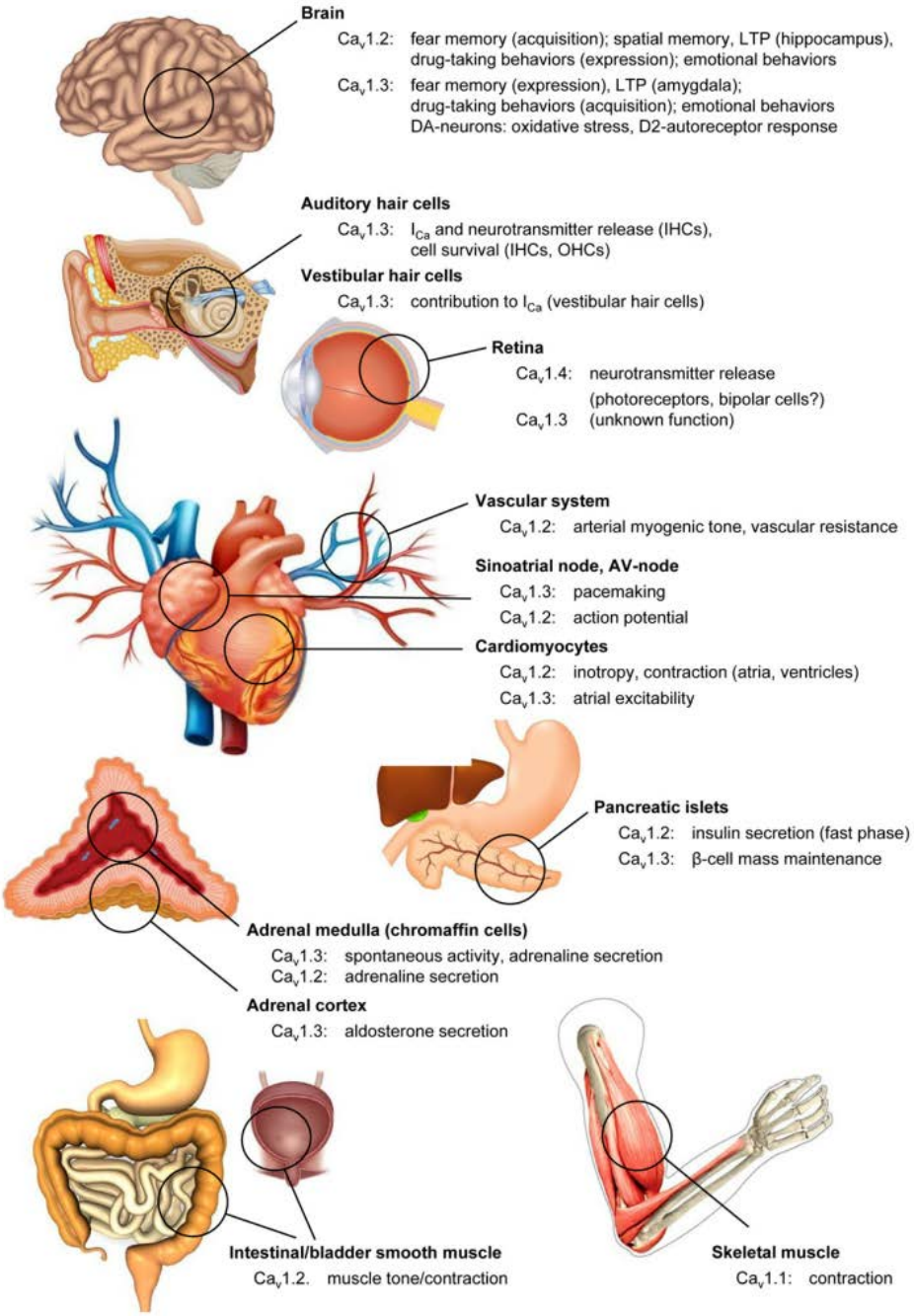

Figure 2. **Physiological functions of the various LTCC isoforms.** Whereas R-, Q-, P- and N-type Ca^{2+} currents are predominantly recorded in neurons, L-type Ca^{2+} currents are recorded in a wide range of cell types. (Image source: *Zamponi et al., 2015*)

The expression of $Ca_V1.4$ channels is primarily restricted to the retina, where they are expressed in synapses of the outer and inner plexiform layer and on photoreceptor cell bodies. In so-called ribbon synapses they are necessary for Ca^{2+} entry that triggers the exocytosis of neurotransmitters. The importance of proper $Ca_V1.4$ function is evident, since both loss or gain of channel function leads to alterations in photoreceptor synapse function and hence visual disorders (Stockner and Koschak, 2013, Zamponi et al., 2015). Studies also demonstrate the expression of $Ca_V1.4$ channels in dorsal-root ganglia neurons (Murakami et al., 2001), T-lymphocytes (Kotturi and Jefferies, 2005) and mast cells (McRory et al., 2004).

1.1.2 Channelopathies in Ca_V 1.4 channels

Several X-linked visual disorders such as X-linked retinal disorder, cone-rod dystrophy (Jalkanen et al., 2006), night-blindness-associated transient tonic gaze (Simonsz et al., 2009), Åland Island eye disease (Jalkanen et al., 2007) and incomplete congenital stationary night blindness (Bech-Hansen et al., 1998) are linked to mutations in the CACNA1F gene coding for the $Ca_V1.4$ channel α1 subunit. Distinguishing between these visual disorders is difficult because of the similarity of their symptoms and the variability within their clinical pictures (Burtscher et al., 2014). Subsequently, the distinction of the different disorders is made using electroretinograms (ERGs) (Striessnig et al., 2010).

1.1.2.1 Congenital stationary night blindness type 2

Most mutations in the gene CACNA1F coding for the voltage-gated calcium channel $Ca_V1.4$ cause incomplete congenital stationary night blindness type 2 (CSNB2), which is an X-linked form of congenital stationary night blindness (OMIM: 300110). In contrast to CSNB1, however, which is caused by mutations in the metabotropic glutamate receptor 6 or by defects in the nyctalopin, night blindness is not the primary symptom in CSNB2 (Striessnig et al., 2010). The range of mutations affecting the CACNA1F gene includes missense or truncation mutations. Furthermore, both loss and gain of function mutations may result in

CSNB2. Altered gating properties of the $Ca_V1.4$ channel thus lead to an elimination or decrease of the channel-mediated Ca^{2+} entry which is necessary for normal photoreceptor signaling (Stockner and Koschak, 2013, Zamponi et al., 2015). Meanwhile, a variety of mutations causing CSNB2 have been functionally characterized (Figure 3) (Hemara-Wahanui et al., 2005, Hoda et al., 2005, Hoda et al., 2006, McRory et al., 2004, Peloquin et al., 2007, Singh et al., 2006). Loss as well as gain of function mutations in the CACNA1F gene can also result in alterations of photoreceptor synapse formation.

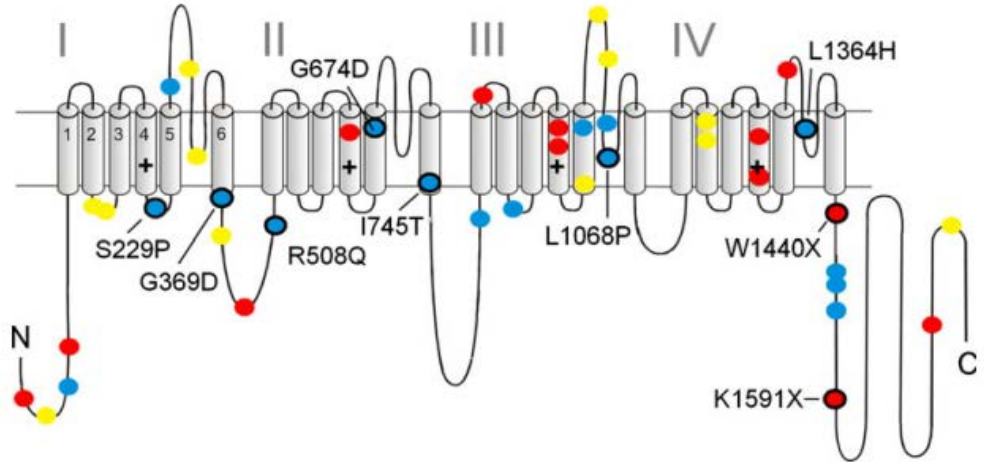

***Figure 3*. Identified mutations in the $Ca_V1.4$ α1 subunit in patients suffering from CSNB2.** Positions of CSNB2 mutations are indicated. Structural changes are indicated by colors: *blue* = single missense mutation; *red* = mutation that leads to a truncated protein; *yellow* = in-frame deletion or insertion; *black circles* = mutations that are functionally characterized (Image source: *Striessnig et al., 2010*)

The symptoms associated with CSNB2 are myopia, nystagmus, strabismus, decreased visual acuity and night blindness. However, the severity of night blindness is variable (Bech-Hansen et al., 1998). CSNB2 mainly affects male individuals, due to its X-linked nature, though homozygote and in some cases even heterozygote females can be affected (Hope et al., 2005).

Generally, the clinical picture of CSNB2 is quite variable. The disease is therefore diagnosed through alterations in the ERG. Patients suffering from CSNB2 display a dim scotopic ERG and a characteristic negative bright-flash ERG which possesses large a-waves but reduced b-waves (Striessnig et al., 2010). This data from the ERG hints at a defect in neurotransmission between photoreceptors and second-order neurons (Striessnig et al., 2010, Tremblay et al., 1995).

1.1.2.2 Mouse model for congenital stationary night blindness type 2

A new mutation—first described in a New Zealand family—in the CACNA1F gene causes a special form of CSNB2. Male individuals in particular have severe symptoms including abnormal color vision and in some cases even intellectual disability. Furthermore, heterozygote female individuals suffer from this severe form of CSNB2, which is contradictory with previous descriptions of female heterozygotes with CSNB2. Examinations of the fundus, however, point to a normal appearance of the optic discs and vessels (Hope et al., 2005).

The mutation underlying this phenotype was identified as the I745T mutation in the CACNA1F gene. The I745T mutation is responsible for shifting the voltage-dependent activation of the $Ca_V1.4$ channel. This shift is approximately -30 mV. The mutation also causes slower inactivation kinetics of the $Ca_V1.4$ channel. The exceptional phenotype described in the New Zealand family is therefore due to an increase in the $Ca_V1.4$ channel activity (Hemara-Wahanui et al., 2005).

To investigate the functional phenotype of patients with gain-of-function mutations in the CACNA1F gene, the $Ca_V1.4$ IT mouse line is used. Knoflach and colleagues demonstrate that the IT mouse line is a very suitable model to characterize the functional phenotype of patients harboring the $Ca_V1.4$ I745T point mutation. In line with human patients, the IT mouse line shows a loss in visual function, which was confirmed with the visual task of the Morris Water maze (Knoflach et al., 2013). Furthermore, recent evidence indicates that the gain-of-function I745T mutation leads to immature photoreceptor synapse in affected mice (Zabouri and Haverkamp, 2013). The IT mouse line shows an increased response latency upon light exposure, which is explained by an increased basal calcium level in photoreceptors that in turn increases the time needed to diminish glutamate release. The reason for the higher Ca^{2+} levels in the photoreceptors is the leftward shift in the activation curve of the mutated $Ca_V1.4$ channel. Knoflach and colleagues also analyzed the behavioral phenotype of $Ca_V1.4$ IT mice and detected no differences in anxiety-related parameters or in learning- and memory-related processes (Knoflach et al., 2013).

1.2 The circadian clock

The earth's rotation around the sun creates a 24-hour solar cycle, and to maximize fitness, animals have organized their physiology to this day/night cycle (Allada et al., 2001). Most light-sensitive organisms possess a time-measuring device called the circadian clock which allows them to achieve such organization. It has been speculated that there are as many clocks as there are cells in the body. However, a hierarchy exists between these clocks (Dibner et al., 2010). At the top are the suprachiasmatic nuclei (SCN) of the hypothalamus which operate as a master pacemaker by synchronizing the peripheral clocks. The result of this plethora of endogenous clocks is a circadian rhythm. These clocks are entrained by recurring environmental cues, with light being the most potent stimulus (Reppert and Weaver, 2001).

Genome-wide transcriptome profiling studies indicate that 2% of the detected genes are subjected to a circadian expression (Duffield et al., 2002). Nevertheless, there are assumptions that even 50% of mammalian genes are expressed periodically in one or more tissue (Welsh et al., 2010). The cyclic gene expression influences the sleep-wake cycle, xenobiotic detoxification, cell proliferation and metabolism (Schibler, 2007). Disturbances in the circadian rhythm are linked to a variety of diseases such as metabolic syndrome, obesity and neuropsychiatric disorders, but also cancer (Bray and Young, 2009).

1.2.1 The molecular core of the circadian clock

In animals, circadian behavior is an integrative system where genes produce a behavioral output. The core of this molecular clockwork is a transcriptional–translational feedback loop (TTFL). In mammals, this TTFL is based on what are known as core clock genes, which create an auto-regulatory feedback loop by interacting with each other (Albrecht and Ripperger, 2009). However, hundreds of downstream target genes show circadian expression (Takahashi, 2004). Additionally, posttranslational modifications such as phosphorylation contribute to the creation of the circadian rhythm (Lee et al., 2001).The TTFL consists of a positive and a negative limb. The positive limb includes the transcription factors

circadian locomotor output cycles kaput (Clock) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (Bmal1). These basic helix-loop-helix (bHLH)-period-Arnt-single-minded (PAS) transcription factors form heterodimers that induce the rhythmic expression of the target genes Period (Per 1-3) and Cryptochrome (Cry1 and Cry2) as well as circadian output genes (Ko and Takahashi, 2006), by binding to E-box enhancers with the nucleotide sequence CACGTG (Gekakis et al., 1998, Reppert and Weaver, 2002). In mice with a conditional CLOCK knockout, however, the circadian rhythm does not chase. There is evidence that NPAS2 can likewise heterodimerize with BMAL1 and largely compensate the loss of CLOCK (DeBruyne et al., 2007).

As summarized in figure 4, gene transcription is ascendant until the gene products of Cry and Per accumulate and form heterodimers. The heterodimers translocate back to the nucleus, where they act on CLOCK(NPAS1):BMAL1 complex and thereby inhibit their own transcription (Ko and Takahashi, 2006).

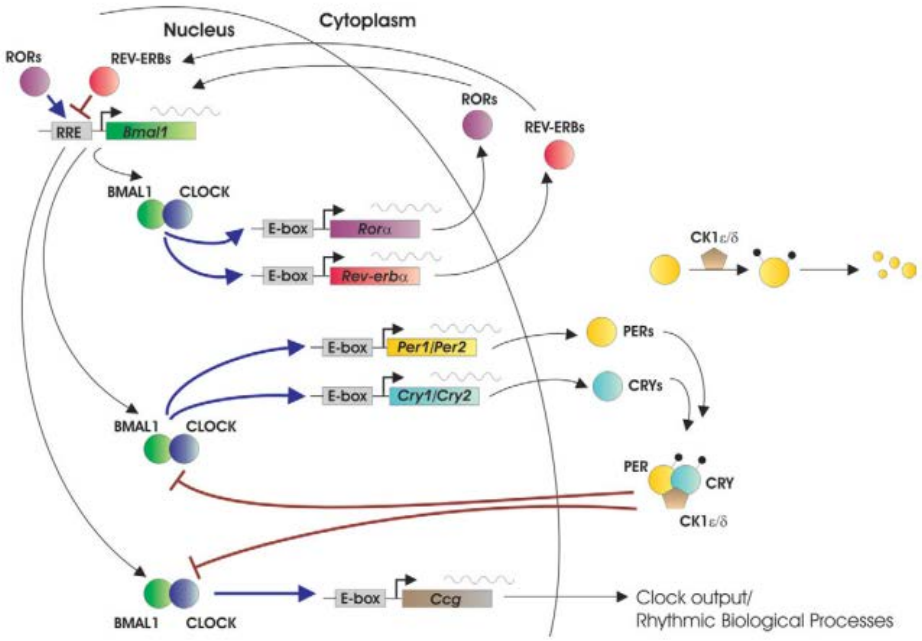

***Figure 4*. The transcriptional–translational feedback loops of the mammalian circadian clock.** The autoregulatory feedback loop involves the activators Clock and Bmal1. They activate the transcription of their target genes Per1, Per2, Cry1 and Cry2. Their gene products, on the other hand, form a repressor complex constituting for the negative limb of the TTFL. The heterodimer Clock:Bmal1 also drives the transcription of numerous target genes, called clock-controlled genes (Ccg). Members of Ror (α, β and γ) and Rev-erb (α and β) form a regulatory loop in which Rors act as activators and Rev-erbs as repressors of Bmal1 transcription. Besides transcription, nuclear import and posttranslational modification of clock factors both play crucial roles in the regulation of the feedback loop (Image source: *Ko and Takahashi, 2006*)

For a new cycle to begin the inhibitory proteins that inhibit Clock:Bmal1 must be removed. Phosphorylation and subsequent degradation are mainly responsible for the elimination of Per and Cry (Gallego and Virshup, 2007).

There is an additional feedback loop which connects the positive and negative limbs of the TTFL. This loop involves reverse erythroblastosis virus (Rev-erbs α and β) and related orphan receptor (Rors α, β and γ), which are transcriptionally regulated by Clock:Bmal1. In this modulatory feedback loop Rev-Erbs inhibit and Rors activate the transcription of Bmal1 (Albrecht, 2012).

1.2.2 Posttranslational mechanisms

Posttranslational modifications of clock genes are essential because they are responsible for fine tuning the circadian rhythm. Hence, phosphorylation, sumoylation, histone acetylation and methylation of the mentioned regulators control the biological length of the day. Taken together, posttranslational modifications are necessary to give the Clock a 24-hour duration, because they ensure a delay between activation and repression of transcription. Generally, reversible phosphorylation is a key step in the regulation of critical processes such as nuclear entry and the formation of protein complexes as well as protein degradation (Gallego and Virshup, 2007). Since the phosphorylation is reversible, both kinase and phosphoprotein phosphatase play a role in the regulation of the circadian clock (Gallego and Virshup, 2007). As summarized in figure 5, Casein kinase I ε (CKI ε) and Casein kinase I δ (CKIδ) have distinct regulatory roles. Depending on the position and the pattern of the phosphorylation, clock proteins have a different fate (Virshup et al., 2007). The familial advanced sleep-phase syndrome (FASPS) demonstrates the importance of CKI in the regulation of the circadian rhythm. Mutations in CKI and in CKI phosphorylation sites of PER2 are the cause of FASPS (Shanware et al., 2011). Individuals suffering from FASPS have a shorter period length (~23.3 hours) and hence are associated with phase-advanced sleep–wake rhythms (Jones et al., 1999)

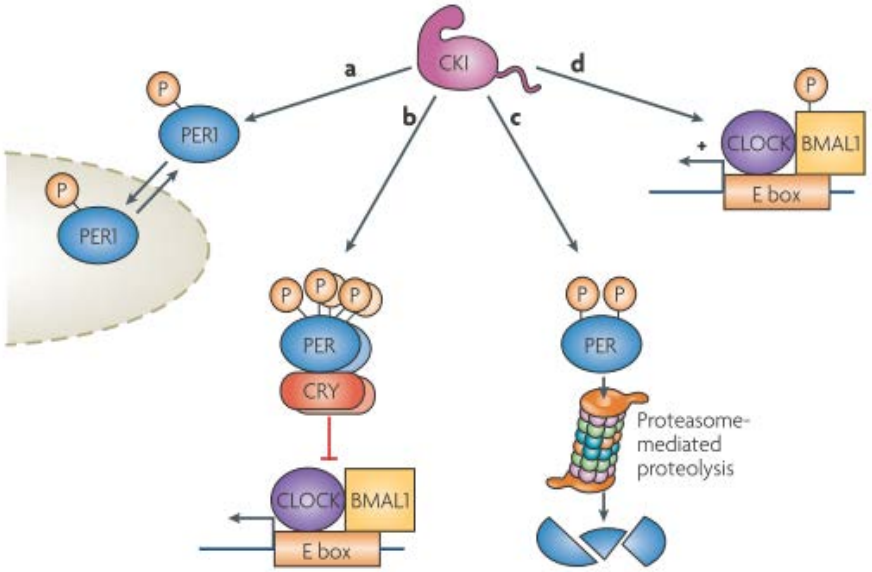

***Figure 5*. Roles of CKI in the circadian clock.** CKI has distinct roles in the circadian clock. **a.** In some cells CKI promotes the cytoplasmic accumulation of PER, whereas in others it promotes nuclear translocation of PER. **b.** The phosphorylation of PER is the highest with maximal repression of CLOCK:BMAL1. A link may therefore exist between the phosphorylation of PER and transcriptional inhibition. **c.** Phosphorylation of distinct sites of PER targets these proteins for degradation. **d.** CKI can also phosphorylate BMAL1 and thereby enhance its transcriptional activity. (Image source: *Gallego and Virshup, 2007*)

Although CKI seems to play an important role in regulating the circadian clock there are also other kinases which interact with the circadian clock like mitogen-activated protein kinase (MAPK) or glycogen synthase kinase-3β (GSK-3β). MAPK phosphorylates Bmal1, resulting in a negative regulation of Clock:Bmal1 mediated transcription (Sanada et al., 2002). GSK-3β engages in the regulation of the circadian clock through Per2 nuclear localization. Subsequently, an overexpression of GSK-3β leads to a phase advance, whereas decreased function results in a phase delay of the molecular oscillators (Iitaka et al., 2005). Additionally, GSK-3β is able to phosphorylate REV-ERBα. Due to phosphorylation REV-ERBα is stabilized and transcription of BMAL1 is thus inhibited (Yin et al., 2006). As research in this area progresses, there is increasing evidence that GSK-3β is capable of phosphorylating most clock proteins. In general, the complexity of the interplay of posttranslational phosphorylation is being revealed, as is the fact that further kinases appear to play a role in it (Figure 6) (Bellet and Sassone-Corsi, 2010).

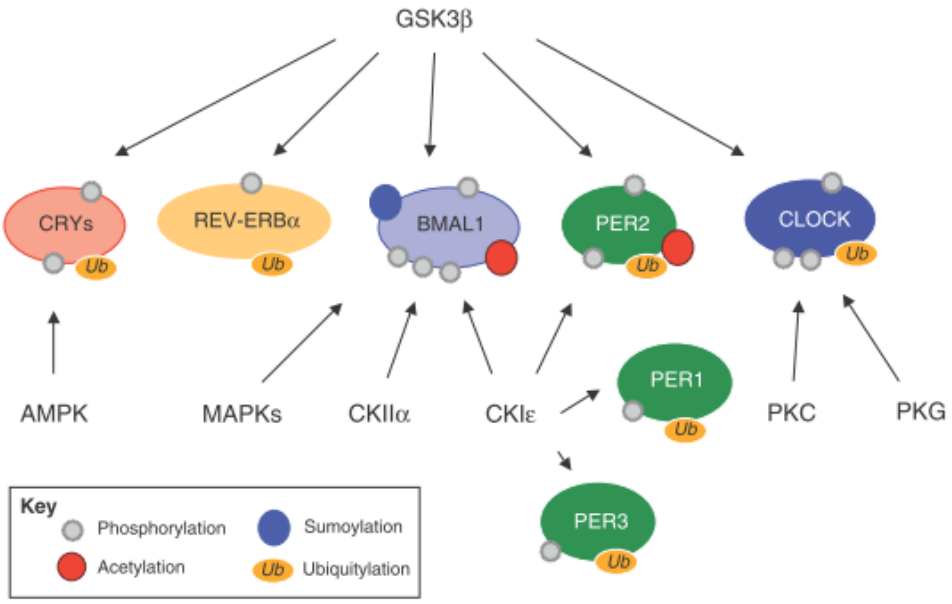

***Figure 6*. Posttranslational modification of clock proteins.** Phosphorylation is a common modification executed by a number of kinases, thereby controlling protein stability, turnover and subcellular localization. Acetylation of BMAL1 is essential for circadian rhythmicity. Ubiquitylation and Sumoylation are further modifications of certain clock regulators. (Image source: *Bellet and Sassone-Corsi, 2010*)

The most abundant serine/threonine phosphatases in the cell are protein phosphatase 1 (PP1) and PP2A (Yang et al., 2004), which also play an important role in the regulation of the clock (Gallego and Virshup, 2007). As mentioned above, phosphorylation balanced by regulated dephosphorylation determines the level for protein degradation of the core clock proteins. Proteasomes therefore also affect the clock's precision. Proteins must be tagged with ubiquitin polypeptides in order to be recognized by the proteasome. Ubiquitin ligase is responsible for the ubiquitination of target proteins (Gatfield and Schibler, 2007). The phosphorylation of Per through CKI on a specific site introduces a recognition site for the ubiquitin ligase β-TrCP. As a result, PER is ubiquitinylated and subsequently degraded by proteasome-dependent proteolysis (Virshup et al., 2007). In the case of CRY the necessary ubiquitin ligase is Fbxl3 (Gatfield and Schibler, 2007).

Another posttranslational modification that affects the circadian rhythm is sumoylation. *In vivo* BMAL1 is rhythmically sumoylated, thereby regulating the turnover of BMAL1 (Cardone et al., 2005). Furthermore, BMAL1 undergoes extensive posttranslational modification and is also a substrate for ubiquitination

(Lee et al., 2008) and acetylation. Interestingly, Bmal1 is acetylated through Clock, which possess intrinsic histone acetyl transferase (HAT) activity. This acetylation is essential for the circadian transcriptional program (Hirayama et al., 2007).

In conclusion, posttranslational modifications are essential for the clock machinery to function in a precise and robust way. Additionally, posttranscriptional regulation (Kojima et al., 2003) and chromatin remodeling (Etchegaray et al., 2003) also play a role in fine tuning the circadian clock.

1.2.3 The suprachiasmatic nucleus

The SCN is located in the anteroventral hypothalamus right above the optic chiasma. It surrounds the third ventricle and is a paired neuronal structure. In mice, the individual unilateral SCN contains ~10.000 neurons which are divided into two anatomic subunits: a ventral core region and a dorsal shell region (Welsh et al., 2010).

Like peripheral cells, SCN neurons are capable of generating an autonomous circadian rhythm. However, SCN neurons possess some special qualities. First, neurons from the SCN receive photic input from the retina which makes synchronization with the day/night cycle possible (Morin and Allen, 2006). Second, they have a mechanism which allows them to synchronize with each other, which means they remain synchronized even in constant darkness. This intercellular coupling generally confers robustness against perturbations (Aton and Herzog, 2005). The dominant neurotransmitter of synapses among SCN neurons is gamma-Aminobytyric acid (GABA) (Strecker et al., 1997). Third, they are able to synchronize peripheral cells throughout the body through variable direct and indirect output pathways (Gachon et al., 2004). In summary, the SCN is entrained to the light/dark cycle and additionally synchronizes subsidiary cellular oscillators (Welsh et al., 2010). As already mentioned, the SCN is the predominant pacemaker in mammals since complete SCN lesions cause the circadian periodicity in behavioral and endocrine variables to abolish (Moore and Eichler, 1972, Stephan and Zucker, 1972).

Nevertheless, the SCN are not the only structure to display daily oscillations in the brain. Nuclei in the thalamus and hypothalamus, habenula, hippocampus, amygdala, and the olfactory bulbs show such oscillations too. Projections between these non-SCN brain regions may facilitate the maintenance of circadian rhythms via neuronal circuits (Albrecht, 2012).

1.2.4 Peripheral circadian clocks

As outlined in section 1.2.1, most peripheral tissues show a cyclic expression of clock genes and hence possess a circadian clock (Balsalobre et al., 1998). We know that circadian timing is organized into a hierarchy of oscillators. In contrast to SCN oscillators, peripheral “slave” clocks can only sustain rhythmicity for a couple of days in constant darkness. To maintain 24-hour oscillations they need input from the master clock. Guo et al. showed that SCN neurons have to synchronize each hepatocyte on a daily basis to achieve phase coherence in the liver (Guo et al., 2006). This hypothesis was also confirmed by Shin Yamazaki and colleagues, who showed that SCN neurons adjust much faster to light-induced phase shifts than the slower-shifting peripheral oscillators (Yamazaki et al., 2000). SCN neurons synchronize slave oscillators, which in turn adjust local rhythm in physiology and behavior (Reppert and Weaver, 2002). However, the molecular mechanism underlying the timing mechanisms in the SCN clock and peripheral oscillators is quite similar. The actual mechanism which distinguishes them is unknown, but there are suggestions that it concerns differences in clock protein levels and kinetics, rather than the presence of a specific gene/protein expressed only in the SCN (Reppert and Weaver, 2002, Yagita et al., 2001).

The peripheral oscillators are synchronized through various lines of communication by the SCN. The feeding rhythm, which is influenced by the rest-activity cycle, is a potent *Zeitgeber* for peripheral organs (Damiola et al., 2000). Body-temperature cycles, directly influenced by the SCN, also play a role in resetting peripheral organs (Brown et al., 2002). Furthermore, the SCN utilize more direct timing cues such as humoral and neuronal signals in order to entrain peripheral clocks (Vujovic et al., 2008). With regard to humoral signals,

glucocorticoids seem to be an important entrainment signal from the SCN. Interestingly, virtually all cell types except SCN neurons express the glucocorticoid receptor, while plasma-glucocorticoid hormone levels also show robust daily rhythmicity. These alterations are directly influenced by the SCN via the hypothalamic-pit-adrenal axis (Balsalobre et al., 2000, Dibner et al., 2010).

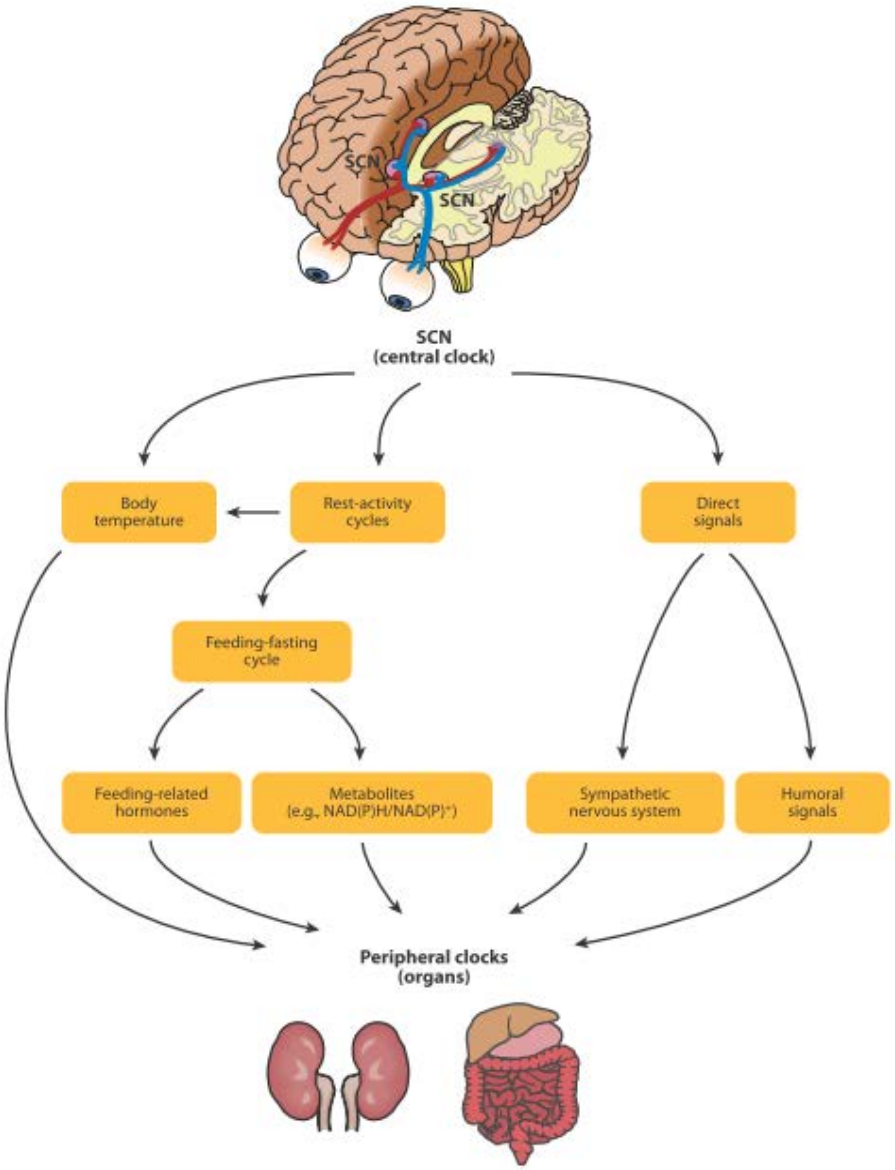

***Figure 7*. Peripheral clock entrainment pathways.** The suprachiasmatic nuclei utilize many routes to achieve phase coherence in the periphery. (Image source: *Dibner et al., 2010)*

1.2.5 Synchronization of the endogenous clock

The circadian timekeeping system can be envisaged as three compartments: the inputs, the circadian clock, and the outputs (Liu et al., 2007). Having discussed the circadian clock in detail, we now briefly analyze how the synchronization process works. As mentioned above, light is the main resetting cue for animals. Herein we therefore focus on light perception in the SCN, as illustrated in figure 8, although other environmental, non-photic cues perform subordinate roles (Hastings et al., 1997).

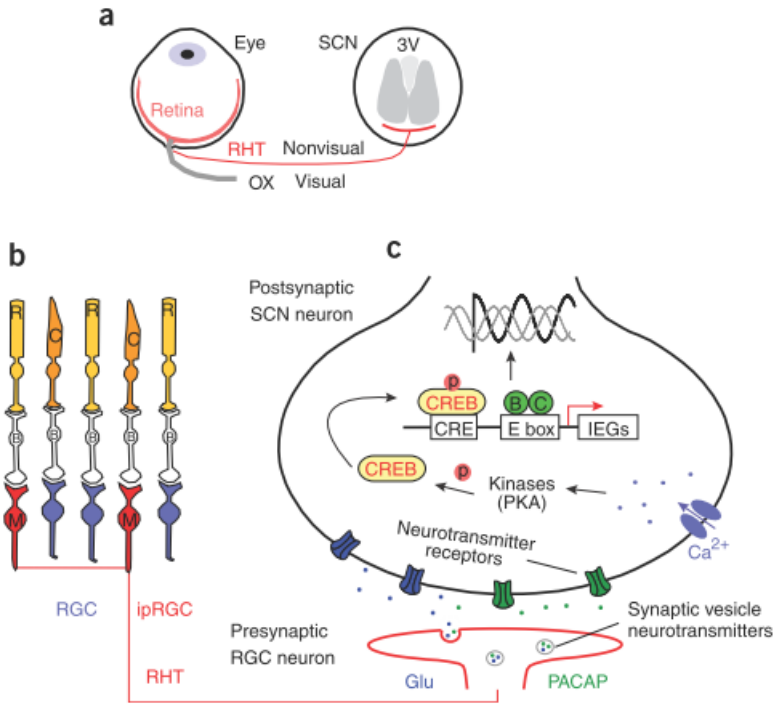

Figure 8. **Light entrainment of the SCN. a.** Light inputs from the retina to the SCN. The SCN reside in the hypothalamus above the optic chiasm (OX). ipRGCs form the RHT in the OX, which projects to neurons in the SCN. **b.** ipRGCs also receive light input from rods (R) and cones (C) through bipolar (B) and amacrine cells. **c.** Glutamate (Glu) and pituitary adenylate cyclase activating peptide (PACAP) are the central neurotransmitters released from presynaptic RGCs. They bind to their respective receptors, resulting in a depolarization and a Ca^{2+} influx. The increase in Ca^{2+} concentration and changes in cyclic adenosine monophosphate (cAMP) levels lead—in postsynaptic SCN neurons—to an activation of multiple kinases and finally to the phosphorylation of cAMP response element (CREB); phosphor-CREB (CREB-p) activates through cAMP response elements (CRE) immediate early genes (IEG), resulting in a new circadian phase. (Image source: *Liu et al., 2007)*

The pathways involved in circadian photoreception differ from those responsible for image formation. This has been shown in studies where rodless and coneless mice were still able to reset their activity rhythm and suppress melatonin in response to light (Lucas et al., 2001). Studies hint at melanopsin being the functional photopigment responsible for circadian photoreception (Kofuji et al., 2016, Gooley et al., 2001). Retinal ganglion cells (RGCs) are neurons residing in the inner retina. They receive signals from the rods and cones. However, a small subgroup of RGCs contain the photopigment melanopsin, which renders them intrinsically photosensitive (designated ipRGCs). Accordingly, ipRGCs fire independently of rods and cones in response to light (Liu et al., 2007). Intrinsically photosensitive retinal ganglion cells project through the retinohypothalamic tract (RHT) to the SCN, conveying light signals perceived by the retina to the retinorecipient neurons of the SCN.

Activation of NMDA receptors by glutamate is predominantly responsible for the physiological response of the SCN to light. This activation increases the firing rate of the retinorecipient cells. Additionally, it activates multiple intracellular cascades, such as an increase in intracellular calcium, changed cyclic adenosine monophosphate (cAMP) levels, activation of various kinases and finally the phosphorylation of CERB, which activates so-called immediate early genes (IEGs) (Antle et al., 2009). IEGs include among others Per1, Per2 and c-fos. Induction of IEGs is subsequently necessary to induce phase shifts (Liu et al., 2007).

The absence of rods, cones and melanopsin leads to a total loss of photic entrainment (Hattar et al., 2003, Panda et al., 2003). However, normal light entrainment is observed in mice lacking either rod and cones or melanopsin alone (Liu et al., 2007) It would therefore appear that both rod and cones as well as melanopsin seem to play a role in light entrainment.

The SCN is thus synchronized to an environmental rhythm and are then capable of synchronizing peripheral oscillators utilizing the pathways described. Subsequently, this plethora of internal clocks influences a variety of physiological processes such as proper timing of hormone release, sleep-wake cycles, feeding behavior, and body temperature (Takahashi et al., 2008). Furthermore, a variety

of pathways under circadian regulation are embedded in essential metabolic pathways. It is also evident that metabolism itself affects the circadian clock (Bellet and Sassone-Corsi, 2010, Takahashi et al., 2008). Findings suggest that the cell cycle is under circadian modulation (Matsuo et al., 2003, Miller et al., 2007). Moreover, circadian regulations play a role in xenobiotic detoxification. Hence, the endogenous clock is also responsible for the circadian sensitivity to chemotherapeutic agents (Schibler, 2007, Takahashi et al., 2008).

1.2.6 Circadian rhythm and disease

In modern living, external timing cues clash with our internal circadian physiology, which leads to phase disturbance. This desynchrony contributes to the morbidity and well-being of human health. As research progresses it is becoming clear that circadian variations feature in a variety of diseases such as cardiovascular diseases, Alzheimer's disease, metabolic diseases, and cancer (Hastings et al., 2003). Possibly the most obvious connection between circadian rhythm and disease are disturbances in the sleep–wake cycle such as the familial advanced sleep-phase syndrome, or the delayed sleep-phase syndrome (Takahashi et al., 2008). Furthermore, mood-related disorders are closely connected to alterations in the circadian rhythm. In many cases it is not clear whether circadian desynchrony is the reason for a disease or is instead just a symptom of a disease (Albrecht, 2013). Additionally, further research is needed to understand whether mutations in core clock genes or rhythmicity contribute to disease etiology (Takahashi et al., 2008).

2 Aim of the thesis and research design

The aim of this master project was to investigate whether there is a difference in the behavioral circadian rhythm of Cav1.4 IT mice and wild-type mice. Locomotor activity was therefore assessed by recording wheel running behavior using the experimental set-up depicted in Figure 9. Mice were initially placed in light:dark (LD) 12:12 conditions for 21 days for the investigation of the light-entrained circadian rhythm. Next, the assessment of the free-running circadian activity was determined by changing the conditions to constant darkness (DD) for 10 days. On day 33 mice were exposed to a light pulse (30 minutes, 300 lux) for the induction of a phase shift response. The light pulse was administered at circadian time (CT) 16, four hours after activity onset. After 8 more days of DD, conditions were changes to LD for 7 more days. Finally, mice were sacrificed on day 48 between 11 a.m. and 1 p.m.

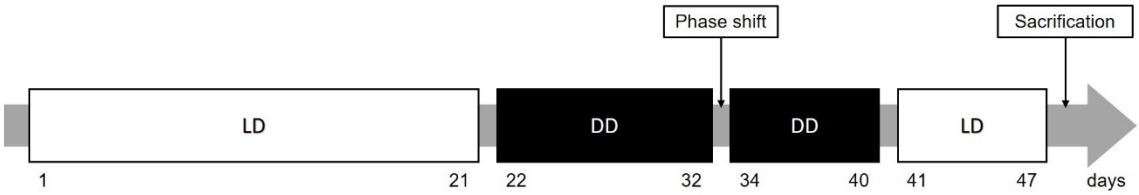

***Figure 9*. Study design.** Light protocol to assess the circadian wheel-running activity in Cav1.4 IT mice and wild-type mice; light/dark (LD): 12hrs light and 12hrs darkness; dark/dark (DD): 24hr constant darkness.

To investigate possible changes at the molecular level, qRT-PCR was applied in order to determine the expressional level of 13 clock genes and clock-controlled genes in SCN tissue of Cav1.4 IT mice and wild-type mice.

3 Materials and Methods

3.1 Animals

Animals of the mouse line containing the I745T mutation in the CACNA1F gene (IT) were kindly provided by Prof. Alexandra Koschak, Department of Pharmacology and Toxicology - Institute of Pharmacy, University of Innsbruck, Austria). Male, adult (10-12 weeks of age) IT and wild-type littermate control animals were used for all experiments. The genotype of all mice was assured by PCR genotyping from mouse tail biopsies as previously described (Knoflach et al., 2013) Animals were housed individually in Nalgene cages containing a running wheel (15cm in diameter; Actrimetrics, Evanston, IL). Regular mouse chow and tap water were offered *ad libitum*. The animal room was sound-attenuated and the temperature was held constant at 23 ± 2 °C. Before the beginning of the experiment, mice were kept on a light (12hr):dark (12hr) cycle. During the light phase, light intensity was 200 lux at all times. Under the DD condition, cage cleaning and animal care were performed under dim red light (15 lux). Experiments were designed to reduce animal suffering and limit the number of animals used. The experiments described were approved by the national ethical committee on animal care and use (Bundesministerium für Wissenschaft, Forschung und Wirtschaft) and conducted in accordance with international laws and policies.

3.2 Evaluation of circadian wheel-running activity

To measure wheel running activity, wheel revolutions were detected and recorded using the ClockLab software (Actimetrics) using 1 minute sampling epochs. The ClockLab software package (Actimetrics, Evanstone, IL) was further used for the analysis of wheel-running activity including activity onsets, offsets, bout detection and evaluation of the phase shift response using standard parameters previously described by our group (Griesauer et al., 2014, Schaufler et al., 2016). All parameters, except for the phase shift response, were evaluated for each animal under LD and DD conditions.

3.3 Gene expression

3.3.1 Dissection

Animals were sacrificed by neck dislocation during the light phase of the circadian cycle (CT 5). Brains were quickly dissected out and placed into optimal cutting temperature compound (OCT). Samples were frozen in liquid nitrogen and stored at -80°C until used for further analysis

In order to isolate the SCN, brains were sliced into 250 µm-thick slices with a cryostate (Microtom). The coronal section at bregma -50 mm was used for manual microdissection of the suprachiasmatic nuclei (SCN) according to established anatomical landmarks (Watson, 2010). To this end, the sections were placed on dry ice and the isolated SCN samples were transferred to RNase-free Eppendorf tubes containing RNAlater® (Ambion, Austin, TX, USA) and stored at -20°C until used for further analysis.

3.3.2 RNA isolation

For the isolation of RNA from the obtained samples the Qiagen miRNA Micro Kit (cat.no. 217084) was utilized. The extraction was carried out as described in the supplied protocol. In brief, the SCN samples were gently thawed, retried from the RNAlater solution and placed into 700 µL QIAzol® Lysis Reagent. Subsequently, the samples were homogenized utilizing a mini hand mixer. After a five-minute incubation period at room temperature (RT) 140 µL of chloroform was added to the tubes containing the homogenate. The tubes were then vortexed vigorously and incubated at RT for three minutes, followed by 15 minutes of centrifugation at 12,000 x g and 4°C. The upper aqueous phase was subsequently transferred into a new Eppendorf tube and 1,5 volumes of 100% ethanol were added. The samples were pipetted to an RNeasy MinElute spin column and centrifuged at 10,000 rpm for 15 seconds at RT. The flow-through was discarded and 700 µL RWT buffer were added to the RNeasy MinElute spin column. Next, the tubes were centrifuged at 10,000 rpm for 14 seconds at RT and the flow-through was discarded. Adjacently, 500 µL RPE buffer were pipetted

onto the columns, followed by 15 seconds of centrifugation at 10,000 rpm and RT. Flow-through was then discarded and 500 µL of 80% ethanol were added to the columns. The tubes were centrifuged at 10,000 rpm for 2 minutes at RT. Flow-through was discarded and the RNeasy MinElute spin columns were placed onto new collection tubes to be centrifuged for five minutes at full speed with open lids, at RT. To elute the RNA, the RNeasy MinElute spin columns were placed in new 1,5 ml Eppendorf tubes, 14 µL RNase-free water were added and they were centrifuged at RT for 1 minute at full speed. Finally, the purity and concentration of the RNA was measured by using a Nanodrop photometer (NanoPhotometer™, IMPLEN, 7122 V2.3.1). Subsequently, samples were frozen at -80°C until utilized for further analysis.

3.3.3 cDNA synthesis

The cDNA synthesis was performed using the DyNAmo cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's handbook. Briefly, samples were thawed on ice and 900 ng of RNA (obtained from a pool of three samples) were transferred into a new tube. RNase-free water was added to obtain a volume of 7 µL. A mastermix was prepared according to the ingredients in Table 1, and 13 µL of mastermix were added to each sample. Finally, the samples were placed into a thermocycler and the program was set according to the steps listed in Table 2.

Table 2. Mastermix for qRT-PCR reaction

	1x
RT buffer	10 µL
Random hexamer primer set (300 ng/µL)	1 µL
M-MuLV RNase H^+ reverse transcriptase	2 µL
Total	**13 µL**

Table 3. Incubation protocol for reverse transcription

	Temperature	**Duration**
Primer extension	25°C	10 minutes
cDNA synthesis	37°C	30 minutes
Reaction termination	85°C	5 minutes
Cooling of the sample	4°C	Hold

3.3.4 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

To analyze gene expression a qRT-PCR using the Fast SYBR® Green Mastermix (Applied Biosystems, Foster City, CA) was carried out. The diluted cDNA was thawed on ice and a primer-specific mastermix of the ingredients listed in Table 4 was prepared, after which 2 µL of the sample's cDNA and 13 µL of mastermix were pipetted into a 96-well PCR plate. After mixing the plate smoothly it was sealed with a PCR plate foil and centrifuged at 1,000 rpm for five minutes. It was then placed into a StepOnePlus real-time PCR system (Applied Biosystems®, life technologies™) and the program (StepOne Software v2.2.2 Applied Biosystems®, life technologies™) was set - as indicated in Table 5 - for 40 cycles. Reactions were carried out in duplicates. The primer sequences for the analyzed clock genes are listed in Table 6.

To calculate $\Delta C(t)$ as an indicator for the relative amounts of target mRNA in each sample, $C(t)$ values for β-actin were used. The $\Delta C(t)$ values allow the calculation of $\Delta\Delta C(t)$, which reveals the mRNA fold change levels between Cav1.4 IT mice and wild-type mice using the following formula (Livak and Schmittgen, 2001):

$$2^{-(\Delta\Delta C(t))}$$

Table 4. qRT-PCR mastermix

	1x
2 x precision mastermix (SybrGreen)	7,5 µL
Specific forward primer (20µM)	0,15 µL
Specific reverse primer (20µM)	0,15 µL
dH_2O	5,2 µL
Total	**13 µL**

Table 5. qRT-PCR protocol

	Temperature	**Duration**
Initial denaturation	95°C	10 mins
Denaturation	95°C	15 secs
Annealing	60°C	30 secs
Elongation	60°C	30 secs

Table 6. Sequence of primers used

Primer Name	Primer length (bp)	sequence (5' to 3')
mus_Clock_fwd	21	GGC GTT GTT GAT TGG ACT AGG
mus_Clock_rev	21	GAA TGG AGT CTC CAA CAC CCA
mus_Npas2_fwd	21	ACG CAG ATG TTC GAG TGG AAA
mus_Npas2_rew	19	CGC CCA TGT CAA GTG CAT T
mus_Bmal1_fwd	20	AAC CTT CCC GCA GCT AAC AG
mus_Bmal1_rev	20	AGT CCT CTT TGG GCC ACC TT
mus_Cry1_fwd	21	AGG AGG ACA GAT CCC AAT GGA
mus_Cry1-rev	21	GCA ACC TTC TGG ATG CCT TCT
mus_Cry2_fwd	21	AGC TGA TGT GTT CCC AAG GCT
mus_Cry2_rev	20	CAT AAT GGC TGC ATC CCG TT
mus_Per1_fwd	24	CCA GAT TGG TGG AGG TTA CTG AGT
mus_Per1_rev	24	GCG AGA GTC TTC TTG GAG CAG TAG
mus_Per2_fwd	21	AGA ACG CGG ATA TGT TTG CTG
mus_Per2_rev	21	ATC TAA GCC GCT GCA CAC ACT
mus_Per3_fwd	20	CCG CCC CTA CAG TCA GAA AG
mus_Per3_rev	20	GCC CCA CGT GCT TAA ATC CT
mus_Rev-erbα _fwd	23	CCC TGG ACT CCA ATA ACA ACA CA
mus_Rev-erbα_rev	22	GCC ATT GGA GCT GTC ACT GTA G
mus_Rev-erbβ_fwd	19	GGA ACG GAC CGT CAC CTT T
mus_Rev-erbβ_rev	19	TCC CCT GCT CCC ATT GAG T
mus_ROR-α_fwd	19	TTG CCA AAC GCA TTG ATG G
mus_ROR-α_rev	21	TTC TGA GAG TCA AAG GCA CGG
mus_ROR-β_fwd	20	ATG GCA GAC CCA CAC CTA CG
mus_ROR-β_rev	19	TAT CCG CTT GGC GAA CTC C
mus_ROR-γ_fwd	21	CGA GAT GCT GTC AAG TTT GGC
mus_ROR-γ_rev	21	TGT AAG TGT GTC TGC TCC GCG

3.4 Statistical analysis

Microsoft Excel 2016 or IBM SPSS Statistics 24 software package were used for data analysis. In order to test for significant differences between two groups a two-tailed student's t-test was carried out. The level of significance was set as $p \leq 0.05$ at all instances.

4 Results

4.1 Cav 1.4 IT show a shorter period length under free running conditions

In order to examine potential alterations of the circadian behavior in Ca_V 1.4 IT mice show we compared the wheel-running rhythms between Ca_V 1.4 IT and wild-type control mice under LD and DD conditions (see Figure 9 for reference of the light protocol). Under light-entrained conditions no differences were observed (Figure 10A). However, Ca_V 1.4 IT mice showed a significantly shorter circadian period (*tau*) under free-running conditions (constant darkness) compared to wild-type mice ($p < 0.05$) (Figure 10B).

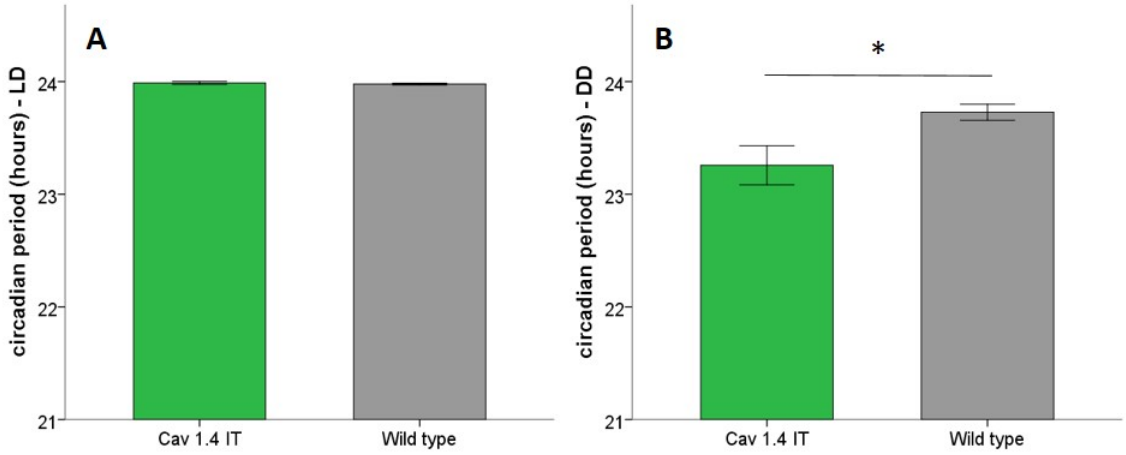

***Figure 10*. Circadian period** (tau) **in Cav 1.4 IT (n=10) and wild-type mice (n=11). A.** No differences in the circadian period are observed under light-entrained conditions (LD). **B.** Cav 1.4 IT mice show a significantly shorter circadian period under free running conditions (DD). *p < 0.05; data displayed as mean ± SEM

4.2 Ca_V 1.4 IT mice display more activity during inactivity phase

As revealed in Figure 11, no difference in the total wheel-running revolutions was observed between the groups, either under LD or DD conditions. Hence, differences in the circadian period in Ca_V 1.4 IT mice do not result from alterations in the overall locomotor activity. However, more wheel revolutions were counted in the Ca_V 1.4 IT group during their inactive (rho) phase under LD and DD conditions respectively while no differences during the active (alpha) phases were observed.

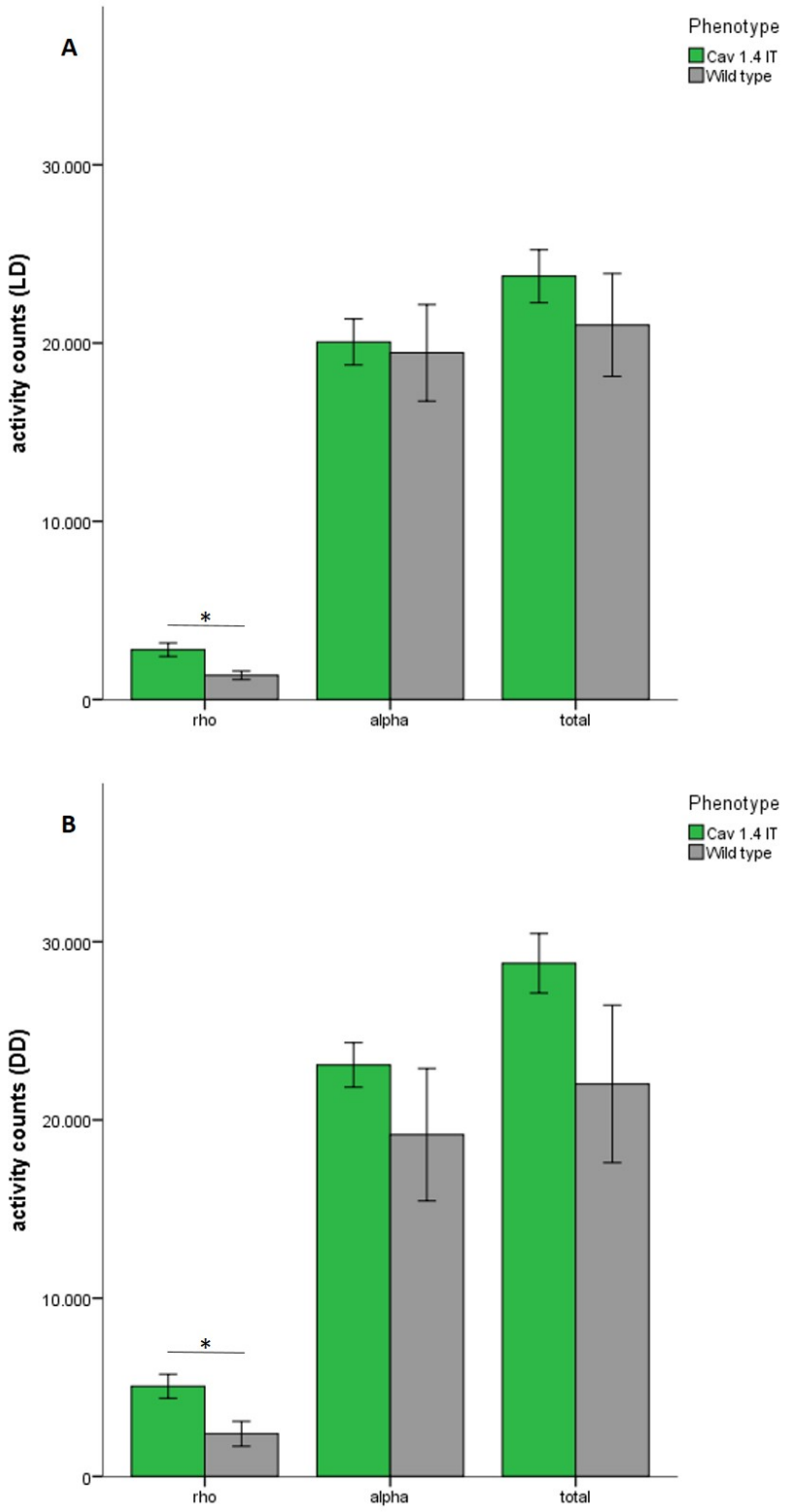

***Figure 11*. Wheel-running activity of Cav 1.4 IT (n=10) and wild-type mice (n=11). A.** No significant difference in the total amount of wheel-running activity under LD conditions. However, Cav 1.4 IT mice show a significantly higher amount of wheel-running activity during their inactive (rho) phase. **B.** No difference in total amount of wheel-running activity is observed under free running conditions. Nevertheless, a significantly higher amount of wheel-running activity can be seen in the inactive phase of Cav 1.4 IT mice. *p < 0,05; data displayed as mean ± SEM

4.3 Ultradiem structure of circadian profiles under LD and DD conditions

An actogram is produced by plotting the 24 hours wheel-running activity for consecutive days (Albrecht and Eichele, 2003). Comparing the actograms obtained from Ca_v 1.4 mice and wild-type mice suggest some differences in the ultradiem structural organization of the activity rhythm (Figure 12B). Hence, we analyzed the activity bouts of both groups, but no statistically significant differences between genotypes for the number of activity bouts under either LD or DD conditions were observed (Figure 13)

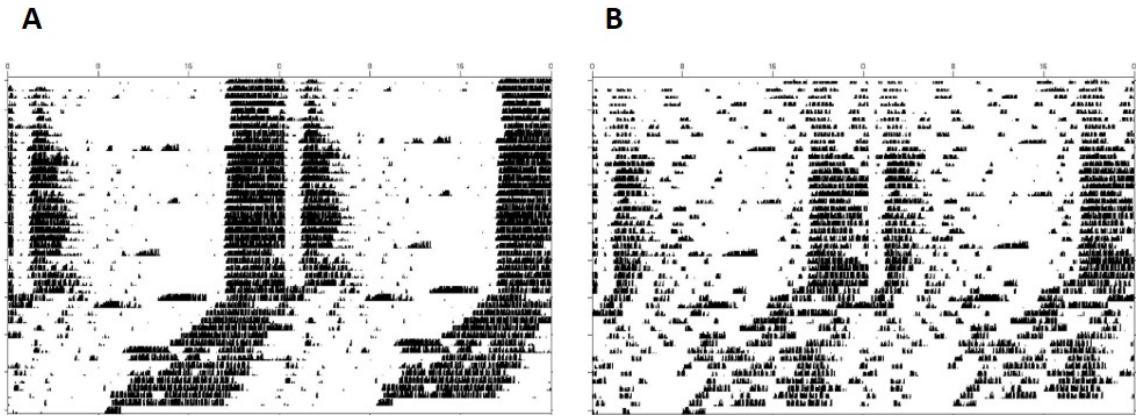

Figure 12. **Sample actograms of Cav 1.4 IT and wild-type mice.** The depicted actograms illustrate the wheel-running activity of **A.** wild-type mice and **B.** Cav 1.4 IT mice.

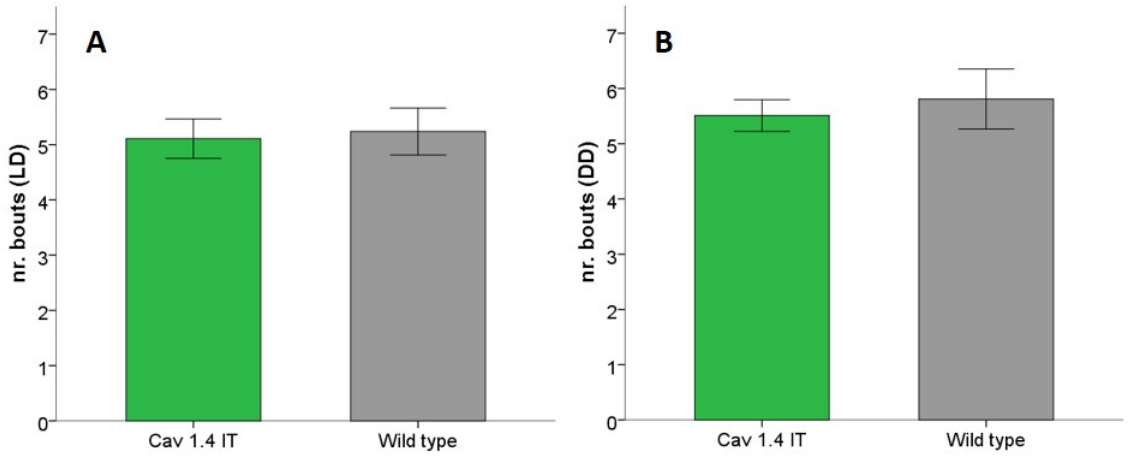

Figure 13. **Activity bouts in Cav 1.4 IT (n=10) and wild-type mice (n=11).** No difference in the amount of activity bouts between Cav 1.4 IT and wild-type mice either under **A.** LD or **B.** DD conditions; data displayed as mean ± SEM

4.4 Greater phase delay of Ca_V 1.4 mice in response to light entrainment

We used the light-induced phase shift as a paradigm in order to investigate the responsiveness of the endogenous clock to exogenous *zeitgeber*. To this end mice who had been held under constant darkness were exposed to a short light pulse (30 minutes, 300 lux) during the subjective early night (CT 16) in order to induce a phase delay (Albrecht and Eichele, 2003). The mean-phase delay of Ca_V 1.4 IT mice is greater than that of wild-type mice, just reaching the threshold for statistical significance (p = 0,05; Figure 14).

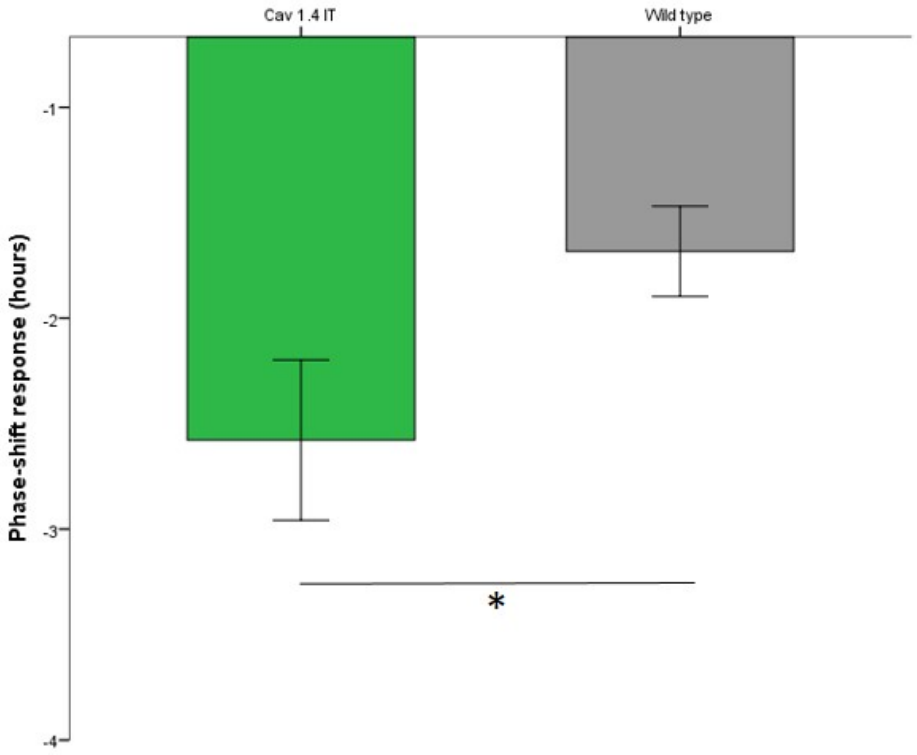

***Figure 14*. Phase-shift response.** The brief light impulse at CT16 leads to a greater phase delay in Cav 1.4 IT (n= 10) mice compared to wild-type mice (n=11); p = 0,05; data displayed as mean ± SEM

4.5 No differences in the expression level of clock genes

In a next step, we investigated the expression of core clock genes in the SCN - the body's central pacemaker (Welsh et al., 2010) – as potential molecular correlate to the circadian behavioral phenotype displayed by Ca_V 1.4 IT mice. We first focused on essential genes of the positive and negative limb of the TTFL: *Clock, Npas2, Bmal1, Per1-3 and Cry 1-2*. As illustrated in Table 7, no significant differences in the mRNA levels of any of these genes were found in SCN tissues of Ca_V 1.4 IT mice and wild-type mice. In the next step we analyzed expression of genes constituting the interlocking feedback loop, namely: *Rev-erbα, Rev-erbβ, Rorα, Rorβ, Rorγ.* No differences in the expressional levels between genotypes were observed for any of the genes analyzed (Table 7).

Table 7. Relative clock gene and clock-controlled genes mRNA expression levels in the suprachiasmatic nuclei of Cav1.4 IT mice relative to wild-type mice.

mRNA	fold change relative to wild-type	p value
Clock	1,071 ± 0,082	0,3771
Bmal1	1,066 ± 0,073	0,3370
Per1	1,016 ± 0,090	0,9090
Per2	1,202 ± 0,115	0,2062
Per3	1,012 ± 0,093	0,8880
Cry1	1,032 ± 0,079	0,7108
Cry2	0,985 ± 0,084	0,8601
Rev-erbα	1,161 ± 0,045	0,1520
Rev-erbβ	0,949 ± 0,030	0,3726
Rorα	0,902 ± 0,167	0,5797
Rorβ	1,076 ± 0,115	0,6318
Rorγ	1,074 ± 0,178	0,6012
Npas2	1,191 ± 0,065	0,0797

Note. The table shows the fold change of clock gene and clock-controlled genes mRNA expression of Cav1.4 IT mice (n = 5) compared to wild-type mice (n = 5) in the suprachiasmatic nuclei. Data are displayed as mean ± SEM.

5 Discussion

The I745T gain of function mutation in the CACNA1F gene coding for the $Ca_V1.4$ channel leads to a certain form of X-linked CSNB2 (Hemara-Wahanui et al., 2005). This non-progressive retinal disorder causes night-blindness, a decrease in visual acuity, nystagmus, myopia and abnormal ERG response (Hope et al., 2005). Since light and light perception are the main entrainment cues for the circadian clock it would be reasonable to speculate that patients suffering from CSNB2 could present with abnormalities in their circadian rhythm due to their visual impairment. We here tested this possibility using a genetic mouse model of the disease and assessing circadian locomotor activity as behavioral display of circadian rhythmicity and assessed the expression of clock genes as potentially related molecular signature.

Interestingly, $Ca_V1.4$.IT mice show a significantly shortened circadian period under free running conditions but not under light-entrained conditions. These data suggest that the reason for the altered circadian rhythm most likely relates to disturbances in the endogenous clock, since the circadian behavior under DD conditions is directly dependent on the endogenous molecular organization of the circadian clock (Albrecht and Eichele, 2003). Taking the wheel-running activity as the measurement for the circadian phenotype, mutations in the following circadian clock genes have been associated with period shortening: *Per1*, *Per 2* (Zheng et al., 2001), Cry1 (van der Horst et al., 1999, Reppert and Weaver, 2002) as well as *Rev-erbα* and *Rev-erbβ* (Preitner et al., 2002). Mutations in *CKI* ε also seem to shorten *tau* (Lowrey et al., 2000). Cry1 appears to offer a promising target, because mutations in its gene lead to an altered sleep pattern (Ko and Takahashi, 2006). This alteration can be observed in $Ca_V1.4$ IT mice, which show a significantly higher amount of activity during their inactive phase compared to wild-type mice possibly pointing towards a modulation in sleep patterns. Hence, *Cry1* may constitute a potential molecular link involved in the circadian alterations observed in $Ca_V1.4$ IT mice.

Nevertheless, it can be assumed that the circadian phenotype of $Ca_V1.4$ IT mice was not due to limitations of the general locomotor activity of these mice, since no differences in the total wheel running activity between groups were observed

While at first sight the visual inspection of actograms obtained from $Ca_V1.4$ mice pointed towards a possible fragmentation of the ultradiem structure in $Ca_V1.4$ mice, no statistically significant differences in the amount of activity bouts between genotypes was found. Hence it is possible that the perceived differences in the presentation of the actograms may rather relate to the finding of increased activity of $Ca_V1.4$ mice during their inactive phases and provide another hint toward a modulated sleep pattern, which remains to be analyzed in future experiments.

The finding that $Ca_V1.4$ IT mice respond to the light pulse at the subjective early night with a phase delay, as would be expected from the literature (Daan and Pittendrigh, 1976, Albrecht and Eichele, 2003), evidences that their endogenous molecular circadian machinery is receptive to the resetting stimuli of an external *zeitgeber* (light). The observation that a greater phase delay is observed in $Ca_V1.4$ IT mice compared to the control group suggests an enhanced sensitivity of $Ca_V1.4$ IT mice under these conditions which requires further investigation.

Hence, while $Ca_V1.4$ IT mice suffer from visual impairment, their circadian machinery can successfully synchronize to light. This finding matches research establishing that even blind mice can be entrained by light as long as their ipRGCs still function. The ipRGCs transmit light signal to the SCN in a cone-and-rod independent pathway via the RHT (Liu et al., 2007). Hence, the visual impairment may not necessarily be the explanation for the altered circadian phenotype of $Ca_V1.4$ IT mice and the relationship between CSNB2 and circadian alterations remains to be fully explored. Further investigations are needed to elucidate whether the I745T mutation only plays a role in ribbon synapses or in other areas too.

Analysis of the gene expression of important clock genes and clock-controlled genes suggests no differential levels in SCN tissue between $Ca_V1.4$ IT mice and wild-type mice in the current analysis. However, from these data it is not possible to conclude that variances in the expression levels of clock genes of $Ca_V1.4$ IT mice and wild-type mice do not exist and further experiments and refinements in the experimental design will aid in addressing the remaining open question in the future.

First, the method of isolating the SCN could be improved and laser-capture microdissection could significantly enhance the specificity of the dissection as compared to the manual microdissection used for the present study. Additionally, other elegant methods for the isolation of SCN tissue exist and might be applied in future approaches (Savelyev et al., 2011).

Second, clock-gene expression oscillates in a 24-hour rhythm. It is therefore not sufficient to analyze gene expression at a single time point, as was done in the current pilot experiment which constituted a snap shot approach, due to the lack of additional samples at that time. To more coherently assess diurnal oscillation of clock gene expression the examination of at least four or preferably six time points would be needed. Only then can definite conclusions about phase differences and changes in amplitude or the period of gene expression between the two groups be drawn. As Table 8 also indicates, the expression of distinct clock genes peaks at different time points (Ko and Takahashi, 2006).

Table 8. Circadian clock and clock-controlled genes

Gene	Average circadian time at peak transcript level	
	SCN	**Periphery**
Bmal1	15 – 21	22 – 02
Clock	Constitutive	21 – 03
Per1	4 – 8	10 – 16
Per2	6 – 12	14 – 18
Per3	4 – 9	10 – 14
Cry1	8 – 14	14 – 18
Cry2	8 – 14	8 – 12
Rev-erbα	2 – 6	4 – 10
Rorα	6 – 10	Arrhythmic
Rorβ	4 – 8	18 – 22
Rorγ	no data available	16 – 20
NPAS2	no data available	0 – 4
CKIε	Constitutive	Constitutive
CKIδ	Constitutive	Constitutive

Note. Circadian time (CT) at peak transcript level of circadian clock and clock-controlled genes of wild-type mice in the SCN and in peripheral circadian oscillators. (adopted from *Ko and Takahashi, 2006*)

Third, it would be interesting to see whether there are any changes in the protein levels of clock and clock-controlled genes in $Ca_V1.4$ IT mice and wild-type mice, since posttranscriptional and -translational protein modifactions play an important role in regulating the endogenous clock system (Lee et al., 2001). Changes in protein stability, location and turnover of clock gene products might therefore also contribute the circadian phenotype of $Ca_V1.4$ IT mice.

All of the above mentioned experiments are planned in future cohorts of aninmals once the respective animal numbers for each genotypes are available. At the current state of knowledge one can, however, derive from the herein presented data that the I745T mutation in the CACNA1F gene significantly alters circadian locomotor activity rhythms in mice the molecular basis for which remains to be further elucidated in pending follow-up experiments.

6 Conclusion

Even though the molecular basis for the circadian phenotype of the $Ca_V1.4$ IT mouse model for CSNB2 still needs to be clarified, the finding that the mutation leads to alterations in circadian rhythmicity is a first important lead. While caution needs to be taken when applying conclusions obtained from data derived from animal models to the human population, the present study can be considered as translational impulse suggesting further research in people in order to investigate whether patients suffering from CSNB2 may also present with circadian disturbances including deranged sleep patterns. Furthermore, it could be clinically relevant to explore whether CSNB2 patients consequently have a higher risk for the development of pathological conditions associated with disturbances in the circadian rhythm, such as mood disorders or metabolic diseases.

7 Abbreviations

AVN	atrioventricular node
bHLH	basic helix-loop-helix
Bmal1	brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
Ca2+	calcium
cAMP	cyclic adenosine monophosphate
Ccg	clock-controlled genes
CKI	casein kinase
Clock	circadian locomotor output cycles kaput
CRE	cAMP response elements
CREB	cAMP response element-binding protein
Cry	cryptochrome
CSNB1	congenital stationary night blindness type 1
CSNB2	congenital stationary night blindness type 2
CT	circadian time
DD	constant darkness
ERG	electroretinogram
FASPS	familial advanced sleep-phase syndrome
GABA	gamma-Aminobytyric acid
Glu	glutamate
GSK-3β	glycogen synthase kinase-3β
HAT	histone acetyl transferase
I	isoleucine
IEG	immediate early genes
ipRGC	intrinsically photosensitive retinal ganglion cells
LD	light:dark
LTCC	L-type calcium channel
MAPK	mitogen-activated protein kinase
Npas2	neuronal PAS domain-containing protein 2
O.C.T	optimal cutting temperature compound
OX	optic chiasm
PACAP	pituitary adenylate cyclase activating peptide

PAS	Period-Arnt-single minded
Per	period
PP	protein phosphatase
Rev-erb	reverse erythroblastosis virus
RGC	retinal ganglion cells
RHT	retinohypothalamic tract
Ror	related orphan receptor
RT	room temperature
RyRs	ryanodine sensitive Ca2+ channels
SAN	sinoatrial node
SCN	suprachiasmatic nuclei
SNARE	N-ethylmaleimide-sensitive-factor attachment receptor
T	threonine
TTFL	transcriptional–translational feedback loop
VGCC	voltage-gated calcium channels
β-TrCP	beta-transducin repeat containing E3 ubiquitin protein ligase

8 Bibliography

ALBRECHT, U. 2012. Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron,* 74**,** 246-60.

ALBRECHT, U. 2013. Circadian clocks and mood-related behaviors. *Handb Exp Pharmacol***,** 227-39.

ALBRECHT, U. & EICHELE, G. 2003. The mammalian circadian clock. *Curr Opin Genet Dev,* 13**,** 271-7.

ALBRECHT, U. & RIPPERGER, J. A. 2009. Clock Genes. *In:* BINDER, M. D., HIROKAWA, N. & WINDHORST, U. (eds.) *Encyclopedia of Neuroscience.* Berlin, Heidelberg: Springer Berlin Heidelberg.

ALLADA, R., EMERY, P., TAKAHASHI, J. S. & ROSBASH, M. 2001. Stopping time: the genetics of fly and mouse circadian clocks. *Annu Rev Neurosci,* 24**,** 1091-119.

ANTLE, M. C., SMITH, V. M., STERNICZUK, R., YAMAKAWA, G. R. & RAKAI, B. D. 2009. Physiological responses of the circadian clock to acute light exposure at night. *Reviews in Endocrine and Metabolic Disorders,* 10**,** 279-291.

ATON, S. J. & HERZOG, E. D. 2005. Come together, right...now: synchronization of rhythms in a mammalian circadian clock. *Neuron,* 48**,** 531-4.

BAIG, S. M., KOSCHAK, A., LIEB, A., GEBHART, M., DAFINGER, C., NURNBERG, G., ALI, A., AHMAD, I., SINNEGGER-BRAUNS, M. J., BRANDT, N., ENGEL, J., MANGONI, M. E., FAROOQ, M., KHAN, H. U., NURNBERG, P., STRIESSNIG, J. & BOLZ, H. J. 2011. Loss of Ca(v)1.3 (CACNA1D) function in a human channelopathy with bradycardia and congenital deafness. *Nat Neurosci,* 14**,** 77-84.

BALSALOBRE, A., BROWN, S. A., MARCACCI, L., TRONCHE, F., KELLENDONK, C., REICHARDT, H. M., SCHUTZ, G. & SCHIBLER, U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science,* 289**,** 2344-7.

BALSALOBRE, A., DAMIOLA, F. & SCHIBLER, U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell,* 93**,** 929-37.

BECH-HANSEN, N. T., NAYLOR, M. J., MAYBAUM, T. A., PEARCE, W. G., KOOP, B., FISHMAN, G. A., METS, M., MUSARELLA, M. A. & BOYCOTT, K. M. 1998. Loss-of-function mutations in a calcium-channel alpha1-subunit gene in Xp11.23 cause incomplete X-linked congenital stationary night blindness. *Nat Genet,* 19**,** 264-7.

BELLET, M. M. & SASSONE-CORSI, P. 2010. Mammalian circadian clock and metabolism - the epigenetic link. *J Cell Sci,* 123**,** 3837-48.

BEZANILLA, F. 2008. How membrane proteins sense voltage. *Nat Rev Mol Cell Biol,* 9**,** 323-32.

BRAY, M. S. & YOUNG, M. E. 2009. The role of cell-specific circadian clocks in metabolism and disease. *Obes Rev,* 10 Suppl 2**,** 6-13.

BROWN, S. A., ZUMBRUNN, G., FLEURY-OLELA, F., PREITNER, N. & SCHIBLER, U. 2002. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr Biol,* 12**,** 1574-83.

BURTSCHER, V., SCHICKER, K., NOVIKOVA, E., POHN, B., STOCKNER, T., KUGLER, C., SINGH, A., ZEITZ, C., LANCELOT, M. E., AUDO, I., LEROY, B. P., FREISSMUTH, M., HERZIG, S., MATTHES, J. & KOSCHAK, A. 2014. Spectrum of Cav1.4 dysfunction in congenital stationary night blindness type 2. *Biochim Biophys Acta,* 1838**,** 2053-65.

BUSQUET, P., HETZENAUER, A., SINNEGGER-BRAUNS, M. J., STRIESSNIG, J. & SINGEWALD, N. 2008. Role of L-type Ca(2+) channel isoforms in the extinction of conditioned fear. *Learning & Memory,* 15**,** 378-386.

BUSQUET, P., NGUYEN, N. K., SCHMID, E., TANIMOTO, N., SEELIGER, M. W., BEN-YOSEF, T., MIZUNO, F., AKOPIAN, A., STRIESSNIG, J. & SINGEWALD, N. 2010. CaV1.3 L-type Ca2+ channels modulate depression-like behaviour in mice independent of deaf phenotype. *Int J Neuropsychopharmacol,* 13**,** 499-513.

CARDONE, L., HIRAYAMA, J., GIORDANO, F., TAMARU, T., PALVIMO, J. J. & SASSONE-CORSI, P. 2005. Circadian clock control by SUMOylation of BMAL1. *Science,* 309**,** 1390-4.

CATTERALL, W. A. 2000. Structure and regulation of voltage-gated Ca2+ channels. *Annu Rev Cell Dev Biol,* 16**,** 521-55.

CATTERALL, W. A. 2011. Voltage-gated calcium channels. *Cold Spring Harb Perspect Biol,* 3**,** a003947.

CATTERALL, W. A., PEREZ-REYES, E., SNUTCH, T. P. & STRIESSNIG, J. 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol Rev,* 57**,** 411-25.

CHAN, C. S., GUZMAN, J. N., ILIJIC, E., MERCER, J. N., RICK, C., TKATCH, T., MEREDITH, G. E. & SURMEIER, D. J. 2007. 'Rejuvenation' protects neurons in mouse models of Parkinson's disease. *Nature,* 447**,** 1081-6.

CURTIS, B. M. & CATTERALL, W. A. 1984. Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. *Biochemistry,* 23**,** 2113-8.

DAAN, S. & PITTENDRIGH, C. S. 1976. A Functional analysis of circadian pacemakers in nocturnal rodents. *Journal of comparative physiology,* 106**,** 253-266.

DAMIOLA, F., LE MINH, N., PREITNER, N., KORNMANN, B., FLEURY-OLELA, F. & SCHIBLER, U. 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev,* 14**,** 2950-61.

DEBRUYNE, J. P., WEAVER, D. R. & REPPERT, S. M. 2007. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci,* 10**,** 543-5.

DIBNER, C., SCHIBLER, U. & ALBRECHT, U. 2010. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol,* 72**,** 517-49.

DRAGICEVIC, E., POETSCHKE, C., DUDA, J., SCHLAUDRAFF, F., LAMMEL, S., SCHIEMANN, J., FAULER, M., HETZEL, A., WATANABE, M., LUJAN, R., MALENKA, R. C., STRIESSNIG, J. & LISS, B. 2014. Cav1.3 channels control D2-autoreceptor responses via NCS-1 in substantia nigra dopamine neurons. *Brain,* 137**,** 2287-302.

DUFFIELD, G. E., BEST, J. D., MEURERS, B. H., BITTNER, A., LOROS, J. J. & DUNLAP, J. C. 2002. Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells. *Curr Biol,* 12**,** 551-7.

ERTEL, E. A., CAMPBELL, K. P., HARPOLD, M. M., HOFMANN, F., MORI, Y., PEREZ-REYES, E., SCHWARTZ, A., SNUTCH, T. P., TANABE, T., BIRNBAUMER, L., TSIEN, R. W. & CATTERALL, W. A. 2000. Nomenclature of voltage-gated calcium channels. *Neuron,* 25**,** 533-5.

ETCHEGARAY, J. P., LEE, C., WADE, P. A. & REPPERT, S. M. 2003. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature,* 421**,** 177-82.

GACHON, F., NAGOSHI, E., BROWN, S. A., RIPPERGER, J. & SCHIBLER, U. 2004. The mammalian circadian timing system: from gene expression to physiology. *Chromosoma,* 113**,** 103-12.

GALLEGO, M. & VIRSHUP, D. M. 2007. Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol,* 8**,** 139-48.

GATFIELD, D. & SCHIBLER, U. 2007. Physiology. Proteasomes keep the circadian clock ticking. *Science,* 316**,** 1135-6.

GEKAKIS, N., STAKNIS, D., NGUYEN, H. B., DAVIS, F. C., WILSBACHER, L. D., KING, D. P., TAKAHASHI, J. S. & WEITZ, C. J. 1998. Role of the CLOCK protein in the mammalian circadian mechanism. *Science,* 280**,** 1564-9.

GOOLEY, J. J., LU, J., CHOU, T. C., SCAMMELL, T. E. & SAPER, C. B. 2001. Melanopsin in cells of origin of the retinohypothalamic tract. *Nat Neurosci,* 4**,** 1165.

GRIESAUER, I., DIAO, W., RONOVSKY, M., ELBAU, I., SARTORI, S., SINGEWALD, N. & POLLAK, D. D. 2014. Circadian abnormalities in a mouse model of high trait anxiety and depression. *Ann Med,* 46**,** 148-54.

GUO, H., BREWER, J. M., LEHMAN, M. N. & BITTMAN, E. L. 2006. Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. *J Neurosci,* 26**,** 6406-12.

HASTINGS, M. H., DUFFIELD, G. E., EBLING, F. J., KIDD, A., MAYWOOD, E. S. & SCHUROV, I. 1997. Non-photic signalling in the suprachiasmatic nucleus. *Biol Cell,* 89**,** 495-503.

HASTINGS, M. H., REDDY, A. B. & MAYWOOD, E. S. 2003. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci,* 4**,** 649-61.

HATTAR, S., LUCAS, R. J., MROSOVSKY, N., THOMPSON, S., DOUGLAS, R. H., HANKINS, M. W., LEM, J., BIEL, M., HOFMANN, F., FOSTER, R. G. & YAU, K. W. 2003. Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature,* 424**,** 76-81.

HEMARA-WAHANUI, A., BERJUKOW, S., HOPE, C. I., DEARDEN, P. K., WU, S. B., WILSON-WHEELER, J., SHARP, D. M., LUNDON-TREWEEK, P., CLOVER, G. M., HODA, J. C., STRIESSNIG, J., MARKSTEINER, R., HERING, S. & MAW, M. A. 2005. A CACNA1F mutation identified in an X-linked retinal disorder shifts the voltage dependence of Cav1.4 channel activation. *Proc Natl Acad Sci U S A,* 102**,** 7553-8.

HIRAYAMA, J., SAHAR, S., GRIMALDI, B., TAMARU, T., TAKAMATSU, K., NAKAHATA, Y. & SASSONE-CORSI, P. 2007. CLOCK-mediated acetylation of BMAL1 controls circadian function. *Nature,* 450**,** 1086-90.

HODA, J. C., ZAGHETTO, F., KOSCHAK, A. & STRIESSNIG, J. 2005. Congenital stationary night blindness type 2 mutations S229P, G369D, L1068P, and W1440X alter channel gating or functional expression of Ca(v)1.4 L-type Ca2+ channels. *J Neurosci,* 25**,** 252-9.

HODA, J. C., ZAGHETTO, F., SINGH, A., KOSCHAK, A. & STRIESSNIG, J. 2006. Effects of congenital stationary night blindness type 2 mutations R508Q and L1364H on Cav1.4 L-type Ca2+ channel function and expression. *J Neurochem,* 96**,** 1648-58.

HOPE, C. I., SHARP, D. M., HEMARA-WAHANUI, A., SISSINGH, J. I., LUNDON, P., MITCHELL, E. A., MAW, M. A. & CLOVER, G. M. 2005. Clinical manifestations of a unique X-linked retinal disorder in a large New Zealand

family with a novel mutation in CACNA1F, the gene responsible for CSNB2. *Clin Exp Ophthalmol,* 33**,** 129-36.

IITAKA, C., MIYAZAKI, K., AKAIKE, T. & ISHIDA, N. 2005. A role for glycogen synthase kinase-3beta in the mammalian circadian clock. *J Biol Chem,* 280**,** 29397-402.

JALKANEN, R., BECH-HANSEN, N. T., TOBIAS, R., SANKILA, E. M., MANTYJARVI, M., FORSIUS, H., DE LA CHAPELLE, A. & ALITALO, T. 2007. A novel CACNA1F gene mutation causes Aland Island eye disease. *Invest Ophthalmol Vis Sci,* 48**,** 2498-502.

JALKANEN, R., MANTYJARVI, M., TOBIAS, R., ISOSOMPPI, J., SANKILA, E. M., ALITALO, T. & BECH-HANSEN, N. T. 2006. X linked cone-rod dystrophy, CORDX3, is caused by a mutation in the CACNA1F gene. *J Med Genet,* 43**,** 699-704.

JONES, C. R., CAMPBELL, S. S., ZONE, S. E., COOPER, F., DESANO, A., MURPHY, P. J., JONES, B., CZAJKOWSKI, L. & PTACEK, L. J. 1999. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. *Nat Med,* 5**,** 1062-5.

KNOFLACH, D., KEROV, V., SARTORI, S. B., OBERMAIR, G. J., SCHMUCKERMAIR, C., LIU, X., SOTHILINGAM, V., GARCIA GARRIDO, M., BAKER, S. A., GLOSMANN, M., SCHICKER, K., SEELIGER, M., LEE, A. & KOSCHAK, A. 2013. Cav1.4 IT mouse as model for vision impairment in human congenital stationary night blindness type 2. *Channels (Austin),* 7**,** 503-13.

KO, C. H. & TAKAHASHI, J. S. 2006. Molecular components of the mammalian circadian clock. *Human Molecular Genetics,* 15**,** R271-R277.

KOFUJI, P., MURE, L. S., MASSMAN, L. J., PURRIER, N., PANDA, S. & ENGELAND, W. C. 2016. Intrinsically Photosensitive Retinal Ganglion Cells (ipRGCs) Are Necessary for Light Entrainment of Peripheral Clocks. *PLoS One,* 11**,** e0168651.

KOJIMA, S., HIROSE, M., TOKUNAGA, K., SAKAKI, Y. & TEI, H. 2003. Structural and functional analysis of 3' untranslated region of mouse Period1 mRNA. *Biochem Biophys Res Commun,* 301**,** 1-7.

KOTTURI, M. F. & JEFFERIES, W. A. 2005. Molecular characterization of L-type calcium channel splice variants expressed in human T lymphocytes. *Mol Immunol,* 42**,** 1461-74.

LEE, A. S., RA, S., RAJADHYAKSHA, A. M., BRITT, J. K., DE JESUS-CORTES, H., GONZALES, K. L., LEE, A., MOOSMANG, S., HOFMANN, F., PIEPER, A. A. & RAJADHYAKSHA, A. M. 2012. Forebrain elimination of cacna1c mediates anxiety-like behavior in mice. *Mol Psychiatry,* 17**,** 1054-5.

LEE, C., ETCHEGARAY, J. P., CAGAMPANG, F. R., LOUDON, A. S. & REPPERT, S. M. 2001. Posttranslational mechanisms regulate the mammalian circadian clock. *Cell,* 107**,** 855-67.

LEE, J., KIM, D. & SHIN, H. S. 2004. Lack of delta waves and sleep disturbances during non-rapid eye movement sleep in mice lacking alpha1G-subunit of T-type calcium channels. *Proc Natl Acad Sci U S A,* 101**,** 18195-9.

LEE, J., LEE, Y., LEE, M. J., PARK, E., KANG, S. H., CHUNG, C. H., LEE, K. H. & KIM, K. 2008. Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. *Mol Cell Biol,* 28**,** 6056-65.

LIU, A. C., LEWIS, W. G. & KAY, S. A. 2007. Mammalian circadian signaling networks and therapeutic targets. *Nat Chem Biol,* 3**,** 630-639.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods,* 25**,** 402-408.

LOWREY, P. L., SHIMOMURA, K., ANTOCH, M. P., YAMAZAKI, S., ZEMENIDES, P. D., RALPH, M. R., MENAKER, M. & TAKAHASHI, J. S. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science,* 288**,** 483-92.

LUCAS, R. J., FREEDMAN, M. S., LUPI, D., MUNOZ, M., DAVID-GRAY, Z. K. & FOSTER, R. G. 2001. Identifying the photoreceptive inputs to the mammalian circadian system using transgenic and retinally degenerate mice. *Behav Brain Res,* 125**,** 97-102.

MANGONI, M. E., COUETTE, B., BOURINET, E., PLATZER, J., REIMER, D., STRIESSNIG, J. & NARGEOT, J. 2003. Functional role of L-type Cav1.3 Ca2+ channels in cardiac pacemaker activity. *Proc Natl Acad Sci U S A,* 100**,** 5543-8.

MANGONI, M. E., TRABOULSIE, A., LEONI, A. L., COUETTE, B., MARGER, L., LE QUANG, K., KUPFER, E., COHEN-SOLAL, A., VILAR, J., SHIN, H. S., ESCANDE, D., CHARPENTIER, F., NARGEOT, J. & LORY, P. 2006. Bradycardia and slowing of the atrioventricular conduction in mice lacking CaV3.1/alpha1G T-type calcium channels. *Circ Res,* 98**,** 1422-30.

MARCANTONI, A., VANDAEL, D. H., MAHAPATRA, S., CARABELLI, V., SINNEGGER-BRAUNS, M. J., STRIESSNIG, J. & CARBONE, E. 2010. Loss of Cav1.3 channels reveals the critical role of L-type and BK channel coupling in pacemaking mouse adrenal chromaffin cells. *J Neurosci,* 30**,** 491-504.

MATSUO, T., YAMAGUCHI, S., MITSUI, S., EMI, A., SHIMODA, F. & OKAMURA, H. 2003. Control mechanism of the circadian clock for timing of cell division in vivo. *Science,* 302**,** 255-9.

MCRORY, J. E., HAMID, J., DOERING, C. J., GARCIA, E., PARKER, R., HAMMING, K., CHEN, L., HILDEBRAND, M., BEEDLE, A. M., FELDCAMP, L., ZAMPONI, G. W. & SNUTCH, T. P. 2004. The CACNA1F gene encodes an L-type calcium channel with unique biophysical properties and tissue distribution. *J Neurosci,* 24**,** 1707-18.

MILLER, B. H., MCDEARMON, E. L., PANDA, S., HAYES, K. R., ZHANG, J., ANDREWS, J. L., ANTOCH, M. P., WALKER, J. R., ESSER, K. A., HOGENESCH, J. B. & TAKAHASHI, J. S. 2007. Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci U S A,* 104**,** 3342-7.

MOORE, R. Y. & EICHLER, V. B. 1972. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res,* 42**,** 201-6.

MOOSMANG, S., HAIDER, N., KLUGBAUER, N., ADELSBERGER, H., LANGWIESER, N., MULLER, J., STIESS, M., MARAIS, E., SCHULLA, V., LACINOVA, L., GOEBBELS, S., NAVE, K. A., STORM, D. R., HOFMANN, F. & KLEPPISCH, T. 2005. Role of hippocampal Cav1.2 Ca2+ channels in NMDA receptor-independent synaptic plasticity and spatial memory. *J Neurosci,* 25**,** 9883-92.

MORIN, L. P. & ALLEN, C. N. 2006. The circadian visual system, 2005. *Brain Research Reviews,* 51**,** 1-60.

MURAKAMI, M., NAKAGAWASAI, O., FUJII, S., KAMEYAMA, K., MURAKAMI, S., HOZUMI, S., ESASHI, A., TANIGUCHI, R., YANAGISAWA, T., TAN-NO, K., TADANO, T., KITAMURA, K. & KISARA, K. 2001. Antinociceptive action of amlodipine blocking N-type Ca2+ channels at the primary afferent neurons in mice. *Eur J Pharmacol,* 419**,** 175-81.

NAMKUNG, Y., SKRYPNYK, N., JEONG, M. J., LEE, T., LEE, M. S., KIM, H. L., CHIN, H., SUH, P. G., KIM, S. S. & SHIN, H. S. 2001. Requirement for the L-type Ca(2+) channel alpha(1D) subunit in postnatal pancreatic beta cell generation. *J Clin Invest,* 108**,** 1015-22.

PANDA, S., PROVENCIO, I., TU, D. C., PIRES, S. S., ROLLAG, M. D., CASTRUCCI, A. M., PLETCHER, M. T., SATO, T. K., WILTSHIRE, T., ANDAHAZY, M., KAY, S. A., VAN GELDER, R. N. & HOGENESCH, J. B. 2003. Melanopsin is required for non-image-forming photic responses in blind mice. *Science,* 301**,** 525-7.

PELOQUIN, J. B., REHAK, R., DOERING, C. J. & MCRORY, J. E. 2007. Functional analysis of congenital stationary night blindness type-2 CACNA1F mutations F742C, G1007R, and R1049W. *Neuroscience,* 150**,** 335-45.

PEREZ-REYES, E., KIM, H. S., LACERDA, A. E., HORNE, W., WEI, X. Y., RAMPE, D., CAMPBELL, K. P., BROWN, A. M. & BIRNBAUMER, L. 1989.

Induction of calcium currents by the expression of the alpha 1-subunit of the dihydropyridine receptor from skeletal muscle. *Nature,* 340**,** 233-6.

PREITNER, N., DAMIOLA, F., LOPEZ-MOLINA, L., ZAKANY, J., DUBOULE, D., ALBRECHT, U. & SCHIBLER, U. 2002. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell,* 110**,** 251-60.

REPPERT, S. M. & WEAVER, D. R. 2001. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol,* 63**,** 647-76.

REPPERT, S. M. & WEAVER, D. R. 2002. Coordination of circadian timing in mammals. *Nature,* 418**,** 935-41.

REUTER, H. 1979. Properties of two inward membrane currents in the heart. *Annu Rev Physiol,* 41**,** 413-24.

SANADA, K., OKANO, T. & FUKADA, Y. 2002. Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *J Biol Chem,* 277**,** 267-71.

SAVELYEV, S. A., LARSSON, K. C., JOHANSSON, A.-S. & LUNDKVIST, G. B. S. 2011. Slice Preparation, Organotypic Tissue Culturing and Luciferase Recording of Clock Gene Activity in the Suprachiasmatic Nucleus. *Journal of Visualized Experiments : JoVE***,** 2439.

SCHAUFLER, J., RONOVSKY, M., SAVALLI, G., CABATIC, M., SARTORI, S. B., SINGEWALD, N. & POLLAK, D. D. 2016. Fluoxetine normalizes disrupted light-induced entrainment, fragmented ultradian rhythms and altered hippocampal clock gene expression in an animal model of high trait anxiety- and depression-related behavior. *Annals of Medicine,* 48**,** 17-27.

SCHIBLER, U. 2007. The daily timing of gene expression and physiology in mammals. *Dialogues Clin Neurosci,* 9**,** 257-72.

SHANWARE, N. P., HUTCHINSON, J. A., KIM, S. H., ZHAN, L., BOWLER, M. J. & TIBBETTS, R. S. 2011. Casein kinase 1-dependent phosphorylation of familial advanced sleep phase syndrome-associated residues controls PERIOD 2 stability. *J Biol Chem,* 286**,** 12766-74.

SIMONSZ, H. J., FLORIJN, R. J., VAN MINDERHOUT, H. M., BERGEN, A. A. & KAMERMANS, M. 2009. Nightblindness-associated transient tonic downgaze (NATTD) in infant boys with chin-up head posture. *Strabismus,* 17**,** 158-64.

SINGH, A., HAMEDINGER, D., HODA, J. C., GEBHART, M., KOSCHAK, A., ROMANIN, C. & STRIESSNIG, J. 2006. C-terminal modulator controls Ca2+-dependent gating of Ca(v)1.4 L-type Ca2+ channels. *Nat Neurosci,* 9**,** 1108-16.

SINNEGGER-BRAUNS, M. J., HETZENAUER, A., HUBER, I. G., RENSTROM, E., WIETZORREK, G., BERJUKOV, S., CAVALLI, M., WALTER, D., KOSCHAK, A., WALDSCHUTZ, R., HERING, S., BOVA, S., RORSMAN, P., PONGS, O., SINGEWALD, N. & STRIESSNIG, J. 2004. Isoform-specific regulation of mood behavior and pancreatic beta cell and cardiovascular function by L-type Ca 2+ channels. *J Clin Invest,* 113**,** 1430-9.

STEPHAN, F. K. & ZUCKER, I. 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci U S A,* 69**,** 1583-6.

STOCKNER, T. & KOSCHAK, A. 2013. What can naturally occurring mutations tell us about Ca(v)1.x channel function? *Biochim Biophys Acta,* 1828**,** 1598-607.

STRECKER, G. J., WUARIN, J. P. & DUDEK, F. E. 1997. GABAA-mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. *J Neurophysiol,* 78**,** 2217-20.

STRIESSNIG, J., BOLZ, H. J. & KOSCHAK, A. 2010. Channelopathies in Cav1.1, Cav1.3, and Cav1.4 voltage-gated L-type Ca2+ channels. *Pflügers Archiv - European Journal of Physiology,* 460**,** 361-374.

STRIESSNIG, J., PINGGERA, A., KAUR, G., BOCK, G. & TULUC, P. 2014. L-type Ca2+ channels in heart and brain. *Wiley Interdiscip Rev Membr Transp Signal,* 3**,** 15-38.

SURMEIER, D. J., GUZMAN, J. N., SANCHEZ-PADILLA, J. & GOLDBERG, J. A. 2011. The origins of oxidant stress in Parkinson's disease and therapeutic strategies. *Antioxid Redox Signal,* 14**,** 1289-301.

TAKAHASHI, J. S. 2004. Finding New Clock Components: Past and Future. *Journal of Biological Rhythms,* 19**,** 339-347.

TAKAHASHI, J. S., HONG, H. K., KO, C. H. & MCDEARMON, E. L. 2008. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet,* 9**,** 764-75.

TAKAHASHI, M., SEAGAR, M. J., JONES, J. F., REBER, B. F. & CATTERALL, W. A. 1987. Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *Proc Natl Acad Sci U S A,* 84**,** 5478-82.

TANABE, T., TAKESHIMA, H., MIKAMI, A., FLOCKERZI, V., TAKAHASHI, H., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature,* 328**,** 313-8.

TREMBLAY, F., LAROCHE, R. G. & DE BECKER, I. 1995. The electroretinographic diagnosis of the incomplete form of congenital stationary night blindness. *Vision Res,* 35**,** 2383-93.

VAN DER HORST, G. T., MUIJTJENS, M., KOBAYASHI, K., TAKANO, R., KANNO, S., TAKAO, M., DE WIT, J., VERKERK, A., EKER, A. P., VAN LEENEN, D., BUIJS, R., BOOTSMA, D., HOEIJMAKERS, J. H. & YASUI, A. 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature,* 398**,** 627-30.

VIRSHUP, D. M., EIDE, E. J., FORGER, D. B., GALLEGO, M. & HARNISH, E. V. 2007. Reversible protein phosphorylation regulates circadian rhythms. *Cold Spring Harb Symp Quant Biol,* 72**,** 413-20.

VUJOVIC, N., DAVIDSON, A. J. & MENAKER, M. 2008. Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. *Am J Physiol Regul Integr Comp Physiol,* 295**,** R355-60.

WATSON, C. 2010. Chemoarchitectonic atlas of the mouse brain. *In:* PAXINOS, G. (ed.) 1st ed. ed. Amsterdam ;: Academic Press.

WELSH, D. K., TAKAHASHI, J. S. & KAY, S. A. 2010. Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol,* 72**,** 551-77.

WHITE, J. A., MCKINNEY, B. C., JOHN, M. C., POWERS, P. A., KAMP, T. J. & MURPHY, G. G. 2008. Conditional forebrain deletion of the L-type calcium channel Ca V 1.2 disrupts remote spatial memories in mice. *Learn Mem,* 15**,** 1-5.

YAGITA, K., TAMANINI, F., VAN DER HORST, G. T. & OKAMURA, H. 2001. Molecular mechanisms of the biological clock in cultured fibroblasts. *Science,* 292**,** 278-81.

YAMAZAKI, S., NUMANO, R., ABE, M., HIDA, A., TAKAHASHI, R.-I., UEDA, M., BLOCK, G. D., SAKAKI, Y., MENAKER, M. & TEI, H. 2000. Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats. *Science,* 288**,** 682-685.

YANG, Y., HE, Q., CHENG, P., WRAGE, P., YARDEN, O. & LIU, Y. 2004. Distinct roles for PP1 and PP2A in the Neurospora circadian clock. *Genes Dev,* 18**,** 255-60.

YIN, L., WANG, J., KLEIN, P. S. & LAZAR, M. A. 2006. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. *Science,* 311**,** 1002-5.

ZABOURI, N. & HAVERKAMP, S. 2013. Calcium channel-dependent molecular maturation of photoreceptor synapses. *PLoS One,* 8**,** e63853.

ZAMPONI, G. W., STRIESSNIG, J., KOSCHAK, A. & DOLPHIN, A. C. 2015. The Physiology, Pathology, and Pharmacology of Voltage-Gated Calcium Channels and Their Future Therapeutic Potential. *Pharmacol Rev,* 67**,** 821-70.

ZHENG, B., ALBRECHT, U., KAASIK, K., SAGE, M., LU, W., VAISHNAV, S., LI, Q., SUN, Z. S., EICHELE, G., BRADLEY, A. & LEE, C. C. 2001. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell,* 105**,** 683-94.

9 Appendix

9.1 Abstract

9.1.1 English

The I745T mutation in the CACNA1F gene leads to a particularly severe phenotype of congenitally stationary night blindness type 2 (CSNB2). CACNA1F codes for the $Ca_V1.4$ channel which is important for proper neurotransmission between photoreceptors and second-order neurons. This mutation was firstly described in a New Zealand family and is characterized by the severity of its symptoms including myopia, low visual acuity, nystagmus, variable degrees of night blindness and in some cases even intellectual disability. Due to its X-linked character CSNB2 normally affects only male individuals. However, this severe phenotype also affects heterozygote females. The $Ca_V1.4$ IT mouse line serves as a suitable model to investigate the disease. Herein, we show the consequences of this gain of function mutation on the circadian rhythm of IT mice.

Therefore, we recorded the wheel running activity of $Ca_V1.4$ IT mice and wild-type mice under light entrained and free running conditions. Thereby, the wheel revolutions served as a read out of the rhythmicity generated by the circadian clock. The results implicate that the I745T mutation leads to a significant shortened circadian period which is not due to restriction in the locomotor activity because wild-type mice and $Ca_V1.4$ IT mice show the same total activity.

In order to find a molecular reason underlying the circadian phenotype we analyzed the gene expression level of clock genes and clock controlled genes in the SCN. However, we did not find significant differences of the expression levels of these genes between $Ca_V1.4$ IT mice and wild-type mice.

In summary, we were able to show that the gain of function mutation I745T results in abnormalities of the circadian rhythm in fact in a shortening of the circadian period. However, the molecular reason underlying this phenotype still need to be elucidated.

9.1.2 German

Die I745T Mutation führt zu einer besonders schweren Form der kongenital stationären Nachtblindheit Typ 2 (CSNB2). Das CACNA1F Gen kodiert für den $Ca_V1.4$ Kanal, welcher für die Neurotransmission zwischen Photorezeptoren und Biboplarzellen essentiell ist. Erstmals wurde diese Mutation in einer neuseeländischen Familie beschrieben. Diese Art der Nachtblindheit zeichnet sich durch die Schwere ihrer Symptome, unter anderem Myopie, Nystagmus, Sehschwäche sowie eine variable Ausprägung der Nachtblindheit, aus. In einigen Fällen wurde sogar über eine mentale Retardierung der betroffenen Patienten berichtet. Da das betroffene Gen am X-Chromosom liegt, sind normalerweise nur Männer oder homozygote Frauen betroffen. Allerdings sind bei dieser Mutation auch heterozygote Frauen betroffen. Zur Untersuchung dieser Form der Nachtblindheit dient ein Mausmodell, welches die I745T Mutation besitzt, die sogenannte $Ca_V1.4$ IT Mauslinie. In dieser Arbeit zeigen wir welche Auswirkung diese Mutation auf den zirkadianen Rhythmus der Mäuse hat.

Dazu wurde das Laufverhalten von Wildtyp Mäusen mit dem von $Ca_V1.4$ IT Mäusen verglichen. Hierzu wurde ihre Laufradaktivität analysiert, einmal unter Tag (12 Stunden Licht) – Nacht (12 Stunden Dunkelheit) Bedingungen und einmal unter Bedingungen absoluter Finsternis (24 Stunden Dunkelheit). Dabei dienten die Laufradumdrehungen als Output der durch die zirkadiane Uhr erzeugten Rhythmizität. Die Ergebnisse deuten darauf hin, dass die I745T Mutation zu einer signifikanten Verkürzung der zirkadianen Periode führt, wobei eine Verkürzung auf Grund lokomotorischer Einschränkungen ausgeschlossen werden konnte, da wild typ Mäuse und $Ca_V1.4$ IT Mäuse gesamt gesehen die gleiche Aktivität zeigten.

Um die molekulare Ursache dieses zirkadianen Phänotyps zu finden analysierten wir die Genexpression essentieller „clock genes“ im Suprachiasmatischen Nukleus. Dabei konnten wir keine Unterschiede zwischen wild typ Mäusen und $Ca_V1.4$ IT Mäuse feststellen.

Zusammenfassend kann gesagt werden, dass die I745T Mutation zu signifikanten Veränderungen im zirkadianen Rhythmus führt. Für die Aufklärung der molekularen Ursache sind weitere Untersuchungen notwendig.

9.2 Index of Figures

9.3 Index of Tables

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