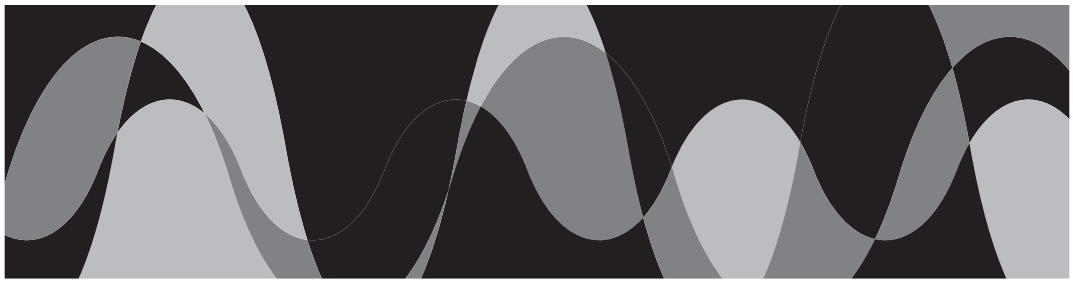

Tenth Meeting

Society for Research on Biological Rhythms

Program and Abstracts

SRBR

May 21–25, 2006

Sandestin Golf and Beach Resort

Sandestin, FL

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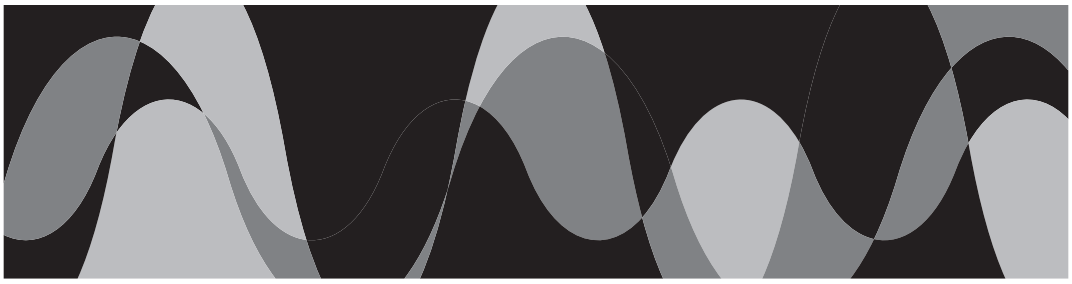

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The SRBR wishes to thank the following for their contributions:

Actigraph

Alliance Pharmaceuticals

Alpco Diagnostics

NINDS (National Institute of Neurological Disease and Stroke)

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General Information

Registration

Meeting registration will take place on Sunday, May 21, from 15:00 until 19:00 and on May 22–25 from 8:00 until 11:00 and 14:30 until 16:30 May 22–24 in the Foyer of the Baytowne Conference Centre.

Early Registration:
Postmarked by April 5, 2006

SRBR Member: $325.00

Non-Member: $375.00

SRBR Student Member: $165.00

Non-Member Student: $190.00

Guest Registration: $75.00

Late Registration:
Postmarked after April 5, 2006

SRBR Member: $350.00

Non-Member: $400.00

SRBR Student Member: $175.00

Non-Member Student: $200.00

Guest Registration: $100.00

Membership rates apply to individuals who have joined SRBR before the meeting and have paid current dues. Student rates apply to registrants who will not have received their Ph.D. or M.D. degree at the time of the meeting. Registered participants and guests are invited to the opening reception and the closing banquet.

President's Welcome

Twenty years ago, on November 12, 1986, Fred Turek, Dave Hudson, Joe Takahashi, and Gene Block founded the Society for Research on Biological Rhythms. Who would have imagined that today we are analyzing the structural biology of bacterial clock proteins? Or that we are performing real-time measurements of rhythmic gene expression within individual cells in a dish? Or that we are delineating the neurobiology of a neurotransmitter that underlies narcolepsy? And that's just for starters. Every other year, we have been gathering at this meeting to announce, debate, and celebrate these advances, in presentations that cut across disciplines, approaches, and organisms.

Welcome to the 10th Biennial Meeting of the Society for Research on Biological Rhythms. I wish to gratefully thank Carla Green and her Program Committee for expertly organizing the scientific treat you are about to enjoy, Ken Wright, Jr. and his committee for awarding our trainee travel fellowships, Wendy Pickering and Karen Nichols at Conferences and Institutes for their fantastic work to ensure that the meeting runs smoothly, and our government and corporate sponsors for making it all possible.

And, of course, I extend my special thanks to all of you, presenters and participants, for sharing your passion, energy, and intelligence to make the meeting such a biennial success. To everyone, my best wishes for a fruitful week of sun, sand, and circadian—and ultradian and infradian—biology.

William J. Schwartz
President, SRBR, 2004–2006

SRBR Information and Message Desk

The Society will maintain an information desk in the Foyer of the Baytowne Conference Centre from 8:00 to 11:00 on May 22-25, and from 14:30 to 16:30 on May 22–24. Late arrivals can register during these times. A message board will be located next to the information desk. Meeting participants are asked to check the message board routinely for mail, notes, and telephone messages.

Conference Center Information

The Baytowne Conference Center is conveniently located within walking distance of all properties. All SRBR scientific sessions and social events will take place at the Center.

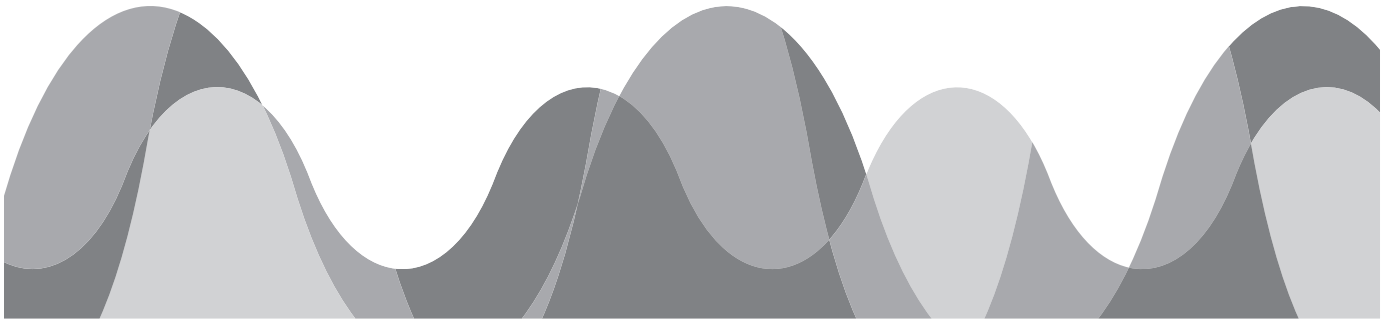
Instructions to Presenters

All posters will be displayed throughout the meeting, with even numbered posters presented on Monday, May 22 and odd numbered posters presented on Wednesday, May 24, 2006. Please hang posters on Monday morning (May 22) between 8:00 and 10:00. All posters must by removed by 10:00 on Thursday, May 25.

Computer projectors will be set up in each meeting room. PowerPoint presenters should bring their data on a computer or shared computer.

Social Events

The SRBR Opening Reception will begin at 19:00 on Sunday, May 21 on the Grand Lawn. A banquet for conference attendees and guests will be held in the Magnolia Ballroom on Thursday, May 25 at 20:00.



Meeting at a Glance

All events will take place in the Sandestin Conference Center unless otherwise noted

Sunday May 21

19:00–22:00 **Opening Reception**—*Grand Lawn*

Monday May 22

08:00–10:00 **Poster Session Setup**—*Magnolia ABC*

08:30–10:30 **Symposium 1: Molecular Regulation of Circadian Rhythms**—*Azalea I*

Symposium 2: Neuronal Pacemaker Organization and Disorganization—*Azalea II*

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Sessions A–C**

A) Mammalian Clock Genes I—*Azalea I*

B) Photoperiodic Timers and Development—*Azalea II*

C) Clocks and Disease—*Azalea III*

12:30–16:30 Free Time

16:30–18:30 **Symposium 3: Circadian Clock Mechanisms I**—*Azalea I*

Symposium 4: Circadian Clocks and Cognition—*Azalea II*

Symposium 5: Extra-SCN Oscillators—*Azalea III*

Workshop I: Assessment of Chronotype: Tools and Tricks—*Camellia II*

20:00–22:30 **Poster Session I:** Even numbered posters—*Magnolia ABC*

Tuesday May 23

8:30–10:30 **Symposium 6: Sleep and Human Circadian Biology**—*Azalea I*

Symposium 7: Transcription/Translation Feedback Loops and Neuronal Electrochemical Signaling—*Azalea II*

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Sessions D–F**

D) Drosophila Clock Genes—*Azalea I*

E) SCN—*Azalea II*

F) Peripheral, Oscillators/Endocrine—*Azalea III*

12:30–16:30 Free Time

16:30–18:00 **Presidential Symposium: Simple Principles for Complex Systems**—*Azalea I*

18:00 Free Night

Wednesday May 24

8:30–10:30 **Symposium 8: Organization of the Mammalian Circadian Timing System: Centrifugal or Centripetal?**—*Azalea I*

Symposium 9: 'Green' Clocks: Conservation and Diversity of Circadian Architectures

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Sessions G–I**

G) Entrainment—*Azalea I*

H) Clocks and Metabolism—*Azalea II*

I) Sleep—*Azalea III*

12:30–16:30 Free Time

16:30–20:30 **Symposium 10: Mathematical Modelling**—*Azalea I*

Symposium 11: Circadian Clock Mechanisms II—*Azalea II*

Symposium 12: Shedding Light on Human Clock Function Disorders—*Azalea III*

Workshop II: Comparative Anatomy of Circadian Pacemaker Networks in Arthropod Brains—*Camellia II*

20:00–22:30 **Poster Session II:** Odd numbered posters—*Magnolia ABC*

Thursday May 25

8:30–10:30 **Symposium 13: Highlights from New Investigators**—*Azalea I*

Symposium 14: Non-Image Forming Photoreception and Photoentrainment—*Azalea II*

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Session J–l**

J) Mammalian Clock Genes II—*Azalea I*

K) Plant and Neurospora Clock Genes—*Azalea II*

L) Human Circadian Rhythms—*Azalea III*

12:30–16:30 Free Time

16:30–17:30 **Business Meeting**—*Azalea I*

17:45–1750 **A Tribute to Erwin Bünning (1906–1990) on his 100th Birthday**—*Azalea I*

17:50–19:00 **Pittendrigh/Aschoff Lecture: Michael Young (Rockefeller University)**

New Roles for Old Proteins in the Drosophila Clock—*Azalea I*

20:00–23:00 **Banquet**—*Magnolia ABC*

Program for 2006 SRBR Meeting

Sunday May 21

19:00–22:00 Opening Reception

Monday May 22

08:00–11:00 **Poster Session Setup**—*Magnolia ABC*

08:30–10:00 **Symposium 1**—*Azalea I*

Molecular Regulation of Circadian Rhythms

Chair: Joseph Takahashi (Northwestern University)

08:30 **A Conditionally Active Liver Clock: Inputs and Outputs** • Ueli Schibler, University of Geneva

09:00 **Nuclear Receptor Expression Links the Circadian Clock to Metabolism** • Ronald Evans, Salk Institute

09:30 **The Role of Peripheral Clocks in Cardiovascular and Metabolic Function** • Garret FitzGerald, University of Pennsylvania

10:00 **From Analysis to Synthesis of Mammalian Circadian Clocks** • Hiroki Ueda, RIKEN

Symposium 2—*Azalea II*

Neuronal Pacemaker Organization and Disorganization

Chair: Rae Silver (Columbia University)

08:30 **Mechanisms Mediating Circadian Rhythm Amplitude and Synchrony in the SCN** • Erik Herzog, Washington University

09:00 **Disorganizing Pacemakers with Odd LD Cycles** • Horacio de la Iglesia, University of Washington

09:30 **Encoding Daylength: Single Cell or Network Property?** • Johanna Meijer, Leiden University

10:00 **The Circadian Network and Light in *Drosophila*** • Michael Rosbash, Brandeis University

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Session A**—*Azalea I*

Mammalian Clock Genes I

Chair: David Weaver (University of Massachusetts Medical School)

11:00 **1 • Use of the Tetracycline System in Circadian Biology: Conditional and Tissue-Specific-Induction of Clock** • Hee-Kyung Hong, Northwestern University

11:15 **2 • Tetracycline-Inducible Expression of Wld-Type and Tau Mutant Casein-kinase 1 epsilon in Mice** • Caroline H. Ko, Northwestern University/University of Toronto

11:30 **3 • Mechanisms of Cyclic Transcriptional Regulation Through C-terminal Domain of BMAL1 Mammalian Circadian Clock Protein** • Kazuhiro Yagita, Nagoya University

11:45 **4 • A Clock Shock: Mouse CLOCK is not Required for Circadian Oscillator Function** • Jason P. DeBruyne, University of Massachusetts Medical School

12:00 **5 • CRYPTOCHROMES Alter CLOCK/BMAL1 Co-Dependent Phosphorylation: A Mechanism for Inhibition of Circadian Transcriptional Activation** • Nicolas Cermakian, Douglas Hospital Research Centre

12:15 **6 • Cryptochromes and Photolyases: Interchangeable Core Domains?** • Inês Chaves, Erasmus University Medical Center

Slide Session B—*Azalea II*

Photoperiodic Timers and Development

Chair: Gregory Cahill (University of Houston)

11:00 **7 • Circadian Clocks and Photoperiodic Timers–From Geography to Gene Expression** • William E. Bradshaw, Center for Ecology & Evolutionary Biology

11:15 **8 • Molecular Links between Development and the Circadian Oscillator in Drosophila** • Nicholas R.J. Glossop, The University of Manchester

11:30 **9 • CLK Expression and Regulation during Development in Drosophila** • Paul E. Hardin, Texas A&M University

11:45 **10 • The Clk Gene Has a Broad Ability to Induce Circadian Cycling in Non-Clock Cells** • Valerie L. Kilman, Center for Sleep and Circadian Biology

12:00 **11 • Development of the Zebrafish Pineal Gland Clock** • Yoav Gothilf, Tel Aviv University

12:15 **12 • Atp6v1e1 Is Regulated by OTX5, CLOCK1 and BMAL1, and Affects the Pineal Formation in Zebrafish** • Han Wang, University of Oklahoma

Slide Session C—*Azalea III*

Clocks and Disease

Chair: David Earnest (Texas A&M)

11:00 **13 • Determination of Circadian Period Length in Delay Sleep Phase Syndrome Patient Fibroblasts** • Filippo Tamanini, Erasmus MC

11:15 **14 • Molecular Basis for the Human Familial Advanced Sleep Phase Syndrome Phenotype** • Katja Vanselow Charite, Humboldt-University Berlin

11:30 **15 • Improved Tumor Control and Cell Cycle Inhibition Through Circadian Clock Induction** • Ida Iurisci, INSERM

11:45 **16 • The Role of the Circadian Clock in Mammary Gland Development and Breast Cancer** • Richard P. Metz, Texas A&M University

12:00 **17 • Circadian Regulation of Immune Functions in Splenic Macrophages** • Bert Maier Charite, Humboldt-University Berlin

12:15 **18 • The Molecular Clock and Circadian Variability in Thrombosis** • Elizabeth J. Westgate, University of Pennsylvania

16:30–18:30 **Symposium 3**—*Azalea I*

Circadian Clock Mechanisms I

Chair: Elizabeth Maywood (MRC Laboratory of Molecular Biology)

16:30 **Negative and Positive Feedback Loops of the Circadian Clock of *Neurospora*** • Michael Brunner, University of Heidelberg

16:55 **How Do Drosophila Pacemaker Neurons Process Light Signals?** • Justin Blau, New York University

17:30 **Transcriptional Control of the Circadian Clock** • Steven Reppert, University of Massachusetts

17:45 **Circadian Clock Function in the Mammalian Retina** • Charles Weitz, Harvard Medical School

18:10 **Hot Topic Presentation: A Chromatin Remodeling** • Paolo Sassone-Corsi, University of California, Irvine

Symposium 4—*Azalea II*

Circadian Clocks and Cognition

Chair: Martin Ralph (University of Toronto)

16:30 **Cognitive Performance Rhythms in Social Children** • Mary Carskadon, Brown University

16:55 **Implicit Time Memory** • Martin Ralph, University of Toronto

17:20 **The Roles of Sleep States in Memory Consolidation** • Carlyle Smith, Trent University

17:45 **A Neuronal Circuit for Rhythmic Motivation and Learning** • Carolina Escobar, Nacional Automa de Mexico

18:10 **Diurnal Rhythms and Executive Function** • Constanze Hahn, University of Toronto

Symposium 5—*Azalea III*

Extra-SCN Oscillators

Chair: Theresa Lee (University of Michigan)

16:30 **Clock Gene Dependent and Independent Mechanisms of Methamphetamine-Induced Circadian Rhythms** • Sato Honma, Hokkaido University

17:00 **Localizing a Food-Entrainable Pacemaker in Mammals** • Ralph Mistlberger, Simon Fraiser University

17:30 **The Methamphetamine-Sensitive Circadian Oscillator in Mice** • Michael Menaker, University of Virginia

18:00 **Where is the Food—Entrainable Circadian Oscillator(s)** • Masashi Yanigasawa, University of Texas, Southwestern

Workshop I—*Camellia II*

Assessment of Chronotype: Tools and Tricks

Chair: Derk-Jan Dijk (University of Surrey)

16:30 **Diurral Preferences: It's Not All in Your Genes** • Simon Archer, University of Surrey

16:40 **Assessing Social Jetlag with the Munich ChronoType Questionnaire, MCTQ** • Till Roenneberg, University of Munich

16:50 **Chronobiological Studies in Early and Late Chronotypes Selected with the Munich ChronoType Questionnaire, MCTQ** • Marijke Gordijn, University of Groningen

17:00 **The Forced Desynchrony Protocol for Assessing Chronotypes** • Jeanne Duffy, Harvard Medical School

17:10 **What Do We Assess with Chronotype?** • Marie Dumont, University of Montreal

17:20 **Diurnal Preference: From Phenotype to Genotype and Back** • Derk-Jan Dijk, University of Surrey

20:00–22:30 **Poster Session I:** Even numbered posters—*Magnolia ABC*

Tuesday May 23

08:30–10:30 **Symposium 6**—*Azalea I*

Sleep and Human Circadian Biology

Chair: Louis Ptacek (University of California–San Francisco)

08:30 **Circadian Contributions to Cumulative Homeostatic Sleep Drive and Differential Vulnerability to Sleep Loss** • David Dinges, University of Pennsylvania

09:00 Charles Czeisler, Harvard University Medical

09:30 **Modeling of Human Circadian Mutations** • Ying-Hui Fu, University of California–San Francisco

10:00 **Restless Legs Syndrome and Iron Regulation** • Christopher Earley, Johns Hopkins School of Medicine

Symposium 7—*Azalea II*

Transcription/Translation Feedback Loops and Neuronal Electrochemical Signaling

Chair: Gene Block (University of Virginia)

08:30 **Cellular Physiology of Circadian Clock Neurons** • Michael Nitabach, Yale University School of Medicine

09:00 **Ionic Mechanisms underlying Circadian Rhythms: A Role for the fDR Current** • Chris Colwell, University of California–Los Angeles

09:30 **BK Channels Regulate Spontaneous Firing in the SCN and Circadian Behavioral Rhythms** • Andrea Meredith, University of Maryland

10:00 **Mechanism of Spontaneous Electrical Activity of SCN Neurons** • Bruce Bean, Harvard University

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

11:00–12:30 **Slide Session D**—*Azalea I*

Drosophila Clock Genes

Chair: Ravi Allada (Northwestern University)

11:00 **19 • Hyperexcitation of Pacemaker Neurons Leads to the Emergence of a Stable Bimodal Oscillation in Prolonged Constant Darkness** • Sheeba Vasu, New York University

11:15 **20 • Structural and Biochemical Characterization of PAS Domain Interactions in Drosophila PERIOD** • Eva B. Wolf, Max-Planck Institute for Molecular Physiology

11:30 **21 • A Mutational Analysis of Drosophila DBT and Vertebrate CKI Delta Demonstrates an Evolutionary Conserved Mechanism for Circadian Period Determination by These Protein Kinases** • Jeffrey L. Price, University of Missouri-Kansas City

11:45 **22 • PAR Domain Protein 1? Function in the Drosophila Circadian Clock** • Juliana M. Benito, University of Houston

12:00 **23 • Molecular and Neural Basis of Photoadaptation in the Circadian Network** • Dan Stoleru, Brandeis University

12:15 **24 • PER Dependent Rhythms in CLK Phosphorylation and E-box Binding Regulate Circadian Transcription** • Wangjie Yu, Texas A&M University

Slide Session E—*Azalea II*

SCN

Chair: Shelley Tisch Kau (University of Illinois)

11:00 **25 • Pathways of Metabolic Non-Photic Input to the Suprachiasmatic Nucleus Interfere with Light Input to the SCN** • Ruud M. Buijs University of Vera Cruz/ Netherlands Institute for Neuroscience

11:15 **26 • Molecular Traces of Dawn and Dusk in Different SCN Regions** • Roelof A. Hut, University of Groningen

11:30 **27 • Complex Cellular Activities in the Rodent Suprachiasmatic Clock** • Hugh D. Piggins, University of Manchester

11:45 **28 • Cyclic AMP-Dependent Signals Sustain Molecular Time-Keeping in Mammals and Determine Circadian Period** • John S. O'Neill, MRC LMB

12:00 **29 • Differential Function of the Mitogen–Activated Protein Kinase (MAPK) Targets MSK and RSK in the Suprachiasmatic Nuclei (SCN) of Mice** • Karl Obrietan, The Ohio State University

12:15 **30 • Single-Cell Bioluminescence Imaging of PER2 Expression in SCN Cultures from Wild Type and Mutant Mice: SCN Neurons Are Not Always Autonomous Clocks** • David K. Welsh, The Scripps Research Institute

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Peripheral Oscillators/Endocrine

Chair: Michael Menaker (University of Virginia)

11:00 **31 • Tissue-Specific Rescue of Circadian Rhythmicity and Activity Levels in Bmal1–/– Mice** • Erin L. McDearmon, HHMI/Northwestern University

11:15 **32 • Analysis of Mouse Ocular Per2 Circadian Rhythms** • David T. Lourim, Medical College of Wisconsin

11:30 **33 • Tissue-Specific Nature of Adrenergic Signaling in Peripheral Circadian Timing** • Dermot F. Reilly, University of Pennsylvania

11:45 **34 • Circadian Oscillations in Calcineurin Activity and MCIP1/DSCR1 Regulate Diurnal Changes in Cardiac Contractility** • Nita Sachan, University of Texas Southwestern Medical Centre

12:00 **35 • Corticosterone Response to a Light Pulse Is Additive with Restraint Stress and Does Not Change with Repeated Exposure** • Jennifer A. Mohawk, University of Michigan

12:15 **36 • The Relationship between LH Interpulse Interval and Amplitude Is Influenced by Cycle Phase, but Not by Sleep in Adult Women** • Elizabeth B. Klerman, Brigham and Womens Hospital

16:30–18:00 **Presidential Symposium**—*Azalea I*

Simple Principles for Complex Systems

Chair: William Schwartz

16:30 **Exploring Complex Networks** • Steve Strogatz, Cornell University

17:00 **Falling Off the Limit Cycle** • David Paydarfar, University of Massachusetts Medical School

17:30 **Network Dynamics and Cell Physiology: Lessons from Modeling the Cell Cycle** • John Tyson, Virginia Polytechnic Institute & State University

20:00–23:00 Free

Wednesday May 24

08:30–10:30 **Symposium 8**—*Azalea I*

Organization of the Mammalian Circadian Timing System: Centrifugal or Centripetal?

Chair: Michael Hastings (MRC Laboratory of Molecular Biology)

08:30 **The Tau Mutation in Mice and Hamsters** • Andrew Loudon, The University of Manchester

09:00 **Reductionist Approaches to the Study of Circadian Behavior and Clock Mechanism** • Steve Brown, Humboldt University, Charité Hospital

09:30 **Food Entrainment of Circadian Rhythms: Where is the Oscillator?** • Clifford Saper, Beth Israel Hospital, Harvard

10:00 **Functional Interplay Between Circadian and Stress Response Systems** • Marina Antoch, The Cleveland Clinic

Symposium 9—*Azalea II*

'Green' Clocks: Conservation and Diversity of Circadian Architectures

Chair: Takao Kondo (Nagoya University)

8:30 **Cyanobacterial Circadian Clocks in Cells, in Vitro, and in Modeling** • Tokitaka Oyam, Nagoya University

9:00 **Multiple-Loop Architecture of the *Arabidopsis* Circadian Clock** • Andrew Millar, University of Edinburgh

9:30 **Functional Genomics Approaches to Decoding Transcriptional Regulation in *Arabidopsis* Circadian Clocks** • Steve Kay, The Scripps Research Institute

10:00 **FLOWERING LOCUS T (FT) and Long-Distance Flowering Signal (Florigen) in *Arabidopsis*** • Takashi Araki, Kyoto University

10:30–11:00 **Coffee Break**—*Magnolia Foyer*

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Entrainment

Chair: Gianluca Tosini (Morehouse School of Medicine)

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11:15 **38 • The Molecular Gatekeeper Dexras1 Sculpts the Photic and Nonphotic Responsiveness of the Mammalian Circadian Clock** • Hai-Ying Mary Cheng, Ohio State University

11:30 **39 • A Switch from Nocturnal to Diurnal Entrainment in a Mouse Model of Photoreceptor Dysfunction** • Susan E. Doyle, University of Virginia

11:45 **40 • The Eye-Attached SCN Slice Preparation: An in Vitro Mammalian Circadian Visual System** • Kwoon Y. Wong, Brown University

12:00 **41 • Single Unit Responses of SCN Neurons to Irradiance, Duration and Wavelength** • Howard M. Cooper, INSERM U371

12:15 **42 • Evolution of Inner Retinal Photoreceptors: Mammals Have Lost a Melanopsin Gene** • Robert J. Lucas, University of Manchester

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Clocks and Metabolism

Chair: Veli Schibler (University of Geneva)

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11:15 **44 • Loss of Nocturnin, a Circadian Deadenylase, Confers Resistance to Diet Induced Obesity** • Nicholas Douris, University of Virginia

11:30 **45 • The Cross-Talk of Cholesterogenesis and the Circadian Regulation** • Damjana Rozman, Faculty of Medicine, University of Ljubljana

11:45 **46 • The Circadian Clock Protein BMAL1 Affects Steroidgenesis and Is Necessary for Fertility** • J.D. Alvarez, University of Pennsylvania

12:00 **47 • Altered Sleep and Diurnal Regulation in Leptin-Deficient Mice** • Aaron D. Laposky, Northwestern University

12:15 **48 • A Network of Autonomic Clock Outputs** • Andries Kalsbeek, Netherlands Institute for Neuroscience

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Sleep

Chair: Marie Dumont (University of Montreal)

11:00 **49 • Effect of Sleep Fragmentation on Spectral Activity of Sleep EEG in Morning-type and Evening-Type Individuals** • Valérie Mongrain, Chronobiology Laboratory

11:15 **50 • Diurnal Sex Differences in the Sleep-Wake Cycle of Mice Are Dependent on Gonadal Function** • Ketema N. Paul, Northwestern University

11:30 **51 • Effects of Bedpartners on the Sleep–Wake Cycle** • Gerhard Kloesch, University of Vienna

11:45 **52 • Circadian Effect On Degree of Sleeep Inertia Present After Awakening** • Frank A.J.L. Scheer, Brigham and Womens Hospital Harvard Medical School

12:00 **53 • Diurnal Patterns of Sleep Tendency in Children and Adolescents as a Function of Circadian Phase Angle** • Stephanie J. Crowley, Brown University

12:15 **54 • Circadian Rhythm of Alertness in Delayed Sleep Phase** • Kathryn J. Reid, Northwestern University Feinberg School of Medicine

16:30–18:30 **Symposium 10**—*Azalea I*

Mathematical Modelling

Chair: Danny Forger (University of Michigan)

16:30 **Modelling the Neurospora Circadian Clock** • Peter Ruoff, Stavanger University

16:55 **Directing Experiments on Plant Clocks Using Modeling** • James Locke, University of Warwick

17:20 **Tonic and Phasic Effects of Light: A Reappraisal** • Serge Daan, University of Groningen

17:45 **Synthetic Biology: Developing Model Systems for Quantitative Studies of Complex Genetic Regulation** • Alex Ninfa, University of Michigan

18:10 **Modeling Reveals Opposite Function of Tau and Other Short-Period Circadian Mutants** • Daniel Forger, University of Michigan

Symposium 11—*Azalea II*

Circadian Clock Mechanisms II

Chair: Martha Merrow (University of Groningen)

16:30 **Evolution of Clock Molecules** • Charlambos Kyriacou, Leicester University

17:00 **Cell Based Models in the Study of the Mammalian Circadian Clock** • John Hogenesch, Scripps, Florida

17:30 **Putting on and Taking Off; Tracking Daily Time with a Balance of Protein Kinases and Phosphatases** • Isaac Edery, Rutgers University

18:00 **Genetic Dissection of Temperature Compensation Reveals a Role for Proteins Common to Many Circadian Systems** • Jay Dunlap, Dartmouth University

Symposium 12—*Azalea III*

Shedding Light on Human Clock Function Disorders

Chair: Anna Wirz-Justice (Psychiatric University Clinics, Basel)

16:30 **Clock-Gene Polymorphisms and Circadian Rhythm Sleep Disorders** • Takashi Ebisawa, The University of Tokyo

17:00 **Advancing and Delaying the Blind with Melatonin** • Debra Skene, University of Surrey

17:30 **Long-Term Light and Melatonin Treatment in Dementia** • Eus van Someren, Netherlands Institute for Neurosciences and VU University Medical Center

18:00 **Circadian Rhythms and Bipolar Disorder: A Genetic Perspective** • Vishwajit L Nimgaonkar,University of Pittsburgh School of Medicine

Workshop II—*Camellia II*

Comparative Anatomy of Circadian Pacemaker Networks in Arthropod Brains

Chair: Paul Taghert (Washington University)

16:30 **The Neuro-Anatomical Basis of Circadian Rhythms in Drosophila Melanogaster** • Orie Shafer,Washington University

17:00 **Molecular and Neuroanatomical Organization of the Circadian Clock in Honey Bees *(Apis mellifera)*** • Guy Bloch, The Hebrew University of Jerusalem

17:30 **The Silkworm, Bombyx Mori, as a Model System for Circadian Structure** • Makio Takeda, Kobe University

18:00 **Overview** • Charlotte Helfrich-Foerster, University of Regensburg

20:00–22:30 **Poster Session II:** Odd numbered posters—*Magnolia ABC*

Thursday May 25

08:30–10:30 **Symposium 13**—*Azalea I*

Highlights from New Investigators

Chair: Martha Gillette (University of Illinois)

8:30 **A KaiC-binding His-to-Asp Phospho-Relay System Mediating Signals from the Kai-Based Protein Oscillator to Gene Expression in Cyanobacteria** • Hideo Iwasaki, Waseda University

8:55 **Circadian Regulation of Hormone Signaling** • Stacey Harmer, University of California-Davis

9:20 **Role of Retinal Photoreceptors in Detecting Light for Non-Image-Forming Visual Functions** • Samer Hattar, Johns Hopkins University

9:45 **QTL Analysis of the Circadian Clock of Arabidopsis** • Harriet McWatters, Oxford University

10:10 **Potential Roles of Id (Inhibitor of DNA binding) Genes in the Mammalian Circadian System** • Giles Duffield, Dartmouth Medical School

Symposium 14—*Azalea II*

Non-Image Forming Photoreception and Photoentrainment

Chair: Ignacio Provencio (University of Virginia)

08:30 **Dial and Lunar Photoperiodicity in a Marine Polychaete, and Evolution of the Neuroendocrine Brain** • Detlev Arendt, EMBL Heidelberg

09:00 **Characterization of Melanopsin: Insight Into Evolutionary Linkage between Vertebrate Circadian and Invertebrate Visual Pigments** • Akihisa Terakita, University of Kyoto

09:30 **Ganglion-Cell Photoreceptors: Morphing Melanopsin's Message** • David Berson, Brown University

10:00 **ipRGCs Encode Color and Irradiance** • Dennis Dacey, University of Washington

10:30–11:00 **Coffee Break**

11:00–12:30 **Slide Session J**—*Azalea I*

Mammalian Clock Genes II

Chair: Choogon Lee (Florida State University)

11:00 **55 • A Large-Scale Forward Genetics Screen to Identify Circadian Rhythm Mutants in the Mouse** • Sandra M. Siepka, Northwestern University

11:15 **56 • Impact of a Novel ENU-Induced Long Period Circadian Mutant on Molecular timekeeping in the SCN and Periphery** • Liz Maywood, MRC Laboratory of Molecular Biology

11:30 **57 • The Tau Mutation in CKIe Is an In Vivo Gain of Function Mutation on PER1 and PER2** • David M.Virshup, University of Utah

11:45 **58 • Oscillator Network Interactions Compensate for Circadian Clock Gene Defects in Suprachiasmatic Nuclei** • Andrew C. Liu The Scripps Research Institute

12:00 **59 • Mouse Phenome Project: A Systematic Comparison of Circadian Activity Rhythms in 34 Inbred Mouse Strains** • Kazuhiro Shimomura, Northwestern University

12:15 **60 • Circadian Transcriptional Mechanism of Molecular Clocks** • Toru Takumi, Osaka Bioscience Institute

Slide Session K—*Azalea II*

Plant and Neurospora Clock Genes

Chair: Deborah Bell-Pedersen (Texas A&M)

11:00 **61 • Constitutive Overexpression of PRR7 Weakens Circadian Rhythms in *Arabidopsis thaliana*** • Eva M. Farre* and Steve A. Kay, The Scripps Research Institute

11:15 **62 • A Novel Computational Model of the Circadian Clock in Arabidopsis that Incorporates PRR7 and PRR9** • Francis J. Doyle, University of California–Santa Barbara

11:30 **63 • Genetic and Molecular Characterization of the *Neurospora Crassa* BD Mutation: The Molecular Basis of the Overt Circadian Rhythms in Neurospora** • Jay C. Dunlap, Dartmouth Medical School

11:45 **64 • Molecular Mechanism of Circadian Singularity Behavior** • Yi Liu, UT Southwestern Medical Center

12:00 **65 • A Genetic Screen for Loss of FRQ-Based Negative Feedback in *Neurospora Crassa*** • Mi Shi, Dartmouth Medical School

12:15 **66 • Genes Involved in Temperature Compensation of the *N. Crassa* Clock** • Arun Mehra, Dartmouth Medical School

Slide Session L—*Azalea III*

Human Circadian Rhythms

Chair: Elizabeth Klerman (Harvard Medical School)

11:00 **67 • Phase-Advancing Human Circadian Rhythms with the Dual MT1/MT2 Melatonin Agonist VEC-162** • Shantha M.W. Rajaratnam, Brigham and Womens Hospital

11:15 **68 • Melatonin Entrains Free-Running Blind Individuals with Circadian Periods Less Than 24 Hours** • Jonathan S. Emens, Oregon Health & Science University

11:30 **69 • Amplitude and Phase of 6-Sulphatoxymelatonin During Night and Day Shifts Offshore** • Michelle Gibbs, University of Surrey Centre for Chronobiology

11:45 **70 • Evaluation of Melatonin as a Countermeasure for Entrainment to a Shorter-than-24-Hour Wakefulness–Sleep Schedule** • Kenneth P. Wright, University of Colorado at Boulder

12:00 **71 • Circadian and Homeostatic Regulation of Sleep In Blind Individuals with No Conscious Light Perception (NPL)** • Joseph T. Hull, University of Surrey, UK, in collaboration with Brigham and Womens Hospital,

12:15 **72 • Superiority of Blue (470 NM) Light In Eliciting Non-Image Forming Brain Responses during Auditory Working Memory In Humans: A fMRI Study** • Gilles Vandewalle, Cyclotron Research Centre, University of Liege

16:30–17:30 **Business Meeting**—*Azalea I*

17:45–17:50 **A Tribute to Erwin Bünning (1906–1990) on his 100th Birthday**—*Azalea I*

Professor Bünning, the plant physiologist, considered endogenous biological rhythms as one of the fundamental components of life. His research began with the sensory physiology and movements of plants in the 1920s and went on to the analysis of basic properties of their circadian system. He described light responses, humidity effects, and temperature compensation. From there, he went on to pioneer studies in plant photoperiodism and morphogenesis. These, together with his book, The Physiological Clock (1963), were landmarks in the development of research on biological rhythms. We wish to commemorate him on the anniversary of his one-hundreth birthday as one of the fathers of modern chronobiological research.

Charlotte Helfrich-Forster (Regensburg), John Dittami (Vienna) and Wolfgang Engelmann (Tübingen)

17:50–19:00 **Pittendrigh/Aschoff Lecture: Michael Young (Rockefeller University)**—*Azalea I*

Chair: Carla Green (University of Virginia)

New roles for old proteins in the Drosophila clock

20:00–23:00 **Banquet**—*Magnolia Ballroom*

Friday May 26

Depart

Poster Session 72–282

Even numbered posters will be presented on Monday, May 22, 2006
Odd numbered posters will be presented on Wednesday, May 24, 2006

SCN

72A • Suppression of Grp-Induced C-Fos Expression in the Hamster SCN by a Serotonin Agonist and an NMDA Antagonist • Eric M. Mintz, Kent State University

73 • Disrupted Neuronal Rhythms in the Suprachiasmatic Nuclei of Vasoactive Intestinal Polypeptide-Deficient Mice • Timothy M. Brown, University of Manchester

74 • PAI-1/Vitronectin Modulation of Glutamate-Induced Phase-Sshifts of the SCN Circadian Clock • Xiang Mou, University of Tennessee-Knoxville

75 • Cholinergic Projections to the Suprachiasmatic Nucleus (SCN) of the Diurnal Grass Rat • Alexandra Castillo-Ruiz, SRBR Member

76 • Five Muscarinic Receptor Subtypes are Expressed in the Rat SCN • Jose L. Chavez-Juarez, Instituto de Fisiologia Celular, Universidad Nacionale Autónoma de Mexico

77 • Light-Induced Changes in Gene Expression in the Hamster SCN as Assessed by Microarray and Quantitative Real-Time PCR • Veronica M. Porterfield, Kent State University

78 • Daily Rhythms in PER1 within and beyond the SCN of Female Grass Rats *(Arvicanthis niloticus)* • Chidambaram Ramanathan, Michigan State University

79 • Effect of Aging on 5-HT7 mRNA Expression in Neuroanatomical Components of the Circadian Timing System • Marilyn J. Duncan, University off Kentucky Medical Center

80 • Microinjection of GRP into the SCN Region during the Late Night Increases PER1 Gene Activity in the Entire SCN of the Mouse • Karen L. Gamble, Vanderbilt University

81 • A Characterization of Cells that Rhythmically Express mPER1 in the Mouse SCN • Veronika Garga, University of Houston

82 • Experience-Dependent Filamentous (F)-Actin Reorganization in the Rat SCN • Martha U. Gillette, University of Illinois at Urbana-Champaign

83 • Intrinsic Scale-Invariant Patterns of Locomotor Activity: Influence of the Circadian Pacemaker across a Wide Range of Time Scales Spanning 4–24 Hours • Kun Hu, Brigham and Womens Hospital, Harvard Medical School

84 • A Comparison of Period Gene Expression in the Nocturnal Rat and Diurnal Degu: The Suprachiasmatic Nucleus, Striatum, Cingulate, and Parietal Cortices • Megan H. Hagenauer, University of Michigan

85 • Genetic Analysis of Ca2+ Signaling in Circadian Clock Neurons • Marie C. Harrisingh, Yale School of Medicine

86 • Neuron or Glia? Involvement of Two Different Cell Types in Circadian Rhythm Generation in the SCN • NaotoHayasaka, Kinki University School of Medicine

87 • Photoperiodic Encoding in the SCN: From Neurons to Networks • Henk Tjebbe vanderLeest, Leiden University Medical Center

88 • Two Methods of Uncoupling a Circadian Clock Reveal Sustained and Damped Oscillator Types • Alexis B. Webb, Washington University

89 • The Phase Shifting Effects of Gastrin-Releasing Peptide Require Concurrent Activation of NMDA Receptors • George J. Kallingal, Kent State University

90 • Tethered k-ACTX-Hv1C Expression in Clock Neurons Disrupts Circadian Locomotor Activity • Ying Wu, Yale School of Medicine

91 • Perturbing Vesicle Traffic Reveals Potent Effects on Circadian Period in Drosophila • Luoying Zhang, Northwestern University

92 • Attenuated Circadian Rhythms in Mice Lacking the Prokineticin 2 Gene • Qun-yong zhou, University of California-Irvine

93 • Potentiation of The Resetting Effects of Light on Circadian Rhythms of Hamsters Using Serotonin and Neuropeptide Y Receptor Antagonists • Mary E. Harrington, Smith College

94 • Simulations Of Day-Length Encoding in the SCN: From Single Cells to Tissue Level Organization • Joseph H.T. Rohling, Leiden University Medical Center

95 • A Sex Difference in Rhythms of mRNA for Vasoactive Intestinal Polypeptide and its Receptor, but not of Vasopressin mRNA,in the Suprachiasmatic Nucleus of a Diurnal Rodent • Megan M.Mahoney, University of Michigan

97 • Glutamatergic Synaptic Inputs to the SCN from the Paraventricular Thalamus Are Mediated by Ampa-Kainate Receptors • Javier Alamilla-Gonzalez, Universidad Nacionale Autónoma de Mexico

98 • Transduction of Hypothalamic Neurons with Lentiviral Constructs • Maria M. Canal, University of Manchester

99 • Evidence for Circadian Synchronization between Astrocytes • Luciano Marpegan, Washington University

Peripheral Oscillators

100 • Expression of HPER1 in Human Peripheral Blood Mononuclear Cells throughout the Sleep–Wake Cycle • Francine O. James, McGill University

101 • Imaging mPER1 Gene Expression in Mouse Midbrain Cultures • Daniel J. Hiler, Bowling Green State University

102 • Control of PER1-Luc Expression Rhythms in Ovaries: Neural versus Humoral Signals • TomokoYoshikawa, University of Virginia

103 • Differential Effects of Cryptochrome (Cry) Mutations on Central and Peripheral Circadian Expression of PERIOD2::LUCIFERASE in Mice • Caroline H. Ko, Northwestern University/ University of Toronto

104 • Expression of the c-Fos and PER1 Immunoreactive in the Limbic System in Rats under Restricted Feeding Shedules • Manuel Angeles-Castellanos, Universidad Nacional Autónoma de Mexico

105 • A Retinal Diffusible Factor Modulates the Pineal Gland Clock in Zebrafish • Nancy E. Hernandez de Borsetti, University of Houston

106 • Lighting-Induced Circadian Disruption: Simultaneous Effects on Mammary and Liver Clock Gene Expression • John D. Bullough, Rensselaer Polytechnic Institute

107 • Effects of Denervation on Circadian Rhythms in Salivary Glands • Nina Vujovic, University of Virginia

108 • Zebrafish Brain Contain Multiple Dampened Oscillators • Hugo M. Borsetti, University of Houston

109 • Circadian Rhythm of Glycogen Synthase Kinase 3 (GSK 3) in the Murine Heart • Vishnu Chintalgattu, East Carolina University

110 • c-Jun N-Terminal Kinase Modulates the Period length of Period1-Bioluminescence Rhythms in Rat-1 Fibroblasts • Chiaki Fukuhara, Morehouse School of Medicine

111 • Ontogenesis of the Circadian Clock within the Rat Liver • Alena Sumova, Academy of Sciences

112 • Circadian Function In The Olfactory Bulb • Daniel Granados-Fuentes, Washington University

113 • Influence of Altered Gravity on Clock Gene Expression in Rat-1 Fibroblasts • Sonia Vadruci, Space Biology Groupe

Behavior/Outputs

114 • Scheduled Wheel-Running Stabilizes Behavioural Rhythms of Vipr 2–/– Mice • Andrea Power, University of Manchester

115 • Diurnal Body Temperature Rhythms as a Measure of Welfare in Pasture-Based Cattle • Paul E. Kendall, AgResearch Limited

116 • Genetic Analysis of Tail Suspension Behavior in Genetical Rhythm Splitting Mice (CS Strain) • Shigeru Tomida, Nagoya University

117 • Ear Temperature as a Function of Time Of Day, Cognitive Demand, and Chronotype • Martin R. Ralph, University of Toronto

118 • Circadian Rhythms of Conditioned Avoidance Behaviors in Rodents do not Require the Suprachiasmatic Nucleus • Martin R Ralph, University of Toronto

119 • Combined Infrared and Wheel-Running Monitoring Reveals Unconsolidated Locomotor Behaviors in VIP KO Mice • Christopher M. Ciarleglio, Vanderbilt University

120 • Effects of Tuberomammillary Nucleus Ablation on Food Anticipatory Circadian Rhythms in Rats • Glenn J. Landry, Simon Fraser University

121 • Splitting of Circadian Rhythms in Locomotor Activity and SCN Expression of c-Fos and pERK in the SCN of the Djungarian Hamster *(Phodopus sungorus)* • Cheryl D. Waring, University of Manchester

122 • Locomotor Activity Recording in Adult Zebrafish • Adi Tovin, Tel Aviv University

123 • Spontaneous Internal Desynchronization of Locomotor Activity and Body Temperature Rhythms from Plasma Melatonin Rhythm in Rats Exposed to Constant Dim Light • Gianluca Tosini, Neuroscience Institute

124 • Characterization of Behavioral and Metabolic Rhythms in a Model of Nocturnal Work (Night Workers) in Rats • Roberto Carlos Salgado, Fac de Medicina

125 • Behavioral and Neuronal Activity in Rats Entrained by Regular and Palatable Food • Katia Rodríguez, Universidad Nacional Autónoma de Mexico

126 • A Circadian Rhythm in Memory Consolidation in Zebrafish • Oliver Rawashdeh, University of Houston

Entrainment

127 • An Inhibitor of Casein Kinase Ie Induces Robust Phase Delays in The Activity Onsets of Telemeterized Rats under Free-Running and Entrained Conditions as a Function of Dose, Time of Dosing, and Duration of Dosing • Jeffery Sprouse, Pfizer Global Research & Development

128 • Circadian Photoreception in *Drosophila melanogaster* • Nicolai S.D. Peschel, Universität Regensburg

129 • A Behavioural Analysis of the Relative Contribution of the Novel and Classical Photoreceptors to Circadian Entrainment • Sarah L. Jones, Imperial College

130 • Photic Entrainment of the Dorsal SCN • KazutoWatanabe, Dokkyo Medical University School of Medicine

131 • Human Pheromones Accelerate Reentrainment Following Trans-Meridian Travel • Tammy J. Jechura, Albion College

132 • Immunocytochemical Localization of Short Wave-Light-Sensitive Molecules in Nonvisual Photoreceptors. Can Blue-Light Filtering Reduce Known Pathological Effects of Night-Illumination? • Bela Vigh, Semmelweis University

133 • Characterization of the Effects of Light Flashes on the Hamster Circadian System • Luis A. Vidal, Stony Brook University

134 • *Cephalochordate melanopsin*: Evolutionary Linkage between Vertebrate Circadian Photopigment and Invertebrate Visual Pigment • Mitsumasa Koyanagi, Osaka University

135 • Dark Pulses and NPY Decrease P-ERK Levels in the Subjective Day • Paola C. Yannielli, Universidad Nacional de Quilmes

136 • Accelerated Re-Entrainment in BALB/cJ Mice • Tara A. LeGates, Rider University

137 • Effects of Acute In Vitro Ethanol Treatment on Glutamate-Induced Phase Shifts of the Mammalian Circadian Clock • Andy Mangrum, University of Tennessee

138 • The Selective 5-HT7 Receptor Antagonist Can Block the Inhibitory Effects of 8-OH-DPAT on Light in Syrian Hamsters • Maria Gardani, University of Glasgow

139 • Individual Variability and Photic Entrainment of Circadian Rhythms in Golden Spiny Mice • Rotem Cohen, Tel Aviv University

140 • Melanopsin Structure and Function: A Comparative Analysis • Susana S. Pires, Imperial College

141 • Alteration of Photic Responses of Circadian Timing System and Pupillary Light Reflex to Previous Light Exposure • Ludovic S. Mure, INSERM U371

142 • Melanopsin-IR Co-Localizes with GnRH in the Hamster Brain • J.H.Blanchard* and L.P. Morin, Stony Brook University

143 • PACAP Is Necessary for the Expression of Normal Light-Induced Phase Advances • Jennifer W. Mitchell, University of Illinois at Urbana-Champaign

144 • Variability of Diurnality in Laboratory Rodents • Roberto Refinetti, University of South Carolina

145 • Phase Response Curves in Mice: The Effects Of Light Pulse Duration and of Skeleton Pulses • Maria Comas, Rijksuniversiteit Groningen

146 • Light as a Circadian Stimulus for Nocturnal Rodents Used in Human Cancer Research • Mariana G. Figueiro, Rensselaer Polytechnic Institute

147 • The Role of Mid-Wavelength Cones in Non-Visual Responses to Light • Ouria Dkhissi-Benyahya, INSERM U371

148 • Mouse Photic Entrainment Involves Modulations of Light Responsiveness as Well as Circadian Period and Activity Duration • Dwight E. Nelson, University of St. Thomas

149 • Circadian Organization in Royal College Surgeon Rats • Gianluca Tosini, Neuroscience Institute

150 • Behavioral and Metabolic Food Entrainment Are Driven by Different Oscillatory Systems • Carolina Escobar, Universidad Nacional Autonoma de Mexico

151 • Photoentrainment, Phase Shift by Chemical Signal, and Their Effects On Per1 Expression in the Golden Spiny Mouse *(Acomys russatus)* Brain • Rachel Ben-Shlomo

152 • The HLH Transcription Factor Inhibitor of DNA Binding 2 (Id2) Gene Is Involved in the Photoentrainment Mechanism of the Mammalian Circadian Clock • Giles E. Duffield, Dartmouth Medical School

153 • The Dorsomedial Hypothalamic Nucleus Is Critical for the Expression of Food-Entrainable Circadian Rhythms • Joshua J.Gooley, Beth Israel Deaconess Medical Center

154 • An Auditory Stimulus Phase Advances Circadian Rhythms In The Early Subjective Day • Namni Goel, Wesleyan University

155 • Impact Of Conflicting Zeitgebers on Reentrainment: Behavior-Mediated Responses? • Menno P. Gerkema, University of Groningen

156 • The Role of the 5-HT1A Receptor in Serotonergic Enhancement of Photic Phase Shifts • Victoria M. Smith, University of Calgary

156A • Advanced Nonphotic Entrainment: A New Automated Approach • Robert Dallmann, University of Toronto

Photoperiodism

157 • Ontogenesis of the Seasonally Varying Activity Pattern in European Hamster • Stefanie Monecke, University of Stuttgart

158 • NRe Lesions Delay the Reproductive Response to Long Day Lengths • Brett J. Teubner, University of Memphis

159 • A Common Mechanism of Circadian Coupling Promotes Changes in Circadian Waveform across Different Entrainment Paradigms • Jennifer A. Evans, University of California-San Diego

160 • Phase Shift Responses of LDLD Split Rhythms in Syrian Hamsters • Jeffrey A. Elliott, University of California-San Diego

161 • Investigation of Transcriptional Pathways Driving the Photoperiodic Control of Seasonal Rhythms in Mammals • Sandrine M. Dupre, University of Manchester

162 • Consequences of Exotic Entrainment for Reproduction and Development in Hamsters • Evan E. Raiewski, University of California-San Diego

163 • Adjustment to Long Photoperiods in Circadian Gene Knockout Mice • Maria Comas, Rijksuniversiteit Groningen

164 • Seasonal Changes in Pelage Thickness and Growth Dynamics of Photorefractory Siberian Hamsters • Matthew J. Paul, University of Massachusetts Medical School

165 • Identification and Characterization of Thyroid Hormone Transporter Mediating Photoperiodic Response in Birds • Nobuhiro Nakao, Nagoya University

166 • Homeostasis of Tau Revisited: Do Aftereffects of Photoperiod Depend upon Casein Kinase 1e? • Eric L.Bittman, University of Massachusetts

Invertebrates

167 • Application of cAMP Mimicks the Effects of Pigment-Dispersing Factor on the Electrical Activity of Accessory Medulla Neurons in the Cockroach *Leucophaea maderae* • Monika Stengl, Philipps-University of Marburg

168 • Molecular Mechanisms that Regulate Circadian Olfactory Responses in Drosophila • Parthasarathy Krishnan, Texas A&M University

169 • Modeling of Some Circadian Properties in Crayfish • Miguel Lara-Aparicio, Nacional Autonoma de Mexico

170 • The Circadian Clock Suppresses Long-term Memory Formation at Night by Inhibiting Learning-Induced Transcription • Lisa C. Lyons, University of Houston

171 • Beta-Pigment-Dispersing Hormone-expressing Nneurons in *Cancer productus* Brain and Optic Ganglia • Yun-Wei A. Hsu, University of Washington

172 • Daily Expression of Discs-Large in the Photoreceptor Terminals of Drosophila Visual System • Elzbieta M. Pyza, Jagiellonian University

173 • Moonlight during the Night Provokes a Complete Change in the Activity Pattern of Fruit Flies • Wolfgang M. Bachleitner, University of Regensburg

174 • Pigment-Dispersing Hormone Induces Periodic Changes in Excitability of Photoreceptor Cells in Crayfish • Carolina Barriga-Montoya, Universidad Nacional Autonoma de Mexico

175 • Daily Variation of Effect of Melatonin upon Excitability of Photoreceptor Cells in Crayfish • Leonor Mendoza, Universidad Nacional Autónoma de Mexico

176 • Circadian Rhythms in Olfactory Receptor Neurons • Terry L. Page, Vanderbilt University

177 • The Phase Response Curve of the Electroretinogram Circadian Rhythm of Crayfish, by Applying Exogenous Melatonin • Hector Solis, Universidad Nacional Autónoma de Mexico

Endocrinology

178 • The Zinc Finger Protein EGR-1 (Early Growth Response- 1) Mediates the Induction of mPer1 Expression by Gonadotropin-Releasing Hormone (GnRH) in T3-1 Pituitary Gonadotrope Cells • James Olcese, Florida State University College of Medicine

179 • The Circadian Machinery in the Pituitary Gland • Xavier Bonnefont, Institut de Génomique Fonctionnelle

180 • Effects of Ovarian Steroid Hormones on Per1 Expression in the SCN • Michael T. Sellix, University of Virginia

181 • Exogenous T3 Elicits Long-Day Reproductive Responses in Short-Day Housed Siberian Hamsters • David A. Freeman, University of Memphis

182 • T3 Implantation into the Mediobasal Hypothalamus Mimics Seasonal Morphological Changes in the Median Eminence of Japanese Quail • Takashi Yamamura, Nagoya University

183 • Long-Day Suppressed Expression of Type-2 Deiodinase in the Mediobasal Hypothalamus of the Saanen Goat, a Short-Day Breeder • Shinobu Yasuo, Johann Wolfgang Goethe University

184 • Porcine Stress Hormone Circadian Rhythm in Intensive Care • Mary Anne M. Vincent, University of Texas Health Science Center

185 • Effects of Time of Feed Delivery on Daily Rhythms in Glucose and Insulin in Blood Plasma and Glucose Tolerance in Dairy Cows • Alma D Kennedy, University of Manitoba

Melatonin

186 • Activation Of Mt1 or Mt2 Melatonin Receptors Phase Shift Distinct Circadian Rhythms in the C3H/Hen Mouse • Margarita L. Dubocovich, Northwestern University Feinberg School of Medicine

187 • Melatonin Duration Mediates the Photoperiodic Regulation of Song Control Nuclei in the House Sparrow, *Passer domesticus* • Paul A. Bartell, Texas A&M University

188 • The Rate of Reentrainment Is Associated with the Timing of Melatonin Onset • Jimo Borjigin, University of Michigan Medical School

189 • Melatonin Stimulates Growth of Primary Chick Astrocytes in Culture • Jiffin K. Paulose, Texas A&M University

Human Circadian Rhythms

190 • Daytime Light Exposure Advances Melatonin Onset in Humans • Marina C. Giménez, University of Groningen

191 • Blue-Enriched versus White Light for Circadian Phase Delays • Mark R.Smith, Rush University Medical Center

192 • Phase Response Curve to Single One-Hour Pulses Of 10,000 Lux Bright White Light In Humans • Steven W. Lockley, Brigham and Womens Hospital, Harvard Medical School

193 • Beyond Blue Light: Effects Of Manipulating Light Intensity and Wavelength on the Human Circadian System • Claude Gronfier, INSERM U371

194 • Measuring Light as a Stimulus for the Human Circadian System • Mark S. Rea, Rensselaer Polytechnic Institute

195 • Time-Of-Day Effects of Caffeine Administration on Salivary Melatonin and Cortisol Levels: Preliminary Results • Rebecca Robillard, Hôpital du Sacré-Coeur de Montréal

196 • Circadian Phase-Shifting Effects of Daily Ramelteon in Healthy Adults • Gary Richardson, Henry Ford Hospital

197 • Learning-Related Changes in Daytime Sleep EEG Parameters Depend on the Nature of Word-Pair Associates • Christina Schmidt, University of Liège

198 • Daily Wrist Activity Rhythms: Sex, Symmetry and Handedness • Bernard P. Possidente, Skidmore College

199 • Circadian Rhythms in Phonological and Visuospatial Storage Components of Working Memory • Candelaria Ramirez, Universidad Autónoma de Nuevo Leon

199A • Adaptation to the 24.65 Hour Martian Day Alters the Human Circadian Pacemaker • Frank Scheer, Harvard Medical School

Sleep

200 • Short Sleep Durations Reduce Phase Shifts to Light • Helen J. Burgess, Rush University Medical Center

201 • Sexual Cohabitation and Sexual Activity Effects on Cognitive Sleep Reward • Marietta P. Keckeis, University of Vienna

202 • Sleep Response to 24-Hour Total Sleep Deprivation in Young and Old Rats • Youngsoo Kim, Northwestern University

203 • Daytime Recovery Sleep Is More Sensitive to the Effects of Caffeine than Nocturnal Sleep • Julie Carrier, Université de Montréal

204 • Physiological and Behavioral Correlates of a PER3 Polymorphism in Humans • Antoine U. Viola, University of Surrey

205 • Phase Relationship between Melatonin and Sleep in Delayed Sleep Phase • Kathryn J. Reid, Northwestern University Feinberg School of Medicine

206 • Sleep Deprivation Down Regulates ERK I/II Phosphorylation within the Suprachiasmatic Nuclei of the Syrian Hamster • Michael C Antle, University of Calgary

207 • The Differential Effects of Age and Task-Type on Performance Recovery from Sleep Deprivation • Bryce Mander, Northwestern University

208 • Sleep–Wake Patterns of *Octodon Degus* • Jamie I. Perryman, University of Michigan

209 • Forced Desynchronization of REM And Nonrem Sleep in the Rat • Horacio O. de la Iglesia, University of Washington

210 • Association of Melatonin Phase and Recovery Sleep Following Prolonged Sleep Restriction in Adolescents • Eliza Van Reen, Brown University

Disease, Aging, Cancer, Metabolism

211 • Potentiation of Dioxin Responses when the Clock's Not Ticking • David J. Earnest, Texas A&M University Health Science Center

212 • Effects of Experimental Chronic Jet-Lag on Oxaliplatin Toxicity in Mice • Elisabeth S. Filipski, INSERM

213 • Dietary Fatty Acids Induce BMAL1 Expression in Mice Adipose Tissue but Not in Liver • Shigeki Shimba, Nihon University

214 • Circadian Rhythmicity in a Mouse Model of Alzheimer's Disease • Roxanne Sterniczuk, University of Calgary

215 • Circadian Rest-Activity Cycles in Antepartum Depression during Light Therapy • Katharina Wulff, Imperial College London

216 • Circadian Clocks Regulate Mammalian Energy Homeostasis • Katja A. Lamia, Harvard Medical School

217 • Timing of Rest–Activity, Light Exposure, and Melatonin in Patients with Schizophrenia and Unemployed Subjects • Katharina Wulff, Imperial College

218 • Molecular Chronopharmacology of Cyclin-Dependent Kinase Inhibitor Seliciclib(R-Roscovitine) on Mouse Liver • Francis A. Lévi, INSERM

219 • The Effect of Alcohol on Jet Lag Recovery in the Diurnal Rodent, *Octodon degus* • Cameron B. Harris, Albion College

220 • An Endogenous Circadian Rhythm in an Index of Cardiac Vulnerability Confirmed with a Constant Routine Protocol • Kun Hu, Brigham & Womens Hospital, Harvard Medical School

221 • Acute Systemic Inflammation Induces Upregulation of Circadian Clock Genes Per2 and Bmal1 in Equine Peripheral Blood • Barbara A. Murphy, University of Kentucky

222 • Repeated Phase-Advances of the Light Cycle Increase Mortality in Aged Mice • Alec J. Davidson, University of Virginia

223 • Neonatal Alcohol Exposure Alters Anatomical Components of the Photoentrainment Pathway in Adult Rat • David J. Earnest, Texas A&M University Health Science Center

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Abstracts

1

Use of the Tetracycline System in Circadian Biology: Conditional and Tissue-Specific Induction of Clock

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We utilized the in vivo inducible transgene tetracycline transactivator (tTA) system to regulate Clock gene expression conditionally in a tissue-specific and temporal manner. This "tet" system consists of two components that control gene transcription: a tetracycline-dependent transactivator (tTA) driven by a specific promoter and tet operator sequences next to a minimal promoter fused to the gene of interest. As a proof of principle of the system in circadian behavior, we generated an SCN/brain enriched transactivator that drives the expression of a dominant negative mutant Clock (Clock?19) in wild-type mice. The SCN/brain enriched transactivator line was constructed using a 10 kb promoter region of a neuroendocrine/neuronal secretory protein, secretogranin-II. We tested the TET-OFF system where mice will require administration of doxycyline to inactivate the transactivator protein tTA, consequently silencing the Clock?19 transgene. Double transgenic mice showed long period rhythms, an average of 24.5 hours, similar to Clock heterozygous mice in constant conditions. Upon treatment with doxycyline in the drinking water, the circadian period of these mice immediately returned to a wild-type circadian period, an average of 23.7 hours, indicating the transgenically induced Clock?19 was "turned off". We also found that double transgenic mice showed altered phase response curve amplitudes similar to that observed in Clock heterozygous mice. These results demonstrate that expression in the SCN and brain is sufficient to drive running wheel activity behavior in a conditional and reversible manner. Our transgenic strategy offers a promising approach to investigate how the central and peripheral oscillators contribute to the normal physiological function and integration of the circadian system to regulate behavioral state.

2

Tetracycline-Inducible Expression of Wild-Type and Tau Mutant Casein-Kinase 1 Epsilon in Mice

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The tau mutation is a semidominant, autosomal allele that dramatically shortens the period of circadian activity rhythms in Syrian hamsters. The tau locus encodes the gene Casein kinase 1 epsilon (Csnk1e),

a homolog of the Drosophila circadian gene double-time. Previous studies have demonstrated in vitro that CSNK1E protein interacts with other components of the circadian clock, and that its role in posttranslational modifications is important in regulating circadian period. To investigate further the role of Csnk1e in circadian regulation of mammalian behavior, we generated several lines of transgenic mice in which either the wild-type Csnk1e allele or the tau mutant Csnk1e allele was inducibly expressed. Expression of Csnk1e (wild-type or tau) was controlled in an anatomically- and a temporally-specific manner via a tetracycline transactivator regulatory system. Over-expression of the tau mutant Csnk1e allele in the suprachiasmatic nucleus (SCN) shortened the period of circadian locomotor activity rhythm by approximately 30-40 minutes. When the tau transgene expression was subsequently repressed with doxycyline treatment, a wild-type free-running period was restored. A shortening of the circadian period of locomotor activity was not observed when tau mutant Csnk1e was expressed in brain regions other than the SCN. Furthermore, we did not observe any aberrant circadian phenotypes in transgenic mice in which wild-type Csnk1e was over-expressed. These results demonstrate an inducible, tissue-specific, and reversible dominant-negative effect of the tau mutation on mammalian circadian behavior.

3

Cyclic Post-Transcriptional Regulation of the Circadian Clock Protein mPER2 in Mammalian Cells

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Post-transcriptional/-translational regulations are important mechanisms regulating the circadian clock system of many organisms including mammals. Expression of the essential clock protein mPER2 dramatically oscillates in the master oscillator cells of the suprachiasmatic nucleus (SCN) and peripheral cells. Although post-translational modifications, such as phosphorylation and ubiquitination, are likely to involve in the regulation of mPER2 stability, these mechanisms are believed to elicit the delayed intracellular accumulation rhythm of mPER2 from its mRNA oscillation rather than to generate the protein cycle itself. Here, we show that the bioluminescence from an mPER2-Luciferase fusion protein apparently oscillates in a circadian pattern even in cells constitutively expressing mPer2-Luc mRNA. This suggests that a post-transcriptional/-translational mechanism itself is capable of generating the circadian mPER2 accumulation cycle, and thus the cyclic post-transcriptional/-translational regulation may function in the circadian clock system in mammals.

4

A Clock Shock: Mouse CLOCK Is not Required for Circadian Oscillator Function.

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The circadian clock mechanism in the mouse is composed of interlocking transcriptional feedback loops. Two transcription factors, CLOCK and BMAL1, are believed to be essential components of the circadian clock. We have used the Cre-LoxP system to generate whole animal knockouts of CLOCK and evaluated the resultant circadian phenotypes. Surprisingly, CLOCK-deficient mice continue to express robust circadian rhythms in locomotor activity, although they do have altered responses to light. At the

molecular and biochemical levels, clock gene mRNA and protein levels in both the master clock in the suprachiasmatic nuclei and a peripheral clock in the liver show alterations in CLOCK-deficient animals, although the molecular feedback loops continue to function. Our data challenge a central feature of the current mammalian circadian clock model regarding the necessity of CLOCK:BMAL1 heterodimers for clock function.

5

Cryptochromes Alter CLOCK/BMAL1 Co-Dependent Phosphorylation: A Mechanism for Inhibition of Circadian Transcriptional Activation

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Circadian clocks, based on interlocked molecular feedback loops, are a pervasive feature in virtually all kingdoms. CLOCK and BMAL1 are central components of the molecular clock in mammals and belong to the bHLH/PAS family. They drive circadian transcription of clock genes and clock-controlled genes, in a tissue-specific manner. Features of the dimerization of both partners, proposed to rely on the PAS domains, have not been investigated. Here, we demonstrate that PAS domains function requires domains extending over the short core motifs. Strikingly, while deleting core PAS domains does not overtly affect dimerization, it abolishes transcriptional activity of the heterodimer. Interestingly, these deletions also abolish phosphorylated forms of CLOCK and BMAL1, suggesting a link between the phosphorylation status of the heterodimer and its transactivation capability. If phosphorylated forms of CLOCK/BMAL1 are those enabling transcriptional activation, then transcriptional repressors such as CRY1-2 and PER1-3 proteins may directly affect phosphorylation. In agreement with this hypothesis, we find that CRY1-2, potent repressors of CLOCK/BMAL1, affect phosphorylation of both proteins and shift the phosphorylated/unphosphorylated ratio of BMAL1 towards a predominant unphosphorylated form. In contrast, PER proteins, which are weak repressors, are without effect. From these data, we propose a mechanism for the inhibition of circadian transcriptional activation by CRY1-2.

6

Cryptochromes and Photolyases: Interchangeable Core Domains?

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Members of the cryptochrome (CRY) and photolyase (PhL) family are composed of a common core domain and a distinguishing C-terminal extension (CT). While the core domain is highly homologous between different species, the CT is variable in sequence and length (absent in PhLs, ~125 aa in mCRY1). The differences in the CT are accompanied by functional differences: mammalian CRYs are major components of the circadian oscillator, plant CRYs are involved in light sensing, and PhLs are enzymes that repair UV-induced DNA damage. It is likely that different CTs have contributed to the functional diversity among the cryptochrome/photolyase family.

Functional analysis of mCRY1 revealed two distinct domains within the CT: a putative coiled-coil domain, involved in both nuclear localization and binding to mPERs and BMAL1, and an NLS necessary for correct nuclear localization of the mCRY/mPER complex (Chaves et al., *Mol.Cell.Biol.*, in press). Importantly, the CT of mCRY1 is necessary but not sufficient for inhibition of CLOCK/BMAL1-

mediated transcription, one of the major functions of mCRY1. In line with these data, when a PhL is fused to the extended CT, it gains the ability to inhibit CLOCK/BMAL1.

The above study was performed with the (6-4PP)-PhL from A.thaliana, which has 48% aa identity to mCRY1. To further understand the functional interchange between the core domains of CRYs and PhLs, we extended the study to the CPD-PhL from P.tridactylus, a more distant relative with only 21% aa identity. Interestingly, preliminary results suggest that this PhL can I) inhibit CLOCK/BMAL1-mediated transcription, and II) affect the wheel-running behavior of transgenic mice.

7

Circadian Clocks and Photoperiodic Timers—From Geography to Gene Expression

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Despite decades of research, the relationship between the circadian clock regulating daily activities and the photoperiodic timer regulating seasonal activities remains unresolved in many animals, including arthropods. Using evolved differences among populations of the pitcher-plant mosquito, Wyeomyia smithii, we have probed the genetic mechanisms underlying variation in circadian rhythmicity and photoperiodic time measurement. Critical photoperiod is positively and closely correlated (R^2 >90%) with latitude and altitude from the Gulf of Mexico to Canada. Over this same range, critical photoperiod is not correlated with either the period or amplitude of the rhythmic response to the Nanda-Hamner protocol, an experimental procedure historically used to infer a circadian basis of photoperiodic time measurement. It is therefore difficult to argue that circadian rhythmicity has been a causal, necessary factor in the evolution of photoperiodic time measurement in W. Smithii. We propose that Nanda-Hamner periodicity is an expression of basic circadian rhythmicity, but that photoperiodic time measurement is a separate physiological mechanism. Further, the observed geographic variation in Nanda-Hamner periodicity implies that the circadian clock itself is genetically variable among populations. Indeed, we have evidence that transcription of the circadian rhythm gene, timeless, varies between populations from different latitudes. Variation among populations both in Nanda-Hamner periodicity and the expression of timeless show that the circadian clock has evolved over the climatic gradient of North America; However, the lack of correlation between Nanda-Hamner periodicity and critical photoperiod indicates that the circadian clock mediating daily activities has evolved independently of the photoperiodic timer mediating seasonal activities.

8

Molecular Links between Development and the Circadian Oscillator in Drosophila.

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We know a lot about how the circadian oscillator sustains rhythms once initiated in a number of organisms, including Drosophila. In contrast, we know little about how or when the oscillator starts ticking in any organism.

In Drosophila, the basic-Helix-Loop-Helix-PAS transcription factors CLOCK (CLK) and CYCLE (CYC) activate most of the other core oscillator components (i.e., period, timeless, vrille, par-domain-protein 1) so the simplest way to start the oscillator would be to activate CLK and CYC.

The fly compound eye was used as a model system to identify factors that lie upstream of the oscillator mechanism. Current in vitro and in vivo results show that: (1) the Clk locus contains multiple-target-sites for factors that shape photoreceptor development, (2) CLK is expressed in photoreceptor precursors prior to their differentiation, and (3) the onset of the full-molecular oscillator does not occur until several days after initial CLK expression.

The implication of these results for molecular developmental chronobiology research in flies and mammals will be discussed.

9

CLK Expression and Regulation During Development in Drosophila

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The Drosophila circadian timekeeping mechanism is comprised of interlocked transcriptional feedback loops in which CLOCK-CYCLE (CLK-CYC) activate their feedback regulators via E-box-mediated transcription. These feedback loops operate in neuronal and non-neuronal/oscillator' cells, but how this pattern of expression is determined during development isn't known. Since CLK-CYC initiates feedback loop function, understanding when and where CLK and CYC are expressed during development and how their expression is activated should shed light on oscillator cell determination. We recently showed that, in adults, CLK is expressed in oscillator cells throughout the head and body and non-oscillator cells such as Kenyon cells (KCs), which mediate olfactory learning and memory. Here we show that CLK is expressed in oscillator and non-oscillator cells in the larval CNS. Expression of CLK in non-oscillator cells includes developing KCs, as revealed by DACHSHUND (DAC) co-localization. Surprisingly, DAC appears to be expressed in all CLK-expressing cells in larvae except oscillator cells. CLK and DAC co-expression is also seen in embryos, before functional oscillators can be detected. Genetic analysis reveals that DAC is required for CLK expression in non-oscillator cells in the larval CNS and in the eye imaginal disc, which will give rise to photoreceptor oscillators in adults. These studies demonstrate that CLK is expressed in oscillator cells and DAC-expressing non-oscillator cells in the larval CNS, that CLK is expressed in embryos before oscillator cells are detected, and that DAC is necessary for CLK expression in non-oscillator and certain oscillator cells during development.

10

The Clk Gene has a Broad Ability to Induce Circadian Cycling in Non-Clock Cells

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In flies, Clock is expressed in all six groups of clock cells. Previously, we showed ectopic expression of Clk can induce molecular clock gene cycling in non-clock cells. We were interested in how general was Clk's ability to induce molecular cycling across different cell types. In larvae, we used the GAL4-UAS system

to express Clk in a variety of cell types and tissues, including different neural subsets, glia, and epithelial cells. We found ectopic, sometimes widespread, expression of PER, even in clearly non-neural tissues such as the salivary glands. With some drivers, PER abundance cycled similar to controls while with others, the phase was atypical. Currently, we are assessing the degree of overlap of GAL4 expression with PER cycling. Given that many different cell types can show clock oscillations when Clk is expressed during development, we asked whether Clk expression could turn differentiated adult cells into clocks. We drove ubiquitous expression of a temperature-sensitive GAL80 (repressor of GAL4) with the tubulin promoter. At low temperatures, we were able to repress ectopic Clk driven by cryptochromeGAL4. Upon switching adult flies to 29°, we saw de-repression of GAL4 and induction of ectopic PER cycling. We are now using this strategy to further test ectopic clock induction and maintainence. These data and our previous results indicating that Clk uniquely induces ectopic cycling argue that Clk may play a pivotal role in the developmental induction of the core molecular feedback loop that defines all clock cells.

11

Development of the Zebrafish Pineal Gland Clock

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Arylalkylamine-N-acetyltransferase (AANAT) is a key enzyme in the melatonin-rhythm- generating system in the pineal gland. In zebrafish, pineal Aanat2 is a clock-controlled gene, exhibiting rhythmic expression in the pineal gland as early as the second and third day of development. Light exposure is mandatory for the development of the pineal circadian clock that drives the Aanat2 rhythms. Additionally, light induces the expression of Period2 (Per2), mainly in the pineal gland, through a light-responsive enhancer in the Per2 promoter. Functional knockdown of PER2 abolishes Aanat2 rhythms, indicating that light-induced Per2 expression is an important event in the development of the pineal circadian clock.

Interestingly, light exposure and light-induced expression of Per2 at early developmental stages, prior to pineal gland formation (mid-blastula to early segmentation; 4-16 hr post-fertilization), are able to set the phase of the circadian clock as evident by clock-controlled rhythms of Aanat2 expression two–three days later when the pineal gland forms.

These findings suggest that early embryonic cells possess independent circadian oscillators. Light exposure entrains or synchronizes these oscillators, and the 24-h rhythm is then maintained throughout development. The implication is that the circadian oscillator is maintained during a period of rapid proliferation and, remarkably, differentiation, that take place during development.

12

Atp6v1e1 Is Regulated by OTX5, CLOCK1 and BMAL1, and Affects the Pineal Formation in Zebrafish

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To investigate possible roles of the circadian clock during developmental processes, we conducted an EST (Expressed Sequence Tags) analysis of zebrafish circadian oscillators, eyes, and brain, including the

pineal gland. Among the genes screened, atp6v1e1, encoding ATPase, H+ transporting, lysosomal, V1 subunit E, isoform 1, exhibits rhythmic pineal expression: larvae from two consecutive days (72–116 hours post-fertilization) under LD (Light/Dark 14/10 hours), DD (constant dark) and LL (constant light) all display nighttime-specific atp6v1e1 pineal expression. The atp6v1e1 pineal expression is abolished in bmal1, clock1, and otx5 antisense Morpholinos-perturbed morphants, suggesting OTX5 and CLOCK1, BMAL1 proteins act together to regulate the atp6v1e1 rhythmic pineal expression. In an atp6v1e1 mutant where atp6v1e1 is inactivated by the retrovirus insertion, pineal expression of annat2, rev-erba, irbp (interphotoreceptor retinoid binding protein), and exo-rhodopsin is all altered, suggesting that atp6v1e1affect the pineal development. Additionally, pineal expression of atp6v1e1 is restricted in floating head (flh-/-) but enlarged in masterblind (mbl-/-). These results suggest that OTX5 and CLOCK1, BMAL1 proteins interact to control the atp6v1e1 rhythmic pineal expression, and atp6v1e1 represents a group of previously uncharacterized genes through which the circadian clock regulates development.

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13

Determination of Circadian Period Length in Delay Sleep Phase Syndrome Patient Fibroblasts

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Circadian rhythms are generated by cell-autonomous, transcription-translation feedback loops that involve a defined set of clock genes. It is believed that defects in key components of this circuit underlie circadian diseases such as Advanced and Delayed Sleep Phase Syndrome (ASPS and DSPS, respectively). DSPS individuals are characterized by a shifted sleep-wake cycle, which correlates with a delayed melatonin rhythm onset. The functionality of the molecular oscillator in DSPS has never been studied at the cellular level. By combining a real-time clock read out system (based on Per2::luciferase) with RNAi knock-down of key genes of the core oscillator (Bmal1, Clock, Cry2) in cultured mouse and human fibroblasts, we validated an assay that recapitulates important circadian parameters observed in vivo. The subsequent analysis of human skin primary fibroblasts, infected with a Per2::luc lentivirus, revealed a large variation in period length in the control group (n=8) and failed to identify alterations in the circadian system of a set of DSPS individuals (n=18). The lack of correlation between time of melatonin onset and cellular period length in the DSPS group suggests that the major cause of this disorder is unlikely to be clock gene mutations that affect periodicity of the core oscillator. Similar experiments have been performed on fibroblasts from "owls" and "larks" selected on the basis of the "Munich/Groningen" chrono-questionary, and those results will be discussed.

14

Molecular Basis for the Human Familial Advanced Sleep Phase Syndrome Phenotype

Katja Vanselow* and Achim Kramer

Familial advanced sleep phase syndrome (FASPS) is a dominant human phenotype characterized by a four-hour advance of sleep, temperature, and melatonin rhythms. Genetically, FASPS is correlated with single nucleotide changes in the clock genes hPer2 or hCKIdelta. In hPer2, this mutation results in a serine to glycine substitution at the first amino acid in a putative cluster of phosphorylation sites for CKIepsilon/delta, leading to hypophosphorylation of hPER2. Since phosphorylation of mPER2 targets the protein for proteasomal degradation, it has been speculated that hypophosphorylation such as that predicted in FASPS might stabilize hPER2. Here, we show that the FASPS-mutation alters the properties of mPER2 and thereby the dynamics of the circadian oscillator. Surprisingly, rather than being stabilized, FASPS-mutated mPER2 is less stable than wild-type mPER2. This effect is even more pronounced in an mPER2 variant where the whole phosphorylation motif is mutated. Immunocytochemistry data indicate that the destabilizing effect of the FASPS-mutation is correlated with a reduced nuclear localization of the protein. To test potential dominant effects of the FASPS-mutation on circadian dynamics and entrainment behavior, we established a NIH3T3 cell line harbouring a recombination cassette that allows the comparative analysis of different mPER2 variant-induced phenotypes. For the FASPS-mPER2 expressing cells, we find an early phase after dexamethasone synchronisation and a drastically altered phase angle in a temperature entrainment regime. Based on these and additional data we propose a new model for the interaction of PER2 with CRY1/2 and CKIepsilon/delta and its consequences for the molecular clock mechanism.

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15

Improved Tumor Control and Cell Cycle Inhibition through Circadian Clock Induction

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A close link exists between the circadian timing system and the cell-division cycle, whose deregulation which is the main feature in cancer cells. We investigated whether seliciclib, a cyclin-dependent kinase inhibitor, improves the crosstalks between the circadian clock and the cell-division cycle in a mouse tumor model.

Mice (n=132) bearing Glasgow osteosarcoma (GOS) received seliciclib (300 mg/kg/day) or vehicle for 5 days at ZT3, 11 or 19. One day later, mice were sacrified in DD at six different circadian times every four hours. Tumor mRNA expression patterns were determined for clock and clock-controlled cell cycle genes with quantitative RT-PCR. The targets of Seliciclib in tumors were investigated by affinity chromatography on immobilized seliciclib.

Seliciclib reduced tumor growth by 55% following dosing at ZT3 or ZT11 and by 35% at ZT19 as compared to controls ($p<0.001$). No significant rhythmic transcription was found for Rev-erba, Per2, and Bmal1 in the tumors of controls. Seliciclib administration induced circadian expression of all the three genes with normal phase relations only after ZT3 dosing. c-Myc and Wee1 mRNAs displayed circadian rhythms in controls ($p=0.003$ and 0.001 respectively). Seliciclib ablated these rhythms and enhanced Wee1 expression, supporting improved control of G2/M gating. Seliciclib further modified CK1Δ and CK1E activity patterns according to dosing time, thereby providing a likely mechanism for the dosing time dependency of tumor clock induction and antitumor efficacy.

The induction of a functional molecular clock in tumor was associated with best antitumor activity of seliciclib. The circadian clock and its upstream regulators represent relevant targets for cancer therapeutics.

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The Role of the Circadian Clock in Mammary Gland Development and Breast Cancer

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Several epidemiological studies have suggested circadian rhythm perturbations are a contributing factor to breast cancer risk. Despite its potential importance, very little is known about the mammary circadian clock and what impact it has on breast cancer. We have recently reported that the mammary gland contains a developmentally regulated peripheral circadian oscillator, but the consequences of this clock are unknown.

To begin addressing what role the circadian clock plays in breast cancer, we analyzed a panel of human breast- and breast cancer-derived cell lines and matched normal breast and tumor mRNAs from clinical samples for clock gene expression by real time RT-PCR. In general, clock gene expression was reduced in human breast cancer samples, and was inversely related to invasiveness of the breast-derived cell lines.

To determine if the mammary circadian clock affects mammary gland development, we analyzed virgin Per1/Per2 double knockout mouse (PerKO) mammary glands by whole mount analysis. Mammary gland development was significantly impaired in PerKO mice, and the phenotype was intrinsic to the gland as PerKO glands transplanted into wild type mice displayed similar defects. Interestingly, introduction of Per1 siRNA into mouse mammary epithelial cells (HC-11) prevented prolactin-stimulated differentiation suggesting that PER1 is required for lactation.

By analyzing developmental stage-time courses for mammatrophic gene expression, we found that circadian expression of several genes governing mammary gland development were also developmentally regulated. These data provide support for the hypothesis that circadian disruption is associated with alterations in cellular homeostasis and may contribute to mammary tumorigenesis.

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Circadian Regulation of Immune functions in splenic macrophages

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Circadian rhythms are well known in a variety of immunological parameters and functions. Most prominent are rhythms in absolute and relative numbers of circulating white blood cells and their subsets, cytokine levels and serum cortisol. Circadian effects on susceptibility, course of disease (e.g., rheumatoid arthritis, asthma), clinical diagnostics, and pharmacological therapy underline the importance to clinical medicine. An in vivo model of macrophage function with lipopolysacharide (LPS)-challenged mice exhibits a high amplitude circadian pattern in mortality. Cortisol is thought to drive these rhythms via glucocorticoid receptors expressed in many cells types of the immune system. However, recent data from other peripheral tissues where a self-sustained circadian clockwork is responsible for rhythm generation raise questions about this common model. Here, we show that circadian clock genes are rhythmically expressed in spleen and lymph nodes in vivo. Long-term recordings with slice or suspension cultured splenocytes from PER2::LUC mice demonstrate that these rhythms are independent of cortisol levels or other extrinsic zeitgebers. In addition, we show that TNF-alpha is secreted in a circadian manner from LPS-stimulated spleen cells. The impact of a circadian clock in the spleen on the survival rate of LPS-challenged mice is the aim of future studies. This work is supported by the Deutsche Forschungsgemeinschaft, NIH grant 63104 and the 6th Framework Project EUCLOCK.

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The Molecular Clock and Circadian Variability in Thrombosis

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Cardiovascular physiology and pathophysiology undergo diurnal variation, which may be under the control of master and/or peripheral clocks, diurnal activity, and stress, or both. ClkMut mice have disrupted circadian gene expression but normal locomotor activity, allowing us to study direct affects of the oscillator without the confounding affect of activity. Genes relevant to vascular injury and integrity oscillate in WT mouse aorta; therefore, we explored the influence of the clock in conditioning the response to a thrombogenic injury in WT and ClkMut mice. Laser-induced photochemical injury in WT mice at 9:00 AM and 9:00PM led to significantly faster occlusion times (18.8 +/- 1.6 and 20.2 +/- 2.9min, respectively) compared to injury at 3:00 PM (36.7 +/- 4.0min, $p<0.01$ and $p<0.05$, respectively). ClkMut mice lacked this time-dependent response, with occlusion times of 28.4 +/- 3.1min at 3:00 PM and 36.1 +/- 3.7min at 9:00 PM. Vessel occlusion was significantly faster for WT mice versus ClkMut mice at 9:00 PM ($p<0.05$). We have confirmed oscillations in platelet aggregation and activation, plasma PAI-1, active PAI-1, and tPA in WT mice, which may condition the circadian thrombotic response to injury. Previous studies have shown loss of rhythmic Pai-1 expression and lower plasma PAI-1 in ClkMut animals, which may be responsible for our observed increase in occlusion time. These studies show, for the first time, a time-dependent injury response to a thrombogenic stimulus in vivo, which is lost with Clk mutation; the first evidence for a role of the molecular circadian clock in driving a cardiovascular pathophysiological response in mice.

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Hyperexcitation of Pacemaker Neurons Leads to the Emergence of a Stable Bimodal Oscillation Prolonged Constant Darkness

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Electrical activity of clock neurons has been implicated in the circadian regulation of behavior in Drosophila melanogaster. Previous studies from our laboratory have shown that hyperexcitation of ventral lateral neurons (vLNs) by ectopic expression of bacterial sodium channel NaChBac (pdfGAL4<NaChBac) disrupts free-running locomotor activity behavior and the molecular oscillations assayed after five days in constant darkness (DD). Further analysis of this behavior reveals the emergence of two components of free-running activity, with fast and slow periodicities, following an early episode of arrhythmicity for the first five days, whereas the activity bouts of the control flies had a steady single periodicity. To understand the underlying molecular basis of multi-stable behavior induced by hyperexcitation of vLNs, we assayed the expression of PER in different clock neuronal subgroups: vLN, dorsal lateral neurons (dLN), and dorsal neurons 1, and 2 (DN1 and 2) after 14 days of DD exposure. Unlike the initial desynchrony between the vLN and the DN subgroups, observed on day-5, a new stable synchronous pattern of PER expression emerges after prolonged exposure under DD. Two peaks of PER in the vLN corresponds with the troughs in the DN1 and DN2 subgroups. A prominent PER peak, in-phase with the peaks in the DNs, and 6 hours out-of phase with those in the small vLN, emerges in the dLN of the NaChBac flies whereas, in the control flies, the PER levels flatten out. Electrophysiological recordings confirmed that the vLNs of the NaChBac flies are hyperexcited, showing a bi-modal pattern of electrical activity. One of the patterns is characterized by rapidly rising depolarizations (-80mV) with plateau potentials and a second pattern where the plateau potentials are either shorter or completely absent. vLNs of the control flies exhibit spontaneous slow membrane potentials (10 mV in amplitude) with small, non-overshooting spikelets riding the peaks. Therefore, hyperexcitation of the vLNs modulates plasticity of the circadian pacemaker circuit, resulting in a new long-term stable synchronous molecular oscillation along with a stable split behavioral activity rhythm.

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Structural and Biochemical Characterization of PAS Domain Interactions in Drosophila Period

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Period Proteins are central components of the Drosophila and mammalian circadian clock. To provide insights into molecular mechanisms underlying PER protein functions and interactions, we have solved the crystal structure of a Drosophila PERIOD (dPER) fragment including the two PAS domains (PAS-A and PAS-B) and two ??helices (?E and ?F) located C-terminal to PAS-B within the conserved C-domain. The structure has revealed a dPER homodimer, which is stabilized by interactions of PAS-A with PAS-B and helix ?F. The PAS-A-?F dimer interface includes the 29h perL mutation site (V243D), suggesting

its functional relevance in the clock. To establish the existence of both PAS-A dimer interfaces (PAS-A-PAS-B and PAS-A-?F) in solution and quantify their relative contribution to dimer formation, we have analysed a number of interface mutants by analytical gel filtration and analytical ultracentrifugation. We have also solved the crystal structures of two additional dPER PAS domain fragments with and without helix ?F. In agreement with our solution studies, the fragment without ?F (termed dPER??F) crystallizes as a monomer. Our structural and biochemical studies on dPER PAS domain variants will be presented.

21

A Mutational Analysis of Drosphila DBT and Vertebrate CKI Delta Demonstrates an Evolutionarily Conserved Mechanism for Circadian Period Determination by These Protein Kinases

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Drosophila doubletime protein (DBT) and its vertebrate orthologs casein kinase I (CKI) epsilon or delta establish are circadian periodicity by regulating the nuclear accumulation of period protein (PER). Here, we address two questions: 1) How does enzymatic activity of DBT affect PER's temporal program and circadian behavior, and 2) How evolutionarily conserved is the circadian function of Drosophila DBT and its vertebrate CKI orthologs? Mutant of Drosophila DBT and vertebrate CKI delta which have lowered enzymatic activities, have been assayed for their effects on PER and on circadian behavior by introducing them as transgenes into Drosophila cultured cells or flies. Expression of a kinase-inactive, dominant negative form of Drosophila DBT leads to constitutively high levels and hypophosphorylation of PER, and behavioral arrhythmicity or very long-period rhythms. The results indicate that kinase activity is essential for DBT-dependent PER degradation and behavioral rhythmicity, but that kinase-inactive DBT retains enough function to antagonize wild type DBT. Expression of vertebrate CKI delta transgenes carrying the same mutations found in short- or long-period Drosophila dbt mutants or in the short-period hamster tau mutant produce period changes in flies as similar to those found in the original mutant animals. These results indicate that CKI kinases with apparently lowered kinase activity can produce the same period shortening or lengthening in the context of either the fly or vertebrate protein, and that there is evolutionary conservation of CKI-dependent period determination. An initial in vivo analysis of DBT mutants that are more active than wild-type DBT will also be presented.

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PAR Domain Protein 1? Function in the Drosophila Circadian Clock

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The circadian timekeeping mechanism in *Drosophila melanogaster* consists of two interlocked transcriptional feedback loops that are activated by CLOCK-CYCLE (CLK-CYC) heterodimers. In one loop, CLK-CYC activates period (per) and timeless (tim) transcription, then PER-TIM heterodimers accumulate and feed back to inhibit CLK-CYC activity. In the other loop, CLK-CYC activates vrille (vri) and PAR domain protein 1E (Pdp1E) transcription, whereupon sequential VRI repression and PDP1E activation mediate rhythms in Clk transcription. However, Clk mRNA is at peak levels in ClkJrk mutant flies even though little PDP1E is expressed, which suggests that PDP1E is not the only activator of Clk transcription. Since PDP1 null mutants are lethal, we used RNA interference and overexpression

approaches to determine whether PDP1E is necessary for circadian oscillator function in adults. In these experiments, tim-Gal4 was used to drive UAS-PDP1E RNAi and UAS- PDP1E expression in oscillator cells of wild-type flies, resulting in a >80% reduction or >10-fold increase in PDP1E levels, respectively. Reducing or increasing PDP1E had little or no effect on the overall level or cycling of Clk mRNA, VRI and PER. However, flies with reduced or increased levels of PDP1E showed a high degree of behavioral arrhythmicity despite persistent oscillator function in brain pacemaker neurons. These results argue that PDP1E functions to control oscillator output, but not as a component of the oscillator.

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Molecular and Neural Basis of Photoadaptation in the Circadian Network

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Many animals, including mammals and Drosophila, manifest morning and evening locomotor activity bouts to take advantage of and even anticipate favorable environmental conditions associated with these times of day. At least in flies, the timing of these events is controlled in large part at a brain network level. A group of clock neurons expresses the neuropeptide PDF and controls morning activity (small LNv, M-cells), whereas another oscillator group controls evening activity (E-cells). Our recent results also suggested that M-cells influence the phase of evening cells in absence of light, whereas E-cells can time the output of morning cells under normal lighting conditions. Nonetheless, little is known about how the network adjusts its function to variations in environmental conditions such as those typically occurring during seasonal progression. To this end, we have investigated the mechanisms used to detect the most relevant environmental cue, i.e., light. We first refined our understanding of the main Drosophila photoreceptor protein CRYPTOCHROME (CRY) and its relationship to the core pacemaker. We next constructed transgenic animals in which CRY-mediated photoreception was disrupted in a cell-specific fashion. Surprisingly, we found that entrainment to light stimuli is a feature of E-cells. These results and others indicate that both E- and M-cells send reciprocal signals under light–dark conditions, in one direction to convey lights-on (dawn) information and in the other to convey lights-off (dusk) information. Such functional organization of the circadian network suggests a new model for seasonal adaptation different from that of Pittendrigh and Daan.

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PER-Dependent Rhythms in CLK Phosphorylation and E-box Binding Regulate Circadian Transcription

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The Drosophila circadian timekeeping mechanism is composed of transcriptional feedback loops in which CLK-CYC-dependent activation and PER-TIM-DBT-dependent repression directly or indirectly control all circadian transcription. However, the mechanism by which PER-TIM-DBT feeds back to inhibit CLK-CYC transcriptional activity is not well understood. We have employed chromatin immunoprecipitation assays to show that interactions between PER containing complexes and CLK-CYC inhibit E-box binding and, consequently, transcriptional activity. Previous western results demonstrate that CLK is

a phosphoprotein that, paradoxically, accumulates to high levels when per and tim transcription is low and falls to low levels when per and tim transcription is high. Using a more stringent protein extraction procedure, we find that the overall levels of CLK are relatively constant, but that CLK cycles between hyperphosphorylated and hypophosphorylated forms. Hyperphosphorylated CLK accumulates in concert with hyperphosphorylated PER, PER dependent complexes containing DBT kinase and CLK, and transcriptional inhibition. CLK and PER accumulate to high levels in dbt mutants and dbt RNAi treated S2 cells, which suggests that DBT destabilizes both PER and CLK. Once hyperphosphoylated CLK and PER are removed, a novel hypophosphorylated form of CLK accumulates in parallel with the transcription of per, tim, and other CLK-CYC target genes. These results suggest a model for regulating circadian transcription whereby PER-TIM-DBT complex-dependent phosphorylation of CLK inhibits E-box binding and destabilizes CLK, thus coordinately removing hyperphosphorylated CLK and PER and enabling the accumulation of hypophsophorylated CLK to initiate the next round of CLK-CYC-dependent transcription.

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Pathways of Metabolic Non-Photic Input to the Suprachiasmatic Nucleus Interfere with Light Input to the SCN

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Most research on non-photic input to the suprachiasmatic nucleus (SCN) has focused on the intergeniculate leaflet and raphe nuclei. In the present study, we have investigated how metabolic information may reach and influence the SCN. Hereto we have used an anatomical analysis of the arcuate nucleus (vmARC), the dorsomedial hypothalamus (DMH) and entrainment to food as technical and experimental approach. The results show an extensive reciprocal relationship between these structures and the SCN, whereby especially the ventral SCN is densely innervated. Food entrainment or ghrelin (GHRP-6) that activate, respectively, the DMH or the vmARC inhibited the activity of ventral SCN neurons as demonstrated by Fos immunocytochemistry. In addition, GHRP-6 injection at ZT22 could inhibit the light-induced Fos immunoreactivity in the SCN and the light-induced phase shift. These results demonstrate that metabolic information may reach the SCN both via the circulation mediated by the vmARC or by viscero-sensory input mediated by the DMH. Since daytime and light-induced neuronal activity of the SCN is known to inhibit locomotor activity in rodents, it is proposed that under food anticipatory conditions the vmARC (NPY-AGRP) and DMH-NPFF input to the ventral, retina termination, site of SCN, is involved in inhibiting SCN neuronal activity, allowing locomotor activity during the light period.

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Molecular Traces of Dawn and Dusk in Different SCN Regions

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Life on earth must cope with large seasonal changes in food availability and temperature. Animals adaptively anticipate such fluctuations by annual changes in behaviour and physiology. Migration, hibernation, reproduction, and behavioural rhythmicity often show strong annual changes that are driven

primarily with a timing system that responds to changes in day length (photoperiod). Important in physiological and reproductive photoperiodic responses is the duration of the nocturnal melatonin signal, which mirrors day length and is shaped by signalling from the circadian pacemaker (SCN). Behavioural responses, such as activity compression under long days in nocturnal mammals, are also likely to be under the influence of the SCN. The precise mechanism by which the SCN codes for day length is largely unknown. Recently, it was shown that in a strongly photoperiodic mammal, the Siberian hamster, the expression of several clock genes under changing day length follows dawn in the caudal SCN while it follows dusk in the rostral SCN. Phase angle differences between rostral and caudal SCN gene expression could potentially form the basis for photoperiodic signalling from the SCN. Here, we provide evidence that also in a weakly photoperiodic mammal, the house mouse, caudal and rostral clock gene expression in the SCN follows, respectively, dawn and dusk. We hypothesise, that differences in the amount of rostral–caudal differentiation as a function of day length may partly explain the variation in behavioural or physiological responses to changing day length in strongly-and-weakly photoperiodic species.

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Complex Cellular Activities in the Rodent Suprachiasmatic Clock

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The suprachiasmatic nuclei (SCN) function as the most pervasive of the brain's circadian clocks, but how cells and anatomical subdivisions within this structure are organized to produce synchronized coherent outputs remains unclear. One powerful model posits that the rodent SCN clock is composed of dawn and dusk oscillators, but the anatomical correlates of this remain to be identified. Results from electrophysiological recordings of SCN neurons in vitro as well as studies on transgenic and wild-type animals in vivo suggest that multioscillatory rather than dual'= oscillatory activities define the circadian clock in this structure. For example, with exposure to constant light, many Djungarian hamsters show behavioural decoupling or splitting, and within the SCN of these split animals, there are distinct differences in the patterns of c-Fos staining, both between left and right SCN as well as within one side of the SCN. This suggests that the splitting phenomenon is accompanied by complex re-organization of SCN cellular activity. Adult mice deficient in neuropeptide signalling [VIP deficient; VIP/PHI-/- or lacking the VPAC2 receptor (Vipr2-/-)] have accelerated behavioural rhythms (periods of 22-23h) or are arrhythmic, but in vitro recordings from the SCN of these mice reveal that the range of estimated periods in SCN neuronal activity is much greater in the VIP/PHI-/- mice. These studies indicate that very different patterns of SCN neuronal activity can accompany apparently similar behavioural rhythms and suggest that caution should be exercised in inferring SCN organization from behavioural observations alone.

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Cyclic AMP-Dependent Signals Sustain Molecular Time-Keeping in Mammals and Determine Circadian Period

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Circadian timing in mammalian cells is based upon an auto-regulatory transcriptional/ post-translational feedback loop, pivoted around the rhythmic expression of Period and Cryptochrome genes. Although circadian activation of various second-messenger signalling cascades (including cyclic nucleotides, MAPK,

and calcium) has been widely observed, their role within the clockwork has been viewed primarily in terms of entrainment, most obviously via induction of Per expression. In VIP2 receptor knockout mice (Vip2r-/-), however, the molecular clockwork is suspended in most suprachiasmatic nucleus (SCN) neurons. This receptor signals via adenylyl cyclase (AC). We, therefore, sought to test the role of AC signalling in maintaining circadian time-keeping in mammals, using real-time bioluminescent recording of circadian gene expression.

Inhibition of the Gsalpha-binding site of AC caused a dose-dependent, reversible, dampening of circadian gene expression from Per1::luciferase organotypic SCN slice cultures, monitored by photomultiplier tubes. To determine the generality of this effect, we examined 3T3 fibroblast cells. Abrogation of the endogenous oscillation of cAMP concentration stopped circadian gene expression as reported by Bmal1::luciferase reporter constructs. P-site AC inhibition prolonged fibroblast period from ca. 21 hours to ca. 30 hours. Comparable treatment of Per1::luciferase SCN slices lengthened circadian period up to 30 hours.

Loss of cAMP signalling may account for the loss of molecular timekeeping in the SCN of the Vip2r-/- mutant mouse. More generally, cAMP signalling pathways are essential to sustain, and regulate period of, the mammalian circadian clockwork, both in SCN and in peripheral cells.

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Differential Function of the Mitogen-Activated Protein Kinase (MAPK) Targets MSK and RSK in the Suprachiasmatic Nuclei (SCN) of Mice.

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The mitogen-activated protein kinase (MAPK) cascade couples photic stimulation to entrainment of the mammalian circadian clock. MAPK signaling-regulates transcription via the direct action of extracellular signal regulated kinase (ERK) 1/2 on transcription factors and indirectly through ERK-regulated kinases. Two such downstream targets of ERK are the 90 kDa ribosomal S6 kinases (RSKs) and mitogen- and stress-activated protein kinases (MSKs). Both families of kinases are known to phosphorylate the transcription factor CREB, a central mediator of entrainment. Thus we sought to characterize and differentiate the function of RSK1 and MSK1 in the suprachiasmatic nucleus (SCN), the location of the mammalian circadian clock. Here we report that RSK1 and MSK1 are maximally activated by light at discreet phases of the subjective night. This data suggests the two kinases may differentially contribute to the expression of clock genes and behavioral phase advancing and delaying of the clock.

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Single Cell Bioluminescence Imaging of PER2 Expression in SCN Cultures from Wild-Type and Mutant Mice: SCN Neurons Are Not Always Autonomous Clocks

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The SCN contains a heterogeneous population of cells, and it is not clear how these cells or their interactions contribute to pacemaker function. We cultured SCN cells in slice and dissociated preparations from mice expressing a PERIOD2::LUCIFERASE fusion protein (Yoo, PNAS, 2004), allowing bioluminescence imaging of PER2 expression in single cells (Welsh, Curr Biol, 2004). SCN slices show strong circadian rhythms of PER2 expression, most prominent in the SCN shell and the periventricular zone dorsal to the SCN. Cells outside the SCN show a much lower level of expression, but are also rhythmic, sometimes in antiphase to SCN. In dissociated SCN cultures, circadian rhythms of PER2 expression are seen in most (but not all) luminescent neurons (~85%), and are not synchronized even among adjacent cells. Astrocytes have much lower levels of expression, but also appear rhythmic. Thus, we confirm that, like fibroblasts, single SCN neurons are autonomous clocks. Surprisingly, however, this cellular autonomy does not always apply to the same extent in clock gene mutants. We dissociated cells from PER2::LUC mice lacking functional Cry1, Cry2, or Per1 genes, and found that Cry2-/- SCN neurons (and fibroblasts) express strong PER2 rhythms with long periods, concordant with behavioral and SCN slice phenotypes. However, Cry1-/- and Per1-/- SCN neurons (and fibroblasts) are largely arrhythmic, in contrast to the strong rhythms observed in SCN slices and behavior. Thus, whereas most SCN neurons from wild type mice are autonomous oscillators, with genetic perturbations the rhythmicity of many SCN cells depends on intercellular interactions. [Supported in part by K08 MH067657 (DKW).]

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Tissue-Specific Rescue of Circadian Rhythmicity and Activity Levels in Bmal1-/- Mice

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BMAL1 (MOP3) is an essential component of the mammalian circadian pacemaker that transactivates expression of negative feedback loop members Per and Cry. Mice with targeted deletion of Bmal1 alleles exhibit loss of circadian rhythmicity, but also display decreased wheel-running activity, decreased body weight, reproductive defects, progressive arthopathy, and shortened life span. Therefore, BMAL1 is implicated in a variety of physiological functions that may be distinct from its role in circadian rhythm regulation, as well as dependent on the tissue type in which Bmal1 is expressed. To elucidate these diverse functions of BMAL1, we produced transgenic mice that either ubiquitously overexpress a BAC clone containing genomic sequence of Bmal1, or constitutively overexpress Bmal1 cDNA in a brain- or muscle-specific manner. We then examined the effects of rescued gene expression in a Bmal1-/- background. We first determined that BAC-rescued Bmal1-/- mice exhibited complete restoration of circadian rhythm,

wheel running activity levels, fertility, and long-term survival. Next, we examined the brain- or muscle-rescued Bmal1-/- mice and observed distinct tissue-specific functions of BMAL1. For example, circadian rhythm of wheel-running activity was restored in brain-rescued Bmal1-/- mice; however, total activity levels and body weight were still significantly lower than in wild-type mice. On the other hand, muscle-rescued Bmal1-/- mice exhibited activity levels and body weight similar to wild-type mice, yet remained behaviorally arrhythmic. Gene expression of known BMAL1 targets and output genes was also examined in these mouse lines. Our results not only elucidate the roles BMAL1 plays in various functions but also refine our understanding of tissue-specific regulation of holistic physiology.

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Analysis of Mouse Ocular Per2 Circadian Rhythms.

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In African clawed frog, retinal Per2 is expressed principally in photoreceptors and is regulated by light and dopamine. We have used Per2::luc knockin mice along with quantitative situ hybridization (ISH) and immunofluorescence (IF) to analyze Per2 expression in mouse eyes. Rhythmic luciferase activity in explants of neural retina from Per2::luc mice was not detected. ISH and IF analysis showed a high level of near constitutive expression of Per2 in the ganglion and inner nuclear layers. In contrast, ISH detected a lower level of rhythmically expressed Per2 mRNA in the photoreceptor layer. This suggests that Per2::luc rhythms in photoreceptors may be obscured by relatively high, near constitutive Per2 expression in the inner retina. Explants of RPE-choroid, ciliary body and cornea from Per2::luc mice showed high-amplitude luciferase rhythms. In each tissue, rhythmicity was confirmed in intact eyes by ISH, and IF revealed rhythmic changes in subcellular localization of Per2. Interestingly, phase differences were detected among different ocular tissues in the same eye. Our results reveal circadian rhythms of Per2 in photoreceptors, RPE-choroid, ciliary body, and cornea as well as an unexpected, near constitutive expression pattern in the inner retina. Different phases among ocular tissues suggests complexity in the mechanisms for phase control within the eye, while near constitutive Per2 in the inner retina suggests dominance of a non-circadian control mechanism driving Per2 expression.

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Tissue Specific Nature of Adrenergic Signaling in Peripheral Circadian Timing

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The incidence of heart attack and stroke undergo diurnal variation, raising the possibility of interplay between circadian gene expression and asynchronous cues such as exercise and psychological stress. However, it is largely unknown how the SCN entrains peripheral oscillators and how asynchronous cues are assimilated into hormonal mechanisms under circadian control. Norepinephrine and epinephrine, added to human and mouse aortic smooth muscle cells (ASMC) in-vitro, altered Per1, E4bp4, and dbp, expression via pathways activated by ligation of β2 and α1 adrenergic receptors (ARs). Agonists of these ARs phase advance, by 2-4 hours, oscillations of clock genes induced in vitro in vascular smooth muscle cells by serum shock. However, oscillations of Per1, E4bp4, dbp and Per2 were preserved ex vivo in the aorta, heart, and liver harvested from Dopamine beta-hydroxylase knockout mice (Dbh-/-) that cannot

synthesize either norepinephrine or epinephrine. Furthermore, clock gene oscillations in liver, heart, and white adipose tissue phase shifted identically in Dbh-/- mice and in Dbh+/- controls in response to daytime restriction of feeding. Significant differences in clock gene expression between Dbh+/- and Dbh-/- in food-restricted mice were apparent only in brown adipose tissue. Overall, these results suggest that while adrenergic signaling can influence circadian timing in vitro, its absence in vivo does not adversely affect rhythms in most peripheral tissues.

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Circadian Oscillations in Calcineurin Activity and MCIP1/DSCR1 Regulate Diurnal Changes in Cardiac Contractility

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Many factors within the cardiovascular system, including blood pressure, heart rate, neurohormonal levels, and the incidence of fatal cardiovascular events, oscillate with 24-hour periodicity. Our laboratory has recently observed that levels of a cardioprotective protein, modulatory calcineurin-interacting protein (MCIP1, also known as DSCR1 for Down Syndrome Critical Region 1), fluctuate with a 24-hour periodicity in the heart. MCIP1 is an endogenous feedback inhibitor of the protein phosphatase calcineurin. MCIP1 mRNA and protein levels were highest around ZT=0 and lowest at ZT=9. Calcineurin-regulated transcription factor, NFAT, is found in the nucleus at ZT=0 but not in the evening. These data demonstrate that there are robust diurnal fluctuations in calcineurin activity in a normal, healthy heart. This is important, as calcineurin activity has been implicated in the progression of hypertrophy and heart failure. We hypothesize that circadian fluctuations in calcineurin activity are beneficial and help to coordinate cardiac function over a 24-hour period. Consistent with this hypothesis, we observed increased phosphorylation of numerous components involved in increased cardiac contractility, including phospholamban and the protein phosphatase1 (PP1) inhibitor I-1, oscillating in opposite phase to calcineurin activity. Together, these data point to coordinated circadian regulation of gene transcription, Ca2+ handling, and calcineurin activity in the heart.

We have also observed robust circadian oscillations in MCIP1 expression in multiple tissues. MCIP1-/- mice display a lengthening of their innate circadian day length and a shift in peak Per2 and BMAL1 expression suggesting that, in addition to cardiac effects, calcineurin/MCIP1 signaling may influence circadian activities in other tissues.

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Corticosterone Response to a Light Pulse is Additive with Restraint Stress and Does Not Change with Repeated Exposure

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Recent experiments have demonstrated that circadian recovery following a phase-shift of the light–dark (LD) cycle is altered by restraint stress. We sought to determine whether a phase-shifting light pulse, with or without concurrent restraint stress, results in alterations in corticosterone (CORT) levels. In the first study, we measured the effect of a one-hour light pulse, occurring six hours before the normal lights-on (ZT18), on CORT in male rats. The CORT levels of these animals were compared to those of rats experiencing no manipulation (control), a one hour bout of restraint stress, or a combination of light and

restraint. Restraint or light alone resulted in greater CORT than in control animals ($p < 0.01$). CORT was highest in animals experiencing both stressors simultaneously ($p < 0.01$). Repeated, acute exposure to these stimuli did not greatly alter the CORT response. Upon three separate exposures to a one hour light pulse (with two weeks between stimulus presentations), CORT was maintained at the same level ($p > 0.05$). The same effect held true for animals exposed to three separate bouts of restraint. Animals repeatedly exposed to light and restraint showed a slight alteration in CORT concentration between stressor presentations, but had CORT levels consistently higher than control animals or those experiencing light or restraint alone ($p < 0.01$). These data suggest that a light pulse at an unexpected time of day is a stressor.

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The Relationship between LH Interpulse Interval and Amplitude Is Influenced By Cycle Phase, But Not By Sleep in Adult Women

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Complex effects of sleep on Luteinizing Hormone (LH) secretion depending on the age and gender of the subject have been reported, including stimulation of LH pulse amplitude by sleep during puberty and suppression of LH secretion by sleep in specific menstrual cycle phases in adult women. As part of a four-month study examining the effects of sleep disruption on menstrual cycle dynamics, pulsatile secretion of LH was studied in the luteal phase (LP) and the early follicular phase (EFP) of the menstrual cycle.

Healthy non-obese, young women (19-35 years) with regular menstrual cycles, and on no medications affecting reproductive hormones or sleep were studied in the EFP (n=7) and LP (n=8). LH pulses were analyzed (http://dsm.bwh.harvard.edu/bmu/sb_pulse/) and the inter-pulse interval (IPI) was calculated as the time between pulse onsets. Data were analyzed using paired t-tests and regression analysis.

There were fewer pulses during sleep than wake in both the EFP ($p=0.003$) and LP ($p=0.025$). There was a significant correlation of amplitude with preceding IPI in both cycle phases. The slope of this relationship was steeper for LP (7.4 IU/L/hr) than EFP (1.8 IU/L/hr), but was not different between sleep and wake. Sleep-related slowing of pulsatile LH secretion occurs in the LP and EFP. The relationship of LH pulse amplitude to previous IPI is significantly accentuated in the LP, but is not affected by sleep. These data suggest that the effect of sleep on pulsatile LH secretion during the normal menstrual cycle is not mediated by inhibition of LH pulse amplitude.

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Temperature as a Zeitgeber for the Circadian Clock

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Clocks are aligned to the environment via inputs from both daily light and temperature cycles. Previous molecular and behavioral studies in Drosophila have largely focused on light-dependent regulation of circadian clocks and their outputs. Although light is the strongest and best understood Zeitgeber for the circadian clock, temperature is also an important input. This work aims to better understand the role of temperature on gene expression and behavior in the fly. We generated genome-wide expression profiles of transcripts during temperature entrainment and subsequent constant conditions in both wild-type and arrhythmic mutant backgrounds, and used Northern, Western, and behavioral analyses to confirm and extend these data. The results indicate that two types of daily transcript rhythms are induced in response to temperature cycles: clock-independent temperature-driven oscillations and clock-dependent circadian oscillations. The genome-wide expression profiles of transcripts oscillating after temperature entrainment show a significant overlap with their light-entrained counterparts. The most reliable clock-controlled expression profiles maintain the same mutual phase relationships after entrainment by temperature or light. That is, the phase observed at the onset of the thermophase is systematically advanced by about six hours relative to the phase at the onset of light. A similar phase relationship is observed at the level of protein expression and locomotor activity behavior. These observations indicate that entrainment by light and temperature would occur cooperatively under natural circumstances, given the size of the delay that is commonly found between environmental temperature profiles and light/dark cycles.

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The Molecular Gatekeeper Dexras1 Sculpts the Photic and Nonphotic Responsiveness of the Mammalian Circadian Clock

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The mammalian master clock, located in the suprachiasmatic nucleus, is exquisitely sensitive to photic timing cues, but the key molecular events that sculpt both the phasing and magnitude of responsiveness are not understood. Here we show that the Ras-like G protein, Dexras1, is a critical factor in these processes. Dexras1-deficient mice (dexras1-/-) exhibit a restructured nighttime phase response curve and a loss of gating to photic resetting during the day. Dexras1 affects the photic sensitivity by repressing or activating time-of-day-specific signaling pathways that regulate ERK/MAPK. During the late night, Dexras1 limits the capacity of PACAP/PAC1 to affect ERK/MAPK, and in the early night, light-induced phase delays, which are mediated predominantly by NMDA receptors, are reduced as reported previously. Daytime photic-phase advances are mediated by a novel signaling pathway that does not affect the SCN core, but rather stimulates ERK/MAPK in the SCN shell and triggers down-regulation of clock protein expression.

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A Switch from Nocturnal to Diurnal Entrainment in a Mouse Model of Photoreceptor Dysfunction

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We report here a reversal of the normal nocturnal activity rhythm in a mouse model of photoreceptor dysfunction. Mice lacking both RPE65, a key protein in retinal chromophore recycling, and melanopsin (OPN4), retain only a small amount of rod function. In a 12-hour light: 12-hour dark cycle (light, 150μW/cm2), 85% of Rpe65-/-;Opn4-/- mice (n=14) oriented their activity such that it was coincident with the light phase while 15% free-ran. Rpe65-/-;Opn4-/- mice also showed reversed masking responses to light. However, when day-active mice were released into constant darkness, circadian locomotor activity rhythms free-ran from the time of lights on, indicating that the activity rhythms were diurnally entrained and not simply masked. In order to assess the phase of clock gene expression within the SCN and peripheral tissues, we crossed Rpe65-/-;Opn4-/- mice with mPer2luciferase (mPer2luc) knockin mice. Rhythms of PER2::LUC bioluminescence from cultured SCN of diurnally entrained double knockout mice (n=4) peaked approximately nine hours out of phase from those of sibling controls (n=5). Phase relationships between the SCN and peripheral tissues did not differ from those of controls. These results demonstrate that the signal responsible for the nocturnal to diurnal activity switch in Rpe65-/-;Opn4-/- mice lies upstream from the SCN, and therefore that changes in retinal input to the SCN can have dramatic effects on temporal niche preference.

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The Eye-Attached SCN Slice Preparation: An in vitro Mammalian Circadian Visual System

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The suprachiasmatic nucleus (SCN) receives information about ambient light levels through the retinohypothalamic tract. This information is used to reset the molecular clock of individual SCN neurons, leading to entrainment of overt animal behavior and physiology. Due to difficulties in recording in vivo light-evoked responses in the SCN, little is known about the organization and function of these connections and how they lead to resetting of the biological clock. To circumvent this issue, we have developed a novel in vitro SCN brain-slice preparation that maintains functional connectivity to both retinas, enabling patch-clamp recordings from visually identified SCN neurons that are still capable of responding to light. This allows us to conduct experiments concerning light processing in the SCN with unprecedented control and knowledge of the cells we record from. We have recorded light-evoked responses from numerous SCN cells using this preparation and demonstrated the following: 1) Some SCN cells derive their light responses from both the melanopsin photosensory system and classical photoreceptors, while others appear to be driven only by melanopsin. 2) SCN neurons show adaptation in their light-evoked responses, consistent with recent findings of adaptation in melanopsin retinal ganglion cells. 3) Light responses in the core neurons reverse near zero mV and are thus probably mediated mainly by glutamate receptors. These data reveal previously unknown properties of SCN cell function and processing of photic information.

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Single Unit Responses of SCN Neurons To Irradiance, Duration and Wavelength

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In mammals, non-visual responses to light including phase shifts of activity rhythms, melatonin suppression, pupil reflexes, and masking have been shown to involve intrinsically photosensitive retinal ganglion cells, which express melanopsin (ipRGC). These ipRGCs also receive input from rods and cones via amacrine and cone bipolar cells and convey photic information to the master circadian clock in the suprachiasmatic nucleus (SCN). We studied the responses of single neurons in the SCN to variations in irradiance, duration, and wavelength by recording extracellular single neuron activity in anesthetized animals. Experimental sequences and response patterns were done using a computerized system, and activity of individual neurons was distinguished using a spike wave-form discriminator. SCN light-responsive neurons are typically characterized by phasic ON responses to light followed by a sustained response to continued light exposure. At light extinction, an OFF response is observed followed by a long delay to recover the basal firing rate (post-stimulation persistence), the amplitude of which depends on irradiance. The sustained response is very robust and does not decrease even after long (5-60 min) exposures to bright light. SCN neurons are most sensitive to short wavelength light and fail to respond to wavelengths longer than 620 nm. In addition, the responses of SCN neurons are significantly altered by previous light exposure and suggest distinct contributions of rods and/or cones as well as melanopsin ipRGCs.

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Evolution of Inner Retinal Photoreceptors: Mammals Have Lost' a Melanopsin Gene

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Recent work has demonstrated the importance of a new functional photopigment, melanopsin (OPN4), in entraining the mammalian circadian clock. Using a combination of in silico and classical molecular biology approaches, we set out to examine the evolution of melanopsin in vertebrates. In the process, we have identified a novel melanopsin gene in zebrafish, chicken, and Xenopus that appears to be the true ortholog of mammalian melanopsin. This new melanopsin (which we have termed Opn4m) shares >70% amino acid identity with human melanopsin across the transmembrane domains compared with ~58% identity to the original Xenopus melanopsin (termed Opn4x). RT-PCR analysis in zebrafish, chicken and Xenopus laevis identifies expression of both Opn4m and Opn4x genes only in tissues known to be photosensitive (eye, brain and skin). In the eye of 14 day old chickens, Opn4m is found in a subset of cells in the inner nuclear, outer nuclear and ganglion cell layers, the vast majority of which also express Opn4x. A comprehensive in silico analysis of vertebrate genomes indicates that while most vertebrate species have both melanopsin genes, chromosomal reorganisation events early in mammalian evolution have resulted in the loss of Opn4x

from all members of this class studied to date. Our findings show for the first time that non-mammalian vertebrates retain two melanopsin photopigments, while mammals have just one. These data raise important questions regarding the functional differences between Opn4x and Opn4m pigments, the associated adaptive advantages for most vertebrate species in retaining both melanopsins, and the implications for mammalian biology of lacking an Opn4x orthologue.

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Circadian Orchestration of the Hepatic Proteome

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Recent studies have highlighted the pivotal contribution of the circadian transcriptome to rhythmic, tissue-specific physiology. Analysis of gene expression alone, however, inevitably provides an incomplete picture of metabolic regulation. We therefore conducted proteomic analyses of mouse liver, employing two dimensional differential gel electrophoresis, combined with peptide mass fingerprinting (MALDI-TOF mass spectrometry), or liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Across the matrix of analyses, 642 protein "spots" were reliably detected, and of these 61 (i.e. 9.5%) and 136 (i.e., 21%) exhibited a highly significant circadian variation, with ANOVA $p<0.01$ and $p<0.05$, respectively. Thus, 10–20% of robustly expressed soluble liver proteins are under circadian regulation, comparing favourably with previous estimates of 5–10% made for the circadian transcriptome. In some cases, several distinct spots represented multiple isoforms of the same circadian protein derived from a single gene. We therefore established a final list of 38 identified genes for which the corresponding 50 protein products were rhythmic ($p<0.01$), with peak expression across a range of circadian phases. Circadian regulation of selected proteins was validated by Western blots of independent samples from wild-type and circadian mutant mice. The circadian proteome includes rate-limiting factors in vital hepatic pathways such as urea formation and sugar, alcohol, and bile acid metabolism. We used quantitative PCR to test whether circadian proteins were subject to transcriptional regulation. Unexpectedly, almost half of the cycling proteins detected did not have a corresponding cycling transcript. Our studies reveal the importance of post-transcriptional and post-translational mechanisms as circadian control points within global hepatic physiology.

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Loss of Nocturnin, a Circadian Deadenylase, Confers Resistance to Diet-Induced Obesity

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Nocturnin is a deadenylase that is hypothesized to control mRNA expression in a circadian manner by degrading the poly-A tail of target RNAs, which leads to mRNA turnover or translational silencing. We generated a mouse with a targeted disruption of the nocturnin gene. Initial characterization of our

mouse showed no alterations in development, reproduction, or behavior, including no change in tau. Since nocturnin is highly expressed in the liver, we performed histological analysis of this tissue and established that nocturnin knockout mice had decreased lipid accumulation. Because of this fat accumulation deficit, we next measured the body weights of these mice on both standard and a high fat "Western" diet. Although there was no difference in weight while mice were maintained on standard diet, when transferred to a Western diet, the knockout mice showed significantly less weight gain than the wild-type controls. This "lean phenotype" was not the result of increased activity or decreased foot intake. Further studies demonstrated that the knockout mice on standard diet exhibited glucose intolerance, but increased insulin sensitivity when on a normal diet. Interestingly, on the Western diet, the knockouts, like the wild-type mice, become insulin resistant, therefore demonstrating that resistance to weight gain does not necessarily confer resistance to other deleterious effects of high fat diet. These findings point to an alteration in the knockout's ability to regulate glucose homeostasis and lipid metabolism. We propose that nocturnin has a role in the circadian regulation of metabolic genes through post-transcriptional modification of targeted transcripts.

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The Cross-Talk of Cholesterogenesis and the Circadian Regulation

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Regulation of genes encoding enzymes of cholesterol biosynthesis and uptake is mediated generally by the negative cholesterol feedback loop and sterol regulatory element binding protein (SREBP) transcription factors. SREBPs bind to modified E-box binding sites SRE (sterol regulatory elements). Lanosterol 14alpha-demethylase (CYP51) is one of the cholesterogenic genes activated by transcription factors SREBP. In the liver, the SREBP pathway seems to play a major role in cholesterol homeostasis. However, the liver is also a circadian organ with about 10% of genes showing circadian behaviour. Among these are circadian transcription factors as well as genes involved in cholesterogenesis. Several lines of evidence suggest that the expression of CYP51 is circadian. The aim of our study was to investigate whether: 1) circadian transcription factors influence the expression of cholesterogenic CYP51 from the proximal promoter containing a SRE site; 2) SREBP transcription factors influence the expression of Bmal1, Per1, and Per2 from their proximal promoters. The overexpressed SREBP2 (but not SREBP1) isoforms activate the expression of Bmal1 and Per1 promoter-reporters in transfection assays, whereas there is no effect on Per2. In accordance to that, the Bmal reporter is repressed in lipid rich medium. Overexpressed Clock/Bmal proteins do not influence the expression of CYP51-promoter-reporter whereas overexpressed Per1/Cry1 lead to repression. Per1/Cry1 also attenuate the SREBP2-induced expression of the CYP51 promoter-reporter. These data suggest that the cross-talk of cholesterol synthesis and the circadian regulation might be mediated by interacting of Per1/Cry1 by SREBP2 as well as by modulating the expression of circadian transcription factors by SREBP2.

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The Circadian Clock Protein BMAL1 Affects Steroidogenesis and Is Necessary for Fertility

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Reproduction is profoundly affected by circadian rhythms. For instance, in mammals the serum concentrations of many reproductive hormones oscillate with a daily rhythm, and the ablation of brain regions necessary for circadian rhythms generally leads to sterility. We found that BMAL1 is necessary for fertility in mice. Bmal1 knockout (Bmal1 KO) mice, of both sexes, are completely infertile. Although the reproductive systems of male and female Bmal1 KO mice are intact, gross and microscopic analysis suggests that hormonal disruptions in the Bmal1 KO mice underlie the infertility. Male Bmal1 KO mice have low sperm counts and low concentrations of serum testosterone, possibly due to a defect in the Leydig cells of the testis. Female Bmal1 KO mice can ovulate, but suffer very early pregnancy loss possibly due to failure of the corpora lutea. Why the deletion of Bmal1 leads to these hormonal defects is unclear, but given the function of BMAL1 as a transcription activator, one possibility is that expression of genes necessary for the production of reproductive hormones is disrupted. Consistently, we found reduced expression of steroidogenic genes in both the testis and the ovary of Bmal1 KO mice. In contrast, Clock mutant mice have normal expression of these genes in the testis. We hypothesize that BMAL1 regulation of steroidogenic gene expression allows it to modulate steroidogenesis. Ongoing work is focused on understanding the mechanism by which BMAL1 regulates this expression.

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Altered Sleep and Diurnal Regulation in Leptin-Deficient Mice

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The homozygous Clock mutant mouse exhibits decreased sleep time and dramatic impairments in energy metabolism, including obesity and hyperphagia. These findings suggest that circadian, metabolic, and sleep systems are linked by common genetic mechanisms. In this study, we tested the hypothesis that animals with a genetic deficiency in leptin (ob/ob mice), a major hormone signal of adiposity, would exhibit impairments in the diurnal and homeostatic regulation of sleep. Adult male ob/ob (n=9) and wild-type control (n=8) mice were studied under entrained (12:12 L:D) conditions. Ob/ob mice exhibited increased amounts of 24-hr NREM sleep (wt, 601.5±10.8 vs. ob/ob, 669.2±13.4 minutes, $p<.001$). Ob/ob mice also had significantly disrupted sleep architecture with an elevated number of arousals from sleep (wt, 108.2±7.2 vs. ob/ob, 148.4±4.5, $p<.001$) and increased stage shifts (wt, 519.1±25.2 vs. ob/ob, 748.0±38.8, $p<.001$). Ob/ob mice had overall lower body temperature (wt, 35.1±0.2 vs. ob/ob, 33.4±0.2 ˚C, $p<.001$) and locomotor activity counts (wt, 25,125±2137 vs. ob/ob, 5219±1759, $p<.001$). Interestingly, ob/ob mice also displayed a notable attenuation in the diurnal organization of sleep-wake stages, NREM delta power, body temperature, and locomotor activity. Following sleep deprivation, ob/ob mice had smaller amounts of NREM and REM recovery sleep in terms of, both the magnitude and the duration of the recovery response. In combination, these results indicate that leptin deficiency not only impairs metabolic function, but also disrupts the regulation of sleep architecture and diurnal rhythmicity. These data provide another example of overlapping regulatory mechanisms involved in metabolic, sleep, and circadian function.

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A Network of Autonomic Clock Outputs

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The most detailed information on neural pathways employed by the SCN to transmit its rhythmic signal is available for the adrenal and pineal gland hormones, i.e., corticosterone and melatonin. These data indicate an important role for the connections of the SCN with the autonomic nervous system (ANS), more specifically, with its sympathetic part. In order to investigate if the circadian control of the SCN also comprises the parasympathetic part of ANS, we extended our studies to the circadian control of glucose homeostasis. Daily rhythms in peripheral glucose uptake and hepatic glucose production were found. The daily rhythm in glucose production proved to be modulated by an SCN effect on sympathetic pre-autonomic neurons in the PVN. Euglycemic clamp experiments are ongoing to locate the SCN target area for the effects on peripheral glucose uptake. Thus far, our results show that the pre-autonomic neurons in the PVN are controlled by the combined effect of a continuous glutamatergic and a rhythmic GABAergic input from the SCN. Hypothalamic stimulation also changed hepatic clock gene expression. On the other hand, hepatic clock-gene rhythms are not dependent on an intact sympathetic input and not sufficient to sustain a daily plasma glucose rhythm. Finally, when studying the effects of our hypothalamic manipulations on plasma insulin levels, i.e., a hormone from the endocrine pancreas, the first indications for a circadian control mechanism of the parasympathetic part of the ANS were revealed. Moreover, our data revealed an organ-specific differentiation of (light-induced) SCN output.

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Effect of Sleep Fragmentation on Spectral Activity of Sleep EEG in Morning-type and Evening-Type Individuals

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We have shown that morning types (M-types) have a faster dissipation rate of homeostatic sleep pressure than evening types (E-types). Here, we tested whether there was also a difference in the homeostatic response to sleep disruption. The sleep of 12 M-types and 12 E-types (19-34 y.), was recorded by polysomnography for five consecutive nights according to each subjects habitual sleep schedule: adaptation night, baseline night, two nights of sleep fragmentation (5 min of awakening every half-hour), and recovery night. Sleep EEG recorded from the Fz/linked ears derivation was digitized at 256 Hz. Spectral analysis (FFT) was performed in NREM sleep and REM sleep on 4-sec artifact-free sections for six frequency bands: SWA (1–5 Hz), theta (4–8 Hz), alpha (8–12 Hz), low sigma (12–14 Hz), high sigma (14–16 Hz) and beta (16-24 Hz). Activity (uV2/Hz) in each band was compared using Group-by-Night ANOVAs. In NREM sleep, significant Group-by-Night interactions were found for SWA and theta ($F_{3,66} > 2.8$, $p < 0.05$). The increase in SWA and theta activity between baseline and recovery nights, as well as the increase in SWA between the two fragmented nights, were higher in M-types than in E-types (simple effect: $p < 0.02$). In REM sleep, a trend for a similar Group-by-Night interaction was also found for SWA ($F_{3,66} = 2.5$, $p = 0.08$). No significant effect appeared in other frequency bands. These results add further evidence to the hypothesis of a difference in homeostatic sleep regulation between chronotypes.

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Diurnal Sex Differences in the Sleep–Wake Cycle of Mice Are Dependent on Gonadal Function

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Sex is an important determinant of the pathophysiology of several disorders that influence and/or impair the sleep–wake cycle. To date, few studies have examined either the role of sex or the gonadal hormones on sleep and wakefulness. The difficulty in performing well-controlled clinical experiments on sex and sleep underscores the need for effective animal models to investigate the influence of the gonadal hormones on sleep–wake states. This study describes the influence of sex on sleep and wakefulness in mice, the primary mammalian genetic model for sleep analysis, and tests the hypothesis that gonadal function drives sex differences in sleep–wake states. EEG/EMG sleep–wake patterns were recorded in intact and gonadectomized male and female C57Bl/J6 mice maintained on a 14-hour light:10-hour dark schedule. Following a 24-hour baseline recording, mice were sleep deprived during the light phase by gentle handling, and given a 10-hour recovery opportunity during the immediate dark phase. Intact female mice spent more time awake than intact males during 24 hours of baseline recording at the expense of NREM sleep. Although the recovery response of NREM sleep was similar between males and females, when examined in reference to baseline levels, females exhibited a more robust recovery response. Gonadectomy in males and females reduced or eliminated the majority of sex differences in sleep architecture and homeostasis. These data demonstrate that the gonadal hormones influence the amount, distribution, and intensity of sleep, but do not account for all sex differences in the sleep/wake cycle.

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Effects of Bedpartners on the Sleep–Wake Cycle

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The study has been designed to examine changes in sleep-wake cycles that occur with sexual cohabitation (bed partners). Subject were young unmarried couples (mean age: 25; range: 22–26) with no children and no history of sleep disturbances who regularly slept alone or with their partners. They were given a standardized entrance examination (sleep anamnesis, standardized sleep quality questionnairesl, and a questionnaire to evaluate morning- or evening- chronotypes). Subjects documented their sleep-wake pattern in sleep diaries over a period of four weeks. Their sleep-wake cycles were monitored by wrist-worn actigraphs. During the investigation period, subjects were asked to spend at least 10 nights together and 10 separately. Preliminary results demonstrate significant reductions in subjective and objective (actigraph) sleep quality and in subjective sleep efficiency (sleep log data) in the co-sleeping condition as compared to the nights spent alone. This tendency was observed in both females and males. Moreover, in the co-sleeping condition, both sexes increased their sleep-onset latency, although this was more pronounced in females. When sleeping alone, women have more sleep epochs associated with movements than men. On nights spent together, the mean activity score increased in both females and males, but the changes were not significant. The current investigation reveals complex interaction between sleep quality, sleep habits, and "sleep needs" in bedpartners and contributes to new insights on gender differences during sleep and

wakefulness. The preliminary results indicate that these interactions become even more complex when nocturnal cohabitation is accompanied by sexual contact.

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Circadian Effect on Degree of Sleep Inertia Present After Awakening

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Sleep inertia is the sleepiness or impaired cognitive performance experienced immediately upon awakening, the majority of which dissipates within an hour. We tested whether there exists a circadian rhythm in the degree of sleep inertia.

Seven adult subjects were studied throughout a 10-day protocol performed in dim light during which subjects slept across all circadian phases, achieved by scheduling a recurring 28-h day. Subjects were awakened using a standardized auditory stimulus three times each sleep period, and immediately performed a computerized two-minute serial addition test, and reported subjective sleepiness (Karolinska Sleepiness Scale). Sleep inertia was quantified as the change within 20 min of awakening. Data are presented only following awakenings from Stage II sleep. Core body temperature was used as a circadian phase marker.

Immediately upon awakening, subjects attempted an average of 22 additions, which improved to 26 after 20 minutes. There was a significant circadian rhythm in sleep inertia of cognitive performance ($P<0.001$), with peak improvement during the biological night (circadian phase bin 300°), no improvement during the biological day (180°), and a peak-to-trough difference of ~110% (5 additions). There was no significant circadian rhythm in sleep inertia of subjective sleepiness. There is a clear circadian rhythm in the degree of sleep inertia of cognitive performance but not in the sleep inertia of subjective sleepiness. These findings may have important implications for professions requiring decision-making immediately upon awakening, e.g., on-call medical professionals.

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Diurnal Patterns of Sleep Tendency in Children and Adolescents as a Function of Circadian Phase Angle

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We examined phase angle in association with diurnal patterns of sleep tendency after in children nominal and restricted sleep conditions. Our hypotheses are based on predictions of Carskadon and Acebo (2002, Sleep).

Thirty-two participants (17 boys) ages 10–15 years reported morning (n=20) or evening (n=12) phase preference (Smith et al. [1989] J. *Applied Psychology*). Participants kept a stable sleep schedule for 2.5 weeks at home ("nominal") followed by a symmetrically restricted (70%) sleep schedule for one week. Following each sleep condition, participants maintained the same sleep schedule for one night in the laboratory. Sleep tendency was measured every two hours the following day using the Multiple Sleep Latency Test (MSLT). Saliva was also collected in dim light (<15 lux), and melatonin onset (DLMO) phase was determined using

linear interpolation with a 4 pg/mL threshold. Nominal and restricted phase angles ("PA") were defined as the time interval between DLMO phase and nominal or restricted wake-up times, respectively.

Following nominal sleep, wider PAs were associated with shorter evening sleep latency (2100 SLT: r=-.498, p<.05, n=23). Following restricted sleep, wider PAs were associated with longer morning sleep latency (0700 SLT: r=.446, p<.05, n=31; 0900 SLT: r=.375, p<.05; 1100 SLT: r=.467, p<.01).

These data are consistent with the prediction that waking at a later circadian phase (wide PA) may promote greater evening sleep tendency compared to waking on an early circadian phase (narrow PA). A wide PA also protects morning alertness in restricted sleep conditions. PA may predict diurnal patterns of sleepiness in children.

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Circadian Rhythm of Alertness in Delayed Sleep Phase

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Potential causes of Delayed Sleep Phase (DSP) have been suggested such as an unusually long circadian period or altered homeostatic drive for sleep. Patients with DSP also report increased levels of alertness in the evening that make it difficult to fall asleep earlier. The aim of this study was to determine if there are changes in the timing of alertness between patients with DSP and controls.

Nine DSP and five age–and–gender matched controls completed a four-day inpatient study. DSP patients were diagnosed according to ICSD criteria. Subjects kept a habitual sleep–wake schedule for 3–5 weeks prior to entering the laboratory. In the laboratory, subjects kept, this habitual schedule. Testing began at circadian time 2. A Multiple Sleep Latency Test (MSLT), and Visual Analogue Scales (VAS) were completed at intervals of two hours during 28 hours of wakefulness.

For both the MSLT and subjective alertness, there was a significant time effect ($p<0.0001$) but no group or interaction effect during the 28 hrs of wakefulness. Alertness levels decreased the longer the subjects were awake.

When compared at the same circadian time there was no significant difference in alertness between patients with DSP and controls, whereas, when aligned to clock time, a higher level of evening alertness was seen in the DSP group. These findings confirm a delay in the timing of the circadian rhythm of sleep and wake propensity, relative to clock time. Furthermore, these results do not lend support to the hypothesis of altered homeostatic sleep regulation in DSP.

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A Large-Scale Forward Genetics Screen to Identify Circadian Rhythm Mutants in the Mouse

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In the last decade, tremendous progress has been made in identifying genes and gene products that are components of the mammalian circadian clock. In spite of this progress, it appears likely that additional clock components exist.

Protein interaction assays, microarray analysis, and genetic quantitative trait analysis all suggest that there are more clock components yet to be identified. However, there are few candidates with definitive functional or genetic evidence available. Furthermore, genetic screens for circadian mutants in mammals have been limited in both scale and scope. For these reasons, we have continued to screen for circadian rhythm mutants in mice.

We are currently engaged in a large-scale ENU mutagenesis screen for recessive mutations affecting the nervous system and behavior (see http://genome.northwestern.edu/neuro). One of the five domains of primary interest in this project involves circadian behavior. The goal of this screen is to identify both new circadian rhythm genes and additional mutant alleles of known circadian rhythm genes.

To date, we have screened over 13,000 mutant mice for abnormal circadian locomotor activity and have identified over 50 circadian rhythm mutants. These mutants include mice with altered free running periods (~30 mutants), mice with variable activity onsets (~25 mutants) and mice with abnormal entrainment patterns (1 mutant). Efforts are currently underway to map these mutations to specific gene loci. These mutants represent important genetic tools that will provide insights into the mechanism and regulation of the mammalian circadian clock.

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Impact of a Novel ENU-Induced Long Period Circadian Mutant on Molecular Timekeeping in the SCN and Periphery

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Circadian timekeeping in mammals is driven by transcriptional/post-translational feedback loops that are active within both peripheral tissues and the circadian pacemaker of the suprachiasmatic nuclei (SCN). As part of a large-scale chemical mutagenesis screen, mice were measured or a wheel-running activity to assess for circadian phenotype. One mutant line (PLAY68) was identified as having a long circadian period with homozygotes having a period of ca. 27h, heterozygotes ca. 24.5h compared with ca 23.5h in the wild-type (WT) littermates. This lengthened period phenotype is robustly inherited in a semi-dominant fashion. To test the role of PLAY68 in molecular timekeeping within the SCN and peripheral tissues, real-time recordings of circadian gene expression were made in PLAY68 mutants carrying an mPER2::luciferase transgene as a reporter of activity within the core circadian feedback loop. Photomultiplier recordings of luciferase activity from organotypic SCN slices of WT mice revealed high amplitude, precisely-defined

circadian cycles of gene expression. Consistent with the lengthened circadian wheel-running behaviour of heterozygous mice, PLAY68mut/+ slices also exhibited well-defined circadian cycles of bioluminescence, which were significantly longer than WT SCN slices (25.83h ± 0.26h vs 24.17h ± 0.3h). In addition to the SCN, recordings from organotypic slices taken from peripheral tissues of these mice revealed that the impact of this mutation was widespread. Using real-time imaging of circadian gene expression across lung, heart, and kidney slice cultures, we show that tissues from PLAY68mut/+ mice had significantly longer circadian cycles of bioluminescence compared with WT controls. These results confirm the global importance of the PLAY68 gene in regulating circadian timekeeping.

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TheT au Mutation in CKIe Is an in Vivo Gain of Function Mutation on PER1 and PER2

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The tau mutation in casein kinase I (CKIetau) causes a decrease of kinase activity in vitro, but it has been difficult to reconcile this loss of function with the current model of circadian clock function. The Forger-Peskin model, a detailed quantitative model of the mammalian circadian clock, predicts the opposite: that CKIetau must be a gain of function mutation. We have verified this prediction experimentally by testing CKI function in NIH 3T3 cells. We find that the CKIetau produces a gain of function on PER proteins. CKIt expressed in cells increases the in vivo phosphorylation and shortens the half-life of both PER1 and PER2. Phosphopeptide mapping shows that the increase in phosphorylation is restricted to specific sites on PER, probably those that stimulate the bTrCP-dependent proteasomal degradation. However, consistent with published data, CKIetau remains less active than wild-type CKIe?on casein and BMAL1 and is a loss of function in the Wnt signaling pathway. The quantitative model predicts that all short- period mutations in CKI family members will produce a gain of function. Interestingly, the Drosophila DbtS mutation and the human CKId(T44A), reported to be loss of function mutations, also enhance PER phosphorylation in cells. These findings experimentally validate the systems biology approach and provide a remarkable example of how a specific mutation can be both a loss and a gain of function, depending on the substrate.

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Oscillator Network Interactions Compensate for Circadian Clock Gene Defects in Suprachiasmatic Nuclei

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In mammals, the circadian timing system is a hierarchical multioscillator network consisting of a master clock in the suprachiasmatic nuclei (SCN) that coordinates oscillators in many peripheral tissues. Inference concerning the relative contributions of core clock components has been based primarily on genetic disruption and subsequent molecular and behavioral analyses. In this study, we used real-time

bioluminescence imaging to monitor the dynamics and persistence of clock gene expression in tissue- and cell-autonomous preparations from circadian mutant mice harboring mPER2::LUC reporter. We showed that Cry1 and Per1 are indispensable in the intracellular clockworks of both individual SCN neurons and fibroblasts derived from knockout mice, whereas Cry2 and Per3 mutants only have period length defects and are not essential for sustained rhythmicity. These results suggest that the intracellular mechanism in the peripheral oscillators is similar to that in the SCN. However, intercellular interactions in the SCN, but not in peripheral tissues, can sustain circadian rhythms in cells deficient for Cry1 or Per1. Thus, intercellular synchronization provides a fail-safe mechanism in the SCN to retain robustness despite genetic perturbations, distinguishing the SCN from peripheral tissue oscillators. We propose that clock gene function should be examined not only at the behavioral level but also at the cellular level, whereby tissue- and cell-autonomous properties of the intracellular clockwork can be revealed.

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Mouse Phenome Project: A Systematic Comparison of Circadian Activity Rhythms in 34 Inbred Mouse Strains.

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A large number of inbred laboratory mouse strains originate from a mixed but limited founder population in a few laboratories. Indeed, recent SNP-based haplotype analysis of laboratory mouse strains has shown that the genome is composed of a mosaic of ancestral haplotypes that originate from *M. m. musculus, M. m. domesticus, M. m. molossinus* and *M. m. castaneus* subspecies. The average haplotype block size is ~1-2 MB. When any two laboratory inbred strains are compared, ~2/3 of the genome has no or very little difference (~1SNP/20Kbp) and the remaining of 1/3 is divergent (~1 SNP/0.2Kbp). These results support the idea that inbred strains may be a powerful resource for high-resolution mapping for quantitative trait loci (QTL). In order to test this hypothesis, we measured and analyzed circadian behavior in 34 different inbred mouse strains. As expected, there are significant strain differences in several aspects of circadian locomotor behavior, such as free running period, phase angle of entrainment, and amplitude of circadian rhythms. We then performed a genome-wide haplotype association study with ~150,000 SNPs. We are now testing each locus identified in experimental F2 crosses derived from two strains with extreme phenotypes. This strategy is one of the most powerful approaches to achieve high-resolution mapping of QTLs because it utilizes the existing, historical accumulation of ancestral recombinations in the creation of inbred strains to resolve loci rather than by the generation of new experimental crosses and congenic strains, as practiced previously.

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Circadian Transcriptional Mechanism of Molecular Clocks

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Molecular mechanism of circadian oscillation is based on transcriptional negative feedback loops of clock and clock-controlled genes. A hetero-complex of bHLH-type transcription factors, CLOCK and BMAL1,

binds to the E-box of the Per promoters and activates their transcription. The proteins translated inhibit their own transcription. This model is based mainly on a study using Per1 promoter by overexpression-based transient reporter assay because Per1 is the first cloned mammalian period homologue. Recent works using knock-out mice or with human genetics revealed that Per2 is more a rather core-clock molecule than Per1. Also Bmal1 mRNA expression displays a robust circadian rhythm, which is anti-phase to Per2, and BMAL1 seems to form another core-clock molecule. We have established an in vitro real-time oscillation monitoring system by using photomultipliers. Using this system combined with the use of knock-out mice, we revealed that a non-canonical clock E-box in Per2 promoter and ROREs in Bmal1 promoter are essential for their robust oscillation of Per2 and Bmal1 transcription. Further, the circadian rhythm is related to various phenomena, and I will discuss its relationship to other brain functions such as stress response and mental states.

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Constitutive Overexpression of PRR7 Weakens Circadian Rhythms in Arabidopsis Thaliana

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PRR7 is a member of the circadian regulated pseudo-response regulators and has been shown to play a role in the circadian clock in *Arabidopsis*. We present data on the circadian phenotype of transgenic plants overexpressing PRR7. Although a weak constitutive expression of PRR7 coding region in the prr7 mutant background is able to complement the mutant phenotype, stronger overexpression in the wild-type background weakens circadian rhythms under constant conditions. In addition, the expression of PRR7 coding region under control of its own promoter is also able to complement the prr7-3 phenotype. These results indicate that circadian-regulated transcription of PRR7 is not necessary for clock function and that there are likely to be additional posttranscriptional regulation mechanisms of PRR7. Indeed, we observe that the level of PRR7 protein is regulated postranscriptionally under light-dark cycles.

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A Novel Computational Model of the Circadian Clock in Arabidopsis that Incorporates PRR7 and PRR9

Melanie N. Zeilinger*, Eva M. Farré, Steve A. Kay, and Francis J. Doyle III

In plants, as in animals, the core mechanism to retain rhythmic gene expression relies on the interaction of multiple feedback loops. In recent years, molecular genetic techniques revealed a complex network of clock components in *Arabidopsis*. To gain insight into the dynamics of these interactions, new components need to be integrated into the mathematical model of the plant clock. We present a method to accelerate the iterative process of model identification, to incorporate new components, and to systematically test different proposed structural hypotheses. Recent studies indicate that the pseudo-response regulators PRR7 and PRR9 play a key role in the core clock of *Arabidopsis*. We incorporate PRR7 and PRR9 into an existing model involving the transcription factors TOC1, LHY and CCA1. We propose candidate models based on experimental hypotheses and identify the computational models with the application of an optimization routine. Validation is accomplished through systematic analysis of various mutant phenotypes. We introduce and apply sensitivity analysis as a novel tool for analyzing and distinguishing

the characteristics of proposed architectures, which also allows for further validation of the hypothesized structures.

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Genetic and Molecular Characterization of the Neurospora crassa *bd Mutation: The Molecular Basis of the Overt Circadian Rhythms in Neurospora*

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For the past 40 years, the bd mutation in *Neurospora crassa* has been an integral tool in the study of circadian rhythms. This semidominant mutation allows the clear visualization of the circadianly-regulated conidiation (banding) pattern. By developing a detailed SNP map in the vicinity of bd, we have identified bd as a single-point mutation changing T79 to I in the RAS-1 protein. Extensive literature for Ras biology has previously led to a connection between ras and reactive oxygen species; other evidence supports the hypothesis that hyperoxidant states may trigger cell differentiation (Hansberg and Aguirre, J. Theor. Biol. 142:201-221,1990). This led us to suggest that the bd mutation might lead to an imbalance in ROS (Reactive Oxygen Species) levels, which in turn, was responsible for the banding phenotype observed on race tubes. This hypothesis places ras-1 in the position of governing a global developmental switch affecting many developmental processes, prompting additional experiments in which levels of ROS in cells were manipulated and the effects on the overt rhythm followed. Through such manipulations, we find we can phenocopy the band mutation in wt cells with simple chemical treatments, and suppress the banding phenotype in bd mutants. This places Ras signaling and ROS balance as important determinants of circadian output in *N. crassa*.

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Molecular Mechanism of Circadian Singularity Behavior

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Circadian singularity behavior is a phenomenon characterized by the abolishment of circadian rhythmicities with a critical phase-resetting stimulus. The molecular mechanism is still uncharacterized despite its discovery more than 35 years ago and the fact that it is observed in organisms ranging from single-cell organisms to humans. In this study, we were able to demonstrate that both temperature step up and light pause, stimuli that activate the expression of the Neurospora circadian clock gene frequency (frq), are able to trigger singularity behavior in this organism when applied at a critical time during the declining phase of FRQ expression. The arrhythmicity is transient and is followed by the resumption of rhythm in randomly distributed phases after a few days, suggesting that the Neurospora circadian clock is a limited cycle based oscillator in which FRQ is a state variable. We show that the mutations of frq lead to changes in the amplitude of FRQ oscillation, which determines the amplitude of the clock and the sensitivity of the clock to phase-resetting cues. Our results further suggest that the singularity behavior in this organism is due to the loss of rhythm in all cells and not due to the desynchronization of the cell population. Together, these data are consistent with a model in which the singularity behavior is due to a circadian negative feedback loop driven to a steady state (equilibrium) after the critical treatment.

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A Genetic Screen for Loss of FRQ-Based Negative Feedback in Neurospora crassa

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Negative feedback is a central aspect of circadian oscillation mechanism in nearly all organisms. In Neurospora, FRQ (FREQUENCY) and FRH (FRQ Helicase) complex transiently bind with the WCC (White Collar Complex) to inhibit its transcriptional activity. Thus, a circadian clock is formed with an oscillation of WCC activity. To investigate components in this feedback loop, a genetic screen was designed to collect mutants with loss of negative feedback loop, and application of this screen has yielded a new frq allele, frqLN(frq loss of the negative feedback allele). FRQLN can bind with FRH, but not with WC-2, indicating that FRQLN harbors a mutation in a potential WCC binding domain that breaks the negative feed back loop. Consequently, WCC activity is maintained at a high level in the mutant strain, with a high level of frqLN RNA expression in constant darkness. Interestingly, the stability of the FRQLN mutant protein is increased, which suggests that the interaction between WCC and FRQ might promote FRQ degradation consistent with a model where negative feedback itself might induce the degradation of negative elements to promote a more robust oscillation.

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Genes Involved in Temperature Compensation of the N. Crassa **Clock**

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Temperature compensation of the clock, the relative invariance of circadian period as a function of temperature, is a defining but poorly understood property. We have used a genetic approach to dissect temperature compensation: identification, cloning, and molecular analysis of alleles of two genes displaying altered temperature compensation has suggested a model for how temperature compensation might work. We have experimentally validated the model by demonstrating that controlled dosing of the relevant gene products results in predictable alterations of temperature compensation. These data have, in turn, fostered further development of our mathematical model for the function of these loci in establishing compensation. The identification of these genes and their roles in temperature compensation provides an entree to understanding the molecular bases of this indispensable clock property.

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Phase-Advancing Human Circadian Rhythms with the Dual MT1/MT2 Melatonin Agonist VEC-162

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Appropriately timed melatonin administration is reported to phase-advance human circadian rhythms. However, the claim that melatonin can effectively treat circadian rhythm sleep disorders is controversial. We studied the effects of a dual MT1/MT2 melatonin agonist, VEC-162 (Vanda Pharmaceuticals), on the plasma melatonin rhythm, performance and sleepiness and on polysomnographic sleep in humans exposed to a simulated time zone transition, achieved by a five-hour phase advance of the light-dark (LD) and sleep–wake cycles. Thirty-nine subjects (20 females) ages 18–50 years, completed a randomized, double-blind, placebo-controlled, parallel groups design to assess efficacy and safety of nightly oral doses (10 mg, 20 mg, 50 mg or 100 mg) of VEC-162 compared to placebo (n=7-9/group) administered 30 minutes prior to bedtime. Subjects were healthy and medication-free. Subjects maintained a fixed sleep-wake schedule for at least twooutpatient weeks. The laboratory study included three days baseline with placebo lead-in, 19-hour constant posture protocol for pre-treatment circadian phase assessment, three days of a sleep-wake cycle phase advanced by 5 hours treated with VEC-162 or placebo, and a 24-hour constant posture for post-treatment phase assessment. Plasma melatonin was measured by LC/MS. Performance, subjective sleepiness, and mood were evaluated every two–three h with the Psychomotor Vigilance Task, Karolinska Sleepiness Scale, and visual analog scales, respectively. VEC-162, compared with placebo, phase advanced the onset of plasma melatonin by up to five hours on the first administration. The phase-shifting properties of VEC-162 may be utilized in the treatment of circadian rhythm sleep disorders.

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Melatonin Entrains Free-Running Blind Individuals with Circadian Periods Less than 24 Hours

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Most totally blind individuals lack photic input to the circadian pacemaker and have rhythms that free-run with observed periods (taus) greater than 24 hours. In such blind free-runners (BFRs), exogenous melatonin induces corrective phase advances, resulting in entrainment. We studied two BFRs (BL-62 and BL-64) with taus less than 24 hours, saliva was collected for 14–25 h at two-week intervals and was assayed for melatonin. The melatonin onset (MO) was assessed using a three pg/ml threshold. Free-running taus were calculated by linear regression through a series of MOs. Melatonin (0.3 mg) was administered at 6:30 (BL-62) and 20:00–21:00 (BL-64). Criteria for entrainment required that the 95% confidence intervals overlap with 24.00 for a regression line drawn through at least four MOs. Free-running taus (± 95% CI) were 23.66 ± 0.05 h (BL-62) and 23.81 ± 0.08 h (BL-64). Both entrained to 0.3 mg melatonin, with post-

entrainment periods of 23.98 ± 0.02 h (BL-62, 99 days) and 24.01 ± 0.09 h (BL-64, 76 days). The average entrained MO occurred at 21:00 (BL-62) and 14:52 (BL-64). After 112 days, tau in BL-62 shortened to 23.978 ± 0.016 h; thus, long-term entrainment in this subject remains to be demonstrated. Melatonin can entrain BFRs with taus less than 24 hours by inducing corrective phase delays. In such individuals, morning administration may be preferable to achieve entrainment at the normal phase. These results have implications for the treatment of other circadian rhythm sleep disorders where phase delays may be required.

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Amplitude and Phase of 6-Sulphatoxymelationin During Night and Day Shifts Offshore

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Different shift schedules are operated by the U.K. offshore oil industry. Circadian adaptation is seen for14-nights (14N), 1800–0600h schedule. No circadian phase (PHI) change is seen with 14 days (14D), 0600–1800h. During 7N7D, 18000600/0600–1800h, subjects adapt to nights but show variable direction and rate of adaptation back to days. Possible changing amplitude (AMP) of melatonin during shiftwork is of interest in relation to health risks. PHI and AMP (6-sulphatoxymelatonin RIA, cosinor analysis) in sequential urine samples for 14 days were measured in male offshore shiftworkers working: 14D (0600–1800h), n=18, 14N (1800–0600h), n=8, 7N7D (1800–0600/0600–1800h), n=23. Light exposure was measured by ActiwatchL (Cambridge Neurotechnology Ltd). Statistics: ANOVArm and/or t-test as appropriate, using D2-D13. Results: 14D, no PHI change (mean ± SD, 3.55±0.24h), decrease in AMP ($p=0.02$) D8-13. 14N, adaptive delay (PHI 5.24±1.66h to 12.69±1.74h), AMP increase ($p<0.01$) D8-13. 7N7D, variable response: 1) adapt to nightshift (N) not dayshift (D), N=8, AMP lower during D, $p<0.01$; 2) adapt to N, delay to D, N=6, AMP lower during D, $p<0.01$; 3) adapt to N advance to D, N=6, no AMP change; 4) no change, N=3. For 7N7D, PHI on D2 of shift but not D7 (end of nights) predicted response to days, mean PHI 1) 4.71±1.64h, 2) 6.34±1.12h 3) 2.43±0.65h, $p<0.001$. Light exposure was greater during day work (153±59 lux) than night work (80±10 lux), mean ± SEM, 7N7D, n=12. Conclusions: most offshore nightshift workers changing to days, and dayworkers with an early start, show lowering of aMT6s production.

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Evaluation of Melatonin as a Countermeasure for Entrainment to a Shorter-than-24-Hour Wakefulness–Sleep Schedule

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Due to orbital mechanics, shuttle astronauts are commonly exposed to day lengths that are, on average, shorter than 24 hours. We tested the hypotheses that 1) daily administration of melatonin (5 mg) 60 min prior to scheduled bedtime would entrain the human circadian pacemaker in sighted subjects to a 23.5-h day (15.67-h scheduled wakefulness in ~1.5 lux/0.0048W/m2 in the angle of gaze:7.83-h scheduled sleep

in darkness), and that 2) melatonin would be an effective countermeasure for performance impairments on this schedule. Five healthy male subjects (34 +/- 7.25; mean +/- SD) were studied for up to 50 days each in the laboratory. Subjects were administered melatonin or placebo for 18 days in a double blind crossover design. Constant routines were used to assess circadian phase prior to and following treatments. Four of five subjects in the placebo condition clearly did not entrain to the 23.5-h day. Two of three subjects who began with placebo did not complete the study. Melatonin treatment maintained normal-phase relationships between the temperature rhythm and scheduled sleep-wake cycle for two of the remaining three subjects. Cognitive performance continued to improve (i.e., learning occurred) in the melatonin but not the placebo condition (Condition x Day, $P<0.05$). Subjective sleepiness was lower near the end of the protocol for the melatonin compared to the placebo condition ($P<0.05$). The current findings suggest that melatonin may be useful as a countermeasure to entrain the circadian pacemaker of some sighted humans to non-24-hour day lengths that require a daily phase advance.

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Circadian and Homeostatic Regulation of Sleep in Blind Individuals with No Conscious Light Perception (NPL).

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The circadian pacemaker in a majority of NPL blind individuals is not entrained to the 24 hour day, and consequently these individuals exhibit non-24-hour melatonin and sleep rhythms. The aim of the current analysis was to assess the contribution of circadian phase and time awake on the sleep structure in NPL blind subjects using polysomnography (PSG). PSG (C3-A2, O1-A2, EOG, EMG, ECG) and plasma melatonin samples were measured in ten NPL blind subjects (8M, 2F; mean age ± SD = 49 ± 11 yrs) during a 30-day forced desynchrony laboratory protocol in which subjects lived on a scheduled 28-hour day' (18.67 h wake: 9.33 h bed-rest). Scheduled bed-rest was distributed across circadian phase while prior wakefulness remained constant. REM, NREM, Wake, and Sleep Efficiency (SE) were calculated as a function of circadian phase based on melatonin peaks (45° or ~3 h bins) and of time in bed during scheduled bed-rest (3.11 h bins). NREM as a percentage of total sleep time (TST), Wake as a percentage of total recording time (TRT) and SE varied significantly with both circadian phase and time in bed. REM as a percentage of TST varied significantly only by circadian phase. SE, REM, and Stage 1 were maximal, and SWS, Stage-2 and Wake were minimal near to the melatonin peak. SWS and SE significantly declined whereas percentage of awake, Stage 1, and Stage 2 rose with increasing time in bed. These results are consistent with the two-process model for determining sleep duration and structure. Under normally entrained conditions, the homeostatic drive for wakefulness during the latter part of the night is counteracted by the increasing circadian drive for sleep. However, in NPL blind subjects who are not entrained to the 24 h day in society, these processes become desynchronized, resulting in non-24-h sleep-wake disorder.

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Superiority of Blue (470 NM) Light in Eliciting Non-Image Forming Brian Responses During Auditory During Auditory Working Memory in Humans: A fMRI STUDY

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Light exerts profound effects on physiology and behavior in humans. Some of these effects may be mediated in part by a recently discovered, blue (470nm) light-sensitive photoreceptor system. Healthy volunteers (n=18) participated in a fMRI experiment during two visits, two days apart. On both visits, subjects were maintained in dim-light (<5 lux) for 3h before performing auditory 2-back task sessions in a 3T Allegra MR scan (Siemens, Germany) (32 slices, voxel size: 3.4x3.4x3mm, TR: 2130ms, TE: 40ms, FA: 90°). Sessions were acquired before (<0.01 lux), during, and after (<0.01 lux) one eye was exposed to a monochromatic light (~3x10^13ph/cm2/s). Visits were identical except for light condition (blue-470nm or green-550nm) and were counterbalanced. We show that, while participants perform an auditory working memory task during the day, blue monochromatic light elicits greater Non-Image Forming (NIF) responses than green monochromatic light in areas involved in working memory (left middle frontal gyrus, right insula, left intraparietal sulcus, left inferior parietal lobule), and in the left thalamus implicated in the modulation of cognition by arousal. As expected, we observe these effects without behavioral modification, excluding confounding effects of variations in alertness and performance. This is the first report of greater NIF responses to blue compared to green light exposure in brain structures involved in higher cognitive functions. The data suggest that the melanopsin photoreception system modulates subcortical structures, which in turn affects a widespread set of cortical areas. Data also have implications for lighting systems design.

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Superiority of GRP-Induced C-Fos Expression in the Hamster SCN by a Serotonin Agonist and an NMDA Antagonist

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The neuroanatomical routes by which photic information is processed in the suprachiasmatic nucleus (SCN) have been the subject of extensive experimental investigation. Within the SCN, gastrin-releasing peptide (GRP) has been identified as having a potential role in regulating the size of light-induced behavioral phase shifts of circadian rhythms. Microinjection of GRP into the SCN or third ventricle induces light-like phase shifts of the circadian clock, and microinjection into the third ventricle also induces c-fos and p-ERK expression in the SCN. In this study, the ability of GRP to induce c-fos was tested in the presence of the NMDA antagonist, AP5, and the serotonin agonist, 8-OH-DPAT (DPAT). Adult male Syrian hamsters equipped with guide cannulas aimed at the third ventricle were housed in DD

until stable free-running rhythms were observed. Each animal received a microinjection at CT 13.5 of GRP, GRP+AP5, or GRP+DPAT. One hour later, animals were given an overdose of sodium pentobarbital anesthesia, perfused, and post-fixed. Forty μm thick brain sections were immunostained for c-fos, and the number of c-fos positive nuclei in the SCN was counted. Both AP5 and DPAT significantly reduced the number of c-fos positive cells in the SCN. Further analysis revealed that the suppressive effects of AP5 and DPAT were primarily localized to the dorsal and medial SCN, while little effect of AP5 or DPAT was seen on c-fos expression in the ventral SCN. These data suggest that cells in the SCN that respond to GRP are also influenced by serotonergic and glutamatergic input.

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Disrupted Neuronal Rhythms in the Suprachiasmatic Nuclei of Vasoactive Intestinal Polypeptide-Deficient Mice

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Vasoactive intestinal polypeptide (VIP) is a key signalling molecule within the suprachiasmatic nuclei (SCN). VIP-deficient mice (VIP/PHI-/-) display abnormal wheel-running rhythms, and neonatal VIP/PHI-/- neurons exhibit reduced electrical rhythmicity compared to WT. It is unclear, however, how the loss of VIP affects on the physiology of the SCN in adult animals.Using suction electrodes, we recorded extracellular multiunit activity (MUA) for 48-90h from adult SCN slices prepared during early projected day from male and female mice housed under a 12h:12h light:dark cycle. Subsequently, we discriminated the activity of individual neurons from these MUA recordings using spike2 software (CED, UK). Consistent with behavioural data, two thirds of VIP/PHI-/- slices (7/11) and all VIP/PHI-/+ SCN slices (n=7) exhibited circadian MUA rhythms. The majority of individual SCN neurons recorded from both VIP/PHI-/+ (~81%, n=16) and VIP/PHI-/- (~67%, n=33) slices also expressed detectable discharge rhythms. However, amplitudes of SCN cellular rhythms in homozygous animals were ~50% of those in VIP/PHI-/+ neurons. Further, VIP/PHI-/+ neurons consistently expressed near-24h periods (mean: ~23.6h) with peaks during the projected day, whereas, VIP/PHI-/- neuronal rhythms typically peaked during the projected night, with many expressing accelerated periods (~22.5h) and a smaller population with very slow oscillations (~29h).

We demonstrate that, in parallel with the reported deficits in mouse behaviour, VIP-deficiency causes less severe disruptions of adult SCN rhythmicity than previously observed in VIP-receptor mutants, where only ~30% of neurons sustain rhythms. We suggest that loss of VIP disturbs SCN cellular physiology less profoundly than loss of its receptor.

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PAI-1/Vitronectin Modulation of Glutamate-Induced Phase-Shifts of the SCN Circadian Clock

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Mammalian circadian rhythms are controlled by a clock located in the suprachiasmatic nucleus (SCN). The mechanisms through which light shifts the SCN circadian clock are similar to those in the hippocampus

underlying memory formation and long-term potentiation. Two secretory proteins, tissue plasminogen activator (tPA) and brain-derived neurotrophic factor (BDNF), have been implicated in this process, but their relationship is unclear. Through the extracellular protease plasmin, tPA can generate mature BDNF (mBDNF), which is critical for LTP expression. We are investigating whether tPA could have similar actions in the SCN. SCN neuronal activity in vitro exhibits a circadian rhythm with a peak during mid-day. In vitro treatments that shift the clock shift the time of peak activity. We recorded neuronal activity from SCN brain slices of adult male mice. Glutamate applied in early subjective night causes phase delays in SCN in vitro neuronal activity (-2.26+0.2hr; mean phase shift+SER; n=3). Pretreatment with the tPA inhibitor, plasminogen activator inhibitor-1 (PAI-1), blocks glutamate-induced phase shifts (-0.67+0.23hr, n=6). This blockage is prevented by co-applying exogenous plasmin (-4.00+0.00hr, n=2). Futher, we show that PAI-1 does not block glutamate-induced phase shifts in vitronectin knockout mice (VN-/-) (-2.5+0.33hr, n=4). Vitronectin protein binds to and stabilizes PAI-1 in its active form, thereby enhancing its activity. In control experiments, PAI-1 alone has no effect (-1.00+0.00hr, n=3), and glutamate induces normal phase-shifts in VN-/- mice (-2.5+0.71hr, n=2). These results strongly suggest that tPA modulates glutamate-induced phase shifts in the SCN through plasmin-associated actions.

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Cholinergic Projections to the Suprachiasmatic Nucleus (SCN) of the Diurnal Grass Rat Alexandra Castillo-Ruiz, Laura Smale, and Antonio A. Nunez.*

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The SCN of the laboratory rat receives inputs from the cholinergic systems of the basal forebrain and brain stem that are active during REM sleep and/or wakefulness. All the available data are consistent with the view that the molecular clock of the SCN and its phase relation to the light-dark cycle are the same in diurnal and nocturnal rodents. Since nocturnal and diurnal species display sleep and wakefulness at different phases of the light–dark cycle, any cholinergic stimulation of the SCN associated with stages of vigilance should affect the clock at opposite phases of its cycle, depending on whether an animal is diurnal or nocturnal and could result in different behavioral outcomes across species. Since the cholinergic inputs to the SCN have not been described in diurnal mammals, this study sought to determine the presence cholinergic fibers in the SCN of the diurnal grass rats. Using immunocytochemical approaches, we have confirmed the presence of cholinergic fibers in the SCN of grass rats as well as in the lower sub-paraventricular zone (LSPV) of grass rats and laboratory rats. We are now investigating the pattern of daily neural activity in cholinergic systems of the grass rats with projections to the SCN and the LSPV.

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Five Muscarinic-Receptor Subtypes are Expressed in the RAT SNC

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Although entrainment of circadian rhythms to light occurs mainly by activation of NMDA receptors in the SCN, there is also evidence that acetylcholine plays some role in the entrainment of the SCN to light pulses. Since the genes sequences for five muscarinic receptors in the rat brain have been characterized, we

sought to determine its relative amount in the SCN at three different times by Real time RT-PCR, using GADPH as reference gene. Coronal hypothalamic slices (400 mm) were obtained at ZT 06, ZT 12 and ZT 23. The SCN from six animals were placed in 1.5 ml eppendorf tubes. Each of these tubes were used as a single data point. The relative amount of mRNA for the each of the five muscarinic receptors is as follows.

	M1	M2	M3	M4	M5
ZT06	0.01±0.005	0.015±0.04	0.052±0.006	0.035±0.01	0.018±0.01
ZT12	0.03±0.02	0.011±0.09	0.049±0.04	0.017±0.005	0.017±0.01
ZT23	0.03±0.03	0.016±0.09	0.046±0.02	0.005±0.005	0.034±0.02

We further profiled the muscarinic receptors gene expression in nine single cells recorded between (ZT 9 to-12) and we found more expression from M2 receptors. These results clearly indicate: mRNA for the five known types of muscarinic receptors are present in the SNC and independently regulated by the circadian cycle, supporting the hypothesis of cholinergic modulation of SCN entranment.

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Light-Induced Changes in Gene Expression in the Hamster SCN as Assessed By Microarray and Quantitative Real-Time PCR

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Light pulses given during early subjective night cause phase delays in hamsters. Several genes have been identified that are rapidly induced by light, such as egr1 and c-fos. In the current study, we attempted to identify light-induced genes in the suprachiasmatic nucleus (SCN) of Syrian hamsters, using microarray analysis. Adult male Syrian hamsters were housed in constant dark for four to seven days. Light (300 lux) or dark pulses were administered at CT13 for 30 minutes. Immediately following the treatment, the hamsters were sacrificed, and their brains were removed and frozen in cold isopentane. Twelve μm thick sections of the SCN were microdissected using laser capture microscopy, and the total RNA was extracted, purified, and checked for quality on an Agilent Bioanalyzer. The RNA was then processed through two rounds of linear amplification and hybridized to Affymetrix Mouse 430A 2.0 genechips. Only genes which showed at least 50% increase or decrease in expression and had a significant T-test for change in expression were considered to have shown a significant change in expression. A total of 8 up and 9 downregulated candidate genes were identified. Primers were designed for quantitative real-time PCR (qRT-PCR) analysis, and the experiment was repeated using qRT-PCR to confirm the results of these genes. This analysis has correctly identified two genes previously known to be upregulated by light: egr1 and c-fos. We are currently working to confirm other genes that were shown to have a significant change in gene expression.

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Daily Rhythms in PER1 Within and Beyond the SCN of Female Grass Rats (Arvicanthis niloticus).

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Rhythms in expression of Per genes are very similar in the SCN of diurnal and nocturnal species. Little is known, however, about clock gene expression in brain regions beyond the SCN in species other than nocturnal rodents. In this study, we sought to determine if the coupling between molecular rhythms within the SCN and those present in other brain regions might differ in day- and night-active species. Specifically, we evaluated numbers of cells containing immunoreactive PER1 in the SCN, in cells that contain tyrosine hydroxylase (TH) and in other regions of the forebrain in female grass rats kept in 12:12/LD and sacrificed at different times of day (ZT 2, 6, 10, 14, 18, and 22; n=5/group). A rhythm in PER1 expression, with a peak in the early subjective night was evident in the SCN. A rhythm was also apparent in the lower subparaventricular zone, where the peak occurred at ZT 10. Rhythms in PER1 were also seen in the lateral subnucleus of the bed nucleus of the stria terminalis and in the central nucleus of the amygdala , with peaks early in the light period. Finally, we saw a rhythm in the percent of TH+ cells in the A14 group that expressed PER1; this rhythm peaked at ZT 22. Taken together, these results suggest that molecular oscillators exist in numerous regions of the grass rat brain, and that coupling between molecular oscillators within and beyond the SCN may be quite different in nocturnal and diurnal species.

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Effect of Aging on 5-HT7 mRNA Expression in Neuroanatomical Components of the Circadian Timing System

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Previous studies have shown that aging decreases responsiveness of the circadian timing system to several zeitgebers, including the serotonergic drug, 8-OH-DPAT. The dorsal raphe nucleus (DRN) exhibits an age-related attenuation of both 5-HT7 receptor binding and functional responses (i.e., phase shifts to 8-OH-DPAT microinjection). To further investigate the mechanisms underlying the aging decrease in 5-HT7 receptors, we investigated aging modulation of 5-HT7 receptor mRNA expression in the DRN and other circadian substrates, the suprachiasmatic nucleus (SCN), and median raphe nucleus (MRN). A 592 base pair region from nucleotides 748-1340 of the 5-HT7 receptor was generated by RT-PCR from hamster-brain RNA. Resulting DNA was cloned into transcription vector pBSKS(+), and an anti-sense cRNA probe was synthesized and labeled with 35S. Using this probe, in situ hybridization was performed on coronal sections prepared from the brains of young, middle-aged, and old male Syrian hamsters. Image analysis of X-ray film autoradiograms showed that the 5-HT7 receptor mRNA signal in both the DRN and MRN was faint and was not affected by aging. In conjunction with other studies, these data suggest that most 5-HT7 receptors in the DRN may be localized to afferent terminals rather than cell bodies. In contrast to the DRN, 5-HT7 receptor mRNA expression in the SCN, which was restricted to a small subregion of the ventral core, was significantly decreased by aging ($P<0.005$).

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Microinjection of GRP into the SCN Region During the Late Night Increases Per1 Gene Activity in the Entire SCN of the Mouse

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Circadian rhythmicity in the primary mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus, is maintained by transcriptional and translational feedback loops involving the gene Per1. During photic stimulation, Per1 expression is initially observed in the ventral, "core" region of the SCN, which contains immunoreactivity for gastrin releasing peptide (GRP) and vasoactive intestinal polypeptide (VIP). Subsequent Per1 expression is observed in the rhythm-generating, dorsal, "shell" region of the SCN, which contains immunoreactivity for arginine vasopressin (AVP). Previous evidence suggests that GRP may be involved in mediating photic information from retinorecipient cells in the core to the rhythm-generating clock cells of the shell. This study tested whether GRP application to the SCN region in vivo results in Per1 activation in AVP- or VIP- producing neurons of the SCN using a transgenic mouse in which a green fluorescent protein (GFP) reports Per1 gene activation. Per1::GFP mice housed in a light–dark cycle received 200-nl injections of GRP or vehicle into the SCN region three hours before lights on (ZT 21) and were sacrificed and perfused at ZT24. Double label immunocytochemistry for AVP-GFP or VIP-GFP revealed that GRP-injected mice had 70% more GFP-single labeled cells throughout the SCN than vehicle controls. While GRP-injected animals had significantly more AVP-GFP double labeled cells than controls, AVP-GFP and VIP-GFP cells together were only ~21% of GFP single-labeled cells. These results indicate that GRP application in the late night induces Per1 gene activity throughout the SCN; however, the peptidergic phenotype of these GRP-responsive cells remains unknown.

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A Characterization of Cells that Rhythmically Express mPER1 in the Mouse SCN

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The suprachiasmatic nucleus (SCN), the site of localization of the light-entrainable circadian clock in mammals, is comprised of a multitude of neurochemically-distinct cell populations. Although the neuroanatomical diversity of the SCN has been well described, little is known about the functional organization of the nucleus. In the current study, we have begun a functional characterization of SCN cells that display circadian expression of the canonical clock protein, mPER1.

MPER1::eGFP transgenic mice, which express an unstable form of green fluorescent protein (GFP) driven by a 3 kb fragment of the mper1 promoter (Kuhlman et al., 2000), were bred and raised under LD 12:12. In the first experiment, the distribution and circadian pattern of expression of mPER1::eGFP and c-FOS protein (FOS) among SCN neurons was determined. Peak expression occurred at CT10, with 4965 +/- 900 GFP fluorescent cells/SCN (n=3) and 1787+/-524 (n=3) FOS-immunofluorescent cells/SCN observed. Only 4.2% of mPER1::eGFP cells (283 +/- 96 cells/SCN) co-expressed FOS. Electrophysiological recordings of mPER1::eGFP neurons performed between ZT5-12 in the hypothalamic slice preparation indicated that at least 50% of this cell population is retinorecipient, compared with approximately 25% of non-fluorescent SCN neurons. Finally, injection of fluorescent microspheres into the paraventricular nucleus (PVN), a major target of SCN projections, revealed that very few (<5%) of mPER1::eGFP cells project to the PVN. These results indicate that mPER1::eGFP cells 1) are distinct from spontaneously expressing FOS cells, 2) receive retinal innervation, and 3) are probably not SCN output cells.

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Experience-Dependent Filamentous (F)-Actin Reorganization in the Rat SCN

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The circadian clock in the suprachiasmatic nucleus (SCN) comprises a dynamic, tightly-controlled cycle of cellular events that repeats roughly every 24 hours. The effect of signals from the eye to the SCN depends upon clock state. In the early night, light/glutamate (Glu) signals reset the clock via an NMDAR/nitric oxide (NO) pathway. Delays in clock phase require release of stored Ca2+i through neuronal ryanodine receptors (RyR). Paradoxically, at the very circadian time that Glu/RyR-activation induces phase delay, a cholinergic/cGMP/IP3- mediated pathway causes phase advance. Thus, the clock is sensitive to signals with different spatial and temporal Ca2+ dynamics in early night. Ca2+i fluxes can alter structural elements within cells. Cytoskeletal actin filaments (F-actin) are dynamic structures that form sub-membranous networks, interacting with cell surface receptors, channels and intracellular effectors. F-actin changes contribute to cell migration and neuronal plasticity. Therefore, we tested the hypothesis that F-actin reorganization contributes critically to the phase shifts induced by Glu signaling during early night. We used fluorescently-labeled phalloidin, which stains F-actin specifically, and an F-/ G-(globular) actin separation assay. Our data show that Glu induces F-actin depolymerization, but carbachol does not. We found that Jasplakinolide, an F-actin stabilizer, blocks Glu-induced phase delay, but not carbachol-induced phase advance. Moreover, F-actin depolymerizers, Latrunculin A and Cytochalasin D, applied in early night mimic Glu-induced clock-resetting. Together, our results implicate actin-based cytoskeletal reorganization as pivotal to SCN state changes during light/Glu-stimulated phase delay.

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Intrinsic Scale-Invariant Patterns of Locomotor Activity: Influence of the Circadian Pacemaker Across a Wide Range of Time Scales Spanning 4–24 Hours

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Control of human activity is complex, being influenced by many factors both extrinsic (work, recreation, reaction to unforeseen events) and intrinsic (the circadian pacemaker that influences our sleep/wake cycle and ultradian oscillators with shorter time scales. We recently discovered that human activity possesses additional intrinsic scale-invariant patterns at time scales from minutes to four hours, suggesting an activity control system that orchestrates all influences on activity to produce an overall fractal pattern across this time scale. We tested whether or not such scale-invariant patterns in activity; 1) exist across even wider time scales, up to 24 hours; 2) also exist in a mammalian species that is nocturnally active, i.e., Wistar rats; and 3) are influenced by the internal circadian pacemaker(supra-chiasmatic nuclei; SCN). Activity was recorded for 10 days in: 1) humans living in constant dim light; 2) control rats living under 24-hour light–dark cycles, and again under constant darkness; and 3) rats with SCN lesions living under 24-hour light/dark cycles, and again under constant darkness. We found that the scale-invariant pattern of activity occurs in humans across a time scale that is six times wider than previously detected: from minutes up to 24 hours. Moreover, the scale-invariant pattern of activity also occurs in nocturnally active rats with

identical properties across the same time scale up to 24 hours. In addition, this scale-invariant pattern was very robust, being unaffected by the environmental light-dark cycle. However, in rats with SCN lesion, the scale-invariant pattern of activity completely breaks down at time scales >4 hours resulting in dramatically altered activity fluctuations resembling white noise without feedback control. We conclude that the SCN imparts scale-invariant patterns of activity across a wide range of time scales spanning 4 to 24 hours. A different neuro-anatomical source must be responsible for the scale-invariant patterns of activity from minutes to four hours. Our findings suggest a common mechanism of scale-invariant activity control for humans and rats and demonstrate a previously unknown role of the SCN in activity control at multiple time scales rather than solely at a period of ~24 hours

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A Comparison of Period Gene Expression in the Nocturnal Rat and Diurnal Degu: the Suprachiasmatic Nucleus, Striatum, Cingulate, and Parietal Cortices.

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Nocturnal and diurnal mammals have similar core molecular feedback loops in the suprachiasmatic nucleus (SCN) based on examination of period gene rhythms. It is unknown if these findings can be generalized to period gene rhythms outside of the SCN. This study examined the 24-hour oscillatory patterns of Per1, Per2 and Per3 in the brains of Octodon degus (degu), a diurnal rodent, and the Sprague-Dawley rat, a nocturnal rodent. We hypothesized that in the degu, similar to other diurnal and nocturnal rodents, period gene transcript levels in the SCN would have monophasic 24 h expression, peaking in the light phase and decreasing into the dark phase. However, we hypothesized that period gene levels in the striatum, cingulate, and parietal cortices would follow a 24-hour rhythm that is 12-hour out of phase from that of nocturnal rodents. Brains of 55 adult male degus and 60 adult male rats were collected every two hours in LD 12:12 and processed for period gene mRNA via in situ hybridization. In degus, the oscillatory pattern of period gene mRNA was similar to that seen in other rodents, but with an earlier decline of Per1 and Per2 during the transition from light to dark. The degu striatum had a 24-hour pattern in Per2 that is 12-hour out of phase from that of nocturnal rodents, with mRNA peaking during the light phase. These results suggest that clock gene expression may differ between diurnal and nocturnal species inside and outside of the SCN.

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Genetic Analysis of Ca2+ Signaling in Circadian Clock Neurons

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Our previous work indicates that depolarization-activated conductances in the plasma membrane of circadian pacemaker neurons play an essential role in free-running cellular oscillation. To investigate whether intracellular Ca2+ signals are also necessary for normal free-running cellular oscillation, we have generated transgenic flies that express the Ca2+ buffer protein parvalbumin (PV) in clock neurons. PV expression disrupts normal temporal patterns of cytoplasmic Ca2+ accumulation and, therefore, allows us to test for a role of Ca2+ signaling in circadian clock function.

Expression of PV in LNv pacemaker neurons induces dose-dependent lengthening of the period of free-running rhythms of locomotor activity. In addition, the free-running rhythms of PV-expressing flies show

an increase in period length over time, then weaken and become arrhythmic. While accumulation of PDF in the dorsomedial terminals of LNvs of PV-expressing flies remains cyclic in both LD and DD conditions, PV expression delays the phase of peak accumulation of the clock protein PDP1 under free-running conditions. These results indicate that disrupting Ca2+ signals in pacemaker neurons slows, destabilizes, and ultimately stops circadian oscillation, suggesting that intracellular Ca2+ signals are an essential component of the core cellular clock mechanism.

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Neuron or Glia? Involvement of Two Different Cell-Types in Circadian Rhythm Generation in the SCN

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Circadian rhythmicity of cultured SCN slices in Per2::luc transgenic rats persists for a month or longer without any external stimulus. This is in contrast with the damping rhythms observed in cell culture, but the mechanism by which circadian rhythmicity is sustained in the SCN is still unclear. In addition, whereas the circadian firing rhythms in the individual neurons in the SCN have been reported, contribution of the glial cells in the SCN to the rhythm generation/sustentation is largely unknown. To study roles of neurons and glias in the circadian clock in the SCN, we used SCN slices and neurons/astrocytes isolated from the SCN or cerebrum and analyzed their characteristics. We found that SCN slices still retain both neurons and astrocytes after several months of culture, although the number of each cell-type was markedly reduced. We also observed that the limited number of the neurons extended their axons and made new networks that seemed to cover the whole SCN, whereas astrocytes were sparsely distributed in the SCN. SCN slices from newborn animals, which have much larger number of neurons than adult SCNs after several months of culture, showed more robust Per2::luc rhythmicity than adult SCNs. These data suggest that neuronal networks in the SCN are important for rhythmicity, and also imply that the number of the neurons affects the amplitude of the rhythmicity. We also observed prolonged Per2::luc rhythms in several astrocyte cell lines form SCN compared to lines from cerebrum. The result suggests that astrocytes in the SCN are also involved in the rhythm extension.

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Photoperiodic Encoding in the SCN: From Neurons to Networks

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The circadian pacemaker of the suprachiasmatic nucleus (SCN) plays a major role in the adjustment of animal behaviour to both the daily and the annual cycle.To investigate how SCN neurons adapt to and code for seasonal changes in the environmental light–dark cycle, we housed mice under long (LD 16:8) and short (LD 8:16) photoperiods and monitored their wheel running activity for at least four weeks. Electrophysiological recordings of multiunit activity (MUA) were performed in acutely prepared

hypothalamic slices (400 mm) using stationary electrodes. The results show that the peak width of the MUA-rhythm in the SCN was 11.71 ± 0.36h (n=22) in long days, and 8.14 ± 0.34h (n=20) in short days ($p<0.001$). Subpopulation activity was extracted by off-line analysis of digitally recorded neuronal activity. The duration of subpopulation activity was shorter than the MUA and did not differ between long and short day ($p>0.25$), both for animals in both long and short photoperiods (pooled mean: 4.15 ± 0.21h). For animals kept in long photoperiods, the time of maximal activity of the subpopulations showed a broad distribution whereas for those in short photoperiods, the distribution was narrower. This shows that the differences in electrical output from the SCN observed under short and long photoperiods result from the different spacing of electrical activity patterns of neuronal subpopulations.

The data indicate that seasonal changes in MUA pattern observed at the tissue level are generated by out-of-phase oscillating neurons that, as an ensemble, code for the photoperiod.

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Two Methods of Uncoupling a Circadian Clock Reveal Sustained and Damped Oscillator Types

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The population of individual circadian oscillators in the suprachiasmatic nucleus (SCN) of the hypothalamus is not homogenous. Recent studies highlight that SCN neurons vary in their pacemaking ability, neuropeptidergic content, and response to timing information from the environment. We hypothesize that SCN neurons fall into distinct classes based on these criteria. Period2-mediated luciferase expression recorded from individual SCN neurons plated at very low densities indicated that some cells sustain periodicity, while others are damped oscillators or show no intrinsic rhythmicity. Since the majority of cells show sustained rhythms in the SCN explant, intercellular communication likely maintains circadian synchrony and rhythm amplitude. Similarly, SCN synchrony and rhythmicity are impaired during disinhibition of adenylyl cyclase (AC) activity by pertussis toxin (PTX) in high density and slice cultures. AC mediates cyclic-AMP formation, which can modulate CREB levels and Period gene induction. This provides a potential intracellular pathway by which intercellular signals could affect circadian phasing among pacemaking cells. Since the circadian phenotype of low density and PTX-treated cultures is similar to that of VIP-/- or VPAC2-/- SCN, we reason that VIP signaling between SCN neurons may modulate circadian rhythmicity and synchrony in a heterogeneous oscillator population via a PTX-sensitive intercellular pathway.

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The Phase-Shifting Effects of Gastrin Releasing Peptide Require Concurrent Activation of NMDA Receptors

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Previous studies have shown that microinjection of gastrin-releasing peptide (GRP) into the SCN region or third ventricle causes circadian phase shifts similar to those produced by light pulses. Activation of NMDA receptors in the SCN region also produces light-like phase shifts. This study was designed to test the effects of (±)-2-Amino-5-phosphonopentanoic acid (AP5), an NMDA antagonist, and L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC), a glutamate reuptake inhibitor, on GRP-induced phase shifts. Adult male Syrian hamsters equipped with surgically implanted guide cannula aimed at the 3rd ventricle were housed in constant darkness until stable free-running rhythms of wheel-running activity were apparent. Microinjection of GRP into the third ventricle at CT 13.5 induced large phase delays. These GRP-induced phase delays were blocked by coadministration of AP5, suggesting that GRP-induced phase delays require concurrent activation of NMDA receptors. Microinjection of AP5 alone did not induce significant phase shifts. A second experiment was designed to test whether GRP-induced phase shifts would be enhanced by PDC. Co-administration of PDC and GRP elicited significantly larger phase delays at CT 13.5 than GRP alone. However, administration of PDC alone did not induce a significant phase shift. Finally, when administered just prior to a light pulse, PDC elicited significantly larger phase delays than light pulse plus vehicle controls. These data suggest that GRP's effects on circadian clock phase are highly dependent on the level of excitation provided by activated NMDA receptors.

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Tethered k-ACTX-Hv1C Expression in Clock Neurons Disrupts Circadian Locomotor Activity

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Drosophila clock neurons exhibit self-sustaining cellular oscillations that rely in part on transcriptional feedback loops. Our previous work indicates that electrical silencing of pacemaker neurons (LNVs) abolishes free-running rhythms of locomotor activity and stops free-running oscillation of PER, TIM and PDP1 proteins in the LNVs. This suggests that depolarization-activated ionic conductances in clock neurons are also core components of the cellular circadian oscillator. To identify the particular ionic conductances necessary for cellular oscillation, we interfered cell-autonomously with ionic conductances in clock neurons via transgenic expression of subtype-specific ion channel toxins tethered to the plasma membrane via a glycophosphatidylinositol anchor. Our studies reveal that the expression of k-ACTX-Hv1C, a K+ channel blocker from spider venom, disrupts circadian behavior. k-ACTX-Hv1C causes pupal lethality in flies expressing the toxin with a pan-neuronal driver, elav-GAL4. When expressed specifically in all clock neurons using a tim-GAL4 driver, k-ACTX-Hv1c induces behavioral arrhythmicity in free-running conditions. Using a pdf-GAL4 driver, which drives toxin expression only in LNV pacemaker neurons, k-ACTX-Hv1c lengthens free-running behavioral period at low doses and induces arrhythmicity at high doses. Furthermore, k-ACTX-Hv1c expression significantly damps PDP1 protein oscillation in

clock neurons of flies expressing k-ACTX-Hv1c in all clock neurons. These results suggest that k-ACTX-HV1c-sensitive ion channels of clock neurons play an important role in circadian clock function.

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Perturbing Vesicle Traffic Reveals Potent Effects on Circadian Period in Drosophila

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Circadian clocks are thought to consists principally of intracellular transcriptional feedback loops. Emerging evidence implicates a role for intercellular communication in synchronizing these cellular clocks which in turn drives a 24-hour coherent circadian behavior. To investigate the role of neuronal communication in Drosophila circadian function, we expressed a dominant negative shibire (shi ts1), which is the Drosophila homologue of human dynamin, in a tissue-specific manner. Expression in circadian pacemaker neurons resulted in robust long periods (>28 hours). Co-expression of components involved in trafficking clathrin-coated vesicles and cAMP signaling pathway enhanced or rescued this effect, respectively. This effect required the neuropeptide pigment dispersing factor (PDF), a major neurotransmitter of a core subset of pacemaker neurons. Expression of shi ts1 reduced PDF immunostaining. Taken together, our data present compelling evidence for a novel role of intercellular signaling in timing, not just synchronizing disparate neural pacemakers.

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Attenuated Circadian Rhythms in Mice Lacking the Prokineticin 2 Gene

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Circadian clocks drive daily rhythms in virtually all organisms. In mammals, the suprachiasmatic nucleus (SCN) is recognized as the master clock that synchronizes other central and peripheral oscillators to evoke circadian rhythms of diverse physiology and behavior. Prokineticin 2 (PK2), a secreted protein that is encoded by a clock-controlled gene, has been indicated as a candidate SCN clock output signal in regulating circadian locomotor rhythm. Here we show that PK2-null mice exhibit reduced rhythmicity for a variety of physiological and behavioral parameters, including locomotor activity, body temperature, feeding behavior, sleep-wake cycle, glucocorticoid and glucose levels, as well as the expression of peripheral clock genes. These studies demonstrate the critical role of PK2 signal for the maintenance of robust circadian rhythms.

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Potentiation of the Resetting Effects of Light on Circadian Rhythms of Hamsters, Using Serotonin and Neuropeptide Y-Receptor Antagonists

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In hamsters, the effects of light on circadian rhythms can be modulated by serotonergic input from the raphe nuclei and by neuropeptide Y containing afferents from the intergeniculate leaflet in the thalamus. In this study we measured effects of several compounds acting on serotonergic receptors and a neuropeptide Y Y5 receptor antagonist to determine if combined serotonergic-neuropeptide Y acting treatments could synergistically potentiate effects of light on rhythms. Hamsters were treated with either BMY 7378 or NAN-190 and the Y5 antagonist CP-760,542. Replicating prior work, we found that pretreatment with either drug alone increased the phase shift to light at CT19. The combined effect of BMY 7378 and CP-760,542 given prior to a light pulse at CT 19 was to further potentiate the subsequent phase shift in wheel-running rhythms (the phase shift was 317% of controls; light alone: 1.35 h phase shift vs. BMY 7378, CP-760,542, and light: 4.27 h phase shift). Combined treatment with NAN-190 and CP-760,542 produced a light-induced phase shift 576% of controls (phase shift to light alone: 1.23 h vs. NAN-190, CP-760,542, and light: 7.1 h phase shift). These results suggest that the resetting effects of light on circadian rhythms can be greatly potentiated in hamsters by using pharmacological treatments that block both serotonergic and neuropeptide Y afferents to the suprachiasmatic nuclei.

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Simulations Of Day-Length Encoding in the SCN: From Single Cells to Tissue Level Organization

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The circadian pacemaker of the suprachiasmatic nuclei (SCN) is a heterogeneous structure containing many single-cell oscillators that display phase differences in gene expression and electrical activity rhythms. Thus far, it is unknown how single neurons contribute to the population signal measured from the SCN. We used single-unit electrical activity patterns that have previously been recorded in SCN slices and investigated in simulation studies how changes in pattern shape and distribution alter the ensemble activity rhythm of the SCN. The results were compared with recorded ensemble rhythms. The simulations show that single units should be distributed in phase to render the recorded multiunit waveform, and that different distributions can account for the multiunit pattern of the SCN.

Photoperiodic encoding relies on changes in the waveform of the neuronal output from the SCN. We show that these changes may be based on changes in phase differences between neurons, as well as from changes in the circadian pattern of individual neurons. Both mechanisms give rise to different outcomes at the population level, leading to testable predictions. If single units broaden their activity pattern in long days, the maximum frequency of the multiunit activity should increase, whereas an increase in phase difference between the single unit activity bouts should decrease the amplitude of the neuronal activity rhythm in the SCN. The results show the usefulness of simulation studies in which it is possible to test several configurations of single cell oscillators for understanding the function of the multioscillator organization within the SCN.

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A Sex Difference in Rhythms of mRNA for Vasoactive Intestinal Polypeptide and its Receptor, but not of Vasopressin mRNA, in the Suprachiasmatic Nucleus of a Diurnal Rodent

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Diurnal and nocturnal animals differ dramatically with respect to the timing of estrous-related events. The circadian system and suprachiasmatic nucleus (SCN) may regulate the occurrence of these events by communicating temporal information via rhythms in vasoactive intestinal polypeptide (VIP), vasopressin (VP), or a receptor for an SCN signal (i.e.. VIP receptor VIP2R). The rhythm in VIP mRNA is 9 hrs out of phase in female relative to male rats. Here, we sought to determine if this is also the case in diurnal grass rats (*Arvicanthis niloticus*) and if rhythms in VP, VIP, and/or VIP2R mRNA are different in grass rats and lab rats. Intact females, ovariectomized (OVX) females, OVX females treated with estradiol (E), and males were sacrificed at (ZT 1, 5, 9, 13, 17, and 21; ZT 0=lights-on, ZT 12=lights-off). Tissue was processed for in situ hybridization, and density of SCN signal was measured. There were significant effects of time on VIP, VIP2R, and VP mRNA. There was also a sex difference in the VIP mRNA rhythm, and the VIP2R rhythm was affected by sex and by ovariectomy. VP mRNA rhythms did not differ across groups. Interestingly, SCN rhythms were the same as those reported for nocturnal lab rats. These data provide further evidence that 1) VIP mRNA rhythms may be related to sex differences in the regulation of hormone secretion and 2) SCN function is the same in nocturnal and diurnal species, despite very different temporal patterns of reproduction and daily activity.

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Glutamatergic Synaptic Inputs to the SCN from the Paraventricular Thalamus Are Mediated by Ampa-Kainate Receptors

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There is evidence that the paraventricular thalamus (PVT) projects to the suprachiasmatic nucleus (SCN). We previously found that electric or chemical stimulation of the PVT has similar effects on the phase response curve (PRC) to the light. To characterize synaptic potentials evoked by PVT on SCN neurons, we recorded in whole cell patch clamp in sagital slices. The stimulation of the PVT evoked synaptic potentials of 5.5±1.1 mV in amplitude and a decay time of 25.0±5.4 msec. Synaptic potentials were completely abolished by DNQX (10μ) but not APV (50μ), which contrast with the retino-hypothalamic tract (RHT) evoked synaptic activity (4.8±1.7 mV in amplitude, 228.5±94.9 msec in decay time) which were decreased in 80% by administration of APV. Application of bicuculine (10μ) had no effect on the synaptic potential induced by stimulation of RHT or PVT. These data suggest than synaptic potentials evoked by the stimulation of PVT were mediated by AMPA-KAINATE glutamate receptors in contrast to RHT, which showed both components—NMDA and AMPA-KAINATE— as previously reported. The use of a similar neurotransmitter to the SCN from PVT and RHT may explain the similarities between the PRC induced by PVT or TRH stimulation.

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Transduction of Hypothalamic Neurons with Lentiviral Constructs

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Over the past few years, HIV-derived (lentiviral) vectors have been widely used in the gene therapy field, mainly because of their capacity to infect both dividing and quiescent cells. Lentiviral vectors enable the transduction of a great variety of cell types otherwise resistant to gene transfer, including neurons. For example, dispersed cortical and hippocampal neurons have been successfully transduced with lentiviral vectors. Currently, lentiviral constructs have not been extensively tested on neurons in intact brain slices, and optimisation for discrete brain regions remains a significant issue.

Here, we developed a lentivirus tagged with green fluorescent protein (LV-GFP) and used this to transduce a hypothalamus-derived neuron cell line-N38. Neurons were incubated with the LV-GFP for 24 hours. The cells were then fixed and stained with the nuclear marker 46-diamidino-2-phenylindole (DAPI). Using fluorescence imaging, we observed that DAPI staining (blue) was localized in the nuclei of all cells, whereas GFP staining (green) was universally localized in the cell, but only in positively virally-transduced neurons. The majority of cells were successfully transduced, and the percentage of positive cells was proportional to the virus titer.

We are presently adapting this technique to transduce hypothalamic slices containing the suprachiasmatic nuclei, site of the master circadian clock in mammals. If this is successful, then numerous possibilities open up for the manipulation and study of the circadian system.

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Evidence for Circadian Synchronization between Astrocytes.

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Evidence from in vivo and in vitro studies indicate that intercellular communication modulates the rhythmic physiology of the suprachiasmatic nuclei (SCN), the master circadian clock in mammals. These nuclei, characterized by a highly heterogeneous population of neurons, contain a dense network of astrocytes, cells that can modulate intercellular communication within the brain. We previously showed that astrocyte cultures display circadian rhythms that are synchronized by various stimuli and are influenced by SCN co-cultures. As a first step to study whether astrocytes communicate circadian information to each other, we studied the effects of the number of plated cells or plating density on rhythmicity. We obtained astrocytes from knockin mice containing the luciferase gene downstream of the Period2 gene. We measured bioluminescence from the population of glia, using a photomultiplier tube or from individual glial cells with an ultrasensitive CCD camera. We found that increasing the number of plated cells from 5x103 to 1.2x105 increased the number of recorded circadian cycles from 1 to 6 without significantly affecting period or amplitude. Cultures plated at low density (100 cells/mm2) showed longer periods (23.9h ± SD) and more cycles (8 ± 1.5) than those plated at densities between 400 to 1000 cells/mm2 (23.1h± SD, 5 ±1.1 cycles). These changes in circadian parameters may arise from inter- or intracellular changes that depend on the number and proximity of neighboring astroglia.

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Expression of HPER1 in Human Peripheral Blood Mononuclear Cells throughout the Sleep–Wake Cycle

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We have previously reported the circadian expression of HPER1 in peripheral blood mononuclear cells (PBMCs) under constant routine (CR) conditions. We describe here the diurnal pattern of HPER1 expression under a 16:8 light-darkness (LD) schedule. Six healthy, drug-free, young men (n=4) and women (n=2, studied in follicular phase) ages 18–30 years (mean age ± SEM: 23.7 ± 1.6 years) maintained a regular eight-hour sleep episode for two weeks prior to the study. Upon admission to the laboratory, subjects maintained their habitual sleep–wakeschedule (mean: 23:23-07:23), were exposed to ~150 lux of full-spectrum light during wake periods, and slept in darkness. PBMCs were isolated from whole blood samples drawn from an indwelling catheter every ~120 minutes. The expression of HPER1 was determined relative to HCDK4 via real-time PCR. Dual-harmonic regression analyses were performed on individual expression profiles in order to establish the presence of a significant oscillation and the time of fitted maximum. All six subjects displayed a statistically significant expression of HPER1 under LD conditions. Regression analyses revealed that mean peak expression occurred (±SEM) 3.9 ?1.9 hours after the time of habitual awakening. Two male subjects displayed later peaks of HPER1 expression where maximal levels were detected 11.5 and 8.2 hours after the time of habitual awakening. While the phase alignment of peak HPER1 expression in LD resembles that observed in our prior study, the present results suggest some inter-individual variability in the expression in HPER1 in PBMCs.

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Imaging MPER1 Gene Expression in Mouse Midbrain Cultures

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Circadian pacemakers have been identified in several organs and brain regions through bioluminescence recordings of tissue explant cultures from transgenic mice or rats expressing the firefly luciferase gene luc. One useful transgenic mouse contains the mPer1 gene promoter controlling luc (mPer1::luc). Luminometry that is used to identify circadian rhythms in mPer1::luc explant cultures detects total light emitted, but it may not reveal circadian rhythms in some areas because of signal from neighboring bright non-rhythmic cells. For this reason, brain slice cultures from mPer1::luc mice were imaged with a cooled-CCD camera to record from brain nuclei that are not distinguished as rhythmic through luminometry. Coronal sections from midbrain and brainstem displayed a wide range of luminescent structures. Surprisingly, the mesencephalic trigeminal nuclei (Me5) were particularly bright and were visible along their rostral-caudal distribution. These unusual sensory cells might reveal a circadian rhythm during longer recordings. The Me5 receive proprioceptive signals from the oral cavity and help control jaw movement and feeding rate. Me5 neurons are also unique in the central nervous system because they arise from the neural crest. Me5 cells interact with hypothalamic nuclei controlling feeding behavior and satiety, and any circadian pacemaker in the Me5 might be linked to the food-entrainable circadian oscillator.

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Control of Per1-Luc Expression Rhythms in Ovaries: Neural versus Humoral Signals

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Most peripheral tissues contain circadian oscillators whose phase relationships are coordinated by oscillators in the central nervous system. Although evidence has accumulated for both neural and humoral controls of peripheral rhythms, the pathways by which central oscillators control peripheral ones are far from fully described. To understand these pathways, it is important to investigate peripheral oscillators in vivo in the absence of normal circulatory and/or neural inputs. We evaluated the importance of the sympathetic control of circadian clock in the ovary. The two major sympathetic nerve inputs of one ovary of Per1-luciferase transgenic rats were sectioned. LD cycles to which the rats were entrained were shifted by six hours to compare the rate of reentrainment in the sectioned and control ovaries. Peak phase of Per1-luc expression of the nerve sectioned and intact ovaries shifted at a similar rate, indicating that either the sympathetic inputs were not necessary for control of the ovarian clock or other inputs were capable of the control. In the next experiment, we eliminated all possible nerve inputs to the ovary by packing ovaries in a dialysis membrane. After the same regimen of the six-hour phase shifts the Per1-luc rhythm of the encapsulated ovary shifted as fast as the control ovary. This suggests that humoral signals alone are capable of regulating the phase of the circadian clock in the ovary.

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Differential Effects of Cryptochrome (Cry) Mutations on Central and Peripheral circadian Expression of PERIOD2::LUCIFERASE in Mice

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The Cryptochrome 1 and 2 genes (Cry1 and Cry2) are components of the negative feedback loop in the mammalian circadian clock, and mutations in these genes result in aberrant period of circadian locomotor activity rhythms. Cry1-/- mice display ~1-hour shorter and Cry2-/- mice display ~1-hour longer free-running periods than the wild-type mice. Mice deficient in both Cry genes exhibit arrhythmic behavior. Circadian oscillations in Cry1 and Cry2 expression are observed in a central circadian pacemaker, the suprachiasmatic nucleus (SCN), as well as in peripheral tissues. Previous studies have reported that peripheral tissues express self-sustained circadian rhythms in absence of the SCN. To understand further the dynamics of Cry components in the central and peripheral oscillators, we monitored real-time circadian expression of PERIOD2::LUCIFERASE bioluminescence in the SCN, cornea, pituitary, liver and lung explants from wild-type, Cry1-/-, Cry2-/-, and Cry1-/-Cry2-/- mutant mice. In general, the periodicity of PERIOD2::LUCIFERASE oscillation in the SCN explants was representative of behavioral characteristics of each mutation in mice. The peripheral tissue explants from Cry1-/- and Cry2-/- maintained shortened and lengthened periods, respectively. The bioluminescence from Cry1-/-Cry2-/- peripheral explants were arrhythmic. Importantly, Cry1-/- peripheral explants consistently showed damped bioluminescence rhythms; whereas, Cry2-/- peripheral explants showed persistent circadian rhythms. These

results reveal differential effects of the Cry mutations on central versus peripheral oscillators and highlight a dominant role for Cry1 in the persistence of the peripheral rhythms.

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Expression of the c-Fos and PER1 Immunoreactive in the Limbic System in Rats under Restricted Feeding Schedules

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Entrainment by daily restricted feeding schedules (RFS) in the rats produces a high motivational state, manifested by increased arousal, augmented locomotion, exploration and foraging starting two to three hours prior to meal time. The daily food anticipatory activity (FAA) may rely on motivational processes regulated by limbic system structures. In order to better define the role of the limbic system during food entrainment, we identified the structures expressing c-Fos during FFA. In the restricted feeding group, the amygdalar nuclei (CEA and BLA), prefrontal cortex (PFC), lateral septum (LS), paraventricular thalamic nucleus (PVT), and bed nucleus of stria terminalis (BNST) showed increased c-Fos when rats were exhibiting FAA. Peak values started two hours prior to meal time. In the same structures we evaluated PER1 expression under restricted feeding schedules. In the PFC, LS, and PVT, acrophases were shifted and showed peak values between ZT 6 and ZT12. In the CEA and BNST, peak values were not modified and remained at the same ZT. These data confirm that the limbic system is involved in FAA and that RFS impose phase to clock genes in the limbic system. This may allow the daily generation of the motivational state for FAA previous to food access.

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A Retinal Diffusible Factor Modulates the Pineal Gland Clock in Zebrafish

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Two photoreceptive organs that secret melatonin rhythmically, pineal gland and eyes, were originally described in teleost. Several reports demonstrated a clock responsive to light controlling melatonin levels at about 24 hours postfertilization, while retina is not yet differentiated, suggesting pineal gland as a candidate for an early clock structure in embryos. The larval pineal gland can function as an input structure and also as an oscillator when it secretes melatonin. However, little is known about how both structures interact in adult zebrafish. Considering that both structures posses an intrinsic clock responsive to light regulating their melatonin secretion communication between them is to be expected. We approached that possible communication by co-culturing pineal glands from adult Per-3-Luc transgenic zebrafish with retina from adult wild type zebrafish. The rhythmic expression of pineal gland Per-3-luc was measured during seven days by topcount scintillation. As a result of the co-culture pineal gland exhibit an about one-hour shorter period of Per-3-luc rhythmicity when compared with the rhythmicity of pineal gland alone. The experiment was repeated using for co-cultures retinas from cryptocrome2a (cry2a) mutant zebrafish. Cryptochrome2a (Cry2a) protein is highly expressed in retina and the mutation results in a truncated Cry2a protein lacking its C-terminal. Retinas from cry2a zebrafish failed to induce the shortening of Per-3-luc pineal gland period remaining unaffected.

We conclude that retina can modulate the pineal gland intrinsic clock through a diffusible factor which is absent in retinas deficient in cry2a. Then, the C-terminal of Cry2a is required for the production or release of that diffusible factor.

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Lighting-Induced Circadian Disruption: Simultaneous Effects on Mammary and Liver Clock Gene Expression

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Functional clock genes exist in liver and mammary tissue and could influence development and cancer risk via light-induced circadian disruption. To investigate lightings potential role, three groups (A-C) of 18 female rats were exposed to one of three lighting treatments for 14 days: A—standard (12L:12D) photoperiod using 3500K fluorescent lamps (~70 microW/cm2); B—standard photoperiod using light-emitting diodes (LEDs, peak wavelength 525 nm, ~5 microW/cm2); C—disrupted photoperiod (phase-reversed every 2 days) using LEDs. On the 14th day, three animals per group were sacrificed every four hours over 24 hours. Mammary and liver Per1-2-3, Cry1-2, Clock, Bhlhb2-3 and Bmal clock gene expression was measured by real-time RT-PCR. Group-A animals exhibited significant rhythmic peaks of gene expression in both tissues for all genes. Group-B and -C animals, respectively, displayed successively fewer peaks for liver genes; all mammary genes, however, maintained significant peaks. Temporal correlations of clock gene expression within and across tissues revealed similar patterns. Findings suggest a differential, organ-specific role of lighting in clock gene expression patterns. Specifically, lower light levels and photoperiod disruption each decrease clock gene rhythmicity in liver tissue and reduce clock gene synchrony between liver and mammary tissues. The same treatments appeared to strengthen the internal clock gene synchrony within mammary tissue. Since clock gene expression is emerging as an important factor in cell-cycle regulation and development, circadian organization could have implications for breast cancer etiology. This research was supported by NCI and NIEHS grants U01 ES012771-Supl. 2, R21 ES11659).

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Effects of Denervation on Circadian Rhythms in Salivary Glands

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The circadian clock in the rat suprachiasmatic nucleus (SCN) is thought to maintain phase synchrony among circadian oscillators throughout the organism. The SCN responds to environmental light signals, but time-limited meal access can act as a competing, more salient time cue to some peripheral tissues. Little is known about the mechanisms by which the SCN or food access controls the phase of these oscillators. The submaxillary salivary gland in the rat receives sympathetic and parasympathetic innervation, some of which originates in the SCN. To assess the role of this innervation in phase control of the gland, we performed unilateral sympathetic denervation and measured the response of this tissue to light and feeding schedules.

In salivary gland explants from intact Period1-luciferase rats, robust Per1-luc rhythms in vitro peaked at ZT 20. Surprisingly, phase was not affected in rats fed only during 4 hours at night (ZT 16-20) or day (ZT 4-8), however only 50% of salivary gland explants from day-fed rats were rhythmic. This finding suggests that the feeding schedule may compete weakly with signals originating in the SCN for phase control over salivary gland tissue.

Following unilateral dorsal-vagal complex transection, all submaxillary gland explants from day-fed rats were rhythmic with a dramatically different phase, peaking at ZT 9. One interpretation of this result is that the fibers, which were cut normally, contribute to synchronization of salivary gland rhythms to the external light cycle, but when the sympathetic input is removed, the glands become more responsive to food entrainment cues.

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Zebrafish Brain Contain Multiple Dampened Oscillators

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Zebrafish present a circadian model recently described as having multiple peripheral oscillators; indeed the circadian clock was demonstrated even at the cellular level and they can respond not only to light, but also to temperature. Zebrafish pineal gland clock has been previously reported at the melatonin secretion level. However, no central pacemaker has been reported in zebrafish. Zebrafish brain can be divided anatomically in: telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon, and medulla spinalis. Neural tissues from these brain areas were dissected from zebrafish carrying the Per3-luciferase transgene and were cultured to monitor the intrinsic Per3 expression patterns in the different brain areas. The rhythmic expression of Per-3-luc was measured during seven days by topcount scintillation at 24˚C in constant darkness condition. Circadian rhythmicity was observed in vitro in most of the brain areas studied, but in all the cases was also observed a dampening along the time. Some brain areas (such as telencephalon or optic tectum) exhibited at least three cycles of oscillations; while other areas (such as the olfactory bulb) expressed rhythmicity that persisted during the whole recording period. However, as previously observed pineal gland exhibited a robust and stable oscillation with no dampening during the recording time.

We conclude that zebrafish brain contains multiple dampened circadian oscillators

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Circadian Rhythm of Glycogen Synthase Kinase 3 (GSK 3) in the Murine Heart

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Glycogen synthase kinase 3 beta (GSK 3 beta) is a serine/threonine kinase that regulates a wide spectrum of functions, including cardiovascular function. Previous studies suggest that circadian clock genes, intrinsic to the heart, may mediate the diurnal variation in the responsiveness of the cardiovascular system to external stimuli. In addition, GSK 3 is thought to regulate circadian clock gene expression in drosophila (Cell. 2001, 105:769-79). Thus, we sought to investigate whether GSK 3 shows diurnal variation in the heart. Male C57BL/6J mice (6–8 week old, Harlan) were housed in 12-hour light: 12 hour dark cycles in controlled room temperature (72 ± 4o F) for at least two weeks before tissue collection. Heart samples

(n=3) were taken every other hour of the 24-hour circadian cycle. Since GSK 3 activity is regulated by phosphorylation at its own serine 9 residue (Ser9-pGSK), we measured the immunoreactivity of pGSK and total GSK 3 beta using the Western blot. While the immunoreactivity of the total GSK 3 beta remains constant over the 24 h cycle, that of the pGSK exhibits a robust circadian oscillation, peaking during late night in the heart. Our data suggest that GSK may be involved in the regulation of circadian clock gene expression in the heart, similar to previous findings in the SCN and the liver (Journal of Biological Chemistry 2005, 280: 29397-402).

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c-Jun N-Terminal Kinase Modulates the Period Length of Period1-bBoluminescence Rhythms in Rat-1 Fibroblasts

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Recent, growing evidence has shown the involvement of protein kinases in the mechanism of circadian rhythm generation. One of the mitogen-activated protein kinases (MAPKs), p38MAPK, has been shown to regulate the period length of circadian rhythms. In the present study, we investigated whether another MAPK, c-Jun N-terminal kinase (JNK), is involved in the regulation of circadian rhythm expression using rat-1 fibroblasts stably transfected with Period1-luciferase reporter gene construct, and a real-time bioluminescence monitoring system. Western blot analysis showed that JNK1, not JNK2, phosphorylation levels showed circadian rhythms in synchronized fibroblasts. Further, inhibition of JNK, using a specific inhibitor SP600125, decreased JNK phosphorylation levels. In the presence of SP600125, the period length of Period1-bioluminescence rhythm was increased from 24.28 ± 0.20 to 31.48 ± 0.07 hrs, whereas the negative JNK inhibitor showed no significant effect. We then found that the effects of SP600125 were reversible and dose-dependent. The period length of Period1-bioluminescence rhythm was similarly increased in the suprachiasmatic nucleus and peripheral tissues-lungs, pituitary gland, and pineal gland-of Period1-luciferase transgenic mice by SP600125 treatment. Period length of Period1-bioluminescence rhythm was significantly decreased in cell lines in which JNK1 is constitutively expressed. Our preliminary observation suggests that valproate hyperactivates JNKs and decreases period length, whereas lithium, which is known to increase period length, likely decreases JNK phosphorylation levels in fibroblasts. Our results suggest that JNK may play a role in the modulation of period length in the central and peripheral molecular clockworks.

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Ontogenesis of the Circadian Clock within the Rat Liver

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In mammals, the principal circadian oscillator within the suprachiasmatic nuclei (SCN) entrains circadian clocks in numerous peripheral tissues. The molecular mechanism underlying daily time measurement is basically shared by the central and peripheral clocks. In the rat SCN, the rhythmical expression of clock genes develops gradually during postnatal ontogenesis. The aim of the present work was to reveal when during postnatal ontogenesis the rhythmical expression of clock genes starts to develop within the rat

peripheral clock in liver. Circadian profiles of mRNA expression of clock genes Per1, Per2, Cry1, Clock, Rev-Erb and Bmal1 were analyzed in livers of 2-, 10- and 20-day-old pups and adult rats by the real-time RT-PCR. In 20-day-old pups, the profiles in clock genes expression resembled roughly those in adults; clear circadian rhythms of all studied genes with high amplitudes and typical anti-phase relationship between Per/Cry/Rev-Erb and Bmal1 expression were detected. In 10-day-old rats, the rhythmical profiles of clock genes expression exhibited lower amplitudes and their phases were shifted by about eight hours when compared to adult rats. In two-day-old rats, the amount of all clock genes transcripts was significantly lower than in older rats. In summary, our data indicate gradual development of the circadian rhythmicity in liver during postnatal ontogenesis.

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Circadian Function in the Olfactory Bulb

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Recently, it has been shown that multiple cell types can express daily rhythms in gene expression. The major circadian pacemaker in mammals, the suprachiasmatic nucleus (SCN), has been shown to drive rhythms including sleep–wake, locomotion, and hormone release. It is however, not known if rhythms in other tissues regulate circadian physiology. We have shown that the main olfactory bulb (OB) fulfills the criteria of a circadian pacemaker that regulates daily rhythms in firing rate and Period 1 gene activity. To test whether this circadian clock mediates daily changes in olfaction, we measured induction of c-Fos protein by the odorant Cedar Oil (CO). Mice were maintained in constant darkness and, at one of six specified times in the locomotor activity cycle, stimulated with 0.01% CO in mineral oil for five minutes. c-Fos was induced approximately twice as many cells by CO during the subjective night, but not during the subjective day, in both the OB and the Piriform Cortex (PC). This circadian rhythm persisted in the OB and PC of SCN-lesioned animals which showed no overt circadian rhythms in locomotor activity. Spontaneous c-Fos expression it was also rhythmic, peaking around subjective dawn in the OB and PC. Rhythms observed in the PC were abolished by complete bulbectomies (OBX) and, following unilateral OBX, in the PC ipsilateral to the lesion. These data are consistent with a circadian clock in the OB regulating olfactory sensitivity on a daily basis and that also controls other brain areas independent of the SCN.

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Influence of Aaltered Gravity on Clock Gene Expression in Rat-1 Fibroblasts

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Mammalian circadian rhythms, like core body temperature fluctuation or sleep–wake cycle, are controlled by a central pacemaker residing in the suprachiasmatic nucleus (SCN) of the hypothalamus. A key feature of the SCN is its capability of rhythm entrainment to the ambient light–dark cycle evoked by the earth rotation.Investigations on rodents, monkeys, and humans clearly indicated that space travel disturbs circadian rhythms, pointing out that further geophysical parameters may also account for an accurate adjustment of the endogenous rhythm to exogenous time cues. Circadian rhythms are based mainly on the expression pattern of clock genes, which have been found not only in the SCN but also in peripheral

tissues. Cultured rat-1 fibroblasts, a model cell system of the peripheral clock, have been shown to exhibit robust daily oscillations in the expression of clock genes for several days after a serum shock. Studies have demonstrated that such a treatment elicits a surge of expression of the clock genes Per1 and Per2, similar to that observed in the SCN of animals being subjected to a light pulse.

In our study, we tested the hypothesis that a low-gravity environment leads to modifications in the clock genes expression pattern of rat-1 fibroblasts following serum shock treatment. Our results indicate distinct rhythm differences in the clock gene expression between rat-1 fibroblasts in modeled low-gravity and stationary control cells. These data illustrate for the first time the sensitivity of peripheral clock genes to gravity.

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Scheduled Wheel-Running Stabilizes Behavioural Rhythms of Vipr2-/- Mice

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Vasoactive intestinal polypeptide and its VPAC2 receptor are important for normal functioning of the suprachiasmatic nuclei circadian pacemaker. Mice lacking the VPAC2 receptor (Vipr2-/-) exhibit altered behavioural rhythms and impaired synchronization to light. Entrainment to non-photic stimuli has yet to be tested in these animals and here we assessed the effects of scheduled exercise on locomotor and drinking rhythms in wildtype (C57BL/6J) and Vipr2-/- mice. Mice were individually housed under a 12-hour:12-hour light–dark cycle for two weeks and then released into constant dark (DD) for the remainder of the experiment. After 14–16 days, all wild-type mice were strongly rhythmic (mean tau±SE; 23.52±0.08h; n=9), while of the nine Vipr2-/- animals, five were weakly rhythmic (22.33±0.18h), and 4 were arrhythmic. Wheels were then immobilized so that mice could only run in them for a fixed six hours a day. This scheduled wheel-locking continued for 50 days, during which both genotypes showed apparent entrainment (tau of ~24 hours) of drinking rhythms. Subsequently, mice were allowed to freely exercise in the wheels for another 14–21 days. Compared to the first free-run in DD, both genotypes of mice manifested significantly lengthened periods in wheel-running rhythms. All nine wild-type mice were rhythmic (23.75±0.09h) and six of nine Vipr2-/- mice were tightly rhythmic (23.85±0.07h). Notably, one previously arrhythmic Vipr2-/- mouse now expressed robust ~24-hour behavioural rhythmicity. These results suggest that, unlike light, a non-photic Zeitgeber such as scheduled voluntary exercise, stabilizes behavioural rhythmicity in mice with deficient neuropeptide signalling.

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Diurnal Body Temperature Rhythms as a Measure of Welfare in Pasture-Based Cattle

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Cattle remain outdoors on pasture for the entire year in New Zealand. Consequently, they may be exposed to sudden changes in weather associated with periods of cold and often windy and wet conditions in the winter, and hot and humid conditions in the summer. Such short-term changes in climate may make adaptation difficult and potentially have a detrimental effect on the welfare of cattle. In a series of experiments, we investigated the welfare of cows in winter and summer conditions using changes in the diurnal rhythm of vaginal temperature (TV) as indicators of stress. In winter, the amplitude of the TV

rhythm was greater in cows exposed to the cold compared to cows under shelter, and this effect was greater in thinner cows relative to fatter cows. In cows exposed to cold conditions, the time of the minimum in the TV rhythm was later, the maximum of the TV rhythm was earlier, and rates of change in TV greater, in comparison to sheltered cows. In summer, the provision of shade and/or sprinklers reduced the maximum of the rhythm of TV, and the magnitude of the response was greater under hot and humid conditions. In conclusion, cattle maintained a relatively constant TV with a characteristic diurnal rhythm. However, the amplitude and time of the nadir and peak, and the rates of change of the TV rhythm were affected by thermal stress and may be useful indicators of welfare.

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Genetic Analysis of Tail Suspension Behavior in Genetical Rhythm Splitting Mice (CS strain)

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CS mice show variety of abnormal behavior, including circadian rhythms, sleep properties, tail suspension (TS), and forced swimming (FS) behavior. TS and FS behavior, widely used for screening antidepressants, are exceptionally abnormal in CS mice, with these mice showing almost no immobility time. In the present study, therefore, we tried to identify the genes controlling TS behavior, with a forward genetic approach. To identify the gene, we first analyzed F2 mice from an intercross between C57BL/6J and CS strains. Interval mapping revealed one major QTL affecting immobility in the TS behavior. Next, we generated a congenic strain including relatively wide candidate region that retains CS phenotype and also several subcongenic strains between the congenic strain and C57BL/6J. The analysis of these subcongenic strains revealed that the gene responsible for abnormal TS is located within a few Mb region on a certain chromosome.

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Ear Temperature as a Function of Time of Day, Cognitive Demand, and Chronotype

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Cognitive performance is associated with daily rhythms of arousal in human beings. Performance on explicit and implicit recall tasks is linked to subject chronotype in that performance is best at the subjects' optimal and non-optimal times, respectively. Inasmuch as body temperature is a measure of circadian rhythmicity, we tested the degree to which performance was associated with temperature as assessed by a standard infrared tympanic thermometer. Two experiments were run in parallel. The first assessed temperature and executive function (EF), the second addressed temperature and cognition with emotional reactivity. Subjects were assessed for chronotype using CMEQ or MEQ and were tested at both optimal

and non-optimal times. Similar differences in ear temp. were found on both tasks: 1) Morning temperature < Evening temperature; 2) Morning chronotype < evening chronotype. No interactions were found.

Finally, ear temperature changed significantly over the course of both tests, but in opposite directions — temperature decreased for all groups on the EF tasks and increased for all groups on the emotionally charged task. Therefore, time of day, chronotype, and the emotional content of the cognitive task affect ear temperature independently.

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Circadian Rhythms of Conditioned Avoidance Behaviors in Rodents doo not Require the Suprachiasmatic Nucleus

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In rodents, maximal performance on passive avoidance (PA), conditioned place preference (CPP), and conditioned place avoidance (CPA) tasks is obtained only when the clock times of testing and prior training are the same. For CPP, the time stamp persists after SCN ablation. We now report that 24-hour rhythms of performance on PA and CPA also are produced in arrhythmic, SCN-lesioned hamsters. Maximal performance occurs at 24-hour intervals following the last training session. Rhythm expression, but not learning per se, is sensitive to stimulus magnitude (foot shock) in mice, and to the presence of the period mutation, tau, in hamsters. Ablation of the SCN in tau mutant hamsters rescues the wild-type 24- hour rhythmic expression of learning. Because time is not an explicit discriminatory cue in these experiments, the results indicate that animals are predisposed to express these conditioned behaviors at 24-hour intervals. The phenomenon may underlie 24 hr anticipatory behavior in rodents. This research was supported by NSERC, Canada.

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Combined Infrared and Wheel-Running Monitoring Reveals Unconsolidated Locomotor Behaviors in VIP KO Mice

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Mammalian circadian locomotor rhythms are often measured using wheel-running activity. However, infrared motion detection may be useful when animals are unable or unwilling to run, and as a measure of locomotor activity not influenced by feedback from wheel running. Here we have constructed cages in which we can simultaneously monitor wheel running and locomotion by an infrared motion sensor (Visonic Spy 2). Using combined wheel-running and infrared (IR) motion detection to monitor C57Bl/6J mice, we found that activity rhythms recorded by the two systems were almost identical. However, in vasoactive intestinal peptide knockout (VIP-/-) mice, a strain known for irregular rhythms, infrared monitoring revealed significant "off-wheel" locomotor activity distributed across the circadian cycle in 12:12 light–dark cycles. This suggests that VIP-/- mice may have altered masking and/or locomotor feedback. Our combined wheel-IR cages offer the means to study wheel reinforcement on behavior by simply locking the wheel and continuing data collection on the IR channel. Our data suggest that combining traditional

wheel running with IR motion detectors offers a reliable and flexible measurement of circadian locomotor activity.

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Effects of Tuberomammillary Nucleus Ablation on Food Anticipatory Circadian Rhythms in Rats

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Rodents can anticipate a daily mealtime by entrainment of a circadian oscillator that is anatomically distinct from the master light-entrainable pacemaker located in the suprachiasmatic nucleus. The location of food-entrainable oscillators driving food-anticipatory activity (FAA) rhythms is unknown. Recently, FAA has been shown to coincide with activation of the histaminergic E2 and E4 subgroups of the hypothalamic tuberomammillary nucleus (TMN), while behavioral activation during feeding coincides with activation of the perifornical hypothalamic area (Meynard et al., 2005). To determine whether TMN activation is a necessary component of the mechanism driving FAA, rats with lesions of the E2 or E4 TMN subgroups were maintained on a three-hour/day meal provided during the middle of the light period (LD 12:12). Activity was recorded using food-bin monitors and overhead motion sensors. Lesions were assessed using a Nissl stain and adenosine deaminase immunostaining (a marker for histamine neurons). Rats with damage to either the E2 or E4 subgroups exhibited robust FAA in both measures. Assessment of combined lesions of these subgroups and other histamine subgroups of the TMN will also be reported.

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Splitting of Circadian Rhythms in Locomotor Activity and SCN Expression of c-Fos and pERK in the SCN of the Djungarian Hamster (Phodopus sungorus).

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Behavioural splitting is a phenomenon in which the single daily sustained rhythm of rodent locomotor activity (LA) dissociates into two distinct components that free-run with different periods until they reach a stable phase relationship ~ 12 hours apart. This phenomenon has been studied extensively in Syrian hamsters, where exposure to constant light (LL) (200-1000 lux) for six to eight weeks causes ~60% of animals to exhibit split behavioural rhythms. Unilateral expression of c-Fos and pERK (phosphorylated extracellular regulated kinase) proteins in the suprachiasmatic nuclei (SCN), suggests that splitting is accompanied by a desynchronisation between the left and right SCN. Little is known about splitting in other species, and here we examined the effect of LL exposure on LA and SCN expression of c-Fos and pERK in Djungarian hamsters. Transfer to LL (~55 lux) significantly reduced the intensity (21.98±2.25 wheel revolutions/min) and duration (3.27±0.28 h) of LA, compared to that under a light–dark cycle (16hours light, 8 hours dark) (36.78±1.34 wheel revolutions/min, 8.11±0.09 h) and caused 81% (44/54) of animals to display split LA rhythms within three weeks. In these split animals, c-Fos and pERK were expressed unilaterally in the rostromedial SCN. In the caudal SCN, the pattern of pERK expression was similar to the rostromedial SCN, however the pattern of c-Fos expression was flipped in almost 60% of

animals, with high c-Fos on the side contralateral to high c-Fos expression in the rostromedial SCN. These data support the current view that multiple populations of oscillators within the SCN control circadian rhythmicity.

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Locomotor Activity Recording in Adult Zebrafish

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Zebrafish provide a powerful experimental model for the genetic analysis of development; genetic and molecular techniques and bioinformatics tools, including the entire genomic sequence, are available. Consequently it is emerging as an important model for studying the vertebrate circadian clock system.

In order to investigate the effect of external cues on the circadian clock of adult zebrafish, a locomotor activity recording device was developed. This device— two parallel poles, on each side of an aquarium, containing multiple infrared LEDs and photo detectors — is able to detect the motion of fish passing through the cross section of an aquarium. Each activity event is translated to an electric pulse that is logged on a computer. Activity rhythms under different conditions are recorded and analyzed by CLOCK LAB analysis program.

Five Aquariums in a water recirculation system were placed in a light-controlled room; each tank was equipped with an activity recording device and installed with an automated feeder. Different but constant temperature conditions (temperature range 10^{o}c – 30^{o}c) were maintaining in each aquarium by utilizing a water chiller and heaters and by regulating water flow. The effects of photoperiodic conditions, feeding schedule, and water temperature on rhythmic locomotor activity of adult zebrafish are presented.

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Spontaneous Internal Desynchronization of Locomotor Activity and Body Temperature Rhythms from Plasma Melatonin Rhythm In Rats Exposed to Constant Dim Light

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A previous study has reported that spontaneous internal desynchronization between the locomotor activity rhythms and the melatonin rhythms may occur in rats (30% of tested animals) when they are maintained in constant dim red light (LLdim, 1?W/cm2, lower wavelength cutoff at 640 nm) for 60 days (Fukuhara et al., 2005, Neurosignals 14: 117-125). Previous work has also shown that melatonin plays an important role in the modulation of body temperature. The aim of the present study investigation was to investigate the effect that desynchronization of the melatonin rhythm may produce on the circadian rhythm of body temperature (Tb). Circadian rhythms in locomotion (i.e., running-wheel activity, RW) and Tb were continuously monitored for 60 days in 22 adult males maintained in LLdim. Twenty-one animals showed clear circadian rhythms in RW and Tb, whereas one animal was arrhythmic. RW and Tb rhythms were always strictly associated and we did not observe desynchronization between these two rhythms. On day 60, blood samples were collected six times over 24 hours, and melatonin levels in the serum were measured by radioimmunoassay. Marked inter-individual differences in the melatonin rhythm were observed

(values at the peak level ranged from 104 to 1,007 pg/ml). In seven animals, the melatonin profile was desynchronized from RW and Tb. Our data also indicate that the free-running period and the amplitude of RW and Tb were not different between desynchronized and non-desynchronized rats, thus suggesting that the circadian rhythm of plasma melatonin plays a marginal role in the regulation of the RW and Tb.

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Characterization of Behavioral and Metabolic Rhythms in a Model of Nocturnal Work (Night Workers) in Rats

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Organisms present daily behavioral and physiological fluctuations associated with the light-dark alternation. The suprachiasmatic nucleus (SCN) is the master clock that gives a temporal order and keeps the peripheral oscillators coupled. Under special situations, such as aging, transmeridionals trips, and nocturnal work schedules, the phase relation among circadian rhythms is alteraded due to uncoupling of the peripheral oscillators with the SCN, known as internal desyncronization. Models used to study the effects of shift work have used shifts of the light–dark cycle. Here, we present a model of nocturnal work in which the animals are forced to be active during their rest period. Locomotor activity and food-and-water intake of the rats was continuously recorded; the movement counts were collected using the automatic monitoring system designed in our laboratory. Rats were maintained in baseline for eight days. In subsequent days, they were placed in wheels for eight hours of forced activity during the rest phase for 30 days, with food and water ad libitum. After each session of forced work, they were returned to their cages. Rats submitted to nocturnal schedules of work have a significant decrement of the nocturnal activity. The consumption of food and water changed towards the day. The levels of plasmatic glucose showed a loss of rhythmicity, and the triacylglycerols were in antiphase with regard to the control rats. Rats showed arrhythmic activity during the weekends. These results demonstrate that this forced work manipulation can be a good experimental model of nocturnal work.

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Behavioral and Neuronal Activity in Rats Entrained by Regular and Palatable Food

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Anticipatory activity (AA), is the increase in locomotion previous to food access in animals under daily food restriction. Food entrainment involves at least two systems: metabolic and motivational. Daily administration of a palatable mealunder stable metabolic conditions produces AA without food restriction. This study explored in rats which component, the metabolic or the motivational, predominates when both are presented in competition. The behavioral response and the neuronal activity of central structures were characterized in a counter balanced design of entrainment with two meals daily: A first group (FC) was given chow food (20g) at 1000 hours and chocolate (5g) at 1600 hours a second group (CF) received

chocolate at 1000 and chow at 1600 hours. During the first two weeks of entrainment, the rats showed AA and more intensity in neuronal activity of the Dorsomedial hypothalamic nucleus (DMH) and Lateral Septum (LS) to the chocolate. After a month of entrainment, the rats showed more AA, DMH and LS neuronal activity pior to food access. Both groups showed phase shift of their general daily activity towards the light phase, and decrementing in the dark phase. Present data indicate that animals are able to discriminate between the metabolic and the motivational component of a stimulus and anticipate differentially to both stimulus. In the long term, the chronic food restriction overrides the hedonic influence of chocolate.

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A Circadian Rhythm in Memory Consolidation in Zebrafish

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Various behaviors such as locomotor activity in zebrafish have been shown to exhibit a circadian rhythm. We were interested in studying whether memory consolidation in zebrafish is also circadian modulated. Zebrafish were trained using an active avoidance paradigm in a shuttle box. In this learning paradigm, animals had to cross a hurdle to avoid an electric shock administrated after the onset of a light signal. Animals learned the avoidance task whether they were trained during the day or the night. However, only animals trained during the daytime displayed good retention of the learned avoidance task as compared to animals trained during the night-time. To test whether the rhythm was circadian, animals were trained on the third day of constant darkness during the subjective day as well as during the subjective night and tested 24 hours later. The results demonstrated that only animals that were trained during the subjective day showed good retention of the learned task, suggesting its modulation by a circadian clock. To distinguish whether memory consolidation or the recall of memory was circadian modulated, animals were trained during both the subjective day and the subjective night and tested 36 hours later in the opposite subjective daytime. The results showed that the time of training rather than the time of testing determined whether the animals showed good retention. This suggested that the rhythm in the retention of the learned avoidance task represents a circadian rhythm in memory consolidation rather than in the recall of memory.

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An Inhibitor Of Casein Kinase Ie Induces Robust Phase Delays in the Activity Onsets of Telemeterized Rats under Free-Running and Entrained Conditions as a Function of Dose, Time of Dosing, and Duration of Dosing.

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CKIe is an essential regulator of the biological clock, serving to modulate the phosphorylation and turnover of PER proteins within the SCN and other neural and peripheral tissues. In the present study, the effects of enzyme inhibition on the behavioral expression of circadian rhythmicity were evaluated in telemeterized male rats. All animals were dosed with vehicle or a potent and selective CKIe inhibitor, PF-670462. In rats maintained in 12:12 LD and released into DD following dosing, PF-670462 induced significant phase delays in a dose-dependent fashion with a magnitude of 3.60 ± 0.16 hours (mean ± SEM) at the highest

dose tested (100 mg/kg sc). Phase-response curves conducted using a lower dose (50 mg/kg) revealed either little change in phase (CT 0, 3) or sizable delays (CT 9, 12). In animals maintained in LD throughout the study, phase delays to PF-670462 (50, 100 mg/kg, ZT12) remained robust; four to five days were typically required for re-entrainment. Chronic dosing (30 mg/kg ip) for 20 days yielded phase delays that accumulated daily despite the LD cycles; drug levels were strictly pulsatile, given the rapid entry into the brain and short t1/2 (0.55 ± 0.11 h). These behavioral effects are supported by in vitro data demonstrating the ability of this compound to inhibit PER phosphorylation and its nuclear translocation. In all, these results suggest that inhibition of CKIe delays the circadian clock, that the largest phase delays are found at the LD transition, and that these changes persist under LD conditions and accumulate with chronic dosing.

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Circadian Photoreception in Drosophila melanogaster

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Most of the fundamental facts about the circadian rhythms were first revealed in the model organism *Drosophila melanogaster*. Currently, almost all the major genes that are involved in generating these circadian rhythms, (e.g., period, timeless, clock, cycle), have been discovered. But we are still far away from having a complete model of the circadian clock. Many more proteins that interact with the core clock and thus are playing only a minor but nevertheless important role in the whole clockwork are still awaiting discovery.

One of those new proteins is the QUASIMODO (QSM) protein, that was found in an enhancer trap screen. This new protein is under the transcriptional control of the clock and can be found in some of the major clock neurons, such as the large and small LNvs. Further experiments with RNAi mediated knockdowns or with overexpression of the qsm gene revealed an interesting new role for QSM in the clock. Immunohistologic and genetic approaches support the theory that this protein is involved in the circadian photoreception and that QSM somehow has an important function for the release of the Pigment-dispersing factor PDF.

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A Behavioural Analysis of the Relative Contribution of the Novel and Classical Photoreceptors to Circadian Entrainment

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Melanopsin has recently been identified as a novel photopigment of the mammalian retina, which, along with rod and cone photopigments, provides the circadian system with environmental light information. One function of this light input is to allow entrainment of the circadian system to environmental time. The aim of this project is to determine how the novel and classical photoreceptive pathways interact allowing photoentrainment.

The ability of wild-type and rd/rd cl animals to entrain to 26-hour (13L:13D) and 22-hour (11L:11D) T-cycles at gradually reducing irradiances was determined. Tau was measured using the chi-squared periodogram, and stable entrainment was established using phase angle.

When exposed to 11L:11D rd/rd cl, animals are able to entrain when wild-type mice show free-running behaviour. For example, at ?100 lux wild-type animals were unable to entrain (n = 12) whereas 50% of rd/rd cl mice were able to entrain at this irradiance (n = 10) and 20% still showed entrainment at 10 lux (n = 10). Under a 13L:13D cycle, rd/rd cl animals were again better able to entrain than wild-type mice. At 100 lux, six of nine rd/rd cl mice tested showed stable entrainment whereas all wild-type animals were incapable of entrainment. Neither genotype showed entrainment below 100 lux.

These findings suggest that both the advancing and delaying limbs of the phase response curve are enhanced by the loss of rods and cones. One explanation is a difference in clock gene induction in the SCN, and our future studies aim to test this hypothesis.

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Photic Entrainment of the Dorsal SCN

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Mammalian circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The pacemaker is entrained to environmental light–dark cycle; the photic information is transmitted primarily via the retino-hypothalamic tract (RHT). RHT fibers end on the ventral part of the SCN, where vasoactive intestinal polypeptide (VIP)-immunopositive neurons are localized. They send their axons into the dorsal SCN, where most of the vasopressinergic (AVP) neurons are located. Previous studies show that the ventral part of the SCN responds to light at first, and then the dorsal part of the SCN is gradually entrained to the ventral. However, the mechanism of the entrainment is unknown. The dorsal part of the SCN exhibits an endogenous rhythm of AVP in vivo and in vitro. Measuring AVP releasing rhythm in the SCN slice culture, we examined phase-shifting effect of N-methyl-D-aspartate (NMDA) on the rhythm of the dorsal SCN. The pulse application of NMDA induced phase delays at early subjective night and phase advances at late subjective night. Even in the presence of bicucullin, a GABA-A antagonist, NMDA also induced phase shift of the rhythm. On the other hand, VIP antagonist inhibited NMDA-induced phase shift. These results suggest that VIP but not GABA would concern the photic entrainment of the dorsal SCN.

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Human Pheromones Accelerate Reentrainment Following Trans-Meridian Travel: A Novel Treatment for Jet Lag?

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Jet travel and the accompanying "jet lag" are common experiences for many people, including vacationers, business travelers, athletes, and military personnel. Jet lag results from a desynchronization of internal circadian rhythms.Symptoms can include fatigue, irritability, insomnia, intestinal distress, changes in appetite, a decrease in mental acuity, and poor athletic performance. Social, diurnal rodents resynchronize activity and temperature rhythms with environmental time cues faster with exposure to odors of conspecifics after a six-hour advance of the light–dark cycle. Data also clearly show that humans are responsive to particular olfactory cues and experience physiological changes with exposure to olfactory steroidal stimuli. We hypothesized that humans would respond to putative human pheromones with a reduction in the length of time required to reentrain body temperature after a phase shift. Using a double-blind technique, human participants were exposed to olfactory stimuli or a control of vehicle only following

a flight that created a 6-hour advance of the light:dark cycle. Exposure to the olfactory stimuli resulted in significantly accelerated reentrainment of body temperature rhythms following eastward jet travel across six times zones. Possible underlying mechanisms are discussed with implications for practical applications and future research.

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Immunocytochemical Localization of Short Wave-Light-Sensitive Molecules inn Nonvisual Photoreceptors. Can Blue-Light Filtering Reduce Known Pathological Effects of Night-Illumination?

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OS-2, pinopsin, cryptochrome I and II, being short wavelength-sensitive molecules, were localized immunocytochemically in retinal, pineal, and deep brain nonvisual photoreceptors regulating day–night cycle of various vertebrates and human. In the retina cryptochrome II was demonstrated in the cytoplasm of a subpopulation of photoreceptors. Cryptochrome II was localized in the nuclei of some photoreceptors and in the inner and outer granular layer. In the pineal organ–among other photoreceptor molecules–OS-2, pinopsin and cryptochrome II were found. Pinopsin was also demonstrated in submammalian preoptic nuclei representing deep encephalic photoreceptors.

Low wavelenght light was found to markedly supress melatonin secretion of the pineal organ at night and may be a factor producing pathological events by night illumination (Csernus et al.,1999; Copinschi et al., 2000; Kayumov et al., 2005). As the molecules immunocytochemically localized in the present work are blue-light sensitive, their presence in nonvisual photoreceptors strengthens the view that filtered, short wave-light-free illumination or wearing light filtering glasses may reduce known health risks (such ase gastrointestinal or cardiovascular disorders, brest and colorectal cancer, oveweight, depression, disturbed sexual activity) caused by low melatonin secretion during night illumination.

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Characterization of the Effects of Light Flashes on the Hamster Circadian System

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We have shown that very brief, very bright flashes of light can phase shift the circadian activity rhythm of hamsters (Vidal and Morin, Society of Neuroscience Abstracts, 2004). Furthermore, these same flashes can induce Fos protein expression in cells of the hamster suprachiasmatic nucleus (SCN). We have completed a series of experiments that sought to determine the optimal parameters of flash presentation for eliciting a robust phase response (i.e., number of flashes versus time interval). Various protocols of flash presentations that proved effective at eliciting phase shifts of locomotor activity were also tested for their capacity at inducing Fos protein expression in the SCN. Finally, an effective phase-shifting flash protocol was selected to conduct an irradiance response test. When white light flashes are presented over a five-minute interval at circadian time (CT) 19, at least 10 equally-spaced flashes are necessary to elicit a consistent and robust phase advance. Increasing the number of flashes over the same interval has a significant effect on the

magnitude of the phase shift. For a one-hour stimulus interval, five flashes presented at CT19 are enough to elicit a phase shift of approximately one hour. A significant increase in phase shift magnitude results from increasing the number of flashes over the same interval. Fos expression in the SCN following 10 or more flashes is significantly greater than after no light or 1 flash. A protocol of 60 flashes over five minutes yielded an unusually large amount of Fos expression. The irradiance response test showed that the flash response is an "all or none" type, as flashes of an irradiance of less than 107 uW/cm2 were incapable of producing significant phase shifts. The results suggest that (1 light flashes are as effective as longer duration light pulses in phase shifting the locomomtor activity rhythm and at activating SCN neurons; (2 some component of the circadian system is integrating these short-duration flashes and equating them to a light pulse of longer duration; and 3) due to the brevity of the stimulus, it is unlikely that melanopsin retinal ganglion cells are involved in the phase response to flashes.

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Cephalochordate Melanopsin: *Evolutionary Linkage between Vertebrate Circadian Photopigment and Invertebrate Visual Pigment*

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Melanopsin is a photopigment in the photosensitive retinal ganglion cells of vertebrates and involved in the circadian photoentrainment. Since photopigment properties, such as absorption maximum, photochemical behavior, and signal transduction cascade, relate to characteristics of the photoreceptor cells, it is of interest to elucidate molecular properties of melanopsin. We tried to express melanopsins of several animals in cultured cells and successfully obtained the functional melanopsin of cephalochordate amphioxus, which is the closest living invertebrate to the vertebrate. Spectroscopic analyses revealed that the amphioxus melanopsin has an absorption maximum at 485 nm, which shows good agreement with that of action spectra electrophysiologically obtained from the mammalian melanopsin-expressing native retinal ganglion cells and cultured cells in previous reports (~480 nm). We also found that the melanopsin is a bistable pigment that converts to the stable photoproduct upon light absorption and reverts to the original pigment by additional light absorption. Furthermore, immunohistochemical analyses revealed that the amphioxus melanopsin is colocalized with the Gq type G protein, demonstrating that the melanopsin triggers the Gq-mediated signal transduction cascade in vivo. These results indicate that biochemical and photochemical properties, not just primary structure, of the melanopsin are considerably similar to those of the rhodopsins in the rhabdomeric visual cells of higher invertebrates. Taken together with the fact that the amphioxus melanopsin is expressed in the rhabdomeric photoreceptor cells, the cephalochordate rhabdomeric photoreceptor represents an evolutionary link between the vertebrate photosensitive ganglion cell and the invertebrate rhabdomeric visual cell.

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Dark Pulses and NPY Decrease P-ERK Levels in the Subjective Day

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High levels of phosphorylated ERK1/2 (P-Erk) have been associated with the light phase of the daily cycle and with photic entrainment. P-Erk is higher during the day under DD as well as LL conditions, whereas total ERK levels remain constant. Recently, it has been reported that dark pulses reduce the levels of P-Erk in the SCN. Also, short dark pulses around the time of light transition are capable of inducing phase advances in vivo. We previously reported that a five-hour exposure to darkness with a novel wheel (CT 4-9) in animals kept under LL induce a phase advance of the spontaneous electrical rhythm in the SCN when the slices are prepared immediately after the treatment, while three hours of exposure are not effective. Here we present preliminary data showing that P-Erk levels are significantly reduced in the SCN (as measured by western blot methods) after five hours of dark exposure (CT 4-9). However, short pulses (15 or 30 min) of dark at CT 11 do not alter P-Erk levels. Also, NPY (200 uM) applied to the SCN in vitro is capable of decreasing the levels of P-Erk at CT6 but not at CT 19. Thus, current data suggest that phase shifts induced by dark exposure during the subjective day may involve the MAPK pathway. NPY might be one of the candidates that regulate the levels of P-Erk in the middle of the light phase.

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Accelerated Re-Entrainment in Balb/Cj Mice

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Re-entrainment of circadian activity rhythms in mammals after large shifts of the light-dark (LD) cycle usually takes place over multiple cycles due to as-yet-unidentified factors limiting shifts of the circadian system in vivo. In our experiments, running wheel rhythms of BALB/cJ mice re-entrained in one ti twodays, while C57BL/6J mice re-entrained to six-hour advances of the light–dark cycle within five to seven days. A one-day shift of the LD cycle followed by release into constant darkness yielded 5.0 ± 2.6 hour phase advances in BALB/cJ, and 1.9 ± 0.7 hour advances in C57BL/6J mice. Simple release into constant darkness yielded 1.6 ± 1.1 hour and 0.7 ± 0.6 hour advances for BALB/c and C57BL/6J mice, respectively. Rapid re-entrainment by BALB/cJ mice was not dependent on running activity, as experiments measuring activity with infrared motion detectors yielded similar results. A one-day six-hour shift of the LD cycle advanced the circadian system in BALB/cJ, but not C57BL/6J mice, sufficiently enough to see light-induced Fos expression in the SCN at ZT10 relative to the previous LD cycle, a time at which light would not normally induce Fos. Data support the hypothesis that rapidly advanced activity rhythms and suprachiasmatic sensitivity to light reflect an accelerated rate of re-entrainment of the SCN in BALB/cJ mice.

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Effects of Acute In Vitro Ethanol Treatment on Glutamate-Induced Phase-Shifts of the Mammalian Circadian Clock

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Light is the primary entraining signal for the mammalian circadian clock located in the suprachiasmatic nucleus (SCN). Light entering the eye leads to release of glutamate in the SCN. Glutamate binds to NMDA receptors initiating a cascade of cellular processes that ultimately modulates clock phase. SCN neurons show a 24-hour rhythm in neuronal activity that peaks in the middle of the day when isolated in a brain slice preparation. Treatments that phase-shift the SCN clock in vivo have been shown similarly to shift this rhythm of neuronal activity in vitro. Here, we have investigated the effects of ethanol on circadian rhythms in SCN brain slices.

Application of glutamate (1mM) in the early night to mouse SCN brain slices causes a phase-delay (2.0 ± 0.5h, n=3). Co-application of ethanol (20mM) blocks these phase-shifts (0.25 ± .35h, n=2). The effects of ethanol are dose-dependent, with an ED50 of 9mM.

Glycine, a cofactor of the NMDAR, appears to partially prevent the ethanol inhibition in a non-competitive manner when co-applied with glutamate and EtOH (5uM to 1mM). Experiments investigating the mechanism of glycine's actions suggest that glycine receptors in the SCN may be playing a role in modulating clock phase, since glycine can phase advance the clock when applied in early night. Early studies indicate that strychnine inhibits the phase-shifting actions of glycine. Taken together, our data suggest that glycine inhibition of ethanol effects may not be due to enhancing NMDA receptor activation.

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The Selective 5-HT7 Receptor Antagonist Can Block the Inhibitory Effects of 8-OH-DPAT on Light in Syrian Hamsters

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The mammalian circadian clock can be synchronised to the environmental light–dark cycle by daily adjustments of the pacemaker to light, which is the major temporal stimulus. Light results in advances during the late night and delays in the early portion of the active phase in nocturnal animals, such as hamsters. Other than light input, the circadian timing system receives afferent serotonergic projections, which are involved in the modulation of the circadian response to photic information. Serotonin (5-hydroxytraptime or 5-HT) is a widespread neurotransmitter in the mammalian circadian system. Serotonin agonists, such as the 5-HT1A/7 agonist 8-hydroxy-2- (di-n-propylamino) tetralin (8-OH-DPAT), can dose-dependently attenuate the circadian phase shifts to light in hamsters at both advances and delays. Consistent with previous studies we found that systemic administration of 8-OH-DPAT (5 mg/kg in 9% saline) in hamsters (n=8) 15 minutes prior to photic stimulation at CT14 inhibits the light-induced circadian phase delays as well as the advances at CT19 (n=16). Pre-treatment with the selective 5-HT7 antagonist SB-269970-A (3 mg/kg in 9% saline) can attenuate the inhibitory effects of the 5-HT1A/7 agonist 8-OH-DPAT on light-induced phase delays at CT14 but not on advances at CT19. Results of administration of the 5-HT7 antagonist with light were not significantly different from light alone at either circadian time. These findings indicate that the 5-HT7 receptor subtype may be involved in

mediating the established inhibitory effects of the 5-HT1A/7 agonist on photic responses of the circadian timing system.

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Individual Variability and Photic Entrainment of Circadian Rhythms in Golden Spiny Mice

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Golden spiny mice are diurnally active in most of their natural habitat. Their diurnal activity is ascribed to non-photic cues: competitive exclusion from the nocturnal niche, or thermoregulatory considerations. Here, we studied the entrainment of golden spiny mice to light (n=29).

In the laboratory, golden spiny mice were primarily nocturnal and displayed an unusual variety of rhythm patterns, with activity bursts occurring during both activity and rest periods. Occasional, spontaneous shifts of activity rhythms between light phases were recorded. In all cases but one, body temperature shifted in parallel with activity. Under DD conditions, the free running period (tau) of all individuals but one was shorter than 24 hours, and in all individuals but the same one it was shorter than tau under LL conditions.

In response to a six-hour phase delay, all individuals entrained to the new LD cycle in a relatively uniform way. During phase advance, four out of the 12 individuals further delayed their activity and body temperature rhythms, and eight individuals advanced their activity rhythm, but the re-entrainment took them over twice as long as to re-entrain to the phase delay.

We suggest that the golden spiny mouse is a nocturnal rodent whose circadian system developed the flexibility to be nocturnal or diurnal according to environmental conditions, or a nocturnal rodent in the process of turning diurnal, and that it has low sensitivity to the immediate masking effect of light on activity.

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Melanopsin Structure and Function: A Comparative Analysis

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The aim of this project is to understand how the melanopsin gene (Opn4) sequence correlates with function and to what extent the resulting protein contributes to abnormal circadian timing. We have taken a comparative approach and examined both intra and inter-species variations in melanopsin.

We are interested in the role that variations in melanopsin may have in human circadian rhythm abnormalities. To this end, we are screening the Opn4 gene for single nucleotide polymorphisms (SNPs) that we can then use in association studies in different patient groups, for example, schizophrenia, seasonal affective disorder and bipolar disease.

Analysis of the melanopsin gene sequence in patients expressing abnormal circadian regulation (such as schizophrenia patients) and animals living in extreme environments and separated by large spans of evolutionary time (such as the deep-sea fish *Coryphaenoides profundicola* and the marsupial mouse *Sminthopsis crassicaudata*) will allow us to identify some key functional elements in the melanopsin protein and to relate its molecular structure to the possible function of this recently identified opsin.

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Alteration of Photic Responses of Circadian Timing System and Pupiullary Light Reflex to Previous Light Exposure

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In mammals, non visual responses to light involve intrinsically photosensitive retinal ganglion cells that express melanopsin (ipRGC) and that receive modulatory inputs from rods and cones. The ipRGCs project to the suprachiasmatic nucleus (SCN), involved in photic entrainment of the circadian clock, and to the olivary pretectal nucleus (OPN), involved in the pupillary light reflex (PLR). Several studies in animals and in humans suggest that previous light experience can affect photic responses of the circadian timing system. We studied the sensitivity of the response for phase shifts and of the PLR following prior exposures to different irradiances and wavelengths of light or to equivalent durations in darkness. The results show that, depending on the wavelength and the irradiance of the light during the pre-exposure period, the amplitude of the subsequent photically-induced phase shift and the PLR can be significantly altered compared to those of the controls exposed to only darkness. Our findings support the idea that the circadian timing system shows a certain degree of adaptation of photic responses to light. However, the roles of the different photopigments in adaptive processes are still unkown.

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Melanopsin-IR Co-Localizes with GnRH in the Hamster Brain.

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Melanopsin, a novel photopigment described in retinal ganglion cells projecting to the SCN, is not usually evident in the brain of mammals. However, using antibody PA1-781 from Affinity BioReagents, we have identified a melanopsin-IR network in the hamster brain. The same antibody does not reveal a similar network in mouse or rat brain. The melanopsin-IR fibers are limited in distribution to the olfactory system, preoptic area, lateral septum, organum vasculosum lamina terminalis, SCN, subparaventricular hypothalamus, supramammillary descussation, habenula, and a few other regions including a dense fiber bundle extending into the pituitary gland. Comparison of the melanopsin-IR system with the GnRH system shows that they are very similar, and, double-label analysis indicates that there is essentially complete localization of GnRH- and melanopsin-IR fibers. Cells immunoreactive to GnRH and melanopsin are visible in the basal forebrain. It is not yet fully certain that the peptide recognized by antibody PA1-781 is melanopsin. However, blocking studies for fiber staining have been successful. Pre-adsorption of antibody with 1 ug/mlof melanopsin (peptide from ABR against which the antibody was made) partially blocked immunoreactivity and it was completely blocked by 10 ug/ml melanopsin. Several publications show that olfactory bulbectomy and hypophysectomy alter hamster circadian period, and bulbectomy renders rats photoperiodic. These phenomena do not yet have adequate explanation, but may be related to the present observations.

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PACAP Is Necessary for the Expression of Normal Light-Induced Phase Advances

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Pituitary adenylate cyclase-activating peptide (PACAP) likely contributes to the normal entrainment of the mammalian circadian clock. It is co-stored with glutamate (GLU) in terminals of the retino-hypothalamic tract (RHT) and can shift the clock in a time-dependent manner, either alone or in combination with glutamate. In order to examine the potential role of PACAP in light-stimulated phase resetting, we examined the circadian activity of transgenic mice with a genetic lesion for PACAP, but not for PACAP-related peptide (PRP). The wheel-running activity rhythms of the mice were monitored under 12:12 light–dark (LD), in constant darkness (DD), after single light pulses, under a 23-hour T-cycle, and with six-hour shifts in the phase of the LD schedule. Additionally, the responses of neural activity rhythms in SCN slices to glutamate were measured in both early and late night. The PACAP-null mice expressed normal endogenous rhythms and entrainment to 12:12 LD cycles. Phase delays to light and glutamate also were unaltered. However, in the absence of PACAP, neither light nor glutamate induced phase advances. Entrainment to exotic light schedules that required phase advance was changed significantly. Our findings suggest that the interaction between PACAP and light- and glutamate-activated pathways is necessary to generate normal phase advances. This, in contrast to some previous results, suggests that PACAP may be more than a modulator of glutamate-activated effects. These data suggest that PACAP contributes a necessary role in the integration of the phase-advancing light signal.

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Variability of Diurnality in Laboratory Rodents

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Although the difference between diurnal and nocturnal organisms may often be quite obvious (i.e., diurnal organisms are active mostly during the day and nocturnal organisms are active mostly during the night), very little is known about what makes diurnal organisms different from nocturnal ones. In this study, the locomotor activity rhythms of domestic mice, laboratory rats, Syrian hamsters, Siberian hamsters, Mongolian gerbils, degus, and Nile grass rats was compared. Running-wheel activity was monitored under a light–dark cycle of 12 hours of light and 12 hours of darkness per day. Nile grass rats were found to be reliably diurnal, whereas laboratory rats, Siberian hamsters, domestic mice, and Syrian hamsters were reliably nocturnal. Both diurnal and nocturnal subgroups were observed in Mongolian gerbils and degus. A downward gradient of diurnality was observed from Mongolian gerbils classified as diurnal, degus classified as diurnal, gerbils classified as nocturnal, and degus classified as nocturnal. Nocturnal degus remained nocturnal when tested with an infrared motion detector without running wheels. Thus, although the diurnal–nocturnal dichotomy could be applied to some of the species, it was not appropriate for others. The dichotomy may reflect researchers' needs for systematization more than a natural distinction between

species. Through mechanisms as yet poorly understood, the balance between entraining and masking processes seems to generate a gradient of temporal niches that runs from predominantly diurnal species to predominantly nocturnal species with many chronotypes in between, including species that exhibit wide intra-species gradients of temporal niche.

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Phase Response Curves in Mice: The Effects of Light Pulse Duration and of Skeleton Pulses

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The extent to which continuous, parametric action of light, and non-parametric action of the transitions from darkness to light and light to darkness contribute to circadian entrainment remains an unsolved puzzle.We tackled this issue by studying the phase responses of laboratory mice (C57BL6J//OlaHsd) to single light pulses of seven different durations (1, 3, 4, 6, 9, 12, 18 hours) given every 11 days in otherwise constant darkness (DD). When PRCs were plotted using the onset, midpoint, or the end of the pulse as a phase reference, they corresponded best for the mid pulse as a reference (with the best fit in fact at 36% of the light pulse). This result suggested that both onset and end of the light pulse had an effect on the response of the system. In order to test this hypothesis, we performed a second series of experiments where double light pulses (1h duration) with 1, 2, 4, 7, 10 and 16 hours in between were given. The exposure to double light pulses with 1, 2 and 4 hours of darkness in between, compared to the 3-4 and 6-hour light pulses, respectively, induce the same PRC. Thus, for light pulses up to six hours duration the middle part of the light pulse seemed not to play a role, and we attributed the response to the beginning and end of the light. In addition, there is a continuous effect of light pulses on the system since light pulse duration affected both amplitude and shape of the phase response curve (PRC). Assuming that photoreceptor adaptation reduces the effect of additional light after the first hour, we fitted models based on the 1h PRC to the data for all light pulses. The best overall fit was obtained when the effect of additional light was reduced to 22 % of the effect of the first hour. For these predicted PRCs, the light action centered on average at 38% of the light pulse, close to the empirical percentage of 36%. The result is thus compatible with an initial major (non-parametric) contribution of the onset of the light pulse followed by a reduced continuous (parametric) effect of light responsible for the differences between PRCs for different duration pulses.

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Light as a Circadian Stimulus for Nocturnal Rodents Used in Human Cancer Research

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Nocturnal rodents are common models in human cancer research. Recent evidence suggests that circadian disruption affects cancer etiology in nocturnal rodents and humans. Light is the primary exogenous stimulus for regulating and disrupting the circadian system, yet the absolute and spectral sensitivities of the

circadian systems of nocturnal rodents and of humans differ profoundly. In humans, the circadian system apparently shares photoreceptors and neurons in the outerplexiform layer of the retina with those used in color vision. Thus, the human circadian system exhibits a form of spectral opponency, which in turn leads to subadditive responses to polychromatic, as opposed to monochromatic, light. Mice, however, do not have substantial color vision and do not exhibit measurable subadditive circadian responses to light. Rats have modest color vision, but additivity of rat circadian functioning has not been tested. More importantly, the absolute threshold to light by the human circadian system is approximately 3,000 to 10,000 times higher than that of nocturnal rodents. Given the emerging importance of the circadian system in affecting cancer etiology and the need for parametric studies of cancer initiation and growth using nocturnal rodent animal models, it is essential to formally address the fundamental differences in the spectral and absolute sensitivities to light by the circadian systems of humans and of nocturnal rodents. This paper provides a first-order quantitative transformation between light for the circadian system of humans to that of nocturnal rodents, but more research is necessary to provide a completely satisfactory species-specific transformation.

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The Role of Mid-Wavelength Cones in Non-Visual Responses to Light

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Non-visual responses to light, such as photic entrainment of the circadian clock, involve intrinsically light-sensitive melanopsin-expressing ganglion cells as well as rod and cone photoreceptors. However, defining the relative roles and contributions of the different photopigments in non-visual responses has proven to be challenging. To tackle this problem, we used a mouse model deficient in TRb hormone receptors that specifically lacks mid-wavelength (MW) cones. TRb is necessary for the normal expression of MW-opsin, and in mice that are deficient for this factor all cones express SW ospin. MW-coneless mice show normal entrainment in a LD cycle of 100 lux (white) light, attenuated entrainment at 10 lux, and fail to entrain at 1 lux, whereas wild-type mice entrain at all these light levels. Both genotypes show similar phase shifts at 370 and 480 nm for a 15- minute light pulse, but for shorter duration exposures at 480 nm, MW-coneless mice show diminished phase shifts. This genotype also exhibits a pronounced deficit at 530 nm. These results show that MW-cones play a significant role in light entrainment and in phase shifting of the circadian oscillator. Modelling the contributions of the various photoreceptors stresses the importance of considering the particular spectral, temporal, and irradiance response domains of the photopigments when assessing their role and contribution in non-visual responses to light.

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Mouse Photic Entrainment Involves Modulations of Light Responsiveness as well as Circadian Period and Activity Duration

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We have examined the influence of entrainment photoperiod and cycle length on circadian function in mice (C57BL/6J). Using different T-cycles (L:D 12:12; 12.75:12.75) we found photic responsiveness in DD to be modulated immediately following LD. Rapid changes in delay magnitudes (CT/ZT16, induced by 15min, ~500 lux) suggest the responsiveness to light during and following LD are small relative to responses after several cycles in DD. Modulation of responsiveness during LD may limit the range of entrainment. Changes in circadian period (Tau), activity duration (alpha) and responsiveness followed different time courses in DD suggesting that inter-relationships between these parameters are not simple. We also measured the influence of different LD24 photoperiods. Mice were entrained to photoperiods from 20:4 to 4:20 or kept in LL. After seven cycles in DD, we measured responsiveness to CT16 pulses. The LD photoperiod caused significant, long-lasting changes in the photic entrainment pathway. Longer photoperiods (>12h light) were associated with a smaller alpha, shorter Tau, and smaller phase delays at CT16. Some groups in longer light durations (and LL) did not show the smaller delays but may not have been well-entrained prior to DD. Alpha compression and Tau shortening both decayed by ~cycle 21 in DD. In addition to the phase and period adjustments associated with entrainment, the responsiveness of the photic entrainment pathway itself is also modulated during and following LD. This research was suupported by: MH060122

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Circadian Organization in Royal College Surgeon Rats

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Previous studies have shown that classical photoreceptors are not necessary for the entrainment of circadian rhythms in mammals, thus indicating that an undiscovered photoreceptor in the mammalian retina is responsible for the photoentrainment of circadian rhythms. Although Royal College of Surgeon (RCS) rats are a well-established model for studying retinitis pigmentosa in humans, no study has investigated circadian photoreception in these animals. The aim of this study was to examine circadian photoreception in RCS rats. Tan-hooded RCS/N-rdy+ rats homozygous for the normal rdy allele and congenic age-matched RCS/N-rdy homozygotes with retinal dystrophy were used in this study. All animals used for these experiments were 60 +/-2 days old at the time when light-induced phase shift of locomotor activity (15 minutes of light at Circadian Time 15) was measured. RCS/N-rdy and rdy+ did not show any significant phase-shift of the locomotor activity after a 15 minute pulse at the intensity of 1x10-3 μWcm2, whereas significant phase-shifts were observed with the higher light intensities (1x10-1 and 1x101 μWcm2 ; t-test, $P < 0.01$). The magnitude of the light-induced phase-shift of the locomotor activity was not statistically different between RCS/N-rdy and -rdy+ (t-test, $P > 0.05$). Surprisingly, we observed that in RCS/N-rdy the free-running period of the circadian rhythm of locomotor activity was significantly shorter (0.9 hr, $P <0.01$) than in congenic -rdy+. Our data indicate that, in RCS rats photoreceptors, degeneration does not affect circadian photoreception (phase-shift), but it affects the free-running period of the locomotor activity rhythm.

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Behavioral and Metabolic Food Entrainment Are Driven by Different Oscillatory Systems

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Restricted feeding schedules elicit anticipatory activity (FAA) and are strong entraining signals for metabolic variables and peripheral oscillators. Although diverse functions in the organism exhibit simultaneously food-entrained rhythms, the mechanisms underlying food entrainment can be built up by central and peripheral regulatory circuits. In this study, we sought to discern the system underlying metabolic entrainment from that underlying food anticipatory activity. A group of rats was entrained to restricted feeding schedules (RF) with food available for two hours daily. A second group (UF) was exposed to food restriction with daily food available at different and unpredictable times. In DD conditions, RF rats showed FAA, while UF rats did not show activity anticipating the variable feeding schedules. In contrast, both groups showed daily metabolic fluctuations with peaks in phase with the last meal time. At meal time, RF rats showed inhibition of c-Fos in the SCN, while UF rats showed the same c-Fos pattern as ad libitum controls. At meal time, RF and UF showed activation in structures involved in energy balance (DMH and ARC). This study shows that metabolic variables are reset in a daily basis, while behavioral entrainment relies on regular and predictable feeding schedules. Thus FAA and metabolic entrainment are driven by different regulatory systems.

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Photoentrainment, Phase Shift by Cemical Signal, and their Effects on PERr1 Expression in the Golden Spiny Mouse (Acomys russatus) *Brain*

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The spiny mice (Acomys) are common murids inhabiting the arid part of Israel. Two species, the common spiny mouse, *A. cahirinus*, and the golden spiny mouse, *A. russatus*, coexist in the extreme arid and hot part of the Arava Rift Valley. The coexistence of these two species is attained by competitive exclusion of A. russatus from nocturnal activity by *A. cahirinus*, which is nocturnal. Keeping *A. russatus* separately under laboratory conditions results in immediate change to a nocturnal activity pattern. The introduction of heterospecific chemical signals released from *A. cahirinus* urine alters the onset of activity rhythms of *A. russatus*. We studied and compared the response of *A. russatus* to light and chemical signals released by *A. cahirinus* during the subjective night. The daily oxygen consumption values were lowered by both signals. Light pulse at CT14 yielded a clear phase delay, while odor pulse at the same time caused phase advance. We examine changes in the expression of Per1 gene in the hypothalamus, the motor cortex and the piriform cortex as a result of these two phase shifting stimuli. The results will be discussed.

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The HLH Transcription Factor Inhibitor of DNA Binding 2 (Id2) Gene Is Involved in the Photoentrainment Mechanism of the Mammalian Circadian Clock

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Id2 is a helix-loop-helix (HLH) transcription factor identified in a cDNA microarray screen for rhythmically expressed genes in mammalian fibroblasts (Duffield et al., 2002, *Current Biology* 12: 551-557). Studies revealing rhythmic patterns of gene expression within the SCN and peripheral tissues, and in vitro studies of promoter transactivation, suggest a potential role for Id2 in interacting with the canonical clock components BMAL1 and/or CLOCK. Based on these data, we have generated and screened Id2 knockout mice for changes in their circadian behavior, as monitored by their locomotor activity rhythms. We have observed profound changes in their photoentrainment response. Exposed to a 10-hour phase delay of the 12:12 light–dark (LD) photoschedule, wild-type mice show stable re-entrainment to the new LD cycle in 3.75 ± 0.19 days, whereas mutant mice showed a significantly faster response of 1.46 ± 0.26 days ($P < 0.001$). Moreover, immediate transfer from the new photoschedule to constant darkness (DD) revealed a corresponding difference in the magnitude of the phase delay in activity onset produced by exposure to the new photoschedule (wild-type, 3.30 ± 0.60 hr; mutant, 6.09 ± 0.57 hr, $P < 0.01$, mean ± SEM, $n = 11$ mice in each group). In addition, the phase angle of activity onset relative to lights off (ZT12) in stably-entrained mutant mice occurred significantly later (at ZT12.05 ± 0.12 hr) than wild-type controls (at ZT11.6 ± 0.18 hr, $P < 0.05$, mean ± SEM). These data reveal a role for Id2 in the photoentrainment mechanism of the mammalian circadian clock.

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The Dorsomedial Hypothalamic Nucleus Is Critical for the Expression of Food-Entrainable Circadian Rhythms

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Circadian rhythms of behavior and physiology can be entrained by daily cycles of restricted food availability, but the pathways that mediate food entrainment are unknown. The dorsomedial hypothalamic nucleus (DMH) is critical for the expression of circadian rhythms and receives input from systems that monitor food availability. Here, we report that restricted feeding synchronized the daily rhythm of DMH activity in rats such that c-Fos expression in the DMH was highest at scheduled mealtime. During food restriction, unlesioned rats showed a marked preprandial rise in locomotor activity, body temperature, and wakefulness, and these responses were blocked by cell-specific lesions in the DMH. Furthermore, the degree of food entrainment correlated with the number of remaining DMH neurons, and lesions in cell groups surrounding the DMH did not block entrainment by food. These results establish that the neurons of the DMH have a critical role in the expression of food-entrainable circadian rhythms.

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An Auditory Stimulus Phase Advances Circadian Rhyms in the Early Subjective Day

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An auditory stimulus presented in the early subjective night phase delays human circadian melatonin and temperature rhythms, consistent with reports of auditory-derived phase shifts in birds and nonhuman mammals. The present study determined the phase-shifting effects of the same stimulus in the early subjective day. Eleven subjects (ages 18–63, mean age ± SD, 28.0 ± 16.6 y) completed 2 four-day, four-night laboratory sessions in constant dim light (<20 lux). They received two consecutive presentations of either a two-hour auditory or control stimulus from 0600–0800 hour on the second and third mornings while awake. Core body temperature (CBT) was collected in two-min bins throughout and salivary melatonin was obtained every 30 min from 1900–2330 hour on the baseline and post-stimulus/post-control nights. Dim light melatonin onset and CBT minimum were phase markers. The auditory stimulus produced significantly larger phase advances of the circadian melatonin (mean ± SD, 0.87 ± 0.36 vs. 0.24 ± 0.22 h) and CBT (1.08 ± 0.50 vs. 0.43 ± 0.37 h) rhythms than the control. The auditory stimulus also decreased fatigue, suggesting arousing effects. Thus, an auditory stimulus presented during the early subjective day phase advances circadian rhythms, extending previous findings of phase delays elicited in the early subjective night. This nonphotic stimulus may be effective for circadian resynchronization in either the phase advance or delay direction.

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Impact of Conflicting Zeitgebers on Reentrainment: Behavior Mediated Responses?

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Entrainment studies in the 80s by Stephan and Mrososvosky, based on food restriction and non photic stimuli, respectively, have increased the awareness of possible feed back of behavior on phasing of circadian rhythms. Recently, we showed that in voles a peripheral oscillator such as the liver can be made circadian via behavioral interference: clock genes in vole liver activate their circadian expression only after daily food restriction or access to a running wheel. Here, we present evidence that the velocity of reentrainment, as well as the phase position of behavioral activity, strongly depends on whether phase shifts in two circadian zeitgebers (light and food availability) run in parallel or not. The relative strength of both external zeitgebers was tested in an interference experiment using different periods for each zeitgeber, with phasing of behavioural activity as a readout.

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The Role of the 5-HT1A Receptor in Serotonergic Enhancement of Photic Phase Shifts

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The serotonin mixed agonist/antagonist NAN-190 has been shown to potentiate photic phase shifts in hamsters. Although it is assumed that the enhancing effect of NAN-190 is mediated through the 5-HT1A receptor, this drug activates a number of other receptors. Genetic knockout technology in mice provides a method by which to study the contribution of particular receptors to this phenomenon. The goals of the present experiment were to first establish that NAN-190 potentiates the response of normal mice to light, and then to examine the effect of NAN-190 on photic phase shifts in 5-HT1A knockout animals. Adult male mice housed in constant darkness were given an i.p. injection of either NAN-190 (2.5mg/kg) or a vehicle control (DMSO) at CT21, followed one hour later by a 15-minute light pulse. The wild type animals showed a trend towards potentiated photic phase shifts when pretreated with NAN-190. By contrast, the 5-HT1A receptor knockouts demonstrated no enhancement of the photic phase shift by NAN-190, and in fact showed a trend towards an attenuated response. It was also observed that the vehicle-treated knockout animals showed substantially larger phase shifts in response to a light pulse than did the similarly treated wild type mice. These results suggest that NAN-190 enhances photic phase shifts through its activity at the 5-HT1A receptor. Supported by NSERC

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Advanced Nonphotic Entrainment: A New Automated Approach

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Nonphotic events, such as periodic feeding, drug, and locomotor activity, are capable of clock resetting. Wheel running is one of the most studied examples of behavioral influences on circadian rhythms. There are two main challenges that slow progress of understanding in this area: 1 The labor needed to transfer animals to novel wheels, or to block wheels from turning, compared to the ease of programming lighting conditions to change at any time of day or night; and 2 much of the work on nonphotic phase shifting by running in a novel wheel has been done with hamsters, and similar protocols yield only small and less consistent effects in mice. Mice, however, allow additional molecular approaches to investigate the mechanisms underlying nonphotic clock resetting.

In the present study, we describe a simple automated method to precisely schedule wheel access for mice in various T-cycles, which can be easily adjusted to any other rodents. We show that nonphotic entrainment by phase delays can be obtained by this method. More remarkably, we also show that nonphotic entrainment can be achieved by phase advances. Specifically, mice can entrain to a T-cycle of scheduled running wheel access that is shorter than their free-running period, and the phase angle of entrainment suggests that there is an advance portion in the nonphotic phase response curve around activity-onset (CT12). Finally, we also demonstrate an application of this method in a blind mouse model, i.e., Opn4-/-Gnat1-/-Cnga3-/- mice.

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Ontogenesis of the Seasonally Varying Activity Pattern in European Hamster

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One of the adaptations of European hamsters (*Cricetus cricetus*) to the seasonal changes of the photoperiod in its habitat is the strong seasonal variation of the activity pattern. Between mid-May and mid-July, when the animals are sensitive to a shortening of photoperiod, a clear precise activity rhythm is observed, whereas the activity pattern is nearly arrhythmic during all other parts of the year. Young European hamsters are born between early April and late August, i.e. in times when the adults show both the arrhythmic and the rhythmic activity pattern.

Therefore, we investigated when young European hamsters develop the diurnal and the seasonal rhythmicity of the activity pattern depending on the date of birth. After weaning at postnatal day 20-22, running wheel activity was monitored in 36 animals from five litters, born in April, May, June and July, (LD ca. 13:11/15.5:8.5/16:08/summer solstice/15.5:8.5).

The development of the activity rhythm started homogeneously in all litters. After a short phase of consolidation all animals showed the rhythmic activity pattern of adult hamsters sensitive to short photoperiods, unaffected by the date of birth. However, minor photoperiodic variations between litters were evident in the activity duration and activity onset. The ontogenesis of the activity pattern seems to be terminated at postnatal day 70-85. At that age, the young hamsters showed an abrupt change in their activity pattern when switching into the appropriate seasonal activity pattern of the adults.

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NRe Lesions Delay the Reproductive Response to Long Day Lengths

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Siberian hamsters exhibit seasonal rhythms of reproduction that are driven by changes in photoperiod. Long days stimulate and short days inhibit reproduction. The duration of nocturnal melatonin secretion is the endocrine representation of day length. Melatonin target tissues including the nucleus reunions and paraventricular nucleus of the thalamus, and the suprachiasmatic nucleus, mediate its actions upon the reproductive system. Any one of these three target tissues is sufficient to produce gonadal involution in response to short day-like melatonin infusions, but only the suprachiasmatic nucleus is necessary. While the role of these tissues in response to short days has been characterized their role in the response to long-day stimulation of the reproductive axis remains unknown.

Hamsters gestated and raised in short day lengths underwent ablation of either the NRe or PVt at eight weeks of age, prior to transfer to long day lengths. Gonadal and body mass responses were measured weekly for eight weeks in long day lengths. Sham-lesioned control animals and those bearing PVt lesions exhibited rapid gonadal growth in response to long day lengths. Hamsters bearing lesions of the NRe exhibited a three-week delay in gonadal growth as compared to controls. This result suggests that the NRe is necessary for the timely response to long day lengths.

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A Common Mechanism of Circadian Coupling Promotes Changes in Circadian Waveform Cross Different Entrainment Paradigms

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The multi-oscillatory basis of the mammalian pacemaker was first inferred from "split" circadian rhythms and photoperiodic modulation of circadian waveform (i.e., relative length of subjective day versus night). Under these behavioral paradigms, changes in circadian waveform are theorized to reflect the actions of oscillator interactions, and variations in circadian entrainment have been attributed to individual differences in circadian coupling. Despite the use of common concepts, different paradigms that implicate circadian coupling need not engage the same underlying process; rather, circadian oscillators may interact in multiple ways to influence circadian waveform. Here, we examine the relationship between circadian responsiveness under short day lengths and the propensity to split under 24 hour light–dark–light–dark cycles (LDLD) to test whether these disparate coupling paradigms index a common mechanism that differs between individuals. Siberian hamsters were transferred from long to short photoperiods to distinguish short-day responders (SD-R) from short-day nonresponders (SD-NR) by tracking behavioral and physiological changes indicative of a winter phenotype (i.e., expanded nocturnal active phase, gonadal regression, and weight loss). These chronotyped animals were subsequently transferred, along with nonchronotyped controls, to LDLD with dim nocturnal illumination, a procedure demonstrated to split rhythms in this species. Under LDLD, SD-R were more likely to split than SD-NR or nonchronotyped controls. Thus, individual differences in entrainment likely reflect variation in a mechanism of circadian coupling common to both short-day responsiveness and LDLD-induced splitting, which may be used to further investigate oscillator interactions.

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Phase Shift Responses of LDLD-Split Rhythms in Syrian Hamsters

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Under dimly lit (<.05 lux) nights, a majority of Syrian hamsters entrain to multiphasic 24-hour LDLD cycles (e.g., LDLD7:5:7:5) expressing split circadian rhythms of wheel-running activity characterized by two shortened activity bouts entrained ~12 hours apart, each to one of two daily scotophases. Reorganization of the multioscillator SCN pacemaker to give two functional subjective nights per 24 hours is supported by parallel observations in other circadian functions including melatonin, body temperature, and SCN gene expression, and by stable entrainment of splits to T=24 hours by 1hour light pulses (LD1:5). Hypothetically, split entrainment to LD1:5 is mediated by a split light-pulse PRC with two delay and two advance regions per 24 hours. Thus we predicted that light would elicit phase delays early, and advances later, in each of the two split activity bouts. This report will examine responses to 4-hour scotophase deletions, and to 1 hour and 0.25 hour light pulses given to each split activity bout during LDLD8:4:8:4, and to 1 hour light pulses given in the first day of a DD free-run following LDLD7:5:7:5. Because split rhythms rejoin within a few days in DD, the phase shift measured in the first circadian cycle following the stimulus is our primary measure. Preliminary findings indicate delays and advances associated with each bout. Further study will seek to more fully describe the PRCs, evaluate coupling interactions following light perturbations, and determine whether the two bouts of LDLD split rhythms have equivalent or different PRCs.

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Investigation of Transcriptional Pathways Driving the Photoperiodic Control of Seasonal Rhythms in Mammals

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The nocturnal melatonin (Mel) signal acts on the pars tuberalis (PT), located at the interface of the hypothalamus and the pituitary, where it is involved in the seasonal regulation of prolactin secretion. The PT expresses Mel receptors and is believed to relay via a paracrine mechanism photoperiodic signals to pituitary lactotrophs.

Recent studies have shown that the clock genes per1 and cry1 are expressed in a rhythmical fashion in the PT, with per1 tracking light onset/melatonin decline, and cry1 tracking onset of melatonin secretion, and changes in per/cry phasing may provide a mechanism for decoding the melatonin signal in photoperiodism.

To investigate transcriptional pathways involved in this mechanism, we undertook a microarray experiment using PT tRNA from sheep placed in either long-(LP) or short-day photoperiods (SP) and culled at three different time points: ZT4, 12 and 20, hybridized against a normalized 15K brain bovine cDNA library.

Our results show two distinct outlier clusters of only four to five clones in each, differently expressed in LP compared to SP at ZT12. The first one represents clones that are down regulated, and predictably Cry1 is found in this group. The other cluster presents an opposite phase of regulated genes. Three transcripts have been characterized and ovine cDNA sequences cloned for in situ hybridization and real time PCR experiments. Intriguingly, two of these transcripts are known to be involved in insulin regulation, pancreatic development, and insulin pathways, suggesting that this pathway may have been co-opted for photoperiodic time measurement in the PT.

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Consequences of Exotic Entrainment for Reproduction and Development in Hamsters

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A majority of Syrian hamsters exposed to a 24 h LDLD cycles will split locomotor activity between the two daily dark phases and rest in the interpolated light phases. A minority entrains with activity associated with only one of the dark periods. We are evaluating whether this split circadian entrainment pattern might be valuable for human shift workers, who suffer a number of complaints and maladies resulting from a mismatch between work schedules and circadian entrainment state. Thus, we should know whether this paradigm entails any biological complications in our animal model. We assess here whether such an

entrainment paradigm in Syrian hamsters interferes with reproduction and development of offspring, two sensitive markers of physiological stress. Split and unsplit female Syrian hamsters in LDLD8:4:8:4 and unsplit hamsters in LD16:8 were mated with males. The fraction of females that produced litters did not differ between groups. Pups raised in LD16:8 showed similar developmental trajectories as those in LDLD8:4:8:4. Pups of split and unsplit dams in LDLD8:4:8:4 also did not differ. Finally, growth trajectories were not different among pups shown to be split versus unsplit after weaning. Thus, markedly different entrainment patterns do not compromise reproduction or somatic development. Additional data on the melatonin patterns of hamsters under LDLD8:4:8:4 will be presented.

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Adjustment to Long Photoperiods in Circadian Gene Knockout Mice

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The old idea of a dual pacemaking system in mammals, with a M (morning) and an E (evening) component was recently proposed to have a molecular genetic basis. In this hypothesis, Per1/Cry1 are thought to follow dawn while Per2/Cry2 follow dusk as the days lengthen and shorten. Thus, animals might be able to adjust their physiology and behaviour to the time of year. A straightforward prediction from this theory is that animals with a deletion in one of the two oscillators would have deficits in their adjustment to changes in daylength.

We tested this prediction by exposing C57BL6J//OlaHsd wild-type mice, single mutant mice with deletions of Per1, Per2, Cry1, Cry2, and Per2/Cry2 double mutants to three different daylengths (LD18:6, LD6:18 and LD12:12). After one month, they were released into constant darkness. Locomotor activity was recorded throughout the experiments to analyze activity patterns during entrainment and during the subsequent free run. In particular, we wanted to know whether the compression of the daily duration of activity alpha under long photoperiods was compromised by the deletions of these genes as predicted by the hypothesis.

There was no significant difference in alpha among the different genotypes under LD12:12 and LD 6:18. Under LD18:6, Per2-/- mice had significantly longer alpha than the other strains and the wild type ($F=2.82$, $p<0.05$). Indeed, the daily activity time in the Per2-/- mutant was not compressed at all by the long photoperiod. Compression of alpha as seen in the wild-type strain was not compromised in the other mutant strains. In constant darkness, all genotypes except Per2-/- gradually decompressed alpha after release into DD. Per2-/- did not show decompression and started to free run from the onset of activity phase during LD18:6 cycle, which preceded lights-off by as much as seven hours Although this evidence supports the hypothesis that the Per2 mutation specifically interferes with the evening (E) component of the coupled system, the presence of a compression in the other genotypes is at variance with the model's predictions.

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Seasonal Changes in Pelage Thickness and Growth Dynamics of Photorefractory Siberian Hamsters

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In the face of the energetic challenges associated with the approach of winter, many temperate-zone mammals undergo physiological and behavioral changes that conserve energy. The decrease in day length in the weeks after the summer solstice initiates the transition to the winter phenotype, which also can be induced in Siberian hamsters (*Phodopus sungorus*) transferred from long days (LD) to short days (SD) in a laboratory setting. With continued maintenance in SD, however, hamsters develop photorefractoriness and revert to the spring/summer phenotype. Prominent among winter adaptations is an increase in pelage thickness, which also is accompanied by more rapid fur re-growth when hamsters replace lost hair. Here we determined whether the onset of refractoriness is associated with reversion to the spring pelage density, and if so, whether this molt is accompanied by altered pelage growth dynamics. Twenty-seven weeks after transfer to a SD photoperiod (10 h light/day), the weights of four cm2 patches of fur shaved from the hamsters' dorsal and ventral surfaces were greater than those of LD controls (14 h light/day). At all later time-points (weeks 30–49), however, the difference was no longer significant. Furthermore, hair re-growth on both the dorsal and ventral surfaces did not differ between SD and LD hamsters in the first three weeks after shaving at weeks 27–49. We conclude that the transition to the spring pelage is regulated by an interval timer triggered by SD and mediated by the eventual loss of responsiveness to SD.

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Identification and Characterization of Thyroid Hormone Transporter Mediating Photoperiodic Response in Birds

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Previously, we showed that 3,5,3?-triiodothyronine (T3) generated photoperiodically by type 2 iodothyronine deiodinase (Dio2) in the hypothalamus is critical for the photoperiodic response of the gonads in Japanese quail. Although thyroid hormones were long thought to traverse the plasma membrane by passive diffusion due to their lipophilic nature, recent studies demonstrate that several organic anion-transporting polypeptides (Oatp) transport thyroid hormones into target cells. In order to examine the role of thyroid hormone transport systems in avian photoperiodic response, we examined comprehensive expression analysis and functional study of Oatp. We have isolated 10 chicken Oatp genes using database searches. Comprehensive expression analyses using in situ hybridization revealed strong expression of cOatp1c1 and weak expression of cOatp1b1 in the hypothalamus coincided with the expression site of Dio2. In vitro transport studies using cOatp1c1-expressing CHO cells confirmed that cOatp1c1 transported [125I]T4 but not [125I]T3. These results suggest that cOatp1c1 could be involved in the thyroxine transport necessary for the avian photoperiodic response of the gonads. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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Homeostasis of Tau Revisited: Do Aftereffects of Photoperiod Depend upon Casein Kinase?

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The circadian pacemaker is plastic: its endogenous period is altered by long-term entrainment to extreme photoperiods or T cycles. The molecular basis of this phenomenon is unknown, but may lie in up- or down-regulation of the expression of any of several genes that control circadian period. The observation that the tau mutation in hamsters, which results in free running periods of 20 hours or less, is attributable to deficiency in casein kinase 1e led us to hypothesize that this enzyme participates in the generation or maintenance of aftereffects. Wild-type and tau-mutant hamsters were born and raised in 14L:10D, They were placed in DD for assessment of baseline t of locomotor activity. Free running periods were 24.07 + .12 and 19.76 + 0.14h, respectively. Hamsters were next entrained for several weeks to short T cycles whose period was approximately 90% of their free running periods; Wild-type hamsters were exposed to 10L:12D and tau mutants to 9L:9D. Upon release into DD, wild type and tau mutant hamsters exhibited comparable aftereffects. The free running period of wild-types was 98.3 + 0.3% of baseline, and that of tau mutants was 98.3 + 0.6% of baseline. Pittendrigh and Caldarola (1973) observed that individual cockroaches with tau values closer to the limits of the species-typical range showed smaller changes in period in response to temperature steps. They concluded that tau is homeostatically conserved. Our findings are inconsistent with this conclusion, and indicate that CK1e is not necessary for aftereffects of short T.
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Application of cAMP Mimicks the Effects of Pigment-Dispersing Factor on the Electrical Activity of Accessory Medulla Neurons in the Cockroach Leucophaea maderae

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In the cockroach *Leucophaea maderae*, transplantation studies located the circadian pacemaker center, which controls locomotor activity rhythms to the accessory medulla (AMe) with associated pigment-dispersing factor-immunoreactive (PDF-ir) neurons of the optic lobes. Extracellular recordings of the excised AMe revealed that circadian pacemaker candidates of the cockroach produce very regular interspike-intervals. The ultradian oscillating cells are coupled via synaptic (mostly GABAergic) and non-synaptic interactions, forming different assemblies of cells that share the same frequency and the same phase (timing of spikes). Cells belonging to different assemblies differ in phase, but can share the same period. Application of PDF was shown to synchronize phase and period of cells belonging previously to different assemblies mostly via inhibitions of electrical activity. Here, we show that application of membrane-permeable cAMP, but not of cGMP analogues mimicks the effects of PDF application. Thus, we suggest that in the cockroach, PDF-receptors are coupled to an adenylyl cyclase. In current patch clamp studies we are testing this hypothesis.
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Molecular Mechanisms that Regulate Circadian Olfactory Responses in Drosophila

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In Drosophila, circadian oscillators in large basiconic sensillae on the antenna regulate physiological responses to the food odorant ethyl acetate, where robust electroantennogram (EAG) responses are seen during mid-night and weak responses are seen during mid-day. To determine how the circadian clock regulates this physiological output, we investigated molecules that directly or indirectly control olfactory signal transduction, since olfactory sensory neurons (OSNs) are necessary and sufficient for rhythms in olfactory responses. Here, we report preliminary results concerning two putative olfactory signal transduction regulators, the G-protein coupled receptor kinase GRK-2 and the putative odorant receptor chaperone Or83b. Flies that overexpress GRK-2 in OSNs display constant high magnitude EAG responses to ethyl acetate, whereas GRK-2 mutant flies show constant low magnitude EAG responses to ethyl acetate. These results suggest that GRK-2 levels and/or activity are rhythmic, and that GRK-2 activation enhances olfactory transduction. A potential GRK-2 target is Or83b, which is structurally similar to G-protein coupled receptors. Or83b is expressed in ~80% of OSNs, and functions to carry odorant receptors from the cell body to the dendrite. In large basiconic sensillae, Or83b protein accumulates primarily in the dendrites during mid-day and primarily in the cell body during mid-night under both LD and DD conditions. In arrhythmic per0, cyc01, and tim0 mutants, which display constant low magnitude EAG responses, Or83b accumulates primarily in dendrites. These and additional experiments that explore the relationship between GRK-2 and Or83b and how they might function to modulate rhythms in EAG responses will be presented.

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Modeling of Some Circadian Properties in Crayfish

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From experimental facts about circadian rhythms in crayfish, we built a qualitative mathematical model in order to simulate several aspects of such rhythms. By means of the model we could verify the behavior observed in the biological experiments and to establish some biological hypotheses. The biological experiments ranged across aspects such as the ontogeny of a circadian rhythm, from hatching up to adult age, the entrainment of rhythm under periodic signals, the construction of the phase response curve to single light pulses, the absence of synchronization in juvenile animals, the synchronization of adult animals, and so forth. It is worthwhile to emphasize that the mathematical model not only simulated the biological phenomena but lead us to explain and to propose new biological experiments posing interesting although difficult questions of either mathematical or biological character. All the above drive us to think about the process involved in a biomathematical investigation obtaining a scheme that shows the different elements converging in the work of professionals with different specialities.

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The Circadian Clock Suppresses Long-Term Memory Formation at Night by Inhibiting Learning-Induced Transcription

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The circadian clock modulates long-term, but not short-term memory in *Aplysia californica*. Animals demonstrate robust long-term memory when trained during the subjective day, regardless of when tested. However, animals trained during the night exhibit little long-term memory. To determine how the circadian clock suppresses long-term memory, we used an associative, operant paradigm: learning that food is inedible (LFI). We found that LFI training at night, insufficient for long-term memory, resulted in savings at the molecular level that could be converted to behavioral memory with additional training given at night up to 72 hours later. The long-lasting "molecular savings" at night required protein synthesis produced by the initial training session, but not transcription. Animals treated with anisomycin during initial training failed to show long-term memory after two training sessions. In contrast, animals treated with DRB during the initial training expressed robust long-term memory after two sessions. Apparently, circadian inhibition of transcription during the initial training made DRB inhibition of transcription redundant. To test the hypothesis that circadian repression of transcription was a primary limitation to long-term memory at night, we used histone deacetylase inhibitors to facilitate transcription in conjunction with training. Animals treated with TSA or sodium butyrate prior to training at night exhibited robust long-term memory when tested 24 hours later, thus, facilitating transcription during LFI training resulted in long-term memory at times when the circadian clock normally blocks memory formation. Our results indicate that the circadian clock modulates long-term memory formation by regulating learning-induced transcription.

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Beta-Pigment Dispersing Hormone-Expressing Neurons in Cancer Productus Brain and Optic Ganglia

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While most organisms are exposed to daily alternation of light and dark, intertidal marine species are also subjected to tidal cycles. The presence of circadian and circatidal rhythms has been recognized for decades in intertidal crustaceans; however, little is known about the underlying neural basis and output pathways that allow for their overt expression. β-pigment dispersing hormone (β-PDH) regulates pigment dispersion in crustaceans and is a circadian output signal in *Drosophila*. To identify putative neural pathways underlying biological rhythmicity in intertidal organisms, we have started the characterization of the PDH system in the crab, *Cancer productus*. PDH immunostaining revealed labeling in both the brain and the eyestalk, and a visual quantification indicated that eyestalk PDH showed a daily rhythm with a peak towards the end of the day and a trough towards the end of the night. In order to characterize its regulation, we cloned two *pdh* genes, and the sequences of their derived peptides were confirmed via mass spectrometry. In situ hybridization showed *prepro-β-pdh I and II* mRNA to be co-localized only in a cluster of somata between the medulla interna and medulla externa, with only prepro-beta-pdh I present in the retinal photoreceptor cells. Studies are currently ongoing to characterize the temporal pattern of expression of both *β-pdh I and II* mRNA.

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Daily Expression of Discs-Large in the Photoreceptor Terminals of Drosophila Visual System

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In the fly's visual system, the number of tetrad synapses formed between the photoreceptor terminals and monopolar cells L1 and L2 in the first optic neuropil (lamina) changes during the day and night. Moreover, in the housefly, it has been found that changes in the number of tetrad synaptic profiles are correlated with a circadian rhythm of morphological changes of L1 and L2, which swell at the beginning of the day and shrink at night. In *Drosophila melanogaster*, the circadian rhythm in changes of L1 and L2 sizes has also been detected; however, its pattern is different than in the housefly, having two, morning and evening, peaks. Looking for mechanisms involved in cyclical synaptic plasticity and structural changes in neurons of the fly's visual system, we examined expression of the protein Discs-Large (Dlg) that is important for assembling multiprotein complexes at synaptic contacts. It has also been found that Dlg is needed for formation of new plasma membranes during cellularization in early embryogenesis of Drosophila.

We have used the antibody to detect Dlg protein in the brain of Drosophila fixed for immunocytochemistry at: ZT1, ZT4, ZT 13 and ZT16 (ZT0—the beginning of the day and ZT12—the beginning of the night) in LD 12:12.

We found that Dlg is present in the photoreceptor terminals in the lamina and shows cyclical expression which is the highest at ZT13. Moreover, Dlg expression is correlated with changes of L1 and L2 axon sizes, which are the largest at ZT 13.

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Moonlight during the Night Provokes a Complete Change in the Activity Pattern of Fruit Flies

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Under artificial light–dark cycles (LD), fruit flies (*Drosophila melanogaster*) are diurnal, showing two major activity peaks—one in the morning and the second in the evening. Darkness seems to suppress (mask) activity: The evening activity drops rapidly after lights-off and the morning activity rises steeply only after lights-on. To investigate the flies rhythmic behaviour under more natural conditions, we applied dim light (0.03 Lux)— equivalent to half-moonlight—during the night phase. We found the flies became merely nocturnal under these light-moonlight (LM) conditions. The evening activity remained high for several hours after lights-off, then dropped to an intermediate level and rose again to the morning peak level around lights-on. During the light-phase of LM, the activity level showed a broad trough with no activity at all. Despite these severe alterations in locomotor activity, the molecular cycling of the clock protein PER remained basically unchanged (determined in head extracts and in the clock neurons themselves). Our results suggest that the activity level is heavily masked by the conditions of illumination used in the experimental setup: Darkness has a strong negative and moonlight a strong positive masking effect on activity.

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Pigment-Dispersing Hormone Induces Periodic Changes in Excitability of Photoreceptor Cells in Crayfish

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The effect of the pigment dispersing hormone over the receptor potential of the crayfish retinular cells. In the crayfish, the visual photoreceptors have been identified as important structures in the organization of the circadian system responsible for the generation and expression of the electroretinogram circadian rhythm. They are the structures where the circadian rhythm is expressed (effectors) and that transform information from external signals to be conducted to the pacemaker in order to induce adjusments of the ryhthm (synchronizers). The aim of this work is to study the effect of the pigment dispersing hormone (PDH), a key sustance in the machinery of the crustacean and insect circadian rhythms, on the electrical response to light of the visual photoreceptors, at different moments of the circadian time. We show that when isolated eyestalk of crayfish *Procambarus clarkii* to previously adapted cycles LD 12:12 are perfused in a PDH solution, both the fast transient phase and the repolarization phase of the receptor potential (RP) show variations that depends on the circadian time at which the experiment was executed. We also observed a change in the resting membrane potential. Because of the changes produced by the PDH solution on the RP, we can infer that this substance affect, the dynamics of some of the ionic currents that constitute the electrical response to light of the crayfish visual photoreceptors and that the circadian time is an important variable that affect the way the PDH change the shape of the visual response of the photoreceptors.

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Daily Variation of Effect of Melatonin upon Excitability of Photoreceptor Cells in Crayfish

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Several studies have established that, in crayfish, the photoreceptor cells are important structures in the organization of the circadian system responsible for the generation and expression of the electroretinogram circadian rhythm. Indeed, they are effectors and synchronizers of this rhythm. Isolated eyestalks were perfused in a van Harreveld plus melatonin (0.1 mM) solution, and the electrical response to light, the receptor potential (RP) was recorded during-24-hour cycle. Changes in both amplitude and duration of RP were evident. In order to determine if melatonin exerts its action by interacting with membrane receptors, a second group of eyestalks was perfused at CT 4:30, in melatonin plus DH97 (an antagonist of MT2 receptors) solution. There were two moments when the melatonin action was clear: the first one between circadian time (CT) 16:30 and 18:30 and the second one between CT 4:30 and CT 8:30, when the RP amplitude decreases 60%. At CT 4:30, RP amplitude decreased 10% when eyestalks were perfused in the antagonist plus melatonin solution. These changes suggest that the mechanism by which melatonin mediates RP is by interacting with MT2 receptors.

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Circadian Rhythms in Olfactory Receptor Neurons

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Recently, it has been shown that in the cockroach, *Leucophaea maderae*, antennal responses to odorant pulses as measured by electroantennogram (EAG) amplitude, exhibit a circadian rhythm that is driven by a circadian pacemaker in the optic lobe of the brain and is entrained by photoreceptors in the compound eye (Page and Koehling, 2003). Because the EAG measures olfactory receptor potentials along with trans-epithelial potentials, the extent to which the circadian system specifically modulates the sensitivity of olfactory receptor neurons (ORNs) was not clear. To answer this question, the responses of ORNs to odorant pulses were monitored in long-term extracellular recordings from single olfactory sensilla on the antennae of cockroaches restrained in constant darkness and temperature. In these experiments, the number of action potentials elicited in single ORNs in response to a 1.5 sec pulse of ethyl acetate once per hour was monitored over several (3–7 days). Individual ORNs exhibited a clear circadian rhythm with the peak response occurring in phase with the peak of the EAG rhythm (N= 9). Surprisingly we found that while ablation of the optic lobes abolished the rhythm in EAG amplitude, the rhythm in the responses of ORNs persisted (N = 3). These results suggest that circadian modulation of the sensitivity of olfactory receptor neurons can be driven by one or more autonomous circadian oscillators outside the optic lobe. This research was supported by NIH Grant 5RO1-MH069836.

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The Phase Response Curve of the Electroretinogram Circadian Rhythm of Crayfish, by Applying Exogenous Melatonin

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Crayfish *Procambarus clarkii* kept under controlled environmental conditions show a robust circadian rhythm in the amplitude of the electrical response to light (electroretinogram, ERG) of the retinal photoreceptors. In this same species, the circulating level of melatonin shows a daily variation with a peak during the nocturnal phase. In order to know the role of melatonin as a potential non-photic synchronizer of the circadian rhythms in crayfish, a single dose (0.1 ml, 1 microM) of the hormone was applied at different circadian times (CT 0 corresponds to the moment when initiates the activity time) during the recording of the ERG circadian rhythm. The phase shifts, advances or delays, produced by the exogenous melatonin were plotted as a phase response curve (PCR). We obtained a unimodal PCR with advances between CT 18 and CT 4, delays between CT 10 and CT 16, and no changes between CT 6 and CT 8. These results suggest that the peak of melatonin production in the crayfish acts as a synchronizer of the ERG circadian rhythm.

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The Zinc Finger Protein EGR-1 (Early Growth Response-1) Mediates the Induction of mPer1 Expression by Gonadotropin-Releasing Hormone (GnRH) in T3-1 Pituitary Gonadotrope Cells

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The regulation of clock gene expression in cells of the pituitary pars distalis has only recently begun to be explored, despite the firmly established and essential role of these cells in the maintenance of reproductive rhythms. We recently demonstrated that GnRH activates mPer1 expression in immortalized gonadotropes through protein kinase C and p42/44 mitogen-activated protein kinase pathways. In gonadotropes the latter enzyme is known to phosphorylate ELK-1, which upregulates Egr-1, a critical transcription factor for GnRH-induced luteinizing hormone (LH) synthesis. The parallels between the GnRH-LH and the GnRH-Per1 pathways led us to explore whether Egr-1 is involved in the regulation of mPer1 expression in gonadotropes. Of particular interest was the presence of an EGR-1 binding site (ACCGGGGGCGGG) at -83 to -72 of the 5'-flanking sequence of the mPer1 gene. In cell culture experiments, stimulation of αT3-1 cells with the GnRH agonist buserelin (100 nM) induced the rapid induction of Egr-1 mRNA, which was followed by mPer1, as determined by quantitative PCR and luciferase reporter assays. Immunoblotting confirmed that buserelin caused increases in mPER1 and EGR-1 expression. By means of RNA interference experiments, we could demonstrate that silencing of Egr-1 expression (confirmed by qPCR) resulted in markedly lower mPer1 transcript levels. This silencing effect of the Egr-1 siRNA could be rescued by transfecting the cells with an Egr-1 overexpression vector. These data are consistent with a role for the EGR-1 protein in transactivating the mPer1 gene in pituitary gonadotrope cells.

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The Circadian Machinery in the Ptuitary Gland

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The hypothalamic suprachiasmatic nuclei synchronize multiple peripheral oscillators through both nervous and humoral pathways. The pituitary gland is an endocrine interface between the hypothalamus and peripheral targets. Although luciferase-based reporter constructs enabled to evidence sustained oscillations of Period genes in glands maintained for several days in culture, the expression pattern of native clock genes in the pituitary of living animals remains to be investigated.We therefore performed semi-quantitative RT-PCR on pituitaries dissected every four hours from mice housed under LD 12:12. Except for the transcript of Clock that remained constantly expressed throughout the day-night cycle, all the messengers tested exhibited a clear-cut daily rhythm in expression. The mRNA of Bmal1 and Npas2 were barely detectable at ZT12 and peaked around ZT0. Inversely, the Per1, Per2, and Cry1, Cry2 levels reached their zenith at ZT12-16. These profiles were very similar in term of both phase and amplitude to those observed in the liver of the same mice. We verified the actual presence of the PER2 and BMAL1 products by western blot. Interestingly, whereas activation of the circadian machinery in calendar cells of the hypophyseal pars tuberalis requires melatonin, we observed clock genes oscillations in the pars distalis of C57/Bl6 mice that are devoid of melatonin. This suggests that different neuroendocrine signals regulate clock components in the different parts of the pituitary gland.

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Effects of Ovarian Steroid Hormones on Per1 Expression in the SCN

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Ovarian steroid hormones (Estradiol (E) and Progesterone (P)) may provide feedback signals to the suprachiasmatic nucleus (SCN) during various physiological states, including the estrous cycle and pregnancy, to facilitate timed endocrine or neuroendocrine events. Several experiments have shown that ovariectomy followed by steroid hormone replacement modulates the circadian rhythm of locomotor activity in rats, characterized by a shortened free-running period. Although evidence suggests that SCN pacemaker cells express hormone receptors, little is known regarding the potential effects of steroid receptor activation on the circadian clock. We utilized recording of bioluminescence in the SCN from Per1-luciferase transgenic rats with photomultiplier tube assemblies to explore the effects of E, P and E+P on the amplitude and period of PER1 expression in SCN. In contrast with behavioral experiments, treatment with E, P or E+P at doses in the physiological range (200nM-1uM) failed to significantly change the period or amplitude of Per1-expression in vitro. Estradiol treatment at concentrations above 1uM decreased amplitude but failed to affect the period of Per1 expression. We observed a significant decrease in rhythm amplitude and period lengthening in SCN tissue cultures treated with P at doses above 100uM that was attenuated by the addition of E. Given the established effects of steroids on behavior and our inability to replicate this response with steroid treatment in vitro, our results suggest that ovarian steroids may act on targets within the brain outside the SCN that interact with SCN pacemakers to coordinate outputs such as the rhythm of locomotor activity.

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Exogenous T3 Elicits Long Day Reproductive Responses in Short-Day Housed Siberian Hamsters

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Siberian hamsters exhibit seasonal cycles of reproduction driven by changes in day length. Day length is encoded endogenously by the duration of nocturnal melatonin secretion from the pineal gland. Short-duration melatonin signals stimulate and long- duration signals inhibit reproduction. The mechanism by which melatonin signals are decoded at the level of neural target tissues remains uncharacterized. In Siberian hamsters, exposure to short day lengths or injections of melatonin in long days results in a decrease in hypothalamic expression of type 2 iodothyronine deiodinase (Dio2) mRNA. Dio2 catalyzes the conversion of the thyroid hormone thyroxine to triiodothyronine (T3). Thus, exposure to short and long day lengths should decrease and increase hypothalamic T3 concentrations, respectively. We tested the hypothesis that injections of exogenous T3 administered to short-day housed hamsters would mimic exposure to long day lengths with respect to gonadal stimulation. Sixteen hamsters gestated and raised in short day lengths that exhibited photoinhibition of the testes were given daily s.c. injections of T3 or saline vehicle for four weeks beginning at week 12 of life. Testis size, body mass, and pelage scores were obtained weekly for 20 weeks. The results indicate that exogenous T3 induced significant gonadal growth in short day hamsters and delayed spontaneous recrudescence by an interval equal to the number of weeks during which exogenous T3 was administered. These results suggest that T3 mimics long-day exposure in Siberian hamsters and may be involved in decoding the melatonin message.

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T3 Implantation into the Mediobasal Hypothalamus Mimics Seasonal Morphological Changes in the Median Eminence of Japanese Quail

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Recently, it has been shown that photoperiodically generated triiodothyronine (T3) in the mediobasal hypothalamus (MBH) has critical roles in the photoperiodic response of gonads in Japanese quail. In a previous study, we demonstrated seasonal morphological changes in the neuro-glial interaction between GnRH nerve terminals and glial processes in the median eminence (ME). However, a direct relationship between photoperiodically–generated T3 and seasonal neuro-glial plasticity in the ME remained unclear. In the present study, therefore, we have examined the effect of T3-implantation into the MBH on the neuro-glial interaction in the ME. T3 implantation caused testicular growth and reduced encasement of nerve terminals by glial processes in the ME. In contrast, no morphological changes were observed in birds given an excessive dose of T3, which did not cause testicular growth. These results support the hypothesis that thyroid hormone regulates photoperiodic GnRH secretion via neuro-glial plasticity in the ME. This work was supported by grants from the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN; to T. Yo.).

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Long-Day Suppressed Expression of Type 2 Deiodinase in the Mediobasal Hypothalamus of the Saanen Goat, a Short-Day Breeder

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In most animals living in temperate regions, reproduction is under photoperiodic control. In long-day breeders such as Japanese quail and Djungarian hamsters, type-2 deiodinase (Dio2) plays an important role in the mediobasal hypothalamus (MBH), catalyzing the conversion of prohormone thyroxine (T4) to bioactive triiodothyronine (T3) to regulate the photoperiodic gonadal response. However, the molecular basis for seasonal reproduction in short-day breeders remains unclear. In this study, we examined the effect of a long-day stimulus on Dio2 expression in the Saanen goat, a short-day breeder. Dio2 expression was observed in the caudal continuation of the arcuate nucleus, known as the target site for both melatonin and T4 action. In addition, expression of Dio2 and T3 content in the MBH was suppressed by long-day conditions, contrary to results of long-day breeders. These results may provide a mechanism for the reversed effect of long-day signal on reproductive neuroendocrine axis between long- and short-day breeders.

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Porcine Stress Hormone Circadian Rhythm in Intensive Care

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Corticosteroid and catecholamine circadian rhythms have been used as stress indicators in agricultural and intensive care research. The feasibility of a porcine intensive care unit (ICU) experimental model was studied for potentially improved characterization of the effects of the ICU environment, treatment, and critical illness on biological rhythms. Using a prospective observational case-study design, two mechanically ventilated growing-finishing swine cortisol and catecholamine responses were examined and circadian rhythm profiles explored in an experimental ICU setting. Subjects were filtered with invasive catheters under aseptic conditions and anesthesia and placed in ICU beds where they were chemically restrained and cared for with adult ICU clinical protocols for seven days. Blood samples were obtained from indwelling catheters every two hours for 48 hours in Subject 1 and 66 hours in Subject 2 for radioimmunoassay. Additionally, hourly samples were collected from Subject 1 during decompensation at the end of life. Cortisol concentrations varied from 8.3 to 273 nmol/L, epinephrine from 0.112 to 3.19 nmol/L, and norepinephrine from 0.118 to 2.37 nmol/L. Circadian mesors and amplitudes were assessed with cosinor analysis, which revealed that Subject 1 had a norepinephrine rhythm and both subjects had a significant cortisol rhythm ($p < .001$) toward the end of their ICU stay. These circadian rhythm profiles suggest temporal adaptation to the stressors of the porcine experimental ICU.

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Effects of Time of Feed Delivery on Daily Rhythms in Glucose and Insulin in Blood Plasma and Glucose Tolerance in Dairy Cows

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Traditional feeding time for dairy operations is in the early morning. Recent studies have shown beneficial effects of evening feeding in beef cattle and dairy cattle. Studies in humans have shown an early morning poor glucose tolerance. A circadian rhythm in glucose tolerance has not yet been confirmed for dairy cattle. Synchronization of the time of feed delivery with such a rhythm may improve milk fat and energy balance in high producing dairy cows. To examine this possibility, eight Holstein cows were used in a replicated 4x4 Latin Square with four periods. Each period lasted 21 days (14 d adaptation + 7 d sampling). Fresh total mixed ration containing higher concentrate (HC) or lower concentrate (LC) was delivered either at 9:00 AM or at 9 PM. The HC diet contained 62% of dry matter as concentrate. The LC diets contained 51% of dry matter as concentrate. Treatments were 1) HC + AM, 2) HC + PM, 3) LC + AM, and 4) LC + PM. Each sampling week, blood samples were taken every 2 hr for 2 days via jugular catheters. A glucose tolerance test was performed at noon of the last day of periods 2, 3 and 4. Feeding time had a significant effect on daily glucose and insulin rhythms, but had no effect on glucose tolerance or insulin response to glucose injection at noon. The PM- fed cows had a lower glucose and higher insulin level at two hour post-feeding than the AM-fed cows, but from six-eight hours post-feeding, both glucose and insulin were higher in PM-fed cows than in AM-fed cows. These results suggest insulin resistance in dairy cows in the early morning, which is obervable only if the cows are fed in the evening.

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Activation of MT1 or MT2 Melatonin Receptors Phase Shift Distinct Circadian Rhythms in the C3H/HeN Mouse

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This study investigated the effect of genetic deletion of both the MT1 and MT2 melatonin receptors in C3H/HeN mice on melatonin-mediated phase shifts of neuronal firing rhythms in the suprachiasmatic nucleus (SCN) brain slice and of overt circadian activity rhythms. Neuronal firing rate was recorded from SCN brain slices (400 um) using single-unit recording. In slices from wild-type (WT) mice, melatonin (10 pM) applied at CT 10 phase advanced the peak of neuronal firing to CT 3.5 +/- 0.3 (n=3) when compared with vehicle (6.1 +/- 0.1, n=6, $p < 0.005$). A phase advance of identical magnitude was observed in slices from MT1 KO mice (3.4 +/- 0.09, n=3), but not from MT2KO (CT: 5.9 +/- 0.28, n=5 MLT) or double MT1/MT2 KO (6.2 +/- 0.09, n=3) mice. The effect of melatonin (90 ug/mouse, ip) given at CT 10 on wheel-running activity rhythms was assessed in mice kept in constant dark. Melatonin significantly advanced (1.3 +/- 0.11 h, n=12) the onset of activity rhythms when compared with vehicle (0.04 +/- 0.1 h, n=8; $p <0.005$) in W-T mice. This treatment also significantly advanced circadian activity rhythms in MT2 KO (1.3 +/- 0.18h, n=9, $p<0.005$), but not in the MT1KO (0.02 +/- 0.04 h, n=6) or double MT1/MT2 KO (0.11 +/- 0.07h, n=11) mice. These results strongly suggest that the MT1 and MT2 melatonin receptors phase shift distinct rhythms, i.e., the MT1 phase advances overt circadian activity rhythms in vivo, and the MT2 phase advances neuronal firing rhythms in the SCN brain slice in vitro.

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Melatonin Duration Mediates the Photoperiodic Regulation of Song Control Nuclei in the House Sparrow, Passer domesticus

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The pineal gland and its periodic secretion of melatonin are important in the circadian systems of passerines. In songbirds, long photoperiods stimulate an increase in testes size, followed by a subsequent increase in the size of the song-control nuclei. However, the significance of the pineal gland in the regulation of circannual and seasonal events has not been demonstrated; pinealectomy does not affect hypothalamo-gonadal responses to photoperiodic stimulation. Sparrows were pinealectomized to remove their endogenous source of circulating melatonin. Subsequently, birds were housed in DD and cyclically administered melatonin via their drinking water in either a short (8 hours/ day) or long (14 hours/ day) duration, mimicking the effects of a long or short photoperiod, respectively, on cyclical melatonin production. Sparrows entrained to both melatonin schedules, but the sizes of song control nuclei and testes were small in birds from both groups. However, when birds were placed in constant light, which abolishes melatonin production, and cyclically administered melatonin for either a short (8 hours) or long (14 hours) duration, we observed that the song control nuclei were significantly larger from birds administered a short duration of melatonin. Furthermore, the testes of both groups remained regressed. Our data are an initial demonstration that some aspects of photoperiodic induction can be mediated, in part, by the changes in

cyclical melatonin profiles. Furthermore, our data suggest that the effects of melatonin on song control nuclei development are, in part, redundant with the effects of gonadal steroids on these structures.

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The Rate of Reentrainment Is Associated with the Timing of Melatonin Onset

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It is well known that the rate of circadian rhythm reentrainment following time zone changes varies greatly among human individuals. How these individual variations are associated with characteristics of circadian pacemaker properties is not understood. The goal of our study was to assess how the rate of circadian clock resetting is associated with various circadian parameters. Using in vivo on-line pineal microdilaysis, we have conducted detailed analysis of melatonin profiles in rats before and after shifts of the light and dark (LD) cycle. Reentrainment following a one-hour delay or advance shifts of the LD cycle took 2 to 11 days. The majority of rats took more than four days to reentrain to 1h LD shifts in either direction. Large individual differences in the entrained phase angles of melatonin onset/offset, melatonin secretion durations, and the rate of clock resetting were found in rats. Significant correlations were identified between the phase angles of melatonin onset and melatonin secretion duration with the rate of reentrainment (r of 0.91 to 0.99). The extended periods required for rats to reentrain following just a one-hour LD change suggest that clock resetting is not as rapid as previously thought. The finding that the rate of clock resetting is correlated with the entrained phase angles of melatonin onset and with melatonin duration points to a new direction for mechanistic understanding of circadian pacemaker reentrainment.

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Melatonin Stimulates Growth of Primary Chick Astrocytes in Culture

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The avian pineal gland influences rhythmic physiology and behavior in many species of birds via the secretion of melatonin. The sites and mechanisms of melatonin action are not known. Chick diencephalic astrocytes, which express melatonin receptors, have been shown to express rhythmic clock gene expression and glucose metabolism in culture when melatonin is rhythmically administered to these cells. We observe here that primary chick astrocytes also grow at a significantly faster rate in vitro when co-cultured with pinealocytes than when cultured alone. When cultured in the presence of pinealocytes for three days, astrocytes exhibited a two-fold greater cell density than cells grown alone. That trend continued for two more days in vitro. As stated above, astrocytes express melatonin receptors, which regulate clock gene expression and metabolic rhythms in these cells. In order to test whether melatonin alone induces the increase in astrocytic growth, primary astrocytes were administered 10mM melatonin every day at ZT12 for six days. DAPI staining showed an approximately four-fold increase in cell number in melatonin-treated cells compared to vehicle-treated controls by the final day of treatment. These data suggest that melatonin increases mitotic growth of cultured astrocytes, and that the avian pineal gland may be involved in aspects of astrocyte development. This research was supported by NIH P01 NS35846.

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Daytime Light Exposure Advances Melatonin Onset in Humans

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Epidemiological studies demonstrated that earlier timing of sleep correlated with more time spent outdoors (Roenneberg et. al., 2003). Here, we tested whether outdoor light intensities on cloudy days in November in the Netherlands (sunrise 7:48 a.m, sunset 4:46 p.m) are sufficient to shift the circadian pacemaker. Two groups of 15 subjects each (age 21 ± 2) participated in the study for two days. On both days, subjects started with classes from 9:00 AM to 10:30 AM. One group subsequently went and stayed outside until 5:00 PM During the same interval the other group stayed inside in dim light. On the next day the conditions were reversed. On both days, from 5:00 PM. onwards, the subjects stayed in dim light and saliva samples were taken every hour from 6:00 PM. till midnight in the lab and at 3:00 and 6:00 AM at home. Salivary melatonin concentration was determined by radio immuno assay. Melatonin profiles were scaled relative to the maximum amount detected in any of the data points of each person. The time at which the 25% level was reached was taken as dim light melatonin onset (DLMO). Average DLMO was 14 minutes earlier (p=0.03) on the days the subjects had spend outside. These data show that moderate outdoor light intensities are sufficient to advance DLMO, suggesting that early sleep timing on free days could be partially due to outdoor light exposure. In contrast to our expectations, we observed a stronger effect in subjects with earlier melatonin onsets, suggesting higher sensitivity to light for early chronotypes.

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Blue-Enriched Versus White Light for Circadian Phase Delays

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The human circadian system is most sensitive to short wavelength (blue) light. We previously found that bright blue-enriched light produced larger phase advances than bright white light, when sleep/dark was advanced. This study compared the magnitude of phase delays induced by bright blue-enriched light and bright white light, when sleep/dark was delayed. Five 15-minute intermittent light pulses interspersed by 45 minutes of normal room light were delivered on each of two consecutive nights, from 00:45 to 05:00, to coincide with the delay portion of the PRC. Subjects received either blue-enriched light (17,000K, n=10) or white light, (5,095K, n=12). Subjects were exposed to similar luminance from the blue-enriched and white lamps (~3900 vs ~3500 lux), but more total photons (4.6 vs 2.9 x 1015 photons/cm2/sec), and nearly 3 times as many photons in the blue (400-490nm) range (2.0 x 1015 vs 7.4 x 1014 photons/cm2/sec) from the blue-enriched lamps. The dim light melatonin onset (DLMO) and offset (DLMOff) were assessed before and after the light pulses. Mean (± SD) phase delays for the white and blue groups were 3.2 ± 1.3 and 3.2 ± 1.4 hours for the DLMO, and 3.6 ± 1.6 and 1.4 ± 1.5 hours for the DLMOff. Thus, the blue-enriched and white light delayed the DLMO a similar amount, while the white light delayed the DLMOff significantly more. These surprising results will be discussed in terms of putative morning and evening oscillators.

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Phase Response Curve to Single One-Hour Pulses of 10,000 Lux Bright White Light in Humans

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The resetting response of the human circadian pacemaker to white light depends on the phase of exposure and is described by a Phase Response Curve (PRC). The aim of the current study was to test the efficacy of a one-hour exposure to 10,000 lux white light to reset the circadian pacemaker across all circadian phases.

Thirty-six young (18–30 years), healthy male and female subjects were studied for 9–10 days in a time-free environment. Following three baseline days, subjects underwent a 30-52 hour Constant Routine (CR) in <2 lux and, after an eight-hour sleep, were randomized for exposure to one hour of either 10,000 lux white light (4100K) (n = 18) or background light (<2 lux) (n = 18) centered in a 16-hour wake episode, and scheduled at one of 18 circadian phases. Following an eight-hour sleep, subjects began a second CR (33-55 h). Phase shifts were calculated from the difference in plasma (q30-60) or salivary (q60, n = 2) Dim Light Melatonin Onset (DLMO) time between CR1/CR2.

Exposure to a one-hour pulse of 10,000 lux light reset the circadian pacemaker according to a conventional Type 1 PRC with maximal delays and advances of -1.9 and 1.2 h, respectively. Phase delays occurred when the midpoint of light was timed ~6 hours before and after DLMO. There was no discernable PRC in the dim light group. These findings suggest that the duration-response function for circadian phase resetting is non-linear, as the amplitude of the PRC was ~50% of that for a 6.7-h exposure to 10,000 lux (Khalsa et al., J.Physiol. 2003), despite representing only 15% of the duration.

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Beyond Blue Light: Effects of Manipulating Light Intensity and Wavelenght on the Human Circadian System

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Entrainment of the circadian timing system to the 24-hour day is achieved through its daily resetting by light. It is well established that the resetting capacity of light depends on the intensity, timing, duration, pattern and spectral composition of the photic input. Studies in animals have shown that rods, cones and melanopsin-expressing ganglion cells are involved in circadian photoreception. However, the relative contribution of the classical and non-classical photoreceptors is unknown. In order to clarify the responsiveness of the human circadian system to light, we investigated 1) the acute effects of light exposure (spectral sensitivity), 2) the immediate after-effects of light exposure in healthy young males and females, 3) and the effects of combinations of monochromatic lights. The sensitivity of light-induced melatonin suppression was assessed, for each subject, with broadband white light and monochromatic lights of equal photon density (3.16x10e12 photons/cm2/sec) at 9 different wavelengths spread over the visual spectrum (420-620 nm). Blood samples were collected every 15–60 min before, during, and after a 60-min nocturnal light exposure session in subjects with fully dilated pupils. Our results confirm a peak sensitivity of melatonin suppression to wavelengths between 460–500 nm, whereas wavelengths below 460 and above 500 nm are much less effective. From 440–420 nm, we find a steep decrease in the effect of light. The

resumption of melatonin release after light exposure appears to depend on the degree of prior suppression rather than on wavelength alone. Finally, the combination of monochromatic lights gives unexpected results, providing new insight into the mechanisms involved in circadian photoreception. Our results can be of important significance as they could be applied in the treatment of chronobiological and affective disorders.

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Measuring Light as a Stimulus for the Human Circadian System

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Light is the primary exogenous stimulus for circadian regulation and disruption. Nevertheless, light as it affects the circadian system is not defined. Formally, the definition of light is based upon human central (photopic) vision. All commercially available light meters are calibrated in terms of this formal definition. Therefore, the measured and reported quantities of light from the laboratory or in clinical applications can substantially misrepresent the photic stimulus for the human circadian system. Moreover, conventional light meters do not make provision for the temporal characteristics of light exposure (timing and duration), which are fundamental for regulating the circadian system. A prototype head-worn device has been developed and calibrated to provide accurate spectral and temporal representations of the human circadian photic stimulation. The device includes a photopic detector as well as a blue-light detector, both of which are necessary for accurate representation of the circadian photic stimulus. Thus, two channels of time-based photic quantities are available for the duration of a prescribed sampling period. The device also incorporates motion accelerometers to provide continuous profiles of daily activity. The design of the instrument will be described, together with sample circadian light exposure data from people working day or night shifts. We will argue that such data are fundamental for the development of models of circadian regulation and disruption by light as it affects human health.

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Time-of-Day Effects of Caffeine Administration on Salivary Melatonin and Cortisol Levels: Preliminary Results

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Reports on the effects of caffeine on melatonin and cortisol levels are contradictory. Melatonin and cortisol secretions show robust circadian rhythms. Surprisingly, few studies have evaluated if the effects of caffeine on melatonin and cortisol rhythms are modulated by time of day.

Thirty-three moderate caffeine consumers (mean age=38.6y) participated in both a caffeine (200 mg) and a placebo (lactose) conditions in a double-blind crossover design. Twenty-three subjects followed their habitual sleep–wake cycle in the laboratory and received caffeine/placebo three hours (100 mg) and one hour (100 mg) before their habitual bedtime while ten subjects were sleep deprived and received caffeine/placebo two hours (100 mg) and one hour (100 mg) before their habitual waketime. Salivary samples

were collected every 30-min for four consecutive hours starting one hour before caffeine or placebo administration in constant environmental and behavioural conditions.

Compared to cortisol levels in the placebo condition, cortisol concentrations in the last saliva samples were decreased when caffeine was administered in the evening (p=0.005), but were increased when caffeine was administered early in the morning after a night of sleep deprivation (p=0.0003). However, caffeine significantly increased melatonin in the last samples similarly when administered in the evening or at the end of the night (p=0.02).

In this study, time of day modulated cortisol response to caffeine, but not melatonin response. Caffeine increased cortisol levels at the acrophase of its circadian rhythm following a sleep deprivation while it decreased cortisol levels at its nadir. The mechanisms underlying these effects need to be determined.

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Circadian Phase-Shifting Effects of Daily Ramelteon in Healthy Adults

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This study assessed the circadian phase-shifting potential of the chronohypnotic ramelteon, an MT1/MT2-receptor agonist. In a double-blind, placebo-controlled trial, 75 healthy adults (ages 19-44 years) were randomized to receive ramelteon 1mg (n=14), 2mg (n=16), 4mg (n=15), 8mg (n=15), or placebo (n=15). Subjects were confined in a sleep laboratory under low-intensity light for five nights and six days (Day -1, baseline; Days 1-4, treatment; Day 5, final visit). Subjects went to bed five hours earlier (lights-out) than habitual bedtime (starting Day 1). Study drug was given 30 minutes prior to lights-out. Eight hours after lights-out, subjects were awakened, and saliva samples were taken every 60 minutes (±5) for 10 hours. The primary efficacy endpoint was the change from baseline to Day 4 in dim light melatonin secretion offset (DLMoff) time (when melatonin concentration dropped below 3 pg/mL with a negative slope). Standard safety measures were also evaluated. For DLMoff change from baseline, there was a statistically significant mean difference from placebo with ramelteon 1mg (-80.9 min, P=0.002), 2mg (-73.3 min, P=0.003), and 4mg (-83.4 min, P=0.001). Although DLMoff also advanced with 8mg ramelteon (-20.8 min difference from placebo), the difference was not statistically significant. The safety profile of ramelteon 1, 2, 4, and 8mg was similar to that of placebo. During an imposed five-hour advance of the sleep–wake schedule, daily ramelteon administration in healthy adults produced a significantly greater and more rapid circadian phase advance relative to placebo as measured by endogenous melatonin secretion offset time.

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Learning-Related Changes in Daytime Sleep EEG Parameters Depend on the Nature of Word-Pair Associates

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Learning-dependent increases in sleep spindle density have been reported during nocturnal sleep immediately following the learning session. Here, we investigated experience-dependent changes in daytime sleep EEG activity after declarative learning of word-pairs differing in encoding difficulty.

Thirteen male volunteers (21–28 years) spent 3x24hours in the chronobiology facility under constant routine conditions in bed. Around midday, subjects carried out one of two word-pair learning tasks or a matched non-learning task in counterbalanced order. The learning lists differed in concreteness level of the words, resulting in an easy and a difficult encoding condition according to recall performance. After immediate cued recall, subjects were allowed to sleep for four hours. Delayed cued recall was tested 30 min after awakening. Polysomnographical data were subjected to spectral analysis and a sleep spindle detection algorithm.

All volunteers performed better in the easy than in the difficult encoding condition ($p<0.05$). Performance remained stable between recalls in both conditions ($p>0.1$). Compared to the control condition, sleep EEG activity in the low sigma range (11.5-13.25 Hz) was increased after difficult encoding, particularly left frontal ($p<0.05$). Furthermore, the low frequency sleep spindle incidence was enhanced in fronto-central areas ($p<0.05$). After the easy encoding no such modification in the low sigma range was observed.

Our results suggest that changes in daytime sleep EEG oscillations after declarative word-pair learning depend upon the nature of the word-pairs. Low frequency frontal sleep spindles are predominantly increased after learning supporting the hypothesis of an implication of fronto-central located spindles in memory processing.

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Daily Wrist Activity Rhythms: Sex, Symmetry and Handedness

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Actiwatch monitors were worn on both wrists simultaneously by 106 college students for three days. Similar numbers of male, female, left-handed (LH) and right-handed (RH) subjects were recruited. Mean activity levels, peak time of activity and ratio of activity in the light vs. the dark phase of the local photoperiod was measured. RH subjects used their right wrist more than their left ($p< 0.001$), but LH subjects used both equally, and there was no effect of sex or handedness on mean activity. The dominant wrist's activity peaked slightly earlier than the non-dominant wrist ($p<0.04$). Male wrist activity peaked later than female ($p<0.006$), and males were significantly less diurnal ($p<0.01$). These sex differences, however, were dependent on handedness (sex/handedness interactions: $p<0.01$) such that LH males peaked earlier and were more diurnal than RH males, and LH females peaked later and were less diurnal than RH females. These results demonstrate a high degree of symmetry between left and right wrist activity rhythms,

subtle asymmetries in mean level of activity and phase of activity, and sex differences in acrophase that interact with handedness. The latter result suggests that brain differentiation between sexes that generates phase differences in daily rhythms interacts with brain differentiation associated with laterality.

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Circadian Rhythms in Phonological and Visuospatial Storage Components of Working Memory

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Working memory is a basic cognitive process that temporarily maintains the information necessary for the performance of many complex tasks such as reading comprehension, learning, and reasoning. Working memory includes two storage components: phonological and visuospatial, and a central executive control. The objective of this study was to identify possible circadian rhythms in phonological and visuospatial storage components of working memory using a constant routine protocol. Participants were eight female undergraduate students, ages17.5±0.93 years, range=16–19 years. They were recorded in the laboratory in a constant routine protocol during 30–hours. Rectal temperature was recorded every minute; subjective sleepiness and tiredness, as well as phonological and visuospatial working memory tasks were assessed each hour. There were circadian variations in correct responses in phonological (Friedman ANOVA x2= 50.39, $p<0.001$) and visuospatial (Friedman ANOVA x2= 36.88, $p<0.05$) working memory tasks. Cross correlation analysis showed one to two-hour phase delay of working memory tasks with respect to rectal temperature. There were circadian variations in both storage components of working memory, which may be due to circadian variations in the central executive, mechanism responsible for the control of both storage components of working memory. This result may explain the changes in the performance of many complex tasks during the day.

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Adaptation to the 24.65-hour Martian Day Alters the Period of the Human Circadian Pacemaker

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Human expeditions to Mars will require adaptation to the 24.65-hourMartian solar day-night cycle (sol), which is outside the range of entrainment of the human circadian pacemaker under lighting intensities to which astronauts are typically subjected. Lack of entrainment at a normal phase angle leads to disturbed sleep and decrements in wake-time performance. Here, we report that a simple lighting scheme with moderately bright light (~450 lux) that does not impinge upon the nightly sleep episode is more than sufficient to entrain individuals to the Martian sol and to the 23.5-hour average day length required of astronauts during many space shuttle missions. Moreover, given the observation that the genetically-determined period of the circadian pacemaker is affected by prior entrainment in animals, we also tested whether the circadian period of the human circadian pacemaker would change following entrainment to non-24-hour light–dark cycles. We found that the intrinsic period of the human circadian pacemaker

significantly lengthened following two weeks of entrainment to the Martian (24.65-hour) as compared to the shuttle (23.5-hour) day–night cycles, revealing its plasticity. Both the immediate entrainment and the long-term adjustment of circadian period could have important implications for human space exploration and for treatment of circadian rhythm sleep disorders.

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Short Sleep Durations Reduce Phase Shifts to Light

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Short sleep episodes in humans are increasingly common and produce long perceived photoperiods and sleep deprivation. Long photoperiods and sleep deprivation have previously been shown to reduce phase shifts to light in hamsters and mice. We examined the effect of long and short perceived photoperiods (short and long nights), on phase shifts to light in humans. Young healthy subjects slept in dark bedrooms for two weeks of short six-hour nights and two weeks of long nine-hour nights, counterbalanced. There was a circadian phase assessment after each series of nights. After three more days of short or long nights there was a three-day advancing bright light stimulus (n=8) or a two-day delaying bright light stimulus (n=7) and then a final phase assessment. The bright light stimuli consisted of morning or evening intermittent bright light and a gradually advancing or delaying sleep/dark episode. The phase advance and phase delay in the dim light melatonin onset in the short nights were only 48% and 54% of those observed in the long nights. Similarly, the phase advance and phase delay in the dim light melatonin offset in the short nights were only 45% and 71% of those observed in the long nights. These results show for the first time that humans may unwittingly reduce their circadian responsiveness to light when they truncate their sleep. Possible mechanisms include sleep deprivation, the photoperiodic history, and a reduction in photosensitivity during the short nights due to the increased daily duration of ambient light.

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Sexual Cohabitation and Sexual Activity Effects on Cognitive Sleep Reward

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Comparisons of spatial test repetitions from evening sessions to a morning repeat were used to assess the cognitive training associated with sleep. The performance profile in a given test was the currency for training. This paradigm allows one to examine whether sexual sleep environment can alter sleep structure and hence training. In this study, young unmarried couples (mean age: 25) with no children and no history of sleep disturbances were used. All subjects completed the Pittsburgh Sleep Quality Index (PSQI), a standardized sleep anamnesis inventory and the MEQ- questionnaire to evaluate morning-or evening-chronotypes. Sleep–wake patterns were monitored by wrist-worn actigraphs, and all subjects wrote a sleep diary including a standardized self-rating scale for subjective sleep and awakening quality every morning. During the investigation subjects spent at least 10 nights together and 10 separately. Sexual activity was recorded in the sleep log. Five unique spatial test repeats using cube images were done before and one

hour after bedtime. The test repeats were grouped for nights without a partner, nights with a partner and no sexual contact, and nights with sexual contact. The results of both the activity-rest cycles and the cognitive studies were quite surprising in that they demonstrated differences among the three different sleep environments. The differences in the effects of sleep environment on circadian parameters were not the same in males and females. Similarly the effects of sleep environment on cognitive training also show environment and sex dependent patterns in the couples.

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Sleep Response to 24-Hour Total Sleep Deprivation in Young and Old Rats

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It is not clear whether age-related sleep changes are primarily due to alterations in circadian or homeostatic processes. In this study, we examined baseline (BL) sleep as well as the response to sleep deprivation (SD) applied at different times of day in young and old rats.

Young (3 months) and old (20 months) male Fischer (F344) rats underwent a 24-hour (12:12 L:D) BL, 24-hr total SD (slowly rotating wheel), and a 24-hr recovery opportunity beginning either at light onset (young, n=6 and old, n=8) or dark onset (young, n=6 and old, n=8).

In the light onset group, young and old rats had similar amounts of BL wakefulness (58.6 ± 1.5 vs. 56.6 ± 1.2 %), NREM (33.9 ± 1.4 vs. 36.5 ± 0.8 %) and REM sleep (7.5 ± 0.2 vs. 6.9 ± 0.5 %). In response to SD, young and old rats had similar increases in NREM (+32.6 ± 3.5 % vs. +32.7 ± 3.3 %), REM (+59.2 ± 7.1 vs. 64.3 ± 8.6%), and EEG NREM delta power over respective BL levels. With respect to diurnal sleep organization, old rats exhibited less absolute sleep time in the light phase and more sleep in the dark phase compared to young animals during both BL and recovery days. The same results were found for young and old animals in the dark onset group.

Based on these data, we propose that age-related sleep changes in old male F344 rats reflect alterations in circadian regulation rather than homeostatic mechanisms.

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Daytime Recovery Sleep Is More Sensitive to the Effects of Caffeine than Nocturnal Sleep

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Caffeine is often used to fight sleepiness generated by sleep deprivation, jet lag, and shift-work. Consequently, caffeine is consumed at different times of day and night. However, we know little about how homeostatic and circadian processes influence the effects of caffeine on sleep.

Thirty-four moderate caffeine consumers (mean age=38.6y) participated in both a caffeine (200 mg) and a placebo (lactose) condition in a double-blind crossover design. Seventeen subjects slept in the laboratory during the night (Nocturnal) while 17 subjects (sex and age-matched) were sleep deprived for one night (25 hours of wakefulness) and recovery sleep started in the morning (DayRec). All subjects received a capsule of 100 mg of caffeine (or placebo) three hours before bedtime and the remaining dose, one hour before bedtime.

Caffeine lengthened sleep latency, and reduced stage 2 ($p<0.001$) and SWS (P 0.05) similarly in the Nocturnal and DayRec groups. Significant interactions between conditions (Caffeine, Placebo) and time of sleep (Nocturnal, DayRec) were found for total sleep time (P 0.04), sleep efficiency ($p<0.001$) and time spent in REM sleep (P 0.02). Compared to placebo, caffeine reduced sleep efficiency, decreased total sleep time and REM sleep more strongly in the DayRec group than in the Nocturnal group.

The effects of caffeine on sleep are more prominent when caffeine is consumed before daytime recovery sleep than before nocturnal sleep. Caffeine showed stronger effects on sleep at an abnormal circadian phase despite the sleep deprivation. These results have implications for individuals using caffeine during nightime to prevent the effects of sleep deprivation.

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Physiological and Behavioral Correlates of a PER3 Polymorphism in Humans

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Diurnal preference as assessed by the Horne-Östberg scale is associated with a variable number tandem repeat (VNTR) polymorphism in the PER3 gene. The objective of this investigation was to characterise the physiological and behavioural consequences of this polymorphism. Healthy volunteers were selected on the basis of their genotype, independent of diurnal preference. More than 250 volunteers (age 20-35) were genotyped for the PER3 polymorphism. Individuals homozygous for the rarer 5-repeat VNTR (PER3-5/5) were identified and matched for age, gender, ethnicity and body mass index with individuals homozygous for the 4-repeat VNTR (PER3-4/4).

These subjects (n=28) wore actigraphs and completed daily sleep diaries for three weeks prior to a five-day laboratory study. This was completed by 24 subjects and consisted of two baseline sleep episodes followed by an approximately 40-hours constant routine (CR) and a recovery sleep episode.

Preliminary analyses indicate that the two groups did not differ with respect to either their Horne-Östberg score or habitual sleep-wake timing and sleep duration as assessed by actigraphy and sleep diaries ($P>0.05$ in all cases). Preliminary analyses of the daily pattern of wrist activity during the pre-laboratory segment of the study indicated that the rhythm of rest and activity was advanced in the PER3-5/5 individuals by approximately 1.5 hours. Analyses of core body temperature (CBT) data collected during the CR in five PER3-5/5 and 11 PER3-4/4 subjects suggested differences in both the mean and amplitude of the CBT rhythm. These preliminary results support the hypothesis that the PER3 polymorphism modulates circadian physiology and behaviour in humans.

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Phase Relationship between Melatonin and Sleep in Delayed Sleep Phase

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Previous studies have shown alterations in the phase relationship between melatonin and sleep in patients with Delayed Sleep Phase (DSP). The aim of this study was to determine the timing of melatonin rhythm and phase relationships between melatonin and sleep in patients with DSP versus controls.Nine DSP and four age-and gender-matched controls completed a four-day inpatient study. DSP was diagnosed using ICSD criteria. Subjects kept habitual sleep–wake schedules for 3-5 weeks prior to the study. Average sleep and wake times during this period served as sleep and wake times during the study. Plasma samples were collected q30 or 60 for 24 hours under dim-light conditions (<10 lux) and later assayed for melatonin. Melatonin levels were adjusted to a percentage of maximum (average 3 highest values). Compared to controls, DSP patients showed a significant delay in timing of sleep–wake and melatonin rhythm relative to clock time; 50% onset (3.72h). The phase relationship between sleep and melatonin rhythm did not significantly differ between DSP and controls, respectively; sleep onset to 50% onset (-0.9h vs. -1.6h), sleep onset to 50% offset (7.23h vs. 6.96h), 50% onset to wake (9.07h vs. 9.61h), 50% offset to wake (0.77h vs. 1.04h).

The delay in the timing of sleep–wake cycle and circadian melatonin rhythm relative to clock time in DSP confirm previous findings. However, the phase relationship between melatonin and sleep was similar between DSP and controls. These results do not support the hypothesis for internal desychronization of circadian rhythms in DSP.

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Sleep Deprivation Down Regulates ERK I/II Hosphorylation within the Suprachiasmatic Nuclei of the Syrian Hamster

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Non-photic zeitgebers, such as activity and arousal, can reset the circadian clock. These non-photic manipulations decrease the expression of Period and immediate early genes (IEG) in the suprachiasmatic nucleus. The mechanism by which non-photic events lead to such decreases in gene expression is not fully understood. The extracellular signal-regulated kinases I/II (ERK) pathway is linked to regulation of Period and IEG expression. In this study, we evaluate the relationship between behavioral shifts induced by sleep deprivation and phosphorylation of ERK (P-ERK). Hamsters (n=15) were housed in running wheel-equipped cages in a 14:10 LD cycle. On the test day, hamsters were put into dim red light (~1 lux) at zeitgeber time (ZT) 6, and were sleep deprived by gentle handling for 3 hours. Animals were left in DD for three days to assess behavioral phase shifts. Large shifts (>2h) were observed in 8/10 hamsters

("responders"), while 2/10 exhibited small phase shifts within 1 SD of the mean response to DD control ("non-responders"), similar to what has been previously reported. Animals were subjected to a second sleep deprivation, and were perfused at ZT9, and their brains were stained for P-ERK. P-ERK levels were quantified using ROD measurements. Animals that exhibited behavior phase shifts had significantly decreased P-ERK levels compared to DD control hamsters, whereas animals that did not phase shift to sleep deprivation showed no such decrease. These results suggest that sleep deprivation inactivates the ERK signaling pathway only in those animals that phase shift to this non-photic manipulation. This research was supported by NSERC.

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The Differential Effects of Age and Task Type on Performance Recovery from Sleep Deprivation

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While evidence suggests that performance deficits associated with sleep deprivation (SD) subside after one night of sleep in the young, the elderly response remains undefined. Age-related differences in response to SD and recovery sleep were examined using tasks of sustained attention and pre-potent response inhibition to target distinct neurobehavioral networks affected by SD.

Young (25.6±3.7, n=8) and elderly (67.8±5.7, n=9) subjects had a baseline night of 9 hours time in bed (TIB) followed by a 38-hour SD period and 10-hour TIB recovery sleep. Performance was assessed using psychomotor vigilance (PVT) and go/no-go tasks at 8:30, 10:30, 12:30, and 14:30 for baseline, SD, and recovery conditions. Performance was averaged for each condition. PVT lapses were defined as reaction time>500ms.

rmANOVA of PVT lapses indicated significant age ($p<0.001$), condition ($p<0.01$), and age×condition effects ($p=0.05$). Posthoc analysis demonstrated more lapses for both groups in SD versus baseline and recovery conditions. The difference between SD and baseline lapses was larger in the elderly ($p<0.001$). rmANOVA of go/no-go task errors of commission indicated significant condition ($p<0.001$) and age×condition effects ($p<0.05$). Posthoc analyses demonstrated more errors in SD for both groups ($p<0.001$). The elderly did not improve after recovery ($p<0.05$).

These data suggest that while the elderly have more lapses in SD, 10hr TIB is sufficient for both groups to recover from impairments of sustained attention. However, aging may be associated with slower return of inhibitory control. These results indicate that the extent of performance recovery following SD is dependent on age and the type of neurobehavioral networks.

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Sleep–Wake Patterns of Octodon degus

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Octodon degus are diurnal, long-lived, rodents frequently used in circadian research. In contrast to many field and laboratory studies, previous data on sleep in degus suggested they are not diurnal. The aim of

this study was to systematically characterize sleep–wake behavior of these animals in a laboratory setting more typical of temperature and housing conditions in the field. Males (n=3) were individually housed without running wheels in a 12:12 Light:Dark cycle and temperature of 18°C. For 24 hours, we recorded EEG, brain temperature with a thermistor, general activity patterns with infrared devices, and videotaped behavior. Infrared LEDs provided illumination for videotaping in the dark. Sleep–wake brain activity was analyzed in 12 s epochs based on criteria used in the rat. Non-Rapid Eye Movement Sleep (NREMS) was characterized by prominent delta wave activity and locomotor inactivity, while Rapid Eye Movement Sleep (REMS) was categorized by increased theta wave activity, inactivity, and increased brain temperature.

Degus spent 29.75 ± 3.22 % recording time in NREMS during the 12 hours light period, and 39.77 ± 4.21% during the dark period. REMS also occurred more during the dark period (4.99 ± 0.98% vs. 3.17 ± 0.74%). Videotape analysis confirmed more behavioral sleep occurred during the dark. These pilot data of EEG and behavioral activity suggest that degus in cooler laboratory conditions without access to running wheels sleep more during the dark period, similar to other diurnal species. Future work will increase samples sizes and examine the neurobiological mechanisms underlying sleep of these animals.

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Forced Desynchronization of REM and nonREM Sleep in the Rat

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The sleep–wake cycle is among the behavioral and physiological circadian rhythms governed by the master circadian oscillator located in the hypothalamic suprachiasmatic nucleus (SCN). We have recently developed a forced desynchronized rat model in which animals exposed to artificially short light–dark (LD) cycles exhibit independent locomotor activity rhythms with different period within the same individual. These rhythms are respectively associated with the activity of the ventrolateral (VL) and dorsomedial (DM) SCN. This functional division between these SCN subregions raises the possibility that different rhythmic modalities may rely either on the VL- or the DM-SCN. We explored this possibility by performing electroencephalographic recordings in forced desynchronized rats to determine the association between the sleep-wake cycle and the independent activities of the VL- and DM-SCN. Whereas the occurrence of sleep and waking appeared to be associated indistinctly with the activity of either the VL- or the DM-SCN, the occurrence of NREM sleep was predominantly associated with the activity of the VL-SCN. REM sleep, on the other hand, was not associated with LD cycle and was associated predominantly with the activity of the DM-SCN. Our results suggest that the SCN has not only a role in the temporal gating of sleep and waking, but also in the circadian gating of specific sleep stages.

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Association of Melatonin Phase and Recovery Sleep Following Prolonged Sleep Restriction in Adolescents

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We examined whether circadian phase preference or phase angle would influence recovery sleep following chronic sleep restriction.

Twelve healthy adolescents (ages 11–15, mean=13, SD=1.3 years; 4 male) reporting extreme (>2 SD) morning (n=7) or evening (n= 5) phase preference kept a fixed sleep schedule (average about 9.25h) for 2.5 weeks at home ("nominal") followed by a symmetrically restricted sleep schedule (about 70% of nominal) for one week, confirmed by actigraphy and diary. Participants were in the lab after the seventh restricted night for melatonin sampling. Saliva was collected in dim light (< 15 lux), and melatonin onset (DLMO) phase was determined using linear interpolation with a 4 pg/mL threshold. Sleep was then recorded ad lib beginning at the scheduled restricted bedtime. Phase angle was computed as the time interval between DLMO phase and wake-up time on the restricted night. Sleep stages were scored visually in 30-sec epochs using Rechtschaffen & Kales criteria (1968).

No major sleep variable was associated with morning or evening phase preference (t-test). One major sleep variable, minutes of wakefulness after sleep offset, was significantly correlated with phase angle (r=.62, p=.003).

This association indicates that adolescents whose sleep schedule is later relative to their circadian timing system (wider phase angle) are less able to extend sleep even after sleep restriction. Additional cases may provide more explanatory power to these analyses.

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Neonatal Alcohol Exposure Alters Anatomical Components of the Photoentrainment Pathway in Adult Rats

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Neonatal alcohol (EtOH) exposure produces long-term changes in the phase-shifting and entrainment responses of the rat activity rhythm to photic cues. Because the retinohypothalamic tract (RHT) mediates photoentrainment of the circadian clock in the suprachiasmatic nucleus (SCN) and RHT fibers terminate on SCN neurons containing vasoactive intestinal polypeptide (VIP), we separately examined whether neonatal alcohol exposure affects the distribution of RHT fibers and density of VIP neurons in the SCN of adult rats that were gastrostomized and exposed to EtOH (3.0, 4.5 or 6.0g/kg/day) or isocaloric milk formula on postnatal days 4-9. Anterograde tract tracing analysis of RHT fibers innervating the SCN was conducted by intravitreal injection of Alexa Fluor 594-conjugated cholera toxin subunit-B (CTB). Control and EtOH-treated rats showed similar patterns of CTB labeling in the SCN along most of its rostrocaudal axis, although EtOH-related differences in the pattern of RHT innervation were evident in the rostral SCN where the density of CTB-labeled fibers was 2.5-fold greater in EtOH-treated rats relative to controls. The density of VIP-immunoreactive neurons in the SCN was analyzed for evidence of EtOH-induced cell loss using stereological methods. In animals treated with 6.0g/kg/day, the density of VIP neurons in the SCN was reduced by ?35% relative to both control groups. These findings suggest that neonatal EtOH exposure may alter the photic regulation of adult circadian rhythms by interfering with the development of the RHT and/or VIP neurons in the SCN. This research was supported by NIAAA AA13242.

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Effects of Experimental Chronic Jet-Lag on Oxaliplatin Toxicity in Mice

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Both toxicity and efficacy of oxaliplatin were improved with dosing this drug near mid-activity in mice and in synchronized cancer patients. Disrupted circadian coordination accelerates malignant growth in patients and in mice subjected to chronic jet-lag (CJL). We studied CJL effects on clock and pharmacology genes that determine oxaliplatin toxicity in the small intestine, a main target for this drug.

Mice (n=120) were kept in LD 12:12 or submitted to CJL by an 8-h advance of the LD cycle every 2 days for 10 days prior to receiving oxaliplatin (17 mg/kg ip) at one of six circadian times staggered by 4 h. Three weeks later circadian expression of clock genes per2, reverb and bmal1 and ERCC1, GSTP1 and XPA was determined with quantitative PCR in small intestine.

The telemetry-assessed 24-h rest-activity and temperature rhythms were damped or ablated in CJL mice. Oxaliplatin-induced body weight loss was 2.6±0.7 % at ZT15 and 5.3±0.7 % at ZT3 in LD12:12 ($p = 0.009$) and 4.6 ±0.6 % and 8.2 ±0.7 % respectively in CJL ($p = 0.001$).

Per2, Rev-erb? and Bmal1 displayed significant rhythms similar to those known in liver in LD12:12. CJL suppressed clock gene rhythms. ERCC1, GSTP1 and XPA displayed no significant rhythm despite 28, 23 or 16% respective higher expressions during darkness, when oxaliplatin was least toxic. CJL both reduced ERRC1 and XPA over 24 h ($p = 0.008$ and 0.017, respectively) and enhanced oxaliplatin toxicity ($p < 0.001$).

Conclusions: The profound alteration of the molecular clock with CJL revealed its regulatory role on two key molecular determinants of oxaliplatin cytotoxicity.

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Dietary Fatty Acids Induce BMAL1 Expression in Mice Adipose Tissue but not in Liver

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BMAL1 is a transcription factor and plays essential role in the regulation of circadian rhythm. In addition to crucial roles in circadian rhythm regulation, we recently reported that BMAL1 is predominantly expressed in mice mature adipocytes (Aoyagi et al., JHS 2005) and plays role in the regulation of adipogenesis (Shimba et al., PNAS 2005). We show here that the challenge to high fat diet results in significant induction of Bmal1 mRNA in mice adipose tissue. Interestingly, the induction of Bmal1 mRNA by high fat diet is observed only in adipose tissue, but not in other tissues tested. This adipose tissue specific induction of Bmal1 expression accompanied with enhanced expression of its targets genes such as Per and PAI-1. To determine whether dietary fats have a direct impact on Bmal1 expression, cultured adipocytes were treated with various saturated, unsaturated, and trans fatty acids and examined levels of Bmal1 mRNA. One of fatty acids examined was significantly increased Bmal1 mRNA level in cultured adipocytes but not in primary hepatocytes. Inhibition of fatty acid metabolism diminished the effect on Bmal1 expression. Tentative metabolites of the fatty acid increased Bmal1 mRNA level.

The studies with BMAL1 conditional knockout mice (C57 background) and conditional transgenic mice will be discussed.

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Circadian Rhythmicity in a Mouse Model of Alzheimer's Disease

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Alzheimer's Disease (AD) is associated with profound sleep disturbances characterized by fewer restful periods and shorter sleep durations. Disruption in circadian rhythmicity is also observed, with patients exhibiting increased daytime napping, disrupted nighttime sleep, and sundowning. These changes are characteristically different from sleep and circadian disruptions observed with normal aging. The present study examines various circadian phenomena in a mouse model of AD (3xTg), compared to age-matched controls. Preliminary results indicate that 3xTg mice exhibit significantly shorter active phases, as well as significantly shorter freerunning periods, when compared with age-matched controls. Our results also indicate that AD mice had a trend towards larger phase advances and delays to light pulses at CT16 and CT23, respectively. Vasopressin-immunoreactive and vasoactive intestinal peptide-immunoreactive cells, two important components of the mammalian circadian clock, are greatly reduced in the suprachiasmatic nucleus of people with AD. Histological examination revealed that 3xTg mice exhibit a significant decline in the number of cells that contained vasoactive intestinal peptide. However, the number of vasopressin-containing cells was not significantly different from that observed in age-matched controls. An irregular sleep pattern in those with AD is a major cause of institutionalization. Thus, examining the neurological changes associated with AD, specifically with regards to sleep and circadian rhythmicity, will increase our understanding of the underlying mechanisms responsible for AD and its pathology. This research was supported by NSERC (MCA+RHD) and the Scottish Rite Charitable Foundation (RHD)

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Circadian Rest-Activity Cycles in Anteepartum Depression During Light Therapy

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Affective disorder during pregnancy is a difficult situation that requires careful judgment for treating the depression without harm to the fetus. Preliminary trials of light therapy have shown promising results, prompting a randomized controlled trial with larger sample size. Pregnant women who fulfilled DSM-IV criteria for major depressive disorder without seasonal pattern were randomly assigned either to 7000 lux fluorescent bright white light therapy or 70 lux dim red light as a placebo control, administered in the morning upon awakening for 1h/day in a five-week trial. Clinical state was monitored weekly by a validated German version of the 25-item SIGH-ADS interview with a rater blind to conditions. The circadian rest-activity cycle was monitored by an actigraph/lux meter device worn throughout the five weeks. Ten patients

have completed the trial and eight completed actigraphic recordings throughout. SIGH-ADS scores declined significantly from 26.7 ± 4.1 (mean ± SD) at baseline to 16.6 ± 13.2 (p=0.03) after 5 weeks (blind data, placebo dim and bright light combined; 5 full remission, 1 responder). Average activity per hour showed highly interindividual differences in activity levels and distribution. Phase was in stable alignment with time of day (activity onset 7-8h, activity offset more variable 23-2h). Preliminary analyses of relative amplitude, interdaily stability and intradaily variability showed similar estimates before and after treatment, suggesting that clinical improvement was not mirrored in changes in the circadian rest-activity cycle.

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Circadian Clocks Regulate Mammalian Energy Homeostasis

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Mammalian circadian clocks are widely distributed in peripheral tissues and regulate a substantial number of genes in peripheral tissues. The phase of peripheral clocks, in contrast to that of SCN clocks, is determined by feeding time, suggesting that they may play a role in food processing and metabolism. At the molecular level, circadian clocks consist of a network of transcription factors: at the core of this mechanism, the heterodimer of CLOCK and BMAL activates transcription of the period (Per1, Per2 and Per3) and cryptochrome (Cry1 and Cry2) genes and the dimeric complex of their gene products PER and CRY inhibits CLOCK:BMAL-driven transcription. Recent reports have shown that that mice with a hypomorphic Clock allele are hyperphagic and obese and that both these Clock mutant mice and mice lacking expression of Bmal1, the partner of Clock, have altered glucose homeostasis. Another report found different results: Clock mutant mice bred to the ICR genetic background showed attenuated obesity induced by high fat feeding and reduced absorption of dietary fat. We have observed metabolic inefficiency upon loss of circadian clocks by two unrelated genetic disruptions that each abolish circadian rhythmicity: We have examined bmal-/- mice in three genetic backgrounds and Per1-/-;Per2-/- mice in a pure 129S1/SvImJ genetic background.

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Timing of Rest-Activity, Light Exposure and Melatonin in Patients with Schizphrenia and Unemployment Subjects

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Schizophrenia is a severe, chronic and often life-threatening condition. Although disturbed sleep is a common symptom in schizophrenia, it is often dismissed on the basis that these patients stay up late because they can't hold down a job. In a small number of previous studies abnormalities in sleep EEG, sleep onset and maintenance have been reported in patients regardless of either their medication status or the phase of the clinical course. In our ongoing study, we are investigating the extent to which sleep abnormalities are a genuine problem in schizophrenia and whether abnormalities of the circadian system may contribute to the abnormal pattern of sleep often reported in this patient group.

Rest-activity from schizophrenia patients with self-reported sleep complaints were compared to unemployed subjects without self-reported sleep complaints using six-week continuous actigraphy. Light exposure and profiles of six-sulphatoxymelatonin (metabolite of melatonin in urine) were obtained simultaneously from all subjects. The results from actigraphy confirmed sleep abnormalities in all schizophrenia patients. Compared with the unemployed subjects, schizophrenia patients exhibited impaired sleep latency, significantly longer sleep periods, later sleep offsets and the tendency for a delayed melatonin rhythm. The timing of light exposure was closely associated with the phase of activity rather than the natural light dark cycle in both groups. In schizophrenia patients, but not in the unemployed subjects, the melatonin peak positively correlated with a later peak in light exposure. These preliminary results suggest that misaligned circadian sleep-wake phases are involved in self-reported sleep disturbances of schizophrenia patients. Light therapy might provide a helpful adjunct to the treatment of schizophrenia patients with sleep disturbances.

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Molecular Chronopharmacology of Cyclin-Dependent Kinase Inhibitor Seliciclib(R-Roscovitine) on Mouse Liver

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Seliciclib can interfere with both the cell cycle and the circadian clock. The effect of seliciclib dosing time on drug tolerability and on clock genes expression patterns in liver was investigated in mice.

Seliciclib (300 mg/kg/day) was administered for 5 days at one of 6 circadian times staggered by 4 h to a total of 261 mice. Toxicity was assessed with body weight loss, survival and serum transaminase levels (ASAT and ALAT) as markers of liver toxicity. The circadian expression of clock (Rev-erb?, Per2 and Bmal1) and cell cycle genes (c-Myc and Wee1) was determined with quantitative PCR following seliciclib at ZT3, 11 or 19.

Least mortality occurred in mice treated at ZT3 as compared to ZT15 or ZT11 ($p=0.006$). Compared to controls, seliciclib increased mean ASAT (122 vs 75 ui/l, $p=0.001$) and ALAT (194 vs 129 ui/l, $p=0.005$) with least increase at ZT11, a time corresponding to least body weight loss.

Rev-erbα rhythm persisted following seliciclib dosing at ZT3 or ZT11, but it was ablated at ZT19. The circadian patterns in other clock and cell cycle genes were markedly altered or suppressed irrespective of seliciclib dosing time. The drug nearly doubled the expression of c-Myc (ANOVA, $p=0.001$) and nearly halved that of Wee1 (ANOVA, $p<0.001$), suggesting a possible triggering of cell cycling in liver cells.

In conclusion, seliciclib altered the molecular clock in liver in a dosing time dependent fashion that might contribute to the chronotoxicity of this drug.

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The Effect of Alcohol on Jet Lag Recovery in the Diurnal Rodent, Octodon degus

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Circadian rhythms are an important aspect of mammalian biology, mediating daily fluctuations in body temperature, activity and other bodily functions. The onset of a typical circadian rhythm is mediated by environmental factors including light:dark cycle, social interaction, some food cycles, temperature, and other factors. When a radical change in timing of environmental stimuli occurs, circadian rhythms become desynchronized. In humans, travel across time zones causes the phenomenon of "jet lag". Influences on jet lag recovery (known as phase shift reentrainment) have been studied at length and include the use of olfactory cues, exercise, light, and pharmacological treatments. Alcohol has been shown to have a significant impact on circadian rhythms by affecting body temperature and disrupting normal expression of the per gene. While alcohol's interaction with body temperature has been widely studied, the effect that consumption has on reentrainment remains unclear. In recent studies, an excellent animal model for circadian research has emerged, the *Octodon degus*. Unlike most rodents, the degu is diurnal and shows many circadian rhythm similarities to humans. Circadian rhythm research has shown the degu to be an effective and responsive model for the study of reentrainment. In this study, degus were used to examine alcohol's influence on reentrainment after a six-hour phase advance of the light:dark cycle. Data representing the length of time to reentrain following the phase shift in the presence and absence of alcohol ingestion will be presented. Circadian rhythms will be represented through changes in body temperature and activity rhythms as a result of alcohol consumption.

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An Endogenous Circadian Rhythm in an Index Of Cardiac Vulnerability Confirmed with a Constant Routine Protocol

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There is a peak in adverse cardiac events around 10AM. In humans there is a fractal structure in heartbeat fluctuations that changes with cardiovascular pathology. Using a forced desynchrony protocol we recently found that the circadian pacemaker affects the fractal structure of cardiac control. Specifically, the scaling exponent characterizing fractal structures of heartbeat fluctuations increased—bringing it closer to that observed in cardiovascular disease—at the circadian phase corresponding to ~10AM (Hu et al, PNAS, 2004,101:18223-7). Here we test the robustness of this circadian rhythm in heartbeat fluctuations using a different circadian protocol.

Nine healthy adult subjects were studied throughout a constant routine' protocol (38 h wakefulness, with constant posture, room temperature and dim light, and identical snacks every 2 h). Detrended fluctuation analysis of ECG R-R interval data was used for assessment of fractal structures of heartbeat fluctuations in 1 h bins. The resultant scaling exponent data were aligned according to circadian phase (from core body temperature), normalized to account for differences between subjects (z-score), and circadian rhythmicity was assessed (Cosinor analysis).

The scaling exponent characterizing fractal structures of heartbeat fluctuations exhibited a significant circadian rhythm in 8/9 subjects, with peak to trough amplitude between 10-20% of the mean. In the group average, the greatest increase in scaling exponent occurred across the circadian phase corresponding

to 7:00–11:00 AM, peaking soon after that circadian time. There was no significant influence of acute sleep deprivation on the group average scaling exponent.

These results confirm the significant endogenous circadian rhythm in heartbeat dynamics independent from scheduled behaviors, with a similar phase and amplitude as we previously found in a forced desynchrony study. The scaling exponent increased around the circadian phase coinciding with the period of highest cardiac vulnerability determined from epidemiological studies. We speculate that endogenous circadian-mediated influences on cardiac control may be involved in the day-night pattern of adverse cardiac events in vulnerable individuals.

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Acute Systemic Inflammation Indices Upregulation of Circadian Clock Genes Per2 and Bmal1 in Equine Peripheral Blood

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Peripheral oscillators receive timing signals from the master mammalian pacemaker in the suprachiasmatic nucleus (SCN) and function to adaptively anticipate daily changes that might influence local physiology. In contrast to the findings of synchronized clock gene expression in peripheral blood of other species, our recent results demonstrate a lack of such synchronicity in equine blood cells. However, intravenous administration of 0.1µg/lb lipopolysaccharide (LPS) significantly elevates Per2 and Bmal1 expression ($p<0.05$ and $p<0.01$; respectively) compared to vehicle-administered control animals, with peak values at four hours post-treatment. Hyperthermia was observed in treated animals for duration of 6 hrs and the subsequent 24-hours core body temperature rhythm was also significantly disturbed. In addition to entrainment by the SCN, some studies have suggested a relationship between the innate immune response and peripheral clock gene expression. Our current results support this relationship and suggest that activation of the innate immune system may act as a synchronizing signal for equine clock gene expression in peripheral blood cells. This potential immune feedback regulation of an equine peripheral clock may suggest a role of the circadian system in contributing to innate immune reactions and maintaining homeostasis in a tissue that acts as the first line of defense. Further research will be undertaken to elucidate the roles of specific inflammatory markers in this process and to understand how acute inflammation can affect molecular clock mechanisms in this tissue in the horse.

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Repeated Phase-Advances of the Light Cycle Increase Mortality in Aged Mice

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Despite the fact that trans-meridian travel and shiftwork is commonplace in our 24/7 society, few controlled studies have addressed the effects of repeated phase shifts on health outcomes. To begin to assess the effect of clock readjustments on morbidity, 27 and 30 month-old c57bl6 mice were housed in stationaryor phase-shifting 12:12 light-dark cycles. Shifting mice were phase-advanced or phase-delayed by 6h once per week for seven consecutive weeks. Lowest survival (47% in three experiments combined)

was observed in the mice undergoing phase advances. Phase delays were tolerated better than advances (68% survival), followed by the unshifted controls (83%). Potential mechanisms and proximal causes for the shift-related mortality in these aged mice are under investigation.

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Potentiation of Dioxin Responses When the Clock's Not Ticking

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The mammalian circadian clock has been shown to influence other biological processes including development, tumorigenesis and drug metabolism. Because core elements of the clock mechanism, such as PER1, PER2 and BMAL1, share common PAS domains with transcription factors that potentiate,responses to environmental toxins, the present study was conducted to determine whether targeted disruption of specific clock genes affects dioxin-mediated induction of the cytochrome P450, subfamily I, polypeptide 1 (Cyp1A1) gene, a commonly-used biological marker for dioxin response pathways. Real-time PCR methods were used to analyze Cyp1A1 mRNA levels in the mammary glands and liver of wild-type (WT) and mutant mice with targeted disruptions of Per1, Per2 or both Per1 and Per2 that received intraperitoneal injection of either oil or dioxin (10ug/kg.b.w.). Relative to oil-treated controls, dioxin had an inductive effect on tissue-specific expression of Cyp1A1 mRNA in both WT and mutant mice. However, Cyp1A1 induction in the mammary gland and liver was significantly greater in Per1- and double-mutant mice than in WT animals. Dioxin-induced Cyp1A1 expression in the mammary gland and liver of mutant mice was increased by 2- to 8-fold in comparison with the levels observed in WT animals. This observation is noteworthy because elevated Cyp1A1 expression is associated with susceptibility to cancer. The present findings suggest that core elements of the circadian clock may play important roles in the regulation of pathways that mediate toxin responses and carcinogenesis. This research was supported by NIH P01-NS39546(DE).

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Chronobiology of Alcohol: Effects of Chronic and Acute Ethanol Treatments on Circadian Phase-Shifting in the Syrian Hamster

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Chronic ethanol intake is associated with disruptions in sleep and other circadian rhythms in both human alcoholics and experimental animals. Further, recent research in our laboratory has revealed that chronic ethanol intake alters a fundamental property of the circadian pacemaker—its free-running period—in rats. The present experiments were designed to extend these observations by examining the effects of ethanol on photic and non-photic phase shifting in the Syrian hamster, the most popular animal model for studies of circadian phase control. In contrast to typical strains of laboratory rats, hamsters voluntarily consume significant quantities of high-concentration ethanol solutions, even with water freely available. In one experiment, therefore, we examined the phase-shifting effects of brief light pulses and triazolam injections, administered at circadian phases expected to yield maximal phase-advances and phase-delays, in chronic ethanol-exposed and control hamsters. Although we had initially expected opposite effects on photic and non-photic phase shifting, ethanol intake reduced circadian phase-shifting to both stimuli. In a second experiment, we examined the phase-shifting response to acute ethanol and saline control injections.

Ethanol was administered during mid-subjective day, at a time when other GABA-A agonists, including benzodiazepines, yield maximal phase-advances. Surprisingly, despite considerable effort, we were unable to detect reliable ethanol-induced phase shifting across a wide range of doses. These negative results may be related to the hamsters' unusual metabolic processing of ethanol. This research was supported by NIAAA R21 AA013893.

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Mathematical Modeling of Calcium as the Link between Electrophysiology and Molecular Biology in SCN Neurons

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In mammals, circadian (~24-hour) rhythms are generated by a group of ~20,000 pacemaker neurons in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. While there has been much mathematical modeling of the molecular biology of these rhythms and the circadian activity of whole organisms, almost no mathematical modeling has focused on the electrophysiology of pacemaker neurons. These neurons repetitively fire action potentials at ~ 8 Hz during the day and ~ 2.5 Hz during the night. The internal circadian clock can control cytosolic calcium concentrations (Ikeda et al. Neuron 38:253). However, the detailed mechanism linking the internal circadian clock, calcium and the neuronal firing rate is not well-understood. Similar to the Hodgkin-Huxley equations and based on published experimental data (Jackson et al., J Neurosci. 24:7985) we modeled a slowly-inactivating, inward-flowing calcium current and reproduced the experimentally found firing rates of the SCN neurons. Our simulations show that variations in internal calcium concentrations can change SCN neuronal firing rates from ~1 Hz to ~10 Hz or higher. This work suggests a mechanism by which the internal circadian clock within SCN neurons can control the electrophysiological behavior of these cells.

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Dynamics of a Multistage Circadian System

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Tissues throughout the body exhibit circadian rhythms, forming a multi-oscillatory system whose disruption results in jet lag. Our simulations of a multistage circadian system (composed of a pacemaker, an intermediate component, and peripheral oscillators) reveal the flexibility and stability inherent in a multistage system, as well as potential pitfalls. The modeling predicts that jet lag is most severe following an eastward change of 5–8 time zones due to prolonged desynchrony of the system. While this desynchrony is partly due to differing reentrainment rates among peripherals, a greater source of desynchrony is the antidromic reentrainment of some but not all components, triggered by a rapidly shifting pacemaker. We also show how slave oscillators may phase-lead the master pacemaker and how coupling affects relative phase, as well as predicting the differences in behavior of self-sustaining versus damped peripheral oscillators in the system.

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A Mathematical Model for SCN Synchronization in the Mammalian Circadian Clock

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Complex networks, both biological and engineering, often exhibit behaviors as a result of the protocols that link heterogeneous nodes. One behavior of particular interest is synchronization, and while experimental studies have detailed the phenomenon, there are only very limited results for mathematical analyses. The present work details a multicellular molecular model of the mammalian circadian clock that employs vasoactive intestinal polypeptide (VIP) as the synchronizing agent. It is hypothesized that the release of VIP varies rhythmically and is responsive to light. The model includes both intrinsically rhythmic pacemaker cells as well as damped oscillators. Experimental data have shown that individual neurons exhibit a range of periods, when separated, and the model captures this using a range of values for key parameters (notably per transcription rates). Postsynaptic neurons show

time-of-day-dependent responses to VIP binding through a signaling cascade that activates per mRNA transcription. The heterogeneous cell ensemble self-synchronizes, entrains to ambient light-dark cycles, and desynchronizes in constant bright light. The degree of synchronicity observed depends on cell-specific features (e.g., mean and variability of parameters within the rhythm generating loop), in addition to the more commonly studied effect of intercellular coupling strength. These simulations are consistent with experimental data and suggest that intercellular coupling allows coherent timekeeping with relatively heterogeneous cells.

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Modeling Variations in the Circadian Patterns of Cortisol Secretion in Human and Guinea Pig Saliva

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Diurnal patterns in the secretion of cortisol are important outputs of the circadian neural oscillator system implying a wide range of neuromodulatory effects. Assessment of these patterns with a minimum of subject disturbance is essential in both clinical and empirical studies. We have devised mathematical models (using orthonormal trigonometric base functions plus a constant) to predict characteristics of circadian patterns of cortisol saliva in humans and to monitor ultradian dynamics. The model has been applied to 30-minute interval saliva samples of subjects collected over three days in a semi-constant routine. The contribution of harmonics down to 90 minutes in length has been investigated in the models. Amplitudes and phase relationships with the environment and other physiological and behavioral parameters are possible to measure with the technique. A similar approach was taken in the guinea pig but the sampling interval was reduced to 20 minutes. The circadian cortisol rhythm is less robust in the guinea pig than in the human. The data allow us to make predictions about the validity of skeleton sampling paradigms with small numbers of samples and specific phases. They provide a basis for the evaluation of isolated sampling techniques in the Guinea pig and humans. The information is certainly of value for out-patient studies on the changes in circadian cortisol patterns.

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Mechanistic Temperature Compensation of the Circadian Clock

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The internal circadian rhythms of cells and organisms coordinate their physiological properties to the prevailing 24-hour cycle of light and dark on earth. The mechanisms generating circadian rhythms oscillate endogenously with period close to 24 hours, entrain to external signals, suffer phase shifts by aberrant pulses of light or temperature, and compensate for changes in temperature over a range of 10 C or more. Although this mechanism has been intensely studied for decades, exactly how it achieves these characteristics simultaneously is not yet clear. In particular, models of the clock typically do not provide a temperature compensation mechanism that is robust to mutation. Here we discuss alternate ways of achieving temperature compensation that rely on careful construction of the clock mechanism and explore the idea that evolution has optimized the interaction of several feedback loops to produce an oscillator whose period is tightly regulated.

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Addition of a Light Effect to a Physiologically-Based Model of Melatonin

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Plasma melatonin is currently the most accurate marker of human circadian phase. We have developed an analytical tool, MelPhase that utilizes a physiologically-based mathematical model of melatonin (Brown et al., 1997) to estimate melatonin synthesis onset (SynOn) from plasma concentrations. However, SynOn estimates can be confounded by experimental interventions, such as bright light pulses. We updated the model to include a light suppression effect on melatonin.

The first-order kinetic model underlying MelPhase has two compartments representing pineal melatonin concentration and plasma melatonin concentration. The pineal melatonin compartment was modified to incorporate a light effect, which was generated by light preprocessor equations from our light-based circadian model (Kronauer et al. 1999). MelPhase calculates the maximum-likelihood estimates of seven model parameters, including SynOn, using unconstrained nonlinear optimization. We compared the R2 and SynOn estimates of both models (with and without light suppression) by paired t-tests using plasma concentrations sampled every 10–30 minutes from 21 subjects exposed to 6.5-h of continuous bright, intermittent bright, or very dim light pulses (Gronfier et al., 2004).

The average overall R2 for the light suppression model was 0.95, significantly higher ($p=0.01$) than for the model without light suppression (R2=0.77) There was no significant difference in SynOn estimates between the models ($p=0.33$).

The addition of a light effect to the mathematical model of melatonin improved overall model fits to the data but did not significantly affect SynOn estimates. Both models will be tested on other datasets and the results compared with other measures of melatonin phase and amplitude.

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A Mathematical Model of Neurospora Crassa Circadian Rhythms

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The existence of biological clock is observed from cyanobacteria to humans. In all cases, a conserved transcription–translation feedback loop (TTFL, where time-delayed negative feedback plays a major role in the system) is observed. Additional feedback loops are intertwined with this negative feedback loop. In the complexity of multiple feedback loops, a mathematical model may provide a better understanding of the underlying system. In Neurospora crassa, a clock protein FREQUENCY (FRQ) oscillates with a period of about 22 hours. FRQ inhibits its own synthesis by interacting with its transcription factor (TF), WHITE COLLAR 1 (WC-1). Our model recapitulates phase differences observed between frq mRNA and FRQ protein, FRQ and WC-1 proteins, and distinctively different total versus nuclear ratios of FRQ and WC-1 (Schafmeier et al., 2005). The nuclear FRQ is lower than the nuclear WC-1, even though the total FRQ is greater than the total WC-1. Due to the limiting amount of FRQ in the nucleus, it's possible to hypothesize that FRQ would act as a direct or indirect catalytic component inactivating WC-1, as opposed to their non-catalytic physical binding and inactivation of WC-1 (Cheng et al, 2002; Yang et al., 2002). In our model, however, we demonstrate that a non-catalytic, direct linear interaction of FRQ with WC-1 in the nucleus generates robust oscillations, even though the nuclear FRQ is significantly lower than the nuclear WC-1. Our model also simulates the property of temperature compensation and different clock mutants' periods.

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Dynamic Model of a Circadian Oscillator Constructed from its Tau and PRC

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This work introduces a dynamic model of a circadian oscillator that combines features of both continuous and discrete entrainment. It uses knowledge of only the oscillators endogenous period τ and phase-response curve (PRC). The dynamical model is quite simple and accounts for continuous entrainment by allowing the velocity of the oscillator to depend continuously on a phase-modulated entraining signal. To incorporate discrete entrainment, it connects this modification of velocity to a signal that is the modulation between the entraining signal and one depending on oscillator phase. We require this model to be consistent with a circadian oscillators ability to phase-reset and, in doing so, explicitly incorporate the oscillators PRC into the model. We explore the consequences of this model by analyzing the response of the circadian oscillator model to both full and skeleton photoperiods as well as constant light.

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Phenotypic Analysis of kaiC Mutants Showing a Wide Variety of Period Lengths in Synechococcus elongatus *PCC 7942*

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Cyanobacteria are the simplest organisms known to exhibit circadian rhythms. Three clock proteins, KaiA, KaiB, and KaiC, are essential elements of the circadian clock of cyanobacteria. We demonstrated that the oscillation of KaiC phosphorylation is the primary oscillation of cyanobacterial clock. This appears to be consistent with the fact that various kaiC mutants were found with a wide range of circadian phenotypes. To understand the functions of KaiC protein, we performed a PCR-based random mutagenesis on the kaiC gene and screened a library of ~38,000 clones for abnormal phenotypes in the bioluminescence rhythm. We isolated 950 clones that displayed a variety of abnormalities in the properties of circadian rhythm. Among them, approximately 500 mutants exhibited a broad spectrum of periods (between 14 and 66 hours). Mutations of 50 period mutants were mapped to various sites throughout the KaiC sequence, suggesting the determination of the period length involves whole regions of KaiC. Next using these period mutants, we tried to analyze the relationship between period lengths of endogenous circadian clock and environmental responses of the clock. Responses of the period lengths to changes in temperature and light intensities and resetting of the phase by darkness were examined. We will discuss the relationship between period lengths and physiological characteristics of the cyanobacterial circadian clock.

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A Genetic Selection for Circadian Output Pathway Mutations in Neurospora crassa

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To identify components of the circadian clock in *Neurospora crassa*, we have carried out a genetic selection to isolate mutations that alter the expression of clock-controlled gene-1 (ccg-1). The promoter of ccg-1 was fused to the mtr gene (1) to create plasmid pCCG1M. The mtr gene encodes a neutral amino acid permease that allows for both positive and negative selection (2). Loss of MTR function can be selected for based on resistance to the amino acid analog p-flourophenylalanine (FPA). Gain of MTR function can be selected for based on growth of tryptophan auxotrophs on high arginine/low tryptophan (TA) media. The pCCG1M plasmid was transformed into a bd;frq10;mtr;trp-2 strain. Levels of ccg-1 mRNA fluctuate randomly but remain elevated in a frq-null (frq10) strain as a result of the loss of a functional FRQ-based circadian oscillator. The pCCG1M transformed strain, CCG1M, displayed the predicted Mtr+ phenotype: growth on TA media, but not on FPA. Eighty mutant strains were isolated from selective media (FPA) following UV mutagenesis of CCG1M. Analysis of some of these mutations indicates that they reside in an output pathway downstream of the FRQ oscillator. The oscillator components FRQ and WC-1 accumulate with a normal periodicity in the mutant strains; whereas genes known to require a functional FRQ oscillator for rhythmic expression (ccg-1 and ccg-2) are arrhythmic in the mutant background.

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A Region in PERIOD Required for Its Phosphorylation and the Subsequent Protein-Protein Interaction

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Period has been shown to be an essential gene for circadian rhythms regulation in mammals as well as in Drosophila. Its expression exhibits a daily fluctuation at both the RNA and the protein levels. Additionally, the period protein (PER) phosphorylation state also oscillates with ~ 24-hour period and has been shown to be an important feature of circadian rhythm regulation. PER phosphorylation is also probably involved in its molecular functions, i.e., stability, subcellular localization, and transcriptional repression activity, possibly via temporal regulation of protein-protein interactions. This suggests that certain PER phosphorylation states prefer different protein partners for different molecular functions. Despite the importance of PER phosphorylation, many questions remain. For example, how does progressive phosphorylation takes place, and what are the different partners and their functions. In this study, we identified a conserved PER sequence necessary for the progressive phosphorylation in vivo and generated a mutant PER (PERdelta) defective in phosphorylation. PERdelta showed defects in stability, nuclear localization, and repression activity, consistent with previously published observations. By immunoprecipitating PER and PERdelta and comparing the two protein profiles by mass-spectrometry, we identified a few proteins preferentially interacting with PER but not with PERdelta. Results in S2 cells suggest that one of the proteins affects PER stability as well as its activities and also suggests novel biochemical activities. Genetic and behavioral experiments using a deficiency line suggest a genetic interaction between period and this new gene, which indicate that the interaction in S2 cells is of functional consequence in flies.

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A Role for CBP/p300 in the Drosophila Circadian Clock

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The circadian clock regulates a wide variety of physiological, metabolic, and behavioral activities in synchrony with environmental cycles. The heterodimeric complex of Clock (Clk) and cycle (cyc) forms the positive element of the circadian clock that activates transcription from E-box elements and ultimately controls transcription of 5 to 10 % of the genome. However, very little is known about post-transcriptional regulation of CLK/CYC activity. We have found that CLK/CYC activity is regulated through direct phosphorylation of CLK protein by calcium/calmodulin-dependent kinase (CaMK II) and MAPK, identifying the first CLK-kinases. In addition we have found that the CBP/p300 homolog in Drosophila, nejire (nej), is an essential transcriptional co-activator for CLK/CYC-dependent transcription, both in Drosophila cell culture as well as in flies. An increase in nej transcript levels causes behavioral and molecular arrhythmicity and decreased levels of nej cause a long period phenotype. These results identify CBP as a new clock component. CBP is regulated by multiple cell signaling pathways, including CaMK II and MAPK, adding a complex level of post-transcriptional CLK/CYC regulation. We propose that CBP mediates non-photic entrainment of the circadian clock.

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A Roundabout Mutation Alters the Pace of the Circadian Clock

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In recent years, great efforts have been directed to the dissection of the cell-autonomous circadian oscillator in Drosophila. However, less information is available regarding how this molecular oscillator controls one of the most studied clock outputs: the rhythmic rest-activity cycles. We have identified a viable hypomorph allele of roundabout, robohy, where the period of locomotor activity is shortened. From its role in axon pathfinding during embryogenesis, we anticipated developmental defects in clock-relevant neuronal structures. However, robohy produced only subtle defects on the neuronal circuits expressing the pigment dispersing factor, essential for the control of rhythmic behavior, suggesting that alterations are not developmental. The presence of ROBO in the adult fly brain strengthened the possibility of a novel role for ROBO at this post-developmental stage. In fact, the early translocation of PERIOD to the nucleus observed in robohy pacemaker cells indicated that shortened activity rhythms are due to alterations in the molecular oscillator. Behavioral periodicity was also affected by genetic interaction between robohy and abelson, a molecule involved in the canonical ROBO signaling pathway. ABELSON expression within circadian relevant structures suggests a role for molecules involved in this pathway in setting the properties of the molecular oscillator. Unlike all previous reports of period-affecting mutations, we present a novel mechanism affecting clock function associated to a molecule involved in circuit assembly and maintenance.

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A Screen for Deletions that Modify CK2alpha(Tik) in Drosophila Identifies an Independent Role for CK2 in Regulating Circadian Rhythmic Strength

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The protein kinase CK2 regulates circadian periodicity in the fruit fly Drosophila melanogaster. The CK2alpha (Timekeeper) (Tik) mutant results in robust, long-period (26-27 hours) rhythms. We screened 84 lines from the DrosDel collection of isogenic deletions for enhancers and suppressors of the CK2alpa(Tik) circadian phenotype. Initial studies identified deletions that are lethal as heterozygotes in combination with Tik when Tik flies are the mothers, a so-called maternal effect. Using Tik fathers, we identified two independent deletions showing a significant loss in the strength of behavioral rhythmicity but no change in periodicity in a heterozygous Tik background. These effects are allele-specific; heterozygous deletions do not reduce rhythms in a wild-type background. In addition, loss of rhythmicity is not observed in combination with other long-period circadian mutants, indicating a specific interaction between CK2 and gene dosage in the deleted regions. Overexpression of Tik in the ventral lateral pacemaker neurons (LNv) results in significant period lengthening. The deletions reduce rhythmicity in this context, suggesting they modify an LNv function of CK2 and not a more general function. Taken together, these data suggest CK2 plays a role in regulating rhythmic strength in addition to its described role in setting period length. Future studies will use overlapping deletions from the Exelixis isogenic deletion collection as well as the available single-gene mutants to positionally clone the modifiers of CK2alpha(Tik) behavior.

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A Constant Light Screen in Drosophila

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An important property of circadian clocks is their ability to function in the absence of environmental cues. The fruit fly Drosophila melanogaster remains rhythmic in constant darkness (DD). The ventral lateral neurons (vLNs) are the pacemaker cells that maintain circadian behavioral rhythms under these conditions. However, fruit flies rapidly become arrhythmic under constant light (LL). The photoreceptor CRY is required for this circadian response to light: CRY mutant flies (cryb) remain rhythmic in LL with a period of 24 hours, as if they were in DD.

Based on this phenotype, we have undertaken a genetic screen to identify genes influencing circadian light responses. Surprisingly, this screen revealed that overexpression of two pacemaker genes, PER and SLIMB, also results in rhythmicity under constant light. Thus, both loss-of-function mutations in the CRY input pathway and gain-of-function mutations in the circadian pacemaker can make flies resistant to constant light. Unexpectedly, we found that overexpressing PER, SLIMB, and other genes identified in our screen only in the vLNs does not result in rhythmicity in LL. However, flies were rhythmic when we overexpressed these genes in all circadian neurons, except the vLNs. Thus, beside the vLNs, there is another group of circadian neurons that can modulate circadian responses to light and play an important role in maintaining long-term behavioral rhythms. We are conducting experiments to identify these neurons.

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Use of Per Mutants to Analyse the Role by which Phosphorlation Regulates the Drosophila Circadian Clock

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Central to circadian rhythms is a negative feedback mechanism, in which period protein (PER) represses dCLK/CYC-dependent transcripts (including per). Throughout the day, PER protein does not accumulate in the nucleus despite high levels of its mRNAs, and the post-transcriptional events that prevent its accumulation are essential for circadian rhythms. DBT is essential for these effects, but the mechanisms for its effects are not well understood. We have proposed that there are multiple effects of DBT on PER's temporal program, and that these are mediated by different target sites in PER. To address this hypothesis we have generated site-directed mutations in PER. Potential phosphorylation target sites have been changed from a Serine or Threonine to either an Aspartate, which would mimic constitutive phosphorylation, or to an Alanine, which would mimic no phosphorylation at that site. Because DBT may act processively, mutations of one Ser or Thr may alter phosphorylation of downstream target sites as well. Transgenic flies expressing the mutant per genes were constructed to elucidate the effects of the mutations in adult flies. Our results show that many of these mutations lengthen or shorten circadian period. At some of these sites, period alterations are produced by one type of mutation (Ser/Asp or Ser/Ala) but not the other, while at other sites similar period alterations are produced by both types of changes. The results suggest a complex relationship between phosphorylation of sites and period length. The effects of these mutations on different aspects of PER's temporal program are being investigated.

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A Light-Stable cryptochrome in Drosophila

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Cryptochromes are blue-light sensitive proteins identified in various organisms, including Drosophila and mammals. In Drosophila the single light-degraded CRYPTOCHROME (dCRY) acts as a photopigment, which, endogenously, is involved in the entrainment of circadian rhythms both in central and peripheral clock cells. dCRY is also an essential component of the circadian clock in peripheral tissues. In contrast, in mammals, the two CRYs are an integral part of the central pacemaker controlling circadian behaviour and are not degraded after exposure to light. Sequence comparisons between dCRY and mouse CRY1 (mCRY1) reveal that the N-termini of these proteins are well conserved while their C-termini do not show any homology, suggesting a possible explanation to their functional differences. To investigate this further, we engineered a chimeric protein, called dCRY-mCT, where the N-terminus of dCRY (residues 1-492) is fused to the C-terminus of mCRY1 (residues 468-606) and we expressed it in Drosophila clock cells with the GAL4/UAS system. Here we show that the fusion protein is expressed in vivo at constitutively high levels and is not degraded upon light exposure, which is reminiscent of mCRY1 behaviour. Moreover, flies overexpressing dCRY-mCT have a long period of locomotor activity in DD and reduced light sensitivity. These results are possibly explained by a constitutive degradation of TIMELESS and obliteration of its molecular cycles.

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Day, a Putative Signalling Partner of Drosophila Cryptochrome

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In a yest-two-hybrid assay Drosophila CRYPTOCHROME (CRY) binds to TIMELESS (TIM) or PERIOD (PER) under constant light but not dark conditions. Using the same assay we have confirmed a previously described yeast-two-hybrid interaction between CRY and a putative protein CG15803-PA. We have also demonstrated that this interaction occurs in darkness but not in light and we renamed CG15803-PA as DAY (Dark Active in Yeast). We have further shown that DAY binds constitutively to CRYD, a truncated form of CRY missing the regulatory C-terminus, but not to mutated CRY molecules where specific regulatory signals in the C-terminus have been altered. In head extracts we have shown by RT-PCR that day is expressed in vivo. Finally, a CoIP assay carried out in S2 cells coexpressing CRY and DAY revealed that the two proteins do bind in a Drosophila system and that the interaction is stronger under darkness than light. Taken together these results suggest that DAY might participate in the regulation of light signalling in Drosophila.

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Loss of Slowpoke Function Impairs Circadian Pacemaker Circuitry

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In the last decade, substantial progress has been made in elucidating the molecular processes that impart a temporal control over cell metabolism and behavior, but it was not until recently that the hierarchical organization among the different circadian circuits has been addressed. Circadian release of the neuropeptide pigment dispersing factor (PDF) is central to how the clock controls rhythmic rest-activity cycles.

Drosophila mutants in the slowpoke BK potassium channel display an arrhythmic phenotype both under entrained and free running conditions. Lack of SLO function specifically affected PDF staining on projections towards the pars intercerebralis and along the midline. Moreover, not only it impaired PDF circadian release on dorsal projections from pacemaker neurons, but it also altered the normal arborization of the axon terminals at this level, suggesting an impaired contact with PDF postsynaptic targets. We are currently investigating the role of synaptic activity and other factors potentially affecting the structure of this relevant circuit and its connection to rhythmic behavior employing a UAS-gfp reporter tethered to the membrane to follow its morphology under different developmental and temporal conditions.

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Transcriptional Regulation of Cry in Drosophila

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The cryptochrome (cry) gene from Drosophila encodes a blue-light photoreceptor that mediates light input to circadian oscillators throughout the head and body and is required for oscillator function in peripheral tissues. The levels of cry mRNA cycle with a peak at ~ZT5, which is similar to the phase of Drosophila Clock (Clk) mRNA cycling. The rhythm in Clk and cry mRNA abundance is thought to be controlled by PDP1 epsilon and VRI, which activate and repress transcription, respectively. To determine whether VRI and PDP1 epsilon mediate rhythmic cry expression in oscillator cells, we are defining cry spatial and circadian regulatory elements and determining whether VRI and PDP1 epsilon bind to these elements rhythmically in vivo. A series of cry-Gal4 transgenes containing different portions of upstream and intron 1 sequences were tested for spatial and circadian expression. Upstream sequences were sufficient to drive expression in oscillator cells, but intron 1 sequences were necessary to drive circadian expression. Canonical VRI/ PDP1 epsilon binding sites (V/P-boxes) in intron 1 were tested for VRI and PDP1 epsilon binding at different times of the circadian cycle via chromatin immunoprecipitation (ChIP) assays. In wild-type flies, VRI and PDP1 epsilon bind to cry intronic V/P-sites in a rhythmic manner. Additional ChIP analysis of VRI and PDP1 epsilon binding in different clock mutants is currently underway. These results suggest that cry spatial and circadian expression can be independently regulated, and that VRI and PDP1 epsilon may contribute directly to rhythmic cry transcription.

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KaiC-Phosphorylation-Dependent SasA-AnRR16 Two-Component Regulatory System as a Major Circadian-Timing Mediator in Cyanobacteria

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KaiA, KaiB, and KaiC clock proteins and ATP are sufficient to reconstitute the KaiC phosphorylation rhythm in vitro, while almost all gene promoters are under the control of the circadian clock in cyanobacteria. The mechanism by which the KaiC phosphorylation cycle drives global transcription rhythms still remains unknown. Here, we identified a DNA-binding protein, AnRR16, that was found to be a cognate response regulator of the KaiC-interacting sensory histidine kinase, SasA. Circadian transcription was dramatically attenuated in sasA- and AnRR16-mutant cells, and the phospho-transfer activity from SasA to AnRR16 was highly activated by phosphorylated KaiC in vitro. We propose a model in which the SasA-AnRR16 two-component system mediates time signals from the enzymatic oscillator to drive genome-wide transcription rhythms in cyanobacteria. Moreover, our results indicate the presence of secondary output pathways from the clock to transcription control, suggesting multiple pathways ensuring the genome-wide circadian system.

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Identification of Targets of the Clock-Controlled Lark RNA-Binding Protein

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Increased expression or knockdown of the Drosophila LARK RNA-binding protein in Timeless-containing cells lead to arrhythmic behavior, consistent with the hypothesis that the protein functions in a clock output pathway. In an attempt to dissect the molecular pathway downstream of LARK, we have sought in vivo RNA targets of the protein. RNA ligands bound to LARK were captured by immunoprecipitation using an anti-LARK antibody and their identities revealed by microarray-based analysis. These studies identified at least 79 putative LARK targets. Several lines of evidence suggest that these are likely to be bona fide targets of LARK: First, sequence analysis revealed a common sequence element in the 3'UTR of the majority (86%) of the putative targets, suggesting that these RNAs might be similarly regulated, presumably by LARK. Second, for many targets, stready-state abundance is altered in response to changes in LARK amount. Third, mutants of several presumptive LARK targets exhibit abnormal circadian eclosion or locomotor activity rhythms. Finally, we have documented a genetic interaction between a lark null allele and a mutation of one of the putative targets. The identified LARK target RNAs encode products that function in a variety of cellular pathways, including several that might be relevant for circadian control. Further studies of these targets will yield insights about the signaling pathways that mediate clock output.

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CaMK II and Ras/MAPK Regulate CLOCK/CYCLE-Dependent Transcription

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The heterodimeric complex of the transcription factors CLOCK (CLK) and CYCLE (CYC) constitutes the positive element of the circadian clock in Drosophila and mammals. Phosphorylation of clock-proteins forms an essential mechanism to promote and control the molecular oscillator. However, kinases and signaling pathways that regulate CLK/CYC function remain largely elusive. In the present study we performed a chemical screen of kinase inhibitors in a cell culture reporter assay to identify functional regulators of CLK/CYC-dependent gene expression. These studies and analysis of constitutively active forms of kinases revealed that cyclic nucleotide/PKA, calcium/CaMK II, and Ras/MAPK signaling pathways regulate CLK/CYC activity. In vitro phosphorylation analysis showed a direct phosphorylation of CLK by CaMK II and p42-MAPK (ERK2), suggesting that these kinases regulate CLK/CYC-dependent transcription through direct phosphorylation of CLK.

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Functional Role of CREB-Binding Protein in the Circadian Clock System of Drosophila

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Rhythmic histone acetylation underlies the oscillating expression of clock genes in the mammalian circadian clock system. Cellular factors that contain HAT and HDAC activity have been implicated in these processes by direct interactions with clock proteins. However, there was no appropriate animal model for investigating the functional role of HATs or HDACs in the circadian clock system. Here, using transgenic fly models, we show that CBP overexpression in tim-expressing neurons results in arrhythmic circadian behaviors while CBP knockdown in pdf-expressing neurons lengthens the period of adult locomotor rhythm. In contrast to the mammalian circadian clock system, CBP overexpression impaired dCLK/CYC-mediated clock gene expression both in vivo and in vitro. Protein interaction study suggests that CBP may specifically function as a negative regulator of the dCLK/CYC heterodimer in the Drosophila circadian clock system by directly targeting the PAS domain of dCLK protein.

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Lark Activates Post-Transcriptional Expression of a Mammalian Clock Protein, Period1

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Two mouse Lark genes (mLarks), have been identified as structural homologues of the Drosophila clock gene lark (dLark). Like dLark, mLark1 and mLark2 possess two RNA recognition motifs (RRMs) and a CCHC-type zinc finger motif. In addition to the structural resemblance, a strong circadian cycling of the mouse Lark (mLark) protein is observed in suprachiasmatic nuclei (SCN) although the level of its transcript remains unchanged, and the expression profile of the mLark protein coincides with that of the mouse Period1 (mPER1) protein. Furthermore, we found that mLark activates the 5'-CAP/Per1 3' untranslated region (UTR) dependent translation of the mouse Period1 mRNA through the interaction with the 3 UTR. To elucidate the role of Lark in the mammalian circadian system, we analyzed light emission from NIH3T3 cells stably transfected with a Per1::luc reporter gene. A gene knock-down experiment of mLarks resulted in a shorter circadian period in the rhythmicity of bioluminescence, while the overexpression of mLark1 lengthened the crcadian period. These data indicate that mLarks are a novel translational regulators of mammalian circadian clocks.

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An Essential Role for the Protein Kinase CK2 in Drosophila Circadian Rhythms

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Protein phosphorylation plays a critical and highly conserved role in circadian timekeeping. We have described a central role of the protein kinase CK2 in the Drosophila clock (Lin et al., 2002). We identified a dominant mutant allele of the catalytic alpha subunit, CK2alpha(Tik), with a ~26Trina Broussard27 hour period, one of the strongest dominant circadian phenotypes observed in Drosophila. Using the GAL4/UAS system, we overexpressed CK2alpha(Tik) in discrete pacemaker neurons. Consistent with a role as a dominant negative, we observe strikingly long circadian periods (~32 hours). These data indicate that circadian period is highly sensitive to CK2 activity. Long periods could be obtained by expression of CK2alpha(Tik) in the core set of ventral lateral pacemaker neurons (LNv). Behavioral rhythms can be virtually eliminated by increasing CK2alpha(Tik) gene dosage, suggesting an essential role in behavioral rhythmicity. This tool will be valuable in addressing the molecular and network functions of CK2.

CK2 has been previously implicated in regulating the core clock component PERIOD (PER). Here we demonstrate in vivo circadian function of CK2 phosphorylation sites in PER. CK2 phosphorylates PER in vitro and mutations at three serines (S149, S151, and S153) dramatically reduce phosphorylation. Mutation of these sites in vivo results in period lengthening of behavioral rhythms and delayed PER nuclear localization in the core ventral lateral pacemaker neurons, mimicking aspects of the CK2 mutant phenotype. We propose that nuclear entry of PER is triggered by CK2 phosphorylation and contributes to period regulation.

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Biological Function of the PER:PER Homo Dimer in Drosophila

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Two in vitro studies (Huang et al., 1993, 1995) and one recent structural study (Yildiz et al., 2005) suggest the formation of a homo dimer of the Drosophila clock protein PERIOD. But so far, the dimerization has not been shown for the full length protein in vivo and its potential biological function is unknown. We made transformant flies that produce recombinant PER proteins with the HA or MYC tag and no endogenous PER, to test dimerization via CoIP. To validate the PER:PER contact points, resolved by Yildiz et al. (2005) and to reveal the dimers biological function, we also mutated the suggested points of interaction in these proteins to block potential dimerization. The conception and preliminary data of the approach will be presented.

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Mapping of mPER2 Phosphorylation Sites

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Most circadian clock proteins are phosphoproteins in vivo. The circadian nature of their phosphorylation suggests a functional importance of these modifications to the circadian oscillator mechanism. For example, mPER proteins are phosphorylated by CKIepsilon/delta and possibly other kinases. These post-translational modifications are thought to result in cytoplasmatic retention and degradation of mPER proteins, thereby generating a delay in the negative feedback loop considered as essential for the generation of circadian rhythms. However, little is known about the number, location, and function of the phosphorylation sites of clock proteins, nor about the kinases involved. Here, we present a new method for the rapid and comprehensive identification of clock protein phosphorylation sites. mPER2 was stably expressed in HEK293 cells, immunopurified, and subsequently degraded with four different proteases. The resulting mPER2-derived phosphopeptides were enriched via titansphere nanocolumn and subsequently analyzed with nano-liquid chromatography coupled to electrospray tandem mass spectrometry (nanoLC-ESI-MS/MS). mPER2 was found to be phosphorylated on 21 serine or threonine residues. In parallel experiments, the in vitro phosphorylation of previously dephosphorylated mPER2 with candidate kinases such as CKIepsilon/delta, CK2, and GSK 3beta, led to the identification of differential phosphorylation patterns. Taken together, these experiments strongly suggest that a substantial number of the identified mPER2 phosphorylation sites are substrates of kinases other than CKIepsilon/delta. (Our work is supported by the Deutsche Forschungsgemeinschaft and the 6th Framework Project EUCLOCK).

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The Rhythmic Deadenylase Nocturnin Is Acutely Induced By Extracellular Stimuli

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Nocturnin is a circadian-regulated deadenylase that was originally identified in Xenopus and subsequently in several species. The shortening of mRNA polyA length, deadenylation, unleashes messenger degradation and/or translational silencing. These observations support the hypothesis that nocturnin regulates the stability, and/or translational properties, of circadian-related mRNAs.

Mouse nocturnin (mNoc) is rhythmically expressed in multiple tissues suggesting its participation in a broad spectrum of cellular and physiological events. Here we show that mNoc expression is also induced in cell cultures after different extracellular stimuli. Remarkably, no other deadenylase known in mammals showed this acute induction.

In quiescent NIH3T3 cultures, mNoc transcript levels increased dramatically after a serum shock or when the phorbol ester TPA was added to medium. Dexamethasone and forskolin treatments did not induce mNoc message. The half-life of this mRNA was determined to be 30 min. Addition of cycloheximide prevented the mNoc mRNA degradation and resulted in a 30-fold increase in the transcript levels after 4 h of TPA treatment. In addition to the message, protein levels also increment after a serum shock. mRNAs encoding for the other known mammalian deadenylases, CCR4, CAF1, PAN2, and PARN, were not induced in this same paradigm.

These results show that in addition to circadian clocks, other factors can modulate mNoc expression. These data suggest mNoc could be implicated in direct responses to metabolic or physiological changes. Nocturnin may act in turning off the transient expression (inducing messenger decay) of immediate early genes and/or transcription factors that are induced after extracellular stimuli.

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BAC Transgenes Over-Expressing Clock and Bmal1 (Mop3) Reveal that Clock is Rate-Limiting in the Control of Circadian Periodicity

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In previous work on the rescue of the Clock mutation using bacterial artificial chromosome (BAC) transgenes, we found that over-expression of Clock in BAC transgenic mice on a wild-type background had shorter free-running periods than non-transgenic littermates (Antoch et al., Cell 89:655, 1997; Wilsbacher et al., Genom. Res. 10:1928, 2000). Thus, we proposed that Clock is the rate-limiting factor in the positive limb of the circadian oscillator. Here we report a transgenic mouse line containing two Bmal1-encoding BAC clones (BAC 47 and 141, Research Genetics) including 220 kb of mouse chromosome 7. Both BACs contain Bmal1 and approximately 90 kb of surrounding sequence. Southern hybridization revealed the presence of 10 copies of the BAC transgene in a line designated TG76. Bmal1 expression levels in liver of both wild-type and TG76 were in phase. However, TG76 Bmal1 expression was three-fold higher at ZT22 (the peak of endogenous Bmal1 expression); TG76 Bmal1 expression did not increase at the endogenous ZT6-10 trough. Free-running periods of TG76 and wild-type littermates in DD were not

significantly different (TG76 = 23.82±0.1-h and WT = 23.65±0.1-h, p=0.109). Free-running periods of TG55 mice (over-expressing Clock with BAC 54) were slightly shorter than those of wild-type littermates (TG55 = 23.36±0.1-h, p=0.0247). Surprisingly, mice bred to contain both transgenes showed considerably shorter free-running periods than wild-type littermates (TG55/TG76 = 22.14±0.2-h, p<0.0001). The short free-running periods of TG55 mice suggests that Clock is the rate-limiting factor of the CLOCK: BMAL1 heterodimer under normal conditions. However, the additional shortening of period of double transgenic mice suggests that Bmal1 then becomes the rate-limiting factor in Clock over-expression mice. In summary, the over-expression of both CLOCK and BMAL1 is synergistic and leads to an additional period shortening which cannot be seen by over-expression of either CLOCK or BMAL1 alone.

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Analysis for the Robustness of the Chemical Oscillator by Cyanobacterial Clock Proteins

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In the cyanobacterium Synechococcus elongatus PCC7942, kaiA, kaiB, and kaiC were identified as the clock genes, and it was proposed that kaiC played an important role in the circadian clock through the negative feedback on its own transcription/translation regulation. Our recent studies have revealed that temperature-compensated and robust circadian cycling of KaiC phosphorylation/de-phosphorylation can be reconstituted in vitro by incubating KaiC with KaiA, KaiB proteins under the presence of ATP. KaiA enhances the autophosphorylation of KaiC; meanwhile KaiB inhibits the KaiA effect. These processes are likely to generate the robust oscillation of KaiC phosphorylation with a period of about 24 hours. This system can work with simple chemical reactions without biological controls such as in transcription/translation processes. This self-sustained oscillator should involve some machinery that synchronizes the states of the individual clock proteins in solution. The synchronous mechanism would contribute the increase of the robustness for the biological clock system against the intrinsic and extrinsic noises at molecular levels. We are trying to find out the molecular events that would be involved in the mechanisms by comparing characteristics of the oscillatory chemical reactions among each phase. We will discuss the synchronization mechanism from experimental aspect and in the context of the theory of phase dynamics.

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Circadian Expression of AhR and its Signaling Targets and the Role of AhR in Circadian Rhythm

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Aryl hydrocarbon receptor (AhR) belongs to a family of transcriptional regulatory proteins named for their common basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) domain. Many proteins in this group play critical roles in regulating circadian rhythms. Although extensively studied for its role in mediating effects of dioxin-like compounds, a physiological role for AhR remains unclear. Light is an important factor for entraining circadian rhythms and it is also known to activate AhR. Therefore, we hypothesized that light-induced activation of AhR affects the circadian clock pathway through promiscuous binding of AhR with

canonical clock genes. In this study, we show AhR and its signaling target, CYP1A1, have diurnal mRNA expression patterns in both the suprachiasmatic nucleus and the liver. We then tested whether lack of AhR would result in abnormal circadian rhythm by use of AhR knockout (AhRKO) mice. Preliminary results indicated that AhR knockout mice can entrain to 12-hour light shifts similarly to wildtypes and maintain circadian rhythm under constant darkness and light conditions. There was a difference in circadian period between wild-type and AhRKO females following ovariectomy although such differences were not observed in intact males. Although involvement of AhR in circadian clock needs further investigation, rhythmic expression of AhR and its signaling targets suggest a potential role in the circadian effects of light.

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Development of a Conditional Mutant Mouse Bearing the Circadian tau Mutation and Use of Lentiviral Vectors to Report Real-Time Circadian Oscillations in Peripheral Cells

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The tau mutation in casein kinase1 (CK1) shortens the period of circadian rhythms in Syrian hamsters. We have produced both a conditional mutation of tau and a knock-out of CK1 in mice. Behavioural studies of conditional mutant mice (+/tau and tau/tau) reveal an accelerated circadian period of wheel-running, similar to the tau hamster, with gene dose dependency but no significant differences in magnitude of phase-shifts at CT14 and CT22 following a 1h light pulse. +/- and -/- animals reveal free-running periods close to that of wild-type (WT) littermates. To establish the impact of this mutation on peripheral tissues, a 1.7kb Rev-erb? promoter fragment, encompassing 3 E-boxes, was cloned to drive luciferase (Rev::Luc1) and validated for robust circadian rhythmicity in stably-transfected Rat-1 fibroblasts. A lentiviral vector was then developed to stably transduce cultured mitotic and post-mitotic fibroblasts from WT and conditional mutant animals with Rev::Luc1 allowing for assessment of their chronotype by real-time (RT) photon counting. Preliminary data suggest that comparisons of periods of WT vs. +/tau or tau/tau reveal circadian bioluminescence oscillations of similar periods, implying that peripheral oscillators in these cells may not be accelerated by the tau mutation. Primary fibroblasts from tau/tau hamsters did however show accelerated circadian rhythms of RT bioluminescence (~20.5h), closely matching their free-running behaviour. This suggests that the tau mutation may have a complex phenotype, with differing penetrance in peripheral versus CNS tissues, which we are investigating further in a wider range of tissue types.

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Evening Exposure to Blue Light Stimulates the Expression of the Clock Gene PER2 in Humans

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Our aim was to develop a method to measure circadian expression of PER2 expression in humans via non-invasive collection of oral mucosa samples. In a second step, we tested the hypothesis that evening light exposure induces an immediate increase in PER2 expression in mucosa. Non-visual-effects of light are partly mediated by non-classical photoreceptors, containing melanopsin as photopigment with maximum sensitivity in the blue range of visible light. In order to test to what extend the novel photoreceptor system is involved in the regulation of PER2 expression, we applied light at 460 nm and compared its effect to light at a longer wavelength (550 nm). Twelve male healthy volunteers (21-29y) were exposed to blue (460 nm), green (550 nm) and no light (0 lux) for 2 hours in the late evening in a balanced crossover intra-subject design.

PER2 gene expression showed a clear diurnal rhythm with maximal values between 12.00 and 15.00h during the biological day, while minimal levels occurred between 21.00 and 03.00h during the biological night, defined as the period of melatonin secretion. Two hours of evening light exposure (21.30-23.30h) elicited an increase in PER2 expression after blue light ($p=0.032$) but not after green light ($p=0.3$). PER2 levels the next morning at 10.00h tended to be lower after blue light ($p=0.098$) when compared with green light.

Our findings provide the first evidence in humans that a circadian regulator gene, PER2, rapidly responds to light. Importantly, this response to light is clearly wavelength dependent, and indicates that the non-image forming visual system is involved in human circadian gene expression.

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KSRP, an ARE-mRNA-Binding Protein, Is a Binding Partner of Mouse Nocturnin

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Nocturnin, a deadenylase expressed in a circadian manner, is thought to play an important role in circadian clock output pathways by regulating mRNA decay of clock-related gene(s). Previous studies demonstrated that mouse Nocturnin exists in complexes with other proteins. In order to identify the binding partners of Nocturnin that may recruit Nocturnin to its target mRNAs, we performed a large-scale immunoprecipitation screen with mNoc-3XFLAG transfected HEK293 cells. The proteins precipitated with mNoc-3XFLAG were then subjected to Mass Spectroscopy sequencing, which revealed some putative Nocturnin binding proteins. Among them, KSRP (KH homology splicing regulatory protein) is of particular interest. KSRP is an ARE-mRNA (AU-rich element containing mRNA) binding

protein, and has been shown to regulate mRNA turnover by recruiting the mRNA decay machinery to ARE-mRNAs. To verify the interaction between Nocturnin and KSRP, we did immunoprecipitation in reciprocal directions. We found that KSRP can be pulled down with both endogenous and FLAG-tagged mouse Nocturnin, and FLAG-tagged mouse Nocturnin can also be pulled down with endogenous KSRP proteins in HEK293 cells. To further confirm the interaction, we performed a gel filtration analysis with HEK293 cell lysates. Endogenous KSRP and mNoc-3XFLAG both occurred in complexes between 100KD and 200KD. Together, these results prove that KSRP is a Nocturnin binding partner. Since KSRP is an important ARE-specific mRNA decay regulatory factor, we hypothesize that KSRP provides target specificity for Nocturnin, and also provides the bridge between Nocturnin-dependent deadenylation and further mRNA degradation by the exosome.

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CRYs Impair Phosphorylation of Transcriptional Activators in the Molecular Clock: A General Mechanism for Repression?

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CLOCK and BMAL1 are central components of the molecular clock in mammals and belong to the bHLH/PAS family. They drive circadian transcription of clock genes and clock-controlled genes, in a tissue-specific manner. Features of the dimerization of both partners, proposed to rely on the PAS domains, have not been investigated. Here, we demonstrate that PAS domains function requires domains extending over the short core motifs. Interestingly, while deleting core PAS domains does not overtly affect dimerization, it abolishes transcriptional activity of the heterodimer. These deletions also abolish phosphorylated forms of CLOCK and BMAL1, suggesting a link between the phosphorylation status of the heterodimer and its transactivation capability. In agreement with this hypothesis, we find that CRY1-2, potent repressors of CLOCK/BMAL1, affect phosphorylation of both proteins and shift the phosphorylated/unphosphorylated ratio of BMAL1 towards a predominant unphosphorylated form. In contrast, PER proteins, which are weak repressors, are without effect. To clarify whether these effects are specific to the CLOCK/BMAL1 heterodimer or rather reflect a general mechanism in the molecular clock, we assessed phosphorylation status and transactivation capability of other heterodimers involving NPAS2 and/or BMAL2, homologs of CLOCK and BMAL1. From these data, we propose a general mechanism for the inhibition of circadian transcriptional activation by CRY1-2.

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A Functional Genomics Resource for Neuroscientists: The NIH Neurogenomics Project

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The Neurogenomics Project, established by a cooperative agreement with the National Institutes of Health (NIH), is devoted to producing novel mutant mice for neuroscience research and to facilitate the use of mouse genetic tools in understanding the nervous system and behavior. Chemical mutagenesis is used to induce mutations throughout the genome and is combined with phenotypic screens to detect mice with

mutations of neurobiological interest. In order to maximize the genomic coverage, both a dominant G1 and a recessive G3 screen are used. Screening assays focus on recovering mice with alterations in circadian rhythmicity, learning and memory, responses to psychostimulants, novelty-induced behavior, and vision. To date, over 35,000 mice have been screened. Mice showing an abnormal phenotype are bred to test for heritability and to determine mode of inheritance. Once heritability of the trait is confirmed, mutant mice are made available to the scientific community. The Neurogenomics Project participates in the Neuromice.org consortium to distribute these mutant mouse lines (see www.neuromice.org for information on the >200 mutant lines now available, and to place orders). Additional information about the ongoing screens, heritability testing, and available mutant lines can be obtained at the Neurogenomics Project website at www.genome.northwestern.edu/neuro. It is hoped that the study of these mouse models will aid in the functional identification of new genes and pathways in neuroscience-relevant processes.

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Photolyase/Cryptochrome 1 Chimeras Reveal a Functional Domain Important for Repression in Xenopus and Mouse CRY1

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Cryptochrome (CRY) plays an integral role in the negative feedback loop of the circadian clock by repressing the CLOCK:BMAL1 heterodimer. To investigate the relationship between the structure and function of the cryptochromes, we made chimeras of xcry1 and mcry1, which encode repressor-type CRYs found in Xenopus and mouse respectively, with photolyase, a DNA repair enzyme that has high sequence similarity with CRY, but does not share its repressive function. We previously showed that a xCRY1/photolyase chimera, which contains amino acids 1-257 of xCRY1 and 258-stop of xPhotolyase, displayed significantly decreased repression, which could not be explained by disrupted nuclear localization or by decreased protein expression. We report here that 14 of the 46 residues in the second half of the core domain of xCRY1 that are conserved in all repressive CRYs, but not in photolyase, are not sufficient to rescue the disruption in repression seen in our xCRY1/PHOTO chimera. When we made an analogous chimera with mcry1 and xphotolyase, we observed a similarly decreased repression profile. Neither chimeras's disruption in repression could be explained by decreased protein expression or improper subcellular localization. Therefore, we have found that a domain from amino acids 400-499 of CRY1 is important for repression of CLOCK:BMAL1 by CRY1 in Xenopus as well as mouse. Structural predictions indicate that these residues are likely located on a ridge on the surface of the protein and may comprise a protein-protein interaction domain.

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Transcription Factor TIEG1 Represses Bmal1 Gene Expression in Glucose Input Pathway of Rat-1 Fibroblasts

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In mammals, changes in feeding time shift the phases of peripheral clocks in most tissues without affecting the rhythm of central clock localized in the hypothalamic suprachiasmatic nucleus. However,

little is known about the molecular mechanism underlying the feeding-dependent synchronization of the peripheral clocks. Rat-1 fibroblast is a model system for the study of peripheral clock mechanism, and many stimuli have been found to induce (reset) circadian expression of clock genes. We previously found that an application of glucose to the culture medium induced circadian gene expression in rat-1 cells. The rhythm was preceded by slow down-regulation of Per1, Per2, and Bmal1 expression, contrasting to acute up-regulation of these genes usually observed for the rhythm-induction. We identified a gene encoding transcription factor TIEG1 as a glucose-responsive immediate-early gene. The protein level of TIEG1 in rat-1 cells rapidly increased upon glucose treatment. In luciferase reporter assay using HEK293 cells, TIEG1 repressed the transcription from promoter regions of Per1 and Bmal1 genes. The effect of TIEG1 on Bmal1 promoter was dependent on GC box cis-elements located near the transcription initiation site, and EMSA showed that TIEG1 formed a complex with Bmal1 oligonucleotide probe in a GC box-dependent manner. We also found that the Bmal1 promoter activity was independently regulated by TIEG1 and REV-ERBa, a potential repressor of Bmal1 gene. Because the mRNA level of Rev-erva was not up-regulated by the glucose treatment, TIEG1 might act as a specific repressor of Bmal1 gene in the glucose input pathway.

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Mechanism and Role of the Bmal1 Oscillation: A Real-Time Monitoring Study of the Promoter Activities of Clock Genes

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There is a circadian rhythm in *Bmal1* mRNA levels that is antiphase to the *Per1* and *Cry1* mRNA rhythm in the suprachiasmatic nuclei (SCN) and in the peripheral tissues in manmalians. The oscillation of *Bmal1* mRNA is regulated by Rev-Erb alpha and ROR alpha via ROR response elements (ROR-RE) in the *Bmal1* promoter. The expression of *Per1* and *Cry1* gene are regulated by CLOCK/BMAL1 positively, and by PER1/CRY1 negatively through the BMAL1/CLOCK binding to the E-box in these promoters. To investigate the mechanism of these clock gene oscillation, we detected promoter activities of the clock genes by real-time monitoring of bioluminescense of reporter gene. The *Bmal1* oscillation in NIH3T3 cells was damped by overexpresson of a dominant negative Rev-Erb alpha or BMAL1 expression constructs. The circadian oscillation of *Cry1* promoter reporter showed reduced amplitude of the rhythm by transfection of the dominant negative BMAL1 expression vector. But negligibel changes were observed when the dominant negative Rev-Erb alpha expression construct was co-transfected with *Cry1* promoter reporter. These observations indicate that the oscillation of *Bmal1* regulating genes are necessary for the *Bmal1* oscillation and the *Bmal1* oscillation is not necessary for the *Bmal1* regulating genes.

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Quantitative Analysis of Circadian Gene Expression and Signaling Pathways in Fibroblast Cells

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The molecular clock is composed of an interlocked negative feedback loop comprised of at least six core gene products that are driven from critical cis-elements, E-box and RRE. To study the circadian gene expression and regulatory signaling pathways that reset the molecular clock, we established a cell culture-based real-time monitoring system using Rat-1 fibroblast cells and a luciferase reporter that is fused to several clock gene promoters. Using this system, we observed the circadian expression patterns in response to different biochemical stimuli in order to identify pathways that are responsible for "initiating" the rhythmicity (i.e. re-synchronization of fibroblast clocks). To analyze the circadian rhythms in fibroblast cells, we developed a method that statistically quantifies the degree of rhythmicity and oscillatory amplitude by using a modified FFT-NLLS program. This quantitative analysis successfully differentiated the rhythmicity and the strength of the oscillation that was affected by different inducers. The results indicated that there are at least two distinct signaling pathways that can reset the circadian rhythms in fibroblast cells, and that the mechanisms of synchronization by these pathways are likely to be different from each other. We will discuss the significance of our quantitative analysis.

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Temporal Regulation of the Interactions among Kai Proteins for KaiC Phosphorylation Cycle in vitro

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The clock proteins KaiA, KaiB, and KaiC are essential for generation of circadian rhythm in cyanobacteria. Recently, temperature-compensated, robust circadian cycle of KaiC phosphorylation was reconstituted by mixing with KaiA, KaiB and ATP in vitro (Nakajima et al., Science, 2005). This self-sustainable cycle appears to be generated with the associations among the three Kai proteins and ATP. Here we show the temporal profiles of complex formation between the three Kai proteins in vitro. The combination of Pull-down assay and Gel-filtration analysis confirmed that the association dynamics of Kai proteins in vitro is similar to that of in vivo (Kageyama et al., JBC, 2003; Kitayama et al., EMBO J, 2003). These results strongly suggest that circadian rhythm of the KaiC phosphorylation is a master oscillator for the circadian system in cyanobacterial cells.

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Role of Active Nuclear Export of mPER2 in Rhythmic Fibroblasts

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mPERIOD (mPER) and mCRYPTOCHROME (mCRY) are two major components of the mammalian molecular clockwork, and the precise regulation of the activity and cellular localisation of these negative feedback factors is central to the functioning of the circadian clock. Previous studies have used COS-7 cells to study the nuclear trafficking of mPER2 and mCRY1 proteins, and to show that mCRY1 binding leads to retention of mPER2 in the nucleus. Using real-time luminescence reporters in NIH/3T3 fibroblasts, we studied the effects of mPER2 nuclear localisation on cellular circadian timekeeping. When over-expressed in NIH/3T3 fibroblasts, ECFP-tagged mCRY1 is predominantly nuclear, whereas EYFP-tagged mPER2 is exclusively nuclear in only 50% of cells. On co-expression of both these constructs, however, mPER2 is retained in the nucleus (>90% nuclear). mCRY1 therefore promotes nuclear localisation of mPER2 in 3T3 cells. When these constructs were individually transfected into fibroblasts carrying a Per2::dLuc reporter, both mPer2/EYFP and mCry1/ECFP led to a much-dampened bioluminescence rhythm in fibroblasts (30.9 ± 5.93% and 16.4 ± 3.20% of amplitude of control rhythms, respectively), with no significant change in period (22.6 ± 0.42 and 23.3 ± 0.33 vs. 22.7 ± 0.20 h in controls). However, when both mPer2/EYFP and mCry1/ECFP were over-expressed, amplitude was reduced (17.7 ± 2.67% compared to controls), and period significantly increased (24.4 ± 0.45 h, $p < 0.01$). Hence, nuclear retention of over-expressed mPER2 has a significant effect on its function. To investigate the effects of nuclear retention of endogenous mPER2, an inhibitor of the CRM-1/exportin-mediated nuclear export pathway, leptomycin B, was administered to rhythmic fibroblast cultures, at a dose known to cause nuclear retention of over-expressed mPER2/EYFP. This led to significant dampening of rhythms (16.1 ± 4.02% of vehicle controls), but had no significant effect on period (23.5 ± 0.58 vs. 22.7 ± 0.16 h in vehicle controls). These results demonstrate that regulated nuclear trafficking of mPER2 is essential for the functioning of the circadian clock.

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Mechanisms of Cyclic Transcriptional Regulation through C-terminal Domain of BMAL1 Mammalian Circadian Clock Protein

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The BMAL1 transcription factor is an indispensable component of the molecular circadian clock system in mammals. Here, we demonstrated the functional screening assay using the combination of transposon based random 19 amino acids insertion mutagenesis for BMAL1 protein and real-time circadian rhythm monitor system for cultured fibroblasts cell lines. This elucidated that C-terminal domain of BMAL1 played an essential role for the rhythmic transcriptional control depending on the balance of transcriptional activation and suppression for E-box mediated transcription. We identified that the C-terminal region

(the last 43 amino acids) of BMAL1 exactly functioned for transcriptional activation, however, mCRY1 also interacts with BMAL1 at this region depending on the co-existence of CLOCK protein. These results suggest that the C-terminal domain of BMAl1 shares the co-activator interacting transcriptional activating function and mCRY1 interacting transcriptional suppressing function. Moreover, another negative element mPER2 interacts with different part of BMAL1 protein, suggesting that different mechanisms regulate the negative limb of mammalian circadian system between mPER and mCRY.

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Identification of a Non-Canonical E-box that Functions as a Strong Circadian Enhancer in the mDbp Promoter

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In mammals, the expression of nearly 10% of genes occurs with circadian fluctuation in various organs and tissues. This cyclic transcription is thought to be directly or indirectly regulated through circadian transcriptional/translational feedback loops consisting of a set of clock genes. Among the clock genes in mammals, expression of the Dbp mRNA robustly oscillates both in vivo and in culture cells. Here, we show that the mDbp promoter drives reporter gene expression in robust circadian cycles in rat-1cells even though no functional circadian enhancer has been reported. To identify the circadian enhancer generating this robust rhythm, we developed a transposon based enhancer trap vector for in vitro transposition. Using this system, we identified a strong circadian enhancer region containing the CATGTG sequence in the five flanking regionof the mDbp gene; this enhancer region is critical for the ability of the mDbp promoter to drive robust oscillation in living cells. This enhancer is classified as a CANNTG-type non-canonical E-box. These findings strongly suggest that CANNTG type non-canonical E-boxes may contribute, at least in part, to the regulation of robust circadian gene expression. Further, these data may help explain the wider effects of the CLOCK/BMAL1 complex in control of clock output genes.

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Circadian Expression of Luciferase in Bmal1-Luc BAC Transgenic Animals

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Animal models in which the regulatory regions of the Period genes drive the expression of Luciferase have been used successfully in a number of studies. We have now generated bacterial artificial chromosome (BAC)- transgenic mice that express Luciferase under the control of the regulatory sequences of the Bmal1 gene. We present here a first analysis of these mice.

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Beta-Trcp1 and 2 Regulate the Mammalian Circadian Clock

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The PERIOD (PER) proteins are essential clock components that exhibit circadian rhythms in abundance and phosphorylation in both flies and mammals. Mouse PER (mPER) abundance (which depends on both synthesis and degradation) is rate-limiting for initiating negative feedback regulation. Although clearly important, the regulation of mPER function through proteolysis remains largely uncharacterized.

In Drosophila, the SLIMB protein regulates dPER turnover and is essential for a functioning clock. We hypothesize that mammalian homologues of slimb, ?-Trcp1 and 2, play a similarly vital role in the mammalian clockwork. Our preliminary results, using both biochemical methods and analysis of ?-Trcp1 knockout mice, suggest that ?-Trcp1/2 participate in the mammalian clock mechanism by regulating the turnover of mPER and possibly other clock proteins. To unravel the details of how ?-Trcp1/2 regulate the mammalian clockwork, we characterized molecular and behavioral circadian rhythms of ?-Trcp1 knockout mice and the biochemical effects of clock protein interaction with ?-TRCP1/2. We are also evaluating how disruption of both ?-Trcp1 and 2 affects the circadian clock in vivo.

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Differential Effects of Pinealectomy and Enucleation on Clock Gene Expression and 2-DG Uptake in Chick

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Avian circadian organization involves three semiautonomous oscillators: the SCN, pineal, and retina. Each of these structures feedback on one another in a neuroendocrine loop to influence downstream processes and peripheral oscillations. However, the contribution of each structure in driving or synchronizing peripheral oscillators or circadian outputs in avian species is largely unknown. To explore these interactions in chick, we measured mRNA expression of chick clock genes cry1, per2, and per3 in the telencephalon, diencephalon, and liver in chickens that underwent pinealectomy, enucleation, or sham surgery. We also measured 2-deoxy[(14)C]glucose uptake within these tissues. Both enucleation and pinealectomy disrupted or reduced the amplitude of the cry1 mRNA rhythm in diencephalon and telencephalon. Likewise, enucleation reduced, but did not abolish per3 mRNA rhythms in both brain structures. Pinealectomy, however, reduced the rhythm of per3 expression in the diencephalon only. In contrast, the amplitude of 2-DG uptake rhythms was not perturbed by either surgical manipulation in either brain structure; however, the phasing of the rhythm was shifted to an approximately antiphase relationship in animals subjected to either surgical treatment. Interestingly, no clear circadian expression of these clock genes was seen in chick liver, although 2-DG uptake appeared to exhibit a low amplitude rhythm. Moreover, this rhythm was perturbed by surgical treatment in a manner similar to that seen in the brain. In conclusion, these data point to a complex, differential orchestration of central and peripheral oscillators in the chick, and, importantly, indicate a disconnect between canonical clock gene regulation and circadian control of metabolism.

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Identification of Interacting Domain of Mice Cryptochromes with Mice BMAL1 and PEROD2

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Koc University

Circadian rhythms are the endogenous oscillations, occurring with a periodicity of approximately 24 hours, in the biochemical and behavioral functions of organisms. This rhythm is achieved by the clock proteins, which are located suprachiasmatic nuclei (SCN). The clockwork is made up of positive and negative transcriptional regulators. The CLOCK-BMAL1 heterodimer activates transcription of the Per and Cry1 genes. The PER proteins interact with the CRY proteins; central sequences of the PER proteins interact CKI, and the resulting ternary complexes translocate into the nucleus, where they negatively regulate the transcription of Per and Cry genes. The PER and CRY proteins also positively regulate transcription of Bmal1. In this study, interacting domain of cryptochrome with BMAL, and PER has been identified using mammalian two-hybrid system. Based on the mammalian two-hybrid results, it seems to be both mice CRY1 and CRY2 C-termini are the regions that responsible for interaction with BMAL1 and PER2. Further analysis indicated that both BMAL and PER2 C-termini interact with mice CRYs. Finally, it has been shown that CRY-C termini itself cannot inhibit BMAL/CLOCK driven transcription, and CRY N-termini is required for forming stable complex with BMAL/CLOCK.

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Clock and Cycle Play Different Roles to Control the Survival of the Main Drosophila Clock Neurons

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The CLOCK and CYCLE partners act as the positive regulators of the two transcriptional feedback loops of the Drosophila circadian clock. In addition, they have been shown to be required for the expression of the PDF neuropeptide in the small ventral lateral neurons (s-LNv), which are the main pacemaker cells for the control of the rest-activity cycles of the animal. A developmental analysis of the clkjrk and cyc0 mutants was done to understand how the CLK and CYC proteins control PDF expression. Our results indicate that all specific markers of the LNv are gradually lost during brain development in the mutants, showing that CLK and CYC are required for clock cell differenciation. They also show that these two bHLH PAS-domain proteins do not have the same function in this developmental process. First, low temperature can restore PDF expression in the cyc0 but not in the clkjrk mutant. Second, CLK overexpression can partially restore the cyc0 developmental phenotype. Finally, we show that CYC is required for the proper phosphorylation of the CLK protein.

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Functional Characterization of Timeless-2 in Drosophila melanogaster

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Drosophila melanogaster has two timeless genes (tim1 and tim2 or timeout). While the circadian role of tim1 is well demonstrated, the role of its paralogue tim2 is poorly understood. We studied tim2 expression at different developmental stages. RT- and Real Time PCR analyses revealed a unique isoform of tim2 mRNA, expressed at variable levels throughout development (high in embryonic and 3rd instar larval stages, low during pupal life, and high in adult head). Early induction of tim2 specific knockdown (KD) caused lethality at late pupal stages, with mainly head development defects. Recently, a tim2- mutant strain has been generated. Homozygous tim2- flies die during development, at earlier stages with respect to flies carrying the dsRNAi construct. Moreover, cytological analysis of homozygous tim2- larval brains revealed the presence of chromosome breaks and deletions in ~ 30% of metaphases. To investigate the role of TIM2 in the adult circadian clock, we induced TIM2 depletion via dsRNAi triggered by neuronal or circadian drivers (elav-, tim1-, pdfGal4) and evaluated the effects on locomotor activity. We analysed also the locomotor behavior of heterozygous tim2-/+ adult flies. Our data indicated that behavioral periodicity was not affected in either tim2 KD lines or tim2-/+ flies. On the contrary, the responses to light-pulses, given during the night, seemed to be altered in tim2 KD lines, only directed by the elavGal4 driver. These data suggest a possible function for TIM2 in non canonical clock neurons, important for the synchronization of circadian behavior to environmental cues.

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Studies of Cyanobacterial Circadian Oscillator in Vitro and in Vivo

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Nagoya University

Cyanobacteria are the simplest organisms known to have a circadian clock. A circadian clock gene cluster kaiABC was cloned from the cyanobacterium Synechococcus elongatus PCC 7942 and a transcription/translation-based autoregulatory loop of kaiBC gene expression has been proposed to drive circadian rhythms. KaiA and KaiC were proposed as positive and negative regulators of kaiBC expression, respectively. In addition, KaiA-mediated activation of kaiBC expression was KaiC dependent, suggesting that KaiC also functions in a positive feedback process in the molecular oscillatory mechanism. However, we lately showed that KaiC phosphorylation cycle persisted even without transcription or translation. Moreover, self-sustainable oscillation of KaiC phosphorylation was reconstituted in vitro and period of the in vitro oscillation was stable despite temperature change. Therefore, KaiC phosphorylation cycle is assumed to be a basic timing process of the circadian clock. In this study, we investigated cyanobacterial circadian oscillator in vitro and in vivo.

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Normal Circadian Rhythms in Neurospora under Conditions of Choline Starvation

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A long conidiation rhythm of 60 hours or greater can be observed in Neurospora crassa chol-1 mutant strains grown in conditions of choline deficiency. Since this rhythm is neither temperature compensated nor nutritionally compensated, it is not circadian, but some models have suggested that it may be an aspect of the circadian oscillator. To evaluate this, germinated conidiospores grown under a choline depletion condition were transferred to a medium with choline supplementation at different time points within the circadian cycle. If the periods of the circadian clock were distinct in those two conditions, a phase delay would be expected in the supplemented cultures reflecting a longer period of clock under starvation. However, phases of all samples were close to the same, suggesting that a clock with normal period length (~24 hours) operates in both conditions. Consistent with this observation, the FREQUENCY protein also oscillated with two cycles in a two-day time course culture under conditions of choline depletion. Furthermore, a luciferase reporter gene under control of a circadian promoter can maintain an ~24 hour period rhythm for more than ten days in chol-1 strains. These resutls all indicate that there is a wild-type circadian clock operating under conditions of choline depletion. The data suggest that the elongated, non-temperature compensated and non-nutritionally compensated conidiation rhythm observed in race tubes of starved cultures could be a metabolic rhythm that masks expression of the underlying circadian clock or in some other way uncouples the act of conidiation from circadian control.

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Development of a Cycling Transcription Factor Library and Derived Novel Genomic Applications for Circadian Clock Study

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The Scripps Research Institute

The molecular basis of the plant circadian clock consists of interlocking feedback loops involving transcription factors (TF) and different types of putative transcriptional regulators. In addition to the well-studied LHY/CCA1 Myb TFs, recent forward genetic screens have identified another Myb TF called LUX ARRHYTHMO, strengthening our conceptualization of the plant clock as a transcription-based mechanism. Toward understanding the plant circadian clock and the network of TFs sustaining the regulation of several output pathways, we generated a cDNA library containing the 300 Arabidopsis cycling TFs. The library was used in four different applications and generated new interesting leads. In a yeast two-hybrid screen with TOC1, we identify one novel interactor. A second approach, called promoter hiking, involved fusing a series of truncated promoter fragments to the lacZ reporter gene and subsequently, each TFs is assayed in yeast one hybrid (Y1H) for activation. This approach allowed the identification of two new regulators of CCA1. The third approach involved a combination of bioinformatic analyses and Y1H screening. Potential phase-associated DNA elements were identified by mining circadian data from whole genome transcription profiling experiments. Each element was then synthesized in three tandem repeats, fused to the lacZ reporter gene and assayed in yeast one hybrid assays against the TF library. This led to the identification of new circadian regulated elements and potential interacting TFs. The

last approach is an overexpression screen in planta. The effect of each TF driven by the strong constitutive 35S promoter was monitored by analyzing toc1:luc expression. Two new genes affecting the circadian profile of the reporter were identified and are under investigation.

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Spatiotemporal Coordination of Conflicting Functions of the Neurospora Clock Protein Frequency

Michael Brunner

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Frequency (FRQ), the central component of the circadian clock of Neurospora, exerts a negative and a positive role in interconnected feedback loops. In the negative limb it inhibits its transcription factor White Collar Complex (WCC), while it supports accumulation of high levels of WCC in the positive limb. We show that these contradictory functions of FRQ are confined to distinct subcellular compartments and coordinated in temporal fashion. Transcriptional inactivation of WCC requires nuclear FRQ and occurs early in a circadian cycle, i.e. shortly after the onset of FRQ expression. In contrast, support of WCC accumulation depends on cytosolic FRQ. It occurs on a posttranslational level when high amounts of FRQ have accumulated. The transcriptional function of FRQ in the negative limb and its posttranslational function in the positive limb are independent and associated with distinct regions of FRQ. Phosphorylation of two serine residues within the PEST-2 region triggers the maturation of FRQ from a nuclear repressor toward a cytoplasmic activator.

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Two Circadian Timing Circuits in Neurospora crassa *Cells Share Components and Regulate Distinct Rhythmic Processes*

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In *Neurospora crassa*, the circadian clock regulates the production of conidia and the rhythmic expression of a number of genes and proteins. Using microarrays, we identified an evening-specific gene, ccg-16, that has rhythms in mRNA accumulation that persist in the absence of FRQ in constant darkness. In addition, the ccg-16 rhythm is observed in cultures grown in constant light, a condition in which FRQ is expressed at high non-rhythmic levels. The rhythm in ccg-16 mRNA accumulation is temperature compensated, establishing that ccg-16 is controlled by a novel temperature-compensated oscillator that does not require FRQ. However, the oscillator controlling ccg-16 rhythmicity requires WC-1 and WC-2 for activity. We call this oscillator the WC-FLO (WC-dependent FRQ-less oscillator). Rhythmic accumulation of WC-1 protein was also observed in the absence of FRQ and in constant light, suggesting the possibility that WC-FLO components are involved in establishing WC-1 protein rhythms. Together, our data demonstrate that the circadian system of Neurospora involves multiple circadian oscillators that share components and contribute to the diversity and stability of circadian rhythms.

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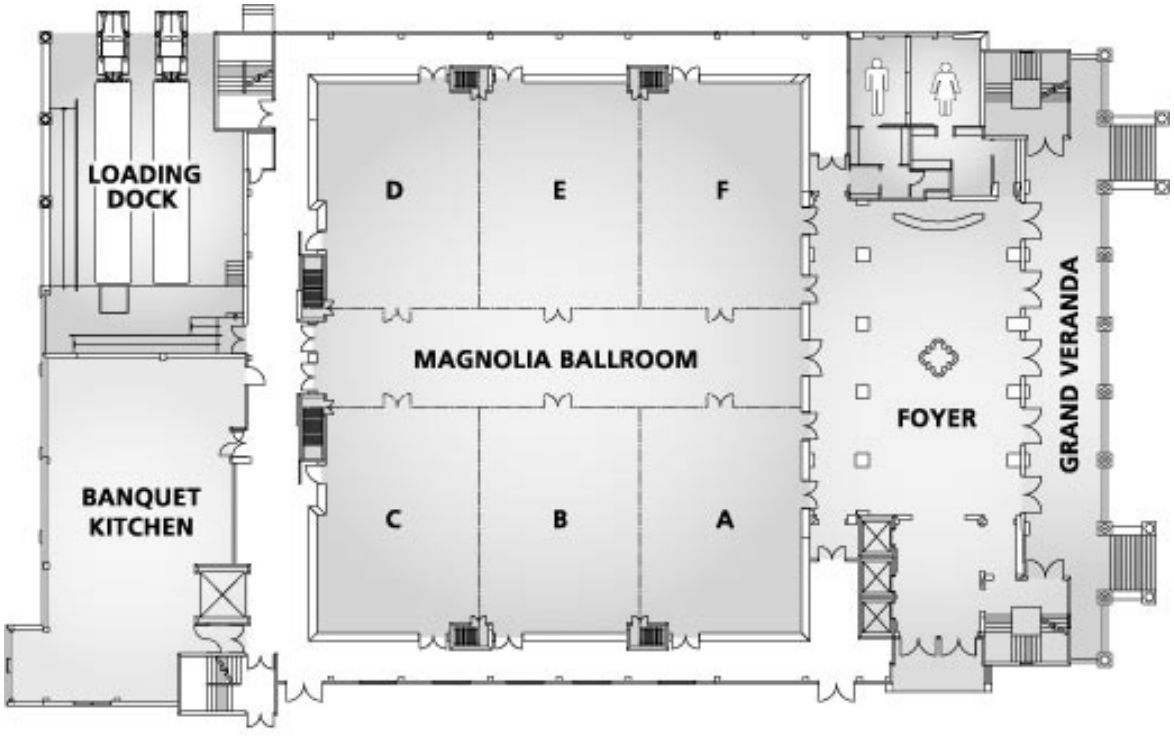
Maps



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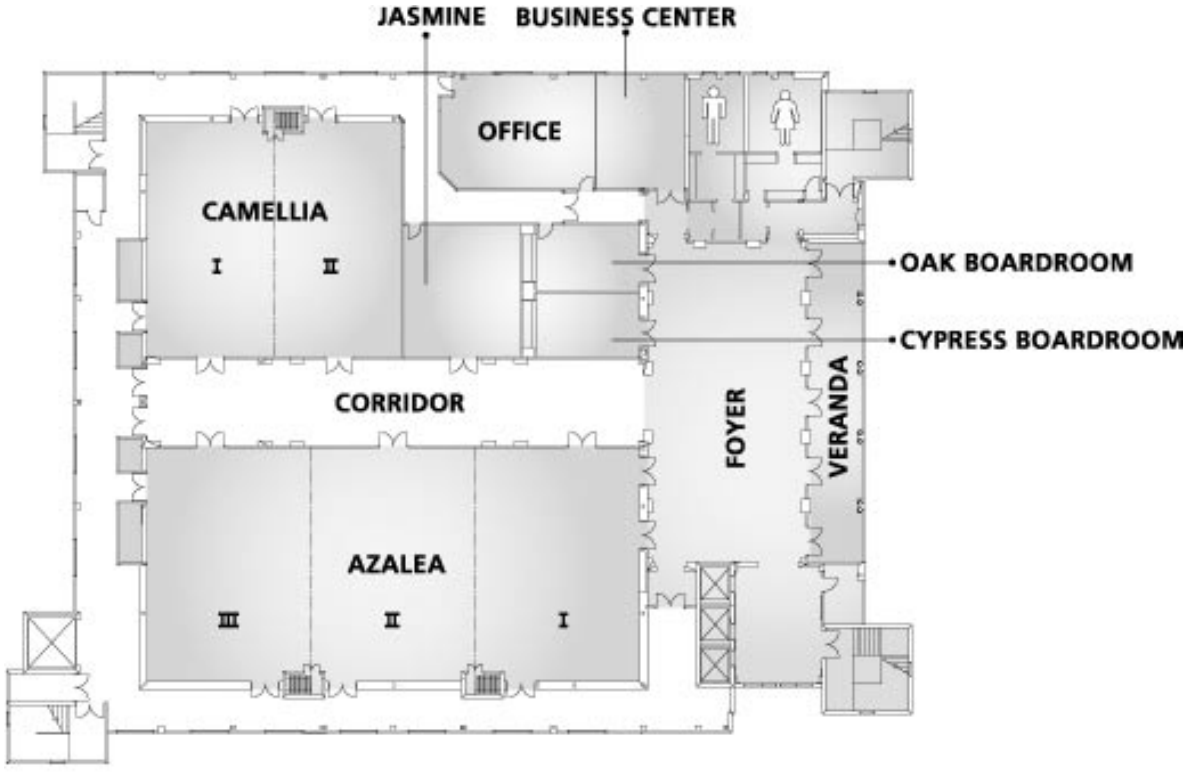

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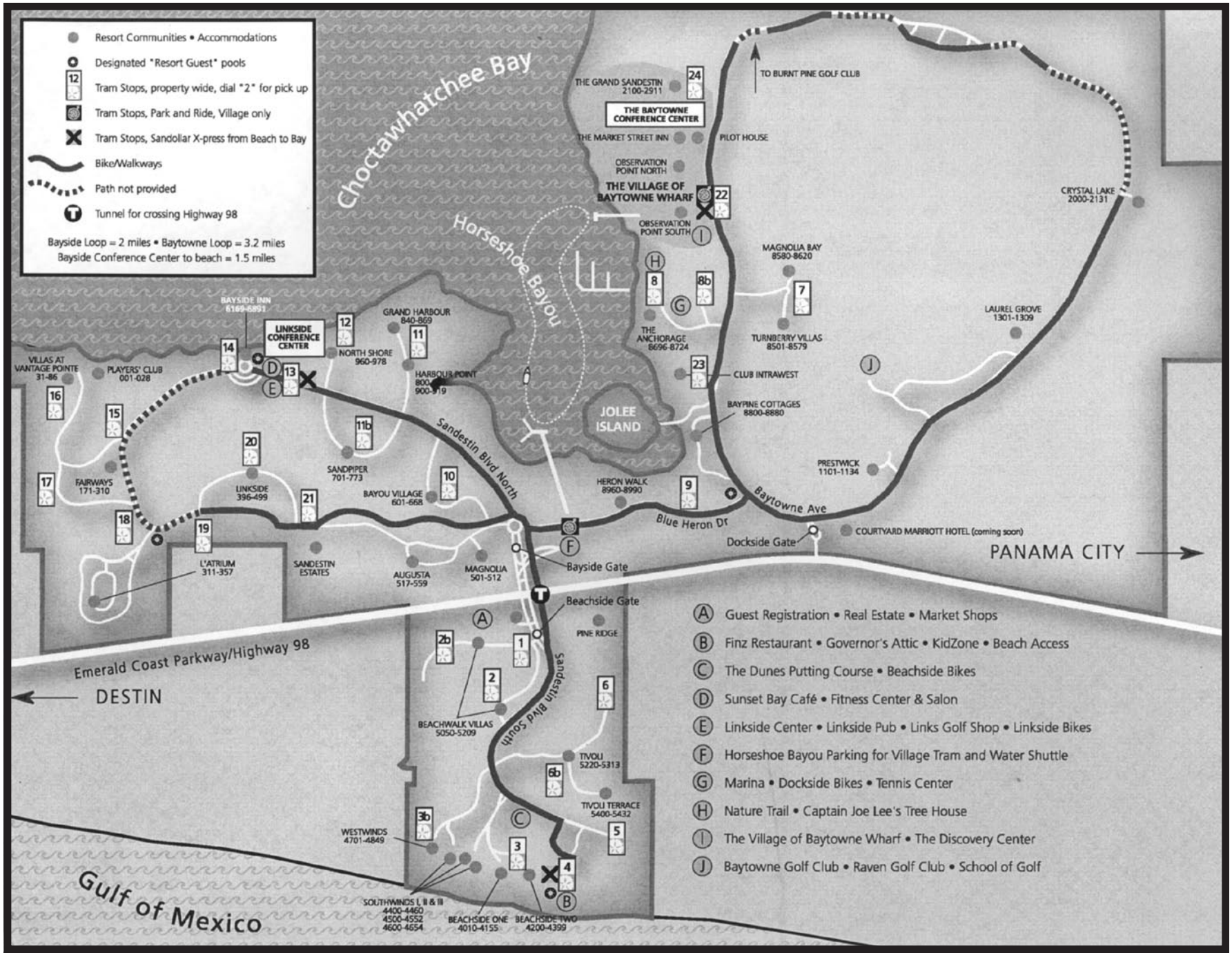
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