Circadian Rhythm: Molecular Mechanisms and Pharmacology

Scott Pedersen
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A circadian rhythm or circadian cycle, is a natural, internal process that regulates the sleep–wake cycle and repeats roughly every 24 hours.

- Responsive to external stimuli (e.g. light, feeding, exercise)
- Widely observed in plants, animals, fungi, and cyanobacteria

*But how does this work?*
Outline

- Discovery of circadian rhythm
- Molecular mechanisms of mammalian circadian rhythms
- Overview of associated diseases and treatment landscape
- Case studies in modulating the circadian machinery
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Circadian rhythms: a timeline

371 BC - First written account of a circadian rhythm

Theophrastus
Greek philosopher + father of botany
371 BC – 287 BC

“... tree with many leaves like the rose, and that this closes at night, but opens at sunrise, and by noon is completely unfolded; and at evening again it closes by degrees and remains shut at night, and the natives say that it goes to sleep.”

Circadian rhythms: a timeline

1729 - first controlled experiment implicating a “biological clock”

Jean-Jeaque de Mairan
French scientist
1678–1771

Mimosa pudica

Circadian rhythms: a timeline

1729 - first controlled experiment implicating a “biological clock”

Jean-Jeaque de Mairan
French scientist
1678–1771

Continued adherence to the daily rhythm in an unlit environment suggested a free running, intrinsic biological timer

1971 - genetic link discovered for the first time

Ron Konopka (left) Seymore Benzer (right)

1971 Konopka and Benzer discover three genetic mutants of *drosophila* with altered circadian rhythms

- Different rhythms in eclosion (hatching) and locomotion were observed
- Mutations mapped to the same locus, subsequently named *period*
Circadian rhythms: a timeline

1984 - drosophila period gene isolated

Key finding: certain subsegments of the per region would restore rhythmicity in circadian locomotor behavior transduced into the genome of arrhythmic flies

Circadian rhythms: a timeline

1984 - drosophila period gene isolated

Wild type: eclosion occurs with rhythmicity

Per mutant: no rhythmicity in eclosion

Rhythmicity restored when per gene transduced into mutant flies

Implication of a transcription/translation feedback loop

Circadian rhythms: a timeline

1984 - drosophila period gene isolated

Per and the circadian clock

- *period* DNA transcribed into *period* mRNA
- *period* mRNA is transported into cytoplasm
- *period* RNA translated into PER protein
- **PER** protein accumulates in cell’s nucleus
- **PER** protein inhibits transcription of *period* gene
- **PER** protein cleared by cell, resetting cycle

Implication of a transcription/translation feedback loop

Circadian rhythms: a timeline

The Nobel Prize in Physiology and Medicine 2017 was awarded jointly to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young

“for their discoveries of molecular mechanisms controlling the circadian rhythm”

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- Case studies in modulating the circadian machinery
The Ubiquitous, Cell-Autonomous Molecular Oscillator

The cell autonomous molecular oscillator controls temporal gene expression in each cell.

Lots of opportunities for fine-tuning circadian machinery!

Suprachiasmatic Nucleus (SCN): The Master Oscillator

Peripheral clocks
- Heart
- Bronchi of lungs
- Liver
- Stomach
- Small intestines
- Adrenal gland
- Kidney
- Large intestine
- Rectum

Neuronal control
- Sympathetic and parasympathetic pathways

Hormonal control
- Glucocorticoids
- Melatonin
- Orexin

Excitatory: Glutamate
- SCN (master oscillator)
- Central clock (~24 hours)
- Excitatory: PACAP
- Inhibitory: GABA

CREB
- Core clock gene expression: PER1 and PER2

Postsynaptic SCN neuron

Melatonin receptor agonism causes sleepiness
Hormonal signaling from the SCN: orexin

Orexin receptor agonism prevents sleep

- Regulation of the transition between wakefulness and the NREM sleep state, and the stabilization of wakefulness (by OX2R)
- Suppression of REM sleep (by both OX1R and OX2R)
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Some common sleep disorders

**Insomnia**

**Jet Lag or Shiftwork**

**Non-24**

Some common sleep disorders

Insomnia: 25% of Americans develop insomnia each year; 10% progress to chronic insomnia.

Jet Lag or Shiftwork: Frequently associated with anxiety, depression, risk of cardiovascular disease, and poor quality of life.

Some common sleep disorders

- Insomnia
- Jet Lag or Shiftwork
- Non-24

Mice subjected to a chronic jet lag paradigm (8-hour phase shift) showed significantly reduced lifespan with disease development, including neurodegeneration, severe ulcerative dermatitis, aging, cystic renal dysplasia, and cancer.

Some common sleep disorders

- **Insomnia**
- **Jet Lag or Shiftwork**
- **Non-24**

**Insomnia**

- chronic steady pattern comprising daily delays in sleep onset and wake times in an individual living in a society

**Jet Lag or Shiftwork**

- Phase advance (six zones east)
- Phase delay (six zones west)

**Non-24**

- >50% of the totally blind suffer from this affliction, rarely occurs in sighted people

Circadian rhythm signaling in disease

Circadian rhythm aberrations are implicated in many chronic and acute illnesses.

Rescuing or repairing circadian rhythm could minimize the progression of or outright prevent a wide range of diseases.

Heart attack/ stroke

Daily headaches

Aging

Alzheimer’s

Current landscape of sleep medicine

**barbiturates (1903)**
- Phenobarbital (Luminal)

**benzodiazepines (1960s)**
- Temazepam (Restoril)
- Triazolam (Halcion)
- Doxepin

**methaqualone (1961)**
- Methaqualone (Quaalude)

**non-benzodiazepines (1980s)**
- Zolpidem (Ambien)
- Zaleplon (Sonada)
- Eszopiclone (Lunesta)

Current landscape of sleep medicine

Why do all of these medicines exhibit similar negative effects?

All either act as GABA_A receptor agonists or positive allosteric modulators
Current landscape of sleep medicine

Why do all of these medicines exhibit similar negative effects?

All either act as GABA\textsubscript{A} receptor agonists or positive allosteric modulators

- Activates chloride ion flux in neurons
- Inhibit the propensity of neurons containing GABA\textsubscript{A} to propagate action potentials
- Change in mood, slowed reaction time, motor deficits, amnestic effects, and respiratory effects


Hormonal signaling opportunities

Melatonin and orexin – two complementary hormones

**Melatonin signalling pathway**

- Melatonin
- MT₁, MT₂
- Inhibition of cAMP
- Inhibition of protein kinase A
- Reduced CREB phosphorylation
- Inhibition of the neuronal firing in the SCN
- Facilitates bedtime sleep onset
- Phase-shifting effects on the circadian rhythm

**Orexin signalling pathway**

- Orexin A, Orexin B
- OX₁R, OX₂R
- Regulation of the transition between wakefulness and the NREM sleep state, and the stabilization of wakefulness (by OX₂R)
- Suppression of REM sleep (by both OX₁R and OX₂R)

**Therapeutic goals**

- Accelerate sleep onset
- Improve sleep duration
- Minimally impact other processes

**Agonism via** melatonin or melatonin analogues

**Antagonism via** novel inhibitors

Melatonin supplementation as a sleep aid

- Approved for medical use in Europe, considered a dietary supplement in the US
- Found to decrease sleep latency by 7.1 minutes and increase sleep duration by 8.3 minutes
- Conclusion: safe, but minimally effective

Note: melatonin in the US is not well quality controlled. Melatonin content has been found to range from -83% to +478% the listed amount

Other melatonin receptor agonists (late 2000s)

Goal: improve pharmacokinetics and half-life of melatonin (20–50 minutes)

- Half life of 1–2.6 hours
  - Sleep latency decreased by 4–7 minutes
  - Meta analyses show mixed impact on total sleep time
  - Approved for treatment of insomnia

- Half life of 55–100 minutes
  - Approved as orphan drug for N24SWD in blind patients
  - Found to increase total sleep time by 60 minutes in patients with 8 hour jet lag from eastward travel, and improve time to sleep
  - Rejected by FDA 3 times between 2014 and 2022 for this indication.

Orexin receptor antagonists (2010s)

- Suvorexant
- Lemborexant
- Daridorexant

Mechanism of action:
- Prevent binding of orexin to promote sleep

Preserve night time arousability (monkey)

Improved daytime cognitive performance
(Compared to other sleep aids, rats)

Beneficial effects in total sleep time, decreases in waking after sleep onset, subjective sleep latency

- No evidence of addiction potential!
- Not as effective as benzodiazepines/ nonbenzodiazepines

Cognitive behavioral therapy for insomnia (CBT-I) is considered the gold standard for treatment. Behavioral treatment aligns with American and European guidelines.

Behavioral treatment by American and European guidelines

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proteasomal degradation

transcription of clock-controlled genes

Well defined signaling architecture—
Lots of opportunity for modulation!

Phenotypic screening for circadian rhythm perturbation

Longdaysin found to shift the period of circadian rhythm gene expression in a dose dependent manner in human U2OS cells

Longdaysin induced period change observed in diverse cell cultures

Ruan, W.; Yuan, X.; Eltzschig, H. K. *PLOS Biol.* 2010, 8(12), e1000559.
What proteins does longdaysin target?

Targets identified to be ~10 kinases with unknown connection to clock mechanisms.
Which of Longdaysin’s targets causes phase delay?

Knockout of individual genes coding for longdaysin did not induce phase delay

Knocking out CK1δ, CK1α, and ERK together recapitulated phase delay of Longdaysin

Key point: no one kinase solely responsible for maintaining circadian rhythm

Ruan, W.; Yuan, X.; Eltzschig, H. K. PLOS Biol. 2010, 8(12), e1000559.
The Ubiquitous, Cell-Autonomous Molecular Oscillator

Long daysin induces phase delay by CK1 inhibition.

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Long days in induces phase delay by CK1 inhibition
The Ubiquitous, Cell-Autonomous Molecular Oscillator

Inhibition of CRY ubiquitination to lengthen circadian period

Will slowing degradation of CRY induce phase delay?

transcription of clock-controlled genes

cytoplasm

nucleus

Cryptochrome ubiquitination inhibition leads to period lengthening

KL001 found to induce phase delay by inhibiting CRY ubiquitination

CRY proteins negatively regulate genes encoding rate-limiting enzymes of gluconeogenesis

Opportunity for development of therapeutics for diabetes treatment

The Ubiquitous, Cell-Autonomous Molecular Oscillator

transcription of clock-controlled genes

proteasomal degradation

REV-ERB agonism to decrease clock amplitude, lowering signaling when awake

Can diminished expression of BMAL1 lower clock amplitude?

REV-ERB agonism successfully shrinks amplitude of circadian signaling without modifying period

Expression of clock dependent genes accordingly down-regulated

**Diminished expression of BMAL1 in mice**

*How does diminished expression of BMAL1 effect the activity of mice?*

- SR9011 lowered activity significantly in mice kept solely in dark
- SR9011 delayed circadian activity by 3 h in mice kept in 12h light/dark cycles

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SR9011 interacts with metabolism

Despite lowered activity, mice were observed to lose weight following dosing with SR9011.

Elevated energy expenditure (by VO₂) coupled with conserved food intake led to weight loss.

Decreased fat mass, plasma triglycerides, and cholesterol levels.

REV-ERB agonists as a treatment for metabolic diseases?

The Ubiquitous, Cell-Autonomous Molecular Oscillator

ROR agonism to increase clock amplitude, raising signaling when awake
Nobiletin as a circadian amplitude enhancer via ROR agonism

Nobiletin treatment increased the amplitude of circadian rhythm gene expression

Nobiletin treatment led to weight loss in mice via increased activity

Nobiletin treated mice were more active regardless of light schedule

Nobiletin treated mice on a high fat diet gained less weight than controls – in a clock dependent manner

Circadian rhythm amplification as a treatment for metabolic disease and/or age related decline?

Circadian rhythm modulators in the treatment of glioblastoma

5–10% survive >5 years after diagnosis

Standard of care is maximal surgical resection, radiation therapy, and chemotherapy

Glioblastoma stem cells found to exhibit
Strong circadian signaling—
A therapeutic opportunity?

Will knocking out key clock transcription factors impact cell growth?

Proof of concept: knockout core clock transcription factors to inhibit glioblastoma growth

Growth in glioblastoma stem cells mitigated when BMAL1 and CLOCK genes were knocked out

Growth in other brain cell cultures maintained upon BMAL1 and CLOCK knockout

Glioblastoma growth uniquely sensitive to clock activity

The Ubiquitous, Cell-Autonomous Molecular Oscillator

Proteasomal degradation

KL001
Slowed Ubiquitination

PERs + CRYs

PERs CRYs CK1s

RORs

REV-ERB

SR9011
Amplitude Dampening

Amplitude can be dampened (SR9011)

Period can be lengthened (KL001)

Transcription of clock-controlled genes

BMAL1

CLOCK

E-box

D-box

NFIL3

DBP

REV-ERB

RORE

Pharmacological intervention in glioblastoma stem cell antagonism

Clock interactions are maintained in glioblastoma cells through dose dependent gene suppression

Pharmacological intervention in glioblastoma stem cell antagonism

Both compounds inhibited GSC growth

Combination approach proved more effective

Clock transcription factor knockout in glioblastoma bearing mice

Core clock transcription factor knockout experiments performed in mice bearing glioblastoma stem cells

Survival of mice with core clock transcription factors BMAL1 and CLOCK knocked out exhibited greatly improved lifespan

Visual examination of mouse brain slices reveals minimal glioblastoma signs in knockout mice compared to control
Perspective and Outlook

Circadian rhythm modulation offers an opportunity for treatment that is mechanistically distinct from classic approaches.

Regulating the circadian machinery is a noteworthy approach for the treatment of a broad range of non-sleep related disorders.

No period-shortening interventions have been published to date.

Questions?