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Detection of polymorphisms in clock genes in patients with dementia, and evaluation of their sleep architecture

Μεταπτυχιακή Διατριβή

Ανίχνευση πολυμορφισμών των clock γονιδίων σε ασθενείς με άνοια και εκτίμηση της αρχιτεκτονικής του ύπνου

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The Disintegration of the Persistence of Memory
Salvador Dali, 1954

ABSTRACT

Introduction: Sleep is more than a state of no-alertness, since its rhythmicity is genetically programmed and regulated by the circadian mechanism. Polymorphisms in clock genes, which constitute the core of the circadian clock, are associated with many sleep and circadian disorders, as well as individual's chronotype.

Aims: Aim of this study was to identify rare variants in clock genes and correlate their presence with extreme sleep phenotypes, as identified by actigraphy measurements and subsequently verified by polysomnography.

Methods: In the present study, the exome of 145 participants of the Thalis Cretan Aging Study Cohort (47 cognitively normal controls and 98 patients diagnosed with dementia), was analyzed in order to detect variants in 14 clock genes: *CLOCK, BMAL1, NPAS2, PER1, PER2, PER3, CRY1, CRY2, BHLHE40, BHLHE41, NR1D1, REV1, RORA, RORB* and one possible regulator of the circadian mechanism, *VDR* gene. These data were coupled with participants' actigraphy recordings.

Results: WES analysis identified 57 known and 34 novel clock gene polymorphisms in this cohort. Analysis of the actigraphy measurements in these participants, showed that participants positive for rs2585405 tended to have a short-lasting night Total Sleep Time (night TST; p=0.051), while rs150149747 was also significantly associated with low TST (p=0.046). Both these variants are located in *PER1*, a crucial regulator of the circadian mechanism. In addition, a *PER3* variation, named rs1776342 was also related with decreased TST (p=0.046). Furthermore, 12 rare polymorphisms were identified in participants with extreme actigraphy phenotypes. Interestingly, 6 of these polymorphisms are novel, indicating that many extreme sleep phenotypes could have a genetic basis, which acts as substrate for sleep disorders' development. For further analysis of participants' sleep quality, studies in Sleep Medicine Laboratory were performed to evaluate changes in sleep architecture that can be correlated with specific variants.

Conclusions: These preliminary results show that sleep patterns in both cognitively normal controls and patients with dementia could have a strong genetic background.

ΠΕΡΙΛΗΨΗ

Εισαγωγή: Ο ύπνος είναι κάτι περισσότερο από μια κατάσταση μη εγρήγορσης, καθώς η ρυθμικότητα του είναι γενετικά προγραμματισμένη και συγχρονισμένη από τον κιρκάδιο μηχανισμό. Οι πολυμορφισμοί στα clock γονίδια, τα οποία αποτελούν τον πυρήνα του κιρκάδιου ρολογιού, συνδέονται με πολλές διαταραχές ύπνου και αλλαγές της κιρκάδιας δραστηριότητας καθώς και με τον χρονότυπο του ατόμου.

Σκοπό: Σκοπός αυτής της μελέτης είναι να αναγνωρίσει σπάνιους πολυμορφισμούς στα clock γονίδια και να συσχετίσει την παρουσία τους με ακραίους φαινότυπους ύπνου, όπως αυτοί αναδύθηκαν μέσα από καταγραφές ακτιγραφίας και στη συνέχεια, επαληθεύτηκαν με τη μέθοδο της πολυπνογραφίας.

Μέθοδοι: Στην παρούσα εργασία αναλύθηκε το έξωμα (exome) από 145 συμμετέχοντες του "Thalis Cretan Aging Study Cohort" (47 γνωστικά υγιείς ως controls and 98 ασθενείς διαγνωσμένοι με άνοια), με σκοπό την ανίχνευση παραλλαγών (variants) σε 14 clock γονίδια: *CLOCK, BMAL1, NPAS2, PER1, PER2, PER3, CRY1, CRY2, BHLHE40, BHLHE41, NR1D1, REV1, RORA, RORB* και ένα γονίδιο, πιθανό ρυθμιστή του κιρκάδιου μηχανισμού, το *VDR*. Αυτά τα γενετικά δεδομένα συνδυάστηκαν με τις καταγραφές ακτιγραφίας των συμμετεχόντων.

Αποτελέσματα: Κατά τη WES ανάλυση αναγνωρίστηκαν 57 γνωστοί και 34 νεοανιχνευθείς πολυμορφισμοί στα clock γονίδια. Η ανάλυση των δεδομένων ακτιγραφίας έδειξε ότι οι συμμετέχοντες που βρέθηκαν θετικοί για το rs2585405 τείνουν να έχουν μικρής διάρκειας νυχτερινό ύπνο (night TST; p=0.051), ενώ ο πολυμορφισμός rs150149747 βρέθηκε συνδεδεμένος, σε σημαντικό βαθμό, με χαμηλής διάρκειας TST (p=0.046). Οι δύο αυτές αλλαγές αφορούν το γονίδιο *PER1*, το οποίο είναι σημαντικός ρυθμιστής του κιρκάδιου μηχανισμού. Επιπρόσθετα, ο πολυμορφισμός rs1776342, ο οποίος εντοπίζεται στο γονίδιο *PER3,* επίσης συσχετίστηκε με μειωμένο ολικό χρόνο ύπνου (TST; p=0.046). Επιπλέον, ανιχνεύθηκαν 12 σπάνιοι πολυμορφισμοί σε συμμετέχοντες με ακραίους φαινοτύπους ύπνου, όπως αναδείχθηκαν με τη χρήση ακτιγραφίας. Είναι ενδιαφέρον το γεγονός ότι 6 από αυτούς τους πολυμορφισμούς δεν έχουν αναφερθεί άλλοτε ξανά στη βιβλιογραφία, επομένως χαρακτηρίζονται ως novel. Τα ευρήματα αυτά αποδεικνύουν ότι πολλοί ακραίοι φαινότυποι ύπνου, πιθανό να έχουν γενετική βάση, η οποία λειτουργεί ως υπόστρωμα για την ανάπτυξη διαταραχών του ύπνου. Για περαιτέρω ανάλυση της ποιότητας του ύπνου των συμμετεχόντων, πραγματοποιήθηκαν μελέτες ύπνου, στο Εργαστήριο Ύπνου του Πανεπιστημίου, με σκοπό την εκτίμηση αλλαγών στην αρχιτεκτονική του ύπνου, οι οποίες πιθανό να συσχετίζονται με συγκεκριμένους πολυμορφισμούς.

Συμπεράσματα: Τα δεδομένα προκαταρκτικά αποτελέσματα δείχνουν ότι οι συνήθειες του ύπνου, τόσο σε γνωστικά υγιείς όσο και σε ασθενείς με άνοια, μπορούν να έχουν ένα ισχυρό γενετικό υπόβαθρο.

CONTENTS

ABSTRACT....3
ΠΕΡΙΛΗΨΗ....4
CONTENTS....5
ABBREVIATIONS....7
PROLOGUE....8
What is sleep?....8
INTRODUCTION....9
Sleep Architecture....9
Polysomnography....10
Wrist actigraphy....11
NREM & REM Characteristics....12
N1....12
N2....12
N3....12
REM....13
Sleep Through Lifetime....14
Homeostatic Sleep Regulation....16
Biological Rhythms....17
Circadian Mechanisms & Mood....19
Functional Profile of SCN Neurons....20
Neurotransmitters of Sleep & Wakefulness....21
Sleep Duration and Brain Calcium....23
Sleep Regulation & Melatonin....23
Sleep Deprivation....24
Genetics of Circadian Clock....24
CLOCK....26
NPAS2....26
BMAL1 or ARNTL1....27
PER genes....27
CRY genes....28
VDR....28
Clock Genes & Their Mutations....29
Sleep & Whole Exome Sequencing....30
Circadian Diseases....31
Clock Genes & Other Diseases....32
HYPOTHESIS....33
MATERIALS & METHODS....34
Cohort....34
Biological Sample Collection....34
DNA Extraction....34
Whole Exome Sequencing....34
Bioinformatics' Analysis....34
Actigraphy Studies....35
Sleep Architecture Studies....35
Statistical Analysis....35
RESULTS....36
WES Analysis....36
Statistical Analysis of Actigraphy Data in Respect with Clock Genes Variations' Distribution....37
PER1 variations....37

PER3 variations.....39
Actigraphy Data Extremities & Rare Polymorphisms.....40
TST.....40
Night TST.....43
Night In Bed-Time.....44
Night Out Bed- Time.....46
Nap TST.....47
Nap Number.....48
Polysomnography Studies.....50
DISCUSSION.....53
APPENDIX.....57
AKNOWLEDGMENTS.....61
REFERENCES.....62
Publications & Books.....62
Web Sites.....66

ABBREVIATIONS

5-HT: serotonin	**Met**: methionine
Ach: acetylcholine	**N-24**: Non-24-H Sleep Wake Syndrome
AD: Alzheimer's disease	**NE**: noradrenaline, norepinephrine
Ala: alanine	**NGS**: Next-generation sequencing
ANS: autonomic nervous system	**NO**: nitric oxide
Arg: arginine	**NPAS2 gene**: Neuronal PAS Domain Protein 2
ARNTL1 or **BMAL1 gene**: Aryl hydrocarbon Receptor Nuclear Translocator-Like1 gene	**NR1D1 gene**: Nuclear Receptor subfamily 1 group D member 1
Asn: asparagine	**NREM**: Non-REM
BD: Bipolar disorders	**NTs**: neurotransmitters
BDNF: brain-derived neurotrophic factor	**NURR1**: nuclear receptor-related 1 protein
BPD: bipolar disorder	**OSAS**: Obstructive Sleep Apnea Syndrome
CLOCK gene: Circadian Locomotor Output Cycles Kaput	**PAS domain**: protein domain first discovered in Per, Arnt, Sim proteins
CNS: central nervous system	**PD**: Parkinson's disease
CRE: cAMP response element	**PER gene**: Period gene
CRSD: Circadian Rhythm Sleep Disorders	**PFC**: prefrontal cortex
CRY gene: Cryptochrome	**PGD**: prostaglandin D
Cys: cysteine	**Phe**: phenylalanine
DA: dopamine	**PNS**: peripheral nervous system
DAergic system: dopaminergic system	**Process C**: Circadian process
DSPS: delayed sleep phase syndrome	**Process S**: Sleep process
E: eveningness	**PSG**: polysomnography
ECG: electrocardiography	**REM**: Rapid Eye Movement
EEG: electroencephalogram	**SAD**: seasonal affective disorder
EMG: electromyography	**SCN**: suprachiasmatic nucleus
EOG: electrooculography	**Ser**: serine
FASPS: Familial Advanced Sleep Phase Syndrome	**SIS**: Sleep Inducing Substance
GHRH: growth-hormone-releasing hormone	**SNP**: single nucleotide polymorphism
GRE: glucocorticoid response element	**SON**: supraoptic nucleus
GWAS: Genome Wide Association Study	**SSNHL**: Sudden sensorineural hearing loss
His: histidine	**SWS**: Slow Wave Sleep
IEG: immediate early gene	**TH gene**: Tyrosine Hydroxylase gene
IL-1: interleukin 1	**Thr**: threonine
Ile: isoleucine	**TNF**: tumor-necrosis factor
KO: knock out	**Trp**: tryptophan
LD cycle: light-dark cycle	**TST**: total sleep time
LDHA: lactate dehydrogenase a	**Tyr**: tyrosine
LTP: long-term potentiation	**Val**: valine
M: morningness	**VDR**: Vitamin D Receptor
MDD: Major depressive disorder	**WES**: Whole Exome Sequencing

What is sleep?

Sleep is more than a state of no-alertness. “It is a function created of the brain, by the brain, for the brain”, as it was first stated by Hobson [1]. Important functions such as memory consolidation, energy storage and development are taking place in the brain during sleep, in order to prepare the body for the next day. This preparation, in addition to consolidate learned information and generation of new memories, also leads to reflexes’ and new movements’ consolidation. Thus, basic brain structures, such as prefrontal cortex, are prepared to perform highest functions, such as consciousness. Selectively, all these silent functions during sleep aim to achieve adjustment and survival in a constantly changing environment.

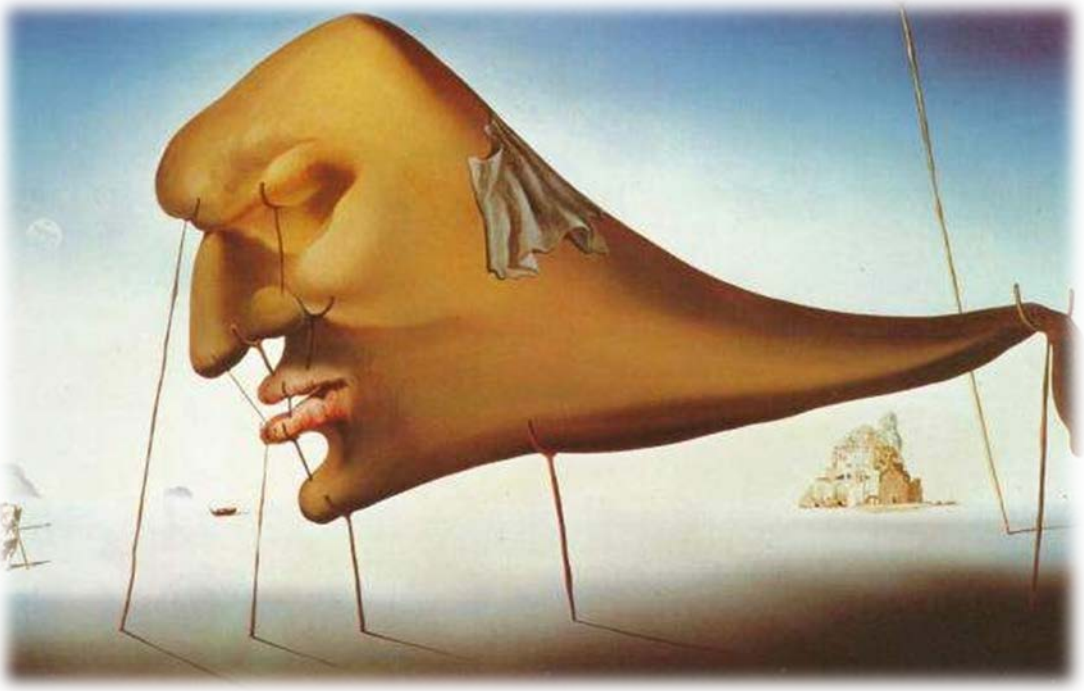

Figure 1. Sleep in art: "Sleep", Salvador Dali, 1937

Sleep Architecture

Sleep is categorized into two basic states; REM and NREM sleep. REM sleep, or as it is commonly referred, Rapid Eye Movement sleep, is the main stage where memory consolidation takes place. Non-REM sleep (or NREM) is sub-categorized into 3 additional stages: N1, N2 and N3. Earlier literature recognized an extra state, namely N4, however nowadays N3 and N4 are grouped together under state N3. [2]

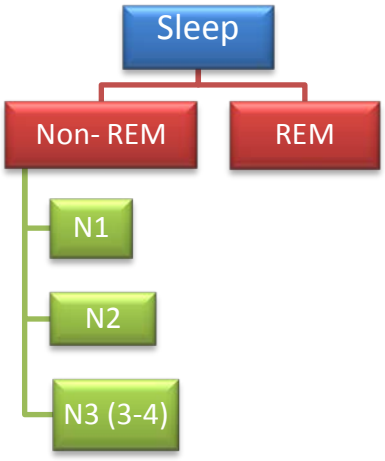

Figure 2. Separation of sleep stages

A typical sleep cycle starts with N1 stage, which is followed by N2 and N3. N3, which is also called deep sleep or Slow Wave Sleep (SWS), is the immediately preceding stage before REM sleep initiation. This sequence of stages repeats approximately 3-4 times per night, resulting accordingly in 3-4 sleep circles. The duration of each circle varies between 90 and 120 min, as it is changing through the night. Notably, the duration of REM sleep increases as the individual proceeds from one cycle to the next one. So, REM sleep increasingly occupies a larger portion of total sleep duration. [2]

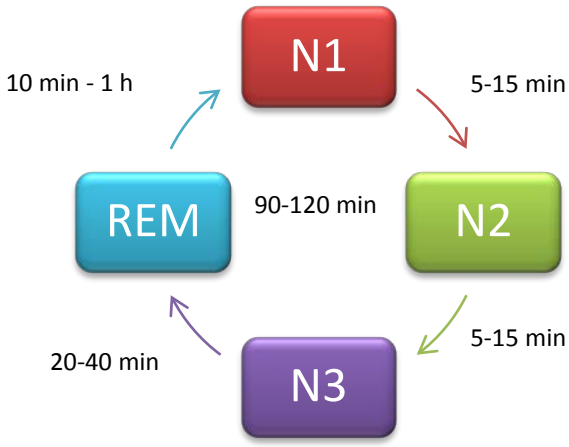

Figure 3. A typical sleep cycle: Total sleep cycle duration varies between 90-120 minutes, while each stage is characterized by specific duration (reported on arrows).

Special sleep laboratories are dedicated to study sleep architecture. The procedure includes the application of electrodes onto each individual's scalp, in order to record electrical waves emitted from different regions of the cerebral cortex. Each of the waves produced display differences in four of the following parameters: the amplitude, frequency, waveform, and distribution. [2]

Amplitude is referred to the height of the wave (amount of voltage) and its measurement unit is μV.

Frequency is the number of waves per second. The number is measured in Hz (Hertz) or cps (cycles per second).

Waveform characterizes the shape of the electrical wave that can be smooth, sinusoidal, regular, irregular or sharp.

Distribution indicates the part of the brain, which generates the waveform, such as frontal, central and occipital regions. Signals generated by a certain region are expected to present the highest amplitude in this area. [2]

During sleep, cortical and thalamic neurons display bursts of action potentials lasting about 500 ms, followed by periods of hyperpolarization lasting about the same length of time. The synchronization of this firing pattern across many neurons can be illustrated as an electroencephalogram (EEG). EEG measurements were first reported from Caton [3] and Berger [4].

POLYSOMNOGRAPHY

In parallel with the EEG-activity recording, electrooculography (EOG), electromyography (EMG), electrocardiography (ECG), respiration and snoring recordings are also measured. A set of all these recordings (*Figure 4*) is called polysomnography (PSG). [2]

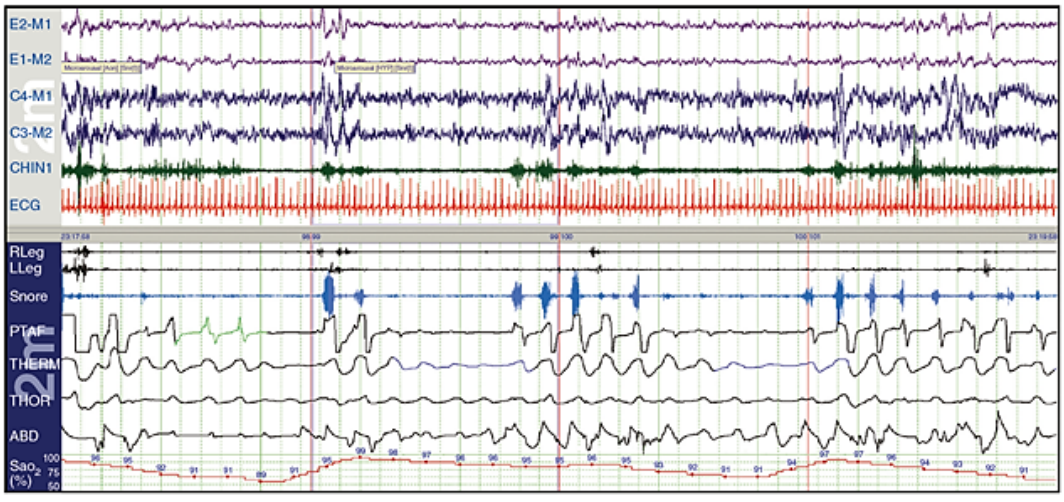

Figure 4. Polysomnography recording: On the left side, upper column (grey) refers to the electrodes that record EEG and face-muscle activity. Different in amplitude, frequency, waveform and distribution waves indicate different sleep state. Lower (blue) column illustrates measurements of the body, such as movements of legs, thoracic activity, snoring, oxygen saturation. [i]

Recording analysis leads to sleep-stages scoring *(Figure 5*), as each stage differs in amplitude, frequency, waveform, and distribution [2]. All trained sleep technicians use the same instructions, rules and terminology when scoring sleep, indicated by "The AASM manual for the Scoring of Sleep and Associated Events". These rules mention the characteristics of each

sleep stages that have universal recognition and usage. The result of sleep scoring provides the individual's sleep-profile. An example is indicated in *Figure 5*, where the sequence and the duration of each stage are illustrated.

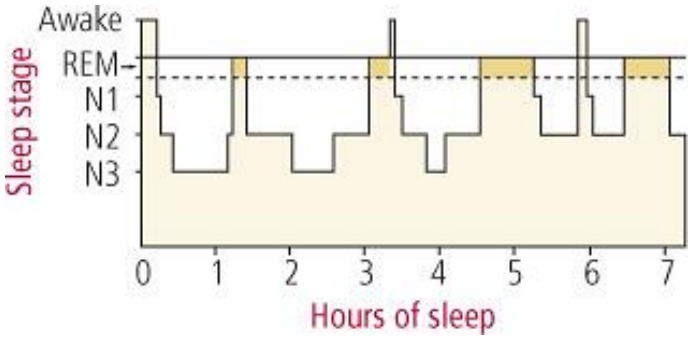

Figure 5. Sequence of sleep stages after polysomnography analysis.[ii]

WRIST ACTIGRAPHY

An additional method for sleep/wake cycle recording is wrist actigraphy, which is a simple and non-invasive measurement of an individual's rest/activity status [5]. Actigraphy devices resemble small electronic watches *(Figure 6*) equipped with accelerometers to measure movements over a period of time. The advantages of this method are:

- ✓ Recording takes place in patient's own environment and
- ✓ Observation can last for many days. [5]

Figure 6. Actigraphy device [iii]

Analysis of actigraphy recordings (*Figure 7*) provides clinicians with valuable information about sleep habits in the patient's natural sleep environment. More specifically, sleep analysis technicians can figure out whether the individual is sleeping or not through the day and what is the duration of their sleep. However, actigraphy cannot indicate abnormalities in sleep architecture. Hence, actigraphy seems to be particularly useful in documenting sleep patterns prior to a polysomnography study, for evaluating circadian rhythm disorders [5].

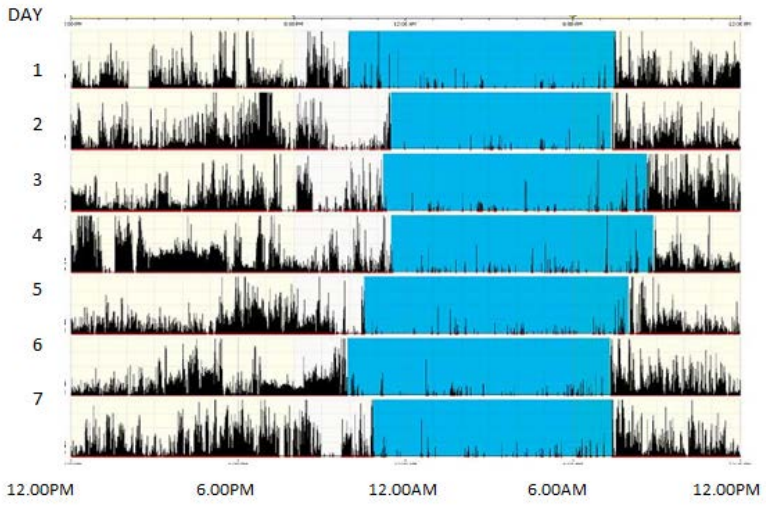

Figure 7. Actigraphy recording: Light blue zones indicate sleep time, where movements (indicated as black waves) decrease [iv].

NREM & REM Characteristics

N1

N1 state is characterized by very light sleep. When an individual switches to N1, he/she has the feeling of soft falling. In fact there is a reduction of muscle activity. The waveforms that can be formed by every cerebral region have a frequency of 4-7 Hz, smooth and sinusoidal morphology and they are called θ rhythm (*Figure 8A*). [2]

N2

N2 is characterized by deeper yet rather light sleep. At this stage the body temperature, the heart rate and breathing pattern decreases. The waveforms are generated from the central and frontal areas and are divided into spindles and K-complexes, respectively. Sleep spindles have a frequency of 11-16 Hz and their form is sinusoidal. On the other hand, K-complexes have a sharp morphology and a frequency less than 2 Hz (*Figure 8B*). [2]

N3

N3, also known as deep sleep, is the restorative phase of sleep. At this stage, breathing is more rhythmic and body repairing is happening. Cerebral signals originate from frontal brain and have a frequency of 0.5- 2 Hz (delta waves). This is why N3 is alternatively named SWS (Slow Wave Sleep). Their waveform varies, thus there is not a characteristic N3 waveform morphology. The duration of N3 increases after intense exercise, as a result of increased cerebral temperature (*Figure 8C*). [2]

Combining all the above mentioned, NREM sleep displays a low EEG activity, where nor eye movements neither dreaming (mental activity) take place. However, during NREM there is emerged muscle tone, resulting from body movements. William Dement, an outstanding sleep physiologist, reports that "NREM is characterized as a state of *an idling brain in a movable body*" [6].

REM

REM (Rapid Eye Movement) is the sleep state where saccadic movements of eyes take place. Its duration is increasing with sleep cycle succession, while dreaming; sexual arousal and heart rate increment are taking place. Brain activity is similar to waking levels (α rhythm: 8-13 Hz) (*Figure 8D*).

More specifically, the EEG pattern becomes sharper, as sawtooth waves (triangular, serrated) of 2-6 Hz are produced from central brain areas (*Figure 8E*). This rhythm is called β and it is present in almost the whole cerebral cortex, except for some parts of PFC, which are "asleep". [2] The role of β rhythm is to "clean the brain", as memory consolidation happens (short term memories turn into long-term). Body movements are absent during REM sleep, so this is a state of "*an awake brain in a paralyzed body*", according to William Dement [6].

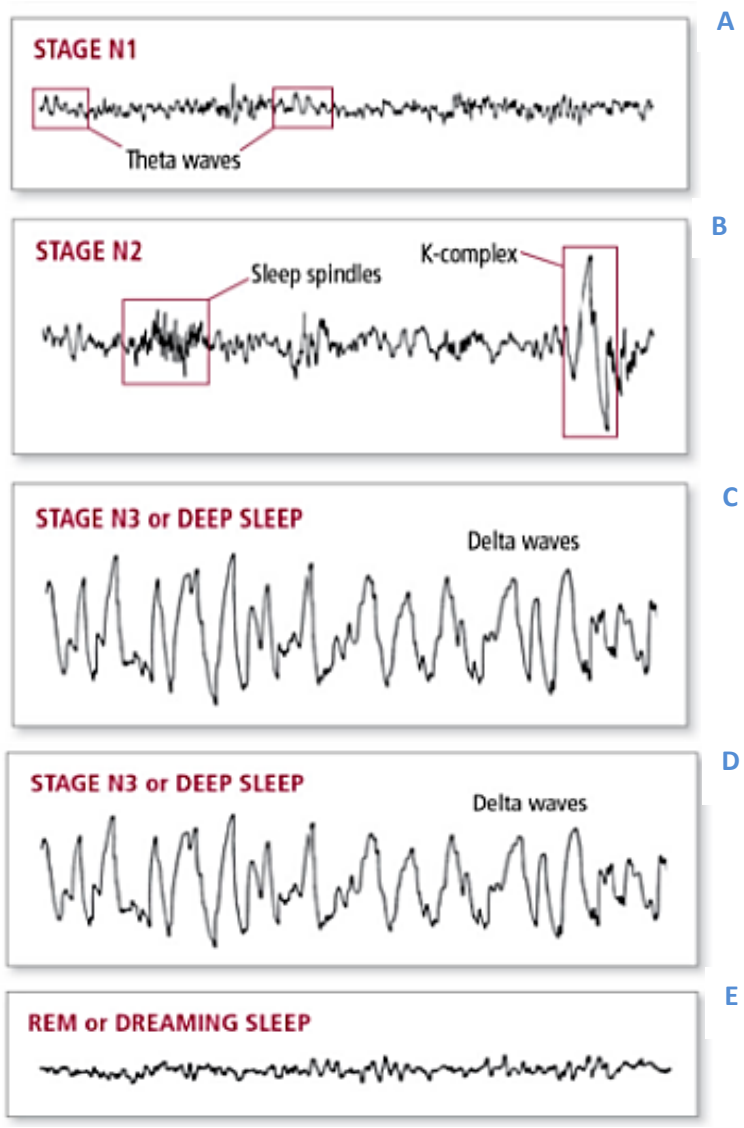

Figure 8 (A-E). EEG wave patterns during sleep. Each sleep stage is characterized by different types of waves [ii]

Sleep Through Lifetime

During deep sleep, growth hormones are released from the pituitary gland, particularly during the first hours of sleeping. Additional hormones whose secretion is coordinated with the advent of sleep are the gonadotropins, which are also secreted by the pituitary gland. Gonadotropins regulate the secretion of sex hormones by the gonads and the appearance of the secondary sex characteristics. The secretion of gonadotropins is not constant, but follows a pulsed release rate instead, occurring every 90 minutes (*Figure 9*). [7, 8]

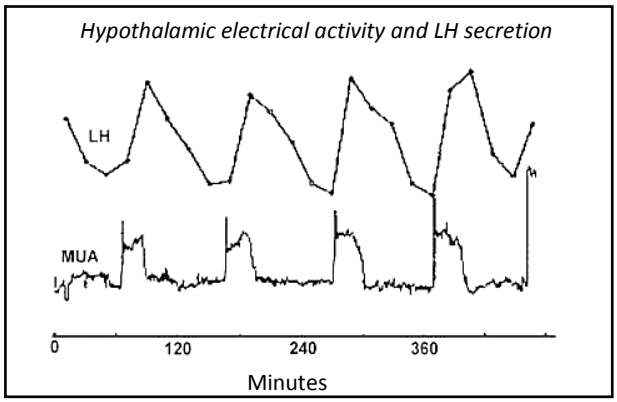

Figure 9. Pulses of LH, the downstream target of GnRH signaling, directly follow episodes of neural activity from the hypothalamus, detected as multiunit activity (MUA) from an implanted electrode. [8, 9]

Sleep does not remain the same throughout lifetime. This is evident from infancy, since infants turn from a loose sleep-wake cycle pattern to a more stable one, as they thrive. Neonatal sleep cycle gradually increases in duration from 30-40 min to 90min. Until then, the synchronization of infant's and parent's sleep-wake cycles does not coincide, resulting in parents' sleep deterioration. Adult-like sleep rhythms are present after the 1st year of life. In the meanwhile, half the sleep time is spend in REM stage. [7, 10]

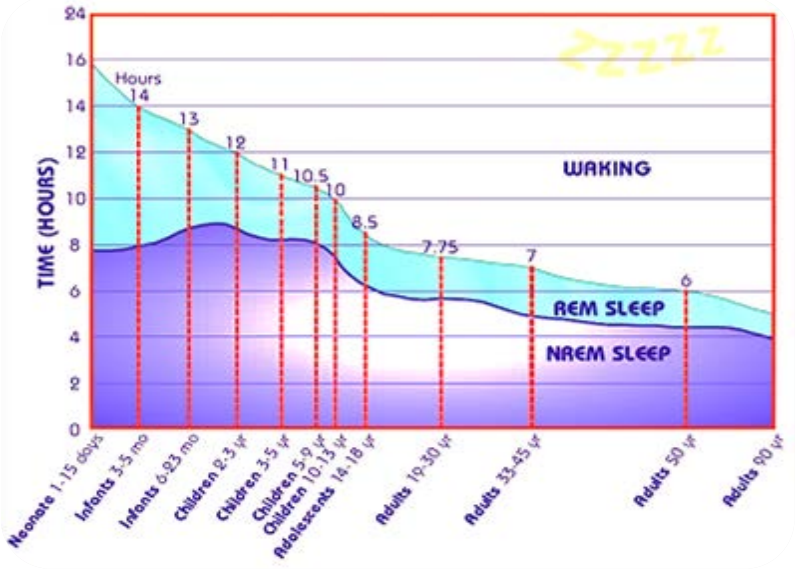

Figure 10. Sleep through aging: As we get older we spent less time sleeping. REM sleep reduces dramatically in elderly, in contrast to infants which enjoy more REM sleep [v].

On the other hand, during adulthood and up to the age of 80, REM sleep usually occupies 20% of total sleep time. Nevertheless, total sleep time per 24h decreases, as we are getting

older (*Figure* 10). Finally, elderly individuals are characterized by a gradual decline of total sleep time and, especially, of restorative sleep (N3) reduction. [7, 10]

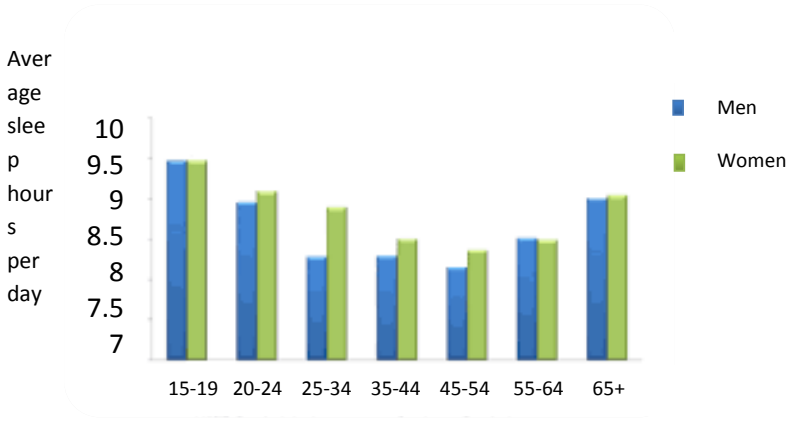

Figure 11. Average sleep time per day, by age range and sex [vi].

Sleep seems to discriminate between sexes too (*Figure 11*), with women of almost all age groups, needing it more [11]. Supporting evidence attribute this phenomenon to the fact that women tend to be more multitask in contrast to men [11]. These pieces of evidence are based on a number of animal studies, which report that longer periods of wakefulness are followed by deeper and/or longer sleep [13-16]

Studies on mice show that aging lengthens the oscillation period (*Figure 12*) and the effect is apparent in a wide range from single-cells to population levels (read also *Functional Profile of SCN Neurons*). However, in the population level, aging attenuates the amplitude of the oscillation and changes the electrical activity, as a result of key genes' expression decline (e.g. *PER2*), and major neurotransmitters' release alterations (e.g. GABA) [17].

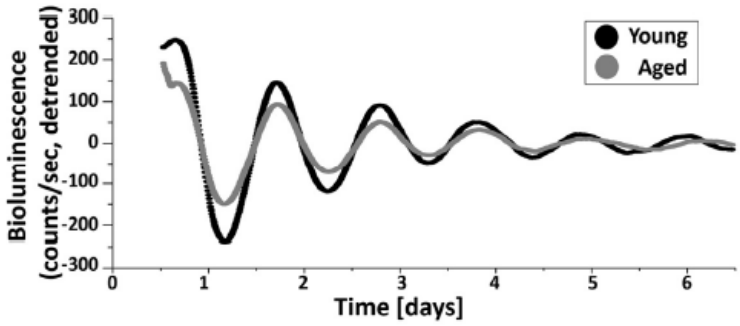

Figure 12. Typical examples of the PER2::LUC rhythm of suprachiasmatic nucleus (SCN) explants from mice maintained under light-dark cycles [17].

Furthermore, aging significantly alters the biological rhythm of expression of many genes, especially in human prefrontal cortex (PFC) [14].This sequence of changes leads to sleep disorders and cognition and nighttime agitation problems in the elderly (*Table 1*) [17, 18].

Table 1. Summary of sleep changes' related to aging.

Increased	Number of awakenings
Increased	Duration of awakenings
Increased	Number of stage shifts
Decreased	Sleep efficiency
Decreased	SWS
Decreased	REM sleep
Decreased	REM latency
Phase advance	Time of sleep

Homeostatic Sleep Regulation

In a sleeping-state, EEG results from synchronized slow oscillations of the membrane potential generated by cortical neurons. These oscillations generate high-amplitude and low-frequency fluctuations, which are characteristic of sleep [13-15, 19]. In contrast, when an individual is awake, EEG consists of low-amplitude and high-frequency fluctuations, as cortical neurons are firing in an irregular pattern [14, 15, 19]. An example of sleep homeostasis is the body's emerged need for sleep recovery after sleep deprivation [20] (read also *Sleep Deprivation*).

Figure 13. Sleep homeostasis is the result of Process C and Process S

Sleep homeostasis is regulated by two separate biological mechanisms; the so-called process C and process S (*Figure 13*) [19].

Process C or Circadian process is sleep-independent and is referred to the rhythmic alterations in an individual's sleep tendency, regulated by cellular circadian oscillators. It is such a fundamental procedure that it is reported even in fly (*Drosophila melanogaster*). [7, 19]

Process S or Sleep process is the increased drive for sleep when an individual remains awake and the declining propensity during sleeping. Process S is directly dependent on

the intensity of activity, as it has been shown in rat studies. Specifically, when the animals were to explore a new environment with many new elements, they activated more neural networks, resulting in longer SWS and EEG recordings. [7, 19]

The above categorization reflects the notion that circadian and sleep processes are separated, but this segregation may not extend to molecular level. On the one hand, some studies support that genes that regulate circadian process (clock genes) are not involved in sleep regulation [21]. On the other hand, the majority of studies indicate that mutations or polymorphisms at clock genes can alter sleep phenotype and cause circadian diseases [21-26], implying that process C is involved in sleep homeostasis.

Biological Rhythms

Biological rhythms are endogenously generated rhythms, which are present in almost every organism (plants, bacteria, animals) [12, 22, 24], whose lifespan is counted in days or greater units, rather than hours that approximate the length of a day and a night [16]. Biological rhythms are separated in:

Ultradian rhythms, which last less than 24h (e.g. REM cycle)
Infradian rhythms, which last more than 24h (e.g. woman's menstrual cycle - 28 days)
Circannial rhythms, which occur yearly or annually (e.g. animals' hibernation and waking pattern)
Circadian rhythms, which are 24h-oscillation of a process (e.g. sleep – wake cycle). Scientists have found approximately 100 circadian rhythms in mammals. [25]

Light is considered to be the most dominant zeitgeber [27], namely it is an important factor that influences the synchronization of the circadian clock to the solar day, tracking of seasonal changes, and sleep regulation [20]. Visible light is a daily stimulus, according to which many organisms have synchronized several of their functions. Human photoreceptors (rods and cones), by activating a number of optic cells, ultimately trigger ganglion cells of the retina. Ganglionic axons conduct the signal to suprachiasmatic nucleus (SCN), a population of neuronal circadian oscillators of the hypothalamus (*Figure 14*). [20] The latter is located in the diencephalon region, below thalamus. SCN also named Master Clock [22], is the core regulator of biological rhythms, as it receives messages from various regions of the nervous system (thalamus, limbic system, neocortex, visual cortex, somatosensory information from brainstem and spinal cord), which it then employs to generate the endogenous biological rhythms. Appropriate integration of these data creates new pieces of information which move from the hypothalamus to distal areas. Therefore, hypothalamus is

- ✓ the headquarter of an organism's functions,
- ✓ responsible for the secretion of many hormones,
- ✓ regulating metabolic and developmental processes,
- ✓ thus connects the CNS with the endocrine system and
- ✓ connected to ANS, PNS and limbic system. [28]

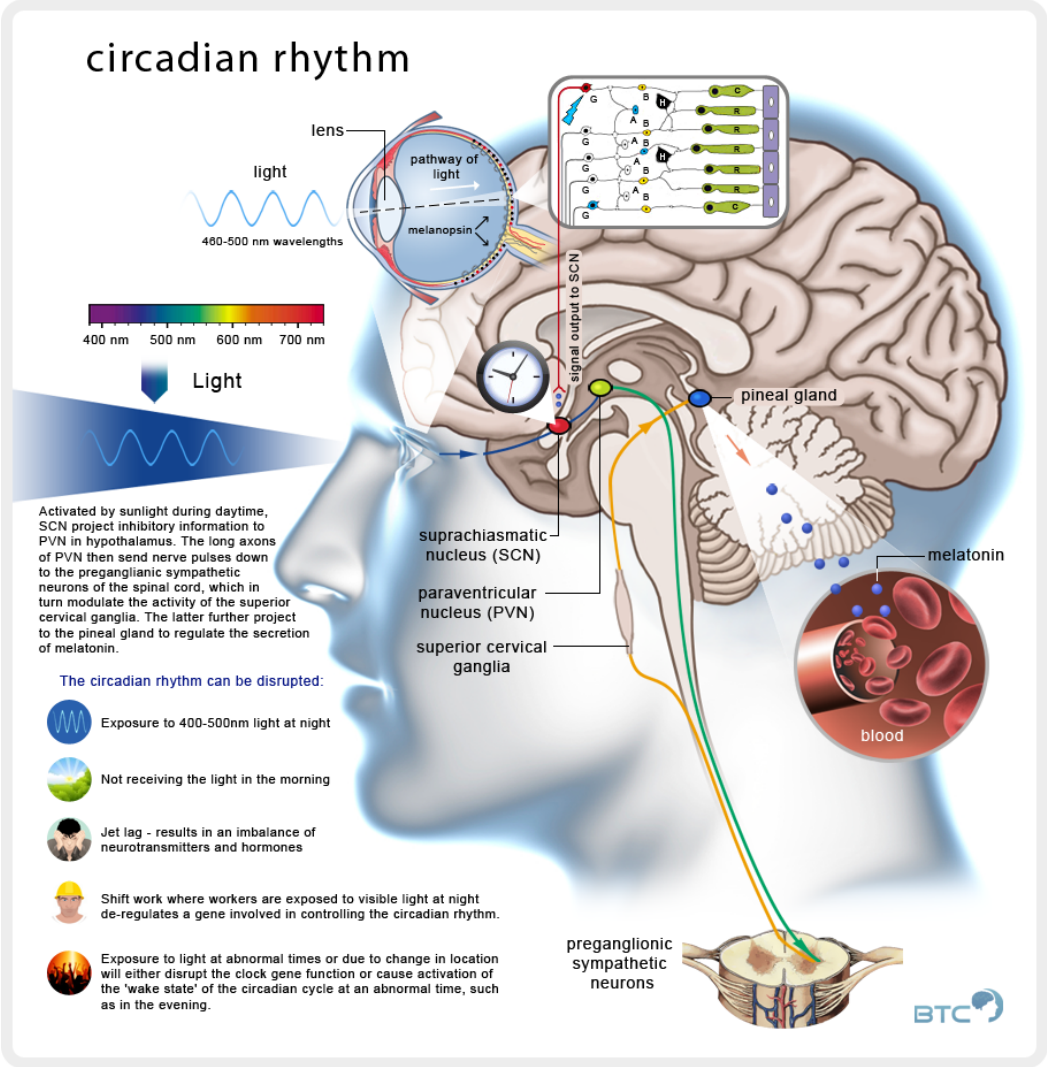

Figure 14. Path of circadian modulation through light: Light activates photoreceptors. Their response is transferred to SCN, which regulates peripheral clocks of the organism, regarding day time and season [vii].

SCN drives rhythms, through environmental light-dark (LD) cycles and photosensitive retinal ganglion cells [17]. These senses participate in organizing temporal architecture of the organism [22]. Apart from the sleep-wake cycle adjustment, SCN is involved in other timetable-related procedures, such as setting meal scheduling, regulating physiological activities and peripheral clocks in different tissues (adipose tissue for lipogenesis and lipolysis, liver for hepatic gluconeogenesis and gastrointestinal functionality etc.).[22] Circadian mechanism has also been attributed other functions, which emphasize its pleiotropic role (*Figure 15*), such as metabolism, glucose homeostasis, cortisol concentration, melatonin concentration, tissue regeneration, bone formation, menstrual cycle and cancer. [22, 23, 25, 29]

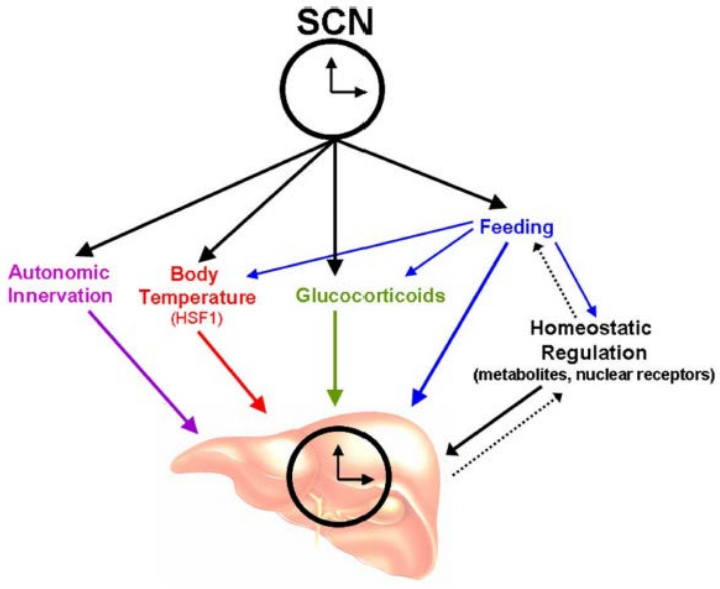

Figure 15. Pathways of peripheral clock entrainment: The master circadian pacemaker within the SCN relays temporal information to peripheral oscillators through autonomic innervation, body temperature, humoral signals (such as glucocorticoids), and feeding-related cues. Local signaling pathways can also affect peripheral oscillators independently from the SCN [22].

Light can also act as mood regulator [20]. Studies in animal models report that circadian behaviour is the result of an integrated system. This system reflects the activation of circadian mechanism by light- stimulus that results in behavioral outputs. [22] Mood modulation can occur indirectly, via sleep and circadian rhythms' regulation, or directly, without sleep and circadian alterations (*Figure 16*). Mood is one of the basic factors that affect learning procedure, while the obtained information is consolidated during sleeping, as explained above. [20]

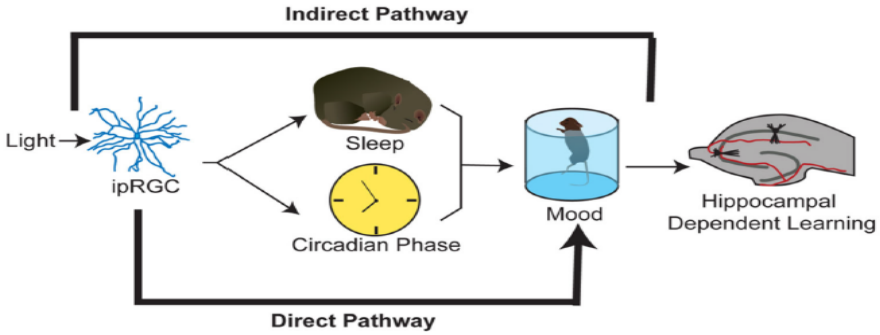

Figure 16. Light as a mood regulator: Both pathways, direct and indirect are illustrated. Each of them results in mood modulation, which, among other roles, it is important for learning processes [20].

Circadian Mechanism & Mood

There are suggestions that circadian rhythms result from the interaction between physiological and psychological processes. The psychological state of a person is considered as mood fluctuations caused from a combination of endogenous and exogenous factors. This type of fluctuations is known to be related with the dopaminergic (DAergic) system, where areas of the limbic brain participate. DAergic system is one of the basic elements, which is impaired, when affecting disorders are present. [30, 31] Scientists recently revealed that circadian nuclear receptors, REV-ERBα are regulators of dopamine (DA) secretion [30].

According to Chung et.al 2014 REV-ERBα may mediate the circadian control of DA biosynthesis through transcriptional regulation of TH gene (*Figure 17*). This molecular connection between circadian mechanism and one of core systems for mood regulation indicates an opportunity for further research, possibly revealing new treatment targets for circadian-related affecting disorders, such as major depressive disorder (MDD), bipolar disorder (BPD), and seasonal affective disorder (SAD) (see sequencing field). Additionally, experiments in mice- report that ClockΔ19 defective allele is associated with maniac-like behavior, as well as with drug addiction-phenotype [30]. Nowadays, literature is rich in evidence that connect elements of DAergic mechanism to clock genes [31-33]. From the psychiatric perspective, this is an initial step towards the molecular explanation of "why patients of affecting disorders have impaired sleep".

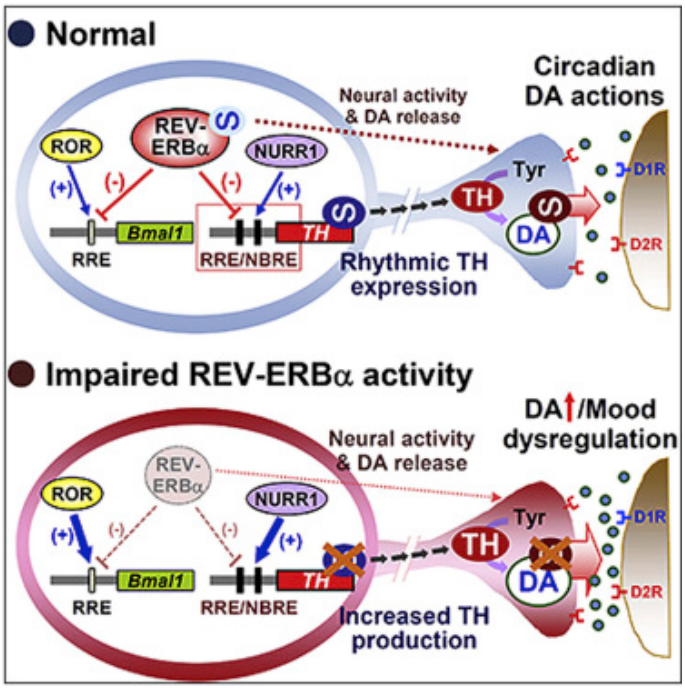

Figure 17. Hypothetical model for rhythmic TH gene expression in DAergic neurons and its relevance to circadian mood regulation. *Normal: REV-ERBα* impacts midbrain dopamine production and mood-related behavior in mice. Also, *REV-ERBα* repressed tyrosine hydroxylase (*TH*) gene transcription via competition with nuclear receptor-related 1 protein (NURR1), another nuclear receptor crucial for dopaminergic neuronal function, thereby driving circadian TH expression through a target-dependent antagonistic mechanism. *Impaired:* Genetic deletion of the *REV-ERBα* gene or pharmacological inhibition of REV-ERBα activity in the ventral midbrain induced mania-like behavior in association with a central hyperdopaminergic state. [30]

Functional Profile of SCN Neurons

Rhythmicity of SCN clock is the result of functional synchronization of hypothalamic neurons. In a single-cell level, SCN neurons exhibit cell autonomous circadian periods that vary from 22 to 30 hours [22]. These periods are high-amplitude and low-frequency oscillations, which imply SWS state [14]. Electrophysiological recordings during SWS, in both intracellular and extracellular level, revealed two separated phases:

- ✓ Depolarizing phase of activated cells, which are bursting and
- ✓ Hyperpolarized phase, where cells remain in a resting state after their activation. [19]

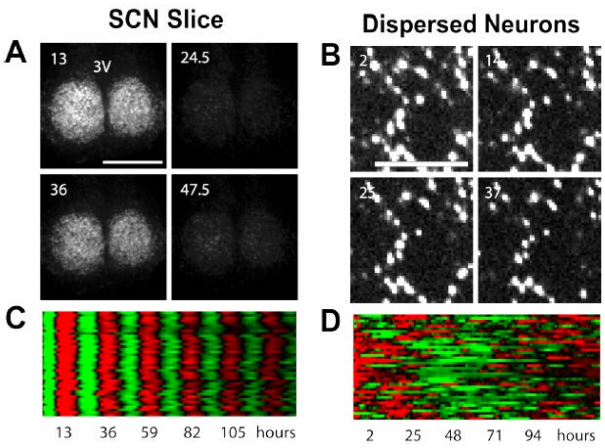

Figure 18. Network and autonomous properties of SCN neurons: Network properties of the SCN can compensate for genetic defects affecting rhythmicity at the cell autonomous level. (A) Bioluminescence images of a Cry1−/− SCN in organotypic slice culture. Note the stable, synchronized oscillations. Numbers indicate hours after start of imaging; 3V indicates the 3rd ventricle. (B) Bioluminescence images of dissociated individual Cry1−/− SCN neurons showing cell-autonomous, largely arrhythmic patterns of high bioluminescence intensity. (C and D) Heatmap representations of bioluminescence intensity of individual Cry1−/− neurons in SCN slice (A) and dispersed culture (B)[22].

24h oscillation results from intercellular bursting coupling among SCN neurons. When the entire SCN population is activated concurrently, circadian period gets narrower, as shown by measurements of animal models' locomotor activity rhythm (*Figure 18*) [6]. Network synchronization transforms mono-cellular heterogeneity in a synchronized intrinsic period indicating processes of phase lability and phase plasticity. In the same research it is reported that cells (fibroblasts or isolated SCN neurons) characterized by *PER* (read also *PER genes*) and *CRY* (read also *CRY* genes) loss-of-function mutations act in a cell autonomous way, which generates arrhythmic oscillations. Intercellular coupling leads to phenotype rescue, albeit their mutant genome [22]. It is ultimately becoming clear that network properties affect rhythmicity at a cell autonomous level. [22, 13-15]

Phase lability refers to the rhythm phases of individual SCN cells that are highly stereotyped and appear as a wave that spreads across the nucleus over time. This function generates cell oscillations that may be of either short period (early phases) or long period (late phases).

Phase plasticity is reflected under different photoperiods, where SCN population displays different waveforms. Therefore, at short photoperiods (e.g. winter) amplitude of SCN oscillation is narrow and high. On the other hand, in long photoperiods (e.g. summer) oscillation waveforms are broad and of low amplitude. [22]

Neurotransmitters of Sleep & Wakefulness

Since sleep is the product of a series of events that occur in the brain, neurotransmitters (NTs) could not be missing from its regulation (*Figure 19*). Biogenic amines, such as dopamine (DA), noradrenaline (NE) and serotonin (5-HT), are the most critical NTs in sleep regulation. Onset of sleep is marked by 5-HT action [34]. The advent of REM sleep is the result of the interaction of two neurotransmitters; increasing acetylcholine (ACh) [35] and decreasing norepinephrine (NE) [36]. The ACh/NE ratio is decisive for REM onset during sleep. In patients with Alzheimer's disease (AD), where cholinergic neurons are impaired, sleep-wake cycle, especially REM onset seems to be disrupted [37]. Furthermore, adenosine

seems to be a promoter of NREM sleep. When caffeine is binding in adenosine receptors, it antagonizes adenosine's action, and as a result it blocks the advent of NREM sleep and it promotes wakefulness. [38] Moreover, DA is a core endogenous activator of wakefulness, while orexin and histamine belong to secondary wakefulness factors [39].

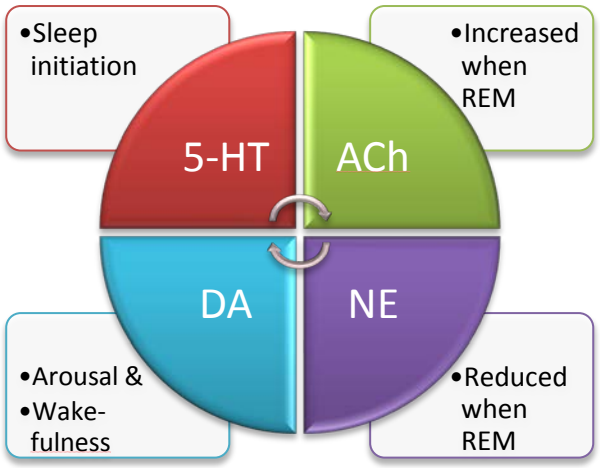

Figure 19. Sleep and wakefulness cycle is the result of different NTs activation.

The "sleep-wake" cycle regulation is a complex process involving a variety of molecules and several brain areas (*Figure 20*). However, the relationship between brain region and neurotransmitter's role in sleep is not absolute. GABA, a basic and mostly inhibitory brain neurotransmitter, has different effects in sleep regulation, depending on the region where is secreted. Thus, GABA promotes sleep when acting in the posterior hypothalamus but it promotes awakening when acting in the pontine reticular formation. [39]

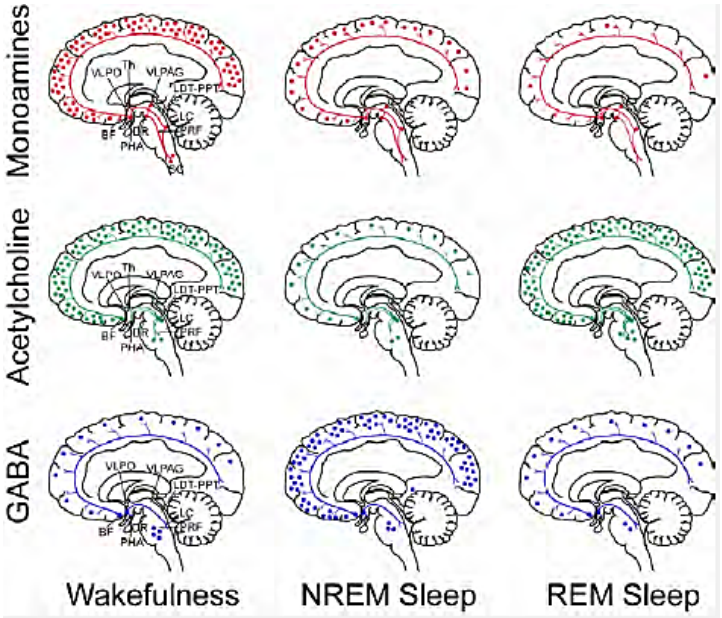

Figure 20. State-specific transmitter release in sleep and wakefulness. Dot densityis proportional to the quantity of an activated neurotrasmitter in the coloured regions for each specific state (Wake, NREM,REM) [40].

Sleep Duration & Brain Calcium

Recently, studies revealed that calcium concentration in mammalian brain controls sleep duration (*Figure 21*). It was specifically reported that SWS is regulated by neural calcium-dependent activity. Indeed, genes involved in calcium hyperpolarization pathway seem to participate in sleep regulation. When these genes were downregulated, using CRISPR, Ca^{+2} hyperpolarization was impaired, and as a result sleep duration was either increased or reduced. Also, on the same research, scientists show that impairment of NMDA receptors' functionality increases of neural excitability, in a way that disrupts functionality of kinases, which play major role in calcium-dependent pathways. This led to elimination of SWS. Thus, process S might be associated with the Ca^{2+}-dependent hyperpolarization pathway. [19] All the above indicate new therapeutical targets for sleep and neurological disorders. Extensive research towards the direction of molecular mechanisms underlying sleep is therefore becoming more and more critical, while new findings pave the way.

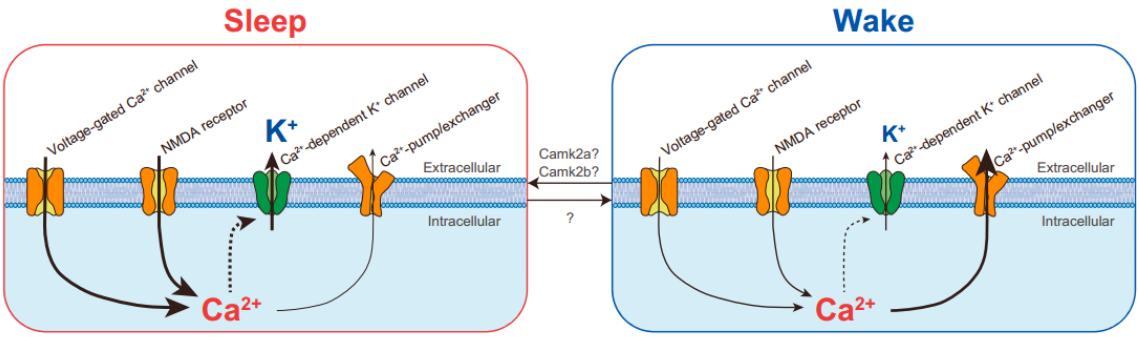

Figure 21. Molecular components of the Ca^{2+}-dependent hyperpolarization pathway are potential molecular targets of the homeostatic sleep regulators. During sleep (left) NMDA receptors and Voltage-gated Ca^{2+} channels are open, so increased amount of Ca^{2+} enters the cell. Wakefulness (right), is an opposite process, in which Ca^{2+} moves to the extracellular environment through Ca^{2+}-pumps. [19]

Sleep Regulation & Melatonin

Melatonin, a hormone released by the pineal gland of diencephalon, has repeatedly been associated with sleep. In fact, melatonin is mainly associated with the feeling of drowsiness than sleep *per ce* [7]. Melatonin secretion is regulated by light stimulus, as it is sensed by retinal receptors [20]. As a result, melatonin release takes place during the night, but not during the day (*Figure 22*). Usually, melatonin secretion is disturbed in transatlantic trips, resulting in passengers' jet lag (when internal clock is not adjusted to environmental clock). Pharmaceutical use of melatonin is a solution to this problem as well as to some psychiatric disorders such as SAD. Melatonin acts on SCN receptors and it mediates phase-advance of the biological clock, but its effectiveness is time-dependent as melatonin receptors have their own circadian rhythm. [6, 7, 20]

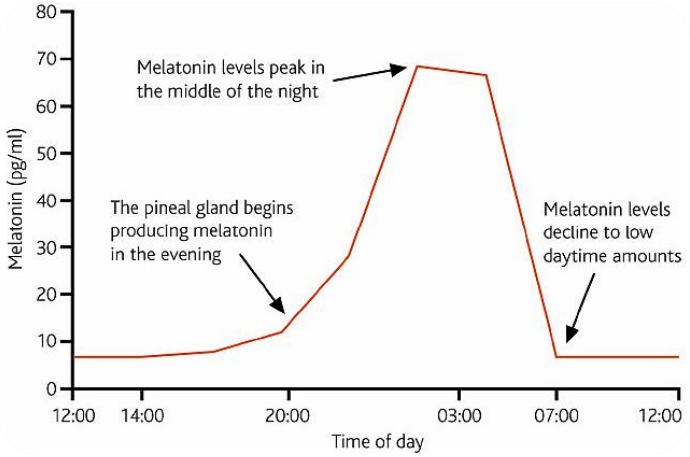

Figure 22. 24h rhythm of melatonin secretion [viii].

Sleep Deprivation

"Sleep deprivation" refers to the state where an individual lacks sleep and, accordingly, its beneficial effects. Cerebral cortex suffers the most from sleep deprivation effects, but it is capable to cope with it for a short period. The rest of the body remains unaffected, because physical restitution of the body is compensated from immobility, which is secondarily dictated by sleep. Only 1/3 of lost sleep can be restored the following day (80% of lost N3 and ½ of lost REM). [41, 42]

Even in cases where sleep duration is decreased to less than 5h, the person's functionality can be significantly impaired[42,43]. A common observation for sleep deprivation implies that when the awakening time is advanced, then N3 is increased at the first part of sleep cycle while REM duration is decreased. Loss of REM sleep leads to inability for learning and performing complex tasks. Moreover, biochemical alterations (blood pressure, glucose levels etc.), mood changes, PFC activity decrement, amygdala activity increase, cellular stress and subsequent downregulation of metabolic processes, complete the sleep deprived phenotype. [41, 42]

Genetics of Circadian Clock

Circadian clock is an internal molecular mechanism of activated clock genes, which is activated every ~24h. This mechanism is well-conserved among species (e.g. photosynthesis in plants) [23]. Actually, it comprises of an auto-regulatory transcriptional network relying on negative feedback [22, 44], indicating that each cell is autonomous and has its own slave oscillators. Circadian clock mechanism controls the rhythmic rise/ fall of gene expression in regions outside of SCN (*Figure 23*) [44]. In early developmental stages, this mechanism is more sensitive to disruption, compared to adulthood. Clock genes participating in this mechanism; belong to a renewable gene list, while many of them are present in organisms beyond mammals [22, 23, 44]. In mammals, the most common clock genes which participate in the circadian mechanism are separated in:

Positive transcriptional activators: *CLOCK, BMAL1 (ARNTL)*
Negative transcriptional regulators: PER1/2/3, CRY1/2
Clock genes' targets: *REV1, NR1D1, RORA, CK1δ/ε, NPAS2, RORB, BHLHE40 (DEC1), BHLHE41 (DEC2)* [17, 18, 22, 23, 44-47]

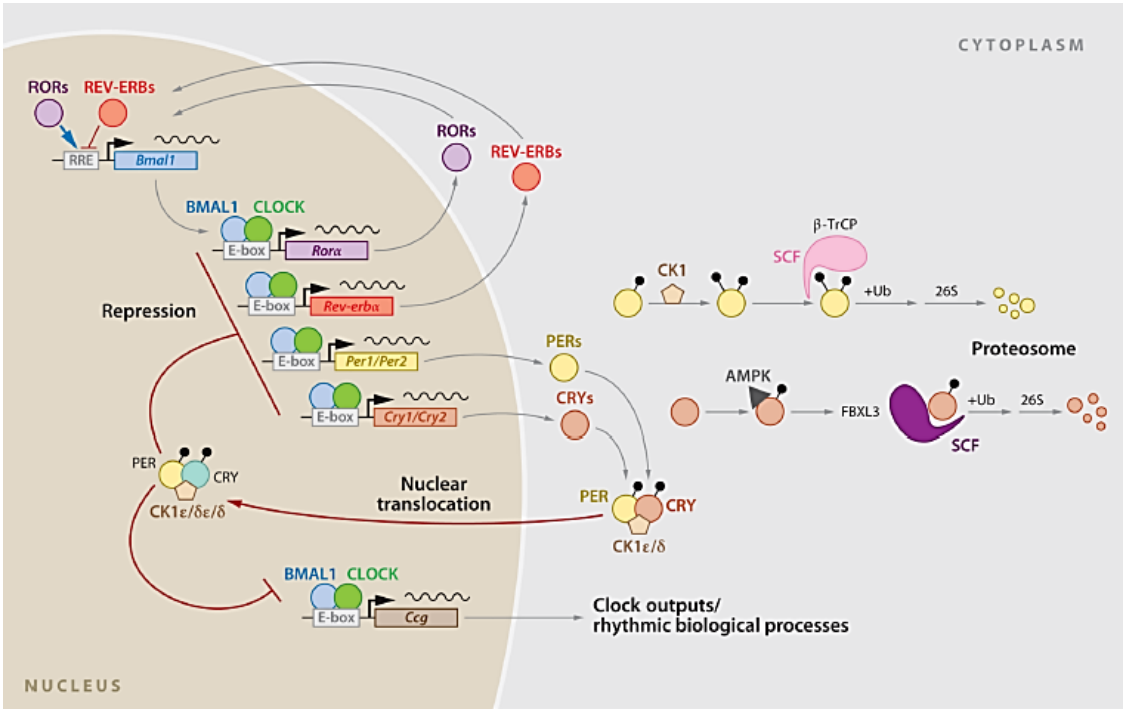

Figure 23. The molecular mechanism of the circadian clock in mammals. An autoregulatory transcriptional feedback loop involving the activators *CLOCK* and *BMAL1*, and their target genes, *PER1, PER2, CRY1* and *CRY2*, whose products form a negative feedback repressor complex, constitute the core circadian clock mechanism. In addition to this core transcriptional feedback loop, there are other feedback loops driven by CLOCK: BMAL1. One feedback loop involving Rev-erbα and Rorα that represses *BMAL1* transcription leads to an antiphase oscillation in *BMAL1* gene expression. *CLOCK: BMAL1* also regulates many downstream target genes, known as clock-controlled genes (Ccg). At the post-transcriptional level, the stability of Per and Cry proteins is regulated by SCF (Skp1-Cullin-F-box protein) - E3 ubiquitin ligase complexes involving β-TrCP and FBXL3, respectively. Casein kinase 1ε/δ (CK1ε/δ) and AMP kinase (AMPK) phosphorylate the Per and Cry proteins, respectively, to promote poly-ubiquitination by their respective E3 ubiquitin ligase complexes, which in turn tag the Per and Cry proteins for degradation by the 26S proteasome complex. [22]

Clock proteins contain one or more PAS signal-sensor protein domains and are differentiated in:

a- class proteins: Per, Npas2 and Clock proteins that act as sensors of environmental and developmental signals (oxygen, redox state, voltage or light)

b- class proteins: Bmal1 protein, which acts as dimerization partner [21]

The list of clock genes is continuously updated as research in sleep and its mechanism is prosperous. Up to now, more than 20 genes with circadian role are recognized.

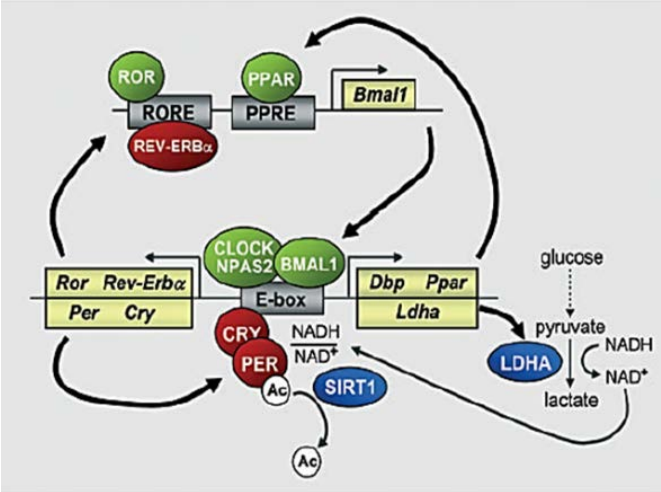

Figure 24. Schematic representation of clock genes' interactions with cellular circadian oscillations. Note that metabolic reactions are also related with circadian mechanism and gene interactions [21].

At the molecular level, energy regulation is mediated by *LDHA* (lactate dehydrogenase a) activation, another *CLOCK/NPAS2* target gene (*Figure 24*). Ldha protein can alter the NADH/NAD$^+$ ratio, affecting intracellular energy. Furthermore, changes in NADH/NAD$^+$ ratio can directly affect *CLOCK*- and *NPAS2*-mediated transcriptional activation and, accordingly, basic elements of circadian mechanism. [21]

CLOCK

Circadian Locomotor Output Cycles Kaput (*CLOCK*) gene is one of major circadian mechanism genes. It is located on 4q12 chromosomal position and its size is 119,238 base pairs [ix]. *CLOCK* product is a basic helix-loop helix transcriptional factor of 846 a.a [ix,20]. In mammals, *CLOCK* is mainly expressed in the SCN and the supraoptic nucleus (SON) of hypothalamus and the cerebellum; in a stable rate, even in sustained darkness [25]. In cooperation with *NPAS2* gene (*Figure 23*), they constitute the core regulatory mechanism of the sleep-wake cycles in mammals and birds. Also, *CLOCK* and *NPAS2* have been associated with timing of sleep onset and offset, as well as sleep duration. [26] A study about *CLOCK* mutant mice revealed that in addition to their disrupted circadian rhythms, these mice presented hyperphagia [24]. As a result, they developed obesity and metabolic syndromes, indicative of their impaired energy balance. This conclusion underlines the central role of clock protein to food intake programming [24].

NPAS2

Neuronal PAS Domain Protein 2 (*NPAS2*) gene, a paralog of *CLOCK* [48], also belongs to the core circadian mechanism (*Figure 23*). *NPAS2* is located on chromosome 2q11.2 and comprises of 176,679 base pairs, that produce a 824 a.a. long protein [ix]. Npas2 forms heterodimers with Clock and Bmal1 proteins [21]. These complexes regulate the expression of 10% of all genome [49], via circadian mechanism. *NPAS2* was firstly identified in the forebrain [21, 49], but nowadays it is also known to be expressed in key metabolic peripheral tissues, such as liver [48, 49]. Binding efficiency of Npas2 to Bmal1 or Clock is an intrinsic regulatory mechanism for the functionality of negative feedback loop. [49] The neuroanatomical distribution of Npas2 is particularly suitable for fulfilling such a function as it is abundantly expressed in the brain with highest expression being detected in thalami nuclei and cerebral cortex, while no noticeable expression is evident in the SCN [21].

In KO studies of *NPAS2* gene, it was showed that there are no severe consequences on behavioral rhythms in mice. However, in double-knockouts of *NPAS2* and *CLOCK*, scientists reported total behavioral arrhythmicity, which would normally be regulated by clock mechanism. Interestingly, this effect seems to be present only in SCN and in the forebrain. Also, studies in peripheral tissues of *CLOCK*$^{-/-}$ mice with suppressed *NPAS2*, revealed circadian arrhythmicity [48]. On the other hand, in CLOCK$^{-/-}$ peripheral cells and tissues, where *NPAS2* is expressed, autonomous circadian rhythms are unimpaired [22]. Therefore, *NPAS2* is able to compensate for the loss of *CLOCK* by rescuing circadian rhythmicity in peripheral cells as well as the SCN [48].

BMAL1 or ARNTL1

Aryl hydrocarbon Receptor Nuclear Translocator-Like1 (*ARNTL1* or *BMAL1*) [17] is another key clock gene [29, 44], located on 11p15.3 chromosomal region [ix]. Its size is 110,615 base pairs, while its product size is 626 a.a. [ix]. *BMAL1* activation follows a 24h rhythm and it takes place in the suprachiasmatic nucleus, hippocampus and neocortex [29]. Bmal1 protein associates with either Clock or Npas2 to form transcriptionally active heterodimers (*Figure 24*). These dimers affect clock machinery by regulating the transcription of Period (*PER* 1, 2) and Cryptochrome (*CRY* 1, 2) genes. [21] In a study where *BMAL1* KO mice where used, it was reported that loss of *BMAL1* gene leads to behavioral deregulation [22]. Regarding protein levels, peripheral blood tissue measurements indicate that in healthy individuals Bmal1 is mostly increased at 8:00 PM [45].

PER genes

Period (*PER*) genes constitute a family of genes (*PER1, PER2, PER3*) that encode proteins with a negative regulatory role in circadian mechanism. Their expression in the forebrain has been positively correlated with sleep need [21].

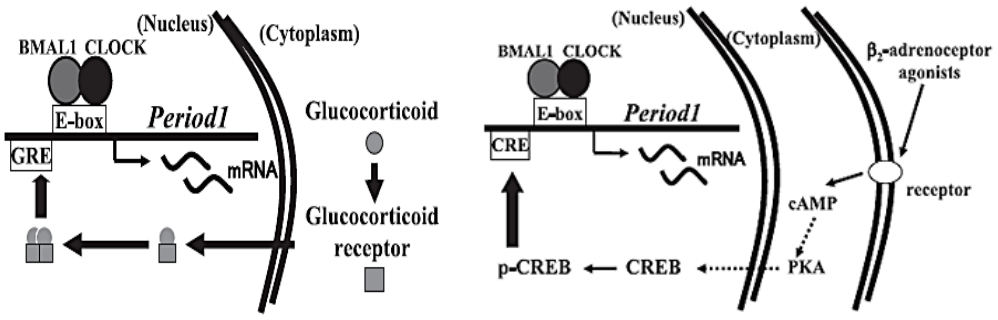

Figure 25. Signaling pathway leading to *PER1* expression via (left) glucocorticoid response element (GRE) and (right) cAMP response element (CRE) [50].

PER1 lies on 17p13.1 chromosomal region and consists of 16,037 base pairs; while Per1 protein comprises of 1290 a.a. [ix].*PER1* has both a glucocorticoid response element (GRE) and a cAMP response element (CRE) in its 5'-upstream sequence *(Figure 25)*. These regions are crucial for its activation mechanism induced by light [50] and by glucocorticoids, IL-6 and a-/b- adrenoceptor agonists [51]. It is noteworthy that light induces Per1 mRNA levels increment [25], and as a result *PER1* expression peaks 0–2 h after sunrise [44]. This expression pattern is reported in both central biological clock regions and in peripheral tissues. Peripheral tissues are vulnerable to *PER1* expression changes because of noradrenaline action. [51] Noradrenaline is a neurotransmitter of the central and peripheral

nervous system. Its peripheral action is mainly during awakening to promote fight or flight behaviour. Noradrenaline levels decrease when the individual is asleep [28].

Period 2 (*PER2*) gene lies on chromosomal position 2q37.3. This sequence of 46,065 base pairs generates a protein product of 1255 a.a. [ix] Per2 induction occurs in response to light [50], causing acute increase in Per2 mRNA levels [25], which peak in the afternoon (*Figure 26)* [44]. Per2 protein acts as a positive regulator of *BMAL1* gene expression, affecting the positive feedback loop of circadian machinery [24].

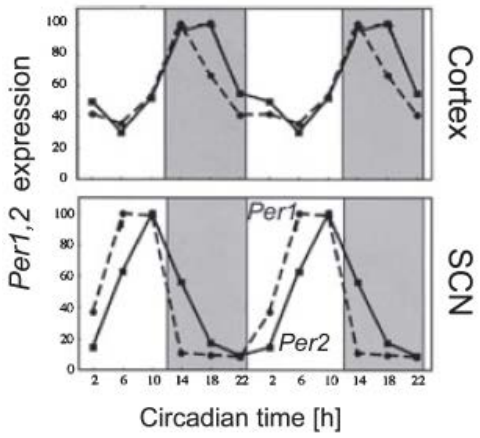

Figure 26. The expression of the circadian clock genes Per1 and Per2 in forebrain areas is driven by the sleep–wake distribution while in the SCN their expression follows a circadian oscillation which is independent from the animal's overt behavioral activity. In the nocturnal mouse Per1 (dashed line) and Per2 (solid line) expression in the SCN peaks in the mid-portion of the subjective light period while in the cerebral cortex Per mRNA levels are highest during the subjective dark phase (grey areas) when animals are awake and active (adopted from Abe et al., 2001). [21]

PER3 gene (60,862 base pairs), located on 1p36.23 chromosomal region, produces a 1201a.a. [ix] protein that forms complexes with Per1/2 and Cry1/2 proteins. These complexes enter the nucleus and suppress the transcription induced by the Bmal1/Clock complex. Per3 levels peak in the interval between Per1 and Per2 peaks (*Figure 26*). [44] *PER3* gene is associated with sleep homeostasis. However, in *PER3* KO studies it was reported that *PER3* activation has a weaker effect on circadian rhythmicity than *PER1* and *PER2*, indicating that *PER3* may act independently [25].

CRY genes

The family of Cryptochrome (*CRY*) genes consists of *CRY1* and *CRY2*. *CRY1* is located on chromosome 12q23.3, while *CRY2* on 11p11.2 [ix]. *CRY1*, a 102,494 base pair sequence, encodes a protein of 586a.a, which acts as inhibitor of its own translation (*Figure 24*). Similarly acts Cry2 protein, which is a 593a.a. peptide. Both are negative regulators of circadian mechanism (*Figure 23*), by making complexes with Pers to form the core transcriptional feedback loop [22]. This loop associates with Clock: Bmal1 and Npas2:Bmal1 dimers and inhibits their transcriptional activation effect on downstream targets (*Figure 24*) such as *ROE, LDHA, REV1* and *NR1D1* genes [21]

VDR

VDR (Vitamin D Receptor) gene is a non-clock gene. However, in the literature vitamin D has been related to many pathological states. Recently, scientists report that vitamin D could repair nerve damage in Multiple Sclerosis, indicating a connection between nervous system

and light-dependent molecules [52]. Cretan population seems to have deficiency in vitamin D, as indicated by studies performed in the Sleep Medicine Laboratory of University of Crete. Notably, this deficiency seems to be more evident in individuals with sleep disorders. Thus, we suspect that vitamin D shortage might be one of the reasons that can cause dysfunction of circadian mechanisms.

Clock Genes & Their Mutations

According to universal assumptions, when a gene variant is present in at least 1% of a population, it is characterized as genetic polymorphism, contrasting rarer alterations, named mutations. Polymorphisms generally refer to small regions of the genome or even to a single nucleotide, in which case they are called single nucleotide polymorphisms, or SNPs. A variety of SNPs have been detected in clock genes and have been associated with a numerous of circadian diseases or other sleep characteristics. Clock gene-polymorphisms in combination with sex and age parameters, can affect the individual's sleep phenotype, regarding aspects of sleep depth, duration of sleep, chronotype [24], and sleep architecture (*Table 2)*. Hence, people are genetically programmed to adopt certain sleep-wake patterns and they seem to sleep and wake up in certain periods of a day [24]. This programming can determine people's working hours, diet, as well as their everyday habits.

It has been reported that mutations in clock genes lead to desynchronization of SCN and peripheral clocks. These mutations decrease the amplitude of molecular oscillations, by increasing the sensitivity to light-and temperature-induced phase shifts. The light-phase shift is not dependent on the strength of the light signal that activates the clock mechanisms. [22] So, the functional alteration in circadian mechanism can cause some of the most common effects of clock-desynchronization, which are mal-adaption of sleep- wake cycle and development of metabolic syndromes [23, 47].

Notably, *PER* or *CRY* single knockouts are thought to alter the circadian period, while functional double KO of *PER1-2* or *Cry1-2* abolish circadian rhythmicity [24]. Furthermore, *BMAL1* and *CLOCK* KO result in behavioral circadian disorganization. Same effects have been reported in are *NPAS2* KO mice. [21]

Although, *PER3* variants are not rare, they usually have weak effects on circadian rhythmicity, by shortening circadian period [24]. Also, a VNTR *PER3* polymorphism has been associated with diurnal chronotype and delayed sleep phase syndrome (DSPS) (read also *Sleep & Whole Exome Sequencing*) [21].

Chronotype is separated to "morningness" (M) or "eveningness" (E). Morningness is when the individual prefers to sleep early in the night and wakes up early in the morning, while eveningness characterizes those people who have opposite preferences.

Table 2. Reported relationship between the clock-variations and human circadian rhythm phenotypes [23].

Clock gene	Circadian phenotype	Association
CLOCK	M-E	+
Per1	M-E	–
Timeless	M-E	–
Per2	FASPS	+
Per3	DSPS	+
CLOCK	DSPS	+?
CLOCK	M-E	–
NPAS2, Per3	Seasonal affective disorder, M-E	+
Per2	FASPS	–
Per3	DSPS, M-E	+
CK1ε	DSPS	+
CK1δ	FASPS	+
Per2	M-E	+

Sleep & Whole Exome Sequencing

Whole Exome Sequencing (WES) is a powerful genetic tool of Next-generation sequencing (NGS) that can be used to analyze an individual's exome in order to identify variants, which might be linked to disease phenotypes. WES output provides base-to-base information about all the coding regions (exons from "EXpressed regiONS") of the expressed genes (exome) of an organism, and also about intron-exon boundaries. Human genome consists of 180,000 exons, which essentially comprise 1% of the total human DNA (approximately 30millions base pairs), yet approximately 90% of all disease-associated variants lie within this small portion of the genome. [53]

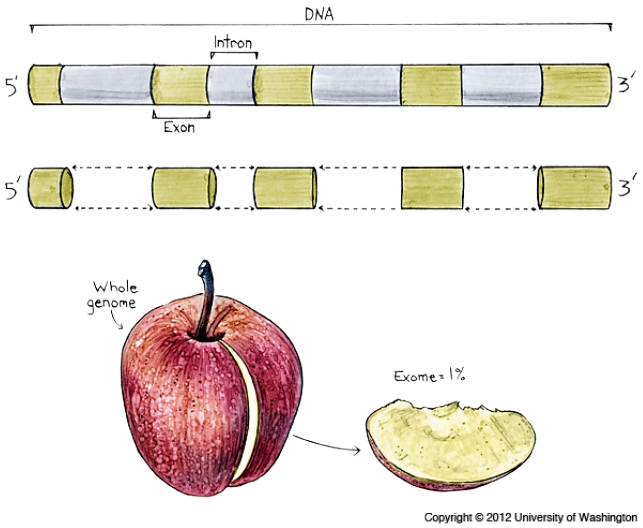

Figure 27. Whole exome is only 1% of whole genome. Exons contain crucial information for organism's functionality [x]

Common variations across the genome can be easily identified through WES. However, a variety of novel variants have been proposed as disease-causing candidates. These variants are usually reported as rare variants, as they are found in lower allele frequencies, due to negative selection pressure over the evolution. Nevertheless, WES can also detect disease associated genetic factors which confer risk in the development of complex disorders [53].

A competitive method for genome analysis is Genome Wide Association Study (GWAS). GWAS analyzes whole genome, in order to examine the whole spectrum of genetic variations, even within intronic and intergenic regions, in different individuals [54]. GWAS is also applied to associate these variants with phenotype traits, which are commonly present in major multifactorial diseases [55]. However, on one hand GWAS is much more costly than WES and on the other hand it reveals a chaotic volume of information which is accordingly laborious to interpret. Hence, GWAS is not as efficient in specifying which gene-variation is causal for a disease [54] whereas, WES is both cost effective and more targeted when seeking answers in the genetic background underlying a pathologic phenotype [55].

WES is a high-throughput method, which is invaluable for the circadian mechanism analysis at the genetic level. SNPs and mutations at clock genes can be easily detected and thereby, sleep abnormalities can be explained on a genetic basis. This piece of information is important for both clinicians, patients and their relatives. Clinicians on one hand can exclude a diagnosis, or to confirm a diagnosis, apply appropriate and individualized treatment to their patients, while the patient's family can be provided with preventive instructions, in order to avoid sleep disturbances, in cases where a well characterized genetic variant is highly heritable.

WES use in research promotes the identification of novel genetic variants of even novel gene candidates, which have not been previously associated with disease (or non-disease) phenotypes. The significance of this possibility lies at the fact that it can give birth to new research projects and even to new research directions. Also, evolutionary, origin and family associations can be revealed. Finally, WES application can be expanded in species with commercial significance [56], agriculture and animal-model improvements [55].

Circadian Diseases

Nowadays life is more time-demanding, resulting in changes in humans' life style. These changes in combination with clock gene-SNPs and/or mutations can cause circadian diseases like Circadian Rhythm Sleep Disorders (CRSD). [23] CRSDs (*Figure 28*) include:

Familial Advanced Sleep Phase Syndrome (FASPS), which is a disorder where sleep onset and offset are persistently advanced, because of causative mutations in clock genes, such as PER2.

Delayed Sleep Phase Syndrome (DSPS), in which sleep onset and offset are persistently delayed. A recently recognized risk factor for DSPS development, is V647G variation in *PER3* gene. This variation seems to be present mostly in DSPS patients and has also been associated with diurnal preference of activity.

Non-24-H Sleep Wake Syndrome (N-24) is a neurological sleep disorder characterized by individual's inability to keep a sleep-wake cycle longer that 24h. [23]

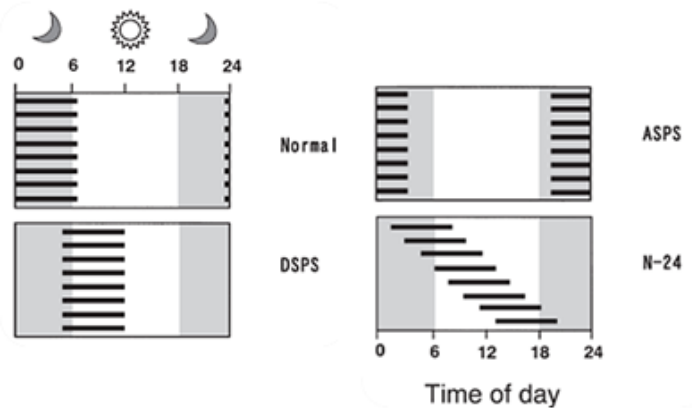

Figure 28. CRSDs compared to normal sleep phenotype. Grey zones illustrate night time and white zone indicates day time. Black bars are for sleep time definition. Normal individuals start sleeping before midnight and get up after 6:00 am. This motif is approximately the same for all the days of a week (see 8 black bars). Each CRSD has its day-night motif, in a weekly basis (DSPS, ASPS) or not (N-24). [23]

Acute Sleep Loss is another circadian disease that results from acute epigenetic remodeling of clock genes, indicating the great impact of environmental factors. Acute sleep loss leads to sleep deprivation phenotype, which can cause impairment of homeostasis (read also *Sleep Deprivation*).

Clock Genes & Other Diseases

Bibliography reports many linkages of clock genes with other diseases. Briefly:

- ✓ Sudden sensorineural hearing loss (SSNHL) with vertigo,
- ✓ Bronchial asthma,
- ✓ Cardiovascular disease,
- ✓ Parkinson's disease (PD),
- ✓ Diabetes,
- ✓ Prostate cancer,
- ✓ Breast cancer,
- ✓ Major depressive disorder (MDD),
- ✓ Seasonal affective disorders (SAD),
- ✓ Bipolar disorder (BD),
- ✓ Obstructive Sleep Apnea Syndrome (OSAS),
- ✓ HIV,
- ✓ Seizures and stroke

This list [11, 18, 21, 26, 44-46, 50, 51] is being daily updated, as research in the field of Sleep becomes more popular. Scientist believe that in many of a total of 80 reported sleep disorders, there is impairment of the circadian mechanism [57]. Hence, sleep regulation mechanism offers a fertile field for research (*Figure 29*), which is also emerging from the fact that 20% of global population suffers from sleep disturbances [58].

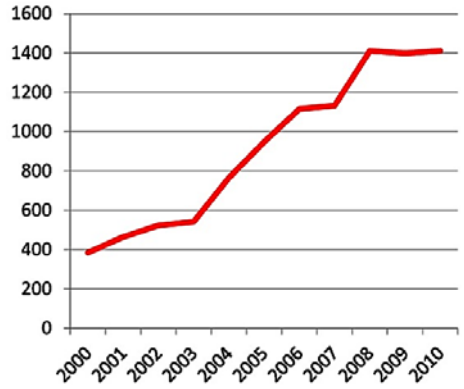

Figure 29. Sleep/insomnia and Epidemiology by year. Vertical axis: Number of PubMed annual publications about sleep research per year. Horizontal axis: Year of publications. [57]

HYPOTHESIS

The present study focuses on “Sleep & Aging”. Up to present, it is known that polymorphisms of clock genes not only affect the time we sleep but also our whole sleep architecture. These effects can be more sensitive in elderly individuals, where sleep is naturally impaired and clock mechanism is decayed. Features such as: low sleep latency, decreased REM state and daytime sleepiness have been observed mainly in this group of patients. Our research aims to detect and investigate certain clock genes’ polymorphisms, in a Cretan Dementia cohort, that are associated with extreme sleep phenotypes, as it is indicated through actigraphy studies. On the second part of our study we examine in depth the most intriguing phenotypes employing polysomnography technology in an attempt to explain how sleep architecture might be impaired from the presence of the respective SNPs.

Figure 30. Sleep in art: "Like sleeping in the Beach", G. Jonathan, 2014

Cohort

Participants for this study were selected from a larger group of 145 Cretan individuals, whose DNA and WES data were available from "Thalis-University of Crete-Interdisciplinary Network for the Study of Alzheimer's Disease" project (Principal Investigator A. Vgontzas, Principal Investigator for the Genetic Substudy I. Zaganas). The present group has the advantage that it originates from Crete, Greece and is thus thought to be genetically homogeneous. From this sample, 47 cognitively healthy individuals and 98 patients diagnosed with dementia have been genetically examined.

Biological Sample Collection

For the genetic analyses, whole peripheral blood from all participants was collected in EDTA vacutainer tubes using 10ml syringes (BD Emerald) or butterfly needles 0,60x19mm company. Samples were stored at -20^{o}C until DNA extraction processing (procedure performed by E. Vogiatzi in the Neurology Laboratory, Medical School).

DNA Extraction

Genomic DNA was obtained from whole peripheral blood, which was processed with QIAamp DNA Blood Mini kit, Qiagen (CA, USA). 200µl whole blood sample was used to obtain a mean of 8.5µg DNA, per case. DNA concentration and purity yield were assessed spectrophotometrically at 260/280 nm. The mean final DNA concentration of our samples was as high as 42.5 ng/µl, while the mean absorption ratio A260/A280, indicating DNA purity, was 1.793. Each DNA sample was properly coded (through unique identifiers), to ensure the anonymity protection of the participants (procedures performed by E. Vogiatzi, G. Gouna, L. Mathioudakis in the Neurology Laboratory, Medical School).

Whole Exome Sequencing

WES was performed at the Unit of Genomics Analysis of Minotech (Principal Investigator D. Kafatzopoulos), at the Institute for Research and Technology (FORTH, Crete). Platform Ion Torrent PROTON (specifications: Ion PI chip v3) was used, after creating the corresponding library and preparing the sequencing reaction with Ion Ampliseq Exome™ kit (MA, USA) and Ion PI Hi-Q OT2 200™ kit (MA, USA), respectively. The bioinformatics processing and data storage was available by the software Ion Torrent Suite™ (MA, USA). The variant called by the software Ion Reporter v.5.0, following comparative analysis of the sample with the reference genome hg19.

Bioinformatics' Analysis

Data analysis was performed either manually on the vcf files containing the raw sequencing data, using targeted gene panels for circadian mechanism or by employing the Ingenuity Variant Analysis software, Qiagen (CA, USA). All 145 participants were examined for 15 clock gene variations (*Table 3*) in an attempt to detect notable SNPs.

Table 3. Genes that were in examined during WES analysis

CLOCK	ARNTL (BMAL1)	NPAS2
PER1	PER2	PER3
REV1	NR1D1	VDR
CRY1	CRY2	BHLHE40
RORA	RORB	BHLHE41

Actigraphy Studies

All participants have undergone actigraphy study for 3 nights (Study performed by A. Vgontzas, M. Basta, I. Koutentaki in the context of the Thalis-MNSAD program). The actigraphy parameters examined were: total sleep time (TST), night TST, night in bed time, night out bed time, nap number and nap TST.

As "total sleep time" is reported the time that participants spend in bed, even if they are not sleeping. "Night TST" refers to sleep duration, which is the total time during which the individual starts to sleep until he/she wakes up. "Night in bed time" is the time when participants go to bed, while "night out bed time" is the waking time. "Naps" refer to short sleeps that are usually taken during the day.

Sleep Architecture Study

Polysomnography (see *Introduction*) took place n Sleep Laboratory of University of Crete (Principal Investigatos S. Schiza) and was performed for 3 participants, 1 control and 2 dementia patients. Sleep architecture sample was limited as many of the participants were unable to visit Sleep Medicine Lab, because of the distance and their severe health status. For sleep architecture study recording machines Alice4 and Alice5 from Philips Respironics were used. Sleep data were illustrated through Alice Sleepware 2.8.78 2010 software. Total sleep duration (TST), percentage of sleep efficiency (SE%), sleep stages analysis, apnea-hypopnea index (AHI), wake after sleep onset (WASO) and number of central apneas are the basic parameters that we examined.

Statistical Analysis

Data were analyzed using SPSS (version 21.0; SPSS Inc., Chicago, IL). Descriptive statistics were used to describe the characteristics of the sample. We used Independent t-test for evaluating the difference between the mean values of the under-examination groups, for every actigraphy parameter (TST, night TST, night in bed time, night out bed time, nap TST and nap number). Significance level is defined for $p \leq 0.05$.

WES Analysis

WES data from 98 demented individuals and 47 normal controls were manually analyzed. Each participant was examined for polymorphisms in genes represented in *Table 3*. Intronic or/and synonymous variants were excluded from further analysis as we focused on variants with possibly damaging effects on the respective gene product. A summary of the identified polymorphisms in each gene examined is represented in *Table 4*. Our analysis revealed 34 novel polymorphisms that have never been reported before. Every novel SNP was given an informal code (*Table 4)*, in which letters refer to the gene origin and the numeric part represent the detection order. Details of the novel variants are represented in *Appendix*. From a total of 91 variations only 16 are reported, at least once in literature. In our genetic cohort, variants of low frequency, being present in less than 5 out of 145 samples, are considered worth studying, as they might be associated with extreme sleep phenotypes.

Table 4. Known and novel polymorphisms depicted from our cohort. The frequency of each polymorphism in our sample of 145 participants is referred as f_{sample}. Bold polymorphisms are reported in association studies.

GENE	POLYMORPHISM	fsample (x/145)	GENE	POLYMORPHISM	fsample (x/145)
CLOCK	rs34897046	8	*PER3*	**rs10462020**	38
	rs767458103	1		**rs10462021**	38
ARNTL	*novel (nBM.1)*	1		rs12023156_rs12033719	78
NPAS2	rs113107029	2		*novel (nP3.1)*	1
	rs11541353	50		rs139315125	1
	rs17025128	6		rs140974114	1
	rs183671025	1		rs143936373	1
	rs201271037	2		rs144178755	1
	rs2305158	49		rs150812083	1
	rs2305160	128		rs1776342	124
PER1	rs112474322	4		**rs228696**	144
	rs143964144	1		**rs228697**	38
	rs150149747	47		rs200038116	2
	rs150726488	1		**rs2640909**	69
	rs2585405	135		**rs35072750**	1
	rs2735611	2		*novel (nP3.2)*	1
	rs370901282	1	*CRY1*	rs201634474	2
	rs74795714	4	*CRY2*	rs11394694	132
	rs754411014	1		*novel (nC2.1)*	1
	novel (nP1.1)	3		rs539729238	3
	novel (nP1.2)	1	*REV1*	rs147467855	1
	novel (nP1.3)	1		**rs3087386**	107
	novel (nP1.4)	1		rs3087399	11
	novel (nP1.5)	1		rs3087401	2
	novel (nP1.6)	1		**rs3087403**	75
	novel (nP1.7)	1		*novel (nR1.1)*	1
	novel (nP1.8)	1		*novel (nR1.2)*	7

	novel (nP1.9)	1		*novel (nR1.3)*	1
	novel (nP1.10)	1		*novel (nR1.4)*	1
	novel (nP1.11)	1		*novel (nR1.5)*	1
	novel (nP1.12)	1		*novel (nR1.6)*	1
PER2	**rs35333999**	8	*NR1D1*	*novel (nNR1.1)*	1
	rs77146655	1		rs148075782	1
	rs78832829	6		rs17616365	1
	novel (nP2.1)	2	*RORA*	rs199616498	2
	novel (Np2.2)	2		rs143635644	1
	novel (nP2.3)	2	*RORB*	rs144902615	1
	novel (nP2.4)	1	*BHLHE 40*	rs182209585	3
	novel (nP2.5)	1		rs2271566	50
	novel (nP2.6)	1		*novel (nB40.1)*	1
	novel (nP2.7)	1	*BHLHE 41*	rs368411883	2
	rs2304672	39		rs372622178	1
	rs934945	62		*novel (nBH41.1)*	8
	rs76355956	1		*novel (nBH41.2)*	1
			VDR	**rs2228570**	127
				novel (nD1)	1
				rs752590757	1

Statistical Analysis of actigraphy data in respect with clock genes variations' distribution

Every polymorphism of the core circadian genes; *CLOCK, BMAL1, NPAS2, PER1, PER2, PER3, CRY1, CRY2,* was statistically analyzed in respect with actigraphy parameters. Because of our small sample with available actigraphy studies (85 participants) we cannot conclude in undeniable associations between specific polymorphisms and actigraphy data. However, we will show some interesting information that could be useful for further research in a larger sample. In the present section only the graphs of the statistically significant polymorphisms are displayed, that is mainly TST related analyses. For additional statistical details read also *Appendix*.

PER1 variations

As shown at *Figure 31* participants baring the polymorphism **rs2585405** in their genome tend to sleep less (mean night TST 441min) than individuals without the variant (mean night TST 496min). Although, this difference is marginally significant (p=0.051) it indicates a differentiation trend between these 2 groups.

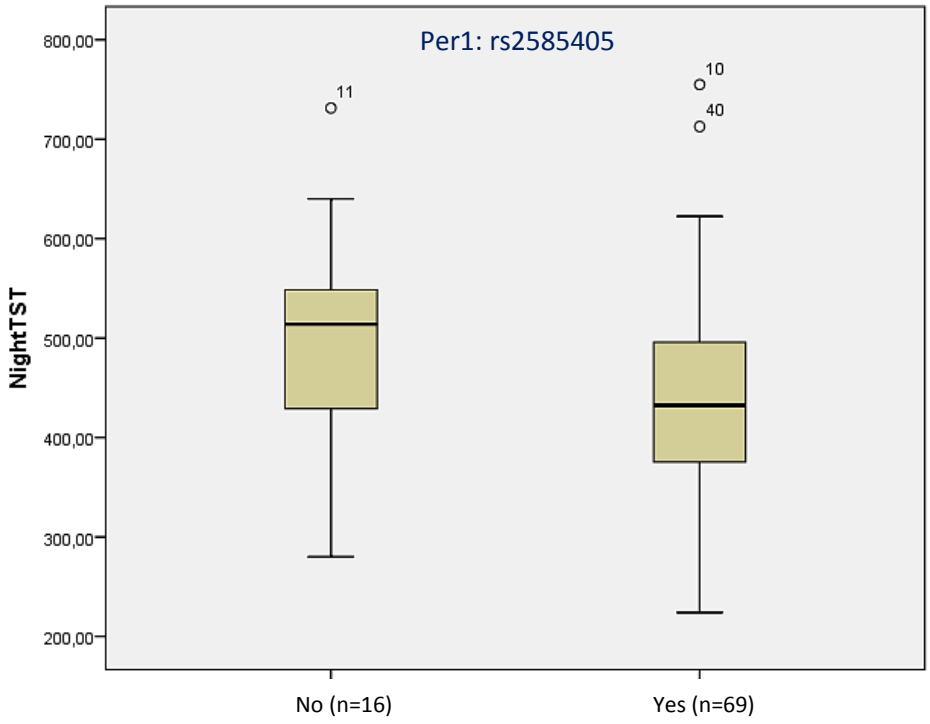

Figure 31. Box Plot for negative and positive for the **rs2585405** participants for night TST parameter.

So far, rs2585405 is known to be a benign variation (Polyphen score: 0.00/1.00) [xi] with a relatively low frequency (0.89%) among Europeans (Non Finnish) according to ExAC browser [xii]. Nevertheless, it has been correlated with prostate cancer risk [59] and higher serum levels of sex hormone-binding globulin levels in Chinese people [60], for whom the respective allele frequency is 0.52% [xii].

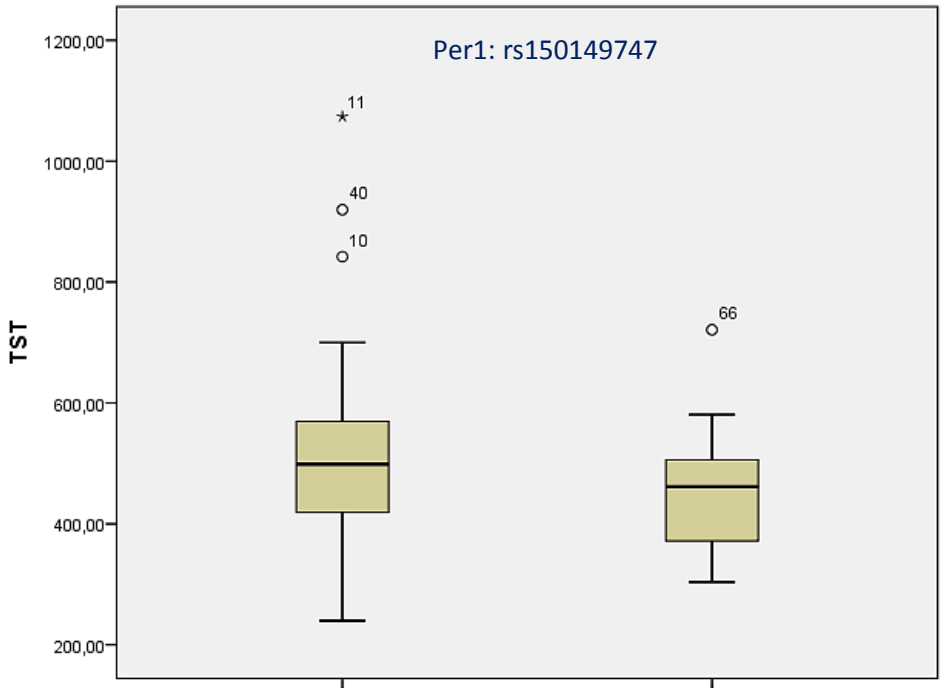

Figure 32. Box Plot for negative and positive for the **rs150149747** participants for TST parameter.

A second *PER1* variation, **rs150149747**, seems to render individuals rather prone to less TST, as there is a significant difference (p=0.045) between the mean TST of the two groups.

Individuals with **rs150149747** have lower TST (452min) than the negative group (513min) (*Figure 32*). According to Polyphen2, this variation is damaging (Polyphen score: 0.99/1.00) [xi] for the protein product, as it leads to a frameshift deletion at the C-terminal domain, which is predicted to interact with other circadian molecules [xiii]. ExAC does not report allele frequency for this polymorphism, indicating that it probably has not been studied. Therefore, we can safely assume a putative association of rs150149747 with low sleep duration, as a result of the impaired Per1 protein and its inability to interact with other molecules of the circadian mechanism.

PER3 variation

Examining *PER3*, we detected a polymorphism (**rs1776342**), which was clustered within individuals with lower TST levels. In *Figure 33* the difference between two TST means may not be so obvious, however, statistical analysis revealed that this difference is significant (p=0.046). More specific, negative group is sleeping 537 min, while positive group is sleeping approximately 1.30h less. Further analysis of this variant revealed that it is a benign alteration (Polyphen score: 0.007/1.00)[xi] that is commonly referred as natural variation [xiii], because of its medium allele frequency (~ 1.5% for Europeans-Non Finnish [xii]). Hence, is not feasible to directly relate this polymorphism with low TST duration. But, it is possible it could confer a risk for the development of such low TST phenotypes.

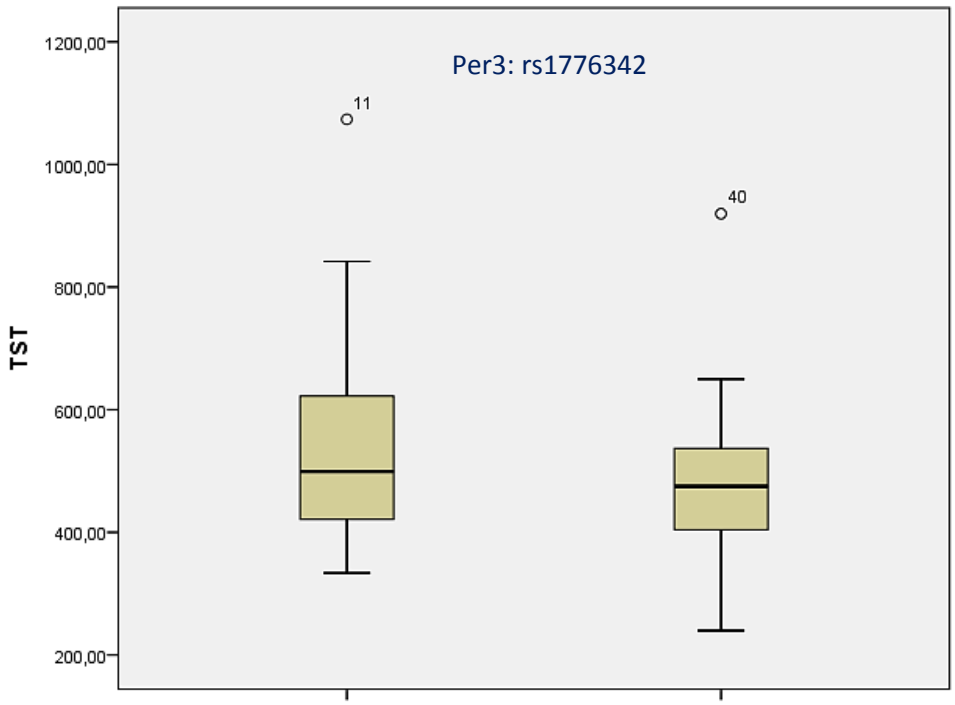

Figure 33. Box Plot for negative and positive for the **rs1776342** participants for TST parameter

Table 5. Genetic information of the statistically significant polymorphisms detected in our sample of 85 individuals with available WES and actigraphy study

Gene	Polymorphism	Coding DNA change	Protein change	Function	European ExAC frequency	Frequency in our sample	Homozygotes
PER1	rs2585405	2884G>C	p.Ala962Pro	missense	0.89	135/145	107/145
PER1	rs150149747	2293_2294delGC or insertion CG|	p.Ala765Arg|	Deletion Frameshift or missense	Not reported	47/145	8/145
PER3	rs1776342	3019G>A	p.Ala1007Thr	missense	~1.5	124/145	1/145

Actigraphy Data Extremities with Rare Polymorphisms

As already mentioned, 85 out of 145 individuals for whom WES data are available, had also undergone actigraphy recordings for 3 subsequent nights. Of those, 7 were controls and 78 were demented. We sought to classify each of them according to the measurements obtained from actigraphy studies and examine whether the extremities of each classification carry rare variants in any of the CLOCK genes we study. For simplicity and anonymity reasons, participants with extreme sleep phenotypes and rare variants in their genome received an informal coding ("P#", P from participant and C for control). All actigraphy recordings were crosschecked with participants' sleep diary data also available in our cohort. The actigraphy parameters we examined are: total sleep time (TST), night TST, night in bed-time, night out bed-time, nap TST, nap number.

TST

TST (total sleep time) refers to the total time the individual spends on sleeping through the day. *Figure 34* indicates mean TST from a 3-day recording for each participant. As our cohort's age range is over 65, we expect mean TST to be between 360-480 minutes. Statistical analysis revealed that mean TST is 495.5 minutes (or 8.25h). However, there are extreme phenotypes (colored bars in *Figure* 34) with a total sleep time lower than 300 or higher than 600 minutes, implying a sleep disorder-phenotype. Specifically, 15 participants, representing 17.64% of our actigraphy sample, are characterized by abnormal TST. However, only 4 out of 15 (*Figure 34*) carry a rare variant in at least one gene from our sleep-gene panel (*Table* 6*)*.

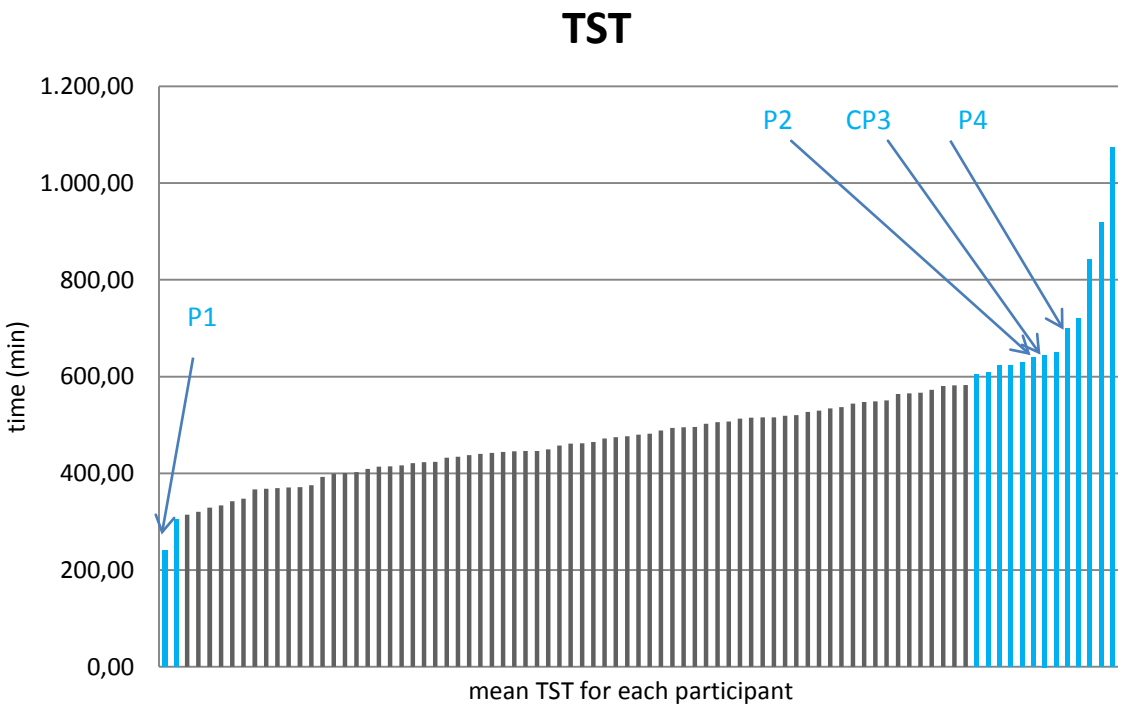

Figure 34. Distribution of the participants according to their mean TST: Horizontal axon indicates the 85 participants. Vertical axon illustrates total sleep time in minutes. Colored bars are participants with extreme TST-phenotype (TST≥600 or TST≤300 min). Arrows indicate participants with extreme phenotype that carry at least one rare polymorphism.

Polymorphisms which are present in 5 or less individuals from our 145 sample cohort were considered as rare variants.

Table 6. Participants with extreme TST-phenotypes and their rare variants.

Participant (informal code)	TST (min)	Polymorphism
P1	239.67	PER1: *nP1.6*
P2	639.33	PER1: rs74795714
CP3	645.00	REV1: *nR1.5*
P4	700.00	NPAS2: rs183671025
		PER2: *nP2.5*

The novel polymorphism ***nP1.6*** is an alteration in *PER1* sequence. This coding DNA change occurs at the 3328th nucleotide and it causes a deletion of a guanine (G) which alters the reading frame of the sequence (frameshift). At the protein level, this alteration causes loss of alanine (Ala) 1110, while by using online prediction programs [xi, xiii], we concluded that this frameshift change generates a smaller protein product missing its last 160a.a. Upon losing its C-terminal domain, Per1 cannot interact with Per2, Per3, Cry1 and Cry2 [ix]. Disruption of Per and Cry complexes could affect circadian mechanism. Another possible functional explanation could be instability of Per1 protein, because of C-terminal lost, as a result to be unable to respond in light stimulus [25]. Both scenarios could putatively lead to elimination of sleep need [21]. Further analyses are needed in order to interpret this possible association.

Participant P2 carries another variant of *PER1*, named **rs74795714.** This is a missense alteration where G at position 718 is replaced by adenine (A), resulting in a valine (Val) 240 isoleucine (Ile) protein alteration. However, this polymorphism cannot be strictly correlated with the extreme TST phenotype of P2, as it is also present in 2 other participants, whose mean TST vary from 414.67 to 582.67 min. Since P2 also presents with increased BMI, it is possible that the observed reduced TST is enhanced by this factor. Literature reports have connected increased BMI with increased risk for OSAS, which is characterized with low sleep duration, loss of restorative sleep and fragmented sleep [61].

Next participant with an extreme TST-phenotype is CP3, who presents with a novel variation in REV1 gene, named ***nR1.5*** hereinafter. *REV1* encodes a protein which carries a special domain for protein-protein interactions [ix]. Previous studies suggest that Rev1 may have a role as a scaffold protein for DNA polymerase recruitment during translation synthesis (TRS) of damaged DNA [62, ix]. This novel polymorphism was identified in the 1715th nucleotide, where cytosine (C) changes to thymine (T). As a result, Ala placed in 572a.a. becomes Val. According to Polyphen2, this novel mutation is probably damaging, because the 572th a.a. is highly conserved among species (Polyphen score 1.00/1.00). Although 572 a.a. is not part of any binding site nor has an apparent significant role, the mutated Rev1 protein may be unable to inhibit Bmal1 activation, resulting in asynchrony of the circadian mechanism (*Figure* 35). Although CP3 has a rather normal BMI, we should take under consideration the existing medication impact [63] (such as benzodiazepine) as well as smoking habits, as these can affect sleep quality [64].

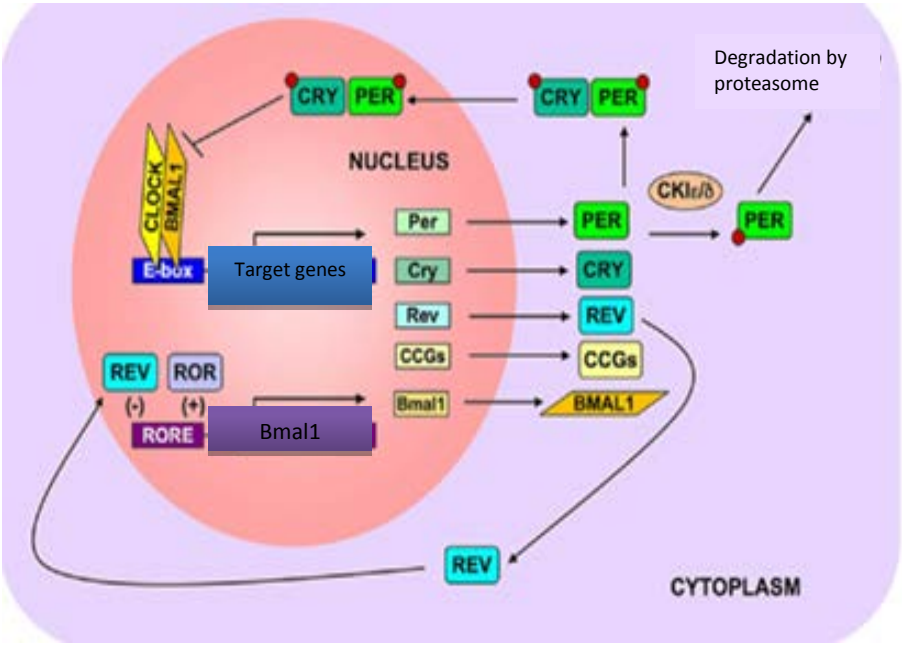

Figure 35. Molecular mechanisms of circadian timing: Core oscillation is augmented and stabilized by a secondary loop involving two orphan nuclear receptor proteins, Rev-Erbα and Rora. Both are activated by *CLOCK* and *BMAL1*, but in turn they affect *BMAL1* expression. While Rora has a positive role in *BMAL1* expression, Rev-Erbα acts as suppressor of *BMAL1.* Rev-Erbα and Rora can coordinate through RORE regulatory sequences [65].

Regarding P4, **rs183671025**, which is an already identified polymorphism of the basic clock gene *NPAS2*, was detected. This variation is located in the 4th exon and has missense functionality. Specifically, it alters nucleotide C to T at cDNA position 191. As a result, Ala64 on the protein sequence is replaced by Val. This a.a. does not participate in any crucial domain of the protein. However, Polyphen2 analysis reports that this variation may be

damaging (Polyphen score 0.89/1.00), because of high Ala64 conservation among species. Hence, disruption of Npas2 protein can lead to dysregulation of the circadian mechanism, as the number of Clock or Npas2/Bmal1 heterodimers might be impaired in a way that the individual sleeps early in the afternoon and for prolonged time. In addition, factors such as the increased BMI and the medication (carvedilol, perindopril, betahistine, furosemide) of the participant could burden the observed extreme TST-phenotype [66].

Another variation, which is present in P4, is ***nP2.5***, which refers to a change on the 19th exon of *PER2*. This novel variation causes a missense G>A alteration at cDNA position 2429. The altered protein product has asparagine (Asn) located at position 810a.a. instead of serine (Ser). Following Polyphen2 analysis, we believe that this alteration is possibly benign (Polyphen2 score 0.001/1.00), implying a small impact on the TST-phenotype of P4.

Night TST

Another actigraphy parameter that we examined is night TST. We essentially measured the neat sleep time, namely the duration of night-time sleep. Normally night TST varies from 360-480 minutes in elderly people. Mean night TST in our actigraphy sample is 451.69 minutes.

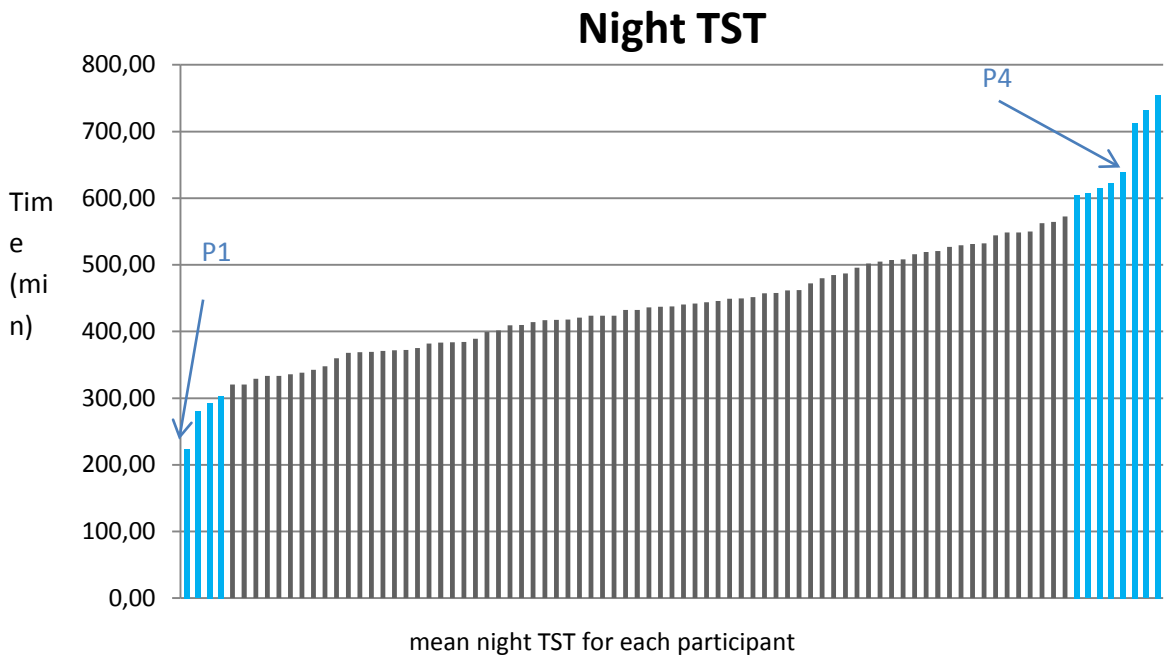

Figure 36. Distribution of the participants' mean night TST: Horizontal axon indicates the 85 participants. Vertical axon illustrates night TST in minutes. Colored bars are participants with extreme night TST- phenotype (night TST≥600 or night TST≤300 min). Arrows indicate participants with extreme phenotype that carry at least one rare polymorphism.

As it was expected, the same participants (P1, P4), who had extreme TST phenotype, present with extreme night TST- phenotype too (*Figure* 36). As extreme night TST- phenotype we considered sleep time less than 5h (300 min) and more than 10h (600min), because it might indicate insomnia or hypersomnia respectively. *Table* 7 illustrates personal mean night TST measurements for each participant that has extreme phenotype. All polymorphisms were analyzed in *TST* section above.

Table 7. Participants with extreme night TST-phenotypes and their rare variants.

Participant (informal code)	Nigh TST (min)	Polymorphism
P1	224.00	PER1: *nP1.6*
P4	640.00	NPAS2: rs183671025
		PER2: *nP2.5*

Night In Bed-Time

"Night in bed-time" is the parameter which represents the time when the individual goes to bed in order to sleep. This parameter is of special importance as it indicates alterations in the circadian rhythm and participant's chronotype. Invariably, older people visit their bed at about 22:00 -00:00. In our actigraphy sample mean value for night in bed- time is 22:25. However, 11 participants are recorded to go to bed too early (before 8:00pm) or too late (after 00:00am) (*Figure* 37).

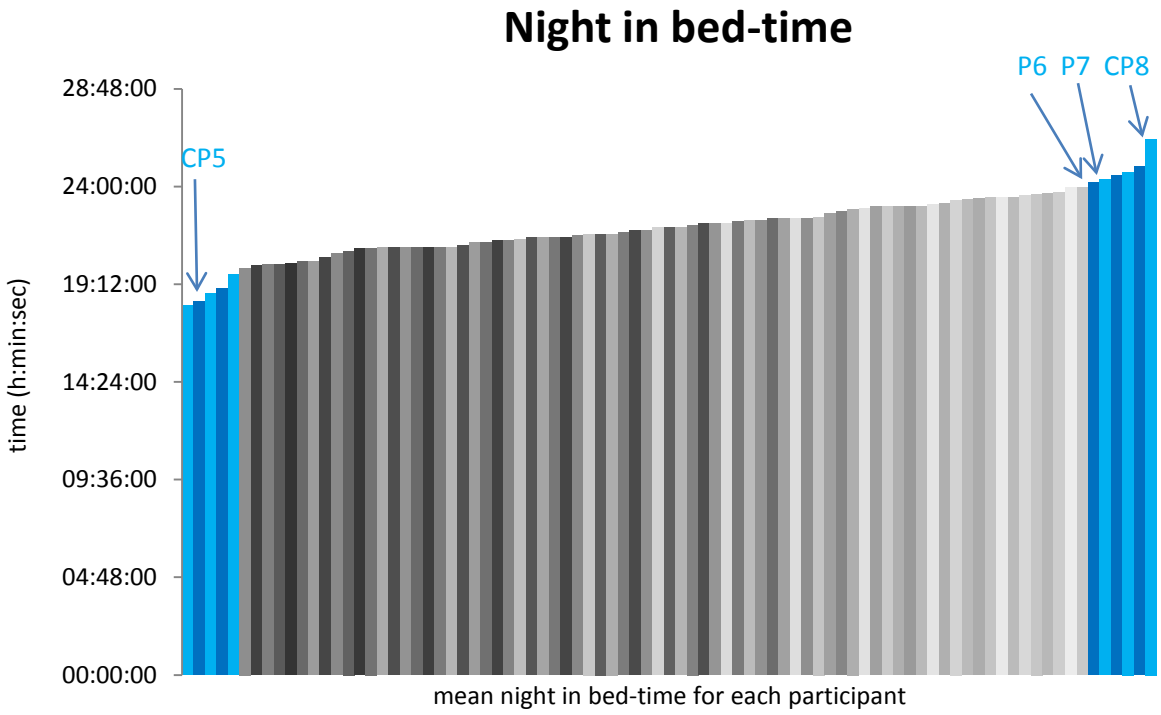

Figure 37. Distribution of the participants' mean night in bed-time: Horizontal axon indicates the 85 participants. Vertical axon illustrates night in bed- time. Colored bars are participants with extreme night in bed- time phenotype (time≥24:00:00 or time≤20:00:00). Arrows indicate participants with extreme phenotype that carry at least one rare polymorphism.

Rare variants are present in only 4 of 11 participants with extreme night in bed-time phenotype (*Table 8*). CP5 and CP8 are reported as controls, because they do not appear cognitive dysfunction and other characteristics, which are present in dementia-diagnosed individuals.

Table 8. Participants with extreme night IN bed time-phenotypes and their rare variants

Participant (informal code)	Nigh IN bed time (h:min:sec)	Polymorphism
CP5	18:20:00	CRY2: rs539729238
		REV1: rs3087386
		REV1: rs3087403
P6	24:11:40	NR1D1: *nNR1.1*
P7	24:21:40	PER2*: nP2.3*
CP8	26:20:00 (or 2:20:00am)	CRY2: rs539729238
		REV1: rs3087386
		REV1: rs3087403

CRY2 is a clock gene that regulates circadian mechanism through a negative feedback loop, as it inactivates its own transcription [22]. **Rs539729238**, which is present at CP5, has already been described but literature provides no information about the functional alterations it causes except for the fact that it is located in the promoter region of this gene. CP5 seems to have increased need for sleep, because he/she drops off very early. This promoter region-alteration might change the transcriptional levels of *CRY2,* possibly resulting in low or high Cry2 proteins levels. Cry proteins form heterodimers with Per proteins [22] in order to repress their own expression, or to affect Bmal1/Clock heterodimers, through a negative feedback loop [21]. Dysfunctional Cry protein could impair the amount of Per/Cry complexes concluding to an altered repression of these targets. Nevertheless, we should not directly relate this variation with the morningness chronotype as it is also present in 2 extra control participants with diverse characteristics. In details, CP8 is characterized by an eveningness chronotype whereas the other participant (CP5), who carries this alteration, has a normal sleep phenotype. Possible additional effectors of CP5's sleep program include smoking habits, increased BMI, medication and the presence of additional polymorphisms (see below) *(Table 8).*

Apart from rs539729238 in Cry2 gene, CP5 also carries two polymorphisms in REV1, **rs3087386** and **rs3087403**. The first variant is too common in our sample (*Table 4*). Therefore, it is not possible to relate it with specific actigraphy phenotype. About literature, Xu *et al. 2013* reports that **rs3087386** variant may be related to lung cancer survival in Chinese people, without revealing any correlation between this variant and sleep disorders [62].

At the same time, **rs3087403** that characterizes CP5 and CP8, is also common in our sample (*Table 4*). In the literature it has been positively related to increased leukopenia risk [67] and it is also significantly associated with overall survival from osteosarcoma [68]. Data from our participants vary in a way that we are unable to associate this REV1 polymorphism with specific chronotypes.

P6 has a novel variation on *NR1D1* gene (Nuclear Receptor subfamily 1 group D member 1), which is a downstream target of the circadian mechanism that encodes a transcription factor, member of the nuclear receptor subfamily 1. The protein product acts as a ligand-sensitive transcription factor, which inhibits the expression of core clock proteins, such as Clock and Bmal1 [ix]. It also regulates genes involved in metabolic functions and macrophage inflammatory response [xiii]. The novel polymorphism ***nNR1*.1** is a missense change located

in exon 4 of *NR1D1* gene. The present change occurs at nucleotide 520, where A turns to C or at the 174 a.a of the respective protein, where Asn becomes histidine (His). This type of alteration is possibly damaging (Polyphen score: 0.606/1.00) for the protein product. The fact that P6 is reported to sleep too late, indicates a possible arrhythmicity in the 24h function of the circadian mechanism, which may result from this impaired protein, his/her increased BMI and his/her multiple medication. Damaged NR1D1 protein may be unable to suppress *CLOCK* and *BMAL1*. Being unregulated, the latter may induce PER and CRY expression more potently, a function that takes place when the individual is awake. Hence, nNR1.1 could act as a substrate for developing an eveningness-like chronotype.

P7 is characterized by a novel variation in *PER2*, named ***nP2.3.*** The current polymorphism has not clear function as WES data show that it is either a missense or a frameshift alteration at exon 19. Its missense function is illustrated as a replacement of A by G at position 2732 or, in a polypeptide-basis, a replacement of a tyrosine (Tyr) 911 by cysteine (Cys).On the other hand, its frameshift action results from the insertion of a C between nucleotides 2731 and 2732. Both alterations seem to have a benign role in P7 phenotype (Polyphen score: 0.022/1.00). Hence, it is not feasible to relate *nP2.3* with the extreme night in bed- time of P7. It is more possible for P7 to experience eveningness because of his/her multiple medication (such as rivastigmine, duloxetine, Valsartan) [63, 66, 70].

Night Out Bed-Time

For studying the wake up-time we use the "night out bed-time" parameter. This parameter is also an indicator of the circadian rhythm and its possible abnormalities. For healthy individuals common wake up time is at approximately 6:00-9:00, according to their daily program. Individuals who wake up before 6:00am or after 9:00pm are characterized by extreme night out-phenotype. In our actigraphy sample mean night out bed time is 7:12.

We found that 13 out of 85 participants have extreme night out bed time (*Figure* 38*)*, but only 5 of them carry rare polymorphisms in their genome (*Table 9*). **Rs74795714** is a benign variant (Polyphen score: 0.001/1.00) according to prediction tools, lying on a region which participates in *PER1* phosphorylation. This polymorphism is detected in two participants P9 and P2, without revealing any information about its role in circadian rhythm regulation, as both participants have opposite night out bed-time phenotype. P2 and P9 have increased BMI and consume multiple pharmaceutical compounds. Thus, both could possibly belong to OSAS cases. A basic differentiation between these two participants, as it is uncovered from all their actigraphy data, is that P9 has a morning-like chronotype in contrast to P2 that has evening-like chronotype. Therefore, it is not feasible to relate rs74795714 with a specific chronotype.

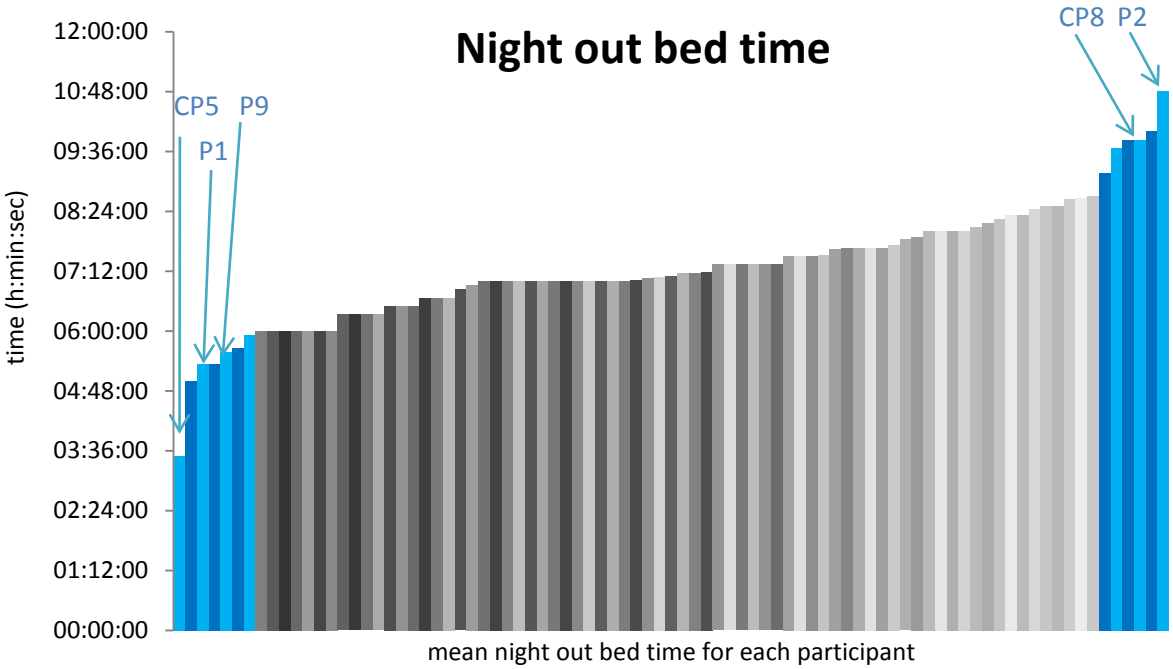

Figure 38. Distribution of the participants' mean night OUT bed time: Horizontal axon indicates the 85 participants. Vertical axon illustrates night OUT bed time. Colored bars are participants with extreme night out bed –time phenotype (time≥9:00:00 or time≤6:00:00). Arrows indicate participants with extreme phenotype that carry at least one rare polymorphism

Table 9. Participants with extreme night out bed-time phenotypes and their rare variants

Participant (informal code)	Nigh OUT bed time (h:min:sec)	Polymorphism
CP5	03:30:00	CRY2: rs539729238
		REV1: rs3087386
		REV1: rs3087403
P1	05:20:00	PER1: *nP1.6*
P9	05:35:00	PER1: rs74795714
CP8	09:50:00	CRY2: rs539729238
		REV1: rs3087386
		REV1: rs3087403
P2	10:48:20	PER1: rs74795714

Nap TST

Nap TST is a parameter that measures total time spent in short restorative sleeps through the day, namely it reflects daytime naps. Scientists declare that a nap lasting 10-20 minutes works as "power banks" while a 60-90 minutes nap improves our memory and cognitive functions [71-73]. In our elderly sample we expect mean nap TST to be impaired because of their lifestyle, medication and comorbid diseases that can influence rhythmicity of the circadian mechanism. Statistical analysis revealed that mean nap TST is 8.45 minutes. However, none of the participants experience extreme nap durations through their daytime (*Figure 39*).

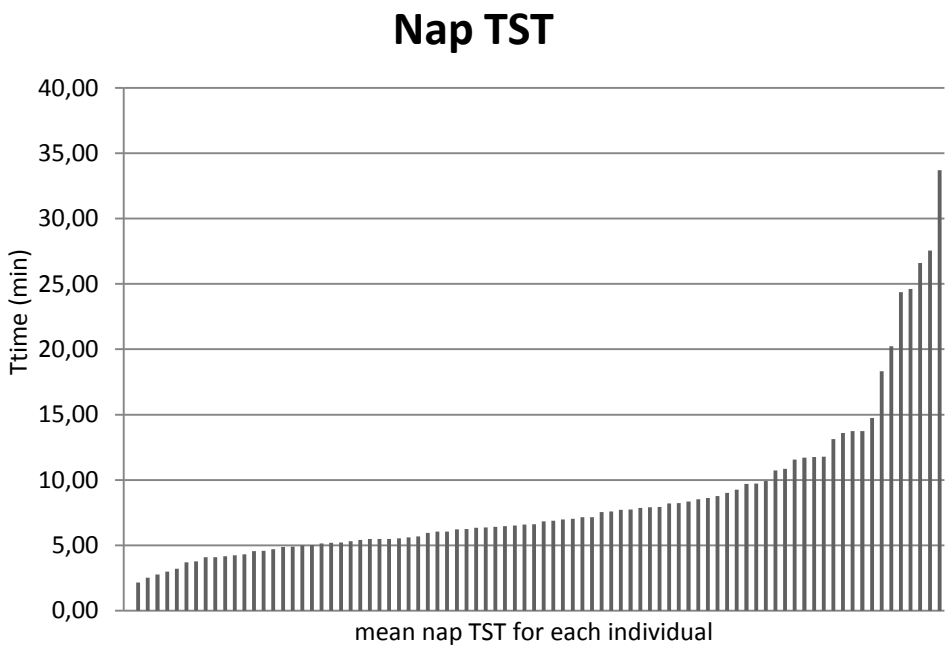

Figure 39. Distribution of the participants' mean nap TST: Horizontal axon indicates the 85 participants. Vertical axon illustrates nap TST. None of the participants has extreme nap TST-phenotype (more than one hour).

Nap Number

"Nap number" information is important as it reveals how many daytime naps our participants have.

Figure 40 indicates that 43.5% of our participants do not have any daytime nap, while 30.58% have 3 naps. If a participant has increased nap number of low duration, this may reflect an underlying pathology, such as OSAS, or it could belong to medication side effects. In our sample, mean nap number of the participants is 1.5 naps per day. Also, 6 participants (*Figure 40*) have extreme nap number (more than 3 naps), but only 2 of them *(Table* 10) carry a rare polymorphism.

Table 10. Participants with extreme nap number-phenotypes and their rare variants

Participant (informal code)	Nap number	Polymorphism
P10	4	BHLHE41: rs372622178
P11	4	PER2: *nP2.7*

P10 presents with a variation of BHLHE41 gene (Class E basic helix-loop-helix protein 41). **Rs372622178** is a missense variation located in the 5th exon of BHLHE41, particularly at nucleotide position 574, where G turns into A. This change leads to a benign protein alteration (Polyphen score: 0.014/1.00), which changes Ala192 to threonine (Thr), without affecting any crucial region of the polypeptide. This protein is categorized as a regulator of the circadian mechanism, as it represses the activity of the circadian transcriptional activator CLOCK or NPAS2/BMAL1 heterodimer, by competing for the binding to E-box found within the promoters of its target genes [ix, xiii]. Defects in this gene are associated with short sleep phenotypes [73]. P10 has a normal bed time schedule and a significantly increased BMI. The latter could contribute to a shorter and more fragmented sleep, resulting in an OSAS-like phenotype. It is true that P10 sleeps approximately 5.3h through the night, consequence originating maybe from SNP rs372622178, or possibly from underlying OSAS or even from and his/her medication (rivastigmine, olmesartan, amlodipine) [66, 70]. Hence,

his/her need for additional sleep is increased, reflecting sleep homeostasis mechanism. Although, it is not safe to associate the present polymorphism with P10's sleep pattern, we could hypothesize that it may act as an additional factor for the extreme nap number-phenotype of P10.

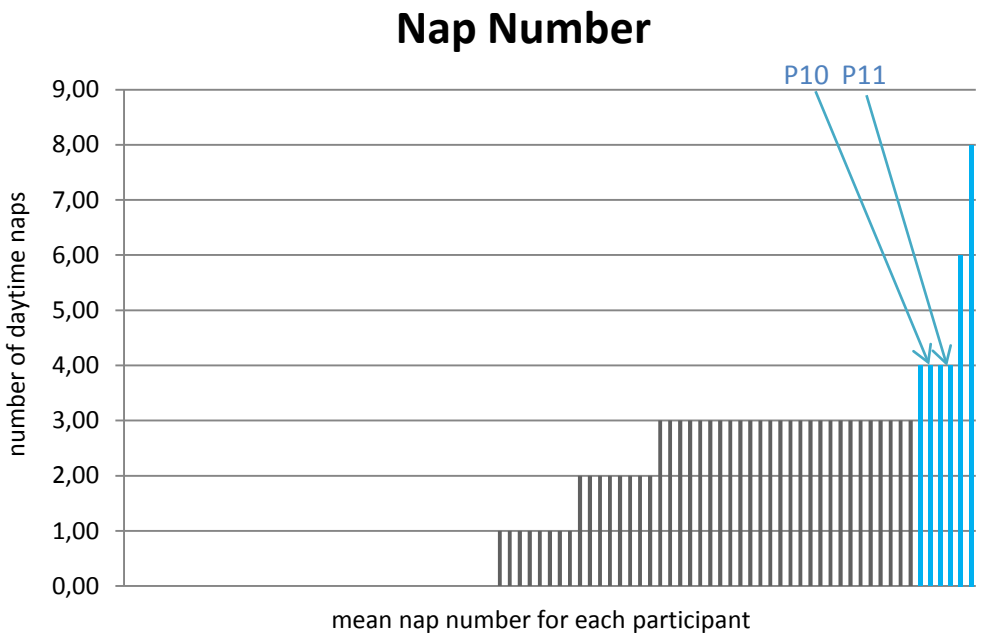

Figure 40. Distribution of the participants' mean nap number: Horizontal axon indicates the 85 participants. Vertical axon illustrates nap number. Colored bars are participants with extreme nap number- phenotype (more than 3 daytime naps). Arrows indicate participants with extreme phenotype that carry at least one rare polymorphism.

P11 has a novel variation at the 19th exon of *PER2* with missense functionality, hereinafter referred as ***nP2.7***. According to the prediction tools, it might be a damaging mutation (Polyphen score: 0.958/1.00). At the protein level, this change turns arginine (Arg)966 into tryptophan (Trp). At the coding DNA level, C at position 2896 is substituted by T. Although the abovementioned protein region has not been attributed a specific function, nP2.7 might disturb the functionality of *Per2* C-terminus. As a result, the mutated protein product might be unable to interact with Cry proteins and *CLOCK*/*BMAL1* targets. However, the rest of P11's actigraphy data indicate a normal sleep phenotype. Therefore, we cannot conclude in a likely association between *nP2.7* and the increased nap number of this individual. It is possible that advanced age, comorbid diseases (like gastroesophageal reflux disease and diabetes) [74, 75] and medication (such as irbesartan, hydrochlorothiazide) [66] of P11 contribute to the observed extreme nap number-phenotype.

Table 11 summarizes our findings on the participants with extreme actigraphy phenotypes. For each participant, rare variants as well as mean measurements in every actigraphy parameter are reported. Values in bold indicate an extreme phenotype for the current actigraphy parameter.

Table 11. Summary of the extreme sleep phenotype participants.

Participants	Rare Polymorphisms	Mean TST (min)	Mean Night TST (min)	Mean Night IN bed time (h:min:sec)	Mean Night OUT bed time (h:min:sec)	Mean Nap TST (min)	Mean Nap number
P1	PER1: *nP1.6*	**239.67**	**224.00**	21:40:00	**05:20:00**	24.61	3
P2	PER1: rs74795714	**639.33**	550.00	23:26:40	**10:48:20**	7.59	3
CP3	REV1: *nR1.5*	**645.00**	562.67	23:25:00	7:00:00	5.62	3
P4	NPAS2: rs183671025	**700.00**	**640.00**	20:20:00	07:50:00	20.22	2
	PER2: *nP2.5*						
CP5	CRY2: rs539729238	425.00	421.00	**18:20:00**	**03:30:00**	4.10	1
	REV1: rs3087386						
	REV1: rs3087403						
P6	NR1D1: *nNR1.1*	446.33	409.33	**24:11:40**	07:53:20	4.55	3
P7	PER2: *nP2.3*	320.33	320.33	**24:21:40**	06:50:00	6.22	0
CP8	CRY2: rs539729238	417.00	414.00	**26:20:00**	**09:50:00**	3.20	1
	REV1: rs3087386						
	REV1: rs3087403						
P9	PER1: rs74795714	414.67	338.33	21:00:00	**05:35:00**	8.36	3
P10	BHLHE41: rs372622178	366.67	320.33	23:06:40	07:00:00	13.12	**4**
P11	PER2: *nP2.7*	547.67	484.67	21:00:00	08:15:00	24.38	**4**

Polysomnography Studies

Sleep architecture studies were performed in only 3 of the 85 actigraphycally-examined participants. For the rest, their severe health condition and the long distance of residence were most common difficulties in visiting Sleep Medicine Laboratory.

Table 12 indicates the actigraphy data of 3 participants that underwent a sleep architecture study. Bold measurements indicate actigraphy extremities in that parameter. P12 was not analyzed before as he/she has not any rare variant. Additionally, P13 has not been prior analyzed, because of absence of actigraphy extremity. *Table 13* summarizes all the polymorphisms that characterize current participants. For each polymorphism its frequency in our 145-individuals cohort (fsample) is indicated. Details for rare variants are mentioned in *Actigraphy Data Extremities and Rare Polymorphisms* section.

Table 12. Actigraphy data of polysomnography participants

Code	TST	Night TST	Night in bed-time	Night out bed-time	Nap TST	Nap number
CP8	417.20	414.00	**26:20:00**	**9:50:00**	3.20	1
P12	**842.00**	**755.00**	**19:00:00**	08:00:00	6.25	1
P13	347.67	347.67	23:25:00	07:00:00	5.22	1

Indicatively, we represent the distribution of individuals with polymorphisms rs539729238, rs17025128, *nBH41.1* or rs78832829 for the parameter TST in appendix (*Figure 44-47*). It is notable that 2 participants with *nBH41.1* and one with rs78832829 have not undergone an actigraphy study, so they are not included in distribution bar charts.

Table 13. Polysomnography participants and their polymorphisms

Code	Gene	Polymorphism	f_{sample} (x/145)
CP8	CRY2	rs539729238	3
	REV1	rs3087386	107
	REV1	rs3087403	75
P12	NPAS2	rs17025128	6
P13	REV1	*nR1.1*	1
	BHLHE41	*nBH41.1*	8
	PER2	rs78832829	5

Genetic background is important for a better evaluation of the polysomnography parameters for which the individuals have been studied. Specifically, we examined a number of indices of sleep quality, such as total sleep time in the lab environment (TST-lab), sleep efficiency (SE%), wake after sleep onset (WASO), apnea-hypopnea index (AHI), number of central apneas and sleep architecture.

Sleep efficiency (SE) is the ratio of time spent in bed to the time someone is actually asleep [2]. It is commonly reported as percentage (SE%), in which the higher the value the better the sleep. WASO is total awakening time after sleep onset [2] and is used to illustrate sleep fragmentation which is usually related to comorbidities [58]. AHI (apnea-hypopnea index) is the number of apneas or hypopneas recorded during the study per hour of sleep, and it is expressed as number of such events per hour. Specifically, it is used for OSAS classification, where 4 phenotypes of OSAS are recognized:

- ✓ Normal: AHI < 5
- ✓ Mild: 5≤AHI<15
- ✓ Moderate: 15≤AHI< 30
- ✓ Severe: 30≤AHI per hour

Concerning apneas, they are classified into 3 types:

Obstructive, which is recognized as respiratory effort throughout the apnea episode
Central, which reflect impairment of the nervous system in sending the required signal for breathing. As a result there is no breathing effort during the apnea episode.
Mixed, which begins as a central apnea and proceeds as an obstructive one. [2]

Table 14. Data assemble of polysomnography participants

							Latency of each stage from lights off (min)						
Code	**Recording Time (min)**	**TST-lab (min)**	**SE%**	**WASO (min)**	**AHI**	**Central Apneas**	**N1**	**N2**	**N3**	**REM**	**NREM (%TST)**	**SWS (%TST)**	**REM (%TST)**
CP8	433	326	75	98	11	0	18	81	198	280	93.1	5.7	6.9
P12	422	156	37	119	11	0	147	152	182	350	94.4	4.2	5.1
P13	443	319	72	319	102	0	22	26	279	329	86	4.1	14

As shown in *Table 14*, CP8 presents a rather satisfying polysomnography study. The number of AHI indicates the presence of mild OSAS, explaining the decreased SE% and WASO measurement. Specifically, because of obstructive apneas, CP8 had fragmented sleep and as a result not adequately efficient. Relative proportions of SWS and REM stages are also shortened in comparison to normal sleep, where 20-25% of sleep is characterized as SWS and 25% as REM [76] (*Figure 43*). It is already known that, in the elderly, restorative sleep is gradually eliminated (reported in *Sleep Through Lifetime*), resulting in an unsatisfactory sleep; herein expressed as SE%. Unfortunately, because of the small sample of polysomnography participants we cannot deduce any correlation between CP8's polymorphisms and sleep characteristics. Especially for the rare variant rs539729238 (*Table 13*), the only assumption we could make is that the altered Cry2 protein may cause a cascade of changes in the circadian mechanism, which can accordingly act as substrate for developing sleep disorders as the individual gets older.

Next participant had a low quality sleep, as it is indicated by the decreased TST-lab, SE, SWS and REM sleep as well as increased WASO. Latency of each step was extremely increased, demonstrating a delay sleep onset phenotype. However, *Table 12* does not confirm this. P12 spends a lot time in bed as actigraphy recording indicates, without necessary being asleep according to *Table 14*. Rs17025128 could not be related with P12's phenotype, as it is present in participants with variabilities in their sleep phenotype (*Figure 45*). Thus, we could assume that age, comorbidities such as dementia and depression, and medication (e.g. SSRI) result P12's sleep phenotype.

P13 polysomnography study was compensatory enough. Most of his/her sleep time was efficient (72%), while 14% of TST was characterized as REM stage. However, increased number of AHI (112), which indicates a severe OSAS phenotype and decreased SWS duration highlight the presence of sleep disorder. For a patient such as P13, who is too old and characterized by dementia, poor sleep quality is expected. This conclusion could not be confirmed only by the actigraphy study (*Table 12*). A unitary variation of *REV1* is present in P13, hereinafter *nR1.1*. According to Polyphen analysis is a damaging (Polyphen score 1.00/1.00) alteration of Ala572 to Val. *REV1* encodes deoxycytidyl transferase involved in DNA repair [xiii]. Hence, any damaging change in Rev1 protein could impair its efficiency as well as further targets of it, such as *BMAL1* (*Figure 35*). About rest of his/her rare polymorphisms (*Table 13*), it is impossible any correlation since they are present in another participants with different characteristics in their sleep (*Figure 46-47*).

Polysomnography recordings are useful tools for simultaneous promotion of qualitative and quantitative characteristics of sleep, especially in individuals with sleep disorders. Therefore, such information completes both genetic and actigraphy studies of these individuals, as they are mentioned in previous sections.

Whole-brain sleep is a result of the homeostatic synchronization of every brain neuronal network [13-15]. Sleep homeostasis depends on the intention of stimulation, which means that long wakefulness periods are followed by enlarged sleep duration. However, sleep deprivation experiments have shown that when sleep homeostasis is impaired, IEGs such as *PER1* and *PER2* are overexpressed. Hence, circadian rhythm alterations putatively affect vital functions like sleep, but also lipid and glucose metabolism. [14]

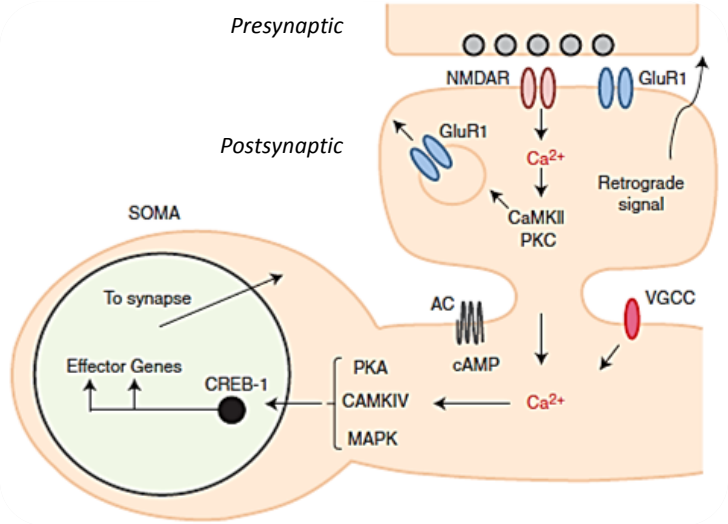

Figure 41. Molecular pathway of BDNF induction: Higher levels of exploratory activities during wakefulness show a stronger induction of effector genes, such as BDNF (brain-derived neurotrophic factor). BDNF plays an important role in synaptic plasticity, specifically in long-term synaptic potentiation after an increase in its levels (or long-term depression after decrease). BDNF facilitates synapse potentiation by regulating spine maintenance, synapse formation after sensory stimulation, and by assisting the process of acquisition of memory tasks. [14]

Sequencing platforms are nowadays widely used in unravelling the genetic background underlying sleep disorders. Specifically, researchers intend to scan thousands of genomes in an attempt to reveal polymorphisms associated with regulation of the sleep/wake cycle. WES is a cost-effective, high-throughput method that detects variants in the coding region of all human genes simultaneously. The benefits of WES include its lower cost and time efficiency analysis as compared to GWAS. Also, WES is more targeted as it is exon-focused. However, by sequencing only the exonic regions, scientists have no access in the intronic information. Although some intergenic structural and some non-coding variants can be elusive by WES, the vast majority of disease-associated variants are known to be located within coding regions.

Literature provides a variety of genetic approaches that have associated specific polymorphisms with sleep characteristics, such as chronotype. According to Jones E. *et al.* (2016), chronotype can act as an indicator of the circadian rhythm. In the present research we analyzed data obtained from the application of WES in 145 participants; 98 dementia-diagnosed and 47 unaffected, focusing our studies on clock genes. We detected 91 polymorphisms, from which 34 were novel and 57 were already reported. Surprisingly, only 16 of these polymorphisms have been associated with specific pathologies in the literature.

To examine the impact of the variants we detected in the quantity and quality of participants' sleep, we performed actigraphy and polysomnography studies respectively.

Specifically, 85 individuals had actigraphy recordings for 3 subsequent days. Initially, we statistically analyzed the relation between polymorphisms in basic clock genes (*CLOCK, BMAL1, NPAS2, PERs and CRYs*) and the actigraphy parameters. As a result, we revealed 2 polymorphisms in *PER1* rs2585405 and rs150149747 that could be related with low TST. In addition, rs1776342 in *PER3* may act as risk factor for reduced TST. Further studies in larger samples are needed in order to in-depth assess these assumptions.

Following our quantitative analysis, we examined if there is any association between rare variants in our sample and actigraphy extremities. As rare variants we considered polymorphisms that present with $f_{sample} \leq 5$. Notably, 6 novel variants were present in individuals characterized by extreme actigraphy phenotype. 4 of these variations possibly have damaging impact for the respective protein, according to Polyphen 2 analysis. This information allows us to assume that the protein product of the mutated gene may be nonfunctional, leading to a cascade of impaired interactions of the circadian mechanism.

Indicatively, individual P6 has a novel variant in *NR1D1* gene, herein nNR1.1. The protein product, which is a ligand-sensitive transcription factor, represses *CLOCK* and *BMAL1* expression [ix]. Due to this mutation, it is possible that the resulting protein is unable exert its inhibitory role. Thus, Clock/Bmal1 heterodimers may induce PER and CRY expression more potently, a function that takes place when the individual is awake. In accordance with our observations, P6 is characterized by an evening-like chronotype. Furthermore, *NR1D1* regulates genes involved in metabolic functions and macrophage inflammatory response [xiii]. Consequently, we could further hypothesize that the accompanying increased BMI of P6 might be a side result of this novel variation. To be more confident regarding our findings, additional research data in individuals bearing this variant are required.

Similar associations between clock genes and chronotypes have already been reported in literature [81-83]. According to Jones E. *et al.* (2016), through GWAS analysis, morningness was related with rs75804782, a *PER2* variant that is connected with iris functionality. [81] Although their study was performed in a large sample (>119,000 individuals), a major disadvantage was that chronotype information was obtained by individual reports, and was not supported by any confirming actigraphy data.

A relation between morningness and a *PER2* polymorphism has been mentioned by Hu Y. and her colleagues, who identified in their cohort of 89,283 individuals, that rs55694368, a variant that is associated with FASPS, is also related with self-reporting morningness. Beside the fact that many loci were significantly associated with morningness; in this GWAS analysis researchers were unable to find clear genetic associations with specific sleep phenotypes, such as insomnia, sleep apnea and sleep need. However, they assumed that BMI and nicotine consumption were significantly related with morning-like chronotype. [82]

Other variants that have been associated with FASPS are *PER3*-P415A and *PER3*-H417R. According to animal model experiments these rare PER3 variants produce unstable protein products that fail to stabilize Per1/Per2 proteins, which are crucial for circadian rhythmicity. As a result, both variants lead to maladaptation of activity rhythms that causes shortening of photoperiods during the winter months. These experimental findings provide indications

about PER3-P415A/H417R role in seasonal affective disorder, as they have previously been associated with Beck Depression Inventory (BDI) as well as with seasonal mood. [83]

Besides chronotype, nap duration and nap number indicate possible nighttime sleep disorders, which affect individuals' functionality. Literature reports agree that naps are "sleep gain" during daytime with significant positive impact on individuals' vigilance, declarative and procedural memory and emotional processing as well as working memory, without affecting subsequent nighttime sleep, at least in young adults. An interesting effect of daytime napping concerns REM sleep. When the individual has a nap with duration over 30min, then he/she enters REM sleep, when memory consolidation is taking place. Therefore, working memory is enhanced, especially in adults, whose habitual nighttime sleep is short. [71]

In our actigraphy sample we depicted rs372622178, a polymorphism of *BHLHE41* gene. Although a benign alteration, it might cause sleep deficit in the carrier of this polymorphism, resulting in increased need of daytime naps. From the molecular aspect, the resulting protein may not be entirely effective in repressing Clock/Bmal1 and Npas2/Bmal1 complexes. As a result, PER and CRY expression will occur, probably keeping the individual awake. The shortening of nighttime sleep duration can cause sleep deprivation resulting in excessive need of daytime restorative sleep, as homeostatic sleep regulation dictates.

Sleep is a whole-brain phenomenon, in which brain is still processing information [13]. Electrophysiological measurements in co-cultures of neurons and glia cells show that the minimum unit for sleep generation is brain neuronal network [13-15]. Hence different sites of the brain can sleep independently in different times [13]. While studying human sleep, we cannot electrophysiologically examine each minimum sleep unit. Therefore we perform EEG studies in order to evaluate sleep quality.

In our cohort only 3 participants accepted to participate in sleep architecture studies. The small sample is a strong limitation that prevent us from relating specific polymorphisms with sleep architecture characteristics. Nevertheless, polysomnography studies confirmed actigraphy assumptions, paving the way for further research.

Sleep is a dynamic state, which can be altered by intrinsic and extrinsic substances, namely somnogens and pharmaceutical factors respectively. Ishimori (1909) was the first to introduce the ''Sleep Inducing Substance (SIS)'' Hypothesis [77]. SIS hypothesis, which is also referred as ''Sleep-Regulatory Substance'' Hypothesis, [78], report that a group of molecules such as adenosine, nitric oxide (NO), prostaglandin D (PGD), tumor-necrosis factor (TNF), interleukin 1 (IL-1), and growth-hormone-releasing hormone (GHRH), are accumulated when the individual is awake and are degraded during sleeping. These molecules are also called somnogens and seem to be connected to process S. [13, 15, 77, 78] More specifically, experiments showed that these molecules are capable to induce sleep when injected in animal models, inducing EEG delta waves production during NREM sleep, which are high-amplitude slow waves (*Figure 42*). In contrast, in the absence of somnogens, low-amplitude fast waves are generated, indicating that neurons are "awake". [13] However, further research on these substances' targets and their electrophysiological impact on the cellular level are imperative [19].

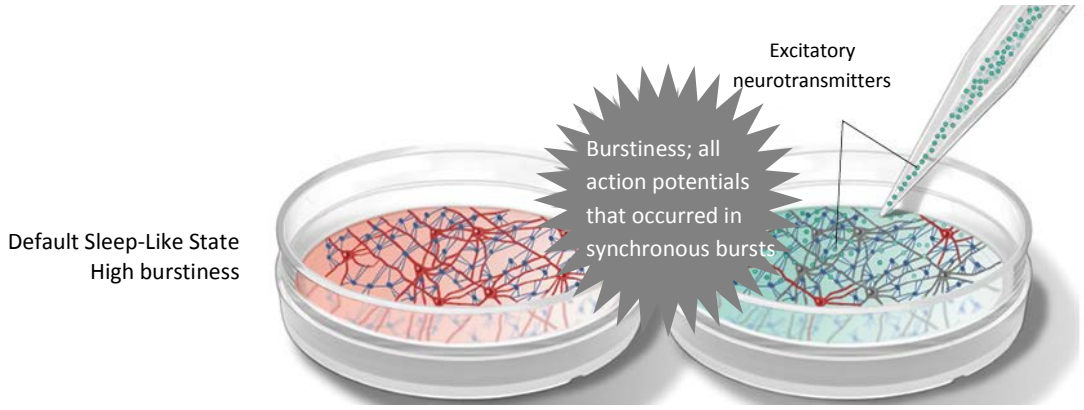

Figure 42. Sleep in a dish: co-cultures of neurons and glia cells indicate that the network's default state is sleep-like. Somnogens can sustain this state for longer periods, as they increase burstiness and delta waves- amplitude. But, when excitatory neurotransmitters are added in the medium, then the synchronized burstiness of neurons is reduced. As a result, network state converts to "awake" and gene expression initiates [13].

Another theory that tries to explain the mystery of molecular basis underlying sleep is "Synaptic Homeostasis Hypothesis" [79]. During wakefulness, strong synaptic changes can occur, resulting in gene expression induction (*Figure 41*). Among these genes, is induced *BDNF*, which is an immediate early gene (IEG) that plays a pivotal role in synaptic plasticity and long-term potentiation (LTP) [19, 80]. BDNF levels are increased during wakefulness and seem to be related to increased slow-wave activity during sleep [80].

It has been proposed that in SWS, depotentiation genes such as calcineurin are activated, in order to cause synaptic downscaling. This finding is also supported by the fact that process C is also related to Ca^{+2}- dependent pathways and its parameters [19]. Hence, alterations in these pathways are capable to change the electrophysiological activity of cortical neurons that affect sleep homeostasis. [19] Thereby, a possible interpretation of synaptic downscaling during sleep is that memory consolidation and energy saving occur at that point. An in-depth examination of synaptic homeostasis hypothesis indicates that sleep is an opportunity for upregulation of genes involved in heme, protein, and lipid synthesis and for encoding the structural constituents of the ribosomes. [80] To date, there is no computational model that could explain this theory, coupling macroscopic (slow homeostatic dynamics, e.g. of process S), with microscopic (fast electrophysiological activity e.g. slow-wave oscillations) data [19].

The above theories indicate that circadian alteration can occur in any basis, molecular, electrophysiological, genetic etc. The inner clock can lose its periodicity in high-amplitude slow waves or burst-pause firing (sleep) and low-amplitude fast waves or transient tonic firing (awaking) oscillations, due to many factors such as sleep deprivation, aging, medication and comorbidities. In our elderly cohort, the latter factors can have a great impact in sleep regulation, in combination with the accompanying genetic variations' impact, all together resulting in an impaired sleep phenotype.

The present research is a novel approach in investigating the effects of clock genes' polymorphisms in sleep quality and quality. Actigraphy and polysomnography data give impetus to study further these polymorphisms in a deeper level, where co-cultures of neural and glia cells, as well as computational models, can emerge circadian arrhythmicity and electrophysiological alterations. These alterations may impair network synchronization of the brain in a way that generates sleep disorders, especially in elderly individuals.

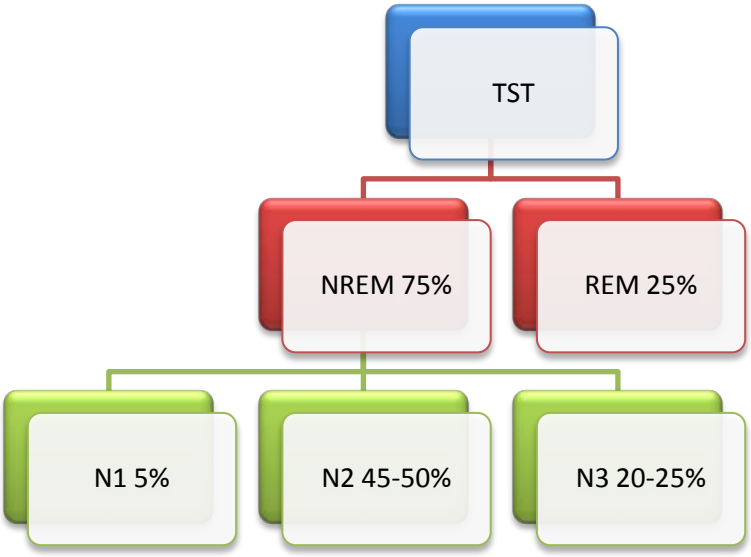

Figure 413. Proportion of sleep stages in normal sleep [76]

Table 15. Values of descriptive and inferential statistics for rs2585405

PER1:rs2585405					
Night TST	Groups	N	Mean	Std. Deviation	Std. Error Mean
	No	16	496,0194	113,86975	28,46744
	Yes	69	441,4228	95,98208	11,55488

Table 16. Values of descriptive and inferential statistics for rs150149747

PER1:rs150149747					
TST	Groups	N	Mean	Std. Deviation	Std. Error Mean
	No	60	513,6445	138,25018	17,84802
	Yes	25	452,1408	93,80636	18,76127

Table 17. Values of descriptive and inferential statistics for rs1776342

PER3:rs1776342					
TST	Groups	N	Mean	Std. Deviation	Std. Error Mean
	No	26	537,5892	160,98339	31,57144
	Yes	59	477,0317	109,19334	14,21576

Table 18. Novel variants and their genetic details

Gene	Novel variant	Chromosomal position	Coding DNA change	Protein change
BMAL1	*nBM.1*	chr11:13398218	c.1354T>G	p.Phe452Val
PER1	*nP1.1*	chr17:8046598	c.3058C>A	p.Pro1020Thr
	nP1.2	chr17:8048287	c.2243C>T|	p.Pro748Leu|
	nP1.3	chr17:8053631	c.280A>G	p.Asn94Asp
	nP1.4	chr17:8045268	c.3455T>C	p.Met1152Thr
	nP1.5	chr17:8045707	c.3328_3328delG	p.Ala1110fs
	nP1.6	chr17:8047027	c.2629T>C	p.Phe877Leu
	nP1.7	chr17:8053978	c.47C>T	p.Pro16Leu
	nP1.8	chr17:8047168	c.2488A>C	p.Ser830Arg
	nP1.9	chr17:8047170	c.2486C>A	p.Pro829Gln
	nP1.10	chr17:8052885	c.748C>T	p.Arg250Trp
	nP1.11	chr17:8047010	c.2646G>C	p.Gln882His
	nP1.12	chr17:8047026	c.2630T>C	p.Phe877Ser
PER2	*nP2.1*	chr2:239155030	c.3754G>A	p.Glu1252Lys
	nP2.2	chr2:239168662	c.1574_1574delA	p.Asn525fs
	nP2.3	chr2:239184471	c.361C>T	p.His121Tyr
	nP2.4	chr2:239171620	c.1126C>T	p.Pro376Ser
	nP2.5	chr2:239162235	c.2429G>A	p.Ser810Asn
	nP2.6	chr2:239161932	c.2732A>G|c.2731_2732insC	p.Tyr911Cys|p.Tyr911fs
	nP2.7	chr2:239161768	c.2896C>T	p.Arg966Trp
PER3	*nP3.1*	chr1:7887324	c.2311T>C	p.Cys771Arg
	nP3.2	chr1:7887201	c.2188C>G	p.Arg730Gly
CRY2	*nC2.1*	chr11:45868967	*(is a utr-5' variation)*	
REV1	*nR1.1*	chr2:100038077	c.1715C>T	p.Ala572Val
	nR1.2	chr2:100019495	c.3240_3241insC	p.Gly1081fs
	nR1.3	chr2:100019230	c.3418G>C	p.Gly1140Arg
	nR1.4	chr2:100019253	c.3395C>T	p.Ser1132Phe
	nR1.5	chr2:100055601	c.675T>A	p.Phe225Leu
	nR1.6	chr2:100022880	c.2521C>G	p.Gln841Glu
NR1D1	*NR1.1*	chr17:38252780	c.520A>C	p.Asn174His
BHLHE40	*nB40.1*	chr3:5021356	*(is a utr-5' variation)*	
BHLHE41	*nBH41.1*	chr12:26275127	c.1321C>T	p.Leu441Phe
	nBH41.2	chr12:26277681	c.32G>C	p.Arg11Thr
VDR	*nD1*	chr12:48250920	c.725C>G	p.Thr242Ser

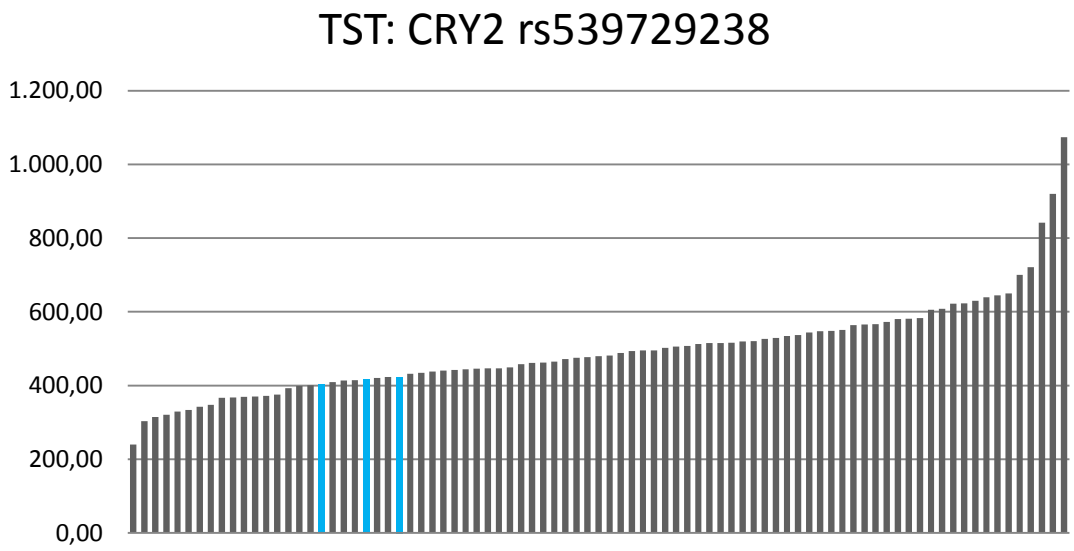

Figure 424. Distribution of mean TST measurements of the individuals with rs539729238 (colored in blue)

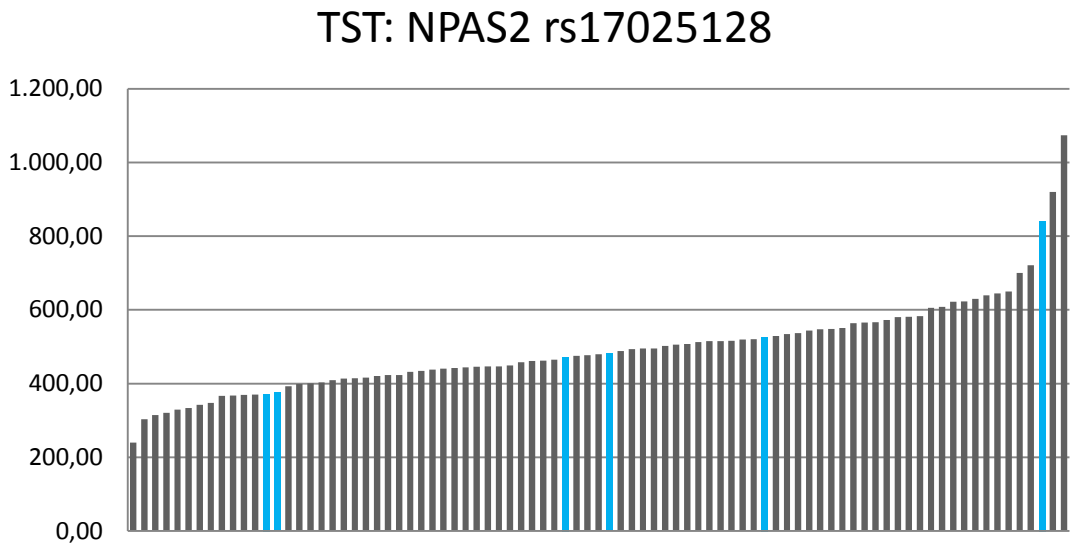

Figure 435. Distribution of mean TST measurements of the individuals with rs17025128 (colored in blue)

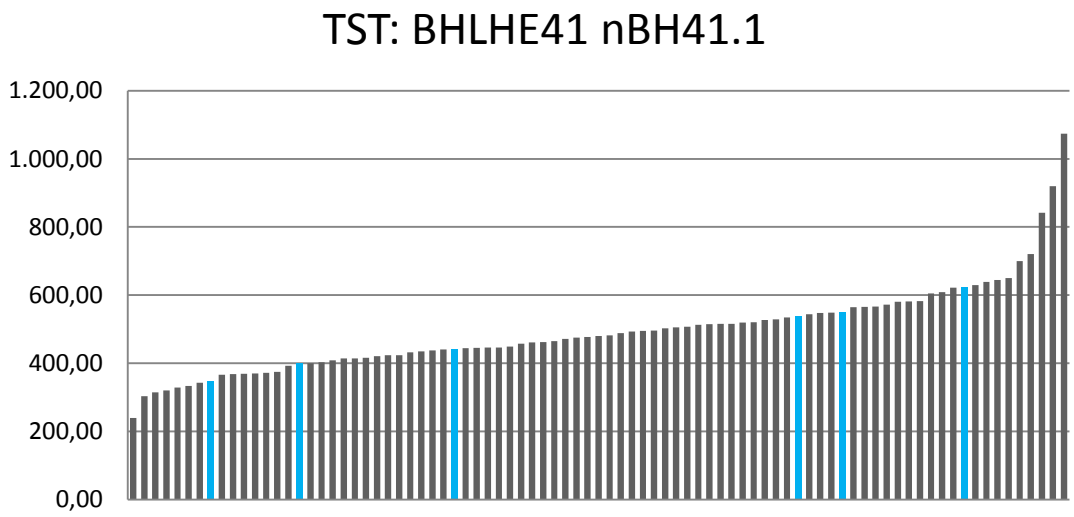

Figure 446. Distribution of mean TST measurements of *nBH41.1* (colored in blue the individuals with)

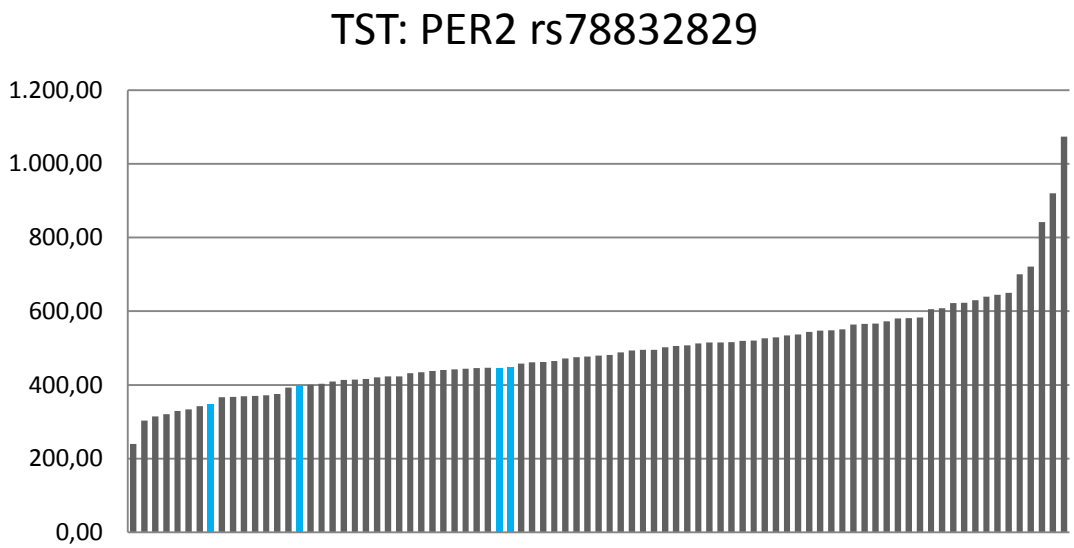

Figure 457. Distribution of mean TST measurements of the individuals with rs78832829 (colored in blue)

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Figure 46. Dreaming in art: “Sleep”, Hadley Hooper, 2007

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Web Sites

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