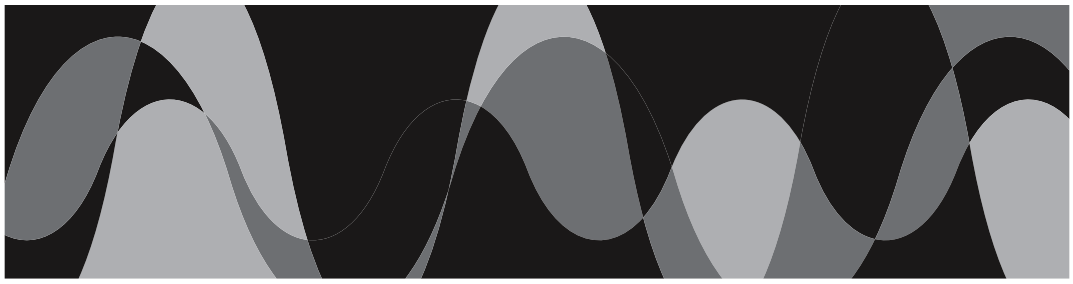

Ninth Meeting

Society for Research on Biological Rhythms

Program and Abstracts

SRS/SRBR

June 23, 2004

SRBR

June 24–26, 2004

Whistler Resort • Whistler, British Columbia

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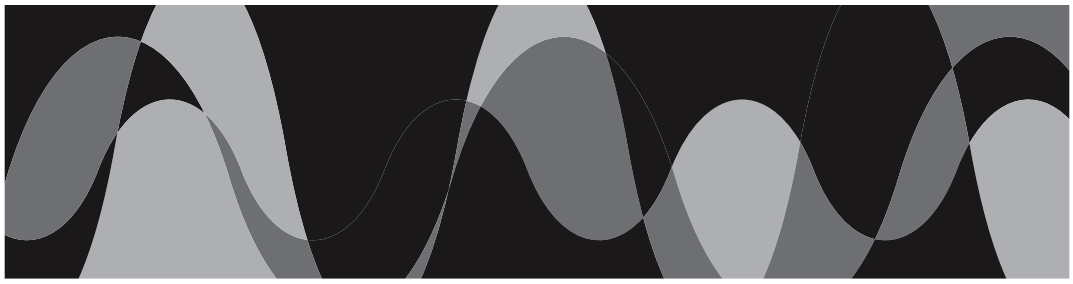

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The SRBR wishes to thank the following for their contributions:

ALA Scientific Instruments
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National Aeronautics and Space Administration
National Institutes of Mental Health
National Science Foundation
Sage Publications

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General Information

Registration

Meeting registration will take place on Wednesday, June 23 from 17:00 until 18:00 and on Thursday, June 24 from 8:00 until 11:00 and 15:00 until 17:00 in the Great Room of the Whistler Conference Centre. Hotel check-in will be at the individual properties.

Early Registration:
Postmarked by May 7, 2004

SRBR Member: $325

Non-Member: $375

SRBR Student Member: $165

Non-Member Student: $190

Guest Registration: $75

Late Registration:
Postmarked after May 7, 2004

SRBR Member: $350

Non-Member: $400

SRBR Student Member: $175

Non-Member Student: $200

Guest Registration: $100

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

The field of circadian biology continues to move forward at a fast pace. The research presented at this year's biennial SRBR meeting reflects the ongoing, important discoveries. Whistler, British Columbia, Canada, is a welcoming and refreshing new venue.

Organizing this meeting would not have been possible without generous contributions from each member of the Organizing Committee. I need to single out Vinnie Cassone for doing more than his share as Program Committee Chair. I also thank Scott Miller and his associates at the Office of Continuing Education, University of Illinois at Urbana-Champaign for providing the necessary infrastructure.

Finally, I want to thank every one in attendance—your participation is what makes this meeting a success. Thank you for your support.

Steven Reppert
President, SRBR

SRBR Information and Message Desk

The Society will maintain an information desk in the Great Room of the Whistler Conference Centre from 8:00 to 11:00 on June 24–26, and from 15:00 to 17:00 on June 24–25. 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.

About the Village

Whistler is nestled amongst the spectacular Coast Mountains of British Columbia, Canada, only 120 kilometers/75 miles north of Vancouver. The charming alpine Village of Whistler is home of Whistler and Blackcomb Mountains.

A cluster of glacier-fed lakes reflects the surrounding mountain peaks that embrace Whistler Village. In addition to famous restaurants, engaging nightlife, and a variety of shops, Whistler also offers a bounty of summer activities.

Conference Centre Information

The Whistler Conference Centre is conveniently located within walking distance of all conference hotels. All SRBR scientific sessions and social events will take place at the Centre.

Instructions to Presenters

A new set of posters will be presented each day. Posters should be set-up on the day of presentation between 8:00–10:00. The poster presentation schedule is listed in the scientific program schedule. Final poster session posters must be removed on Saturday, June 26 by 14:00.

Slide talk presenters should give their slides (clearly numbered and in correct order) to the projectionist 15 minutes before the start of the session.

Computer projectors will be set up in each meeting room. PowerPoint presenters should bring their data on PC-compatible zip disk or CD-rom to the registration area to be loaded onto a presentation laptop computer during the registration period on Thursday, June 24.

Social Events

The SRBR Opening Reception will begin at 19:00 on Wednesday, June 23 in the Great Room of the Whistler Conference Centre. A banquet for conference attendees and guests will be held in the Ballroom of the Whistler Conference Centre on Saturday, June 26 at 20:00.

Schedule

All events will occur in the Whistler Conference Centre unless otherwise noted.

Wednesday, June 23

13:00 **Workshop**

19:00 **Reception**—*Great Room*

Thursday, June 24

08:30–10:30 **National Academy Symposium 1**—*Rainbow Theatre*

10:30–11:00 **Coffee Break**—*Great Room*

11:00–13:00 **Slide Sessions 1–3**

1) Entrainment I—*McGuire*
2) Mathematical/Theoretical Biology of Clocks—*Sky Ballroom B*
3) Circadian Pacemaker Output—*Rainbow Theatre*

13:00–14:30 **Lunch Break**

13:00–14:30 **Poster Session Setup**

Posters 138–223—*Sea Ballroom*

14:30–16:30 **Symposium 3**

Clinical Implications of Clock Function—*Sky Ballroom A*

Symposium 3

From Clocks to Metabolism and Back—*Sky Ballroom B*

13:30–16:30 **Workshop**

National Space Biomedical Research Institute Workshop: Circadian and Sleep Countermeasures for Spac Exploration

NASA: National Space Biomedical Research Institute Workshop: Circadian and Sleep Countermeasures for Space Exploration—*McGuire*

16:30–17:00 **Coffee Break**—*Great Room*

17:00–18:00 **Presidential Lecture**

Rae Silver—*Sky Ballroom B*

18:00–20:00 **Dinner Break**

20:00–22:00 **Poster Session 1**

Posters 138–223—*Sea Ballroom*

Friday, June 25

07:00 **Poster Session Setup**

Posters 224–312—*Sea Ballroom*

08:30–10:30 **Symposium 4**

Circadian Control of Sleep—*Rainbow Theatre*

Symposium 5

Phylogeny and Ecological Consequences of Biological Clocks—*Sky Ballroom B*

10:30–11:00 **Coffee Break**—*Great Room*

11:00–13:00 **Slide Sessions 4–6**

4) Circadian Regulation of Photoreceptor—*McGuire*

5) Infraction Rhythms—*Sky Ballroom A*

6) Integration of Pacemaker Function—*Rainbow Theatre*

7) Genomic and Pharmacological Analyses of Sleep—*Sky Ballroom B*

13:00–14:30 **Lunch Break**

14:30–16:30 **Symposium 6**

Photoperiodic Time Measurement-Molecular Mechanisms—*Rainbow Theatre*

Symposium 6

Non-Clock Functions for Clock Genes—*Sky Ballroom B*

14:30–16:30 **Workshop**

Very Large Phase Shifts of the Circadian Clock—*McGuire*

16:30–17:00 **Coffee Break**—*Great Room*

17:00–18:00 **Aschoff-Pittendrigh Lecture**—*Sky Ballroom B*

18:00–20:00 **Dinner Break**

20:00–22:00 **Poster Session 2**

Posters 224–312—*Sea Ballroom*

Saturday, June 26

08:30–10:30 **Symposium 8**

Circadian System Organization: Hierarchies, Networks or Both—*Rainbow Theatre*

Symposium 9

Neural Outputs of Biological Clocks—*Sky Ballroom B*

10:30–11:00 **Coffee Break**—*Great Room*

11:00–13:00 **Slide Sessions 8–10**

8) Cell Biology—*McGuire*

9) Clinical Applications—*Rainbow Theatre*

10) Genomics and Molecular Genetics—*Sky Ballroom*

13:00–14:30 **Lunch Break**

14:30–16:30 **Symposium 10**

The Next Step: Structure Biology of Clock Proteins—*Sky Ballroom B*

14:30–16:30 **Workshop**

New Methods for Biological Clocks Analysis—*McGuire*

16:30–17:00 **Coffee Break**—*Great Room*

17:00–18:00 **Business Meeting**—*Sky Ballroom B*

20:00–22:00 **Banquet—***Sea Ballroom*

Scientific Program

Thursday June 24

08:30–10:30 **National Academy Symposium 1**—*Rainbow Theatre*

Chairman: Steve Reppert, University of Massachutes

The Enigma of Translational Control in the Gonyaulax Clock Mechanism
Woody Hastings, Harvard University

Drosophila and Circadin Rhythms , 1982–2004: Molecules, Cells and Systems
Michael Rosbash, Brandeis University

Joseph Takahashi Northwestern University

10:30–11:00 **Coffee Break**—*Great Room*

11:00–13:00 **Slide Session 1**—*McGuire*

Entrainment 1

Chair: Shinsuke Kutsuna, Yokohama City University

11:00 **5 • Synchronization by Light-dark Cycle of Circadian Rhythm of Motor Activity in Blind Primate** • John F Araujo,Laboratrio de Cronobiologia, Departamento de Fisiologia, CB/UFRN, Natal, Brasil

11:15 **6 • Entrainment of the Master Circadian Clock by Scheduled Feeding** • Abel Bult-Ito, Behavioral and Evolutionary Neuroscience Laboratory, Alaskan Basic Neuroscience Program, Institute of Arctic Biology

11:30 **7 • Entrainment of the Master Circadian Clock by Feeding Schedules** • Carolina Escobar, Facultad de Medicina,

11:45 **8 • Entrainment of the Human Circadian Pacemaker to Longer-than-24h-days with Intermittent Bright Light** • Claude Gronfier, Division of Sleep Medicine, Harvard Medical School and Brigham and Women's Hospital,

12:00 **9 • Input Pathway of the Circadian Period in Cyanobacterium Synechococcus Elongatus PCC 7942** • Shinsuke Kutsuna,Yokohama City University

12:15 **10 • Initial Steps Towards a Circadian Compromise for Night Shift Workers** • Clara Lee, Eastman CI Biological Rhythms Research Lab, Rush University Medical Center, Chicago

12:30 **11 • Daily Activity Patterns of a Nocturnal and a Diurnal Rodent in a Semi-natural Environment** • Roberto Refinetti,Circadian Rhythm Laboratory, University of South Carolina,

12:45 **12 • A Place for ZTL in Circadian Phototransduction a Place for ZTL in Circadian Phototransduction** • David E. Somers, Ohio State University

11:00–13:00 **Slide Session 2**—*Sky Ballroom B*

Mathematical/Theoretical Biology of Clocks

Chair: Andrew J. Millar, University of Warwick

11:00 **13 • Stochastic Sensitivity Analysis of the Circadian Gene Network** • Neda Bagheri, University of California, Santa Barbara,

11:15 **14 • How Should We Analyze Activity Rhythms?** • Phil Gehrman, Center for Sleep, University of Pennsylvania

11:30 **15 • Is the Mammalian Circadian Clock a Resonant-circuit Oscillator?** • C. V. Hollot, Electrical and Computer Engineering, UMass Amherst

11:45 **16 • A Proposal for Robust Temperature Compensation of Circadian Rhythms** • Christian I. Hong, Dartmouth Medical School

12:00 **17 • Modeling Temperature Compensation in Circadian Rhythm** • Gen Kurosawa, Kyushu University

12:15 **18 • Why Do Clock Mechanisms Have Multiple Loops?** • Andrew J. Millar, Interdisciplinary Programme for Cellular Regulation, Department of Biological Sciences & Mathematics Institute, University of Warwick

12:30 **19 • Martian Circadian Rhythms: a Biosignature?** • Joseph D. Miller, Keck School of Medicine at USC

12:45 **20 • Systems Biology on Mammalian Circadian Rhythms** • Hiroki R. Ueda, Center for Developmental Biology, RIKEN

11:00–13:00 **Slide Session 3—**Rainbow Theatre

Circadian Pacemaker Output

Chair: Gary Pickard, Colorado State University

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11:15 **22 • Do Humoral Signals Regulate Circadian Rhythms of Gene Expression in Mouse Peripheral Organs?** • Eric L. Bittman, Dept of Biology, University of Massachusetts, Amherst

11:30 **23 • SCN Regulation of Circadian Rhythms of Gene Expression in Hamster Peripheral Organs** • Hongnian Guo, Dept of Biology , University of Massachusetts, Amherst MA

11:45 **24 • The Ion Channel, Narrow Abdomen, Functions in Drosophila Pacemaker Neurons to Regulate Circadian Behavior** • Bridget C. Lear, Northwestern University

12:00 **25 • LD and DD Behavioral Circadian Rhythms Are Controlled by Different Clock Neurons in the Drosophila Brain** • Francois Rouyer, Institut de Neurobiologie Alfred Fessard,

12:15 **26 • Evidence That Cardiotrophin-like Cytokine is an Output Signal of the Mammalian Circadian Clock** • Sebastian Kraves, Harvard Medical School

12:30 **27 • Diffusible Factors from Immortalized SCN2.2 Cells Modulate GnRH Secretion from Co-cultured Immortalized GT1-7 Cells** • Rachel White ,Department of Reproductive Medicine, University of California, San Diego, La Jolla

12:45 **28 • Per1-luc Expression Rhythms of Central and Peripheral Oscillators in LL-treated Arrhythmic Rats •** Tomoko Yoshikawa, Department of Biology, University of Virginia, Charlottesville, VA

13:00–14:30 **Break; Put up Posters 138-223—***Sea Ballroom*

13:30–16:30 **Workshop 1**—*Sky Ballroom B*

National Space Biomedical research institute Workshop circadian and Sleep Countermeasures for Space Exploration

Organizer: Gianluca Tosini, Morehouse School of Medicine

Chair: Charles Czeisler, Harvard University

Co-Chair: George Brainard, Thomas Jefferson University

Speakers: Jeffery Sutton, President of the national Space biomedical Research Institute

Chuck Czeisler, Harvard University

George Brainard, Thomas Jefferson University

Michael Menaker, University of Virginia

Naomi Rodgers, NASA

David Sliney, NASA

14:30–16:30 **Symposium 2—***Sky Ballroom*

Clinical Implications of Biological Clock Functions

Chair: Elizabeth Klerman–Harvard Medical School

Speakers: Thomas J. Balkin

Walter Reed, Army Institute of Research
REM Sleep Function: Maybe It's the Rhythm (Not the Tune)

Helen Burgess, Rush University Medical Center
Sleep Freely to Know Your DLMO

Elizabeth Klerman, Division of Sleep Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston MA
Differential Effect of Sleep Disruption on Menstrual Cycle Dynamics

Mark Quigg, University of Virginia
Interactions Between Circadian Rhythms and Epilepsy

Steven A. Shea, Brigham & Women's Hospital
The Circadian Aspects of Noicturnal Asthma

14:30–16:30 **Symposium 3**—Sky Ballroom B

From Clocks to Metabolism and Back

Chair: Jaga Giebultiwicz

Speakers: Ralph Mistlberger, Department of Psychology, Simon Fraser University
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Steve McKnight, McKnight Laboratory Department of Biochemistry UT Southwestern Medical Center
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Robert R Klevecz, Cellular Dynamics Group Beckman Research Institute
Genome-wide Transcriptional Cycles gate DNA Replication and Cell Division in Yeast and Mammals

16:30–17:00 **Coffee Break**—*Great Room*

17:00–18:00 **Presidential Lecture**—*Sky Ballroom B*

Rae Silver

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206 • Molecular Basis of Biorhythmicity and Environmental Signal Transduction in the Annelid Polychaete Nereis Virens (Sars) • Cas Kramer, Department of Genetics, University of Leicester, UK.;

207 • Mechanisms Underlying Circadian Modulation of Memory Formation in Aplysia • Lisa C. Lyons, University of Houston

208 • Feeding Is Not a More Potent Zeitgeber than the Light-dark Cycle in Drosophila • Katsutaka Oishi, Institute of Advanced Industrial Science and Technology

209 • Effects of Light Regimes on Fitness Traits of Drosophila Melanogaster Are Mediated Through Circadian Rhythms • Dhanashree Paranjpe, Jawaharlal Nehru Centre for Advanced Scientific Research

210 • Molecular Targets for the Action of Lithium on Circadian Clocks • Vijay Kumar Sharma, Chronobiology Laboratory, Evolutionary & Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India

211 • Female Odours Affect Rhythmic Mating Frequency and Male Pheromone Response in the Moth Spodoptera Littoralis [Noctuidae] • Germund Silvegren, Lund University

212 • Molecular Basis of Circadian and Circatidal Rhythmicity in a Crustacean, Eurydice Pulchra • Lin Zhang, Department of Genetics, University of Leicester, UK

Mammal

213 • The Circadian Regulator Period2 Functions in Tumor Suppression in vivo • Loning Fu, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas

214 • Photosensibility in the Djungarian Hamster (Phodopus Sungorus) Revealed by Trans-pineal Microdialysis • Annika Herwig, Neurobiologie des Rythmes, , Strasbourg, France; 2 School of Veterenary Medicine, Hannover, Germany

215 • The Role of rPER2 Nuclear Localization Sequence in Mammalian Circadian Clock • Koyomi Miyazaki, National Institute of Advanced Industrial Science and Technology

216 • 24-hour Expression Profiles of Core Clock Components in an Equine Peripheral Tissue • Barbara A. Murphy, University of Kentucky

217 • Appositions Between Orexin Fibers and Neuropeptide-Y cells in the Grass Rat Intergeniculate Leaflet • Joshua P. Nixon, Department of Zoology, Michigan State University, East Lansing, MI

218 • Effects of Simulated Microgravity on Circadian Rhythms of Cardiovascular Physiology and Clock Gene Expression in Laboratory Rats • Hillary M. Olin,

Department of Biology and Center for Biological Clocks Research, Texas A&M University, College Station, TX

219 • Restoration of Rhythmicity in Per2/Cry1 Double Deficient Mice in Constant Light (LL) Conditions • Henrik Oster, Max Planck Institute of Experimental Endocrinology Hannover, Germany;

220 • Circadian Locomotor Rhythms in Mice Are Not Altered by the Anti-tumor Drug, Cyclophosphamide • E. Todd Weber, Rider University, Department of Biology, Lawrenceville, NJ.

Mathematical

221 • InterlockingLoops in the Molecular Mechanism Underlying Circadian Rhythms • Emery Conrad, Departments of Mathematics & Biology, Virginia Polytechnic Institute & State University

222 • Development of Two-dimension Manifolds for the Representation of High Dimension Mathematical Models of the Intra Cellular Mammalian Circadian Clock • Premananda P Indic, Brigham & Women's Hospital/Harvard Medical School, Boston, MA

223 • Theoretical Approach : Coupling Between Circadian Gene Activity and Other Cellular Process • Ivona Jakovljevic , TU Darmstadt

Friday June 25, 2004

7:00 **Poster Set up 224–312—***Sea Ballroom*

8:30–10:00 **Symposium 4**—*Rainbow Theatre*

Circadian Control of Sleep

Chair: Louis Ptacek, HHMI/UCSF

Speakers: Juliette Faraco, Stanford University
Narcolepsy, Hypocretins and Beyond

Louis J. Ptacek, HHMI/UCSF
Genetics of Human Circadian Rhythms.

Chris Jones, Jun LU–Harvard University

8:30–10:00 **Symposium 5**—*Sky Ballroom*

Phylogeny and Ecological Consequences of Biological Clocks

Chair : Charalambos Kyriacou, University of Leicester

Speakers: William Bradshaw, University of Oregon
Evolution of Photoperiodic Time Measurement: Does It Depend on the Circadian Clock?

Eberhard Gwinner, Max-Planck Institute of Ornithology
Circannual Rhythms and Photoperiodism—A Reaction Norm Approach

Carl Hirschie Johnson, Dept. of Biological Sciences, Vanderbilt University, Nashville, TN
Circadian Systems: Adaptive Value and Selective Pressures

Martha Merrow, Institute for Medical Psychology, University of Munich, Germany
Enhanced Phenotyping of Circadian Traits Using Simple Systems

10:30 **Coffee Break**—*Great Room*

11:00–13:00 **Slide Session 4**—*Great Room*

Circadian Regulation of Photoreceptor

11:00 **52 • FKF1: Photoperiodic Blue-light Receptor for Flowering in Arabidopsis** • Takato Imaizumi, The Scripps Research Institute

11:15 **53 • Phototransduction for the Human Retinohypothalamic Tract: High Sensitivity to Short Wavelength Light** • G. Brainard., Rollag Department of Neurology, Thomas Jefferson University, Philadelphia, PA,.

11:30 **54 • Regulation of Photoreceptor Per1 and Per2 by Light, Dopamine, and a Circadian Clock** • Joseph C. Besharse, Medical College of Wisconsin

11:45 **55 • Drosophila CRYPTOCHROME: Photoreception Mechanisms and Unexpected Roles of Its Two Structural Domains** • Ania Busza, University of Massachusetts Medical School

12:00 **56 • Circadian Pacemaker Cells Transmit and Modulate Visual Information to Control a Rapid Behavioral Response** • Ben Collins, New York University

12:15 **57 • Comparing the Effect of an Oral Administration of Melatonin on the Electroretinogram (ERG) of Humans and Dogs** • Mark. Hebert, Department of Ophthalmology, Laval University, Quebec, PQ, Canada.

12:30 **58 • Temporal Response Characteristics of Circadian Photoreceptors** • Camille Rieux, H.M. INSERM U-371, Cerveau et Vision, Bron, France

12:45 **59 • Differential Regulation of Two Arylalkylamine-n-acetyltransferase in the Gilthead Seabream (Sparus Aurata)** • Benny Ron, National Center for Mariculture, Israel Oceanographic and Limnological Research

11:00-13:00 **Slide Session 5**—*Sky Ballroom*

Infradian Rhythms

Chairman:

11:00 **60 The Avian Circadian System is Modified According to Migratory Status** • Paul A. Bartell, Texas A & M University

11:15 **61 • Chronobiological Plasticity Is Associated with Division of Labor in the Bumble Bee Bombus Terrestris** • Guy Bloch, Department of Evolution, Systematics, and Ecology, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

11:30 **62 • Arousal Episodes in Hibernating Golden-mantled Ground Squirrels: Why Wake Up?** • Patricia J. DeCoursey, Department of Biological Science, University of South Carolina, Columbia, SC

11:45 **63 • Diapause and Circadian Clock Gene Expression in the Linden Bug, Pyrrhocoris Apterus** • David Dolezel, Institute of Entomology

12:00 **64 • Feeding Condition Modifies the Photoperiodic Response of Dispersal Characteristics in the Water Strider, Aquarius Paludum** • Tetsuo Harada ,Laboratory of Environmental Physiology, Faculty of Education, Kochi University, Kochi, Japan

12:15 **65 • A Change in Gonadal Hormone Receptor Expression in the SCN Is Related to a Developmental Change in Tau** • Dan Hummer, University of Michigan

12:30 **66 • Competition, Resource Levels, and Temperature Rhythms of Golden Spiny Mice: Do Overt Responses Reflect Entrainment?** • Noga Kronfeld-Schor, Department of Zoology, Tel Aviv University, Tel Aviv, Israel

12:45 **67 • Photoperiodic Regulation of Gene Expression in Major Body Organs of Siberian Hamsters** • Andrew Loudon, University of Manchester

11:00-13:00 **Slide Session 6**—*Rainbow Theatre*

Integration of Pacemaker Function

11:00 **68 • Intra-SCN Signaling: Gastrin-Releasing Peptide Activates "Cap" Cells of the SCN** • Michael C. Antle, Department of Psychology, Columbia University, New York, NY

11:15 **69 • Dexras1 Is a Molecular Coupler of Photic and Non-Photic Cues to the Circadian Clock** • Hai-Ying Mary Cheng, Ontario Cancer Institute/Princess Margaret Hospital

11:30 **70 • Gating of Arylalkylamine N-acetyltransferase Gene Expression in the Mouse Pineal Gland** • Chiaki Fukuhara, Neuroscience Institute, Morehouse School of Medicine, Atlanta GA 30310

11:45 **71 • Circadian Disruptions in Mice with a Targeted Mutation of the Calbindin-D28K Gene** • Lance J. Kriegsfeld, Department of Psychology, Columbia University New York, NY

12:00 **72 • Roles of Vasoactive Intestinal Polypeptide in Circadian Rhythm Processes** • Hugh Piggins, University of Manchester

12:15 **73 • Light and Suprachiasmatic Nucleus Interact in the Regulation of Body Temperature** • FAJL SCHEER*, Department of Hypothalamic Integration Mechanisms, Netherlands Institute for Brain Research, Amsterdam, The Netherlands

12:30 **74 • Single Cell Circadian Rhythms of Luminescence in Cultures from MPER2-LUC Knockin Mice** • David K. Welsh, Dept. Psychiatry, UCSD School of Medicine, La Jolla, CA, Dept. Cell Biology, The Scripps Research Institute, La Jolla, CA

12:45 **75 • Control of Transcript Rhythms in Drosophila by the Circadian Clock and Light** • Herman Wijnen, Laboratory of Genetics The Rockefeller University, New York, NY

11:00-13:00 **Slide Session 7**

Genomic and Pharmacological Analyses of Sleep

11:00 **76 • The Effect of Modafinil on the Circadian and Homeostatic Determinants Underlying the Deterioration of Neurobehavioral Performance Associated with Transmeridian Travel and Sleep Deprivation** • Scott P. Grady, Division of Sleep Medicine, Department of Medicine, Brigham and Women's Hospital and Division of Sleep Medicine, Harvard Medical School, Boston, MA

11:15 **77 • The Molecular Basis of the Interaction between Sleep and Feeding Behaviors in Drosophila Melanogaster** • Susan T. Harbison, University of Pennsylvania

11:30 **78 • Sleep and Circadian Effects of Modafinil during Five Nights of Sleep Restriction •** Timothy H. Monk, University of Pittsburgh

11:45 **79 • Circadian Distribution of the Sleep/Wake Cycle Is Altered in ob/ob Mice •** Jonathan Shelton, Center for Sleep and Circadian Biology, Northwestern University

12:00 **80 • Sleepless: A Mutation that Increases Active Sleep in Neonatal Mice and Wake Time in Adults •** Fred Turek, Northwestern University, Evanston, IL

12:15 **81 • Effects of Sleep Deprivation on Gene Expression and the Immune Response in Drosophila Melanogaster •** Julie A. Williams, HHMI, University of Pennsylvania School of Medicine

12:30 **82 • Modulation of Circadian Light Sensitivity in Drosophila by Serotonin Signaling •** Quan Yuan,Department of Neuroscience, University of Pennsylvania, Philadelphia, PA,

12:45 **83 • Circadian Control and Locomotor Feedback in the Regulation of Hypocretin-1 (orexin A) in Sleep Consolidating Squirrel Monkeys and Polyphasic Rats •** Jamie M. Zeitzer, Department of Psychiatry and Behavioral Sciences; Stanford University; Palo Alto CA

13:00-14:30 Lunch Break

14:30-16:30 **Symposium 6**—*Rainbow Theatre*

Photoperiodic Time Measurement–Molecular Mechanisms

Chair : Shizufumi Ebihara–Nagoya University Japan

Speakers: Takato Imaizumi, University of California–San Deigo

Gerald A. Lincoln, MRC Human Reproductive Sciences Unit, Edinburgh, Scotland
Melatonin-controlled Clock Genes in Calendar Cells

Peter J. Morgan, Molecular Endocrinology Group, Rowett Research Institute, Greenburn Road, Aberdeen, Scotland
Photoperiodically Regulated Gene Expression in the Hypothalamus and Its Link to Body Weight

Takashi Yoshimura, Graduate School of Bioagricultural Sciences, Nagoya University, Japan
Molecular Analysis of Photoperiodic Time Measurement in Birds and Mammals

14:30-16:30 **Symposium 6**—*Sky Ballroom B*

Non-Clock Functions for Clock Genes

Chair : David Earnest–Texas A&M University

Speakers: Urs Albrecht, Dept. of Medicine, Div. Of Biochemistry, University of Fribourg, Switzerland
Circadian Genes and Addiction

Christopher Bradfield, McArdle Laboratory for Cancer Research
Progressive Arthropathy in Mice with a Targeted Disruption of the Mop3 Locus

David Earnest, Texas A&M University Health Science Center
Tick-tox: Clock Gene Expression and Interactions Between the Molecular Pathways for the Regulation of Circadian Rhythms, Toxin Metabolism and Development

Cheng Chi Lee, University of Texas Health Science Center
The Function and Regulation of mPER2

14:30–16:30 Workshop

Very Large Phase Shifts of the Circadian Clock : A SRBR Workshop

Speakers: Joe Miller, University of Southern California

David Glass, Kent State

Marilyn Duncan, University of Kentucky

William Schwartz, University of Massachutes at Wooster

Ralph Mistlberger, Simon Fraser University

Wijesuriya Dayawansa - ???

16:30–17:00 Coffee Break—*Great Room*

17:00–18:00 Aschoff –Pittendrigh Lecture

David Klein–National Institute of Health

18:00-20:00 Dinner Break

20:00-22:00 Poster Session 224–312—*Sea Ballroom*

Molecular

224 • Characterization of the Nocturnin-Containing Deadenylase Complex • Julie E. Baggs, Department of Biology, University of Virginia, Charlottesville, VA

225 • A C-Terminal Truncation Mutation in Zebrafish Cry1c Shortens the Period of Locomotor Rhythms • Hugo M. Borsetti, Dept. of Biology and Biochemistry, University of Houston, Houston, Tx

226 • Does Light Start or Synchronise Circadian Clocks in Culture? • Amanda-Jayne Carr, University College London

227 • Perturbations Caused by Per Mutations: Phenotyping of Per1 and Per2 Deficient Mice • Robert Dallmann, School of Veterinary Medicine Hannover, Germany

228 • New Regulatory Aspects of bZIP Transcription Factor E4BP4: Negative Regulation of E4BP4 by Casein Kinase 1 e • Masao Doi, Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo, Japan,

229 • Effects of Experimental Chronic Jet-lag on Clock and Cell Cycle Gene Expression • Elizabeth Filipski ,INSERM E 0354 Ç Cancer chronotherapeutics È, H™p. P. Brousse, Villejuif, France

230 • Drosophila's Second Clock Loop Is Essential for Temperature Compensation • Daniel Forger, NYU Department of Biology, New York

231 • Photic Entrainment Mechanism of Per1 Rhythm •Akiko Fukuda, Vanderbilt University

232 • Role of p38 Mitogen-activated Protein Kinase in the Chick Pineal Circadian Clock System • Tsuyoshi Hirota, University of Tokyo

233 • A Role for Glycogen Synthase Kinase-3˚Ë in Mammalian Circadian Clock • Chisato Iitaka, National Institute of Advanced Industrial Science and Technology

234 • Real-time Monitoring System in Rat-1 Cell Culture: Toward the SDissection of the Mammalian Circadian Gene Expression • Mariko Izumo, Department of Biological Sciences, Vanderbilt University, Nashville, TN,

235 • Genetic Analysis of Ectopic Clocks in Drosophila • Valerie L. Kilman, Dept of Neurobiology and Physiology, and Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL

236 • Two Coupled Oscillators Control Morning and Evening Locomotor Behavior of Drosophila • Ying Peng, Howard Hughes Medical Institute and Department of Biology, Brandeis University, Waltham, MA

237 • Pas Gene Expression and Interactions Between the Molecular Pathways that Regulate Circadian Rhythms, Toxin Metabolism and Development • Xiaoyu Qu, Texas A&M University

238 • A Kinase-Phosphatase Balance in the Drosophila Circadian Clock • Sriram Sathyanarayanan, Howard Hughes Medical Institute, Univerity of Pennsylvania

239 • Circadian Oscillation of Prokineticin 2 Promoter Activity after Serum Shock • Naoyuki Takashima, Kinki University School of Medicine

240 • Photolyase/cryptochrome Chimeras Reveal Differential Actions of N-terminal Regions in CRY1 and CRY2 • Ellena A. van der Schalie ,Department of Biology, University of Virginia, Charlottesville, VA

241 • Tissue-specific Regulation of mPer Expression • Charlotte von Gall,Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA

242 • Attenuated per1 Expression in Zebrafish bmal1 Morphant Embryos • Han Wang, Department of Zoology, University of Oklahoma, Norman, OK

243 • Long-period Rhythms Caused by Twins-Protein Phosphatase 2a • Meg A. Younger, New York University, New York, NY

Neural

244 • Light-activated IGL Neurons in Rats Are Not NPY-immunoreactive • Benjamin Rusak,Depts. of Psychology, Psychiatry and Pharmacology, Dalhousie University, Halifax, Nova Scotia Canada

245 • Individual Differences in Rhythms of Sleep and Its Neural Substrates in Nile Grass Rats • Michael D. Schwartz, Neuroscience Program, Michigan State University, East Lansing, MI

246 • Fast and Slow Resetting of Circadian Oscillations Within the Suprachiasmatic Nuclei • Mariska J. Vansteensel, Leiden University Medical Center

247 • Ectopic Expression of NaChBac Channels in Drosophila Melanogaster Pacemaker Neurons Alters Circadian Periodicity • Vasu Sheeba, New York University

Non-Mammalian

248 • Circadian Expression of Clock Genes in Cultured Chicken Granulosa Cells • Nobuhiro Nakao , Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya

Nucleus

249 • Protein Kinase G-type Ii Is Required for the Night-to-day Progression of the Mammalian Circadian Clock • Laura A Pace, University of Illinois at Urbana–Champaign

Other

250 • Time Memory in Mammals • Sean W. Cain, University of Toronto

251 • Scheduled Food Deprivation in Mice Reverses Patterns of Immediate-early Gene Expression Induced by Acute Deprivation • Marleen H.M. de Groot, Dalhousie University

Pharmacology

252 • Activation of MT2 Melatonin Receptors Phase Shifts Circadian Rhythms of Neuronal Firing in the SCN Brain Slice But Did Not Affect the Circadian Phase of Running Wheel Activity in the MT1 Knockout Mice • Randall Hudson,University of Illinois at Chicago

253 • Morphine Withdrawal in Per1 and Per2 Mutant Mice • Stephanie Perreau-Lenz, Department of Psychopharmacology, Central Institute of Mental Health, Mannheim, Germany

254 • 5-ht-induced Phase Advances of Scn Neuronal Firing in 5-ht7 Ko Mice: Mediation by 5-ht1a and Possibly 5-ht5a Receptors • Jeffery Sprouse. Pfizer Global Research & Development, Groton, CT

255 • Effects of Chronic Methamphetamine Application on Circadian Wheel Running Activity in C57bl/6j, C3h and Per1-luc Mice • Ozgur Tataroglu, University of Virginia

Photoreceptor

256 • Circadian Responses to Light in the Rpe65 Knockout Mouse • Susan ,Dept. Biology, University of Virginia, Charlottesville, VA

257 • Retinal Input to the SCN : An Electrophysiological Study in Normal and Retinal Degenereted Rats • Elise Drouyer , Institut federatif des Neurosciences (IFNL), Lyon, France

258 • Reduced Light Response of the Neuronal Firing Activity in the Suprachiasmatic Nucleus of Cryptocrome-deficient Mice • Takahiro J. Nakamura, Division of Neurobiology and Behavior, Department of Translational Medical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki

259 • Do M-cones and Rods Contribute to Circadian Photoreception? • Dkhissi-Benyahya Lab. Cerveau et Vision, Bron, France

260 • Classical Photoreceptors Are Required for the Daily and Circadian Expression of Melanopsin mRNA Expression in the Retinal Ganglion Cells of Rats • Katsuhiko Sakamoto, Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA

Pineal Gland

261 • Npy Microinjected into the Scn Suppresses Pineal Melatonin During the Late Night • Karen Gamble, Ctr for Behav Neurosci, Ga State Univ

262 • Functional Genomics of the in vitro Chick Pineal • Stephen Karaganis, Texas A&M University

263 • Investigating the Efficacy of Orange Lens Glasses that Could Be Use by Shift-workers to Block the Undesired Resynchronising Effect of Morning Light • Alexandre Sasseville, CHUL

Plant

264 • Coordination of a Metabolic Pathway by a Circadian-Regulated bHLH • Michael Covington, University of California-Davis,

265 • Conserved Expression Profiles of Circadian Clock-related Genes Between Two Lemna Plants Showing Long-day- and Short-day Photoperiodic Flowering Responses • Tokitaka Oyama, Dept. Biol. Sci., Grad. Sch. Sci., Nagoya Univ.

266 • Roles of the Pseudo Response Regulator Genes in the Arabidopsis Circadian Clock • Patrice A. Salomé, Dartmouth College

Retina

267 • Melatonin Synthesis in Dissociated Rat's Retina • Jacopo Aguzzi, Morehouse School of Medicine

268 • Melanopsin Is Expressed in PACAP Containing Retinal Ganglion Cells of the Human Retinohypothalamic Tract • Jens Hannibal, Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Denmark,

269 • Identifying the Light-Activated Channel in Rat Retinal Ganglion Cells (RGCS) that Project to the Suprachiasmatic Nucleus (SCN) • Erin Warren , Oregon Health & Science University, Portland, OR

Seasonal

270 • Effects of Food Availability and of Testosterone Administration on Compensatory Testicular Hypertrophy (CTH) in the Marsh Rice Rat Oryzomys Palustris • Kent E. Edmonds, Indiana University Southeast

271 • ICV Neuropeptide Y Induces Torpor in Siberian Hamsters • David A. Freeman, University of Memphis

272 • Seaonal Variations in Circadian Rhythms Characterize a Phase of Sensitivity to Short Photoperiods in the European Hamster, Cricetus Cricetus • Stefanie Monecke, University of Stuttgart

273 • Circadian and Photoperiodic Responses of House Sparrow at 270N • Sangeeta Rani, Department of Zoology, University of Lucknow, India

274 • Neural Tissues that Encode Photoperiod Histories in Siberian Hamsters • Brett J.W. Teubner,Department of Biology, University of Memphis

275 • Non-photic Phase-shifting of Golden Hamsters to Induced Activity in Long and Short Photoperiod • Uwe Redlin, University of Stuttgart, Stuttgart, Germany

276 • Sleep-Wake Cycle of a Human Female and Seasonal Photoperiodic and Temperature Change in Warm-Temperate Zone • Tomoko Wakamura, College of Nursing Art & Science, Hyogo, Japan

277 • Seasonal Morphological Changes of GnRH Nerve Terminals and Glial Endfeet in the Median Eminence of Japanese Quail • Takashi Yamamura, Graduate School of Bioagricultural Sciences, Nagoya University

278 • Photoperiodic Regulation of Phase Relationship Between Per2 and Cry1 Genes Expression in the Pars Tuberalis of Japanese Quail • Shinobu Yasuo, Division of Biomodeling, Nagoya University

Sleep

279 • Circadian Gene Expression in Twilight and Square-wave Cycles: Is There Any Difference? • Maria Comas-Soberats, Zoological Laboratory, Biological Center, University of Groningen, , Haren, The Netherlands

280 • The Accurate Measurement of Plasma and Serum Melatonin Without Sample Extraction • Richard E. Conley, ALPCO Diagnostics, Windham, New Hampshire

281 • Genes Affecting Sleep Regulation in Mice • John Hofstetter, VA and Indiana University School of Medicine

282 • Sleepless Mice Display Increased Levels of Daily Locomotor Activity • Paul Ketema Northwestern University, Center for Sleep and Circadian Biology, Evanston, IL

283 • Vasoactive Intestinal Polypeptide (VIP) Contacts on Orexin (ORX) Neurons in Diurnal but Not in Nocturnal Rodents • Gladys S. Martinez ,Psychology Department, Michigan State University, East Lansing, MI

284 • Spectral Analysis of Sleep EEG in Morning-type and Evening-type Individuals • Valerie Mongrain, Chronobiology Laboratory, Sacre-Coeur Hospital & University of Montreal

Suprachiasmatic

285 • Circadian Rhythmicity in a Subset of SCN Neurons and Synchrony Between SCN Neurons Depends on VIP/VPAC2 Signaling • Sara Aton , Departments of Biology, Washington University, St. Louis MO

286 • Effects of 5HT1B Receptor Agonists on Miniature Ipscs in SCN Neurons • Jane Bramley, Colorado State University

287 • Tract-tracing Analysis of Two Candidates for the Suprachiasmatic Nucleus of the Chicken, Gallus Domesticus • Elizabeth L. Cantwell, Center for Research on Biological Clocks and Department of Biology, Texas A&M University, College Station, Texas

288 • GABA-A Receptor Activation by Muscimol Reduces Per1 but Not Per2 mRNA during the Mid-subjective Day in Free-running Syrian Hamsters • J.C. Ehlen1, Biology, Georgia State University, Atlanta, GA,

289 • Removal of Polysialylated Ncam from the Scn Abolishes the Sensitizing Effect of Brain-derived Neurotrophic Factor on Mouse Photic Phase-resetting • J. David Glass, Department of Biological Sciences, Kent State University, Kent, OH

290 • Localization of Serotonergic Receptors Mediating Photic-like Response of the Scn to Quipazine • Caroline Graff, University of Stuttgart, Biological Institute, Germany

291 • Photic Regulation of the Gastrin-releasing Peptide Receptor in Mouse Suprachiasmatic Nucleus • Ilia N. Karatsoreos , Departments of Psychology, Columbia University, New York, NY

292 • Regulation of Glutamate Release from RHT Terminals by Distinct 5-HT Receptor Subtypes in the Rat SCN Slice • Si-Hyun Kim ,CROET, OHSU, Portland, OR

293 • Differential Localization of Prokineticin 2-containing Neurons in the Rat Suprachiasmatic Nucleus • Koh-hei Masumoto, Yamaguchi University

294 • CGMP-dependent Protein Kinase-ibeta Mediates Glutamate Signaling in the Suprachiasmatic Circadian Clock • Jennifer W. Mitchell,University of Illinois at Urbana-Champaign

295 • The Clock in Dorsal SCN Runs Faster Than that in Ventral SCN • Takako Noguchi, Dokkyo

296 • Light and GABA Interact to Alter Period mRNA levels in the SCN of Diurnal Grass Rats • Colleen Novak, Departments of Biology, , Georgia State University, Atlanta, GA

297 • Role of P/Q-type Calcium Channels in the Regulation of Circadian Activity Patterns in Mice • Floor van Oosterhout , Leiden University Medical Center

298 • Attenuated Response to Light in the 5HT1B Receptor Knockout Mouse Results in Phase Delayed Entrainment to Winter-like Photoperiods • Gary E. Pickard, Colorado State University

299 • Circadian Rhythms in Mammalian Glia • Laura M. Prolo, Dept. of Biology, Washington University, St. Louis, MO

300 • Distribution of Immunoreactive PER1 Protein in the Suprachiasmatic Nucleus and Adjacent Hypothalamus of the Diurnal Grass Rat, Arvicanthis Niloticus • Chidambaram Ramanathan, Psychology Department, , Michigan State University, East Lansing, MI

301 • Light Pulses Induce Per1 and c-fos in the Auprachiasmatic Nucleus of Arrhythmic, but not Free-running, Siberian Hamsters • Norman Ruby, Department of Biologcial Sciences, Stanford University, Stanford CA

302 • Transient Dissociation in the Dorsomedial Region of the SCN • Yasufumi Shigeyoshi, Kinki University

303 • Unequal Development of the Clock Gene Expression in the Rat Suprachiasmatic Nucleus • Alena Sumova, Institute of Physiology Academy of Sciences of the Czech Republic, Prague

304 • Astrocytes and Calcium in the Suprachiasmatic Nucleus of Phodopus Sungorus • Nadja Ufer, School of Veterinary Medicine

305 • c-Fos expression in vlSCN of Common Voles Does Not Anticipate Light-dark Changes • Daan van der Veen, University of Groningen

306 • Prokineticin-2 and Prokineticin Receptors in a Diurnal Rodent, Arvicanthis Niloticus • DavidWeaver, Department of Neurobiology and Program in Neuroscience, UMass Med. School, Worcester, MA.

307 • Circadian Oscillation and Light Induction of Immature Per2 mRNA in the Suprachiasmatic Nucleus • Akihito Adachi, Kinki University School of Medicine

308 • The VPAC2 Receptor Is Required For Coordinated Rhythmic Activity of Mouse SCN Neurons in vitro • Tim Brown, University of Manchester

Transcription

309 • Circadian Activity of a Viral Gene Promoter in Tissues of Live Bioluminescent Mice • Michael Geusz, Bowling Green State University

310 • Title??? • Stacey L. Harmer, Section of Plant Biology, University of California, Davis*

311 • Time-dependent Gene Regulation in Human Skeletal Muscle and Evidence of Exercise-induced Modulation • Erin L McDearmon, Northwestern University/HHMI

312 • The Role of Orphan Nuclear Receptor RORa in Clock Gene Transcriptions Demonstrated by a Novel Reporter Assay System • Yoshihiro Nakajima, Cell Dynamics Research Group, National Institute of Advanced Industrial Science and Technology (AIST), Osaka, Japan

June 26, 2004

8:30-10:30 **Symposium 8**—*Rainbow Theatre*

Circadian System Organization: Heirarchies, Networks or Both

Chair: Paul Hardin–University of Houston

Speakers: Andrew Millar, Warwick University

Charlotte Helfrich –Forster, University of Regensbure

Paul E. Hardin, University of Houston, Department of Biology and Biochemistry Houston, TX
Circadian Clocks in Antennal Neurons Are Necessary and Sufficient for Olfaction Rhythms in Drosophila

Michael Menaker, University of Virginia
The Mammalian Circadian Axis

David Whitmore, University College London
Zebrafish Clocks: Embryos, Cells and Light

8:30–10:30 **Symposium 9**—*Rainbow Theatre*

Neural Outputs of Biological Clocks

Chair: Stuart Dryer–University of Houston

Speakers: Charles N. Allen, Oregon Health & Science University
Synaptic Plasticity at SCN Synapses

Ruud M. Buijs, Netherlands Institute for Brain Research
Differentiation of Parasympathetic and Sympathetic Output of the SCN: Its Role in Metabolism

Chris Colwell, Department of Psychiatry, University of California - Los Angeles
Paper Title ?????

Stuart E. Dryer, University of Houston
Mechanisms of Circadian Regulation of Dark Current Channels in Vertebrate Cone Photoreceptors

Paul H. Taghert, Departments of Anatomy & Neurobiology, and Genetics Washington University Medical School
PDF Signaling in Drosophila Circadian Behavior

10:30-11:00 **Coffee break**—*Great Room*

11:00-13:00 **Slide Session 8**—*McGuire*

Cell Biology

11:00 **107 • Evidence for a Role for Endocytosis in the Drosophila Circadian Clock** • Ravi Allada, Department of Neurobiology and Physiology, Northwestern University, Evanston, IL

11:15 **108 • Nuclear Localization of Xenopus Cryptochrome Is Regulated by the C-terminus** • Francesca Conte, Department of Biology, University of Virginia, Charlottesville, VA

11:30 **109 • DBT and SGG Are Opposing Regulators of PER/TIM Nuclear Entry** • Shawn A. Cyran, New York University, New York, NY

11:45 **110 • CKIe AND b-TrCP Regulate Period2 Stability** • Erik J Eide, Department of Oncological Sciences and the Center for Children, Huntsman Cancer Institute

12:00 **111 • Posttranslational Regulation of the Neurospora Circadian Clock** • Yi Liu, Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX

12:15 **112 • Stability of Circadian Oscillator Observed in Individual Cyanobacteria** • Irina Mihalcescu, Lab. de Spectrom trie Physique, Universit Joseph Fourier ö Grenoble I, Cedex, France

12:30 **113 • Pigment-dispersing Factor and GABA Synchronize Insect Circadian Clock Cells** • Monika Stengl, Philipps-University of Marburg, Biology, Animal Physiology, Germany

12:45 **114 • Enhanced Nuclear Import of Mper2 by the C-terminal Tail of Mcry1** • Filippo Tamanini, ErasmusMC

11:00-13:00 **Slide Session 9**—*Rainbow Theatre*

Clinical Applications

11:00 **115 • Circadian and Sleep Homeostatic Aspects of Thermoregulation** • Anna Wirz-Justice, Centre for Chronobiology, Psychiatric University Clinic, Basel, Switzerland

11:15 **116 • Phase Resetting by Long Light Pulses** • Domien Beersma, Zoological Laboratory, University of Groningen

11:30 **117 • Comparison of Melatonin Suppression by Blue Wavelength Light in Young and Older Adults** • Kimberly Green, Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL,

11:45 **118 • Entrainment of the Circadian Pacemaker with Very Low Dose Melatonin** • Alfred J. Lewy, Oregon Health & Science University

12:00 **119 • Sleep and Circadian Rhythmicity Are Essential for Remodeling in the Heart •** Tami Martino, Toronto General Hospital

12:15 **120 • Biological Rhythms in Premenstrual, Pregnant, Postpartum and Menopausal Depression •** Barbara Parry, Department of Psychiatry, University of California, San Diego

12:30 **121 • Immediate and Phase Shifting Effects of Nasal Versus Temporal Illumination of the Human Retina •** Melanie Rÿger, Human Chronobiology Group, Zoological Laboratory, University of Groningen

11:00-13:00 **Slide Session 10**—*Sky Ballroom B*

Genomics and Molecular Genetics

11:00 **122 • A Role for the HLH Transcription Factor Inhibitor of DNA Binding 2 (Id2) in the Mammalian Circadian Clockwork •** Giles E. Duffield, Department of Genetics, Dartmouth Medical School, Hanover, New Hampshire; Department of Visual Neuroscience, Imperial College, Charing Cross Hospital, London U.K.

11:15 **123 • High Throughput Real-time Monitoring of Clock Gene Promoter Activity: A Valuable Tool to Study Clock Gene Function •** Achim Kramer, Charit, Humboldt-University

11:30 **124 • A Genetic Selection for Circadian Output Pathway (COP) Mutations in Neurospora Crassa •** Louis W. Morgan, Center for Biological Clocks Research and Program for the Biology of Filamentous Fungi, Department of Biology, Texas A&M University, College Station, TX

11:45 **125 • High Resolution Mapping for Soc-1 (Suppressor of Clock) Locus in Mouse •** Kazuhiro. Shimomura, Northwestern University

12:00 **126 • Novel ENU-induced Circadian Clock Mutants in Mice •** Sandra M. Siepka, Center for Functional Genomics, Northwestern University, Evanston, IL,

12:15 **127 • Characterization of the Circadian Deadenylase Nocturnin and Its Potential Targets in the Mouse •** Carl Strayer, University of Virginia

12:30 **128 • Natural Polymorphism in the Drosophila Cryptochrome •** Erin Tauber, University of Leiceste

12:45 **129 • Cis-acting Sequences and Transcription Factors Mediating Morning-specific Expression of the Arabidopsis Clock Gene LHY •** Isabelle Carr, Department of Biological Sciences, University of Warwick, Coventry, UK.

13:00–14:30 **Lunch on Own**

14:30–16:30 **Workshop**—*McGuire Room*

New Methods for Biological Clocks Analysis

130 • Real-time Measurement of Circadian Gene Expression in Peripheral Tissues from Transgenic Zebrafish • Gregory M. Cahill, Department of Biology and Biochemistry, University of Houston, Houston, TX

131 • Suitability of AANAT2:EGFP Transgenic Lines as a Model to Study Clock-regulation of the Melatonin Rhythm • Yoav Gothilf1, Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel.

132 • Generation of an Inducible Circadian Clock: Molecular Oscillations and Behavior in Drosophila • Sebastian Kadener, Department of Biology, Howard Hughes Medical Institute, Brandeis University,

14:30–16:30 **Symposium 10**—*Sky Ballroom*

The Next Step: Structural Biology of Clock Proteins

Chair: Susan Golden–Texas A&M University

Speakers: Andy LiWang, Texas A&M

Emil Pai, University of Toronto

Elizabeth Getzoff, Scripps Rush Institute

Carl Johnson, Vanderbilt University

16:30-17:00 **Coffee Break**—*Great Room*

17:00 **Business Meeting**—*Sky Ballroom*

20:00 **Banquet**—*Sea Ballroom*

Abstracts

001

The Enigma of Translational Control in the Gonyaulax Clock Mechanism

Woody Hastings • Harvard University

The striking circadian rhythm of bioluminescence in the dinoflagellate Gonyaulax was shown to be associated with, perhaps due to, a parallel development and activity loss of two proteins, luciferase (LCF), and luciferin (substrate) binding protein (LBP). These and luciferin, are located within scintillons, unique cytoplasmic organelles, visualizable by both immunoLBP and immunoLCF labeling, as well as luciferin fluorescence and bioluminescence. Dunlap and then Johnson definitively demonstrated that this was due to the synthesis and destruction of LCF, and from Northern blots Morse concluded that the circadian regulation of the synthesis of LBP is controlled translationally. Later, several nonbioluminescence CCGs were shown to be also controlled translationally, suggesting a general mechanism in Gonyaulax. Mittag reported a potential regulatory protein binding to a novel 23nt 3[1]UGrepeat in lbpmRNA, but this motif has not been found in any other CCGs. The strong phase shifting by protein synthesis inhibitors and the effects on tau by kinase and phosphatase inhibitors indicates that these steps are central in the core clock mechanism. Microarray analyses in the dinoflagellate Pyrocystis suggested that about 3% of the more than 3,500 ESTs are circadian controlled. More than 50% were identifiable with known genes, none being clock genes from other systems.

002

NAS Symposium

Reppert

003

NAS Symposium

Rosbash

004

NAS Symposium

Takahashi

005

Synchronization by Light-dark Cycle of Circadian Rhythm of Motor Activity in Blind Primate

Mayara M A Silva, Manoella M Alves, Thiago T M Paiva, Vin'cius F Araœjo, Alex M Albuquerque, Ruy M Oliveira Jr, Tyrone C Silva Jr, John F Araujo* • Laboratrio de Cronobiologia, Departamento de Fisiologia, CB/UFRN, Natal, Brasil

Light synchronizes mammalian circadian rhythms with environmental time by modulating retinal input to the circadian pacemaker – the suprachiasmatic nucleus of the hyphotalamus. This photic entrainment does not require rods nor cones, but melanopsin-containing retinal ganglion cells. As we found a couple of blind marmosets (Callithrix jacchus) in our colony (Primate Center of Universidade Federal do Rio Grande do Norte – Natal, Brasil) that oftalmoscopy study showed retinal rods degeneration we have studied the circadian rhythms of these blind primates. Four marmosets (two blind and two normal) were kept in cages with light-dark cycle controlled and attenuated sound. All marmosets were synchronized with 24 hour light-dark cycle (LD) with activity occurring in the light phase. The blind marmosets were entrained with the new LD when light onset was delayed and advance by 6 hours. In freeruning conditions the blind marmosets showed circadian period (tau) of 23,2 hours and the normal marmosets of 23.6 hours. All marmosets, blind and normal, responded to dark pulse in early subjective day with phase delay and with active phase advance after dark pulse in late subjective day. Our results confirmed the hypothesis that, in spite of the retinal degeneration, our blind marmoset are able to synchronize their activity circadian rhythm and this species could be a good primate model circadian photoreception."

006

Entrainment of the Master Circadian Clock by Scheduled Feeding

Abel Bult-Ito*, Marina R. Castillo, Kelly J. Hochstetler, Ronald J. Tavernier, Jr., and Dana M. Greene • Behavioral and Evolutionary Neuroscience Laboratory, Alaskan Basic Neuroscience Program, Institute of Arctic Biology, P.O. Box 757000, University of Alaska Fairbanks, Fairbanks, Alaska 99775-7000, U.S.A.

This study provides important new insights in how non-photic signals affect the mammalian master circadian clock, i.e., suprachiasmatic nuclei. We used a scheduled feeding paradigm which consisted of 6 hours of food availability during the light period (zeitgeber time 4-10) in a 12:12 light-dark cycle, followed by 4-6 months of constant dark. Stable entrainment to scheduled feeding was established 84.9 ± 3.5 (SE; n=20) days after the start of constant dark in 62% of mice tested and lasted for up to 15 weeks. Whether or not mice entrained to scheduled feeding did not depend on free-running period. Using mouse PERIOD2 protein circadian expression profiles in the suprachiasmatic nuclei, we confirmed for the first time that the master circadian clock was in phase with the onset of food availability and entrained by scheduled feeding without caloric restriction, which is in contrast to other published work. Predictable line differences in likelihood of entrainment, i.e., 100% in line 2 versus 4% in line 3, and the distinctive entrainment process provide the opportunity to search for mechanisms and pathways involved in entrainment of the suprachiasmatic nuclei by scheduled feeding and possibly other non-photic entrainment signals.

007

Entrainment of the Master Circadian Clock by Feeding Schedules

Escobar Carolina, Mendoza Jorge, Angeles Manuel • Facultad de Medicina, UNAM, Mexico, DF 04510

Food entrainment can be elicited in rats with bilateral SCN lesions and depends on a circadian oscillator. Evidence obtained with the expression of clock genes has suggested that restricted feeding schedules do not entrain the SCN. However in rats exposed to an hypocaloric diet, restricted feeding schedules modify the response of the SCN to light stimulation. We have reported phase control of the free running period by meal time in chronic malnourished rats. Both studies suggest that the catabolic state induced by a deficient diet may facilitate SCN entrainment by food. In the present study we present evidence of SCN entrainment by feeding schedules in rats maintained in ad libitum conditions and allowed to ingest chocolate daily at a fixed time. Rats (n=8) were maintained in constant darkness and received daily 5 g of chocolate at geographical time 1400h. Rats developed anticipatory activity and when chocolate-time coincided with CT0, free running activity acquired phase and period of chocolate-time and remained expressing the imposed rhythmicity even after 10 days after discontinuing the entraining protocol. Glucose and free fatty acids rhythmicity was modulated by this protocol. This effect was not observed under a light dark cycle. We conclude that under special conditions, deficient diet or lack of light-dark cycle and a high palatable meal, feeding schedules can become a potent zeitgeber on the SCN.Supported by CONACyT 33033-N and DGAPA-UNAM IN203803.

008

Entrainment of the Human Circadian Pacemaker to Longer-than-24h-days with Intermittent Bright Light

C. Gronfier* (1,4), K.P. Wright Jr. (1, 2), R.E. Kronauer (3), and C.A. Czeisler (1) • (1) Division of Sleep Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115; (2) Department of Integrative Physiology, University of Colorado, Boulder, CO 80309; (3) Harvard University, Cambridge, MA 02138; (4) present affiliation: Inserm U371, Bron, France

The endogenous circadian pacemaker is unable to entrain to a 24.6-h light-dark cycle in subjects maintained in very dim light intensity during wakefulness (1). The associated circadian misalignment results in sleep, endocrine, and neurobehavioral impairments. Exposures to brief pulses of bright light have been shown to phase shift the circadian pacemaker with great efficacy (2). Based on these results, we tested the hypothesis that daily exposures to intermittent bright light (IBL) in the evening would establish a normal circadian phase in subjects exposed to a light-dark cycle 1 h longer than their own intrinsic circadian period. Twelve healthy young subjects underwent a 65-day study. Their individual intrinsic circadian period (tau) was determined in a forced-desynchrony procedure. Subsequently, subjects were released into thirty longer-than-24-h days (day length of tau + 1 h) in one of three light conditions: 1) ~25 lux in the angle of gaze [AG]; 2) ~100 lux [AG]; 3) IBL: two 45-min bright light pulses of ~9,500 lux [AG] separated by 60 min of ~100 lux [AG]. Our results reveal that lighting levels of ~25 lux or ~100 lux were insufficient to maintain a normal phase angle of entrainment in all the subjects. They show that exposure to IBL was able to maintain entrainment of the subjects' melatonin rhythms to the imposed sleep-wake cycles. Our results suggest that IBL can be used to entrain the circadian pacemaker to longer-than-24 h days. The implications of these findings are important, as they could be used to treat circadian

misalignment associated with space flight, shiftwork, and circadian rhythm sleep disorders.(1) Wright Jr. KP et al. PNAS 98:14027-32, 2001. (2) Gronfier C et al. Am J Physiol (E), in press.Support: NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute (NSBRI), NASA Grant NAG 5-3952, NIH MO1-RR02635 – GCRC

009

Input Pathway of the Circadian Period in Cyanobacterium Synechococcus Elongatus PCC 7942

Naoki Takai, Shingo Ikeuchi, Katsushi Manabe, and Shinsuke Kutsuna* • Yokohama City University

Environmental light condition modulates the phase and period of the circadian clock. Although several photoreceptor proteins that receive light for the clock regulation were identified, little is known about the input-pathways, which transmitted environmental cues, such as light-signal from the receptor to the clock to reset circadian phase and/or period. Previously, in the model organism cyanobacterium Synechococcus elongatus PCC 7942 a period-extender gene (pex) has been identified as a period lengthening factor about 1-hour in the oscillator. Here we show its role in the input-pathway. pex mRNA and its protein expressed at low level in light, and increased in dark. This derepression repeated under dairy dark period. Even thought, pex-disrupted strain (pex-d) exhibits the short period phenotype, continuous light grown pex-d could coordinate the peak timing of expression of kaiBC operon (kaiBC-timing; used as temporal marker of the clock-movement) at the same time to the one in wild-type (wt) after a 12-hours-dark entrainment, appeared to retain normal function of typical light-entrainment in pex-d. But the pex-d having experience of three diurnal light/dark cycles for 3-days exhibits advanced kaiBC-timing by 3-hours than the one in wt. Consistently, ectopic induction of pex-trans-gene (pex-i) caused the delay in the bioluminescence rhythm. To determin the genetic structure of the input pathway, we examined the expression level of clock genes in the pex-d strain. And we found significant increase of accumulation level of the positive factor KaiA in the mutant. On the other hand, pex-i strain exhibited low level in KaiA level.

010

Initial Steps Towards a Circadian Compromise for Night Shift Workers

Lee C • Eastman CI Biological Rhythms Research Lab, Rush University Medical Center, Chicago

This is the first installment of a multi-part study to determine if circadian adaptation (re-alignment of the temperature minimum (Tmin) to within daytime sleep) can be achieved and maintained while alternating between working night shifts and having days off. Subjects were required to sleep from 23:00 to 07:00 (and up to 1 h later on weekends) for 3 weeks. Following this baseline period, they worked 2 consecutive simulated night shifts (23:00Ð07:00). Experimental subjects (n=11) received five 15-minute intermittent bright light pulses (~3500 lux; ~2200 microW/cm2) during the night shift, wore sunglasses (~12% transmission) while traveling home, and slept in the dark (08:30Ð15:30) after each night shift. Control subjects (n=8) received ordinary room light (~20 lux) throughout the night shift, wore sunglasses (~37% transmission), and slept whenever they wanted after the night shifts. The Tmin (dim light melatonin onset + 7 h) was determined before and after the night shifts. Tmins (mean ± SD in h) for the experimental subjects were 04:24 ± 0.8 and 7:36 ± 1.4, respectively; for control subjects, they were 04:00 ± 1.2 and 4:36 ±

1.4. Thus far, the experimental treatment phase delayed the Tmin by 1.6 h/day. After one more night shift, we expect the Tmin to delay to within daytime sleep. Given a fairly late sleep schedule on days off, this phase position would be an ideal compromise between working nights and having days off. Support: R01 OH003954.

011

Daily Activity Patterns of a Nocturnal and a Diurnal Rodent in a Semi-natural Environment

Roberto Refinetti • Circadian Rhythm Laboratory, University of South Carolina, Walterboro, SC 29488, USA

The entrainment of circadian rhythms by light-dark cycles has been extensively investigated in laboratory studies. In almost all of these studies, organisms have not been allowed to modulate their exposure to the light-dark cycle. In the present study, the rhythm of running-wheel activity was investigated in nocturnal (domestic mice) and diurnal (Nile grass rats) rodents provided with light-tight nest boxes and maintained under long and short photoperiods. Photoperiod length had a significant effect on the duration of the daily active phase (alpha), on the phase angle of entrainment (psi), and on diurnality or nocturnality in both species. The availability of a nest box had a modest effect only on the variability of activity onsets. Although the animals spent most of the inactive phase (rho) inside the nest box, they entered and exited the box many times during both the active and inactive phases. Neither in the nocturnal nor in the diurnal species was there any evidence of entrainment by frequency demultiplication or of entrainment without photic stimulation at either dawn or dusk. These results indicate that, at least in the species studied, the ability of rodents to modulate their exposure to the light-dark cycle does not have a major effect on photic entrainment. Therefore, there is no reason to question the validity of the multitude of previous laboratory studies that utilized inescapable light-dark cycles.

012

A Place for ZTL in Circadian Phototransduction a Place for ZTL in Circadian Phototransduction

Woe-Yeon Kim and David E. Somers* • Ohio State University

The entrainment of the circadian clock in plants is mediated through the red light-sensing phytochrome and blue light-sensing cryptochrome photoreceptors. Fluence rate response curves of mutants in a third class of molecule, ZEITLUPE (ZTL), show enhanced effects on free-running period at low fluence rates in both blue and red light, indicating that this protein also mediates light signaling to the central pacemaker. ZTL is an F-box protein that appears to target a key component of the circadian pacemaker, TOC1, for proteasome-dependent degradation. A novel element of ZTL is an N-terminal flavin-binding motif (LOV domain), which is consistent with the fluence-dependent effect of ztl mutations in blue light, but does not explain the equally strong effect of the mutations in red light. We have created double mutant combinations between ztl-1 and select phytochrome and cryptochrome mutants to determine the nature of the contribution of ZTL to circadian phototransduction, in the context of the known phytochrome and cryptochrome signaling pathways. Preliminary data suggest that ZTL and phytochromeB share parallel or overlapping pathways, as ztl-1 phyB double mutants are more severe than either alone. Results

of additional photoreceptor ztl-1 mutant combinations will be presented. We also examined the effect of ztl mutations in elf3-1 and ELF3 overexpressing backgrounds. ELF3 is linked to many aspects of plant photomorphogenesis, including light-dependent arrhythmicity in elf3-1 mutants. Initial results indicate a surprising epistasis of ELF3 to ZTL in a number of photomorphogenic phenotypes. Our working model of this interaction will be presented.

013

Stochastic Sensitivity Analysis of the Circadian Gene Network

Neda Bagheri*, Rudi Gunawan and Francis J. Doyle III • University of California, Santa Barbara, USA Joerg Stelling Max Planck Institute, Magdeburg, Germany

Traditional methods of representing well-stirred chemically reacting systems are through a set of coupled ordinary differential equations. These equations characterize the evolution of molecular populations as a continuous, deterministic process. Since many cellular processes are tightly regulated with small populations of mRNA (~10s) and protein (~1000s), they are more appropriately described using a stochastic model in the form of a chemical master equation (CME). Thus, classical deterministic analysis no longer applies to these stochastic model fomulations, motivating the development of system theoretic tools for complex stochastic biological networks. Circadian rhythms possess remarkable robustness to changes in environment. Robustness measures the ability of a system to maintian acceptable perfomance under perturbation. The Fisher Information Matrix (FIM) provides a measure of robustness by describing the information content in a signal relative to a set of system parameters. In stochastic models, system outputs are represented by a set of random variables whose density functions are described by the CME. System sensitivities are represented by changes in these density functions due to parameter pertubation. The FIM describes parametric robustness and sensitivity through evaluation of its eigenvalues or relating decompositions. The proposed stochastic FIM analysis allows the investigation of period robustness within the Drosophila circadian network and proves the existence of a sensitivity-based distribution of parameters. Resulting parametric ordering shows groupings of parameters in which system fragility refers to global control variables while system robustness refers to local control variables.

014

How Should We Analyze Activity Rhythms?

P. Gehrman*,1 M. Marler,2 S. Ancoli-Israel,2 • 1Center for Sleep, University of Pennsylvania, 2Department of Psychiatry, University of California San Diego and VASDHS, San Diego, California, USA

Activity rhythms are often collected as a marker of entrained circadian rhythms. This methodology has practical advantages over "pure" markers such as melatonin, including ease of measurement and the ability to collect data over longer time periods. The traditional cosinor model used to analyze these data has been criticized because activity data are more square-wave in shape than sinusoidal. Non-parametric alternatives have been suggested. Three approaches to the analysis of activity rhythms were compared. Subjects were 150 Alzheimer's disease patients (mean (SD) age=84.1(7.8); mean (SD) MMSE=8.5(7.6)). Activity data were recorded with an Actillume (Ambulatory Monitoring, Inc) for three consecutive days and analyzed using the traditional cosinor model, an extension of the cosinor model that allows the fitted model to approximate a square wave, and computation of non-parametric measures. The extended model provided

a significantly better fit of the data than the traditional model in all but 3 subjects. A direct statistical comparison with the non-parametric statistics is not appropriate. Cosinor models can provide insight into underlying neurobiological processes, whereas non-parametric statistics describe specific attributes of the data not captured by cosinor models. In conclusion, the extended cosinor model provides an advantage to the traditional cosinor model in terms of model fit. Choosing between cosinor and non-parametric measures depends on the purposes of the individual study rather than a clear advantage of either approach. Supported by NIA AG08415, NCI CA85264, GCRC grant # M01 RR00827, and the Research Service of the Veterans Affairs San Diego Healthcare System.

015

Is the Mammalian Circadian Clock a Resonant-circuit Oscillator?

Eric Bittman, Biology, UMass Amherst; Yossi Chait, Mechanical Engineering, UMass Amherst; C. V. Hollot*, Electrical and Computer Engineering, UMass Amherst; Mary Harrington, Neuroscience Program, Smith College; Hava Sielgelmann, Computer Science, UMass Amherst

As an alternative to highly-complex computational models for single-cell clock models, we borrow from classical electronics and propose a new paradigm: the resonant-circuit oscillator. While the former involves scores of nonlinear differential equations to model interactions between mRNA-expression and protein-production, a resonant-circuit oscillator is succinctly defined by the feedback interconnection of three functions: resonator, phase-shifter, amplitude-stabilizer. We will demonstrate, via analysis and simulation, that mammalian clock-works can be decomposed along these functions; namely, that: the inhibitive effect of PER-CRY on BMAL1 exhibits resonance. the promotive effect of BMAL1 on PER-CRY produces necessary phase shift, the mass-kinetic dynamic of Per mRNA expression provides amplitude stabilization. This single model explains why cells of the suprachiasmatic nucleus oscillate, while other cells, neighboring and peripheral, exhibit damped behavior. It also relates the parameters of BMAL1 and PER-CRY production to the amplitude and frequency of the cell's free-running oscillation, and, suggests mechanisms for explaining light-entrainment and inter cell-clock coupling.

016

A Proposal for Robust Temperature Compensation of Circadian Rhythms

Christian I. Hong,* Bela Novak, & John J. Tyson • Dartmouth Medical School

The internal circadian rhythms of cells and organisms coordinate their physiological properties to the prevailing 24-hour cycle of light and darkness on earth. The mechanisms generating circadian rhythms have four defining characteristics: they oscillate endogenously with period close to 24 h, entrain to external signals, suffer phase shifts by aberrant pulses of light, and compensate for changes in temperature over a range of 10oC or more. Most theoretical descriptions of circadian rhythms propose that the underlying mechanism generates a stable limit cycle oscillation (in constant darkness or dim light), because limit cycles quite naturally possess the first three defining properties of circadian rhythms. On the other hand, the period of a limit cycle is typically dependent on kinetic rate constants, which increase markedly with temperature. Hence, temperature compensation is not a general property of limit cycle oscillations but must be imposed by some delicate balance of temperature dependent effects. However, if circadian rhythms arise from a switch-like mechanism, which enables the system to alternate between a stable steady state

and a stable limit cycle, then the oscillator period is robustly independent of most kinetic parameters in the mechanism."

017

Modeling Temperature Compensation in Circadian Rhythm

Gen Kurosawa* and Yoh Iwasa • Kyushu University

Circadian clock has a free running period that does not change much with ambient temperature. We study this phenomena, temperature compensation theoretically by using simplified molecular model. When the rate for degradation of proteins or transcription increases, the period of oscillation becomes longer (positive elasticity). In contrast, the period of oscillation becomes shorter (negative elasticity) as the rate for degradation of mRNAs increases. Through both the mathematical analyses and computational analyses for numerous parameter sets, we show that these results hold for most of parameter sets and for a wide range of models, including N-step models, and PER-TIM double oscillator model, provided that the cooperativity of the inhibition of transcription by nuclear proteins is not very large. We then discuss the least costly realization of the temperature compensation by choosing the temperature dependence of reaction rates of the gene-protein network. These results of period sensitivity analyses suggest that high temperature-dependence of degradation of proteins or expression of clock gene causes robust free running period of circadian rhythm

018

Why Do Clock Mechanisms Have Multiple Loops?

Boris Shulgin, Domingo Salazar, Andrew J. Millar*, David A. Rand • Interdisciplinary Programme for Cellular Regulation, Department of Biological Sciences & Mathematics Institute, University of Warwick, Coventry CV4 7AL

A fundamental problem is to understand the relation between the form and the function of biological regulatory networks. The molecular mechanisms of circadian clocks from disparate organisms involve multiple interconnected feedback loops. Mathematical models indicate that a single loop is sufficient to produce robust rhythms with typical circadian characteristics, so the function of the multiple loops is unclear: do they perform species-specific functions, or does an underlying design principle favour interconnected loops? Suggested reasons include increased robustness to stochastic noise or the capacity for circadian output rhythms to track more than one phase but a general explanation is lacking. We argue that the complexity of clock circuits provides flexibility of regulation, in a precise sense, which is necessary for the evolutionary adaptation of multiple circadian properties, simultaneously. These properties include phase tracking and also free-running period, temperature compensation, range of entrainment, and so on. We show that single-loop clocks are generally inflexible, although their many component genes, proteins and biochemical processes may suggest great complexity and apparently provide a wealth of targets for evolutionary adjustments. We introduce the infinitesimal response curve (IRC), which is related to the phase response curve (PRC). IRCs allow us to identify the changes required in order for a clock mechanism to adopt a new set of circadian properties. The flexibility gained by increasing the number of interconnecting loops does not depend on any specific biochemical process and applies to both intracellular and intercellular loops. It provides a general explanation for the diverse mechanisms that are observed in the multiple loops of circadian clocks.

019

Martian Circadian Rhythms: a Biosignature?

Marianne Case, Arash Dini, Gilbert V. Levin, Patricia A. Straat, Hans P. A. Van Dongen and Joseph D. Miller* • Keck School of Medicine at USC

The only life detection experiments performed on another world were those of the Mars Viking Landers in 1976. In the Labeled Release experiment of Levin and Straat, Martian soil samples were mixed with a "cocktail" of 14C-labeled organic substrates in a sealed test cell inside the lander. Evolution of a carbon-containing gas (e.g., CO2 or CH4) was detected with two solid state beta detectors connected to the test cell by a 13 inch swan neck steel tube. A large initial release of labeled gas was observed, followed by strong periodic fluctuations (which continued for 77 days), in the amount of gas detected, superimposed on a slow rise in release. Heat sterilization greatly attenuated the fluctuations. Analyses show, at steady state, the fluctuations exhibited a periodicity of 24.66+/-0.27 hr, statistically indistinguishable from the Martian day. The gas fluctuation appeared synchronized to a 2¼ C periodic fluctuation in internal temperature in the experimental chamber, which in turn was synchronous with almost 50 C daily fluctuations in ambient Mars surface temperature. Further analyses employing the Lomb-Scargle periodogram and harmonic regression show a large decrease in amplitude of this rhythm when the internal temperature cycle amplitude diminished to about 1C. Recent observations of circadian rhythmicity in microorganisms and entrainment of terrestrial circadian rhythms by low amplitude temperature cycles suggest that a Martian circadian rhythm may constitute a biosignature.

020

Systems Biology on Mammalian Circadian Rhythms

Hiroki R. Ueda* • Center for Developmental Biology, RIKEN

Increasing number of genome sequences have been determined in various model organisms. These genome projects have been driving a paradigm shift in life science, from the molecular level to the system level, and accelerating innovations based on genome-scale resources and information. Compared to the increasing needs for system-level understanding of biological phenomena and increasing amounts of genome-scale information, technologies and resources, there are few studies fully utilizing genome-wide research infrastructure toward systematic identification of genetic networks underlying complex and dynamic biological phenomena. Main difficulty in full exploitation of genome-wide research infrastructure is seamless integration of both computational and experimental technologies. We attempt to combine both computational and experimental technologies to develop system identification technologies toward the system-level understanding of biological phenomena and apply them for identification of the genetic network underlying complex and dynamic biological phenomena. In this symposium, we will introduce the outline of system identification strategy including (i) genome-wide gene expression analysis, (ii) genome-wide promoter analysis using mammalian promoter database, (iii) in vitro real-time monitoring of gene expression (iii) high-throughput screening of regulators, (iv) in vitro phenotype analysis. The power of this strategy can be demonstrated by its application for the complex and dynamic biological systems. We applied this strategy to the system identification of transcriptional circuits composed of 16 transcription factors underlying mammalian circadian rhythms, revealed the overall picture of transcriptional circadian circuits with unexpected complexity, and topologically and functionally found Achilles'heel of mammalian circadian clocks.

021

SCN-dependent and SCN-independent Oscillations in the Mammalian Brain

Ute Abraham1*, Daniel Granados-Fuentes1, Julie L. Prior[2], David R. Piwnica-Worms[2] and Erik D. Herzog • [1]Department of Biology, Washington University, St. Louis, MO 63130, [2]MIR, Washington University Medical School, St. Louis, MO 63110

The hierarchical organization of the multi-oscillator, circadian system in the mammalian brain has recently become the subject of debate. For example, the suprachiasmatic nuclei (SCN), considered to be the major pacemaker driving all other oscillations, are not required to sustain rhythmicity in the in vitro olfactory bulb (OB). To confirm that in vitro recordings reflect in vivo rhythmicity, we recorded bioluminescence from the OBs of intact and SCN-lesioned Period1::luciferase transgenic rats. We surgically replaced the skull covering the OB with a window made of agarose and glass. Following applications of luciferin onto the OB every 4 hours for 48 hours, we imaged Per1-driven bioluminescence from the in vivo OB with an ultra-sensitive camera. Initial results indicated that the OBs of intact and SCN-lesioned rats oscillate. The phase of the OB from intact rats was consistent with the phase observed subsequently in vitro. We also found Per1-driven, damped oscillations in cultured explants of the pineal gland, pituitary gland, median eminence, and vascular organ of the lamina terminalis of SCN-lesioned rats. However, the phasing of these rhythms was dependent on the time of surgery. Therefore, rhythmicity might be surgically induced in these tissues. We conclude that the mammalian brain contains circadian oscillators that are SCN-dependent and at least one that is SCN-independent.

022

Do Humoral Signals Regulate Circadian Rhythms of Gene Expression in Mouse Peripheral Organs?

Hongnian Guo, Judy McKinley Brewer, Ameya S. Champhekar, Ruth B. Harris[1], and Eric L. Bittman* • Dept of Biology, University of Massachusetts, Amherst, and [1]Dept of Food and Nutrition, University of Georgia, Athens GA

The suprachiasmatic nucleus (SCN) of the hypothalamus is the central circadian pacemaker in mammals. In both the SCN and peripheral organs, transcriptional – translational feedback loops among critical clock genes including per1, 2 and bmal1 generate circadian rhythms. It has been suggested that the SCN signal controlling peripheral oscillators could be humoral, neural or behavioral. We utilized parabiosis in order to establish the nature of the signal. This technique allows the exchange of blood within pairs of mice in which partners are joined together by fusing their humeri and femurs. If a humoral signal controls the peripheral oscillations, mice made arrhythmic by SCN lesions should show rhythmic per1 and bmal1 gene expression in their peripheral tissues when given intact partners. Mice were kept in 12L:12D for 5 weeks after surgery before transfer to DD. After 3 days they were sacrificed in early subjective day (ZT1) or early subjective night (ZT13). Northern blots revealed high levels of mPer1 and mBmal1 (normalized to mGapdh) at ZT13 and ZT1, respectively, in intact unpaired controls. Similar rhythms were seen in parabiotic pairs in which both mice were intact, indicating that the surgical procedure alone did not alter peripheral rhythms. These oscillations were eliminated by SCN lesions in unpaired mice. When SCN-lesioned mice were parabiotically linked to intact mice, rhythms of mPer1 and mBmal1 were reinstated in both kidney and liver. Thus non-neural (most likely humoral) cues dependent upon the SCN are sufficient to establish rhythms of circadian gene expression in at least some peripheral organs. Supported by NIMH RO1 59166.

023

SCN Regulation of Circadian Rhythms of Gene Expression in Hamster Peripheral Organs

Hongnian Guo*, Judy McKinley Brewer, Michael N. Lehman[1], and Eric L. Bittman • Dept of Biology , University of Massachusetts, Amherst MA 01003, and [1]Neuroscience Program, University of Cincinnati, Cincinnati OH 45267.

The suprachiasmatic nucleus (SCN) is the central circadian pacemaker. Behavioral circadian rhythms are eliminated by ablation and reinstated by transplantation of the SCN. In both the SCN and peripheral organs, critical clock genes generate circadian oscillations. We used Northern blots to examine the influence of day length and role of the SCN in circadian gene expression in hamster peripheral organs. Relative haBmal1 peaked at CT21, haPer1 at CT9-15 and haPer2 at CT15 in liver and kidney of wild type (WT) hamsters after 3 days or 11-13 weeks of DD. In long-term DD hamsters, elevations of haPer1 were extended, consistent with an expansion of ˚„. We ablated the SCN in 47 tau mutant hamsters to determine its role in regulation of peripheral rhythms and the effects of SCN transplants from WT donors. Controls receiving cortex grafts experienced elevated, non-rhythmic expression of haPer1, haPer2 and haBmal1 in liver and kidney. Behaviorally effective SCN grafts reinstated significant rhythms of gene expression in these tissues. Interestingly, SCN grafts that failed to reinstate locomotor rhythms suppressed gene expression to the normal range but failed to restore rhythmicity. The phase of the haPer1, haPer2 and haBmal1 rhythms appeared similar to that of WT intact hamsters. Use of WT hosts and tau donors, and intensive sampling around the times of rise and fall of haPer1, haPer2 and haBmal1, is in progress to test the hypothesis that the SCN entrains peripheral circadian oscillations. Supported by NIMH RO1 59166.

024

The Ion Channel, Narrow Abdomen, Functions in Drosophila Pacemaker Neurons to Regulate Circadian Behavior

Bridget C. Lear* and Ravi Allada • Northwestern University

In flies and mammals, conserved transcriptional feedback loops drive circadian behaviors. However, relatively little is known about how these core intracellular components regulate neuronal activity or in turn, how neuronal function mediates circadian rhythmicity. We have examined Drosophila loss-of-function mutants of a conserved ion channel, narrow abdomen (na). We have shown that na mutants fail to increase activity in anticipation of light-dark (LD) transitions during entrainment conditions and exhibit weak locomotor rhythms during constant darkness (DD), consistent with a circadian function for this channel. We have used tissue-specific rescue experiments to determine the neural requirements for na function. We find that na expression within circadian neurons is sufficient to fully rescue both the LD anticipation and DD rhythmicity phenotypes. Thus, na likely functions within circadian neurons to promote rhythmic behavior. To determine if this channel influences the core molecular oscillator, we have assessed PERIOD expression in circadian neurons. We find that PERIOD oscillations in na mutants occur with a similar phase and amplitude to those of wild-type flies in LD. Taken together, these data indicate that na functions within pacemaker neurons downstream of the core oscillator. We propose that this channel serves as a crucial link between transcriptional feedback loops and circadian regulation of neural activity.

025

LD and DD Behavioral Circadian Rhythms Are Controlled by Different Clock Neurons in the Drosophila Brain

Grima, B., ChŽlot, E., Rouyer, F*. • Institut de Neurobiologie Alfred Fessard, CNRS UPR 2216, av. de la Terrasse, Gif-sur-Yvette, France

LD and DD behavioral circadian rhythms are controlled by different clock neurons in the drosophila brain. The circadian clock that controls activity rhythms of adult drosophila is located in the brain. The PERIOD (PER) protein shows daily oscillations of its abundance in at least five groups of neurons including the ventral lateral neurons (LNv) that express the pigment dispersing factor (PDF). The loss of PDF strongly alters behavioral circadian rhythmicity, which is almost completely lost when the LNv are genetically ablated or when PER oscillations are abolished in these neurons. To understand the role of the different clock neurons in the control of behavioral rhythmicity, we have used the combinatorial Gal4 system to restore per expression in specific neuronal groups of arrhythmic per0 flies. We first show that constitutive per expression restricted to the LNv is sufficient to drive strong PER protein oscillations in these neurons and restore near wild-type 24 hrs behavioral rhythmicity to per0 flies in constant darkness. In LD conditions, wild-type flies display a bimodal activity pattern, whose predominant evening peak is clearly clock-regulated and phase advanced in pdf0 mutants. Targeted PER expression experiments show that the presence of the evening peak is LNv-independent but depends upon PER cyclic expression in another group of clock neurons, the PDF negative dorsal lateral neurons (LNd). These results indicate that LD and DD activity rhythms are controlled by two different sets of clock neurons.

026

Evidence That Cardiotrophin-like Cytokine is an Output Signal of the Mammalian Circadian Clock

Sebastian Kraves*, Charles J. Weitz • Harvard Medical School

The suprachiasmatic nucleus (SCN) drives circadian rhythms of locomotor activity by releasing factors that act locally on receptors near the 3rd ventricle. At least part of this regulation is known to involve periodic inhibition of locomotor activity by the SCN. Cardiotrophin-like cytokine (CLC), an immune system secreted factor not previously documented in the nervous system, satisfies multiple criteria for a role in the circadian inhibition of locomotor behavior by the SCN. CLC is expressed in the SCN in a distinctive subset of neurons, and its transcript shows a robust circadian rhythm, with abundant expression during subjective day and essentially no detectable expression during subjective night, a pattern consistent with a circadian inhibitor of locomotor activity. The receptor for CLC, the ciliary neurotrophic factor receptor (CNTFR), and GP130, its signal-transducing co-receptor, are expressed in the hypothalamus in cells clustered around the 3rd ventricle, as previously inferred for the targets of SCN locomotor regulation. Acute infusion of CLC into the 3rd ventricle resulted in a potent and transient inhibition of running-wheel activity without any effect on the circadian clock itself. Moreover, animals in which running-wheel activity was chronically suppressed by CNTFR activation still exhibited robust circadian rhythms of drinking. Acute or chronic 3rd ventricle infusion of interleukin-6 or interleukin-11, cytokines that act on GP130 receptor complexes different from that for CLC, had no effect on locomotor behavior. These results broadly support a role for CLC in the circadian regulation of behavior by the SCN.

027

Diffusible Factors from Immortalized SCN2.2 Cells Modulate GnRH Secretion from Co-cultured Immortalized GT1-7 Cells

*R.S. White (1), P.L. Mellon (1), D.J. Earnest (2), and P.E. Chappell (1) • (1)Department of Reproductive Medicine, University of California, San Diego, La Jolla, CA 92093-0674, and (2)Department of Human Anatomy, Texas A & M University Health Science Center, College Station, TX 77843-1114.

While previous studies establish the mammalian biological clock as an important regulator of reproductive function, it is currently unknown how the suprachiasmatic nucleus (SCN) modulates GnRH secretion- via monosynaptic neuronal pathways, humoral factors, or a combination of both. Recent studies have shown that immortalized SCN2.2 cells induce circadian rhythms of gene expression and glucose metabolism in fibroblasts, suggesting a diffusible factor responsible for entrainment. Studies from our laboratory indicate that oscillations in clock gene expression regulate GnRH secretion patterns from immortalized GT1-7 cells, suggesting that the SCN may influence reproduction via direct modulation of GnRH release. We used both static co-culture and cell perifusion methodologies to determine if humoral factors released by SCN2.2 cells alter GnRH secretion from GT1-7 cells. GnRH RIA of static media and perifusates revealed that SCN2.2 co-culture or conditioned media elicited rapid and transient increases in basal GnRH secretion, as well as significant increases in GnRH pulse amplitude, approximately 2-4 fold above that observed from GT1-7 cells co-cultured with NIH3T3 cells. Interestingly, a circadian rhythm of GnRH secretion was also observed from SCN2.2-co-cultured GT1-7 cells following the initial transient peak. SCN2.2 cells, in addition to releasing a GnRH-stimulating factor, may also increase extracellular peptidase activity or availability to accelerate the degradation of secreted GnRH, a conclusion supported by an observed rapid decline in exogenous GnRH only following incubation with SCN2.2 cells. These results suggest that the SCN may influence reproductive neurosecretion directly at the level of GnRH release via humoral regulation.

028

Per1-luc Expression Rhythms of Central and Peripheral Oscillators in LL-treated Arrhythmic Rats

Tomoko Yoshikawa[1]*, Shin Yamazaki[2], Michael Menaker[1] • [1]Department of Biology, University of Virginia, Charlottesville, VA 22903, [2]Department of Biological Science, Vanderbilt University, Nashville, TN 37235

The phases of central (SCN) and peripheral circadian oscillators are held in specific relationships under LD cycle, but the oscillators in an arrhythmic animal could be arrhythmic or out of phase each other. Prolonged constant light exposure disrupts circadian rhythms resulting in arrhythmicity in rats, however the mechanism underlying this effect is not known well. In this study, we examined whether circadian oscillators were rhythmic in LL treated arrhythmic rats and, if rhythmic, what were the phase relationships among them. We prepared SCN, pineal gland, pituitary and cornea cultures from transgenic Per1-luc rats whose body temperature and locomotor activity were arrhythmic, and measured light output from the cultured tissues. Most of the cultures from arrhythmic rats were rhythmic in culture. This might reflect in vivo rhythmicity or rhythmicity may have been initiated by the culture procedure. To test this we cultured tissues at two different times 12 hours apart and asked whether phase of the rhythm was related to culture time. The pineal, pituitary and SCN cultures showed culture time dependent phases of the Per1-luc

expression rhythm, while peak phases of the cornea cultures were dispersed around the clock. These result suggest that oscillators in the pineal, pituitary and SCN had been arrhythmic or damped in vivo while the cornea oscillator was free-running. The SCN cultures even from LD entrained rats showed culture time dependent phase shifts suggesting that the SCN may be particularly sensitive to resulting by some aspect of the culture procedure.

029

NASA workshop

George Brainard

030

NASA workshop

Chuck Czeisler

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NASA workshop

Michael Menaker

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Naomi Rodgers

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David Sliney

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NASA workshop

Jeffrey Sutton

035

NASA workshop

TBA

036

REM Sleep Function: Maybe It's the Rhythm (Not the Tune)

Thomas J. Balkin*, Nancy J. Wesensten, Allen Braun, Gregory Belenky • Walter Reed Army Institute of Research

The physiological processes by which sleep restores alertness and cognitive performance are not known, but it is generally thought that these restorative processes require, or are subserved by, the relative brain deactivation that characterizes slow wave sleep. By EEG criteria, however, Rapid Eye Movement (REM) is a state of brain activation (at a level comparable to that seen during wakefulness). Clearly, if the restorative benefits of sleep accrue as a function of brain deactivation, REM sleep must serve a different purpose. The paradoxical nature of REM sleep, and the fact that most dream mentation occurs during REM, has captured the imagination of sleep researchers for the past 51 years÷and a spate of hypotheses regarding possible functions of REM sleep have been proposed. These have ranged from exotic psychoanalytic notions that dreams are somehow necessary for symbolically working through unresolved conflicts, to the mundane÷for example, that REM sleep is necessary for providing "practice" for conjugate eye movements. Currently popular is the notion that REM sleep enhances consolidation of some types of memory. Based on recent findings from functional brain imaging studies during sleep, an alternative function of REM sleep is proposed÷that it serves as a periodic probe to determine the status of the brain (with respect to its readiness for waking), and provides an internal stimulus for awakening when the brain is ready to do so.

037

Sleep Freely to Know Your DLMO

Burgess*, HJ and Eastman, CI • Rush University Medical Center

We analyzed baseline sleep times from 120 young healthy subjects to determine the phase relationship between the DLMO and sleep in subjects following fixed or free sleep schedules. "Free sleepers" (N=60) slept at times of their own choosing and "fixed sleepers" (N=60) slept on a fixed schedule similar to their weekday schedule. Subjects completed sleep logs that were verified by actigraphy. We averaged sleep times from 6 days before we measured the DLMO (threshold=mean+2SD of first 3 low daytime points). The fixed sleepers had earlier DLMOs and sleep times (fixed: DLMO 20:46±1.1 h, bedtime (lights out) 23:17±0.9 h, wake time (lights on) 7:06±1.0 h; free: DLMO 22:41±1.5 h, bedtime 00:56±1.1 h, wake time 9:25±1.3 h, all $p<0.001$). There was no difference in the DLMO to bedtime interval (fixed: 2.5±1.1 h; free: 2.3±1.2 h), but the wake time to DLMO interval was slightly longer in the fixed sleepers (fixed: 13.7±1.1 h, free: 13.3±1.1 h, $p=0.056$). The highest correlation between sleep times and the DLMO was with wake time. Surprisingly, the correlation between wake time and the DLMO was higher in the free sleepers (free: $r=0.70$; fixed: $r=0.44$, $p<0.05$). This is likely because fixed sleepers slept at times determined by social factors, whereas free sleepers slept at times promoted by the circadian clock. Thus maintaining a fixed sleep

schedule does not necessarily improve estimations of the timing of the DLMO. Support: RO1 NR07677, RO1 OH03954.

038

Differential Effect of Sleep Disruption on Menstrual Cycle Dynamics

Elizabeth B. Klerman*[1], Janet E. Hall[2] • [1]Division of Sleep Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston MA, [2]Reproductive Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston MA

We hypothesized that sleep disruption designed to mimic rotating shifts would alter menstrual cycle dynamics, but might have different effects depending on the phase of the cycle in which it occurred. Seven healthy young women (19-34 years) with regular menstrual cycles and on no medications affecting reproductive hormones or sleep were studied. The five-month study included a regular sleep-wake schedule and monthly assessments of day of menses and ovulation as well as a five-day inpatient stay in month 3 during either the follicular (FP; menses –1 to +1 days) or luteal phase (LP; ovulation + 3 to +5 days). The first night, subjects slept for 8 hours at their habitual bedtimes. They were then awake for 24 hours. For the following three days, they slept for 8 hours beginning at their habitual waketime and were awake for the remaining 16 hours. Sleep disruption in the FP resulted in a change in cycle length in all 3 subjects of greater than or equal to 2 days compared with the mean of cycles 1, 2 and 4. This difference was accounted for by an increase in FP length in two subjects and a decrease in FP length in one subject. Sleep disruption in the LP was not associated with changes in cycle length in the cycle of study. However, the following FP was increased in 2 of 4 subjects and decreased in one. Taken together these preliminary results suggest that sleep disruption in either the follicular or luteal phase may affect menstrual cycle dynamics. Support: NIH-R01-HD40291, NIH-NCRR-GCRC-M01-RR02635 and M01-RR01066.

039

Interactions Between Circadian Rhythms and Epilepsy

Mark Quigg • University of Virginia

Circadian rhythms have marked effects on expression of epilepsy, influencing the timing of occurrence of seizures and the characteristics of interictal epileptiform discharges (IEDs). Circadian rhythms are not a singular entity but involve many systems within and without the central nervous system. Therefore, a circadian influence is not likely a single, unified mechanism. Rather, circadian rhythms are better thought of as endogenously–mediated, excitatory and inhibitory influences that vary with time of day and dynamically compete with other seizure precipitants to elevate or depress seizure threshold. I review basic concepts of chronobiology, the organization of the circadian timing system, the effects of epilepsy and seizures on circadian rhythms, and the influences of circadian rhythms on the timing of seizures.

040

The Circadian Aspects of Noicturnal Asthma.

*SA Shea, MF Hilton, H Evoniuk, RT Ayers, SA Shiels, R Sugarbaker, A Malhotra, FAJL Scheer, AF Massaro • Brigham & Women's Hospital

In many patients with asthma, pulmonary function worsens and asthma symptoms increase at night, producing "nocturnal asthma". To assess the relative influences of sleep and circadian rhythms on nocturnal asthma, we performed two complementary protocols in time isolation and dim light: (i) a 10 day 'forced desynchrony' (FD), wherein subjects slept at all phases of the circadian cycle and (ii) a 'constant routine' (CR) wherein subjects remained awake for 40-h under constant conditions. Indices of bronchoconstriction (spirometry, airways resistance) were measured every 2-4 hours. Subjects were took bronchodilator rescue medication (beta2-adrenergic agonist inhaler) based on symptoms alone. Results from both FD and CR protocols revealed a significant intrinsic circadian rhythm in indices of bronchoconstriction. Circadian amplitudes were 2-3 times greater in subjects with asthma than in controls. Spirometry was lowest and airways resistance was highest at a circadian phase that translates to 6:30 AM. FD data were analyzed separately when obtained during established wakefulness and immediately following standardized awakenings from sleep. These FD data revealed a significant interaction between the effect of sleep (or awakening from sleep) and of circadian rhythms: spirometry was substantially lower when sleep occurred in the afternoon when compared to the night. Rescue medication was taken on 54 occasions during the FD (range 0-14 among subjects): 73% occurred in the 12-h circadian period centered around the usual sleep period (only 50% would be expected by chance). Thus, an endogenous circadian rhythm can explain why asthma (pulmonary function and symptoms) is often worst at night. These data also suggest that asthma could worsen if sleep occurs in the daytime. Supported by NIH HL64815.

041

The Serine-threonine Protein Kinase NRC-2 Is Required for Some Circadian Outputs in Neurospora Crassa

*Andrew V. Greene, Alejandro Correa, and Deborah Bell-Pedersen • Program for the Biology of Filamentous Fungi and Department of Biology, Texas A&M University, College Station, TX 77843

While much is known about the nature of the central FRQ-based oscillator in Neurospora crassa, the mechanisms by which this oscillator controls time-of-day specific output are largely unknown. To identify candidate genes involved in the control of circadian output, we first used microarray analyses to catalog genes that are expressed rhythmically in constant darkness. One rhythmic gene identified from the arrays was nrc-2, which encodes a serine-threonine protein kinase that peaks in mRNA abundance during mid to late night. Null mutations of nrc-2 abolish rhythms in several, but not all clock-controlled genes examined. Interestingly, while frq mRNA levels are extremely low in the nrc-2null strain, frq mRNA and FRQ and WC-1 proteins are normally rhythmic, and frq mRNA is light induced to levels comparable to that of wild-type strains. Furthermore, our preliminary data indicate a defect in WC-1 phosphorylation in nrc-2 null strains cultured in constant darkness. Together, these data suggest that NRC-2 functions to control a subset of clock outputs; this may be through a direct signaling mechanism from the FRQ-based oscillator, or through a feedback mechanism involving regulation of the phosphorylation state of WC1.

043

Metabolic Effects on Circadian Rhythms in Mammals: The Role of Behavior

Ralph Mistlberger • Department of Psychology, Simon Fraser University, Burnaby BC

In vitro, changes in cellular redox state can alter the activity of CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers, which regulate transcription of circadian clock genes in the suprachiasmatic nucleus (SCN) and elsewhere. In peripheral tissues, glucose inhibits and insulin induces mPER1,2 expression, and can initiate circadian cycling. Perturbations of metabolism may thus serve as zeitgebers, or modify the effects of other zeitgebers, on central or peripheral circadian clock cells. Perturbations of metabolism can also directly affect the arousal state of behaving organisms; to what extent do metabolic stimuli affect circadian clocks indirectly, via behavior? In rodents, glucoprivation precipitated by insulin, 2-deoxyglucose or 24-48h of food deprivation (FD) can shift circadian phase or attenuate phase shifts to light in the subjective night. These effects are mimicked by stimulating locomotor activity and/or arousal without FD. Where tested, effects of FD are absent if changes in behavioral activity are prevented. Thus, in vivo, metabolic stimuli may affect centrally controlled circadian timing indirectly, by altering behavioral state and activating non-photic input pathways to SCN clock cells. In contrast to acute FD, chronic daytime food restriction entrains a circadian clock outside the SCN that drives food anticipatory circadian behaviors. In mice, NPAS2-KO attenuates food anticipatory rhythms, whereas in rats, extirpation of forebrain structures that express NPAS2 (e.g., neocortex, hippocampus, accumbens) does not. Formal properties of these rhythms, including the 'memory' for diurnal mealtime revealed during FD tests after ad-lib food access has restored nocturnal feeding and phasing of peripheral clocks, present interesting challenges for metabolic models.

043

A Reciprocal Coupling of Biorhythms and Metabolism

Steve McKnight • McKnight Laboratory Department of Biochemistry UT Southwestern Medical Center 5323 Harry Hines Boulevard Dallas, TX 75390-9152

Various lines of evidence, including the interrogation of gene expression patterns via DNA microarrays, have given evidence of cyclic regulation of gene expression as a function of the day:night cycle. In mammals the Clock:BMAL and NPAS2:BMAL heterodimeric transcription factors function as the activating arm of a negative feedback cycle. In addition to regulating expression of the Cryptochrome and Period genes, whose products help form the negative arm of the feedback cycle, the Clock:BMAL and NPAS2:BMAL heterodimers also regulate the expression of genes that specify functional output. Surprisingly, many "output" genes encode enzymes involved in the control of intermediary metabolism. Evidence will be presented indicating that the components forming the central circadian oscillator act to rhythmically control metabolism. Evidence will also be presented indicating that small metabolites feed back to regulate the central oscillator itself, resulting in the reciprocal interlocking between circadian rhythm and metabolism. Finally, the concept of a "metabolic cycle" specified by a central oscillator may translate to biological systems that display ultradian oscillation, including the budding yeast, S. cerevisiae, raising the possibility that the metabolic cycle is universal to eukaryotic cells and organisms.

044

Genome-wide Transcriptional Cycles gate DNA Replication and Cell Division in Yeast and Mammals

Robert R Klevecz • Cellular Dynamics Group Beckman Research Institute, Duarte CA 91010

Cell to cell signaling in yeast results in a stable genome wide transcriptional oscillation and gated cell cycle synchrony in vigorously stirred and aerated cultures. Dynamically, these dense cultures can serve as a model of circadian gating of DNA replication and cell division in mammalian tissues, and may have features in common with quorum sensing in prokaryotes. Although cell cycle times in these cultures can vary from 4 hours to 16 hours, the transcriptional oscillation that organizes cellular functions and underlies and precisely gates DNA replication has a ~40 minute period. In studies of cell kinetics and circadian rhythms in mammalian tissues, DNA replication is gated at 24 hour intervals from populations of cells whose individual cycle times are 5-10 days. For the first time, the dynamic architecture of phenotype can be investigated in gene-by-gene detail through the use of expression microarrays In yeast, period mutants of genes containing PAS domains produce period doubling or period halving of the benchmark ~40-minute oscillation in oxygen consumption. Period doubling of a transcriptional oscillation may represent a unique insight into the organization of the cell and suggests that circadian rhythms arise from period doubling bifurcations in a high frequency transcriptional oscillator.

045

A PERIOD Independent Role for TIMELESS in Drosophila Circadian Behavior

Ben H. Collins, Stephane Dissel, Ezio Rosato and Charalambos P. Kyriacou* • Departments of Genetics and Biology, University of Leicester, Leicester LE1 7RH, UK

The PERIOD (PER) and TIMELESS (TIM) proteins are core components of the Drosophila melanogaster circadian clock, and generate the negative feedback loop that drives circadian oscillations. TIM can stabilise PER, and the PER and TIM proteins cooperate in nuclear entry and retention in key pacemaker cells at night. TIM degrades rapidly in response to light, destabilising PER and releasing the negative regulation that PER provides to per and tim transcription D.melanogaster individuals carrying the null mutation per01 display arrhythmic locomotor activity under constant darkness (DD) while in light-dark (LD) cycles their locomotor activity is elevated under illumination and reduced in darkness, reflecting the 'masking' effect of light. Here we report that the combination of per01 with a mutation in the circadian photoreceptor cryptochrome (cryb) gives remarkably normal circadian locomotor behaviour in 24 h LD cycles. This phenomenon is TIM dependent, revealing a hitherto undiscovered, PER-independent role for TIM in generating behavioural rhythms.

046

Evolution of Photoperiodic Time Measurement: Does It Depend on the Circadian Clock?

William Bradshaw* and Christina Holzapfel • University of Oregon

Only model organisms live in a world of endless summer. Organisms in nature encounter seasonal environments and modify their development and dormancy accordingly. The mosquito, Wyeomyia smithii, ranges from the Gulf of Mexico to northern Canada where it occupies only the water-filled leaves of a single species of pitcher plant. This consistency of microhabitat makes it ideal for studying the regulation and genetics of seasonal activities and their relationship to the circadian clock mediating daily activities. Wyeomyia smithii uses the length of day to initiate, maintain, and terminate larval diapause. Seasonality has selected on critical photoperiod over the range of W. smithii and, concomitantly, we observe geographic variation in response to the Nanda-Hamner protocol, historically used to infer a circadian basis for photoperiodism. Critical photoperiod and the rhythmic response to the Nanda-Hamner protocol are genetically correlated within populations, but not between populations. Hence, these two aspects of photoperiodic time measurement are capable of independent evolution within a single species, showing that the relationship between the circadian clock and the evolution of critical photoperiod may be correlational but is not causal. Indeed, a day-interval (hourglass) timer provides a sufficient mechanism to explain the evolution of critical photoperiod in W. smithii. We have developed a linkage map for W. smithii and are determining whether genes responsible for photoperiodic time measurement map to the same regions as those responsible for circadian rhythmicity.

047

Circannual Rhythms and Photoperiodism—A Reaction Norm Approach

Eberhard Gwinner* and Barbara Helm • Max-Planck Institute of Ornithology

Zeitgebers synchronize endogenous circadian and circannual rhythms by determining their period and phase. The detailed way in which the endogenous system responds to zeitgeber stimuli appears to have evolved such that an optimal adjustment of daily and seasonal activities to the environment is achieved. So far, these "ecological" aspects of synchronization have barely been studied although they may well provide a basis for understanding significant properties of synchronization patterns, expressed, e.g., in phase response curves. In our talk we investigate the way in which photoperiod synchronizes and adaptively modulates circannual rhythms of molt and reproduction in stonechats (Saxicola torquata). These songbirds are equipped with a circannual clock that persists for up to 12 cycles and that is normally synchronized by the annual photoperiodic cycle. Four subspecies of this widely distributet species were studied in "common garden experiments" and subspecies were cross-bred to test whether differences are genetically determined. The results revealed strong evidence for an innate basis of subspecies differences in the responsiveness of certain phases of the circannual clock to its photoperiodic zeitgeber. These differences can best be described in terms of a subspecies specificity in the norm of reaction of the circannual oscillator to photoperiod. It is suggested that such an approach will widen our view of synchronization and, by focussing on the adaptive value of circannual clocks, will allow to place these rhythms into an evolutionary framework.

048

Circadian Systems: Adaptive Value and Selective Pressures

Carl Hirschie Johnson • Dept. of Biological Sciences, Vanderbilt University, Nashville, TN 37235 USA

We addressed the adaptive significance of circadian rhythmicity by testing the relative fitness under competition between various strains of cyanobacteria expressing different circadian periods. Strains that had a circadian period similar to that of the light/dark cycle were favored under competition. Arhythmic strains could not compete well against wild-type strains in light/dark cycles, but they could compete effectively in constant light. In fact, in constant light, the arhythmic strains frequently out-compete wild-type strains (but slowly). These data indicate that fitness is enhanced in cyanobacteria by (1) having a circadian clock, and (2) having a clock with a period that matches that of the environmental cycle. The data also indicate, however, that the clock system in cyanobacteria does not confer an intrinsic advantage that is valuable in constant conditions. The data are interpreted in the context of a model that proposes a rhythmically secreted and light-dependent inhibitor. The "escape from light" hypothesis for an early selective pressure involved in the evolution of circadian clocks will be described as well as experimental support for the hypothesis from Chlamydomonas.

049

Enhanced Phenotyping of Circadian Traits Using Simple Systems

Martha Merrow and Till Roenneberg • Institute for Medical Psychology, University of Munich, Germany

In order to better understand the human circadian system, we recently established a database of chronotype information. The distribution of chronotypes in the human population (judged by self-reported 'phase of entrainment') reflects the clock's complex genetic basis. This, taken together with recent genotyping reports, indicates that the groups of extreme individuals (i.e., larks and owls) will be heterogeneous for clock gene loci. We have thus initiated efforts to use our simpler model systems to devise realistic protocols to enhance the phenotyping of these individuals, with an eye to better genotyping.

The first strategy involves use of a complex, multi-feedback model of a circadian system. We have made a library of mutants, in silico, and used these in simulations of circadian protocols. Interestingly, some sites within the model proved more resistant or sensitive than others to mutant production. For both moderate early and extreme late mutants, almost all can be distinguished by the systematic application of in silico experiments, such as entrainment in extreme photoperiods, systematic changing of phase angles in response to photoperiod differences, or free running period in constant conditions.

The model suggests predictions regarding enhanced phenotyping, and some of these can be tested in Neurospora crassa. To that end, we are extending our understanding of the rules of entrainment in this organism. We have established entrainment behavior in the standard lab strain used for clock research (band), including a molecular profile of clock genes under these conditions. We have also initiated studies on isolates from nature, collected over a wide range of latitudes. First results show a chronotype distribution similar to what the human population yields.

050

Narcolepsy, Hypocretins and Beyond

Juliette Faraco, Ph.D. *, Emmanuel Mignot M.D., Ph.D. • Stanford University

Narcolepsy is a sleep disorder affecting 1:2000 individuals with disabling neurological consequences. Narcolepsy is characterized by excessive daytime sleepiness (EDS), cataplexy and abnormal transitions from wakefulness into REM sleep (SOREMPs). Currently available treatments include amphetamine-like drugs, and antidepressants to enhance monoaminergic activity but do not target the underlying pathophysiology of narcolepsy. Narcolepsy in humans is sporadic, HLA-associated, multigenic, and environmentally influenced. Familial narcolepsy in humans is rare. Using an autosomal recessive model of narcolepsy in canines, we used positional cloning to identify three mutations in the hypocrein (orexin) receptor 2 gene (hcrtr2), a G-protein coupled receptor. Mutations in hypocretin ligand or hypocretin receptor genes are rare in humans. After extensive screening we found only one mutation in the hypocretin ligand gene in a young patient with unusually early onset. Despite this, the majority of sporadic cases are associated with hypocretin peptide deficiency in the CSF. Post mortem studies in narcolepsy brains demonstrate hypocretin neuron loss in the lateral hypothalamus. These results, together with the HLA association suggest an autoimmune process is directed against hypocretin neurons. The results of other experiments indicate that hypocretins are a link to multiple behaviors of importance in neuroscience. Besides their important role in sleep and wakefulness, hypocretins regulate appetite, neuroendocrine, and energy expenditure mechanisms. The Stanford center for narcolepsy research is currently using multiple approaches in rodents, humans and increasingly in zebrafish to study this exciting and functionally important neurochemical system.

051

Genetics of Human Circadian Rhythms

Louis J. Ptacek and Ying-Hui Fu • HHMI/UCSF

Organisms living on our planet have evolved a circadian clock that regulates rhythmic oscillations affecting all aspects of their physiology. Tremendous insights into the molecular basis of circadian rhythmicity have occurred in our growing understanding of organisms from fungi, plants, flies, worms, to rodents. Some of these clock components are conserved across species. However, some organisms are diurnal and others nocturnal. What accounts for this difference? Humans are distinct from other species given the dramatic psychosocial and familio-cultural impositions placed on our sleep schedules. The opportunity to probe the molecular basis of the human circadian clock only recently became possible with the recognition of a Mendelian circadian variant in people (familial advanced sleep-phase syndrome, FASPS). While studying genetics in humans is more challenging than model organisms, one must ultimately study humans to understand functionally conserved elements of the clock and to understand differences between clocks in rodents and humans. We've identified a number of genetic variants in several genes and have begun to probe the in vitro consequences of these variants. Coupling phenotypic, genetic, and biochemical analyses with in vivo studies in various organisms provides an outstanding opportunity for learning about unique elements of human rhythms.

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FKF1: Photoperiodic Blue-light Receptor for Flowering in Arabidopsis

Takato Imaizumi*, and Steve A. Kay • The Scripps Resaerch Institute

In plants, the seasonal day-length changes that are measured by the circadian clock trigger flowering and other important physiological phenomena. Arabidopsis is a facultative long-day plant, thus longer days accelerate flowering. By characterizing a variety of mutants with altered flowering patterns, we, as well as others, have proposed that the long-day specific diurnal expression of a key flowering activator, CONSTANS (CO) gene, is one of the most crucial aspects of the photoperiodic control of flowering. An fkf1 (Flavin binding, Kelch repeat, F-box) mutant was isolated as one of the late-flowering mutants in long days, and FKF1 expression is controlled by the circadian clock, with transcripts peaking in the late afternoon. By analyzing the fkf1 mutants, we showed that the FKF1 expression was controlled directly by the putative core clock components. Additionally, we found that FKF1 protein has an essential role in regulating diurnal CO expression with light dependent manner. The CO expression was observed only when light, especially blue light, coincides with the FKF1 protein expression, indicating that light regulates the FKF1 function. Moreover, it was demonstrated that the recombinant FKF1 protein is a flavoprotein and possesses the properties of a blue-light sensor. Our results indicate that FKF1 is a novel blue-light photoreceptor in Arabidopsis. Both its clock-controlled expression late in the afternoon and its light dependent function are critical for the induction of diurnal CO expression. This leads plants to flower only when the day is getting longer.

053

Phototransduction for the Human Retinohypothalamic Tract: High Sensitivity to Short Wavelength Light

G. Brainard *, D. Sliney, J. Hanifin, G. Glickman, B. Byrne, J. Greeson, S. Jasser, E. Gerner, S. Wengratis, M. • Rollag Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107; Laser Microwave Division, U.S. Army Center for Health Promotion and Preventative Medicine, Aberdeen Proving Ground, Maryland 21010, Department of Anatomy, Physiology and Genetics, Uniformed Services University of Health Sciences, Bethesda, MD 20814.

Two action spectra suggested that a vitamin A1 retinaldehyde-based photopigment may be primarily responsible for melatonin suppression in humans (Brainard et al., *J. Neuroscience* 2001; Thapan et al., *J. Physiology* 2001). More recently, a study has shown that monochromatic 460 nm light is significantly stronger than 555 nm for inducing circadian phase shifts in healthy humans (Lockley et al., *JCEM* 2003). There was poor agreement between the two melatonin suppression action spectra, however, on the relative photosensitivity to monochromatic light at 420-424 nm. This might allow for the possibility that the action spectra match the cryptochrome absorption spectrum. A full fluence-response curve for melatonin suppression has now been completed with 420 nm monochromatic light in 8 healthy male and female subjects (mean age of 24.5 ± 0.3 years). Each volunteer was tested with nine retinal irradiances of 420 nm light (0 to 89.0 $\mu W/cm^2$; 14 nm half-peak bandwidth) with at least one week between each experiment. There were no significant differences between the pre-exposure melatonin values (F=0.69) suggesting circadian stability. The data fit a four parameter sigmoidal curve with a high coefficient of correlation (r^2=0.93) and is univariant with eight other monochromatic wavelengths up to 600 nm (Brainard et al., *J. Neuroscience* 2001). Finally, a within-subjects study using an equal photon density, confirmed that 460 nm

light is significantly stronger than 420 nm light for melatonin suppression ($p<0.04$). These data support the hypothesis that an opsin photopigment mediates photoreception for the retinohypothalamic tract of healthy human subjects. Support: NIH RO1NS36590, National Space Biomedical Res. Inst. under NASA Cooperative Agreement NCC 9-58 and NSF IBN9809916.

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Regulation of Photoreceptor Per1 and Per2 by Light, Dopamine, and a Circadian Clock

Joseph C. Besharse*, Minhong Zhuang, Katie Freeman, Joseph Fogerty • Medical College of Wisconsin

Two Xenopus laevis period genes (xPer1 and xPer2) exhibit different temporal patterns of expression in the retina, and one (xPer2) is directly responsive to light and dopamine. However, period genes are widely expressed different cell types. The goal of this study was to determine the cellular site of light and dopamine regulated xPer2 expression and diurnal xPer1 expression using in situ hybridization. Isolated eyecups were either fixed immediately or treated in vitro at the time of expected light onset to either light or quinpirole, a dopamine agonist, in the presence or absence of the cAMP analog, CPT-cAMP. xPer1 and xPer2 mRNA was analyzed by Northern blot and in situ hybridization. Both xPer1 and xPer2 were expressed in most cell types in the retina. However, light and quinpirole increased xPer2 levels in only in photoreceptors, and the effect of quinpirole, but not light, was blocked by pCPT-cAMP. Comparison of eyecups prepared at light onset (ZT0) and late afternoon (ZT8) show that xPer1 mRNA was high in photoreceptors at ZT0 and decreased at ZT8 while xPer2 mRNA low at ZT0 and high at ZT8. These data show that photoreceptors exhibit antiphasic diurnal changes in xPer1 and xPer2 mRNA as well as acute induction of xPer2 by light and quinpirole, and suggest a critical role of xPer2 in entrainment of the photoreceptor circadian oscillator by light and dopamine.

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Drosophila CRYPTOCHROME: Photoreception Mechanisms and Unexpected Roles of Its Two Structural Domains

Ania Busza*, Myai Emery-Le, Michael Rosbash and Patrick Emery • University of Massachusetts Medical School

Most organisms use circadian rhythms to adapt to daily changes in their environment. The molecular pacemaker underlying these rhythms is synchronized primarily by light and temperature. In Drosophila melanogaster, TIMELESS (TIM) and PERIOD (PER) are two key proteins that feedback negatively on their own transcription to generate circadian oscillations. The primary circadian photoreceptor is CRYPTOCHROME (CRY). When activated by light, CRY resets the pacemaker by triggering the proteasomal degradation of TIM. CRY itself is also degraded by a light-dependent and proteasome-dependent mechanism. Previous in vitro data suggests that CRY might interact directly with PER or with TIM. We present data demonstrating that CRY specifically interacts in a light-dependent manner with TIM in vivo and in S2 cells. This interaction, independent of PER, commits TIM to degradation. However, the CRY-TIM interaction is transient, as CRY itself is not committed to degradation and is thus degraded via a different mechanism than TIM. In addition, analysis of a novel cry allele (crym) reveals the specific

functions of the two CRY structural domains. The photolyase homology domain is sufficient for circadian photosensitivity and phototransduction, whereas the C-terminal domain regulates CRY stability and CRY-TIM interaction. This sharply contrasts with Arabidopsis CRYs and demonstrates that insect and plant cryptochromes use completely different mechanisms.

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Circadian Pacemaker Cells Transmit and Modulate Visual Information to Control a Rapid Behavioral Response

*Ben Collins, Esteban Mazzoni, Claude Desplan and Justin Blau • New York University

Two major assumptions exist regarding circadian pacemaker cells in Drosophila: (i) they modulate animal behavior gradually over 24 h; and (ii) innervation of pacemaker cells by the visual system occurs solely to entrain the clock. We show that both these assumptions are incorrect. When given a choice between light and darkness, Drosophila larvae show a strong preference for darkness, with the larval visual system, Bolwig's organ (BO), required for this behavior: larvae lacking a visual system are blind, and distribute 50:50 between the light and dark. Since BO innervates the pacemaker neurons (LNs), we tested whether the LNs are also part of the neural circuit leading to light avoidance. Either ablation of LNs or inactivation of LN function also made larvae blind, indicating that the circadian pacemaker neurons regulate a rapid behavioral response. In addition, there is a daily cycle in larval light sensitivity which is abolished in clock gene mutants. Since BO itself does not possess a molecular clock, the molecular clock in the LNs must regulate visual sensitivity. Interestingly, mutations in different clock components produce distinct photophobic phenotypes, with mutations in the two different arms of the clock having opposite effects. Null mutations in per or tim render larvae 'blind' to the light stimulus whilst ClkJrk and cyc0 mutants are hypersensitive to light. As per01; cyc0 double mutants are also hypersensitive to light, larval light sensitivity appears dependent on levels of CLK/CYC activity. This presumably involves the CLK driven (and therefore circadian) expression of an as-yet unidentified response factor.

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Comparing the Effect of an Oral Administration of Melatonin on the Electroretinogram (ERG) of Humans and Dogs

*M. Hebert[1], S. Rosolen[2,4],C. Chalier[3] J. Saucet[4] F. Rigaudi re[4], J-. Le Gargasson[4], P. Lachapelle[5], K. Danilenko[6] • [1]Department of Ophthalmology, Laval University, Quebec, PQ, Canada. [2]Clinique Veterinaire Voltaire, Asnieres, France; [3]Aventis-Pharma, Vitry-sur-Seine, France; [4]INSERM U-592, Paris, France; [5]Department of Ophthalmology, McGill University-Montreal Children's Hospital, Montreal, PQ, Canada; [6]Institute of Internal Medicine SB RAMS, Novosibirsk, Russian Federation

Purpose: Compare the effect of oral administration of melatonin on the ERG recorded from beagle dogs and humans. Methods: Photopic and scotopic ERG luminance response functions (20 intensities) were obtained from 7 anaesthetized dogs (3 males, 4 females), once without melatonin (control) and once after an oral administration of melatonin (90mg/dog). Photopic ERG luminance response functions (7 intensities) were obtained from 12 human subjects (6 males and 6 females), before (baseline) and after oral administration of a placebo and before (baseline) and after oral administration of melatonin (15mg). Vmax

(maximal amplitude achieved) and log K (retinal sensitivity) were calculated from the derived luminance response functions. Results: In dogs, 50 minutes after melatonin administration, a significant Vmax amplitude reduction of 43.5% was observed in the photopic condition only, going from 197.3 ±49.6μV to 111.4 ±36μV (mean ±SD; p<0.01). In humans, when compared to the respective baseline, we observed no change in Vmax amplitude after the placebo (86.6 ±9.8μV versus 88.0 ±8.7μV) and a significant decrease of 7.9 %, 50 min after melatonin (87.4 ±13.4μV versus 80.5 ±13.0μV; p<0.01). No change in retinal sensitivity (log K) was observed in dogs or humans. Conclusion: Melatonin administered during the daytime (when least produced naturally) appears to have a negative effect on the cone ERG in both humans and dogs. It has been proposed that in daytime dopamine production favours cone activity and that melatonin and dopamine inhibit each other. Therefore, it is possible that melatonin could have impact cone functioning through a retinal dopamine inhibition.

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Temporal Response Characteristics of Circadian Photoreceptors

Rieux C.*, Drouyer E., Hut R.A., Cooper • H.M. INSERM U-371, Cerveau et Vision, 69675 Bron, France

Institut federatif des Neurosciences (IFNL), Lyon, France In mammals, the 24-hour period of the endogenous pacemaker in the suprachiasmatic nucleus (SCN) is synchronized to the external light-dark cycle through photic information conveyed from the retina. Recent studies suggest that both classical photoreceptors (rods/cones) and light responsive melanopsin expressing ganglion cells are involved in entrainment. A major question concerns the respective contribution of each type of photoreceptor in the construction of the retinal signal in natural conditions. Our strategy is to study the elementary properties of the response of each type of photoreceptor. The circadian response according to wavelength and irradiance has already been investigated. However the temporal aspects of the response have been largely overlooked. We use single unit electrophysiological recording in the SCN as an "on-line" method to study the time course of the response to light stimulation. The neuronal response of SCN neurons has been compared in two conditions: dark-adapted and light adapted retina. Light adaptation allows to significantly decrease the sensitivity of rods and cones. The results suggest that (i) rods and cones display a phasic response and a reduced but tonic response, whereas (ii) melanopsin ganglion cell response exhibits alterations with long time constants and a high amplitude, robust tonic response. These characteristics are very compatible with what has been shown in vitro by (Berson et al., 2002).Support : 5th PCRD (QLK6-CT-2002-02258), ACT INSERM, ACI MRT Marie Curie (#QLK4CT199951420)

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Differential Regulation of Two Arylalkylamine-n-acetyltransferase in the Gilthead Seabream (Sparus Aurata)

Nitzan segev[1], Bina Zilberman-Peled[2], Yoav Gothilf[2] and Benny Ron[1]* • [1]National Center for Mariculture, Israel Oceanographic and Limnological Research, P.O.B. 1212, Eilat 88112, Israel, [2]Department of Zoology, George S. Wise Faculty of Sciences, Tel Aviv University, Tel Aviv, Israel

We identified two AANATs in Sparus aurata: sbAANAT1, related to AANATs found in higher vertebrates, is specifically expressed in the retina; sbAANAT2 is specifically expressed in the pineal organ.

Retinal sbAANAT1 has high substrate affinity and low activity rate while inhibited by high substrate and product concentrations. Nevertheless, pineal sbAANAT2 exhibits low substrate affinity and high activity rate while not inhibited by substrates or products. The two recombinant enzymes also exhibit differential substrate preference. Consequently, we characterized and compared sbAANATs enzymatic activity and melatonin production for over one year while rearing the fish under ambient photoperiod. Since we found distinct differences between the two sbAANATs, we conducted in vivo and in vitro experiments where either fish or pineal gland and retina were exposed to bright light during the night. The results indicate an increase in retinal sbAANAT1 activity in response to light exposure in both in vivo and in vitro experiments. On the other hand, sbAANAT2 activity decreased, as predicted, in response to light exposure of the pineal gland during the in vitro experiment. Interestingly enough, there was sbAANAT2 increase during the in vivo experiments. These results led us to hypothesize that sbAANAAT1 might has an important role in the rapid adjustment of the retina to the appearance of light while not obeying our findings of the proteasomal proteolysis mechanism in the pineal gland. Moreover, the in vivo results convey us to propose a novel hypothesis pointing at an interaction between the retina and the pineal gland in fish.
*Corresponding author. E-mail: ronbenny@agri.huji.ac.il

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The Avian Circadian System is Modified According to Migratory Status

Paul A. Bartell*, Urs Albrecht, Ingrid Schwabl, and Eberhard Gwinner • Texas A & M University

A circannual clock directly controls the dramatic modifications in behavior and physiology observed each Spring and Autumn in migratory songbirds. Conspicuous amongst these modifications are changes in the bird's circadian system, such that these normally day-active birds begin to display "nocturnal migratory restlessness," commonly called Zugunruhe. Zugunruhe is a seasonally specific behavior defined by the presence of nocturnal, directional, stereotypic "wing-whirring". These migratory behaviors can be easily discerned from other locomotor behaviors exhibited by the bird. Because Zugunruhe has been demonstrated to be under the direct control of a circadian oscillator, we investigated whether the observed changes in circadian behavior in the nocturnally migrating Blackcap (Sylvia atricapilla) were concomitant with changes in the central mechanisms of the avian circadian system. We collected blood and tissue samples from Blackcaps (n=48) of different migratory status at different Zeitgeber times under the same photoperiod (12.45:11.15). Using RIA and in situ hybridization technologies, respectively, we observed a decrease in the amplitude and alterations in the phasing of the melatonin signal and a decrease in the amplitude of pineal Period2 expression. The distribution and abundance of additional canonical clock genes (Period2, Cryptochrome1, and BMAL1) in different areas of the brain are currently being analyzed. Our data suggest that the circannual changes in behavior and physiology observed in passerines during migration are, at least in part, dependent upon changes within the central components of the circadian system.

061

Chronobiological Plasticity Is Associated with Division of Labor in the Bumble Bee Bombus Terrestris

Shai Yerushalmi and Guy Bloch* • Department of Evolution, Systematics, and Ecology, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

In honeybees division of labor is associated with plasticity in the circadian system. Bee larvae require constant care and young "nurse" bees work arrhythmically around the clock to provide it. In contrast, older foragers have a highly developed internal clock that is used for sun compass navigation, dance communication, and for timing visits to flowers. Foragers typically have higher average levels and stronger diurnal oscillations of brain period mRNA levels. In this study, we investigated the relationships between division of labor and circadian rhythms in the bumblebee Bombus terrestris. The social organization of bumblebees is simpler and their division of labor does not rely on age as in honeybees. We observed free-flying B. terrestris colonies (n=3) around the clock for 8 days under dim red light (which the bees cannot see). We found that large workers typically perform foraging activities and may start their foraging career as early as at one day of age, whereas small bees start to forage late at life or do not forage at all. Foragers have strong circadian rhythms with reduced activity during the night whereas nurses are active around the clock with weak or no circadian rhythms. In laboratory experiments we placed bee pupae in individual cages and monitored locomotor activity for the emerging adults under constant conditions. We found that large (forager-size) bumblebees developed circadian rhythms significantly earlier than their smaller (nurse-size) sisters. These findings are consistent with the hypothesis that plasticity in circadian rhythms is functionally significant and suggest that ontogeny of adult rhythmicity is influenced by developmental processes at pre-adult stages.

062

Arousal Episodes in Hibernating Golden-mantled Ground Squirrels: Why Wake Up?

*Patricia J. DeCoursey and Sierra Jones • Department of Biological Science, University of South Carolina, Columbia, SC

The profound hibernation of cold climate ground squirrels is a strategy for escaping the rigors of winter. Hibernating, golden-mantled squirrels consistently alternate an extended deep torpor bout with a brief euthermic arousal episode, but the function of the metabolically expensive arousals is not yet clear. The hibernation behavior of 4 golden-mantled squirrels was monitored continuously in a laboratory Hibernaculum by time-lapse videotaping for most of a winter under simulated natural conditions. Implanted Thermochron iButton data loggers recorded body temperature continuously. Arousal episodes were analyzed in terms of 5 behavioral levels of activity intensity: 0=motionless torpor: 1=shivering or rapid breathing; 2=feeding or grooming; 3=active exploration; and 4=very energetic activity. The wake-up progression always proceeded from isolated twitches, to continuous shivering and increasingly rapid breathing, then upright body posture with grooming and feeding, and finally exploration of the cage. The mean arousal episode duration for the four squirrels ranged from 13.4 h to 17.5 h with an overall mean of 16.1 h. The mean % of episode unequivocally spent awake (activity levels 2-4) ranged from 25.2% to 32.4% with a group mean of 29.1%. In some episodes, over 50% of the arousal time was spent awake in level 2-4 categories of activity. The types of behavior during arousal episodes suggest possible adaptiveness with important ecological significance for winter survival of hibernating ground squirrels.

063

Diapause and Circadian Clock Gene Expression in the Linden Bug, Pyrrhocoris Apterus

D. Dolezel*, I. Sauman, M. Hodkova • Institute of Entomology

Although photoperiodism is undoubtedly one of the most important functions of the circadian system, the role of circadian clock genes remains unclear. Molecular cloning of the pivotal clock gene homologs period and Clock from the European linden bug, Pyrrhocoris apterus, enabled us to test their possible role in the bug photoperiodic timing system. We compared the expression of period and Clock genes in the head of animals kept under diapause promoting short days (SD) and diapause preventing long days (LD), using RNase protection assay. There was only a weak diurnal rhythm in both period and Clock mRNA under LD and no detectable rhythm under SD. Under SD, however, the level of period mRNA was about 10-fold and that of Clock mRNA about 2-fold higher than under LD. In a mutant, that does not undergo diapause, even under SD, levels of both transcripts were low in both photoperiods. The block to diapause photoresponsiveness was not associated with defects in the circadian timing, but levels of period and Clock mRNAs were related to the properties of activity rhythms. The differential regulation of the levels of two clock gene transcripts in a photoperiodic mutant strongly indicates a link between photoperiod, the magnitude of clock gene expression, and developmental outputs.

064

Feeding Condition Modifies the Photoperiodic Response of Dispersal Characteristics in the Water Strider, Aquarius Paludum

Tetsuo HARADA* and Tatsuya NISHIMOTO • Laboratory of environmental Physiology, Faculty of Education, Kochi University, Kochi 780-8520, Japan

Objectives This experimental study aims at examining whether photoperiodic responses of dispersal characteristics can be modified by feeding conditions in this species. Material and Methods Diapause adult of A. Paludum were collected from two water ways in Kochi (33°N) at the beginning of October, 2003. After 2 days exposure to low temperature (7°C) for flight muscle maturation, they were kept under 12h light- 12h dark (12L-12D), at 20°C for a week which were similar conditions in late fall of Kochi. Till then the one pair of water striders were fed on adults of Lucilla illustris. The adult water striders were transferred to one of the following conditions. (1) Feeding everyday, 15.5L-8.5D, (2) feeding every two days, 15.5L-8.5D, (3) Feeding every three days, 15.5L-8.5D, (4) Feeding every day, 12L-12D. Each group consisted of 22 pairs of females and males and all survived for the 5 experimental weeks. Flight experiment was performed for the four groups every 10 days till the fifth week. Results Flight propensity was higher under the group (3) than the group (1) in 3-5 weeks (Kruskal-Wallis test, $P<0.01$). The high flight propensity was similar to that under 12L-12D. More percentage (31.8) of the group (3) kept mature fright muscles even 5 weeks after transfer to the long-days than that of the group 1 (4.7%) and group 2 (9.1%) (´š2 test: $P<0.01$) (group 4: 22.7%). Conclusion Long-days promoting of flight muscle histolysis can be canceled under worse feeding condition in A. Paludum.

065

A Change in Gonadal Hormone Receptor Expression in the SCN Is Related to a Developmental Change in Tau

Dan Hummer* & Theresa Lee • University of Michigan

The free-running period (_) of adult male and female Octodon degus (degus) differs by more than 30 minutes (males=23.2h; females=23.75h). This difference first appears after puberty and results from a shortening of _ in males. Exposure to testicular hormones around puberty is necessary for the change in _ to occur. However, a change in circulating testosterone is not a sufficient explanation for the change in _ since there is a 6-month delay between puberty and the decrease in _. We hypothesized that the change of _ in males would be associated with an increase in androgen receptor (AR) expression in the suprachiasmatic nucleus (SCN). Male and female degus were gonadectomized (GDX) or left intact (SHAM) at weaning. was calculated from free-running wheel records collected every other month between 4 and 12-months. Brains were also collected from intact and prepubertally GDX male degus at 2, 4, 6, 8, 10, and 12-months of age. 40µm SCN sections were processed for AR immunocytochemistry. Developmental changes in the number of AR-positive cells within the SCN were examined in relation to the developmental change in _, as well as pubertal changes in circulating testosterone and gonadal morphology. _ did not change in SHAM females, GDX females, or GDX males during development. However, _ shortened significantly between 10 and 12-months of age in SHAM males and was significantly shorter than SHAM females or GDX males at 12-months of age (pÕs<0.05). AR-positive cells increase dramatically in the SCN of intact males between 8 and 12-months of age. These data suggest that sexual differentiation of _ depends in part on the timing of AR expression within the SCN of male degus.

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Competition, Resource Levels, and Temperature Rhythms of Golden Spiny Mice: Do Overt Responses Reflect Entrainment?

Ofir Levy, Tamar Dayan, and Noga Kronfeld-Schor* • Department of Zoology, Tel Aviv University, Tel Aviv 69978, Israel

Nocturnal common spiny mice (Acomys cahirinus) and diurnally active golden spiny mice (A. russatus) coexist in a rocky desert near the Dead Sea. In absence of A. cahirinus, A. russatus are also active during the night, suggesting that they are competitively displaced into diurnal activity. We monitored the effect of A. cahirinus and of food availability on body temperature (Tb) and activity (Ac) rhythms of spiny mice in four 1000 sq m experimental field enclosures: two controls (both species) and two experimental (A. russatus). We implanted the mice with abdominal radio-transmitters, and monitored Tb and Ac with excess food added to the enclosures, and then with no food added. We found no differences in Tb and Ac patterns of A. russatus between control and experimental enclosures. When food was added, A. russatus were active during both day and night in all enclosures. When no food was added, A. russatus turned entirely diurnal both in the control and in the experimental enclosures, suggesting that resource level rather than interference is the proximate cue for temporal partitioning in this system. Next, we trapped all A. russatus individuals, and transferred them to the laboratory, where they were held under constant conditions (27°C, DD). Tb of all individuals revert within one day, showing high Tb during the subjective night, suggesting that diurnal activity of A. russatus in the field reflects a masking effect, or a difference in the way the clock entrains Tb (and Ac) rhythms, rather than the process of entrainment.

067

Photoperiodic Regulation of Gene Expression in Major Body Organs of Siberian Hamsters.

Looby, P., Crawford, F.I., Logunova, L. & *Loudon, A.S.I. • University of Manchester

We have recently shown in Syrian hamsters that melatonin-driven clock gene activation in the pars tuberalis melatonin target site does not alter in short-day (SD) refractory animals, despite altered downstream pituitary signalling driving the LD-like prolactin response (FASEB Journal 17:810-5). In contrast, clock gene rhythms in heart and lung are strongly photoperiod-modulated, but remarkably, they revert to a long-day (LD) like profile in SD-refractory animals (Current Biology 13, 1543-8). Thus, in contrast to neuroendocrine targets, clock genes in major body organs of seasonal mammals may both record photoperiodic time and track seasonal physiology. In the Siberian hamster lung, there is a strong seasonal variation in the phase and amplitude of a number of clock genes, for both mRNA and protein (Western) levels. ICC for PER1 and REV-ERB‡ reveals strong regional localisation within the lung, confined mainly to just two cell types (Clara cells and Type-2 pneumocytes). These cells are putative local-acting stem cells responsible for lung regeneration, and also co-localise for a key rate-limiting enzyme regulating retinoid biosynthesis (RALDH2), essential for normal development. Using heterologous 15K-gene murine arrays, we characterised global photoperiodic-driven changes in gene expression in peripheral tissues. Approximately 17% of transcripts exhibited significantly ($p<0.05$) altered expression (2,545 out of 15,000); intriguingly slightly more (1363) were up-regulated by SD photoperiods. Our data show that peripheral tissues of a seasonal mammal track seasonal photoperiod changes and reveal a surprising extent of seasonal global gene regulation in major body organs.

068

Intra-SCN Signaling: Gastrin-Releasing Peptide Activates "Cap" Cells of the SCN

Michael C. Antle[1],* and Rae Silver[1,2,3] • Departments of Psychology, [1]Columbia University, 1190 Amsterdam Avenue, New York, NY 10027, [2]Barnard College, 3009 Broadway, New York, NY 10027, and [3]Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY

While it is known that some SCN cells receive direct photic input from the RHT, their output signals have yet to be identified. The present study examined whether gastrin-releasing peptide (GRP) serves this function in hamsters. It is known that GRP cells in the SCN respond to phase-shifting light pulses with c-fos expression, and that intra-SCN injections of GRP causes photic-like phase shifts. The present experiments examined responses to 3rd ventricle injections of GRP with the goal of understanding which SCN neurons respond this putative intra-SCN signal. 30 minutes after a GRP injection, cells in the dorsolateral SCN expressed the phosphorylated form of extracellular signal-related kinase 1/2 (P-ERK). This dorsolateral population of GRP-sensitive cells forms a "cap" lying atop the calbindin region. Furthermore, both fos and Per1 mRNA were expressed in this region 90 minutes following a GRP injection. These results suggest that GRP serves as an output signal of light responsive SCN neurons acting on cells of the "cap" region. When integrated with previous studies on SCN heterogeneity, we note that the P-ERK cap cells are distinct from the SCN VIP core, calbindin core and VP shell cells suggesting that the conceptualization of the SCN as having only two compartments is an oversimplification. Supported by NIH grant 37919 to RS and a CIHR fellowship to MCA

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Dexras1 Is a Molecular Coupler of Photic and Non-Photic Cues to the Circadian Clock

Hai-Ying Mary Cheng*, Karl Obrietan, Boyoung Lee, Sean W. Cain, Patricia V. Agostino, Nicholas A. Joza, Mary E. Harrington, Martin R. Ralph, Josef M. Penninger • Ontario Cancer Institute/Princess Margaret Hospital

Many biological processes exhibit circadian rhythmicity generated by an internal clock mechanism that is synchronized to the environment by external photic or non-photic cues. The mechanisms of clock entrainment and the regulation of the clock input pathways are poorly understood. Here we show that the Ras-like G protein, Dexras1, is a critical modulator of the sensitivity of the central pacemaker to photic as well as non-photic inputs. Dexras1-/- mice exhibited (1) advanced phase angle of entrainment to 24 hr light-dark cycles; (2) reduced responsiveness (phase shifts) to pulses of light given in the early night; and (3) diminished response to NMDA. In contrast to reducing the response to photic stimuli, dexras1-/- animals exhibited enhanced responses to non-photic manipulations. These data show that Dexras1 negatively modulates non-photic inputs to the clock, and at the same time positively regulates photic inputs. Unexpectedly, the behavioural rhythms of dexras1-/- mice also exhibited a greater tendency to dissociate into multiple components in constant light. These findings identify Dexras1 as a molecular couple of multiple input pathways to the mammalian circadian clock, modulating the sensitivity of the central pacemaker to photic, non-photic and potentially pacemaker coupling signals.

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Gating of Arylalkylamine N-acetyltransferase Gene Expression in the Mouse Pineal Gland

Liang, J, Chansard, M, Fukuhara, C* • Neuroscience Institute, Morehouse School of Medicine, Atlanta GA 30310

In a previous retinal study we have shown that melatonin is not synthesized following dark exposure in the early morning. In this organ, retinal circadian clock regulates the gating by controlling circadian adenylyl cyclase type 1 (AC1) expression. Previous studies have shown that dark exposure also does not induce melatonin synthesis in the day in the pineal gland. However, it has not yet been elucidated where (the SCN or pineal gland?) melatonin synthesis is inhibited during the daytime following dark exposure. Although most of the inbred mice are melatonin-deficient, we found that Aa-Nat expression in the C57/BL mouse pineal glands are similarly observed to that in rat pineal glands. Since availability of a series of knockout mice, we decided to use mouse pineal glands. In this study we 1) compared Aa-Nat expression in the C57/BL and C3H mouse pineal glands, and investigated 2) whether melatonin synthesis is gated within the pineal gland. Finally we examine 3) whether functional circadian oscillators within the pineal are necessary for gating mechanisms. Aa-Nat mRNA levels in C57/BL mouse pineal gland were measured using real time quantitative PCR method. Our results showed that 1) In both strains, Aa-Nat mRNA levels in the pineal glands showed robust diurnal rhythms, and responded to dark exposure in a same manner. 2) Aa-Nat was not induced by dark exposure in the day, *in vivo*. Beta-adrenergic receptor agonist isoproterenol stimulation did not stimulate Aa-Nat gene expression, *in vivo*, and *in vitro*. Our results suggest that mouse pineal glands are useful model to study regulation of Aa-Nat gene expression. And, gating mechanism is located within the pineal gland. We are currently examining the effect of *Clock* gene mutation on gating mechanisms within the pineal gland. Supported by NINDS grant 5U54NS034194-10 to C.F.

071

Circadian Disruptions in Mice with a Targeted Mutation of the Calbindin-D28K Gene

Lance J. Kriegsfeld(1)*, Dan Feng Mei(2), Lily Yan(1), & Rae Silver(1,2,3) • Departments of Psychology, (1)Columbia University and (2)Barnard College, New York, NY 10027, and (3)Department of Anatomy and Cell Biology, College of Physicians and Surgeons, New York, NY 10032 USA.

The caudal SCN of Syrian hamsters contains calbindin-D28K (CalB) expressing cells that are light-induced, but not rhythmic, in clock gene expression. Cells in this region have a circadian rhythm in the nuclear localization of CalB, with high expression during the day and low expression at night. Importantly, behavioral phase shifts and light-induced Per expression are blocked during the subjective night, and enhanced during the subjective day, following CalB antisense treatment. Finally, we demonstrated that this subregion of the SCN is required for circadian function as measured by behavior (locomotion, drinking, gnawing), physiology (body temperature, heart rate), and hormone secretion (melatonin, cortisol), as targeted microlesions result in loss of rhythmicity. To further explore the role of CalB, we took advantage of the molecular-genetic tools available in a mouse model. Early in postnatal development, CalB is expressed in a core subregion of the SCN reminiscent of the pattern of expression seen in hamsters. We investigated mice deficient in CalB to determine the potential role of this protein in mouse SCN function. The circadian behavior of these animals is severely disrupted, while light-induced shifts in behavior are normal. In agreement with disruptions in free-running behavior, rhythmic PER2 expression is attenuated. In contrast, light-induced Per1 is normal, while light-induced FOS protein is attenuated in CalB mutants. Together, these results suggest that either CalB is necessary for normal mouse SCN development or is an essential component for adult clock function. Supported by NIH Grant NS-37919(RS) and MH-12408(LJK).

072

Roles of Vasoactive Intestinal Polypeptide in Circadian Rhythm Processes

Hugh Piggins*, Tim Brown, and Alun Hughes • University of Manchester

The neuropeptide vasoactive intestinal polypeptide (VIP) and its receptor, VPAC2, are abundant in the rodent suprachiasmatic nuclei (SCN), but it is only recently that the influences of VIP-VPAC2 signalling in circadian pacemaker processes have begun to be understood. Transgenic mice (VIpr2-/-) in which the expression of the VPAC2 receptor gene is impaired fail to manifest overt rhythms in core clock gene expression, electrical discharge in SCN neuronal activity, and wheel-running rhythms (Harmar et al., 2002; Cutler et al., 2003). Mice deficient in VIP also show impairments in free-running behavioral rhythms (Colwell et al., 2003), although the circadian phenotype of these mice is not as severe as that of Vipr2-/- animals. New research from our laboratory has revealed a profound absence of photic gating in the SCN of Vipr2-/- mice (Hughes et al., 2004) indicating that another cardinal trait of the circadian pacemaker is compromised in these mice. However, the SCN circadian pacemaker in Vipr2-/- mice may not be completely ablated. In vitro multiunit recordings from SCN slices of Vipr2-/- indicate that a small proportion of Vipr2-/- SCN neurons maintain low amplitude rhythms in spontaneous discharge activity. These findings suggest that in the absence of VIP-VPAC2 signalling, SCN neurons default to low levels of spontaneous electrical activity, with some neurons sustaining low amplitude oscillations that are not sufficient to orchestrate and synchronize circadian rhythmicity throughout the SCN.

073

Light and Suprachiasmatic Nucleus Interact in the Regulation of Body Temperature

FAJL SCHEER*, C VAN HEIJNINGEN, EJW VAN SOMEREN, RM BUIJS • Department of Hypothalamic Integration Mechanisms, Netherlands Institute for Brain Research, 1105 AZ Amsterdam, The Netherlands

The mammalian biological clock, located in the suprachiasmatic nucleus (SCN), is crucial for circadian rhythms in physiology and behavior. Light is the strongest input to the SCN. However, unequivocal results have been reported on whether the SCN is required for the circadian temperature rhythm and for the acute ('masking') effects of light on temperature. The goal of the present studies was to investigate the interaction between the SCN and light in the regulation of body temperature. All recordings were performed by telemetry in free moving male Wistar rats, including 8 intact and 7 SCN-lesioned rats. Complete lesioning was verified by neurotransmitter staining. Firstly, by analysis only after at least half an hour of behavioral rest, we demonstrated an endogenous circadian rhythm under DD in body temperature independent of locomotor activity. This temperature rhythm was abolished by SCN lesioning. Secondly, we demonstrated a circadian phase-dependent suppressive effect of light on body temperature in intact but not SCN-lesioned animals. Surprisingly, by six months after SCNx, light suppression of body temperature – but not of activity – was restored to the level of intact animals, resulting in a daily body temperature rhythm in LD. The latter finding suggest that, after lesioning of the SCN as main relay station for the acute body temperature-inhibition by light, secondary relay nuclei can eventually fully take over this function of the SCN. The present results provide a possible explanation for the controversy in literature on whether the SCN is required for daily temperature rhythms under DD and LD.

074

Single Cell Circadian Rhythms of Luminescence in Cultures from MPER2-LUC Knockin Mice

David K. Welsh* (1,2), Andrew Liu (2), Seung-Hee Yoo (3), Joseph S. Takahashi (3), Steve A. Kay (1,2) • (1)Dept. Psychiatry, UCSD School of Medicine, La Jolla, CA, (2) Dept. Cell Biology, The Scripps Research Institute, La Jolla, CA, (3) Dept. Neurobiology & Physiology, Northwestern Univ., Evanston, IL

Many mammalian tissues express circadian clock genes rhythmically, but it is not clear precisely how individual cells contribute to circadian oscillations, because detection of single cell rhythms has been elusive. Coronal SCN slices, peripheral tissue explants, and dissociated fibroblasts were cultured from mice expressing a PERIOD2::LUCIFERASE fusion protein (Yoo, PNAS, 2004). Circadian rhythms of luminescence were recorded from whole cultures using a luminometer device (Actimetrics). Cultures were then transferred to the stage of an inverted microscope with an environmental chamber (Solent), for imaging of single cell luminescence with a cooled CCD camera (Hamamatsu Orca II ER). SCN rhythms persist robustly for over 2 months in vitro, with periods of ca. 24-25 hrs. Imaging, however, reveals heterogeneous phasing of single cell circadian rhythms, in some cases organized as rhythmic waves of luminescence moving from side to side, or medial to lateral. Preliminary data also support the hypothesis that damping in whole cultures at least partly reflects cells drifting out of phase, rather than loss of amplitude in individual cells. First, although low density fibroblast cultures show rapid damping after serum shock or medium change, residual rhythmicity persists for at least three weeks in some dense

cultures, where intercellular coupling is presumably stronger. Liver and lung explants also retain low amplitude rhythmicity for several weeks. Second, imaging of nearly arrhythmic liver explants reveals heterogeneously phased circadian rhythms of single cells that have migrated out from the explant. Our data suggest that single peripheral cells may be autonomous circadian clocks. [Supported in part by K08 MH067657 (DKW).]

075

Control of Transcript Rhythms in Drosophila by the Circadian Clock and Light

Herman Wijnen*[1], Catharine Boothroyd[1], Felix Naef[2,3], Michael Young[1] • [1]Laboratory of Genetics and [2]Laboratory of Mathematical Physics, The Rockefeller University, New York, NY; [3]Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland

We describe how the program of circadian transcript oscillations in the adult Drosophila head depends on both the molecular clock circuits and environmental light signals. We have developed a novel global analysis method for detecting oscillatory trends in microarray time series. When used to compare data from wild-type and arrhythmic timeless null-mutant (tim01) flies this method reveals that all circadian transcript profiles are lost in tim01, but that a set of sustained light responses persist. These observations have been independently confirmed using Northern analyses for over 50 transcripts. The novel light-responsive transcripts that we have found are preferentially expressed in the eye and regulated in a manner that depends on the visual transduction component NORPA. Many of the light-responsive transcripts show additional clock-controlled oscillations, suggesting that they may help entrain circadian rhythms in physiology and behavior to the environmental light-dark cycle.

076

The Effect of Modafinil on the Circadian and Homeostatic Determinants Underlying the Deterioration of Neurobehavioral Performance Associated with Transmeridian Travel and Sleep Deprivation

Scott P. Grady[1], Kenneth P. Wright[1,2], Charles A. Czeisler[1] • [1]Division of Sleep Medicine, Department of Medicine, Brigham and Women's Hospital and Division of Sleep Medicine, Harvard Medical School, Boston, MA 02115; [2]Department of Integrative Physiology, University of Colorado, Boulder, CO 80309

Degradation of performance and mood is known to occur in the setting of prolonged wakefulness and increases at circadian phases near the minimum of core body temperature. In this study the ability of the novel wake promoting therapeutic modafinil was tested in a forced desynchrony protocol to quantify the extent to which it counteracted homeostatic and circadian decrements in neurobehavioral performance. Eighteen healthy volunteers were studied in a randomized double-blind placebo-controlled study to determine the effects of modafinil on objective performance (psychomotor vigilance task) and subjective mood and alertness during a month-long 42.85 h forced desynchrony protocol. This protocol dissociates the imposed sleep-wake cycle (14.28h/28.56h) from underlying circadian rhythms allowing separation of the two critical and interacting factors of circadian phase and number of hours awake that together regulate human performance and subjective state. In this study performance on the psychomotor vigilance task varied significantly with both circadian phase and time awake. Treatment with modafinil was associated

with attenuated decrements in performance as measured by lapse rate during psychomotor vigilance testing. Significant condition by hours awake and condition by circadian phase effects were observed (both $P < 0.05$). No difference was noted in subjective alertness between the groups as measured by either the Karolinska Sleepiness Scale or by alertness rated on a visual analog scale. Modafinil significantly attenuated the performance impairing effects of homeostatic sleep drive and circadian phase on psychomotor vigilance performance with no significant effect on subjective alertness.

077

The Molecular Basis of the Interaction between Sleep and Feeding Behaviors in Drosophila Melanogaster

Susan T. Harbison* and Amita Sehgal • University of Pennsylvania

Although the function of sleep remains a mystery, many possible roles have been postulated for its purpose, including tissue restoration and healing, thermal and energy regulation, immune system function, and memory consolidation. A wide body of evidence indicates that sleep is influenced by food intake, supporting the idea that sleep has a role in tissue restoration and/or energy regulation. As recent advances have demonstrated that rest in D. melanogaster meets the behavioral requirements of mammalian sleep, we are using this genetically tractable organism to explore the molecular basis of the sleep-feeding interaction. We have measured sleep phenotypes in a panel of 20 recombinant inbred lines derived from the isogenic parental lines Oregon-R and 2b in order to map quantitative trait loci (QTL) affecting variation in sleep. Preliminary results indicate that baseline sleep times exhibit high levels of sex-specific genetic variation in these lines. We have also subjected the lines to sleep deprivation using light as the depriving stimulus and measured the amount of sleep lost during the deprivation period as well as the subsequent sleep rebound time. The preliminary results of this mapping procedure will be presented, as well as a comparison of sleep QTL locations to those previously identified for starvation resistance.

078

Sleep and Circadian Effects of Modafinil during Five Nights of Sleep Restriction

Timothy H. Monk*, Daniel J. Buysse, David K. Welsh, Jean M. DeGrazia and Robert Y. Moore • University of Pittsburgh

This was a double-blind placebo controlled within-subjects cross-over design study of Modafinil as a countermeasure to five consecutive nights of partial sleep restriction (to 40% of habitual time in bed [TIB]). Subjects experienced both drug and placebo conditions in two week-long time-isolated sessions. The daily routine was specified by technicians. All sleeps were polysomnographically recorded and rectal temperature measured continuously. Bedtime and waketime on the first night (of each week) were at the subject's habitual times. On nights 2 through 6 bedtimes were made later and waketimes earlier (by equal amounts) until TIB was 40% of baseline. A placebo pill was taken at breakfast on the first morning and a pill (either placebo or 200mg. Modafinil) at breakfast on the mornings following nights 2 through 6. Two subjects also took a lunchtime pill. 100-150% of habitual TIB was made available for sleep on night 7 (recovery). Temperature and sleep data are reported here from nine healthy subjects (6f, 3m, age 21-58y). Compared to the placebo condition, Modafinil led to an overall increase (0.15¡C, $p<0.05$) in circadian temperature rhythm amplitude (deeper trough), and a ~7% increase in percent slow wave sleep on nights 5 ($p<0.05$) and 6 ($p=0.06$). On the recovery night 42mins. more total sleep was obtained under drug than

under placebo ($p<0.05$), with 22mins. more delta sleep ($p<0.10$), and a deeper temperature rhythm trough on that night (0.23¡C, $p<0.07$). No effects were found on circadian phase or sleep efficiency.

079

Circadian Distribution of the Sleep/Wake Cycle Is Altered in ob/ob Mice

Jonathan Shelton*, Nicholas Perrino, Joseph Bass, Fred Turek, Aaron Laposky • Center for Sleep and Circadian Biology, Northwestern University

Recent studies have linked pathways regulating sleep, circadian rhythms, and energy expenditure. For example, clock mutant mice develop metabolic complications in addition to their altered sleep and circadian rhythm phenotypes. Therefore in the current study, we utilized the ob/ob mouse, a well-characterized model of the metabolic syndrome, to test the hypothesis that metabolic regulatory pathways influence sleep and circadian rhythms. For this study, ob/ob and wt mice were implanted with EEG/EMG electrodes for sleep/wake characterization. Polysomnographic recordings were visually scored as wake, NREM, or REM. Data were averaged and statistical significance was determined by t-test. The results demonstrate that over 24 hours total sleep time was elevated in ob/ob compared to wt mainly due to an increase in NREM($p=0.002$). In addition, there was a significant increase in arousals and stage shifts. Further findings showed the circadian distribution of the sleep/wake cycle in ob/ob was disrupted as evidenced by a decrease in the light:dark distribution of NREM($p<0.001$) and REM($p<0.05$). In summary, metabolic abnormalities that are hallmarks of the ob/ob mouse are associated with alterations in sleep architecture, dampened circadian rhythmicity of the sleep/wake cycle and severe fragmentation of sleep episodes. Findings from this study provide a foundation to investigate novel interactions between networks controlling sleep, circadian rhythms and energy homeostasis. This work is supported through the NIH (RO1-EH03-116, AG11412, HL59598, and AG-18200).

080

Sleepless: A Mutation that Increases Active Sleep in Neonatal Mice and Wake Time in Adults

Turek FW*, Dugovic C, Laposky AD, Perrino NM, Losee-Olson S • Northwestern University, Evanston, IL

While considerable evidence supports a role for genetic factors in the regulation of the sleep-wake cycle as well as the pathogenesis of sleep disorders, no major regulatory genes for the sleep-wake cycle have been identified. In an attempt to identify genes involved in the regulation of sleep and/or wake, we carried out an intensive mutagenesis program that examined behavioral sleep-wake states in over 5,000 offspring of male mice treatment with a chemical mutagens (ENU). The unique characteristic of this "mutagenesis screen" was the use of baby mice (pups) to screen for abnormal sleep-wake-phenotypes in their number and duration of active sleep (AS), quiet sleep (QS), and wake (W) bouts. Following an initial screen of @ 5,000 G1 offspring, we selected outlier animals for the production of G2 generations. Presently, we are following 11 lines in G3 – G6 generations who are showing an abnormal sleep-wake phenotype as pups; in one of the lines that is most advanced 50% of the offspring show alterations in the total amount of AS and AS bout duration that are 3-5 SD from the wild-type population mean. As adults, these animals show 1-2 hrs less sleep, and we have named the line "Sleepless". This Sleepless line of mutant animals should prove to be useful in not only mapping and identifying a gene that can affect active sleep time in neonates as well as

wake time in adults, but also in developing a better understanding of the development of the sleep-wake states in mammals.

081

Effects of Sleep Deprivation on Gene Expression and the Immune Response in Drosophila Melanogaster

Julie A. Williams*, Sriram Sathyanarayanan, Joan C. Hendricks, and Amita Sehgal • HHMI, University of Pennsylvania School of Medicine

Rest behavior in flies is a sleep like state. Recent studies have implicated a role of components of cAMP-signaling, heat-shock proteins, and the clock gene cycle in sleep homeostasis. To identify other molecular candidates involved in sleep regulation, using microarray analysis we performed a genome-wide screen for changes in gene expression associated with sleep deprivation in flies. We found that three major classes of genes change expression with sleep deprivation. These include genes involved in the immune response, energy and metabolism, and cell signaling, particularly those related to synaptic plasticity. We further analyzed the role of immune related genes in sleep homeostasis. Genetic manipulation of the NF kappa B transcription factor, relish, did not alter rest behavior. However, consistent with the increase in expression of immune related genes, we found that sleep deprivation increased resistance to bacterial infection following an immune challenge. These results demonstrate a correlation between acute sleep deprivation and the immune system indicating that increased expression of genes underlying the immune response is an output of the sleep homeostat.

082

Modulation of Circadian Light Sensitivity in Drosophila by Serotonin Signaling

Quan Yuan*, Fangju Lin, and Amita Sehgal • Department of Neuroscience, University of Pennsylvania, Philadelphia, PA, 19104

Serotonin (5-hydroxytryptamine, 5-HT) plays important roles in the regulation of different behaviors, including control of appetite, sleep, mood, depression, memory and learning. With respect to sleep : wake cycles, sertonin signaling is involved in non-photic phase shifts, photo sensitivity and the regulation of paradoxical sleep in mammals. However, the physiological relevance and underlying molecular mechanisms of these effects are yet to be established. We studied the modification of circadian light responses by serotonin in Drosophila. As in mammals, pharmacological treatments that increase extracellular serotonin, inhibit light responses in flies. The spatial expression profile of the Drosophila serotonin receptor 1B (d5-HT1B) indicated a possible role of this receptor subtype in circadian regulation. Indeed, overexpression of the d5-HT1B receptor in clock neurons reduced the light sensitivity of circadian behavioral rhythms and the effect was synergistic with a mutation in the circadian photoreceptor, cryptochrome. Conversely, decreased levels of d5-HT1B resulted in increased circadian light sensitivity. Consistent with these behavioral observations, light induced TIM degradation was reduced in d5-HT1B overexpressing flies. Furthermore, in both cell culture and in fly heads, we detected the formation of a complex between d5-HT1B and TIM. These findings demonstrate that the inhibitory effect of serotonin on circadian photosensitivity is conserved in mammals and Drosophila. We propose that the modulation of light sensitivity by serotonin constitutes a homeostatic mechanism that regulates the circadian system.

083

Circadian Control and Locomotor Feedback in the Regulation of Hypocretin-1 (orexin A) in Sleep Consolidating Squirrel Monkeys and Polyphasic Rats

Jamie M. Zeitzer*, Shengwen Zhang, Christine L. Buckmaster, David M. Lyons, Emmanuel Mignot • Department of Psychiatry and Behavioral Sciences; Stanford University; Palo Alto CA 94304

Hypocretin-1 (orexin A) is a neuropeptide produced by hypothalamic neurons. Hypocretin-containing nerves innervate a wide variety of cortical and subcortical structures, including those involved in maintaining sleep and wake. Evidence from humans who lack hypocretin (i.e., narcoleptics) suggests that hypocretin may be involved in the expression of the circadian alertness signal. Cisternal CSF hypocretin-1 peaks late during the active period in both sleep-consolidating, diurnal squirrel monkeys (Saimiri sciureus sciureus) and polyphasic, nocturnal rats. In both species, hypocretin-1 can be stimulated by sleep deprivation. We now show that, in the rat, the daily rhythm of cisternal CSF hypocretin-1 is dependent on the presence of an intact suprachiasmatic nucleus (SCN). In SCN lesioned rats, the day/night variation in locomotor activity induced by a light/dark cycle does not produce a rhythm in cisternal CSF hypocretin-1. Despite this, locomotor activity has a small, modulatory influence on hypocretin-1 concentrations in the rat. Likewise, in squirrel monkey, near abolition of movement does not prevent or significantly affect the normal, daily rise of cisternal CSF hypocretin-1. As in the rat, locomotor activity has a small influence on hypocretin-1 concentrations, though the magnitude is smaller than that observed in rats. As rats do not consolidate wake in the same manner as squirrel monkeys, and both the pattern of hypocretin-1 and responsiveness hypocretin-1 is similar in both species, downstream mechanisms of action will need to be examined in future studies. These studies illustrate the complexity of the regulation of hypocretinergic transmission by homeostatic, circadian, and other factors.

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Miller workshop

Wijesuriya Dayawansa

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Miller workshop

Marilyn Duncan

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Miller workshop

David Glass

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Miller workshop

Joe Miller

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Miller workshop

Ralph Mistlberger

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Miller workshop

William Schwartz

090

Circadian Genes and Addiction

Urs Albrecht* • Dept. of Medicine, Div. Of Biochemistry, University of Fribourg, Switzerland

Addiction is a compulsion to repeat a certain behavior. Circadian behavior like sleep is repetitive and we crave for sleep. However, we would not describe sleep as an addictive behavior but a natural drive to recover from daily activities. Interestigly, parallels between addiction and the circadian clock can be drawn. Similarities can be seen between the signal transduction cascades and the molecular adaptations associated with both processes. For example glutamate, which plays a major role in the adaptation of the circadain clock to changing lighting conditions, is important for behavioral sensitization, a measure for addiction. It seems that an altered glutamatergic state in the brains of mice with a defective circadian clock influences addictive behavior.

091

Progressive Arthropathy in Mice with a Targeted Disruption of the Mop3 Locus

Christopher Bradfield • McArdle Laboratory for Cancer Research

Disruption of the murine Mop3/bMal1 locus results in a loss of behavioral and molecular circadian rhythms. Although Mop3 null mice are born in normal numbers, with no obvious anomalies in early development, they do display quantitative reductions in activity during later adulthood. In an effort to explain this decreased activity, we demonstrated that Mop3 null mice display an aging phenotype that is best described as a progressive non-inflammatory arthropathy. The syndrome in these mice is reminiscent

of a human syndrome known as DISH (diffuse idiopathic skeletal hyperostosis). Although little pathology is observed prior to 11 weeks of age, by 35 weeks of age, essentially all Mop3 null animals display marginal joint ankylosis caused by ossification of ligaments and tendons and almost complete immobilization of weight bearing and non-weight bearing joints. Taken in sum, these results explain the decreased activity of Mop3 null mice and suggest the importance of circadian rhythms in normal aging, as well as progressive diseases of bones and joints.

092

Tick-tox: Clock Gene Expression and Interactions Between the Molecular Pathways for the Regulation of Circadian Rhythms, Toxin Metabolism and Development

David Earnest • Texas A&M University Health Science Center

The majority of basic-helix-loop-helix-PAS (bHLH-PAS) proteins interact so as to function as either sensors that detect environmental changes or general partners that potentiate the sensor response. Interactions between transcription factors with bHLH and PAS motifs such as CLOCK, BMAL and PER play a central role in the molecular feedback loop comprising the circadian clockworks in the mammalian suprachiasmatic nucleus (SCN). Many other proteins share bHLH-PAS motifs and some members of this family such as the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) are known mediators of development and responses to environmental toxins (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]). Because many different bHLH-PAS proteins are expressed in the same tissues and cells, this presentation will explore the possibility that AhR activation may influence the circadian clock function of the suprachiasmatic nucleus (SCN) and that CLOCK, BMAL and PER may play some role in responses to environmental toxins. Discussion will focus on studies using an epithelial cell line (HC-11) derived from the mammary gland, a well-characterized model for toxin metabolism, and an immortalized line of rat SCN cells (SCN2.2) because these cells retain the unique oscillatory and pacemaker properties of the SCN in situ. The primary objectives of these studies were to examine the effects of TCDD on clock gene expression in the SCN and mammary gland models for evidence of interactions between the molecular pathways the regulate circadian rhythms, toxin metabolism and development. Supported by NIH P01-NS39546 (DE).

093

The Function and Regulation of mPER2

Krista Kaasik and Cheng Chi Lee* • University of Texas Health Science Center

Mouse and human genetic studies have established PER2 as a major circadian regulator. The loss of mPER2 function results in the dampened expression of core clock genes Bmal1, Cry1 and mPer1 in the SCN and in peripheral tissues. Thus mPER2 is a positive regulator of the mammalian circadian clock mechanism. mPER2 is not known to form an active transcription complex with BMAL1 or CLOCK nor does it show positive regulation of BMAL1/CLOCK transcription complex activity. In the fruit fly, the auto-regulatory mechanism is modeled on a negative feedback function of dPER. However, in the mouse, the CRY proteins are the major negative loop regulators. Thus, the molecular target and function of mPER2 is an enigma. Recent studies suggest that heme and redox regulation plays a role in the regulation

of the circadian transcription complex activity. Our studies shows that Alas1 the rate limiting enzyme for heme biosynthesis is under circadian control suggesting a reciprocal regulation between these two major biological pathways. We have also shown that mPer2-deficient mice also have abnormal sensitivity to genotoxic agents and have an enhanced cell proliferation phenotype. Cell division cycle regulators such as c-Myc, Cyclin D, Cyclin A, Cyclin B, Cdc2, and Wee1 are under circadian control in vivo. Given that the molecular target of mPER2 is unclear, how are mPER2 function linked with the control of cell cycle?

094

Melatonin-controlled Clock Genes in Calendar Cells

Gerald A. Lincoln[1]*, HŒkan Andersson[1] and David G. Hazlerigg[2] • [1]MRC Human Reproductive Sciences Unit, Edinburgh and [2]School of Biological Sciences, University of Aberdeen, Aberdeen, Scotland

Pars tuberalis (PT) cells of the ovine pituitary gland are a model system to investigate how clock genes decode melatonin duration to mediate photoperiodic effects. PT cells are thought to produce a paracrine prolactin-releasing factor that regulates lactotrophs. PT cells express a high density of melatonin MT1 receptors and rhythmically express Clock, Bmal1, Per1, Per2, Cry1, Cry2, CK1e and RevErb_. Peak Cry1 expression occurs after onset of darkness induced by melatonin, and while peak Per1 expression occurs after dawn due to melatonin withdrawal. The Cry/Per interval thus varies with melatonin duration and provides a mechanism for decoding daylength (e.g. through CRY:PER transcriptional control of clock controlled genes). An abrupt switch from short to long days (LD16:8 to 8:16) induces an immediate adjustment of the Cry/Per phasing and an output prolactin response within 24h. Hypothalamo-pituitary disconnected sheep, where the pituitary gland is surgically disconnected form the hypothalamus but the PT-lactotroph relay is still intact, show essentially normal photoperiod/melatonin regulation of prolactin secretion. This includes rapid photoinduction after a light change, photorefractoriness under prolonged fixed photoperiod, and asynchronous circannual prolactin rhythms under constant long days. Chronic blockade of prolactin release does not alter the free-run indicating that the time control is within the PT cell, rather than the lactotroph. We believe that clock gene mechanisms confer calendar function on the PT, allowing it to govern cycles in the prolactin axis, and we speculate that other cell types strategically placed in the hypothalamus function similarly to control other components of seasonal physiology.

095

Photoperiodically Regulated Gene Expression in the Hypothalamus and Its Link to Body Weight

Peter J. Morgan, Alexander Ross, Julian Mercer and Perry Barrett • Molecular Endocrinology Group, Rowett Research Institute, Greenburn Road, Aberdeen, Scotland, AB21 9SB

Considerable progress has been made in understanding the molecular basis of the mammalian circadian clock in the SCN and elsewhere, but our knowledge of the molecular and neural basis of seasonal timing remains more limited. Since the systems involved in seasonal timing interface with some major physiological axes, such as the control of reproduction, energy homeostasis and metabolism, there is considerable potential benefit to be gain from a greater understanding.

Using the Siberian hamster as a model seasonal animal we have explored differential gene expression in the hypothalamus after different periods in either long or short photoperiod, using subtractive hybridisation, microarrays and in situ hybridisation. We have identified a number of genes, which undergo dynamic regulation of gene expression in the dorsal tuberomammillary nucleus (DTM) of the Siberian hamster brain in response to altered photoperiod. This includes genes involved in retinoid acid as well as histaminergic signalling. We have used comparative analysis with other species such as the Syrian hamster and sheep to examine the potential relationships these changes may have with particular physiological end-points.

096

Symposium-photoperiodic Time Measurement-molecular Mechanisms-shizufumi Ebihara (Chair) Molecular Analysis of Photoperiodic Time Measurement in Birds and Mammals

Takashi Yoshimura*[1], Shinobu Yasuo[1], Miwa Watanabe[1], Takashi Yamamura[1], Masayuki Iigo[2], Tsuyoshi Watanabe[1], Nobuhiro Nakao[1], Kanjun Hirunagi[3], Shizufumi Ebihara[1] • [1]Graduate School of Bioagricultural Sciences, Nagoya University, [3]The Nagoya University Museum, Furo-cho, Chikusa-ku, Nagoya 464-8601, [2]Faculty of Agriculture, Utsunomiya University, Mine-machi, Utsunomiya, Tochigi 321-8505, Japan

Japanese quail is an excellent model for studying photoperiodic time measurement (PTM). We have found expression of clock genes in the mediobasal hypothalamus (MBH) where the center for PTM is located. This clock keeps stable time under various light conditions, which seems to enable birds to keep steady state photoinducible phase. In the pars tuberalis of the pituitary, activation of Per2 gene occur in the early day, while activation of Cry1 gene occur in the early night. This temporal association is conserved under different photoperiods. This different phase relationship may decode photoperiodic information. We found that the expression of type 2 deiodinase (Dio2), which catalyses the deiodination of the thyroxine (T4) prohormone to the active triiodothyronine (T3) is induced by long day stimulus. Intracerebroventricular administration of T3 mimics the photoperiodic response of gonads. Since thyroid hormones are essential for the maintenance of seasonal reproduction in a number of mammals, we examined the expression of Dio2 in the Djungarian hamster. In the hamster hypothalamus, Dio2 expression was high under long day conditions, and melatonin administration suppressed Dio2 expression. These results suggest that molecular mechanism for photoperiodism may be conserved among birds and mammals. This work was supported by the PROBRAIN.

097

Organization

Andrew Millar

098

Circadian Clocks in Antennal Neurons Are Necessary and Sufficient for Olfaction Rhythms in Drosophila

Shintaro Tanoue, Parthasarathy Krishnan, Balaji Krishnan, Stuart E. Dryer and Paul E. Hardin* • University of Houston, Department of Biology and Biochemistry, 4800 Calhoun, Houston, TX 77204-5001

The Drosophila circadian clock is controlled by interlocked transcriptional feedback loops that operate in many neuronal and non-neuronal tissues. These clocks are roughly divided into a central clock, which resides in the brain and is known to control rhythms in locomotor activity, and peripheral clocks, which comprise all other tissues and are thought to control other rhythmic outputs. We previously showed that peripheral oscillators are required to mediate rhythmic olfactory responses in the antenna, but the identity and relative autonomy of these peripheral oscillators has not been defined. Targeted ablation of lateral neurons using apoptosis promoting factors and targeted clock disruption in antennal neurons using newly developed dominant negative versions of CLOCK and CYCLE show that antennal neurons, but not central clock cells, are necessary for olfactory rhythms. Targeted rescue of antennal neuron oscillators in cyc01 flies using wild type CYCLE shows that these neurons are also sufficient for olfaction rhythms. These results demonstrate that a peripheral tissue can function as an autonomous pacemaker in Drosophila, reveal differences in the function and organization of circadian oscillators within and between species, and suggest that components of the olfactory signal transduction cascade could be targets of circadian regulation.

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Organization

Forst-Helfrich

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The Mammalian Circadian Axis

Michael Menaker, Tomoko Yoshikawa, Alec Davidson • University of Virginia

Absolute hierarchies are conceptually simple and therefore appealing as explanations of relationships among components of complex biological systems; they are almost always wrong (or at least seriously misleading). Explanations that begin as hierarchies become networks as feedback is uncovered and the autonomous properties of components are revealed. Our view of mammalian circadian organization is currently undergoing such a transformation. In the last year or two the proposed role of the SCN has shifted subtly from "the master oscillator" toward "the conductor of a circadian orchestra composed of autonomous oscillators." I will discuss new data that support this more recent view, derived from experiments in which peripheral tissues are cultured from animals made arrhythmic (or not) by exposure to constant light and SCN lesion. By now, we know more about mammalian circadian organization than about circadian organization in non-mammalian vertebrates (which led the way). There is ample evidence for the existence of multiple distributed circadian oscillators in all vertebrates; however, the links that couple them into functional systems remain mostly undefined and are poorly understood.

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Zebrafish Clocks: Embryos, Cells and Light

David Whitmore • University College London

Previous research has shown that not only do zebrafish tissues contain an endogenous circadian lock, but also that they are directly light responsive. These studies have been extended to examine the development of the circadian clock and light response in zebrafish embryos. Very early stage embryos, 6 hours after fertilization, show changes in the expression of specific genes following a light pulse. These responses, and the presence of a circadian clock, may play a role in regulating DNA repair processes and gating the embryonic cell cycle. Specific zebrafish mutants demonstrate that a functional retina is not required for the majority of these early light responses. Though exposure to light early in development does appear to be required to either start, or synchronize the embryonic circadian clock. The use of light responsive, clock containing cell lines, transfected with luminescent reporter gene constructs, represent a useful tool with which to examine the role of specific molecules within the circadian clock mechanism. The employment of retroviral transfection techniques, cell sorting, and photon counting imaging tedchniques have allowed us to observe circadian oscillations in gene expression in single, and small groups of cells as they phase shift in response to short light pulses.

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Synaptic Plasticity at SCN Synapses

Charles N. Allen* and Heinrich S. Gompf • Oregon Health & Science University

GABAergic synapses within the suprachiasmatic nucleus (SCN) are involved in phase shifting the circadian clock and synchronizing the activity of SCN neurons. The synaptic plasticity of intra-suprachiasmatic nucleus GABAergic synapses was studied by measuring the ratio between pairs of evoked inhibitory postsynaptic currents (IPSCs). Inter-stimulus intervals were chosen to represent the range of spontaneous action potential firing frequencies found in SCN neurons. The majority of synapses studied during the day exhibited paired-pulse depression (PPD), while most synapses studied during the night showed no PPD. The PPD observed could be divided into two types based on stimulus interval that produce the greatest depression. Type 1 PPD expresses the greatest inhibition at shorter inter-stimulus intervals (<125 ms), is predominant in the early morning and is likely due to vesicle depletion. Type 2 showed the greatest inhibition at inter-stimulus intervals between 175-225 ms, is found throughout the day but rarely at night and is likely due to a Ca2+-dependent mechanism that is independent of pertussis toxin sensitive G-proteins. Our results demonstrate the existence of short-term synaptic plasticity in the SCN and imply a role for this mechanism in the integration of temporal information between SCN neurons. Supported by NIH-NS40782.

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Differentiation of Parasympathetic and Sympathetic Output of the SCN: Its Role in Metabolism

Ruud M. Buijs and Andries Kalsbeek • Netherlands Institute for Brain Research

Opposing parasympathetic and sympathetic signals determine the autonomic output of the brain to the body and change in balance over the sleep wake cycle. The suprachiasmatic nucleus (SCN) organizes the activity/inactivity cycle and behavior with it, but it is unclear how the SCN, with its high daytime electrical activity influences this differentiated autonomic balance. Selective retrograde tracing with two unique reporter PRV strains, one injected into the adrenal, the other into the sympathetic denervated liver, demonstrated two separate populations of pre-sympathetic and pre-parasympathetic neurons within the paraventricular nucleus of the hypothalamus. This segregation persists into the SCN, where, as a result, the day-night balance in autonomic function of the organs is affected by specialized pre-sympathetic or pre-parasympathetic SCN neurons. These separate preautonomic SCN neurons provide the anatomical basis for the circadian-driven regulation of the parasympathetic and sympathetic autonomic output, which is responsible for the daily changes in glucose output of the liver and other autonomic output to the organs.

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Chris Colwell • Department of Psychiatry, University of California–Los Angeles

In recent years there has be a resurgence of interest in state-dependent changes in both learned behaviors and in the cellular/molecular events that may underlie learning and memory. In particular, there has been much discussion as to the possible relationship between sleep and learning. Less studied, but perhaps equally important, is the temporal regulation imposed by the circadian timing system based in the SCN. I will first present evidence that the circadian system can regulate learning and memory functions. Examples will be mostly drawn from our own work demonstrating that the circadian system modulates acquisition, recall and extinction of fear conditioning behavior in mice. We have also found that synaptic plasticity as measured by hippocampal long term potentiation (LTP) also varies with the time of day in mice. Both the magnitude and stability of LTP recorded in the CA1 region is larger in night than in the day. These diurnal rhythms continue when mice are held in constant darkness, a key finding that suggests that the LTP is influenced by a circadian oscillator. We believe that this work indicates that the circadian system can play a major role in the temporal organization of learned behaviors and have begun to search for the mechanisms that are be responsible for this temporal regulation.

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Mechanisms of Circadian Regulation of Dark Current Channels in Vertebrate Cone Photoreceptors

Stuart E. Dryer* • University of Houston

Circadian oscillators can exert significant effects on vertebrate visual systems. However, relatively little is known about the molecular output pathways that control phototransduction cascades in vertebrate retina. We have observed that photoreceptor circadian oscillators modulate chick cone cGMP-gated channels (CNGCs) such that they have a higher affinity for cGMP during the subjective night.

Circadian modulation of CNGCs appears to entail posttranslational modification, probably tyrosine phosrphorylation, of the channel complex. A series of biochemical, pharmacological, and molecular experiments indicate that cAMP, protein kinase A (PKA), Ras, and Erk MAP kinase are part of the circadian output pathway controlling CNGCs. Endogenous and exogenous cyclic AMP cause activation of Erk and Ras, which are more active at night in cones, and increase the apparent affinity of CNGCs for cGMP. A dominant-negative form of Ras (RasN17), and a dominant-negative form of the MEK kinase B-Raf block the circadian rhythms in CNGC gating, as also block the effects of cAMP. The activation of Erk by cAMP occurs in minutes, but modulation of CNGCs requires ~ 2 hrs. However, the effect of cAMP on CNGCs is not associated with phase-shifting of the oscillator and does not require protein synthesis or calcium influx. It is likely that multiple steps lie between Erk activation and CNGC modification. Neurotransmitters such as dopamine interact with the circadian output pathways to provide a second layer of control of CNGCs. Moreover, circadian oscillators appear to control the transduction cascades used by dopamine.

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PDF Signaling in Drosophila Circadian Behavior

Paul H. Taghert, Yiing Lin, Gary Stormo, Weihua Li, Susan Renn and Mei Han • Departments of Anatomy & Neurobiology, and Genetics, Washington University Medical School

We are interested in the cellular properties of neuronal pacemakers and how these cells interact to form functional circuits underlying rhythmic behavior. The Drosophila brain contains approximately 100 circadian pacemaker neurons distributed within six identifiable groups. Genetic mosaic analysis has suggested the properties of cells within different groups are varied. We have previously found that a subset of pacemakers, which secrete the neuropeptide PDF, are required for circadian rhythmic behavior and represent critical elements in the circadian neuronal circuits. Recently, the *pdf* gene was also shown to be required for normal geotaxic behavioral response (Toma and Greenspan, 2002, Nature Genetcis). This talk will summarize three sets of experiments aiming to define more precisely the contributions of PDF signaling to those circuits. We have asked and identified which are the critical residues within the proPDF precursor for *pdf* actions on each behavior. We have asked whether there are functional differences among different *pdf*-expressing neurons in their contributions to each of these behaviors – the answer is yes. Finally, we have asked whether the *pdf* circadian phenotype is explained by a lack of molecular oscillations within circadian pacemaker neurons, or alterations in those oscillations – the answer appears to be the latter.

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Evidence for a Role for Endocytosis in the Drosophila Circadian Clock

Ravi Allada*, Elyssa Burg, Luoying Zhang, and Valerie Kilman • Department of Neurobiology and Physiology, Northwestern University, Evanston, IL 60208

Recent evidence suggests a pivotal role for cell-cell communication in sustaining molecular and behavioral oscillations in flies and mammals. In contrast to the core transcriptional oscillator, the molecular aspects of these clock pathways remain unclear. In Drosophila, pacemaker neurons are distinguished by their expression of the neuropeptide, PIGMENT DISPERSING FACTOR (PDF). Genetic inactivation of pdf results in short period behavioral rhythms that dampen over time in constant darkness as well as loss

of transcriptional oscillations in pacemaker neurons. To elucidate PDF-dependent signaling pathways, we expressed a dominant negative homolog of dynamin, shibire (shi(ts)). In a temperature-sensitive manner, shi(ts) dominantly inhibits wild-type shibire, which is essential for receptor endocytosis and synaptic transmission. Broad overexpression of shi(ts) in pacemaker neurons resulted in strikingly long periods (~28 hours; control=24-25 hours). Interestingly, we did not observe significant period lengthening in flies overexpressing the synaptic transmission blocker, tetanus toxin, suggesting that shi(ts) is not working by inhibiting synaptic transmission. The period effects of shi(ts) were completely blocked in pdf01, indicating that shi(ts) is operating through PDF-dependent signaling pathways. Moreover, targeted expression of a clathrin-light chain GFP lengthens period but only when co-expressed with shi(ts), indicating a synergistic interaction between these two critical players in endocytosis. These data are consistent with a fundamental role for endocytic pathways in modulating PDF feedback on the central clock. Future work aims to elucidate the genetic pathways by which shi(ts) is altering clock function.

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Nuclear Localization of Xenopus Cryptochrome Is Regulated by the C-terminus

*Conte, F.E. Zhu, H., Green, C.B. • Department of Biology, University of Virginia, Charlottesville, VA

Vertebrate CRYPTOCHROME (CRY) controls the repressive arm of the circadian molecular loop by blocking CLOCK/BMAL1-driven transcription. This role is conserved in both CRYs (xCRY1 and xCRY2b) of Xenopus laevis. Both xcrys are rhythmically expressed in the photoreceptor layer of Xenopus, where an autonomous clock is localized. Regulation of CRY activity has been an ongoing interest of our lab. We have some evidence that the core region is responsible for repression: we therefore examined the role of the C-terminus. To test the hypothesis that nuclear localization of CRY is regulated by the C-terminus domain, we have generated C-terminal truncation mutants in xCRY1 and xCRY2b by introducing stop codons via site-directed mutagenesis. The constructs were then tested in Cos-7 cells and transgenic tadpoles. While wild type xCRYs transfected into Cos-7 cells are localized to the nucleus, C-terminal truncation mutants are localized to the cytoplasm, and therefore lose repressive activity. The truncation mutants' loss of nuclear localization is also apparent in the photoreceptor layer of transgenic frogs. In addition, truncation of the C-terminus does not affect the repressive function of CRY, because fusion of the truncation mutants with an heterologous nuclear localization signal (NLS) restores nuclear localization and, most importantly, repressive activity. To demonstrate that indeed the C-terminus functions as a NLS, we have cloned it in-frame with Nocturnin, a cytoplasmic protein. Addition of the putative CRY NLS causes Nocturnin to change from mostly cytoplasmic to mostly nuclear. These results demonstrate that, in Xenopus, nuclear localization of CRY but not its repressive ability is regulated by the C-terminus.

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DBT and SGG Are Opposing Regulators of PER/TIM Nuclear Entry

Shawn A. Cyran*, Georgia Yiannoulos, Anna M. Buchsbaum, and Justin Blau • New York University, 100 Washington Square East, New York, NY 10003

Circadian rhythms in Drosophila are under the control of a central clock which is located in the pacemaker lateral neurons in the central nervous system. This is where rhythmic clock gene expression and post-translational modifications occur that keep the molecular clock ticking with a 24-hour period.

The nuclear accumulation of PERIOD (PER) and TIMELESS (TIM) in these clock cells is a major step in determining this daily rhythm. Current models of the clock hypothesize that TIM is the nuclear transporter for PER. In the absence of TIM, PER is degraded rapidly due to phosphorylation by the protein kinase DOUBLE-TIME (DBT) which marks PER for degradation. This model predicts that in the absence of both TIM and DBT, PER should accumulate in the cytoplasm. However using dbt[ar], a DBT mutant which strongly reduces DBT protein kinase activity without affecting its binding to PER, we show nuclear accumulation of PER even in a tim[01] background. Another kinase, SHAGGY (SGG)/GSK-3, has been shown to promote PER/TIM nuclear entry. We show that SGG requires TIM to promote PER nuclear entry. Therefore PER/TIM nuclear entry is a balance of the negative effect of DBT on PER nuclear entry and the positive effect of SGG on TIM. This data provides an increased understanding of the finely tuned regulation of PER/TIM nuclear entry.

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CKIe AND b-TrCP Regulate Period2 Stability

Erik J Eide[1], Heeseog Kang[1] and David M Virshup[1,2] • [1]Department of Oncological Sciences and the Center for Children, Huntsman Cancer Institute, [2]Division of Hematology/Oncology, Department of Pediatrics, University of Utah, Salt Lake City, Utah 84112

Observations from Drosophila and mouse models suggest phosphorylation is a major factor that regulates PER protein stability. Drosophila PER (dPER), mouse PERIOD 1 and 2 (mPER1 and mPER2, respectively) are all phosphorylated prior to their degradation. Fly larvae lacking DOUBLETIME, a serine/threonine protein kinase homologous to human CKIe, have altered circadian rhythm and elevated levels of dPER protein in the clock-containing neurons of the brain. In tissue culture cells, overexpression of CKIe accelerates mPER degradation and inhibition of endogenous CKIe activity stabilizes human PER1. To further study the mechanism by which phosphorylation regulates mPER2 stability, mPER2 hyperphosphorylation was induced by treatment of tissue culture cells with calyculin A, a cell permeable inhibitor of type 1 and 2A serine/threonine protein phosphatases. Hyperphosphorylated mPER2 was rapidly degraded via the ubiquitin-proteasome pathway. We have identified two 25 amino acid sequences within the CKIe binding domain of mPER2, that when deleted, stabilize calyculin A dependent degradation. CKIe is likely to be a bona fide regulator of mPER2 protein stability because the presence of either internal deletion in mPER2 abrogates CKIe binding and IC261, a specific inhibitor of CKIe and CKId, slows the rate of phosphorylation induced protein degradation. We also show that the F-box protein b-TrCP regulates mPER2 stability. Overexpression of a dominant-negative form of b-TrCP (b-TrCP(DF-box)) stabilizes hyperphosphorylated mPER2 and inhibits mPER2 ubiquitination. Either stabilizing internal deletion in mPER2 inhibits b-TrCP binding, similar to CKIe. However, b-TrCP binds a domain adjacent to the CKIe binding sites, suggesting that a stable interaction between b-TrCP and mPER2 requires CKIe activity. Further support for this model came from our observation that mPER2 and b-TrCP bind each other only in the presence of CKIe in vitro. Overall, these results suggest that CKIe regulates mPER2 stability by binding and phosphorylating it. This in turn creates a binding site for b-TrCP which then ubiquitinates mPER2, targeting it for destruction by the 26S proteasome.

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Posttranslational Regulation of the Neurospora Circadian Clock

Yi Liu*, Ping Cheng, Yuhong Yang, & Qun He • Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75390

FREQUENCY (FRQ), WHITE COLLAR-1 (WC-1) and WC-2 proteins are three key components forming the circadian negative feedback loop in Neurospora. FRQ is progressively phosphorylated over time, and its level decreases when it is extensively phosphorylated. Previously, we showed that CKII is an essential clock components, and that the phosphorylation of FRQ by CKII promotes FRQ degradation and is important for the closing of the circadian negative feedback loop. Here, we show that two protein phosphatases, PP1 and PP2A, play distinct roles in the Neurospora clock: PP1 regulating FRQ stability while PP2A is important for the function of the circadian feedback loop. After FRQ is phosphorylated, it is degraded through the ubiquitin-proteasome pathway. Such degradation is mediated by a E3 ligase, FWD-1 (an F-box/WD-40 repeat-containing protein), which is the Neurospora homolog of the Drosophila Slimb protein. The conservation of the posttranslational regulators in the Neurospora and animal circadian systems suggests that the molecules mediating the posttranslational regulation of clock proteins may be the common evolutionary link among distinct eukaryotic circadian systems.

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Stability of Circadian Oscillator Observed in Individual Cyanobacteria

I. Mihalcescu*, Lab. de Spectrom trie Physique, Universit Joseph Fourier – Grenoble I, BP87 38402 St Martin d'Heres Cedex, France, imihalce@spectro.ujf-grenoble.fr, W. Hsing, Department of Molecular Biology, Princeton University, NJ 08544 USA, WHsing@PRDUS.JNJ.COM, S. Leibler, The Rockefeller University, 1230 York Avenue, New York, NY 10021USA

The unicellular photosynthetic bacterium, Synechoccocus elongatus sp. PCC7942, is among the simplest organisms having a circadian clock. In the present study, we managed to measure gene expression under circadian control in a single cell, directly demonstrating that the circadian clock is a property of individual bacteria and not only of a population of cells. We quantified the stability of individual oscillators and their phase dispersion trough successive cell divisions. Finally, we selected neighbouring cells with different initial phases of oscillation and then monitored the time evolution of their descendants. We used for that the bacterial luciferase reporter gene system, a precise and non-perturbing method previously used in many studies of circadian clock in cyanobacteria. We implemented a new experimental set-up, allowing the bioluminescence imaging at high magnification and very low light level (typically 10 photons/minute) for extended periods of time. Our study demonstrates that in addition to usual requirements for the circadian clock (self-sustained oscillations with a period close to 24 hours, temperature compensation and entrainment to 24h period by external factors) the circadian genetic network in cyanobacteria has to deal with the presence of stochastic internal and environmental fluctuations. By measuring the characteristics of individual cellular oscillators, we show that despite such fluctuations the circadian oscillator of these unicellular organisms has a strong stability in time (with a correlation time of several months). Moreover, our results indicate that the genetic network has to insure this stability at the intracellular level, as the interaction between oscillators appears to be negligible.

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Pigment-dispersing Factor and GABA Synchronize Insect Circadian Clock Cells

Nils-Lasse Schneider and Monika Stengl* • Philipps-University of Marburg, Biology, Animal Physiology, Germany, stengl@staff.uni-marburg.de

The accessory medulla with associated pigment-dispersing factor (PDF)-ir neurons is the circadian clock that controlls locomotor activity rhythms in the fruitfly Drosophila melanogaster as well as in the cockroach Leucophaea maderae. Three of the about 12 PDF-ir circadian pacemaker candidates in the cockroach directly connect the bilaterally symmetric circadian clocks and appear to form a synchronizing coupling pathway. Thus, we tested, whether the peptide PDF and GABA, which is the predominant neurotransmitter of accessory medulla interneurons, are circadian coupling signals for clock cells. In extracellular recordings of the excised accessory medulla we show that both, PDF and GABA synchronize accessory medulla neurons via inhibition of action potential activity. We are currently testing which second messenger systems are responsible for the PDF-dependent synchronizations of insect circadian pacemaker candidates. [Supported by DFG STE531/12-1, and Human Science Frontier]

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Enhanced Nuclear Import of Mper2 by the C-terminal Tail of Mcry1

Tamanini F*, Chaves I, Yagita K, Barnhoorn S, and van der Horst GTJ • ErasmusMC

Mammalian CRYPTOCHROMES (mCRY1 and mCRY2) and PERIOD (mPER1 and mPER2) are central proteins in the negative limb of the transcriptional/translational auto regulatory feed-back loop generating circadian rhythms. Although the synchronous nuclear accumulation of mCRYs and mPERs following their mRNA rhythms is a key step of this circuit, the nuclear translocation mechanism has not been fully elucidated. In mammalian cells mCRY1 is a nuclear protein and associates with mPER2, thereby promoting the nuclear accumulation of the latter. mCRY1 differs from the homologous DNA repair enzyme photolyase for its extended carboxy-terminal tail and for the lack of DNA repair activity, however little is known about its functional domains. Here we show that two distinct regions within the C-terminal tail of mCRY1 are required for nuclear import and PER2 association. A functional bi-partite nuclear localization signal (NLS), which is located in the last 20 aminoacids, plays a major role in the nuclear import of mCRY1 in transfected cells. Another domain of mCRY1, the C-terminal coiled-coil (CC2), mediates the association with mPER2 and mPER1 but it is dispensable for nuclear import. Importantly, we demonstrate that the NLS's present in mCRY1 and mPER2 are both required for the effective nuclear translocation of the mCRY/mPER complex, thereby explaining the co-dependency between mCRY1 and mPER2 in this process.

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Circadian and Sleep Homeostatic Aspects of Thermoregulation

K. Kraeuchi, V. Knoblauch, C. Renz, C. Schroeder, A. Wirz-Justice*, C. Cajochen • Centre for Chronobiology, Psychiatric University Clinic, CH-4025 Basel, Switzerland.

The influence of circadian and sleep homeostatic components on thermoregulation was studied in 16 young subjects (20-31y; 8m, 8f) under constant routine conditions (<8 lux), comparing a 40h sleep deprivation (SD, high sleep pressure) with a 40h multiple nap schedule (NAP, low sleep pressure, 150min wake: 75min sleep). Skin temperatures [proximal (PROX) and distal (DIST)] and core body temperature (CBT) were continuously recorded. The circadian waveform of DIST showed an inverse relationship to the circadian profile of CBT and PROX, and the two protocols were not significantly different. In NAP, subjective sleepiness ratings showed a circadian profile parallel to DIST, whereas in SD this circadian pattern was compounded by an additional homeostatic increase. On average, CBT declined by 0.05¡C during the naps, whereas PROX and DIST increased by ca. 0.5¡ and 1.8¡C, respectively, independent of sleep architecture and circadian phase of the nap. The rapid skin temperature rise after lights off is primarily related to a redistribution of heat from core to shell (induced by relaxation and not by sleep per se). Furthermore, sleep during the 8h recovery sleep episodes following the high and low sleep pressure protocols was not related to thermoregulatory changes. Despite the marked differences in EEG slow wave activity during these sleep episodes, CBT, PROX and DIST did not differ significantly. In conclusion, the build-up process of sleep pressure and its dissipation is not related to the thermoregulatory system, but the circadian modulation of sleepiness is. Research supported by the Swiss National Foundation START #3130-054991.98 / 3100-055385.98 to CC

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Phase Resetting by Long Light Pulses

Domien Beersma*, Marian Comas Soberats, and Serge Daan • Zoological Laboratory, University of Groningen

Although natural entrainment of circadian systems occurs in response to light pulses of, on average, 12 hours in duration, most of the knowledge on the responsiveness of circadian systems to light is based on short light pulses. Single short light pulses are neither suitable to investigate adaptation of circadian systems to light, nor can they provide much information about temporal accumulative properties of circadian systems. Existing data in the literature allow the comparison of the effects of short and long light pulses. We present analyses on weak resetting in humans (single pulses between 3 and 6.7 hours in duration) and strong resetting in flesh flies (single pulses between 4 and 20 hours in duration). If adaptation processes dominate the response, we expect that the timing of the onset of the light pulse is most important to the response. In particular for strong phase resetting, in contrast, we expect the endpoint of the light pulse to determine subsequent circadian phase. By comparing the responses to pulses of different duration, both in humans and in flesh flies, we observed that the timing of the midpoint of the light pulse best predicts the phase of the system after the pulse. We conclude from this observation that the timing of the onset and the end of a light pulse are of similar importance to the response, even in pulses of 20h in duration. This conclusion is inconsistent with existing limit cycle models of the circadian pacemaker. (Supported by BRAINTIME, EC QLG3-CT-2002-01829)

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Comparison of Melatonin Suppression by Blue Wavelength Light in Young and Older Adults

*Green K, Wolfe L, Gaskill MB, Goldman N, Zee PC, and Benloucif S • Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, Enlightened Technologies Associates, Inc., Fairfax, VA

Light in the blue (short) wavelength region is the most potent for the circadian timing system. However, with aging the lenticular transmission of shorter wavelengths is reduced and degeneration of melanopsin-containing retinal ganglion cells (RGCs) also occurs. The present study compared the light-induced suppression of melatonin in young and older adults following exposure to different wavelengths of light. Six young (6 F, 26.5 ± 2.5 years) and five older (3F, 2M, 66.6 ± 5.5 years) adults completed 4 overnight stays. They were exposed to light of blue (464nm), green (520nm), red (640nm) wavelengths or control (no light) for 90 minutes with specially designed glasses that delivered light via optical fibers (Somnavue). In young subjects, exposure to blue light suppressed melatonin levels in plasma by 44% ± 48.1 relative to the control night (at 90 minutes, p=0.07). In the older subjects, blue wavelength light suppressed melatonin by only 11% ± 21.9, which was significantly less than the level of suppression in young adults at both 30 minutes (p=0.03) and 90 minutes (p=0.02). Green and red wavelength light slightly suppressed melatonin in both groups (<15%). These results suggest that aging alters the responsiveness of the circadian system to short wavelength (blue) light, possibly due to changes in transmission of shorter wavelengths and/or degeneration of intrinsically photosensitive RGCs. Therefore, the blue wavelength region may not be more advantageous than other light wavelengths for the treatment of circadian rhythm sleep disorders in older adults. Supported by NCRR-00048, R01 HL67604, and P01 AG 11412

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Entrainment of the Circadian Pacemaker with Very Low Dose Melatonin

Alfred J. Lewy*, Jonathan S. Emens, Kyle P. Johnson, Paul T. Giger, Clifford M. Singer, Krista Yuhas, Angie R. Koenig • Oregon Health & Science University

About 15% of the legally blind completely lack light perception. The majority of these have abnormally-phased circadian rhythms and many free-run. Melatonin treatment has been shown to be highly effective in these blind free-runners (BFRs) at doses as low as 0.5 mg, and in one case, 0.05 mg. We report here entrainment of a BFR using 0.025 mg melatonin de novo; entrainment continued when the dose was lowered to 0.020 mg. The subject was a 21 y.o. female college student who became totally blind at age 2 as a result of retinoblastoma. She was bilaterally enucleated. She had asthma and allergic rhinitis but was otherwise healthy. Salivary and plasma melatonin were sampled for 24 hours for assessment of circadian phase every 2 weeks on 26 occasions. The melatonin onset was defined as the interpolated time when levels continuously rose above the threshold of 2 pg/ml (plasma) or 0.7 pg/ml (saliva). She initially free-ran with a period (tau) ± 95% CI of 24.21 ± 0.05 h. She demonstrated relative coordination with a minimum and maximum tau of 24.03 h and 24.57 h, respectively. Melatonin, 0.025 mg, was administered daily initially at 18:00 and later at 21:00. She demonstrated apparent entrainment with a decrease in her tau to 24.02 ± 0.01 h. After 168 days, the dose was decreased to 0.020 mg. She maintained entrainment with an overall treatment tau of 24.007 ± 0.015 h. This is the first report of successful entrainment of a BFR to a dose as low as 0.020 mg.

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Sleep and Circadian Rhythmicity Are Essential for Remodeling in the Heart

Tami Martino*, Nazneen Tata, Gavin Oudit, Martin Ralph, Marty Straume, Denise Belsham, Paul Lee, Peter Backx, Fayez Dawood, Peter Liu, Michael Sole • Toronto General Hospital

Cardiovascular disease is the leading cause of death in the world, and morbidity and mortality are significantly influenced by temporal patterns of sleep cycling. Myocardial infarctions occur more often in early morning than late night. Nocturnal infarcts are larger and more likely to result in heart failure. Shift workers exhibit increased risks of heart attack and sudden death. However, although clinical evidence that sleep rhythmicity plays a critical role in cardiovascular remodeling seems compelling, direct experimental evidence of this hypothesis has never been examined. Since genes critical to cardiovascular growth and renewal pathways are rhythmic across daily sleep/wake cycles in the normal heart, we first investigated whether these genes were rhythmic in heart disease as well. We found that expression of these genes changes significantly in disease, varying in a temporal pattern across the daily cycle, by using microarrays and computational analyses. This prompted us to then investigate the significance in vivo. We used 2 approaches, first by disrupting rhythms in a setting of acquired heart disease in mice, and second by using a model of prolonged rhythm disruption in tau hamsters. Our study demonstrates using multiple assessments of cardiovascular pathophysiology, including gravimetrics, morphometrics, histopathology, gene expression, behavioral analysis, biochemistry, and functional physiology, that sleep and rhythmicity play an essential role in cardiovascular remodeling. Disturbance has significant adverse effects on the heart. These experimental findings have not been previously demonstrated, support our hypothesis, and are relevant to humans with important implications in the clinical setting.

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Biological Rhythms in Premenstrual, Pregnant, Postpartum and Menopausal Depression

*BL Parry, CJ Meliska, N Basavaraj, DL Sorenson, G Zirpoli, LF Martinez, EL Maurer, AM Lopez, RL Hauger, A Gamst, T Wolfson, JA Elliott, DF Kripke • Department of Psychiatry, University of California, San Diego

In depressed patients (DP) with premenstrual dysphoric disorder (PMDD) or a major depressive episode (MDE) during pregnancy, postpartum or menopause, we compared the biological rhythms of plasma melatonin, serum cortisol, thyroid stimulating hormone (TSH), prolactin (collected every 30 minutes in dim/dark light from 18:00-11:00 h) and reproductive hormones (RH)(estradiol, progesterone, follicle stimulating hormone-FSH, luteinizing hormone-LH at 18:00 and 06:00 h), sleep (polysomnography-PSG and subjective measures), activity and illumination (by Actillume) to those of matched normal control (NC) subjects. Melatonin amplitude was lower in PMDD and postpartum DP and higher in pregnant and menopausal DP vs. NC. Cortisol secretion was lower in pregnant DP vs. NC. TSH levels were lower in pregnant and postpartum DP vs. NC. Prolactin secretion was higher in DP vs. NC in all groups except pregnant women. Estradiol and progesterone were lower in pregnant DP vs. NC. PSG recordings showed decreased total sleep time, sleep efficiency, delta sleep and increased wake after sleep onset and Rapid Eye Movement (REM) measures; and poorer subjective sleep quality in all groups of DP vs. NC. Activity and illumination measures were lower in postpartum DP vs. NC. Melatonin rhythms are differentially regulated in PMDD and postpartum DP vs. pregnant or menopausal DP. Decreased cortisol secretion in pregnant DP and decreased TSH in pregnant and postpartum DP contrasts with other studies of MDE. A

consistent finding in all but pregnant groups is elevated prolactin. Sleep quality measures are affected in all groups. Lower illumination might account for higher melatonin in postpartum DP.

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Immediate and Phase Shifting Effects of Nasal Versus Temporal Illumination of the Human Retina

Melanie RŸger*, Marijke Gordijn, Domien Beersma, Bonnie de Vries & Serge Daan • Human Chronobiology Group, Zoological Laboratory, University of Groningen

The Netherlands Objective: The mammalian retina contains both visual and circadian photoreceptors. Nocturnal stimulation of the latter receptors leads to melatonin suppression, which might cause reduced night-time sleepiness. Whether circadian phase shifting in humans is due to the same photoreceptors is not known. By exposing different parts of the retina to light we investigated whether phase shifts and melatonin suppression use the same photoreceptors. Methods: 12 healthy subjects participated in the experiment. They were exposed to 4 hours (12 p.m. till 4 a.m.) of 100 lux temporal or nasal illumination, or dim light (< 10 lux). Hourly measurements included salivary melatonin and subjective sleepiness. Repeated measurement ANOVAs were used to test for differences in melatonin suppression and for differences in circadian phase shifts. Results: Compared to the dim light condition, melatonin secretion was suppressed significantly both in response to the nasal ($p < 0.001$) and to the temporal illumination ($p < 0.005$). Nasal illumination of the retina resulted in significantly greater suppression in melatonin secretion. Nasal illumination induced a significant delay phase shift of DLMO ($p<0.02$), whereas temporal illumination did not. There was no immediate effect on sleepiness. Conclusion: Both the suppression of melatonin and the circadian phase shift of the DLMO are stronger following nasal illumination, suggesting that they may exploit the same photoreceptors, which appear to be more numerous or more sensitive in the nasal part of the retina. The suppression of melatonin was not accompanied by a reduction of subjective sleepiness.

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A Role for the HLH Transcription Factor Inhibitor of DNA Binding 2 (Id2) in the Mammalian Circadian Clockwork

Giles E. Duffield[1,2]*, Nathan P. Watson[3], Stuart N. Peirson2, Mark A. Israel[1,3], Jennifer J. Loros1, Jay C. Dunlap[1] • [1]Department of Genetics, Dartmouth Medical School, Hanover, New Hampshire 03755; [2]Department of Visual Neuroscience, Imperial College, Charing Cross Hospital, St. Dunstan's Road, London W6 8RF, U.K.; [3]Norris Cotton Cancer Center & Department of Pediatrics, 1 Medical Center Drive, Rubin Building, Lebanon, New Hampshire 03756

Id2 (Inhibitor of DNA-binding 2) is a helix loop helix (HLH) transcription factor implicated in development, cell cycle and apoptosis, and was identified in a cDNA microarray screen for rhythmically expressed genes in rat-1 fibroblasts (Duffield et al., 2002, Current Biology 12: 551-557). Subsequent gene expression analysis by real-time quantitative RT-PCR reveals a conserved rhythmic pattern across multiple tissues: in mouse 3T3 cells, and in vivo in other murine peripheral tissues (e.g. heart) and in the suprachiasmatic nucleus (SCN). The peak phase of the Id2 rhythm in all tissues appears to be phase locked to the rhythms of the canonical clock genes, and lags the phase of the bmal1 rhythm by 4-8 hrs.

Id2 is one of a family of four structurally related genes (Id1-4). Examination of the expression profiles of Id1, Id3 and Id4, also revealed rhythmic patterns within the heart and the SCN. In vitro transient transfection studies using an mPer1 promoter linked to a luciferase gene was used to examine the potential of ID2 to interact with the basic HLH clock proteins BMAL1 and CLOCK, and to potentially inhibit their heterodimerization and binding to the mPer1 promoter. Co-transfection with ID2 showed a robust inhibition of CLOCK:BMAL1 transactivation of the mPer1 promoter, thus indicating a potential role for Id2 in regulating central components of the circadian clock. Current studies are focused on examining the affect of inhibiting levels of Id2 mRNA by interference RNA, on the patterns of expression of the canonical clock genes. Research supported by the Wellcome Trust (058332/C/99/Z to G.E.D.) and NIMH (MH44651 to J.C.D. and J.J.L.).

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High Throughput Real-time Monitoring of Clock Gene Promoter Activity: A Valuable Tool to Study Clock Gene Function

Maier B, Reischl S, Loebcke K, Grudziecki A, Vanselow J and Kramer A* • Charit, Humboldt-University

In mammals, the suprachiasmatic nucleus (SCN) as well as almost all peripheral organs contain circadian oscillators. Recent findings suggest that there is no fundamental difference in the clock core mechanism between clock cells in the SCN, in the periphery or in cultured fibroblasts. Real-time monitoring of circadian clock gene promoter activity via luciferase reporter constructs has been shown to allow the estimation of rhythmicity and period length with a formerly unknown precision. Here, we combine RNAi-based gene silencing technology with high throughput real-time imaging of circadian rhythmicity in NIH3T3 fibroblasts using a standard microplate luminometer. To evaluate the concept, we co-transfected NIH3T3 cells with luciferase reporter construct and anti-mCry1 RNAi-vector. In contrast to control siRNA-vectors, transfection with anti-mCry1 siRNA-vector significantly shortened the period of NIH3T3 cell oscillation which is consistent with the shortened period of rhythmic wheel-running behavior of Cry1-/- mice. Since no mouse strain with a loss-of-function mutation for mClock has been described so far, we used siRNA-vectors targeting mClock RNA. In contrast to controls, transfection of anti-mClock siRNA-vector resulted in dose-dependent period lengthening leading to arrythmicity for high vector amounts. Thus, real-time monitoring of clock gene promoter activity combined with gene silencing technologies is a rapid and inexpensive tool for analyzing clock gene function. In addition, the use of a microplate luminometer provides the possibility for high throughput applications, such as forward genetic RNAi-based screens for novel clock components.

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A Genetic Selection for Circadian Output Pathway (COP) Mutations in Neurospora Crassa

Louis W. Morgan*, Michael W. Vitalini, and Deborah Bell-Pedersen • Center for Biological Clocks Research and Program for the Biology of Filamentous Fungi, Department of Biology, Texas A&M University, College Station, TX 77843

In Neurospora crassa, the frq, wc-1 and wc-2 genes encode components of the frq-oscillator. A functional frq-oscillator is required for rhythmic expression of the morning-specific ccg-1 and ccg-2 genes. In frq-null or wc-1 mutant strains, ccg-1 mRNA levels fluctuate near peak levels over the course of the day, whereas ccg-2 mRNA remains at trough levels. We utilized a genetic selection for mutations that affect the regulation of ccg-1 and ccg-2 by the frq-oscillator using the frq-null strain. We find that there is at least one mutant strain, COP1-1 (circadian output pathway derived from ccg-1) that has altered expression of ccg-1 mRNA, but normal ccg-2 expression levels. However, the clock does not appear to simply regulate a repressor of ccg-1 and an activator of ccg-2 in two independent pathways, since our selection identified three mutant strains, COP1-2, COP1-3, and COP1-4, in which a single mutation in each strain affects the expression levels and rhythmicity of both ccg-1 and ccg-2. In the frq+ background the mutations caused alterations in the Neurospora conidiation rhythm. The COP1-2 mutation caused temperature sensitive rhythmicity and the COP1-4 mutation altered temperature compensation suggesting the mutations affect both the function of the clock and control of clock output.

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High Resolution Mapping for Soc-1 (Suppressor of Clock) Locus in Mouse

Kazuhiro. Shimomura*, Deborah, Fenner, Chiaki Omura, Marc S. Richards, Sharon S. Low-Zeddies, Martha H. Vitaterna and Joseph S. Takahashi • Northwestern University

Although nine genes have been proposed to be components of core clock mechanism, using complex trait loci analysis we have recently identified at least 13 additional loci, a number of loci affect the circadian behavior through complex epistatic interaction. This suggests not only that there are more clock relevant genes in the mammalian genome, but also that the function of core clock genes may be well conserved among inbred strains of mice. Natural variation of circadian behavior observed in mammals, including humans, may not be the result of rare mutations in core clock genes but rather allelic variants due to ancestral polymorphisms. To identify the loci that can modify a mutant phenotype is a powerful approach to uncover genes that functionally interact with the mutant locus. The fact that the circadian phenotype of Clock/+ mice can be significantly suppressed by genetic background provides a unique opportunity to identify loci interacting with Clock. Since the protein function of the Clock gene product is known, the function of such modifier loci can be more easily delineate. This would be a key advantage of this type of analysis. We have found an allele-specific suppressor of circadian period in Clock/+ mice that is highly associated with presence of a BALBc/J genotype on mouse chromosome 1 (MMU1). This locus has been named Soc-1 (Suppressor of Clock-1) and has been isolated in congenic strains. With SNP based haplotype analysis among multiple inbred strains, we have mapped the Soc-1 locus to a 1.2 Mb interval on MMU1"

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Novel ENU-induced Circadian Clock Mutants in Mice

Sandra M. Siepka(1)*, Renee McGurk(1), Min Chen(1), Akiko Marusawa(1), Dawn Olson(1), Jennifer Walkowiak(1), Lawrence Pinto(1) and Joseph S. Takahashi(1,2) • (1) Center for Functional Genomics, Northwestern University, Evanston, IL, (2) Howard Hughes Medical Institute, Evanston, IL

One of the goals of the Center for Functional Genomics is to screen 10,000 mice per year to identify both dominant and recessive circadian rhythm mutants. Mutations are generated randomly by N-nitroso-N-ethlylurea (ENU) treatment of male mice. These mice are then mated to produce male founders, each representing a unique mutant line. Each founder is mated to produce daughters, which are then backcrossed to produce mice for phenotyping. These third generation (G3) mice are placed on running wheels and their free running activities recorded. Due to the random nature of ENU mutagenesis we will either identify new mutant alleles of known circadian clock genes or novel components of the mammalian circadian clock. As a pilot study, we conducted a small three-generation recessive mutation screen using BTBR/J mice. Approximately 3600 mice from 217 different mutant lines were placed on running wheels to measure free running activity. Two of these mutant lines produced mice with significantly altered free running periods. The first mutant we identified, part-time, is an autosomal recessive mutant with a free running period approximately 1.5 hours shorter (21.5 hours) than that of wild type mice (23.17 + 0.22 hrs). part-time segregates as a single gene and maps to the distal end of chromosome 10. The second mutant, Overtime, is an autosomal semi-dominant mutant with a free running period more than 2.5 hours longer (25.9 hrs) than that of wild type mice. Overtime also segregates as a single gene and maps to chromosome 14.

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Characterization of the Circadian Deadenylase Nocturnin and Its Potential Targets in the Mouse

*Carl Strayer°, David Lourim, Joe Fogerty, John Walker^, John Hogenesch^, Carla B. Green° and Joseph C. Besharse • °University of Virginia, Medical College of Wisconsin, ^The Genomics Institute of the Novartis Research Foundation

Nocturnin was identified in a screen for rhythmic transcripts expressed in the Xenopus laevis retina. We recently determined that the Nocturnin protein functions as a deadenylase. This suggests a role in regulating gene expression post-transcriptionally via mRNA stability or translatability, a previously unexplored mechanism of circadian regulation. Orthologs have since been described in a variety of animal species, including mouse, where Nocturnin is widely expressed with a high amplitude rhythm. In order to elucidate physiological role of Nocturnin, we have produced a strain of mouse in which the majority of the Nocturnin protein coding sequence, including the deadenylase domain, has been deleted. These mice develop normally, and adults appear healthy and exhibit normal circadian rhythms of locomoter activity. However, preliminary cytological analysis of livers from knock-out mice suggests defects in lipid storage and utilization, including an increase in intracellular lipid droplets. In order to identify genes that were mis-regulated as a consequence of Nocturnin disruption, we used whole-genome gene chips representing >36,000 transcripts to query RNA expression levels in livers from knock-out mice and wild-type littermates. Among the transcripts that were significantly changed in abundance in the knock-out mouse, we found several that are both circadianly regulated and encode proteins that are involved in lipid

metabolism. Current work is directed at determining which of these transcripts with altered accumulation patterns are direct targets of Nocturnin deadenylase activity.

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Natural Polymorphism in the Drosophila Cryptochrome

E. Tauber*, R. Ettourney, G. Grunnel, K. Mongar and C.P. Kyriacou • University of Leiceste

For most organisms light is the main cue for entrainment of the circadian clock. As light conditions vary along latitude, populations from different geographical zones are expected to have different molecular adaptations. Natural polymorphism in the Drosophila Period and Timeless genes have previously been identified. Since Cryptochrome (cry) is the dedicated circadian photoreceptor in Drosophila, we have initiated a screen to identify natural cry alleles. We have sequenced 800 bp of the FAD domain of 18 flies taken from different populations from Africa, the Mediterranean and Europe. Analysis of the sequences, mostly coding DNA from the third and the fourth exons, revealed high level of polymorphism. There were 58 single nucleotide polymorphic sites (SNPs) including four changes that resulted in amino acid substitutions. Out of these four, an A-to-T change resulting in Leucin to Histidine replacement attracted our attention. The significant change in the amino acid properties, and the substantial frequency of the two alleles suggest that the Leu-His polymorphism is maintained by balancing selection, and is functionally important. This was further supported by light pulse experiments where congenic lines with the two different alleles showed a significant difference in their light sensitivity. Finally, we also cloned and sequenced the cry gene from three other Drosophila species and the implications for the evolution of cry will be discussed.

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Cis-acting Sequences and Transcription Factors Mediating Morning-specific Expression of the Arabidopsis Clock Gene LHY

*Isabelle Carr, Jae-Yean Kim, Victoria Larner, Mark Spensley and Bethan Taylor • Department of Biological Sciences, University of Warwick, Coventry, UK.

The MYB transcription factors LHY and CCA1 and the pseudo-response regulator TOC1 form an autoregulatory loop which is believed to function as part of the oscillatory mechanism of the Arabidopsis circadian clock. The LHY and CCA1 proteins, expressed in the morning, act to repress transcription from the TOC1 promoter. TOC1 transcription resumes in the evening as levels of LHY and CCA1 decline. Accumulation of the TOC1 protein at night is believed to promote transcription of LHY and CCA1 in the morning, but the mechanism by which this is achieved is not known. In order to investigate this question, we have undertaken the cis-analysis of the LHY promoter. We report the identification of two elements that contribute differentially to rhythmic transcription. The G-box sequence (CACGTG) was required for rhythmic transcription under red light but not under blue light, whereas a CT-rich region surrounding the transcriptional start site was required in both conditions. Several transcription factors have been shown to form complexes with TOC1, including PIF3 and other members of the bHLH family, and a regulator of seed development and absicic acid responses known as ABI3. Plants carrying an antisense PIF3 transgene exhibited reduced, arrhythmic LHY::luc transcription under red light, yet expression was rhythmic in blue light. In constrast, abi3 mutations altered the level of LHY::luc transcription under both conditions. These

results identify new components of the circadian system of Arabidopsis and suggest distinct mechanisms for rhythmic expression of LHY under red and blue light.

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Real-time Measurement of Circadian Gene Expression in Peripheral Tissues from Transgenic Zebrafish

Maki Kaneko and Gregory M. Cahill* • Department of Biology and Biochemistry, University of Houston, Houston, TX 77204-5001

In vivo recordings of bioluminescence rhythms have been used in several organisms such as cyanobacteria, plants, Drosophila, and mammals with great success in mutagenesis screening as well as studies on physiological aspects of circadian rhythms. In this regard, zebrafish is the most convenient vertebrate species for large-scale mutagenesis. To develop a method for monitoring circadian gene expression in vivo, we have generated transgenic zebrafish in which cyclical expression of firefly luciferase (luc) gene is driven by a clock-gene promoter. We first constructed a transgene in which luc is fused to the promoter of zebrafish Per3 gene whose expression is known to cycle in a circadian manner. Three of five germline transformant lines were found to show bioluminescence rhythms in larvae. This larval rhythm was shown to reflect mRNA cycling of endogenous zPer3, and peaks during the subjective day. The peak phase was shifted earlier in a short-period mutant g4 compared to wild type. Therefore, this system may be used to screen for new circadian mutants. In adults, many peripheral tissues such as the retina, heart, and spleen showed robust oscillations of bioluminescence in culture. These rhythms in cultured tissues were observed in light:dark cycles as well as in constant conditions, and can be entrained by light:dark cycles. The bioluminescence rhythms peak in the middle of the subjective day in several different tissues.

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Suitability of AANAT2:EGFP Transgenic Lines as a Model to Study Clock-regulation of the Melatonin Rhythm

Limor Ziv[1], Adi Tovin[1], Lior Appelbaum[1], Igor B. Dawid[2], David C. Klein[3] and Yoav Gothilf[1]* • [1]Department of Zoology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel, [2]Laboratory of Molecular Genetics and [3]Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

Transgenic (TG) lines that express Enhanced Green Fluorescent Protein (EGFP) under the control of the zfAANAT2 regulatory regions were developed to extend analysis of the mechanisms underlying tissue-specific and rhythmic expression the zebrafish serotonin-N-acetyltransferase-2 gene (zfAANAT2). Here we report on the suitability of these TG lines for these purposes. EGFP fluorescence is detected in the pineal glands of live TG embryos as early as 23 hr post fertilization. Whole mount in situ hybridization analysis reveals that EGFP expression is rhythmic in TG embryos/larvae in both light:dark cycles and constant darkness, indicating that a functional oscillator operates in the pineal gland of TG embryos/larvae. To test whether TG larvae can be used for studying the effect of candidate genes on zfaanat-2 promoter activity, gene knock-down with morpholino-modified antisense oligonucleotides (MO) was attempted. Microinjection of EGFP-directed MO suppressed EGFP expression in a dose dependent manner;

expression was suppressed for a maximum of 12 days. To examine pineal function in EGFP-expressing glands, continuous flow organ culture was used. Adult glands secreted melatonin on a circadian schedule in constant darkness. A light pulse in the beginning of the night immediately suppressed melatonin production and caused a phase-delay. These results indicate that the oscillator, photoreception and phototransduction mechanisms are intact in adult TG pineal glands. These findings indicate that transgenic lines expressing EGFP under control of the zfAANAT2 promoter will be useful for future in vivo and in vitro studies on the mechanisms involved in circadian function in the pineal gland of zebrafish.

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Generation of an Inducible Circadian Clock: Molecular Oscillations and Behavior in Drosophila

Kadener, S., Schoer R., McDonald, M. and Rosbash M. • Department of Biology, Howard Hughes Medical Institute, Brandeis University, WALTHAM, MA, 02454 USA

To address the role of the master gene Clock (Clk) in circadian clock gene expression, we generated transgenic lines carrying a fusion of Clk with the ligand-binding domain of the mouse glucocorticoid receptor (UAS-CLK-GR). Surprisingly we found that the fusion protein interferes with the wild-type molecular clock, only in the absence of dexamethasone. Since the static levels of timeless expression are comparable to wild-type peak levels, the results suggest that CLK's mechanism of action is not just restricted to transcriptional activation. With this goal in view, we are working on the purification of CLK-containing complexes, for partner protein identification. After addition of dexamethasone, the circadian program is almost immediately revived. This activation provides the cleanest example of an in vivo system in which the molecular clock can be initiated. These restored rhythms manifest a huge increase in circadian amplitude compared to wild-type flies and are entrained normally by light. This suggests that the excess CLK activity is incorporated into the circadian circuit and indicates that CLK regulates the amplitude but not the period of the molecular oscillations. We are presently exploiting this in vivo system to confirm and extend the identification of CLK-primary targets, using RNA from different dexamethasone-treated tissues and Affymetrix microarrays. With these approaches, we hope to elucidate two central questions of circadian biology: what is the exact role of the Clk protein in the generation/maintenance of molecular oscillations, and how is the circadian transcriptional network organized?

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Structural

Ellizabeth Getzoff

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Structural

Susan Golden

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Structural

Carl Johnson

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Structural

Andy LiWang

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Anabaena Circadian Clock Proteins KaiA and KaiB Reveal a Potential Common Binding Site to Their Partner KaiC

Robert G Garces[1,3],*, Ning Wu[2,3], Wanda Gillon[2,3] and Emil F Pai[1,2,3,4,5]* • [1]Department of Medical Biophysics, Centres of Excellence, University of Toronto, Toronto, ON, Canada, [2]Department of Biochemistry, Centres of Excellence, University of Toronto, Toronto, ON, Canada, [3]Division of Molecular and Structural Biology, Ontario Cancer Institute, University Health Network, Toronto, ON, Canada, [4]Department of Molecular & Medical Genetics, Centres of Excellence, University of Toronto, Toronto, ON, Canada and [5]Protein Engineering Network, Centres of Excellence, University of Toronto, Toronto, ON, Canada

The cyanobacterial clock proteins KaiA and KaiB are proposed as regulators of the circadian rhythm in cyanobacteria. Mutations in both proteins have been reported to alter or abolish circadian rhythmicity. Here, we present molecular models of both KaiA and KaiB from the cyanobacteria Anabaena sp PCC7120 deduced by crystal structure analysis, and we discuss how clock-changing or abolishing mutations may cause their resulting circadian phenotype. The overall fold of the KaiA monomer is that of a four-helix bundle. KaiB, on the other hand, adopts an alpha-beta meander motif. Both proteins purify and crystallize as dimers. While the folds of the two proteins are clearly different, their size and some surface features of the physiologically relevant dimers are very similar. Notably, the functionally relevant residues Arg 69 of KaiA and Arg 23 of KaiB align well in space. The apparent structural similarities suggest that KaiA and KaiB may compete for a potential common binding site on KaiC.

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Sex Differences in the Duration, Variability, and Heritability of a Seasonal Interval Timer

Brian J. Prendergast(1), Randall A. Renstrom(1), and Randy J. Nelson(2) • (1) Department of Psychology and Institute for Mind and Biology, University of Chicago, Chicago, IL 60637. (2) Departments of Psychology and Neuroscience, The Ohio State University, Columbus, OH 43210.

Seasonal clocks (e.g., circannual clocks, seasonal interval timers) permit anticipation of regularly-occurring environmental events by timing the onsets of seasonal transitions in reproduction, metabolism, and behavior. Implicit in the concept that seasonal clocks reflect adaptations to the local environment is the unexamined assumption that heritable genetic variance exists in the critical features of such clocks, namely, their temporal properties. These experiments quantified the intraspecific variance in, and heritability of, the photorefractoriness interval timer in Siberian hamsters (Phodopus sungorus), a seasonal clock that provides temporal information to mechanisms that regulate seasonal transitions in body weight. Twenty-seven families consisting of 54 parents and 109 offspring were raised in a long-day photoperiod and transferred as adults to an inhibitory photoperiod (continuous darkness; DD). Weekly body weight measurements permitted specification of the interval of responsiveness to DD, a reflection of the duration of the interval timer, in each individual. Body weights of males and females decreased after exposure to DD, and 3-5 months later somatic recrudescence occurred, indicative of photorefractoriness to DD. The interval timer was approximately 5 weeks longer and twice as variable in females relative to males. Analyses of variance of full-siblings revealed an overall intraclass correlation of 0.71±0.04 (0.51±0.10 for male offspring and 0.80±0.06 for female offspring), suggesting a significant family resemblance in the duration of interval timers. Parent-offspring regression analyses yielded an overall heritability estimate of 0.61±0.2; h^2 estimates from parent offspring regression analyses were significant for female offspring (0.91±0.4) but not for male offspring (0.35±0.2), indicating strong additive genetic components for this trait, but primarily in females. In nature, individual differences, both within and between sexes, in the timekeeping properties of seasonal interval timers, and a strong heritable basis thereof, would provide ample substrate for selection to rapidly influence seasonal clocks. Balancing selection in environments where the onset of spring conditions varies from year-to-year could maintain genetic variance in interval timers, and yield seasonal clocks tuned to the local environment.

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Circadian Control of the Cell Cycle in the Dinoflagellate Karenia Brevis: a Role for Blue Light and Characteristics of a Blue Light Receptor

*Brunelle, S.A. and Van Dolah, F.M. • Marine Biotoxins Program, NOS, NOAA and GPMB, College of Charleston

The molecular mechanisms controlling the cell cycle in the Florida red tide dinoflagellate, Karenia brevis, are of interest because they ultimately regulate the rate of formation of toxic algal blooms. Previous work in our laboratory has shown that the cell cycle in K. brevis is phased to the diel cycle, such that cells enter the cell cycle at precise times relative to the onset of light. Here, we demonstrate that the cell cycle is under control of a circadian rhythm that is entrained by the dark/light transition. In a number of organisms, blue light serves to entrain circadian rhythms. Therefore, we next investigated the effect of red and blue light on cell cycle progression. In the presence of blue light, K.brevis appears to enter S-phase early, whereas

in red light, cell cycle progression is delayed in S-phase entry. We have identified four ESTs (expressed sequence tags) in a K. brevis cDNA library with high homology to cryptochromes, blue-light receptors found in bacteria, plants and animals. Three of the four ESTs have highest homology to cryptochrome DASH. Alignments with these ESTs and cryptochrome 1 from Arabidopsis showed the lack of an extended C-terminal region characteristic of cryptochromes 1 and 2. The K.brevis cryptochrome appears to have an extended N-terminal region that is present in cryptochrome DASH. We are currently attempting to obtain the complete gene sequences represented by these ESTs, analyze them with respect to known cryptochromes, and characterize their expression in K. brevis. If these sequences prove to be cryptochrome, this will be the first cryptochrome to be described in a dinoflagellate.

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Food Entrainment Modifies c-Fos Expression Pattern in the Brain Stem of Rats

Manuel Angeles-Castellanos, Jorge Mendoza., Carolina Escobar • Depto. Anatom'a Facultad de Medicina. UNAM. Mexico

When food is restricted to a few hours daily animals increase locomotor activity 2-3 hours preceding food access, which has been termed food anticipatory activity (FAA). Food entrainment has been linked to the expression of a circadian food entrained oscillator (FEO). We have previously described a differential involvement of hypothalamic nuclei in the food entrained process. The communication between the gastrointestinal system and central nervous system is essential for food entrainment. The visceral synaptic input to the brain stem arrives to the dorsal vagal complex and is transmitted directly to hypothalamic nuclei and other areas of the forebrain or via to the parabraquial nucleus (PB). The present study was aimed to characterise the relevance and participation of the brain stem structures as an input pathway involved in food entrainment using c-Fos like expression (c-Fos-IR). The results show an increased c-Fos-IR following mealtime in all brain stem nuclei. The NTS and PB nuclei of entrained animals showed a modulated activation due to entrainment and different from satiated controls. Food entrained temporal patterns did not persist under fasting conditions. Present data suggest that NTS and PB and not the area postrema are involved with the neuronal input of the FEO. Supported by CONACYT 33033-N and DGAPA IN203803

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Intersection of the Hypocretin and Serotonin Neural Systems: Possible Involvement in Circadian Rhythms

Colleen E. Campbell* and Eric M. Mintz • Department of Biological Sciences and School of Biomedical Sciences, Kent State University, Kent, OH 44242

Hypocretins (orexins) are a family of neuropeptides that are involved in sleep regulation. Immunohistochemical studies have shown that hypocretin neurons only sparsely innervate the suprachiasmatic nucleus (SCN), but heavily innervate other cell groups with connections to the SCN. Included among these other groups are the dorsal and median raphe nuclei (DRN and MRN). Both the DRN and MRN are known to have a serotonin mediated influence on circadian rhythmicity. In current studies, we have used confocal microscopy to demonstrate that the serotonin cell bodies and hypocretin fibers are closely associated in both the DRN and the MRN. Since hypocretin exerts an excitatory effect on DRN neurons, it is possible that hypocretin influences circadian rhythms in the SCN by altering

serotonergic activity in the DRN and/or MRN. To examine this possibility we implanted adult male Syrian hamsters with guide cannula aimed at the DRN. Wheel-running rhythms were then monitored in constant darkness. After rhythms stabilized, the animals were microinjected with 500 nl of either hypocretin-1 (1 mg/ml) or 0.9% saline. Preliminary data suggest that single microinjections of hypocretin do not produce phase shifts that are significantly different from saline. Ongoing experiments are examining possible roles for hypocretin in circadian rhythm regulation. Supported by NIH MH67079 and NS043155 to EMM.

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Identification of Orexin-IR Neurons Projecting to the IGL

Luis Vidal, Jane Blanchard and Lawrence Morin • Dept. Psychiatry, HSC, Stony Brook University, Stony Brook, NY 11794

The orexins are a novel family of neuropeptides thought to be modulators of sleep/wake patterns in mammals. A moderate plexus of fibers containing orexin immunoreactivity (ORX-ir) has been described in the hamster intergeniculate leaflet (IGL). To investigate the origin of ORX-ir fibers in the IGL, we injected the tracer cholera toxin -subunit (CTB) into the IGL of the golden hamster. Double-label fluorescent immunohistochemistry for ORX and CTB was examined in the LH region, known to contain a large population of ORX-ir cells. Double labeling was also performed to examine possible co-localization of retrogradely labeled cells with melanin concentrating hormone (MCH), a peptide abundantly present in cells of the LH region close to those containing ORX. Few retrogradely labeled CTB (IGL-projecting) cells were observed. Most were detected in the zona incerta (ZI), sub-incertal zone, and few cells in the most dorsal aspect of the LH. ORX-ir cells were abundant and detected mostly in the medial LH and perifornical region. MCH cells were also abundantly seen in the ZI, in close proximity to CTB cells, but ventral to them. CTB and ORX-ir, cells (estimated total = 10) were present in the dorsal aspect of the LH. This is roughly < 1 % of the total ORX cells. Most CTB cells were located dorsal and lateral to the ORX-ir cells. No CTB-ir cells also contained MCH and no MCH-ir processes were evident in the IGL. The results show that a small number of scattered LH cells containing ORX-ir project to the IGL and contribute in part to the plexus of ORX-ir observed in the IGL. Supported by NS22168 and MH6447101.

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Protein Phosphatase 1 Regulates the Stability of the Circadian Protein PER2

Monica Gallego*, Heeseog Kang and David M. Virshup • University of Utah

The circadian clock is regulated by a transcription/translation negative feedback loop. A key negative regulator of circadian rhythm in mammals is the PER2 protein. Its daily degradation at the end of night accompanies de-repression of transcription. CKIe has been identified as the kinase that phosphorylates PER2, targeting it for ubiquitin-mediated proteasomal degradation. We now report that PER2 degradation is also negatively regulated by protein phosphatase 1 (PP1)-mediated dephosphorylation. In Xenopus egg extract, PP1 inhibition by Inhibitor-2 accelerated mPER2 degradation, and overexpressed PER2 was very efficiently degraded after calyculin A treatment in HEK 293 cells. Since calyculin A mainly inhibits PP1 and PP2A, we tested both enzymes for PER2 binding. Co-immunoprecipitation experiments showed that PER2 bound to PP1c, but not to PP2Ac, in transfected HEK 293 cells. Purified PP1c, as well as PP1 immunoprecipitated from HEK 293 cells, mouse liver and brain, dephosphorylated CKIe-phosphorylated PER2, showing that PER2 is a substrate for PP1 in vitro and in vivo. Moreover, overexpression of a

dominant negative form of PP1c, the D95N mutant, accelerated proteasome-mediated degradation of wildtype, but not mutant PER2, in HEK 293 cells. Thus, PP1 regulates PER2 stability and is therefore a candidate to regulate mammalian circadian rhythm.

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Mapping of Functional Regions in CLOCK and BMAL1 Transcription Factors

Roman V.Kondratov[1]*, Victoria Yu. Gorbacheva[1], Anna A. Kondratova[2], Andrei V. Gudkov[2], and Marina P. Antoch[1] • Departments of [1]Cancer Biology and [2]Molecular Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

Mammalian CLOCK and BMAL1 are two members of bHLH-PAS-containing family of transcription factors that represent the positive elements of circadian autoregulatory feedback loop. Recently we demonstrated that CLOCK/BMAL1 specific heterodimerization initiates a number of posttranslational events including phosphorylation of both proteins, BMAL1-dependent CLOCK nuclear translocation and its subsequent degradation. These posttranslational events are tightly coupled with transcriptional activation of CLOCK/BMAL1-responsive promoters, indicating their functional role in the regulation of the circadian clock. To further characterize the functional significance of dimerization-induced modifications, we used a series of CLOCK and BMAL1 deletion mutants to localize specific regions important for posttranslational regulation. Using these mutants, we identified signals of nuclear localization (NLS) in CLOCK and BMAL1 and showed that both of them are required for translocation of the complex into the nucleus and transactivation of responsive promoters. The analysis of mutants with deleted NLS region that were incapable of nuclear translocation indicated that formation and phosphorylation of CLOCK/BMAL1 complex is initiated in the cytoplasm. We also identified the region within the C-terminal part of protein that is responsible for stability of CLOCK, which is presumably, involved the regulation of CLOCK nuclear degradation. All mapped functional regions of CLOCK/BMAL1 complex are likely to be targets for additional levels of circadian function control.

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Biochemical and Biophysical Characterizations of KaiC Protein in the Cyanobacterial Circadian Clockwork

Tetsuya Mori*, Yao Xu, and Carl H. Johnson • Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

KaiC from Synechococcus elongatus PCC7942 is an essential circadian clock protein in cyanobacteria. Previous sequence analyses suggested its inclusion in the RecA/DnaB superfamily. A characteristic of the proteins of this superfamily is that they form homohexameric complexes that bind DNA. We show that KaiC also forms hexameric ring complexes with a central pore. The association of KaiC molecules into hexamers depends on the presence of ATP. The KaiC sequence does not include the obvious DNA-binding motifs found in RecA or DnaB. Nevertheless, KaiC binds DNA substrates. We also found that the other essential clock proteins KaiA and KaiB modulate the status of KaiC phosphorylation. KaiA inhibits KaiC dephosphorylation and KaiB antagonizes this action of KaiA. Implications for the biochemical and biophysical characteristics of KaiC protein in the circadian clock mechanism will be discussed."

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Investigation for the Role of KaiC Phosphorylation in Circadian Clock System of Synechococcus Elongatus PCC7942

*Taeko Nishiwaki[1,2], Reiko Kiyohara[1], Cheolju Lee[3], Yoshinori Satomi[3], Hakuto Kageyama[1,2], Masato Nakajima[1,2], Yohko Kitayama[1,2], Mioko Temamoto[1], Hideo Iwasaki[1,2], Toshifumi Takao[3] and Takao Kondo[1,2] • [1]Division of Biological Science, Graduate School of Science, Nagoya University, [2]Core Research for Evolutional Science and Technology (CREST), [3]Institute for Protein Science, Osaka University

In the cyanobacterium Synechococcus elongatus PCC 7942, kaiA, kaiB and kaiC have been found to be essential for the circadian rhythm generation. KaiC is proposed as a negative regulator of rhythmic components of all genes in the genome including autoregulatory circadian kaiBC expression. KaiC undergoes circadian Ser/Thr-phosphorylation in vivo and has an autokinase/autophosphatase activity in vitro. The magnitude of KaiC phosphorylation is enhanced and negated by KaiA and KaiB, respectively. However, functions of KaiC phosphorylation in the circadian rhythm generation remain unknown. We identified two autophosphorylation sites using mass spectrometry. By site-directed mutagenesis, these residues were replaced by alanine and the mutations were introduced into kaiC locus in the Synechococcus genome. Phosphorylation of KaiC was reduced in the resultant single mutants and was completely abolished in the double mutant, suggesting that KaiC is phosphorylated at these sites also in vivo. These mutations abolished the circadian rhythm of the PkaiBC activity, indicating that KaiC phosphorylation is essential for the cyanobacterial clock system. It has been known that continuous kaiC overexpression represses the PkaiBC, and temporal kaiC overexpression resets the phase of the rhythm. We found that PkaiBC was repressed only transiently when non-phosphorylatable KaiC was continuously overexpressed. The amount of phase shift induced by temporal overexpression of non-phosphorylatable KaiC was smaller than that of wild-type KaiC. We are also investigating the effect of phosphorylation on KaiC complex formation. Based on these results, possible roles of KaiC phosphorylation in the cyanobacterial circadian system will be discussed.

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A Molecular Analysis of Drosophila Doubletime

Jin-Yuan Fan[1], Fabian Preuss[1], Dara Watkins[1], Edward S. Bjes[1], Shu Bao[2], Madhavi Kalive[2], Eric Schuenemann[1], Michael Muskus[1] and Jeffrey L. Price[1]* • [1]School of Biological Sciences, University of Missouri–Kansas City, Kansas City, MO 64110, and [2]Dept. of Biology, West Virginia University, Morgantown, WV 26506.

Casein kinase I (DBT) is necessary for circadian rhythms and regulates the phosphorylation of the clock protein period (PER) in both mammals and fruit flies. Mutations which shorten (dbtS) and lengthen (dbtL) circadian period have been isolated. Our lab is examining the role of DBT with a molecular analysis in vitro, in flies and in a Drosophila cell culture line (S2). In flies, DBT is expressed at constant levels throughout the day, but its subcellular localization changes. GST pull down assays showed that wild type DBT, DBTS, and DBTL proteins can bind to PER equivalently through an evolutionarily conserved N terminal part of DBT. However, both the dbtS and dbtL mutations reduce the CKI-7-sensitive protein kinase activity of an orthologous Xenopus casein kinase Idelta and of Drosophila DBT immunoprecipitated from transfected S2 cell cultures. These data demonstrate that lowered enzyme activity can be associated with both short-period and long-period phenotypes, and suggest that the period changes

may be produced by some other function of DBT besides its overall kinase activity. However, experiments with a DBT dominant negative showed that kinase activity is essential for mediating degradation of PER in S2 cell cultures. We are currently investigating the hypotheses that the dbt mutations differentially affect the phosphorylation of specific sites in PER and thereby activate or inactivate selected features of PER's circadian program, and that DBT activity may be regulated by proteins which interact with it.

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The Ion Channel, Narrow Abdomen, Functions in Drosophila Pacemaker Neurons to Regulate Circadian Behavior

Bridget C. Lear* and Ravi Allada

In flies and mammals, conserved transcriptional feedback loops drive circadian behaviors. However, relatively little is known about how these core intracellular components regulate neuronal activity or in turn, how neuronal function mediates circadian rhythmicity. We have examined Drosophila loss-of-function mutants of a conserved ion channel, narrow abdomen (na). We have shown that na mutants fail to increase activity in anticipation of light-dark (LD) transitions during entrainment conditions and exhibit weak locomotor rhythms during constant darkness (DD), consistent with a circadian function for this channel. We have used tissue-specific rescue experiments to determine the neural requirements for na function. We find that na expression within circadian neurons is sufficient to fully rescue both the LD anticipation and DD rhythmicity phenotypes. Thus, na likely functions within circadian neurons to promote rhythmic behavior. To determine if this channel influences the core molecular oscillator, we have assessed PERIOD expression in circadian neurons. We find that PERIOD oscillations in na mutants occur with a similar phase and amplitude to those of wild-type flies in LD. Taken together, these data indicate that na functions within pacemaker neurons downstream of the core oscillator. We propose that this channel serves as a crucial link between transcriptional feedback loops and circadian regulation of neural activity.

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CLK and CYC Reveal Clock Specific Spatial Expression

Jerry H. Houl*, Fanny S. Ng, Abigail L. Casillo, Nick R. J. Glossop, Scott M. Dudek, Isaac Edery° and Paul E. Hardin • Department of Biology and Biochemistry, University of Houston, 369 Science and Research Building 2, Houston, TX 77204-5001, Department of Molecular Biology and Biochemistry, Rutgers University, Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey 08854

The identification and analysis of clock genes in Drosophila revealed that the circadian timekeeping mechanism is comprised of two interlocked feedback loops in gene expression, a period (per)/timeless (tim) loop and a Clock (Clk) loop, that are expressed in many neuronal and non-neuronal tissues. CLK and CYCLE (CYC) proteins play a critical role in regulating both of these loops by activating feedback regulators (i.e. per, tim, Pdp1epsilon and vri) that function at specific times during the circadian cycle. Recent studies suggest that Clk and cyc also regulate genes that are not rhythmically expressed, and behavioral processes that are independent of the circadian clock (i.e. drug sensitization and sleep homeostasis). Despite the critical roles Clk and cyc play within and outside the circadian clock, their spatial expression patterns have not been characterized. Using a newly developed CLK antibody and an existing CYC antibody, we detect CLK and CYC in the same 'clock' cells that express PER. In addition,

we find that the Clk and cyc promoters drive expression in the same clock cell pattern as their endogenous counterparts. Taken together, these results indicate that Clk and cyc are co-expressed in canonical clock cells, and that their expression is controlled at the transcriptional level.

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Identifying Cells and Molecules That Control Olfaction Rhythms in Drosophila

Parthasarathy Krishnan*, Shintaro Tanoue, Balaji Krishnan, Gregg Roman°, Stuart E. Dryer and Paul E. Hardin • Department of Biology and Biochemistry, University of Houston, Houston, TX 77204; °Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

Odor induced physiological responses in the antennae of Drosophila melanogaster are regulated by the circadian clock. These olfactory responses, as assayed via electroantennograms (EAGs), require the function of circadian oscillators in peripheral tissues, but the identity of these oscillators and whether they function independent of 'central' lateral neuron (LN) oscillators in the brain has not been determined. We initially assayed EAG responses in flies specifically lacking LNs to determine if these neurons were required for olfaction rhythms. LN deficient flies showed robust EAG rhythms, thereby demonstrating that 'central' oscillators in the brain are not necessary for rhythmic olfactory responses. Since circadian oscillators are present in antennal neurons, we next tested whether EAG rhythms were locally controlled by these neurons. Since ablating antennal neurons would necessarily abolish EAG responses, we developed dominant negative versions of CLK (i.e. CLKD) and CYC (i.e. CYCD) to specifically eliminate circadian oscillator function in these cells. When CLKD or CYCD were expressed specifically in antennal neurons, EAG responses were abolished, demonstrating that antennal neurons are necessary for EAG rhythms. To test whether antennal neurons autonomously controlled olfaction rhythms, we assayed EAG responses in cyc01 flies in which oscillator function was rescued by CYC expression only in antennal neurons. EAG responses in these flies were robustly rhythmic, showing that antennal neurons are sufficient for olfaction rhythms. We are currently identifying output genes required for the circadian modulation of olfactory responses in these neurons.

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Differential Expression of Ionotropic and Metabotropic Glutamate Receptors in mPer2 Mutant Mice: Implications for Synaptic Transmission and Plasticity

Gurudutt Pendyala * and Urs Albrecht • Institute of Biochemistry, University of Fribourg, 1700, Fribourg, Switzerland

The master circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus governs various aspects of mammalian physiology and behavior. mPer2 mutant mice are defective in resetting the clock and become arrhythmic in constant darkness. In light mediated resetting, glutamate plays an important role. Nocturnal stimulation releases glutamate at synapses connecting the eye with the SCN via the Retinohypothalamic tract (RHT). Glutamate binds to its receptors on the SCN neuron, thereby activating common signaling pathways. Perturbation of glutamatergic neurotransmission has been implicated in several neurological diseases. A major step in glutamate neurotransmission is its rapid removal from the synaptic cleft chiefly mediated by Na+ coupled transporters GLAST and GLT-1 localized on the astrocytes and other glutamatergic receptors. We earlier found GLAST down regulated in

the mPer2 mutants and the ninhydrin assay revealed more accumulation of glutamate in the synaptic cleft of these mice. However, it is likely that compensatory mechanisms exist to counteract the excitotoxicity of accumulated glutamate. This led us to examine the expression of the ionotropic receptors AMPA and NMDA whose modulation play a crucial role in the expression of synaptic plasticity, the most well-studied forms being long-term potentiation (LTP) and long-term depression (LTD) in the CNS. We also examined the various subtypes of metabotropic receptors, which are G-protein coupled and are involved in various signal transduction cascades. Western blot analysis performed on the brain extracts of these mice revealed a differential expression of these receptors.

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Melatonin Modulates Diencephalic, but Not Telencephalic, Calcium Waves in Avian and Mammalian Astrocytes

J. L. Peters, R. B. Tjalkens, V. M. Cassone, and M. J. Zoran • Department of Biology, Department of Veterinary Anatomy, and Center for Biological Clocks Research, Texas A&M University, College Station, Texas 77843, U.S.A.

Melatonin, a pineal neurohormone, mediates circadian and seasonal processes in birds and mammals. Diencephalic astrocytes are sites of melatonin action, at least in birds, since they express melatonin receptors and melatonin affects their metabolism. Intercellular calcium waves are also modulated by melatonin in avian diencephalic astroctyes. Here we have demonstrated that calcium waves in mammalian astrocytes spread 2-5 fold farther in culture than waves in avian astrocytes. Nonetheless, mouse diencephalic astrocytes, like those of chicken, were modulated by melatonin. Application of 10 nM melatonin to confluent cultures of mouse diencephalic astrocytes caused a 38% increase in the spread of these waves, similar to the 27% increase this dose of melatonin caused in chick astrocytes. In contrast, melatonin had no effect on calcium waves in either avian or mammalian telencephalic astrocytes. Therefore, the regulation of glial cell physiology by the biological clock, at least as defined by calcium signaling, is regionally restricted within the vertebrate brain. We have also demonstrated fundamental differences in the propagation of calcium waves when comparing diencephalic to telencephalic astrocytes in both birds and mammals. Waves meander among diencephalic astrocytes without a fixed direction to their spread; whereas, waves were more synchronous in their propagation among telencephalic astrocytes, radially spreading from their initiation site. These differences indicate that glia cells communicate via fundamentally different mechanisms in these brain regions.

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Anatomical Localization and Phenotypical Identification of Calpain-activated Cells Mediating Light/glutamate-induced Phase Delays in the Rat Suprachiasmatic Nucleus

S. H. Tyan, C. Beaul* and M.U. Gillette • University of Illinois at Urbana-Champaign

The circadian response to light engages intracellular signaling cascades that result from the activation of N-methyl-d-aspartate (NMDA) receptors within the suprachiasmatic nucleus (SCN). In the early part of the night, light- or glutamate-induced NMDA receptor activation leads to Ca2+ influx which in turn induces Ca2+i release from activated ryanodine receptors. We have previously shown that this secondary Ca2+i flux

activates the Ca2+-dependent neutral cysteine protease, calpain, and that both of its isoforms contribute to light/glutamate-induced phase delays. The present set of experiments was aimed at further characterizing the anatomical localization and peptide phenotype of calpain-activated cells within the rat SCN. Using two-photon confocal microscopy imaging for the fluorescent calpain substrate t-BOC-Leu-Met-CMAC, we observed glutamate-induced increases in calpain activity localized primarily in neuronal processes rather than in neuronal cell bodies. Furthermore, a greater number of calpain-activated cells were identified within the dorsomedial SCN and these cells did not colocalize with neurons containing the calcium-binding protein, calretinin. The distribution of calpain-activated cells within the SCN and their phenotype will be investigated further using calpain imaging combined with immunocytochemistry for several circadian markers, such as vasopressin, vasoactive intestinal polypeptide and calbindin, as well as for the expression of clock genes. Supported by USPHS grant HL 67007 and FQRNT (Canada).

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Effects of Circadian Rhythm Disruption on Radiation-induced Apoptosis

Petit, LM*, Jerry, DJ, Bittman, EL, and Harrington • ME Smith College

Disruption of circadian rhythms may modulate the course of cancer. Epidemiological studies have suggested a link between working at night and increased incidence of breast cancer. One recent study reports that the mPer2 gene functions in tumor suppression by regulating DNA damage-responsive pathways. In the present study, female Balb/c mice were used to examine the effects of disruption of rhythmicity on gamma radiation-induced apoptosis in the mammary and thymus glands. Ovariectomized mice received subcutaneous implants providing estradiol and progesterone one week prior to radiation. Half the mice experienced an 8 hour advance shift in the light/dark cycle (accomplished by shortening one dark period), and half were control unshifted mice (LD12:12; lights on 0700-1900h). Four animals from each group received 5 Grays (500rads) of radiation supplied by a 137Cs irradiator at 1100h and 1900h and sacrificed at 1500h and 2300h, while 2 animals in each group were unirradiated controls. Mammary tissues and thymus glands were removed, formalin-fixed and paraffin-embedded in preparation for TUNEL, using the FragEL DNA Fragmentation kit. Preliminary results indicate that levels of apoptosis in unirradiated controls (~1% of mammary cells TUNEL-positive) were similar to levels described in previous reports. Both control and LD-shifted irradiated groups showed similar incidence of apoptosis (~15% of mammary cells TUNEL-positive). Further analysis of mammary gland results, as well as results from thymus gland will be reported. While our preliminary results suggest that there is little effect of acute light:dark cycle shifts, this does not preclude greater effects from chronic disruption of circadian rhythm entrainment. Supported by a grant from the Mellon Foundation to Smith College (MEH).

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Biolistic Transfection of the Mouse SCN: Fluorescent Proteins Report mPer1 Promoter Activity in Wild-type and Vipr-/- Mice

M.H. Hastings*, A.B. Reddy, A.J. Harmar[2] & E.S. Maywood • MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK; [2]Centre for Neuroscience Research, University of Edinburgh, 1 George Square, Edinburgh, EH8 9JZ, UK

A pivotal molecular mechanism in the circadian loop of the suprachiasmatic nuclei (SCN) is the alternating activation and inhibition of Period gene expression. To develop an assay for this, we have

established a procedure for Biolistic transfection of organotypic SCN slices. The 7.2 kb mPer1 promoter has been sub-cloned into plasmids encoding stable or destabilised, cyan (ECFP) or yellow (EYFP) fluorescent proteins. Plasmid-coated gold microparticles are shot at the slice after 2 weeks in culture. All transfected cells can be identified by the expression of CMV-driven mitochondrial-targetted FP, whilst cells with mPer1 activity are revealed by accumulation throughout the cell of stabilised ECFP or EYFP on the complementary wavelength. Transfection occurred in both neurons and glia, as evidenced by mitochondrial FP. SCN mPer1 activity was almost always in neurons, however, typically bipolar with small perikarya. Co-transfection with mPer1::ECFP and mPer1::ds2YFP allowed for real-time fluorescent imaging of mPer1 activity in individual neurons. The Vipr -/- mouse lacks a cohesive circadian activity cycle. mPer1 expression in the SCN, as evidenced by in situ hybridisation is basal throughout circadian time. Multiple periodicities in the activity-rest cycle suggest either an uncoupling between the SCN and other rhythmically active centres, or uncoupling within the SCN. Alternatively the molecular loop may be inactivated in Vipr -/- mice. To test this, WT and mutant SCN slices were co-transfected with CMV::mitoYFP and mPer1::ECFP and after 10 days expression of ECFP quantified. Both genotypes expressed mPer1::ECFP, with no statistically significant difference in either the frequency of labelled neurons, nor in the intensity of the signal. These results demonstrate that mPer1 activity is retained in the Vipr-/- SCN, and support the interpretation that loss of the VPAC2 receptor uncouples oscillatory SCN neurons.

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Circadian Changes of Mucin Glycosylation in Cells of the Intestinal Crypts of Gastrointestinal Tract in Mice

Andrzej Fiertak and Elzbieta Pyza* • Jagiellonian University, Institute of Zoology

Rythmicity characterizes most functions of the gastrointestinal tract. Significant time-dependent changes have been documented in motility, absorption rates, mucosal enzyme activities, gastric acid secretions, and proliferation of the gastrointestinal mucosa. Glycoconjugates (mucins), produced by different types of intestinal cells, are indicators of physiological changes in the gastrointestinal tract. Their terminal residues change depending on age and food ingested by an animal. Although, the circadian basis and influence of the clock on their expression is unknown. In the present study, adult (3 month old) C57BL mice were used. They were held in LD 12:12, DD or LL and killed at 9:00 hr or at 21:00 hr. Mice in the second (DD) and third (LL) groups were held in DD and LL, respectively for short (3 days) or long (5 days) time. For specific discrimination of glycoconjugates a set of FITC- and TRITC- conjugated lectins was used. In the Paneth's and goblet cells of the intestinal crypts the level of expression of b-D-galactose(1-3)-N-acetylgalactosamine, b-D- galactose (1-4)-N- acetylglucosamine, and b-N-acetylglucosamine was statistically higher at 9:00 hr than at 21:00 hr in mice from LD and DD groups and fed ad libitum. In LL this relation was reversed. There were no differences in glycoconjugate expression between animals held for short and long time in DD or LL. The acquired data showed that the level of mucin glycosylation is regulated by a circadian mechanism.

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Humoral Regulation of the Peripheral Vascular Clock

Dermot F Reilly and Garret A FitzGerald • University of Pennsylvania

The incidence of heart attack and stroke undergo diurnal variation, raising the possibility of interplay between circadian gene expression and external cues related to time of day, such as exercise, feeding, stress and posture. The master clock, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, is driven by transcriptional / translational feedback loops of the major components Per1-3, Cry1-2, Clock and Bmal1. However, most tissues express core clock genes and may serve to amplify or dampen central entrainment of peripheral behaviour. How the master clock in the SCN entrains / resets peripheral oscillators and how asynchronous environmental cues, such as exercise, modulate peripheral rhythms under circadian control is largely unknown. Previously we have shown how the hormone, 9-cis retinoic acid, can reset the vascular clock. We now examine whether humoral signals more directly implicated in cardiovascular function impinge on a vascular oscillator. Components of the circadian clock: Per 2, Per 1, Cry1, Cry2, Rev Erba, Dec1, Dec2 and Bmal1 show rhythmic mRNA expression patterns with a 24hr period following serum shock (t=0hrs) in human aortic vascular smooth muscle cells (hASMC). Putative circadian "output" genes: D site of albumin promoter binding protein (dbp) and plasminogen activator inhibitor (PAI-1) additionally show a rhythmic expression profile with a 24hr period. When angiotensin II (100nM) is added at t=16hrs (but not at t=8hrs or t=24hrs) after induction of rhythms by serum, a marginal phase delay of the circadian peaks in Per2 and dbp expression is observed. Catecholamine (Norepinephrine 10mM and epinephrine 10mM) treatment of serum starved (non cycling) hASMC leads to increased Per1 mRNA expression. Furthermore, when catecholamines are added at t=16hrs, (but not t=8hrs) after induction of rhythms by serum, Per1 expression increases prior to the circadian peak, but the amplitude of subsequent oscillations are dampened significantly. These results suggest increases in vasoactive hormones have distinct effects on this peripheral clock, suggesting that abrupt alterations in circulating hormones may influence circadian timing in vascular cells.

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Diurnal Expression of Clock Genes in Native Gonadotropin-releasing Hormone Neurons

Jason R. Hickok*, Mia E. Layne, Claire R. Zimmerman and Shelley A. Tischkau • University of Illinois

Precisely coordinated timing of neural and endocrine events is required for successful completion of female ovulatory cycles. Bilateral lesion of the hypothalamic suprachiasmatic nucleus (SCN), the site of the primary circadian clock in mammals, disrupts estrous cylicity leading to infertility. While the mechanisms by which the SCN controls ovulation are unknown, it is likely that a daily signal is transmitted from the SCN to neurons that express gonadotropin releasing hormone (GnRH). We hypothesize that GnRH neurons express circadian clock genes, and that rhythmic expression of these genes is driven by the daily signal originating in the SCN. We compared expression of Period1 (mPer1), mPer2, cryptochrome1 and 2 (mCry1-2), Bmal1, Clock (mClk) and timeless (mTim) in an immortalized cell line derived from embryonic GnRH neurons (GT1-7 cells) to expression patterns in an immortalized cell line derived from the rat SCN (SCN2.2 cells). Quantitative real-time PCR indicated a circadian rhythm in the profiles of mPer1, mPer2, mCry2 and mClk in SCN2.2 cells. In contrast, mPer1, mPer2, mCry2 and mClk displayed 2-3 peaks per day in GT1-7 cells. Serum shock induced a circadian pattern of expression in the

GT1-7 cells. GnRH-GFP mice were used to examine clock gene expression in native GnRH neurons. Colocalization of mPer1, mClk, mCry2 and mClk proteins with GFP observed in specific GnRH neurons was restricted to specific times of day, suggesting a circadian pattern of expression of clock genes in native GnRH neurons. These data are the first to reveal ultradian expression patterns of clock gene proteins in isolated GT1-7 cells and localization of clock genes in native GnRH neurons. The results suggest that clock genes express with ultradian periodicity when GnRH neurons are cultured in isolation. However, when given an appropriate signal, expression of clock genes in GnRH neurons changes to a circadian pattern of expression. Further studies will be directed toward examination of the mechanisms underlying SCN-directed function of GnRH neurons. (Supported by a Governor's Venture Technology Fund grant to S.A.T., a Jonathon Baldwin Turner Undergraduate Research Fellowship to C.R.Z. and a Pfizer Minority Undergraduate Research Award to M.E.L).

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Circadian Control of Drug Response: Mouse Sensitivity to Chemotherapeutic Drug Cyclophosphamide is Modulated by the Functional Status of CLOCK/BMAL1 Transactivation Complex

Victoria Yu. Gorbacheva[1], Roman V. Kondratov[1], Renliang Zhang[2], Andrei V. Gudkov[3], Joseph S. Takahashi¤[4], and Marina P. Antoch¤[1]* • Departments of [1]Cancer Biology and [3]Molecular Biology and [2]Mass Spectrometry Core facility, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH and [4]Howard Hughes Medical Institute, Department of Neurobiology and Physiology, Northwestern University, Evanston, IL

There have been numerous indications that the circadian clock can modulate tolerability and efficacy of anticancer therapy. Despite the large number of observations accumulated in the field of chronobiology indicating that variations in highest tolerable doses for many drugs may vary 2- to 8-fold depending on the time of administration, a chronotherapeutic approach has not become a routine clinical practice, perhaps due to the lack of a clear mechanistic rationale. To approach the molecular mechanisms linking circadian system function with responses to cancer therapy, we utilized several mouse models deficient in specific components of the circadian clock. Wild type and mutant mice were treated with a range of doses of a widely used chemotherapeutic drug, cyclophosphamide (CY), at different times of the day; the animals' sensitivity to CY was assessed by drug-induced mortality and body weight loss. First, we confirmed that the drug sensitivity of control C57Bl/6J mice indeed strongly depended on the time of its administration: animals treated at the time of dark-to-light transition (ZT22 – ZT02) were more sensitive to treatment as compared with mice injected at the time of light-to-dark transition (ZT10 – ZT14). Consistently, animals treated at ZT14 were able to tolerate significantly higher doses of CY. Second, we estimated the drug sensitivity of several circadian mutant mice. Interestingly, mice deficient in major circadian transcriptional activators (Clock/Clock mutant and Bmal1-/- knockout animals) were more sensitive to CY than control animals at all times tested. On the contrary, animals deficient in circadian transcriptional repressors (Cry1-/-Cry2-/- double knockout animals) were more resistant to CY therapy compared to their wild-type littermates. Thus, both time-of-day and genotype-dependent variations in response to drug therapy correlate with the functional status of the major circadian CLOCK/BMAL1 transactivation complex. Pharmacokinetic analysis of plasma concentration of different CY metabolites showed that, in contrast to the traditional view, the circadian variations in drug sensitivity cannot be explained by changes in the rates of CY metabolic activation and/or detoxification and likely involves circadian control of the survival of the target cells in tissues that determine viability of the treated organism.

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Expression of Clock Genes in the Hamster SCN During Development

Xiaodong Li* and Fred C. Davis • Northeastern University

Evidence to date indicates that a central transcription/translation feedback loop consisting of multiple clock genes is essential for circadian oscillations at cellular, tissue and organismal levels. We examined the developmental expression of three clock genes (bmal-1,cry1 and per1) in the Syrian hamster to assess rhythmicity in the suprachiasmatic nucleus (SCN) soon after it is formed, from about one day after the completion of neurogenesis to postanltal day 2. Transcript levels for all three genes were rhythmic in the adult SCN. Developmental samples were taken at the dam's circadian times 6, 12, and 18 daily over four days. Sections from each brain were processed in parallel for in situ hybridization using 35S-labeled rRNA probes. Bmal-1 was prominently expressed in the fetal SCN in contrast to per1 and cry1, which were only weakly expressed prenataly. Transcripts of all three genes showed relatively greater abundance just after pups were born. Subsequently, bmal-1 showed a significant decrease, while per1 continued to be greater than prenatal levels. Although some variation in transcript level for each gene was observed across collection times, no consistent circadian rhythm was detected prenatally. These results indicate that molecular oscillations equivalent to those in adults are not required in the developing SCN for the differentiation of an entrainable circadian pacemaker. In fact, an absence of robust oscillations during early SCN development may in part explain the dramatic phase-setting effects of pharmacological agents on the fetal/neonatal clock.

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BMAL1 Regulates Adipose Differentiation

*Shigeki Shimba and Masakatsu Tezuka • Department of Health Science, College of Pharmacy, Nihon University

Adipose differentiation is regulated by several transcription factors, such as a C/EBP family and PPARg2. In this study, we have studied the roles of the BMAL1 in adipogenesis. During adipose differentiation in 3T3-L1 cells, the level of the BMAL mRNA began to increase is highly expressed in differentiated cells. In white adipose tissues isolated from C57BL/6J mice, BMAL1 is predominantly expressed in[a]@adipocytes fraction compared with stromal-vascular fraction. Knock down of BMAL1 mRNA expression by RNAi technique allowed the 3T3-L1 cells to accumulate minimum amounts of lipid droplets in the cells. Consistent with the morphological observations, a minimum amount of adipocytes-related genes was induced in the BMAL 1 knock down cells. Adenovirus-mediated expression of BMAL1 in mature adipocytes induced several genes required for lipid metabolism. Then, we wished to examine if BMAL1 has the ability to promote adipogenesis in NIH 3T3 cells, a relatively nonadipogenic cell line. NIH 3T3 cells were stably transfected with a vector expressing high levels of full length BMAL1 mRNA, or a control vector. Overexpression of BMAL1 in NIH 3T3 cells induced significant amounts of lipids accumulation compared with the control cells by the treatment with a standard differentiation-induction medium containing and PPAR g2 ligands. These results suggest that BMAL1 plays important role in the regulation of adipose differentiation.

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Cyclic Steroid Exposure Modulates GnRH Secretion from Immortalized GT1-7 Cells

*P.E. Chappell, R.S. White, and P.L. Mellon • Department of Reproductive Medicine, University of California, San Diego, La Jolla, CA 92093-0674

Previous studies in rodents have shown that preovulatory gonadotropin-releasing hormone (GnRH) surges require two basic elements: High circulating ovarian estrogen (E2), and a daily neuronal signal, likely originating in the suprachiasmatic nucleus (SCN). In ovariectomized rodents treated with E2, GnRH surges occur on consecutive days with a circadian period. Whereas E2 influences afferent neuronal populations putatively involved in GnRH surge initiation, including the SCN, it is unclear exactly how steroids modulate GnRH secretion at the level of the GnRH neuron. Using immortalized GT1-7 cells, we investigated how varying durations and concentrations of steroids affect GnRH neurosecretion in vitro. GT1-7 cells were perifused with constitutively elevated (100pM) E2, or incrementally increasing concentrations of E2 commensurate with levels observed over the rodent estrous cycle. GT1-7 cells were treated sequentially with estrus, diestrus, and proestrus steroid regimens for 40 hours in 10-hour increments. GnRH radioimmunoassay of perifusates collected during the "proestrus" steroid treatment revealed dramatic (~5-fold) increases in baseline GnRH secretion and mean GnRH pulse amplitude compared to cells exposed to high E2 or ethanol. Late proestrus progesterone titers rapidly decreased GnRH secretion to basal levels. These results suggest that significant components of the GnRH surge mechanism may exist at the level of the GnRH neuron itself, and depend upon both duration and concentration of steroid exposure. Recent studies reveal a functional transcriptional circadian clock within GT1-7 cells that regulates GnRH secretion patterns. In combination, these results suggest a model by which steroids may influence this intracellular clock to modulate infradian reproductive neurosecretion.

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Regulation of Timeless and Mel1a Melatonin Receptor Expression in the Mouse Pars Tuberalis

Shirin Haddady*[1], Charlotte von Gall[2,3], Christopher M. Lambert[2], and David R. Weaver[2] • Departments of [1]Medicine and [2]Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605, and [3]Institute of Anatomy II, J.W Goethe-University, 60590 Frankfurt, Germany

High levels of both mTim gene expression and melatonin Mel1a [MT1] receptor gene expression and binding are present in the mouse pituitary pars tuberalis (PT). Mel1a receptors play an important role in regulating mPER1 in the PT (von Gall et al., Nat. Neurosci 2002). We have utilized genetically modified mice to determine whether there is a functional relationship between mTim and Mel1a receptor gene expression in the PT and suprachiasmatic nucleus (SCN). The mTim gene is necessary for development, and so we were limited to comparing mTim heterozygotes with wild-type mice. However, the level of mTIM protein was not consistently reduced in the PT of mTim+/- mice, relative to wild-type mice of the same (sv129) genetic background. Melatonin receptor binding in the SCN and PT was unaffected by mTim genotype, although the level of Mel1a gene expression in the PT (but not SCN) was reduced in mTim+/- mice. The lack of consistent gene-dosage effect on mTIM levels precludes drawing firm conclusions regarding the role of mTIM in melatonin receptor regulation. Other experiments assessed the impact of melatonin receptor disruption on mTim gene expression and mTIM immunoreactivity.

Mice homozygous for disruption of the Mel1a melatonin receptor gene lacked detectable 2-[125I]-iodomelatonin binding, and had reduced levels of mTim RNA and mTIM protein in the PT, relative to wild-type mice of the same melatonin-producing (C3H) background. mTim/mTIM was unaffected in the SCN. Thus, Mel1a melatonin receptors in the PT influence mTim gene expression and mTIM protein levels, in addition to mPer1/mPER1.

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Stress Response Influences Recovery from Jetlag in the Octodon Degus

Jennifer A. Mohawk* & Theresa M. Lee • University of Michigan

Jetlag can result in an array of detrimental symptoms for the affected individual, making rapid reentrainment of rhythmicity essential. We find that circadian recovery is altered by stress in the diurnal rodent, Octodon degus. In the first experiment, animals were subjected to a 6h phase-advance of the light: dark (LD) cycle. Sixty minutes after the new lights-on, animals underwent 60min of restraint stress. The number of days it took each animal to reentrain its activity rhythms to the new LD cycle was recorded and compared to the number of days it took the animal to reentrain under baseline conditions. Stress significantly delayed the reentrainment of activity rhythms ($p<0.05$). In a second experiment, degus' stress response was suppressed using the glucocorticoid synthesis inhibitor metyrapone. Degus underwent a 6h phase-advance of the LD cycle and were injected with metyrapone for the first five days following the phase shift. The number of days it took each animal to reentrain its rhythms when injected with metyrapone was compared to the number of days it took the animal to reentrain under baseline conditions. Suppression of cortisol synthesis by metyrapone accelerated the reentrainment of activity rhythms in the degu ($p<0.05$). A third experiment demonstrated a positive correlation between cortisol response to a stressor and days to reentrain following a 6h phase advance ($r=0.94$, $p<0.10$). These experiments support the hypothesis that glucocorticoid activation slows recovery from a photic phase shift.

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The Regulation of Long-term Potentiation by Melatonin

L.M. Wang*, D. Chaudhury, D.R. Weaver, C.S. Colwell • Dept. of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, CA, USA

Melatonin is a rhythmically synthesized hormone that has been suggested to function as an output molecule by which the circadian system may transmit temporal information. Melatonin mediates its effects via high-affinity receptors found throughout the nervous system including the hippocampus. This raises the possibility that melatonin in regulates the cellular and molecular properties of hippocampal neurons. The goal of the present study was to characterize the effects of melatonin on long-term potentiation (LTP), a cellular model of hippocampal learning and memory formation. To achieve this, we performed electrophysiological field recordings in the CA1 region of hippocampal brain slices from C3H and C57 mice. Treatment with melatonin dramatically reduced both the magnitude and duration of potentiation induced by a weak-tetanizing protocol. This effect of melatonin was concentration dependent with an EC50 of 100nM. Melatonin failed to inhibit LTP in MT2 receptor KO mice yet had normal effects in MT1 deficient mice. Treatment with melatonin after tetanus also reduced the amplitude of the evoked responses. By itself, melatonin did not interfere with amplitude or duration of the evoked fEPSCs. Finally, a comparison was made between hippocampal slices prepared during the day and those prepared during

the night. Melatonin produced a greater inhibition of LTP during the day than during the night. These findings suggest that melatonin is a circadian modulator of cellular properties in the mouse hippocampus and may potentially act on behalf of the circadian system to regulate learning and memory. Supported by NIH NS43169

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Light Pulse Induced Heme and Iron Associated Transcripts in Mouse Brain—A Microarray Analysis

R. Ben-Shlomo*, R.A. Akhtar, B.H. Collins and C.P. Kyriacou • University of Haifa - Oranim

Synchronization of circadian oscillators with the outside world is achieved because light has acute effects on the levels of one or more of the clock's components. In mammals the PAS transcription factors Clock, NPAS2 and Bmal1 regulates gene expression as a function of the day-night cycle. Both PAS domains of NPAS2 were found to bind heme as a prosthetic group, to form a gas-regulated sensor. The heme status controlled DNA binding in vitro. In a microarray analysis comparing changes in transcripts levels between mice subjected to light pulses during the dark phase with animals maintained in darkness, we traced consistent changes in more than 200 different transcripts. Among them, 20 are associated with heme and iron biosynthesis and catabolism. A model for the pathway of induction of heme and iron homeostasis-related transcripts resulting from light pulses suggest that light signal (as a stressor) induces transcription of heme oxygenase 2 (Hmox2) and Cytochrome P450 oxidoreductase (Por) which may serve as a primary line of cellular defense. HMOX2 protein degrades heme from proteins such as hemoglobin. This degradation generates CO – a signal molecule, and may also: change the redox state of the cell by reducing the NADPH/NADP ratio. This could lead to up-regulation of transcription of globin genes, thereby releasing iron that controls production of ferritins, and further up-regulating aminolevulinate synthase 2 (Alas2).

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Periods of Sensitivity for Melatonin-mediated Phase Shift of Circadian Activity Rhythms and for Melatonin-Promoted Immobility in the Rhesus Monkey

Margarita L. Dubocovich, Tracy D. Brinkman, Irina V. Zhdanova, William T. McKinney • Northwestern University Feinberg School of Medicine

This study assessed the periods of sensitivity for melatonin-induced phase shift of circadian rhythms and immobility in a diurnal mammal, the Rhesus monkey (Macaca mulata). Changes in circadian phase and mobility were assessed by actigraphic recordings of motor activity. Rhesus monkeys (6 male, 10 female; 11-12 years old) were housed in constant dim light (5-12 lux). Treatments [placebo (1 ul/kg ethanol) or melatonin (30 ug/kg)] were given orally in apple juice for 3 consecutive days. Melatonin, given at CT 8.5-11.5 induced a significant phase advance (1.1 ± 0.1 h, n=16) of motor activity onset compared with placebo (0.02 ± 0.04, n=12; $p < 0.001$). By contrast, melatonin given at CT 0.5-3.5 significantly phase delayed (-0.4 + 0.09, n=16) motor activity onset compared with placebo (0.2 + 0.08, n=14; $p < 0.001$). The effect of melatonin on mobility was assessed in monkeys kept in a 14/10 L/D cycle with dim lighting (5-12 lux) during the light cycle. Melatonin given at ZT 9 significantly increased immobility during the second hour after treatment [83.3 + 2.8 %, for melatonin vs. 67.0 + 4.7 %, for placebo, n=14; $p < 0.01$]. By contrast melatonin given at ZT 2 did not significantly affect immobility. We conclude that in the Rhesus

monkey melatonin phase shifts circadian activity rhythms following a phase response curve similar to that described in humans. However, under dim light the effect of melatonin on immobility was restricted to dusk. Supported by MH 63466 to MLD.

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Light Exposure in Morning-type and Evening-type Individuals

Marie Dumont*, Valerie Mongrain, Genevi ve Goulet and Catherine Desrosiers • Chronobiology Laboratory, Sacre-Coeur Hospital & University of Montreal, Montreal (QC), Canada, H4J 1C5

Morning types (M-types) show earlier sleep schedules and earlier diurnal peaks of alertness compared to evening types (E-types). They also have an earlier circadian phase. Since light is the main synchronizer to the 24-h day/night cycle, the present study measured the profile of light exposure in relation to morningness-eveningness. Light exposure and activity were recorded 24-h/day for 7 days in 12 M-type and 12 E-type subjects (19-34 y.). Subjects maintained a regular sleep schedule individually determined according to their preferred bedtime and wake time. After the week of ambulatory recording, core body temperature and salivary melatonin were measured in the laboratory to estimate circadian phase. Final analyses included 9 M-types (4 men and 5 women) and 10 E-types (5 men and 5 women). Compared to E-types, M-types had significantly earlier bedtimes, wake times and circadian phases, but there was no difference for sleep duration. Results showed that M-types were exposed for a longer time to light intensity greater than 1000 lux ($p<0.05$). Group-by-Hour ANOVAs on log lux indicated a significant interaction ($p<0.001$), showing that M-types were exposed to more light in the morning (between 8 and 11 am) than E-types. Conversely, the quantity of light received between 11 pm and 3 am was greater in E-types than in M-types. These results show that M-types are exposed to more bright light than E-types, mainly in the morning. This is consistent with previous laboratory experiments showing that light exposure in the morning produces a phase advance of circadian rhythms. Research supported by CIHR (MD) and NSERC (VM & CD).

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Effects of Pinealectomy on Circadian Entrainment Responses to Skeleton Photoperiods

Jeffrey A. Elliott*, Jennifer A. Evans and Michael R. Gorman • Departments of Psychiatry and Psychology, University of California, San Diego, La Jolla, CA 92093-0667

In mammals photoperiod mediated through the pineal gland regulates seasonal adaptations, yet the role of the pineal in photoperiodic regulation of circadian responses has received relatively little attention. Our previous work in Syrian hamsters shows that entrainment to short photoperiod (SD) increases the size of light induced phase-shifts in free-running rhythms, an effect associated with increased activity time (a). This study examined the influence of pinealectomy on circadian responses to skeleton photoperiods. Male hamsters were pinealectomized (Pinx) or sham-pinealectomized (Sham) and entrained for 4 weeks to a SD skeleton (with dim < 0.1 lx scotophases). A control group was entrained to a long-day skeleton (LD-Sham). Circadian wheel running rhythms were studied during entrainment and during subsequent free-run in dim LL. Entrainment in SD-Sham was characterized by decreased wheel counts, decreased rhythm amplitude (periodogram power), and increased a. All of these SD effects were inhibited by pinealectomy as

verified by BŸhlmann/ALPCO RIA of blood melatonin (samples collected at CT19-20). Phase shifts to light (0.25 h) at CT 14 were enhanced in SD-Pinx and SD-Sham compared to LD-Sham, suggesting that a long duration melatonin signal is not required for this SD effect. A key question to address is whether all of the above pineal effects are gonadally mediated. [Supported by PHS grants HD-36460 and NS 30235].

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Circadian Entrainment and Phase Resetting Differ Markedly under Dimly Illuminated versus Completely Dark Nights

Jennifer A. Evans*, Jeffrey A. Elliott, Michael R. Gorman • Departments of Psychology and Psychiatry, University of California, San Diego

Dim nocturnal illumination, comparable to dim moonlight (0.02 lux) and below established photic thresholds for inducing phase shifts and melatonin suppression, exerts a marked effect on circadian entrainment in the nocturnal hamster. The proportion of animals exhibiting bimodal (i.e. split) activity rhythms is increased by 300% when 24 h light:dark:light:dark cycles incorporate dimly illuminated rather than completely dark scotophases. The present studies assess whether this novel action of light reflects changes in activity levels, nonphotic phase resetting or photic entrainment. Experiment 1 replicated our previous result and demonstrated that dim illumination increases splitting incidence independent of activity levels. In Experiment 2, transfer to running wheels during subjective day elicited comparable novelty-induced activity across groups (1100 ± 531 revolutions/ h). After entrainment to LD 14:10, dim- and dark-exposed hamsters were not differentially phase shifted, whereas significantly greater phase advances were exhibited by dim- versus dark-exposed animals previously entrained to LD 19:5 (3.5 ± 0.7 h versus 0.6 ± 0.07 h, respectively). Under Experiment 3, we exposed hamsters to a series of skeleton photoperiods simulating increasing daylengths and noted when activity "phase jumped" across light pulses. Dim illumination accelerated this response; dim-exposed animals phase-jumped under longer nights than their dark cohorts. Collectively, these results demonstrate diverse effects on entrainment under dimly lit versus completely dark nights, interpreted here in terms of circadian coupling. Properties of the circadian pacemaker may be best studied in the context of dim nocturnal illumination, which more closely simulates the ecological conditions of nocturnal animals.

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The Effects of Altering Serotonin Levels in the Re-entrainment to an Advanced Light Dark Cycle

M. Gardani* and S. M. Biello

Serotonin (5-HT) is a neurotransmitter that mediates a wide range of behavioural and physiological processes and is important in the regulation of the mammalian circadian clock function. Evidence shows the involvement of 5-HT in the re-entrainment of the endogenous clock to a new light dark cycle. MDMA (ŒEcstasy,), which is widely used as a recreational drug of abuse, is a selective serotonin neurotoxin in animals and non-human primates. Previous work has shown that MDMA exposure can alter the circadian clock both in vitro (Biello and Dafters, 2001) and in vivo (Colbron et al, 2002). Untreated animals (n=16) were successfully entrained to a new, 6 hours advanced, light dark cycle within an average of 4.5 ± 0.1 days. Following treatment with MDMA these animals took an average of 8.3 ± 0.1 days to

re-entrain to the shifted environmental cycle. Furthermore, a slower re-entrainment to an advance in the light dark cycle was shown when effectively decreasing levels of serotonin in the circadian clock by using serotonin antagonists. Administration of metergoline (2 mg/kg), which is a non-selective serotonin antagonist to hamsters (n=12), led to a slower re-entrainment to the new light dark cycle (average number of days 6.4+0.8) compare to controls (4.4+0.5). In addition, the selective 5-HT7 receptor antagonist SB-269970 (3mg/kg) attenuated the re-synchronisation to a light dark cycle advanced by 6 hours. Animals synchronised to the new light dark schedule with an average of 6.6 + 0.4 days whereas control animals reached the new cycle within 3.9 + 0.2 days. These results demonstrate the importance of the serotonergic system in the adjustment of the circadian clock to daily and seasonal environmental changes, and implicate the 5-HT7 receptor subtype in at least some of these changes.

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Effects of DMH Lesions on Circadian Entrainment by Restricted Feeding Cycles

Joshua Gooley* and Clifford Saper • Harvard Medical School

Daily restricted meals can entrain circadian rhythms of behavior, body temperature, corticosteroid secretion, and clock gene expression in peripheral tissues. However, the pathways that mediate circadian entrainment by restricted feeding have not been determined. The dorsomedial hypothalamic nucleus (DMH), which receives direct input from the suprachiasmatic nucleus (SCN) and indirect input from the SCN via the subparaventricular zone (SPZ), is critical for the circadian rhythms of sleep-wake, locomotor activity, and plasma corticosteroids. Previously characterized feeding pathways have access to the SPZ and DMH, suggesting that food intake might regulate circadian output in hypothalamic structures downstream of the SCN. To test the hypothesis that restricted feeding entrains circadian rhythms by altering the rhythmic output of the DMH, we are examining the ability of DMH-lesioned rats to entrain to daily meals. In a preliminary set of experiments, large cell-specific lesions were placed in the DMH region using ibotenic acid. DMH-lesioned rats that were given a daily 4-hr restricted meal in the center of the daytime (12:12 light-dark cycle) showed marked deficits in their ability to anticipate the daily meal. Compared to sham-lesioned rats, DMH-lesioned rats showed an 80% reduction in food anticipatory activity in the 3-hr interval preceding the daily meal and failed to show a preprandial rise in body temperature. Also, food restriction-induced phase shifts in body temperature were reduced by 80% in DMH-lesioned rats compared to sham-lesioned animals. We are currently investigating the effects of smaller lesions restricted to the DMH versus lesions in surrounding brain regions.

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Nocturnal Illumination below the Intensity of Moonlight Facilitates Entrainment of Hamsters to Extremely Long T-cycles

Michael R. Gorman*[1], Magdalena E. Kendall[1] and Jeffrey A. Elliott[2]. • Departments of [1]Psychology and [2]Psychiatry, University of California, San Diego, 92093-0109

Our laboratory has recently demonstrated that nighttime illumination (< 0.1 lux) below the threshold necessary to induce phase-resetting or to acutely suppress melatonin secretion nevertheless exerts marked effects on circadian entrainment of hamsters. Here we show that this dim nocturnal illumination greatly facilitates entrainment to extremely long T-cycles. Circadian theory predicts that entrainment fails when the zeitgeber period exceeds the sum of the period of the free-running rhythm and the maximum phase

delay elicited by the zeitgeber. In hamsters held previously in standard long daylengths, brief light pulses elicit a maximum phase delay of 1-2 h, accounting for failed entrainment around a zeitgeber period (T-cycle) of 26 h. Larger phase-shifts, however, can be obtained from hamsters with short-day photoperiod histories, and we showed in Siberian hamsters that short-day entrainment is facilitated under dimly lit versus completely dark scotophases. In the present experiment, we exposed male Syrian hamsters to light cycles lengthening gradually from 24 h to 30 h over ~4 months. Groups differed only in the intensity of scotophase illumination (~ 0.01 lux versus complete darkness). Only 1 of 18 hamsters exposed to completely dark nights entrained to T-cycles > 26 h, whereas a majority of dim-exposed hamsters entrained to T-cycles of 28 h or longer. At the conclusion of the experiment, activity duration in constant conditions was greater for dim- versus dark-exposed hamsters. Dim nocturnal illumination may influence the relative phasing of component circadian oscillators and thereby alter the light pulse PRC to facilitate entrainment to long T-cycles.

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Involvement of Per1 and Per2 Induction in Photic Entrainment of the Circadian Clock

Naoto Hayasaka*, Mamoru Nagano and Yasufumi Shigeyoshi • Department of Anatomy and Neurobiology, Kinki University School of Medicine

Daily photic resetting of a biological clock is one of the fundamental characteristics of the circadian systems to adjust to the environment. Light-induced phase shift is observed exclusively during the subjective night, which coincides with the time when Per1 and Per2 genes are induced by light. To investigate whether induction of the Per genes expression is involved in the photic entrainment, we performed in situ hybridization on the two Per genes in the rat and mouse SCN. We first examined Per1 and Per2 expression in the LD and DD conditions every two hours for 24 hours. At ZT/CT2, Per1 and Per2 were expressed in the ventrolateral portion of the SCN (VLSCN) in LD but not in DD. In addition, different expression patterns of Per1 and Per2 were observed at dawn between LD and DD in the VLSCN, while the dorsomedial portion of the SCN (DMSCN) showed quite similar expression profiles both in LD and DD. In LD, the onset of the Per1 and Per2 genes expression in the VLSCN was first observed at ZT0.5, while VLSCN did not express Per1 or Per2 until CT2 in DD. Therefore, it is likely that the expression of Per1 and Per2 during the early daytime is caused by exposure to light during subjective night. These data suggest the involvement of the Per genes in the daily photic entrainment. This also implies that light exposure both at dawn and dusk contributes to the daily adjustment of the internal clocks to the environment.

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Dissecting the C-terminal Domain of Cryptochrome by Analysis of Drosophila/Mouse Chimerics

Matthew Hemsley[1], Charalambos P Kyriacou[2], Ezio Rosato[1] • [1]Department of Biology, [2]Department of Genetics, University of Leicester, UK

In Drosophila melanogaster, the photopigment CRYPTOCHROME (dCRY) provides a dedicated pathway, which entrains the circadian clock. We have previously shown, using a yeast-two-hybrid assay, that

dCRY interacts with TIMELESS (TIM) and also with PERIOD (PER), in a light-dependent manner. However, on removal of the C-terminal domain, dCRYD binds PER and TIM both under light and dark conditions in yeast. Furthermore, dCRYD confers a constitutive light response when overexpressed in flies. In order to further investigate the role of the C-terminal domain (CT) in cryptochrome signalling, we have generated several chimeric constructs that swap different portions of the CT domains between dCRY and mouse CRYPTOCHROME 1 (mCRY1). mCRY1 has close homology with dCRY, except for the CT, which is unique, and it is not light regulated. The chimeric constructs were tested for their interactions with PER and TIM in light and darkness in a yeast system. Our results suggest that, in both dCRY and mCRY1, a region immediately upstream to the CT is essential for PER/TIM interactions. They also identify a region, within the CT, that is likely the main requirement for light-mediated repression of dCRY.

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Body Weight and Nonphotic Circadian Clock Resetting in Hamsters

Nicholas Cartwright*, Jeral Dennis, Daniel Janik • Biology Department, University of Wisconsin-Eau Claire, Eau Claire, WI 54702

Hamsters awakened in the day and allowed to exercise in a novel running wheel reset their circadian clock by about 3 hr if they run vigorously. About 50% of hamsters show this response. Previous work has shown that food restriction boosts the total amount of activity that hamsters show. We tested the idea that food restriction would lead to more running in a novel wheel and therefore more clock resetting. We selected animals that, under ad-lib feeding, were shown to be poor runners and resetters. When these animals were food restricted for 24 hr, they showed no increase in resetting, but did show an increased after 48 hr of food restriction. We further asked whether increased food intake decreases the amount of running and resetting shown by hamsters that were good runners and resetters under ad-lib feeding conditions. To do this, we fed these hamsters a high calorie diet with a variety of foods that changed on a daily basis. This increased body weight by 10% over that of controls, but there was no decrease in running and resetting. These results suggest that the change in body weight associated with food restriction is not the critical factor increasing running and clock resetting. Supported by NSF 9808866.

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LL-induced Potentiation of Phase Shifts in Syrian Hamsters: Probing Pathway Specificity Using NMDA

Glenn Landry* and Ralph Mistlberger • Simon Fraser University

Phase-response curves for drugs and other stimuli are typically obtained from organisms maintained in constant dark or dim light for several weeks. An alternative approach is to apply stimuli after only 1 or 2 days of constant conditions, which may more closely simulate the natural light exposure patterns of semifossorial rodents, or the 'unnatural' light exposure experienced by humans during transmeridian jet travel, shift rotations or extended duty hours. Recent studies of Syrian hamsters show that a single day of exposure to constant light (LL) can significantly potentiate phase shifts induced by non-photic stimuli, including exercise, 8-OH-DPAT and NPY (Knoch et al, Euro J Neurosci, in press). Potentiation could reflect changes in specific input pathways (e.g., for nonphotic stimuli) to the circadian pacemaker, or changes in a pacemaker parameter, such as its amplitude of oscillation. If a core parameter is involved, then responses to photic inputs (glutamate-mediated) may also be potentiated. To test this, Syrian hamsters

with peri-SCN cannulaes received the glutamate agonist NMDA (200 nl, 10mM) at CT13.5 after 0 or 2 days of LL. Lights were then turned off for several days (modified Aschoff Type II procedure). NMDA induced photic-like delay shifts that were not significantly different between the two lighting conditions. These results suggest that potentiation of phase shifts by LL may be specific to non-photic input pathways. Supported by NSERC.

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Pituitary Adenylate Cyclase-activating Peptide Plays a Time-dependent Role in Light-induced Phase Shifts

*Peder Lindberg, Carol Hamelink, Ruslan Damadzic, Lee Eiden, and Martha Gillette • University of Illinois at Urbana/Champaign

Pituitary adenylate cyclase-activating peptide (PACAP) likely plays a role in the normal resetting of the mammalian circadian clock. It is co-stored with glutamate 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 was monitored under 12:12 light-dark (LD), in constant darkness (DD), and after light pulses. Additionally, the responses of neural activity rhythms in slices to glutamate were measured in both early and late night. Our findings suggest that the interaction of PACAP with light- and glutamate-activated pathways is time-sensitive. This, in contrast to some previous results, suggests that the effect of PACAP may be more complicated than a simple augmentation of some glutamate-activated effects. This study was funded by NIH grants GM07143 (PTL), and #NS22155 (MUG).

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Immune-circadian Cross-talk: Are Astrocytes Involved?

Luciano Marpegan*, Maria Juliana Leone, Tristan Bekinschtein, Monica Costas and Diego A. Golombek • Departamento de Ciencia y Tecnologia, Universidad Nacional de Quilmes, Buenos Aires, Argentina. dgolombek@unq.edu.ar

The circadian system modulates immune variables, which show daily and circadian rhythms. We hypothesize that there is a feedback loop from these immune variables into the circadian system involving classical proinflammatory mediators. This pathway is evidenced by the photic-like PRC induced by bacterial lipopolysaccharide (LPS, 25 mg/kg) in mice. LPS induced phase delays at CT15 (-43±10 min) which showed no additive effects when coadministered with light pulses. Similar delays are observed after i.c.v. administration of IL-1 (30ng/kg) and TNFa (100 ng/kg) at CT15. Sulfasalazine (120 mg/kg), a specific NF-kB inhibitor, blocked LPS-induced phase shifts (-15±13 min). The SCN are enriched in astroglial cells and show high GFAP immunoreactivity (GFAPir), an astrocyte-specific marker. Astrocytes might regulate the inputs into the SCN and act as coupling agents for neuronal activity. Since astrocytes express (and respond to) cytokines, we studied their role as mediators of immune inputs into the central pacemaker. GFAP and NF-kB were colocalized in the SCN slices and in SCN primary glial cell cultures. GFAPir showed higher levels at ZT3 and a trough at ZT21 while no variations were observed for NF-kB. The ability of cultured astrocytes to respond to immune challenges was evaluated measuring the luciferase

activity in glial cultures transfected with a kB-promoter upstream of a luciferase gene. SCN astrocytes responded to LPS, IL-1 and TNFa by activating NF-kB/luc transcription. In summary, immune factors might help in fine-tuning SCN entraining, and this interaction is at least partially mediated by glia.
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Dark Pulses Phase Shift Mouse Behavioural Rhythms

Oliver Marston* and Hugh Piggins • University of Manchester

The phase-shifting actions of light pulses on the behavioural rhythms of mice free-running in constant darkness are well-established. In contrast, responses of the murine circadian system to pulses of darkness on a background on constant light are much less studied. In this investigation, we determined the resetting effects of moderate (2h) or long (6h) duration pulses of darkness on the wheel-running rhythms of adult male C57BL/6J mice (Harlan, UK) in constant light. Dark pulses of either duration evoked significant phase advances when given during the middle to late subjective day/early subjective night, and had no significant resetting effects at other circadian phases. Larger phase advances (~7h) were elicited during the middle to late day by the 6h pulses as compared to the 2h pulses (~1.8h). With 6h pulses beginning at circadian time (CT) 3.5-6.5, there was a significant correlation ($p<0.05$) between wheel-running activity induced by the pulse and the magnitude of the resultant phase shift. These results indicate that murine behavioural responses to dark pulses are similar in many respects to those reported in the Syrian hamster (Boulos & Rusak, 1982; Ellis et al., 1982). Further, induced locomotor activity may be a factor in determining the resetting actions of dark pulses on the mouse circadian system.

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Entrainment of Locomotor Activity and c-Fos Expression by Daily Consumption of a Highly Palatable Meal Without Food Deprivation

Jorge Mendoza *, Manuel Angeles and Carolina Escobar • Departamento de Anatom'a, Facultad de Medicina UNAM, Mexico D.F.

Rats maintained under restricted feeding schedules (RFS) show anticipatory activity (AA) and entrainment of physiological parameters dependent of a food-entrainable oscillator (FEO). Previous evidence has suggested that food entrainment can only be induced when RFS leads animals towards a catabolic state. However, associated with metabolic adjustments, rats develop a motivational drive during AA. In this study we determined the relevance of the motivational drive for food entrainment in absence of a catabolic state. We evaluated whether daily consumption of a highly palatable meal entrains metabolism and behavior without food deprivation. Locomotor activity, glucose (GL) and free fatty acids (FFA) were evaluated in rats maintained in LD 12:12 cycle and free access to water and their regular diet. 5g of chocolate were given daily for 3 weeks at ZT6. Rats showed AA to the palatable meal; however, GL and FFA were not entrained. AA persisted for 4 cycles after interruption of the daily palatable meal, confirming the involvement of a circadian oscillator. c-Fos expression was increased during AA in nuclei of the limbic system and in the hypothalamus. These results suggest that for the expression of FEO a catabolic state is not necessary and that an increase in motivational state by the reward properties of food entrain the FEO.
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Binocular Interactions in the Entrainment and Phase-shifting of Locomotor Activity Rhythms in Syrian Hamsters (Mesocricetus Auratus)

Jin Ho Park*, Matthew J. Paul, Matthew P. Butler, and Irving Zucker • University of California

Binocular interactions in entrainment of circadian rhythms were evaluated in Syrian hamsters maintained in constant light (LL) and subjected to one of four schedules of eye occlusion with opaque contact lenses. In separate groups the opaque lens was inserted into the left or right eye for 12h at the same clock time each day. In additional groups the left and right eyes were alternately occluded for 12h each day, with initial occlusion of the left eye for one group (LR) and the right eye for the other (RL). A majority of hamsters entrained their locomotor activity rhythm when one eye was occluded for 12h. Locomotor rhythms of most animals in the LR and RL groups free-ran for several weeks before entraining with a period of 24h; phase angles of entrainment were unrelated to which eye was initially occluded. A subset of animals generated split locomotor activity rhythms. The artificial light/dark-cycle (LD) imposed on one eye is sufficient to entrain the circadian rhythm of locomotion, even though the other eye was in constant light. Central summation of total luminous flux through the two eyes is implicated in entrainment. The free-running rhythms observed for several weeks in the LR and RL groups indicate that despite the LD cycle imposed on each eye, the central pacemaker is receiving the equivalent of a constant light signal. We speculate that entrainment eventually occurs via interactions between photic and non-photic signals when time of activity onset coincides with time of lensing.

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Feeding Schedule Controls Circadian Timing of Daily Torpor in SCN-ablated Siberian Hamsters

*Matthew J. Paul, Alexander S. Kauffman, and Irving Zucker • Departments of Psychology and Integrative Biology, University of California at Berkeley

Timing of daily torpor was assessed in suprachiasmatic nucleus-ablated (SCNx) and sham-ablated Siberian hamsters fed restricted amounts of food each day either in the light or dark phase of a 14:10 light-dark cycle. Eighty-five percent of sham-ablated and 45% of SCNx hamsters displayed a preferred hour for torpor onset. In each group, time of torpor onset was not random but occurred at a mean hour that differed significantly from chance. Time of food presentation almost completely accounted for the timing of torpor onset in SCNx animals and significantly affected timing of this behavior in intact hamsters. These results suggest that the circadian pacemaker in the SCN controls the time of torpor onset indirectly by affecting timing of food intake, rather than by, or in addition to, direct neural and humoral outputs to relevant target tissues.

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The NMDA Antagonist MK-801 Mimics the Circadian Phase-Shifting Effects of Dark Pulses in Syrian Hamsters

Alan M. Rosenwasser*, Ryan W. Logan, and Matthew E. Fecteau • Department of Psychology, University of Maine, 5742 Little Hall, Orono, ME 04469

The mammalian circadian pacemaker can be phase shifted by a variety of photic and non-photic stimuli, including brief pulses of darkness presented against a constant-light (LL) background. Dark-pulse-induced circadian phase shifting has been interpreted to reflect either a mirror-image photic phase-shifting mechanism, or as non-photic, arousal-dependent phase shifting. Indeed, recent studies from our lab indicate that both photic and non-photic mechanisms contribute to dark-pulse-induced circadian phase shifting in the hamster (Dwyer and Rosenwasser, 2000; Rosenwasser and Dwyer, 2001, 2002). Since the phase shifting effects of light pulses are blocked by the non-competitive NMDA receptor antagonist, MK-801, the present study examined the effects of MK-801 (5 mg/kg, i.p.), either alone or in combination with a 15-min dark pulse, presented at the beginning of subjective night to hamsters free-running in LL (100 lux). At this phase, MK-801 mimicked the phase-advancing effect of a dark pulse, probably by reducing ongoing photic signaling to the circadian pacemaker. Surprisingly, however, combined presentation of MK-801 and a dark pulse resulted in a larger phase advance that appeared to reflect additive effects of the two phase-shifting stimuli. These results indicate that pharmacologic blockade of NMDA receptor-mediated glutamate transmission under LL conditions can evoke circadian phase shifting via an apparent photic mirror-image mechanism. On the other hand, for the stimulus parameters used in this study, neither stimulus in of itself appeared to saturate this mechanism.

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Circadian Phenotype in Mice Mutant for mPer1, mPer2, mCry1 and mCry2: Phase Resetting in Entrainment and Freerun; Period Length, Rhythmicity and Activity in Constant Light

K.Spoelstra*, U.Albrecht, G.T.J.van der Horst and S.Daan • Zoological Laboratory, University of Groningen, the Netherlands

Mutations in each of the genes mPer1, mPer2, mCry1 and mCry2 separately cause the circadian system to deviate from that in wildtype mice, either in period length in constant darkness (DD) or in circadian phase resetting in response to brief light pulses. The phase resetting properties of the circadian system in mice with a functional deletion in any of these genes were studied in entrainment by exposure to brief light pulses in the subjective morning and subjective evening (LD, Aschoff type II protocol); and a full PRC was obtained in freerun (DD, Aschoff type I protocol). Circadian phase shifts were derived from a fully automated and objective calculation procedure. A further characterization of circadian phenotype by measuring period length, rhythmicity and activity levels was made in constant photic conditions (LL) with different light intensity. Functional deletions of mPer2 and mCry2 cause more acceleration and less deceleration of the first circadian cycle in response to brief light pulses than deletions in mPer1 and mCry1, respectively. Constant light of increasing intensity decelerates the freerunning circadian rhythm in mPer1, mCry1, mCry2 mutant mice, but accelerates mPer2 mutants. Simultaneously, such light induces rhythmicity in mPer2 mutants, and reduces it in the other strains. This study was supported by BRAINTIME (EC QLG3-CT-2002-01829)

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Responses of KaiC Protein and kaiBC mRNA to a Phase-Shifting Dark Pulse in Cyanobacteria

Jun Tomita*, Hideo Iwasaki and Takao Kondo • Division of Biological Science, Graduate School of Science, Nagoya University and CREST, JST, Nagoya 464-8602, Japan

The circadian clock can be reset by a several-hour dark-pulse in the cyanobacterium, Synechococcus elongatus PCC 7942. The phase of the rhythm is also reset by transient overinduction of the KaiC clock protein that has been proposed to function mainly in a negative feedback process of the basic oscillation. The levels of accumulation and phosphorylation of KaiC show circadian cycling peaking at early subjective night. To understand the resetting mechanism, the effects of a 5-hour dark pulse on the KaiC and kaiBC mRNA levels were examined. The KaiC accumulation and phosphorylation levels were changed by a dark pulse at CT 7.5-12.5 which altered the phase of kaiBC promoter rhythm. In contrast, they were not affected by a dark pulse at CT 13.5-18.5 when the dark pulse did not shift the phase of the rhythm. The levels of kaiBC mRNA were reduced during the dark pulses at both timings. When cells were released from the 5-hour darkness to LL, kaiBC mRNA was induced within 30 min. The magnitude of this kaiBC induction was negatively correlated with the KaiC amount at the end of the dark pulse, which was consistent with the negative regulation of KaiC. A possible correlation between these KaiC accumulation/phosphorylation and the phase shifting will be discussed.

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Effects of Long-term Exposure to Constant Dim Light on the Circadian System of Rats

Chiaki Fukuhara* & Gianluca Tosini • Neuroscience Institute, Morehouse School of Medicine, Atlanta GA 30310

One the most important aspect of space flight is the absence of geophysical 24-h cycles. In the case of long permanence in space, it is crucial to understand how the whole circadian system would react and behave in such circumstances. In the present study, we investigated the effects of long-term exposure to constant dim red light on the patterns of expression of Period1, Periodd2, and Arylalkylamine N-acetyltranserase (Aa-Nat). Male wistar rats were maintained in 12h-12h light dark cycles (LD), or in constant dim red light for 60 days (CDL). According to running wheel activity records, the animals were sacrificed at 4 hour-intervals, and the suprachiasmatic nucleus (SCN), pineal, retina, heart, skeletal muscles, and liver were collected. Gene expression was measured using real time quantitative PCR, or semi-quantitative in situ hybridization (SCN). In the SCN Period1 and Period2 mRNA expression was rhythmic in LD and CDL. In the retina all the mRNA levels were rhythmic in LD, while the rhythmicity was less clear in CDL. In the pineal gland Period1, Period2, and Aa-Nat mRNA levels were rhythmic in LD. After 60 days in CDL, in about 20 % animals, the expression of these genes was altered. In the muscles, heart, and liver, Period1 and Period2 mRNA levels were rhythmic in LD, and the rhythms became less clear after 60 days in CDL. Our results suggest that long exposure to constant dim red light may induce desynchronization among the SCN and some peripheral oscillators. Supported by NSBRI NCC 9-58 to G.T.

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The Expression of RGS16, Regulator of G-protein Signaling 16 under Restricted Feeding Condition

*Hisanori Wakamatsu, Yoshie Imaizumi, Sadao Yamaoka • Department of Physiology, Dokkyo University School of Medicine, Tochigi, Japan

There are two entrainment stimului of circadian rhythm, scheduled restricted feeding (RF) and light. Light-induced expression of mPer1 and mPer2 in the suprachiasmatic nucleus (SCN), a central pacemaker located in hypothalamus closely correlated with the light-induced entrainment of locomotor activity rhythm. Previously, we investigated the effects of restricted feeding (RF) on the expression of mPer1 and mPer2 mRNA in the paraventricular nucleus (PVN), pyriformcortex, hippocampus and cerebral cortex of mice. In order to examine whether the relation between clock genes function and intraceller signaling in food anticipatory activity (FAA) rhythm, we investigated mRNA of regulator of G-protein signaling 16 (RGS16) on restricted feeding. In situ hybridization revealed the circadian expression of RGS16 mRNA. The expression of the RGS16 mRNA showed a clear peak in SCN at daytime, as well as mPer1 and mPer2. Contrary, a peak in PVN, pyriformcortex, cerebral cortex and hippocampus has seen at nighttime. After 6 days of RF (4 hours in the daytime) RGS16 mRNA level showed a peak in the cerebral cortex, hippocampus, PVN and pyriform cortex in daytime. Scheduled-RF attenuated this rhythmicity outside SCN. However, the expression pattern of RGS16 mRNA in SCN didn't show the phase shift on scheduled-RF. These results suggest that clock genes function and G-protein signaling correlate with FAA rhythm outside SCN regions.

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T-cycle Experiments Demonstrate the Operation of a "Beats" Clock Controlling Circa-semilunar Activity in an Intertidal Isopod

J. F. Cheeseman[1]* R. D. Lewis2 G. R. Warman[1] and M. M. Walker[2] • [1]Department of Anaesthesiology, Faculty of Medical and Health Sciences, [2]School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

The mechanics of lunar and semilunar clocks have not been resolved despite several decades of study. Two hypotheses for the mechanism underlying circa-semilunar (c.15 day) rhythms exist. The first suggests the clock is an independent fifteen day circa-semilunar endogenous oscillator. The second hypothesis is that the fifteen day rhythm is generated by "beats" produced by combined outputs of a circadian clock (c. 24h) and a circa-tidal clock (c.24.8h). The intertidal isopod, Scyphax ornatus, lives on exposed New Zealand beaches and exhibits semilunar patterns of foraging activity, controlled by an endogenous timing mechanism that persists in constant conditions and displays temperature compensation. In the present study, T-cycle experiments (LD12:12, LD11.5:11.5 and LD12.5:12.5) have been conducted to elucidate the mechanism controlling the circa-semilunar rhythms of foraging activity. By varying the T-cycle, potential beat rhythms can be changed from c.15 days to c.6 days (T=23h), or c.60 days (T=25h). Under T=23h, 57% of animals (n=27) showed a predicted beat of 6-7 days. Under T=25 the 15 day rhythm was abolished in 78% of animals (n=18) and no rhythm with a period of less than 60 days was evident in the data. These data provide strong evidence for the "beats" hypothesis of semilunar activity. Additional evidence should be obtained by further light T-cycle experiments and by controlling the entrainment of the tidal component of foraging activity. The T-cycle paradigm employed here may also be useful in determining the mechanistic basis of other marine rhythms such as the circa-tidal rhythm. This work was supported by a University of Auckland, Faculty of Science Fees Bursary to JFC.

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Activation of the Hypocretin System in Syrian Hamsters by Non-photic Stimuli during the Subjective Day

Ian C. Webb*, Dwayne Hamson & Ralph Mistlberger • Simon Fraser University

Circadian rhythms in Syrian hamsters can be phase shifted by arousal procedures that permit locomotor activity (e.g., novel wheel confinement, gentle handling, dominant-subordinate stress interaction), but not by manipulations that prohibit locomotion (e.g., restraint tube, pedestal over water; Mistlberger et al., 2003). The IGL and raphe nuclei have been implicated in clock resetting by nonphotic stimuli, but neural pathways upstream require further elucidation. The hypocretins (hcrts) are recently discovered neuropeptides that project to the IGL and raphe, and have been functionally linked to locomotor activity. To determine if the hcrt system is differentially activated by arousal procedures that permit or prohibit activity, hamsters were subjected to 3h of dark from ZT6-9, with or without wheel confinement (WC) or restraint, and processed for hcrt-1 and Fos immunoreactivity (ir) at ZT9 or 11.5. Some animals also received a light pulse at ZT10 to assess SCN clock resetting. Hamsters in the control condition showed no double-labeled and few Fos+ cells. In contrast, hamsters subjected to WC showed increased double and single-labeled Fos+ cells. Fos-ir in hcrt cells was significantly decreased when sacrifice was delayed until ZT11.5. Hamsters that received a LP after the WC showed a further reduced level of Fos+ cells. The results show that WC in the mid-subjective day is associated with activation of the hcrt system and that light suppresses Fos in non-hcrt lateral hypothalamic cells. Data from physical restraint tests will also be presented. Supported by NSERC.

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Morgue, a Candidate for the Circadian Light Input Pathway in Drosophila Melanogaster

Murad, A*; Schreader, B; Nambu, J; Rosbash, M; Emery, P • University of Massachusetts Medical School

CRYPTOCHROME (CRY) is a circadian cell-autonomous blue-light receptor. It mediates circadian photoreception in the Drosophila pacemaker cells that regulates locomotor activity rhythms – the ventral lateral neurons (vLNs) - and in peripheral oscillators. Light-dependent interactions between CRY and TIMELESS (TIM) result in TIM proteasomal degradation, and CRY itself is also degraded by the proteasome. The details of these mechanisms remain unknown. A mutation in the cry gene, cryb, abolishes most circadian photoresponses. For example, cryb mutants are very strongly rhythmic under constant light while wild type flies are arrhythmic. Based on this observation, a misexpression screen was performed under constant light conditions. One of the lines that remained rhythmic in constant light overexpresses the morgue gene. MORGUE is a protein containing an F-box and an ubiquitin E2 conjugase domain and is therefore an interesting circadian light input pathway candidate. FISH (Fluorescence In Situ Hybridization) reveals that morgue mRNA is present in the vLNs, and over-expression of MORGUE reduces CRY levels. We thus propose that MORGUE influences circadian photoresponses by modulating CRY levels. To strengthen this notion, we are currently studying photoresponses in morgue loss-of-function mutants.

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Circadian Rhythms in Neurospora Crassa: Oscillator Genetics

Stuart Brody*, Michelle Tsukamoto, Laura Lombardi, Kevin Schneider, and Qian Xiao • Molecular Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0116

In Neurospora, the circadian rhythm is expressed as a series of bands (asexual spore-forming areas) on the surface of an agar medium. Although certain null mutations (frq10 or wc-2D) lead to the loss of this rhythm under most conditions, a rhythm can be restored to them by the addition of geraniol or farnesol to the media. Utilizing this restoration as an assay, the effect of other clock mutations can be measured in a frq10 or wc-2D null background after farnesol or geraniol were added. Three types of mutations were found: 1) those such as prd-3, which showed NO effect in the frq10 background; 2) those such as prd-1, or prd-4 which did have a measurable effect in the frq10 background; and, 3) those, such as ult-1 (a new 12 hr. mutant) which suppressed the frq10 effect, i.e. geraniol/farnesol were not then required for a visible rhythm. Additional double mutant strains were constructed, and when these new results are analyzed along with previous studies on double mutants, one can classify these double mutants into three categories: I. Both mutations affect the frq/wc (FWC) oscillator, such as prd-3 or frq10 or wc-2D: II. Both mutations do not affect the FWC oscillator, such as prd-4, prd-1, cel, chol or ult: III. One mutation affects the FWC, one does not, such as frq10 or prd-4. Classifying and assigning mutations to different oscillators/functions is an important first step in unraveling the complexities of a multi-oscillator system. Supported by NSF MCB 0212190.

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Single Nucleotide Polymorphisms in Per2 and Diurnal Preference

J Carpen* (1), M von Schantz (1), D Skene (1), A Williams (2), M Smits (3), S Archer (1) • (1) University of Surrey, Guildford, UK, (2) St Thomas Hospital, London, UK, (3) Hospital de Gelderse Vallei, Ede, the Netherlands

A mutation in the Period 2 gene has been shown to cause advanced sleep phase syndrome. By screening single nucleotide polymorphism (SNP) databases, two SNPs with potential functional relevance were identified. These were a glycine to glutamine substitution in the coding region (position 1244), and a cytosine to guanine substitution located in the 5' untranslated region (UTR). We genotyped subjects (each group n=35) selected for extreme morning or evening preferences, and intermediate diurnal preference (Horne-Ostberg questionnaire, corrected for gender and sex) along with delayed sleep phase syndrome (DSPS) patients (n=30) and a population of 100 control subjects (Coriell Institute). Allele frequencies for the amino acid substitution did not differ significantly between any of the populations studied. The G allele frequency for the 5'-UTR polymorphism was significantly higher in extreme morning preference subjects (0.14) than in extreme evening preference subjects (0.03) (Fisher's exact test, two-sided P value = 0.0307, odds ratio= 5.667). There was no significant difference in G allele frequencies between the extreme morning preference group and the other groups tested. Our results indicate that the 5'-UTR polymorphism is linked to diurnal preference. Its location suggests that it may affect translational efficiency, perhaps by influencing ribosomal binding. This hypothesis is currently being tested.

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Evolutionary Expansion of a Polymorphic Repeat Region in the Per3 Gene that Associates with Diurnal Phenotype in Humans.

A. Jenkins*, M. von Schantz and S. N. Archer • University of Surrey, Guildford, UK.

A region in Per3 was identified that displays a two-nucleotide length polymorphism between the two inbred mouse strains BALB/c and C57BL/6J, which are known to have a difference in free-running circadian period of 0.8 hours. In humans, this region has expanded into a polymorphic tandem repeat, where an 18-amino-acid motif is repeated four or five times. In human volunteers, we have previously shown a significant correlation between the five-repeat region and morning preference and between the four-repeat region and evening preference. Delayed sleep phase syndrome (DSPS), which may be viewed as an extreme form of evening preference, shows an even stronger correlation with the shorter allele. We have now investigated this expansion in different primate species. PCR-based amplification of cDNA from chimpanzee, gorilla and gibbon indicated the presence of a repeat region in all these species. We found one four-repeat allele in both the chimpanzee and gibbon, whereas in the gorilla we have detected two alleles, corresponding in length to three and four repeats of the 18-amino-acid sequence, respectively. Phylogenetic analysis suggests the initial repeat unit was present in rodents, but has expanded since the rodent and primate lineages diverged. We speculate that the interspecific polymorphism may convey an evolutionary advantage and has undergone positive selection within primates.

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Adaptation to Night Shift in Antarctica: Relationship to a Length Polymorphism in Hper3

Ng L[1], Archer S[2], Rieley T[1], Bishop L,[1] Middleton B[2], Arendt J.[2] • [1]British Antarctic Survey Medical Unit, Derriford Hospital, Plymouth, UK; [2]Centre for Chronobiology, University of Surrey, Guildford, UK.

Most Base personnel at Halley Bay (75oS) show circadian adaptation to night shift. This study investigated adaptation in relation to a length polymorphism in hPer3, known to segregate with diurnal preference. Subjects (N=21, 3 F, 18 M), aged 29±1.1 y (X±SEM) worked a week of night shift (2100-0800h) in rotation during 2000 to 2002. 6-sulphatoxymelaonin was measured (RIA) in sequential urine samples (3 days baseline before, and during night shift) followed by cosinor analysis for significant acrophases. Average baseline acrophase was subtracted from day 5 of night shift acrophase to give extent of shift. Participants completed Horne-Ostberg questionnaires. Statistics were linear regression, ANOVA or unpaired Students t-test as appropriate. Average shift by day 5 was 4.72±0.65h (range -1.6 to 10.3h). No significant relationship with the 4/4, 4/5 or 5/5 hPer3 variant was seen, however entrained phase was earliest in 5/5 subjects (morning preference, 4.09±1.01h, N=3), latest in 4/4 subjects (evening preference, 5.92±0.32h, n=7), and intermediate in 4/5 subjects (5.13±0.43h, n=11). 'Eveningness' related positively to extent of phase shift (p=0.01). Conclusions: In this small sample, with no diurnal extremes, the length polymorphism in hPer3 did not relate to speed of adaptation to night shift. Acknowledgements: Supported by the British Antarctic Survey and the BBSRC.

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Prediction of Circadian Phase and Period Using Different Chronotype Questionnaires

K.V. Danilenko, A.A. Putilov`, M. Terman^, A. Wirz-Justice* • Institute of Internal Medicine, and `Institute for Molecular Biology and Biophysics, SB Rams, Novosibirsk, Russia; ^Columbia University & New York State Psychiatric Institute, New York, USA; *Centre for Chronobiology, Psychiatric University Clinic, Basel, Switzerland.

It is widely accepted that the differences between morning and evening chronotypes reflect, at least in part, the difference in the phase of circadian pacemaker. We compared several chronotype questionnaires for their capacity to predict circadian phase and period. The subjects were 16 healthy men and women (19-34y) who participated in chronobiological studies in Novosibirsk in 1997-8 or 2001. The studies comprised two 9-day sessions in laboratory dim light separated by an off-protocol period of 9-22 days. A modified constant routine (CR) protocol was applied at the beginning and end of each session. Circadian phase was estimated with dim light melatonin onset and the downward mid-range crossing time of rectal temperature in the initial CRs; circadian period was derived from data of 9 days in dim light. In 2003-4 the subjects completed the 19-item Horne-…stberg Morningness-Eveningness Questionnaire, revised (MEQ; Terman et al., 2002), the Munich Chronotype Questionnaire (Roenneberg and Merrow, 2002) and the 52-item Sleep-Wake Pattern Assessment Questionnaire (SWPAQ; Putilov, 2000). The SWPAQ includes two separate scales for scoring morningness (12 items) and eveningness (12 items). The only significant correlation was found between the eveningness score of SWPAQ and temperature phase ($r=0.69$, $p<0.01$); correlation with melatonin phase did not reach significance ($r=0.50$), although effect size is high. For the MEQ, correlation coefficients were non-significantly lower than for the SWPAQ (temperature, $r=0.37$; melatonin, $r=0.47$). Circadian period did not correlate significantly with any of the three scales. The more detailed SWPAQ deserves further testing in its ability to predict individual circadian phase.

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Relative Coordination in Free-Running Blind Individuals

Jonathan S. Emens*, Alfred J. Lewy, Bryan J. Lefler, Robert L. Sack • Oregon Health & Science University

In more than half of those blind individuals who completely lack light perception, the absence of photic input via the eye to the circadian pacemaker results in rhythms that free-run. The others are entrained by relatively weak, as-yet-unidentified, zeitgebers. The free-running period in the endogenous melatonin circadian rhythm observed in each blind free-runner (BFR) presumably represents the intrinsic period of pacemaker, unperturbed by such weak time cues. If such time cues do act upon the circadian pacemaker in BFRs, the influence of these zeitgebers should be detected given sufficient resolution. We conducted high-resolution studies in five BFRs: melatonin was sampled for 24 hours for assessment of circadian phase every 2 weeks on 9-17 occasions. The melatonin onset (MO) was defined as the interpolated time when levels continuously rose above the threshold of 2 pg/ml (plasma) or 0.7 pg/ml (saliva). All five individuals free-ran [average linear regression period (tau) ± SD = 24.31 ± 0.06 h], and there was definitive relative coordination in four. These four subjects demonstrated a similar and reproducible pattern of period oscillation: shorter circadian periods when the MO occurred between approximately 20:00 to 08:00 and longer circadian periods between approximately 08:00 to 20:00. The significance of relative coordination for estimation of intrinsic period in BFRs is unclear. The possible influence of weak zeitgebers on estimates

of intrinsic circadian period in BFRs should be considered where obvious period oscillations occur. Nevertheless, BFRs represent the best estimate of the genetically determined, intrinsic circadian period, unperturbed by the aftereffects of entrainment.

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Circadian Adaptation in Night Shift Workers results in Extension of Diurnal Sleep

Francine O. James* and Diane B. Boivin • McGill University

Careful control over the pattern of light exposure is associated with a more appropriate physiological adaptation to a schedule of permanent night shifts, and extended diurnal sleep duration. Ten nurses working permanent night shifts (mean age Á‘ SD: 41.7 Á‘8.8 years) followed a judicious schedule of light/darkness exposure during each night shift including intermittent exposure to bright light during the night shift and shielding from morning light exposure. Nine control group nurses on full-time night shifts (41.98 Á‘7.2 years) worked in their habitual light environments. All workers kept a single 8-hour sleep/darkness episode starting two hours after the end of the each night shift. Diurnal sleep was measured by portable polysomnography or the NightCap device. Circadian phase was evaluated in the laboratory via 36-hour constant routines performed before and after a period of (Á‘SD) 12.1 Á‘ 0.7 night shifts worked over ~20 days. Treatment group workers displayed adaptive phase shifts of the core body temperature rhythm (Á‘SEM) of -9:19 Á‘ 1:04. Control group workers displayed means phase shifts of -4:05 b 1:56. The significantly larger phase shifts of the treatment group (P=0.04) were associated with an extension of total sleep time, where control and treatment group workers slept an average of 6:36 Á‘ 0:12 and 7:12 Á‘ 0:17, respectively (P=0.04). This demonstration of improved sleep duration in the presence of appropriate circadian adaptation to night shift work supports the usefulness of careful control over the pattern of light exposure in this population.

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Light-induced Melatonin Suppression: Age-related Reduction in the Response to Short Wavelength Light

Herljevic, M., Middleton, B.*, Thapan, K and Skene, D.J. • Centre for Chronobiology, School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey, UK

Age-related changes in lens transmittance with reduced transmission of short wavelength light are widely accepted. Light-induced suppression of nocturnal melatonin has been shown to be short wavelength sensitive (< 480 nm) thus whether ageing results in reduced melatonin suppression was tested in the current study. The ability of short and medium wavelength light to suppress night-time melatonin production was investigated in young (n = 13, aged 24 ± 3 yrs, mean + SD) and elderly, postmenopausal (n = 21, aged 57 ± 5 yrs) women. All subjects received 30 mins of monochromatic light (full field, uniform retinal illumination with pupil dilation) of two different wavelengths and irradiances (lmax 456: 3.8 and 9.8 mW/ cm2; lmax 548 nm: 28 and 62 mW/cm2). Melatonin suppression (average plasma melatonin concentrations in the first 45 mins after start of light exposure as a percent of the level before light exposure) was compared across light treatments and between age groups. The elderly subjects showed significantly reduced melatonin suppression with the low intensity (3.8 mW/ cm2) short wavelength (456 nm) light (0.5 ± 4.4%, mean ± SEM) compared to the young subjects (15.3 ± 4.3%) (P<0.05). The other light treatments produced similar melatonin suppression in both age groups (P>0.05). The effect of low intensity short wavelength

light on melatonin is thus reduced with ageing, findings likely to reflect age-related changes in lens transmission. Supported by an EU grant (QLK6-CT-2000-00499).

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The Acute Effect of Light Wavelength on Alertness and Body Temperature

Revell, V.L.*, Dijk, D.J., Arendt, J. and Skene, D.J. • University of Surrey

Whether all irradiance-dependent non-image forming light responses (e.g. circadian photoentrainment, melatonin suppression) utilise the same photoreceptor(s) remains to be proven. The current study aimed to assess the acute effect of light wavelength on core body temperature (CBT) and alertness. Healthy male subjects (n=12, aged 26 + 1 yrs, mean + SEM) were studied in 40, 4-day laboratory study sessions (lighting < 8 lux). On day 3 subjects received a 4-hr monochromatic light pulse (07:15 ö 11:15h) (CT 4). Four wavelengths were assessed at 2 photon densities: 420 and 440 at 2.32 x 10 13 photons/cm2/sec (LPD) and 440, 470 and 600 nm at 6.13 x 10 13 photons/cm2/sec (HPD). Alertness was subjectively assessed (1-9) at 30 min intervals before, during and after light exposure. The change in CBT between day 2 (without light) and day 3 (during light exposure) was calculated. Light exposure increased CBT and although there was no significant difference between the wavelengths the greatest elevating effect was observed with 440nm (HPD). Alertness also increased during light exposure, 420nm light being significantly more alerting than 470nm light ($p < 0.05$). The mean alertness level during the light pulse varied, but not significantly, with wavelength: 440nm (HPD) (most alerting) > 440nm (LPD) and 420nm (LPD) > 470 (HPD) > 600nm (HPD) (least alerting). These data suggest short wavelength sensitivity (420-440nm) of the acute effects of light on alertness and CBT.

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Changes in Plasma Melatonin Profiles during Chronic Nocturnal Sleep Restriction

Naomi L. Rogers*, David F. Dinges • University of Pennsylvania

Sleep deprivation has been reported to have no effect on melatonin secretion, while shifting the timing of sleep-wake behaviour can produce alterations in the phase of secretion. In the present study we examined the effects of shortened nocturnal sleep, with and without daytime naps, on melatonin. N=19 subjects (14m; 5f; aged 21-38y) completed a 14-day in-laboratory study. Following 2 baseline nights (8.2h TIB: 2154h-0606h) subjects were randomly assigned to a 10d chronic sleep restriction condition: 8.2h TIB (2154h-0606h; n=5); 4.2h TIB (2354h-0406h; n=14) with and without a daytime nap (between 0.0h and 2.4h). On baseline day 1 and restriction day 10, subjects were maintained in a constant posture paradigm for 26h, with sleep at the allocated times, and blood samples collected via an indwelling catheter. Plasma melatonin concentrations were determined hourly using RIA. Melatonin profiles were analysed using within-subjects repeated-measures ANOVA comparing day 1 with day 10 across conditions. All subjects demonstrated a circadian rhythm in melatonin across the 26h ($p<0.001$). In the 8.2h condition there were no significant differences in melatonin profiles between assessment days. In the 4.2h sleep-restriction conditions, with dark onset delayed by 2h, there was a significant delay in melatonin onset (approximately 2h; pT0.005). Sleep restriction was able to alter the circadian system, as evidenced by changes in the timing of melatonin secretion. This effect may be due to changes in light-dark exposure and/or the reduced sleep period. Research supported by NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute, NIH M01-RR00040

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Can Melatonin Help the Daytime Sleep of Night Shift Workers?

*Mark R. Smith, Clara Lee, Stephanie J. Crowley, & Charmane I. Eastman • Biological Rhythms Research Laboratory, Rush University Medical Center, Chicago

Exogenous melatonin administration in humans is known to exert both chronobiotic and hypnotic effects. In a previous study, young, healthy, subjects worked five simulated night shifts (23:00 to 07:00) and slept during the day (08:30 to 15:30). Various magnitudes of phase delay were produced by the study interventions, including bright light during the night shifts. As part of this study, subjects ingested either 1.8 mg sustained release melatonin or placebo before daytime sleep. The melatonin did not increase the phase delays, as assessed by the dim light melatonin onset (DLMO) before (baseline) and after (final) the five night shifts. We also hypothesized that melatonin would have a soporific effect. To this end, melatonin (n=18) and placebo (n=18) groups were formed by matching participants with similar baseline and final DLMOs (+/- one hour). Sleep log measurements of total sleep time (TST) and actigraphic measurements of sleep latency, TST, and three movement indices were examined. Although melatonin was associated with improvements in sleep quality and quantity, a repeated measures ANOVA (group x day) indicated that these differences were not statistically significant. Chi Square analyses indicated a trend towards melatonin subjects obtaining more actigraphic TST than their placebo counterparts during the first three hours of day sleep ($p = .06$). Both the melatonin and placebo groups displayed high sleep efficiencies, suggesting that a ceiling effect may have attenuated any group differences.

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Circadian Variation of C-Reactive Protein under Constant Routine Conditions

Kenneth P. Wright Jr.*, Danielle J. Frey, Adam Wertz, and Monika Fleshner • Department of Integrative Physiology, University of Colorado at Boulder

High circulating plasma levels of C-reactive protein (CRP) are reported to be a risk factor for cardiovascular disease. The current study assessed the circadian variation in CRP levels using a constant routine protocol. Twenty-two participants (10 women) aged 28.3 ± 9.3 (SD) years who passed a rigorous health screening participated. None reported shift work for the past three years or transmeridian travel in the previous three months. Participants maintained a routine of 8 h of scheduled sleep for three weeks at home prior to laboratory procedures. Toxicology verified drug free status upon admission. Following three laboratory baseline days participants completed a 40 h constant routine (constant wakefulness, posture, nutrition intake and dim light exposure [< 1.5 lux]). Plasma CRP levels were determined using a high sensitivity ELISA (ALPCO, Windham, NH). Data were z-score transformed and interpolated to provide equal sampling times. Circadian phase was assessed by calculating the dim light melatonin onset (DLMO25%). The DLMO25% was assigned a phase of 0 degrees and CRP data were averaged into 30 degree circadian bins. Repeated measures ANOVA with Greenhouse-Geisser degree of freedom correction factors for sphericity indicated a significant effect of circadian phase on CRP levels ($p < 0.05$). Circulating CRP levels were highest near melatonin onset, decreased across the biological night and increased across the biological day. The current findings indicate that circulating levels of the acute phase protein CRP demonstrate circadian variation. Thus, the circadian clock or down stream effectors appear to influence CRP levels across the 24 h day.

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Circadian Rhythms Control Cadmium Toxicity in Paramecium Tetraurelia: A Model Organism for the Study of Chronotoxicology

Robert D. Hinrichsen* and Joseph Tran • Indiana University of Pennsylvania

While circadian rhythms have been characterized in other species of Paramecium, they have never been confirmed in Paramecium tetraurelia. This study was undertaken to demonstrate a circadian clock that controls the sensitivity of Paramecium to the heavy metal cadmium. Log phase cells (grown in the dark for several years) that were placed in >1 mM CdCl2 died within 20 minutes or less, whereas cells in < 200 uM CdCl2 were capable of surviving for longer than 60 minutes. When cells were grown for two weeks in a 14L:10D cycle at 25a C and tested for survival in 500 uM CdCl2, an oscillation in the sensitivity to CdCl2 of 24 hours was discovered. Cells died within 5 minutes 6:00 AM (CT 18), but were able to survive for 60 minutes or longer at 6:00 PM (CT 6). The circadian rhythm persisted in the dark and also demonstrated temperature compensation; cells grown at 18a, 26a and 30a C had a similar oscillation to the toxic effects of CdCl2. Finally, phase shifting experiments have demonstrated a phase-dependent response to light. To test the hypothesis that levels of a cadmium metallothionein were involved, we have cloned the gene from Paramecium and used it in RNA interference assays. Preliminary data show that a reduction in the levels of cadmium metallothionein protein leads to the elimination of the circadian rhythm. This might implicate a metallothionein as an output protein of the circadian clock in Paramecium tetraurelia and explain the chronotoxic effect of cadmium.

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Transcription Profiling during Cocaine Responses in Wild Type and Circadian Mutant Drosophila

R. George, J. Hirsh* • University of Virginia

Many of the core circadian clock genes of Drosophila are required for normal responses to crack cocaine. These genes are required for sensitization to repeated cocaine exposures, a process linked to human addiction. Since the mouse period 1 gene shows similar alterations in cocaine sensitization, it is of interest to determine how these fly genes are functioning in the cocaine response pathway. One approach is to examine transcriptional profiles from hand-dissected brains of wild type or circadian mutant animals after cocaine exposures. We have generated reliable Affymetrix chip data from RNA preparations extracted from groups of 20 hand-dissected fly brains. We have compared transcript profiles from brains of wild type or circadian mutant flies after either single or repeated cocaine exposures. We find only a small number of genes showing significant changes under these conditions. These studies allow us to categorize genes as potentially involved in immediate versus sensitized cocaine responses. Characterization of transcript levels of these genes in other mutant lines showing altered cocaine responses will help to characterize the pathways altered in these lines. The numerous tools and reagents available in Drosophila will allow validation of these novel targets.

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Molecular Basis of Biorhythmicity and Environmental Signal Transduction in the Annelid Polychaete Nereis Virens (Sars)

Cas Kramer *[1], Thierry Bailhache *[2], Kim S. Last *[3], Pamela Edwards [3], Lin Zhang [1], Ezio Rosato [2], Charalambos P. Kyriacou [1] and Peter J.W. Olive [3] • [1] Department of Genetics, University of Leicester, UK.; [2] Department of Biology, University of Leicester, UK.; [3] School of Marine Sciences and Technology, University of Newcastle, UK.

Marine organisms live in a complex rhythmic environment, influenced principally by daily light/dark (24 h) and tidal high/low (semi-diurnal tidal ~12.4 h, lunar-day tidal ~24.8 h) and spring/neap (semi-lunar tidal ~14.7 days) oscillations. Predicting such oscillations is of vital importance for survival and fitness, particularly for animals living in an intertidal ecosystem, like the polychaete Nereis virens. A multi-channel automated actograph capable of continuous data acquisition has been developed for N. virens. Cultured animals not exposed to tidal stimuli typically show a solar day phenotype (~24h), whereas animals collected from a wild type environment typically exhibit a tidal phenotype (~12.4 h). Whole head mRNA has been extracted from animals showing circadian, circatidal and seasonal behavioural phenotypes. A PCR-based normalisation/subtraction protocol has been set up to generate a normalised cDNA library, 3'UTR-specific and enriched for differentially expressed cDNAs. The normalisation protocol successfully reduced the actin/CK2alpha ratio from >5000 to <5. PCR products from a total of ~40000 individual clones are being spotted onto microarray slides. Analysing temporal gene expression will identify novel cycling genes and help to elucidate the molecular basis of tidal rhythmicity in marine organisms. Furthermore, using conventional screening we have cloned several N. virens clock (-related) genes, among them Clock and Bmal1. Investigating the circadian clock in an ancient phylogenetic group, like the Annelida, will help to answer the question of whether the terrestrial circadian cycle has evolved from a more complex ancestral condition involving both tidal and circadian oscillators.

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Mechanisms Underlying Circadian Modulation of Memory Formation in Aplysia

Lisa C. Lyons*, Maria Sol Collado, Ray I. Fernandez, Omar Khabour and Arnold Eskin • University of Houston

Previously, we showed that the circadian clock modulated the induction of a simple form of long-term memory, long-term sensitization (LTS) of the tail-siphon withdrawal reflex in Aplysia. Animals display robust long-term memory when they are trained during the day, but the formation of memory is significantly suppressed when they are trained at night. The circadian oscillator could modulate memory formation via regulation of serotonergic facilitatory neurons, sensory neurons, motor neurons, and/or other neurons or glia associated with the withdrawal reflex circuit. If circadian modulation occurs through regulation of serotonergic facilitatory neurons, release of serotonin should be modulated. To test this possibility, serotonin release into the hemolymph was measured when animals were trained in the day or the night. LTS training during the day caused a greater increase in serotonin release than LTS training at night. Thus, the circadian clock may modulate the induction of LTS, in part, by modulation of release of serotonin. To determine whether circadian modulation occurs downstream of serotonin release (in sensory or motor neurons), we investigated whether treatment of Aplysia in vivo with serotonin would produce a rhythm of LTS. We found that animals treated with serotonin during the day exhibited greater LTS than animals treated during the night. These results demonstrate that diurnal modulation of LTS also occurs

downstream of the facilitatory neurons. These results set the stage for analysis of the molecular mechanism of circadian modulation of memory formation.

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Feeding Is Not a More Potent Zeitgeber than the Light-dark Cycle in Drosophila

Katsutaka Oishi*, Masayuki Shiota, Katsuhiko Sakamoto, Manami Kasamatsu, Norio Ishida • Institute of Advanced Industrial Science and Technology

Daily scheduled feeding is a potent Zeitgeber that elicits anticipatory activity in mammals. Recent studies have revealed that daytime feeding of nocturnal laboratory rodents completely inverts the phase of circadian gene expression in peripheral tissues such as heart, liver, and kidney independently of environmental light cycles. To investigate whether feeding is a potent time cue for Drosophila, we examined the behavioral activity rhythm and peripheral expression profile of clock genes in Drosophila under 12 h of nighttime restricted feeding (RF). We found that flies could not exhibit food-anticipatory activity rhythms under RF. Moreover, expression profiles of the clock genes, period and timeless, were not affected by either the phase or the amplitude in the periphery. These results suggest that feeding is not a more potent Zeitgeber than the light-dark cycle at either the individual behavioral level or at the peripheral molecular clock levels in Drosophila.

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Effects of Light Regimes on Fitness Traits of Drosophila Melanogaster Are Mediated Through Circadian Rhythms

Dhanashree Paranjpe*, Anitha D., Amitabh Joshi, Vijay Kumar Sharma • Jawaharlal Nehru Centre for Advanced Scientific Research

The ubiquity of circadian clocks has been taken to imply its adaptive significance, but to date there has been no empirical evidence to support it. In order to demonstrate that circadian clocks are adaptive, one needs to establish that organisms can evolve circadian clocks and thereby have superior fitness in their parental environment compared to others who evolved in a different environment. Earlier studies on adaptive significance of circadian rhythms assumed life span as the sole fitness trait, while it has been established that life span is inversely related to reproductive output. We measured egg-to-adult development time, egg-to-adult survivorship, lifetime fecundity, per day fecundity and adult life span of Drosophila melanogaster populations in two constant (LL and DD) and three periodic light regimes (T20, T24, and T28). Development time was shortest in LL (parental regime), followed by DD and T20, and longest in T24 and T28. Egg-to-adult development time was positively correlated with the period of the eclosion rhythm. Egg-to-adult survivorship and lifetime fecundity did not differ in different light regimes. Average number of eggs laid per day in constant environments was significantly greater than in periodic environments, while the life span of adult flies was significantly greater in periodic light regimes than in constant light regimes, thus confirming a well-known trade-off between fecundity and life span. The results of our experiments thus demonstrate that several life history parameters, which may be responsible for superior fitness of the organisms, are influenced by light regimes and such effects are possibly mediated through circadian rhythms.

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Molecular Targets for the Action of Lithium on Circadian Clocks

Vijay Kumar Sharma [1]*, Quasar Saleem Padiath [2], Dhanashree Paranjpe [1], and Sanjeev Jain [3] • [1] Chronobiology Laboratory, Evolutionary & Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, India.; [2] National Centre for Biological Sciences, TIFR Centre, Bangalore, India.; [3] Molecular Biology Unit, Department of Psychiatry, National Institute of Mental Health and Neurosciences, Bangalore, India.

Lithium has been used as a drug in the treatment of bipolar disorder for several decades, and has been shown to influence circadian rhythms by lengthening its period. However, the molecular mechanisms underlying its action are still unclear. It is known that phase and period of circadian rhythm is altered in patents with bipolar disorder, and it is further believed that these rhythms may play an important role in disease mechanisms. It may therefore be possible that some of the therapeutic actions of lithium are related to its effect on circadian clocks. Therefore, identifying the molecular targets for lithium's action on circadian clocks might help in understanding the mechanisms of its therapeutic action and in understanding the disease mechanisms in bipolar disorders. Using Drosophila melanogaster as a model system, we have shown that long-term administration of lithium results in lengthening of the free-running period (t) of circadian locomotor activity rhythm of flies in constant darkness (DD). This effect occurs at concentrations similar to the plasma levels of lithium used in the treatment of bipolar disorder. The lithium treated flies also show reduced activity of one of the previously reported targets of lithium action, Glycogen Synthase Kinase 3b (GSK 3b). GSK 3b has been shown to be involved in the regulation of circadian clocks as the down regulation of this protein results in an elongation of t. The t elongation resembles those seen in a number of organisms including man, after the administration of lithium. Taken together some of the earlier observations, our results suggest that lithium inhibits the activity of GSK 3b to produce its effect on circadian clocks.

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Female Odours Affect Rhythmic Mating Frequency and Male Pheromone Response in the Moth Spodoptera Littoralis [Noctuidae]

Germund Silvegren*, Christer Lefstedt, Wen Qi Rosen • Lund University

Mating in moths is generally mediated by female-produced sex pheromones. Both female pheromone production/release and male pheromone responsiveness show a diurnal variation. Temporal synchronization of mating behaviours is therefore crucial to both sexes. Pairs of the Egyptian cotton leafworm (Spodoptera littoralis) mated significantly less when the males and females were raised in light:dark cycles 10 hours out of phase, though males seemed to have a wider temporal mating window. We showed that males not ready to mate, which were exposed to odours from ready-to-mate females, increased their mating frequency at the same timepoint the following day compared to non-exposed males. A diel rhythm in the behavioural response of male S. littoralis to the female sex pheromone was observed in olfactometer tests. The rhythm was persistent in darkness for at least one day and high response was correlated in time with peak locomotor activity. After 3 days in darkness, no significant differences in olfactometer response were obtained at different timepoints. However, when males in constant darkness experienced daily exposures to pheromone gland extracts, they still showed a temporal rhythm in response after 4 days. This shows that in the absence of external zeitgebers, pheromone gland extracts function to reinforce circadian behavioural rhythms in male S. littoralis.

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Molecular Basis of Circadian and Circatidal Rhythmicity in a Crustacean, Eurydice Pulchra

Lin Zhang*[1], David Wilcockson[2], Cas Kramer[1], Thierry Bailhache[3], Ezio Rosato[3], Michael H. Hastings[4], Simon Webster[2] & Charalambos P. Kyriacou[1] • [1] Department of Genetics, University of Leicester, UK; [2] School of Biological Sciences, University of Wales, UK; [3]Department of Biology, University of Leicester, UK; [4] MRC Laboratory of Molecular Biology, University of Cambridge, UK

The circadian (24h) clock may be considered to be the most important timekeeping device in terrestrial organisms. However, for marine animals, the ability to predict the twice ö daily ebb and flow of the tides, is of equal adaptive significance. The presence of a circatidal (~12.4h) clock, in addition to the circadian clock, has been demonstrated in a wide variety of marine animals. Molecular analysis in terrestrial organisms has revealed that circadian rhythmicity is generated by interlocked transcriptional and translational feedback loops. However, little is known about the molecular mechanisms involved in circadian and circatidal rhythmicity in marine animals. The current study aims to investigate whether the same or similar clock genes are governing both circadian and circatidal rhythmicity; alternatively, a completely novel set of genes may be responsible for circatidal rhythms. To address these questions, initially using a conventional RT-PCR method, we have cloned homologues of circadian related clock genes (such as per, Clk, cyc, cry, dbt and Ck2) from a marine crustacean ö Eurydice pulchra, and their circadian and circatidal expression patterns are being investigated. Although most of circadian related clock genes have been isolated from this organism, the sequencing of these genes is not complete. Full-length of cDNA libraries have been recently constructed separately from circadian and circatial entrained animals in order to isolate full-length cDNAs corresponding to rhythmically expressed genes, which will also be identified from a further global gene expression study by using cDNA microarrays derived from Eurydice cDNA subtractive libraries.

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The Circadian Regulator Period2 Functions in Tumor Suppression in vivo

Loning Fu, Helene Pelicano, Jinsong Liu, Peng Huang, Cheng Chi Lee • Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030 USA

Circadian rhythms are the daily oscillation of a variety of biological processes driven by the endogenous clock. These processes include cell proliferation and apoptosis. Disruption of circadian rhythms has been associated with increased tumor development in mammals. The Period2 (Per2) gene is a key component of mammalian circadian clock. We have found recently that loss of Per2 function results in neoplastic growth in mice. Following g-radiation, these mice show a marked increase in tumor development. We found that the core circadian genes are induced by g-radiation in wild-type mice in a time-dependent manner and such response is deficient in Per2-mutant mice. In addition, the deregulated circadian gene induction in response to g-radiation correlates with a time-dependent apoptotic resistance in Per2-mutant thymocytes. We have also found that although PER2 apparently does not bind to DNA directly, loss of Per2 function leads to deregulation of an array of genes controlling cell proliferation and DNA-damage response. Thus, the circadian regulator PERIOD2 could function as a tumor suppressor in vivo by controlling cell proliferation and DNA damage responsive pathways.

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Photosensibility in the Djungarian Hamster (Phodopus Sungorus) Revealed by Trans-pineal Microdialysis

A. Herwig* [1,2], L. Tritschler[2], B. Bothorel[2], P. PŽvet[2], S. Steinlechner[1], M. Saboureau[2] • [1] Neurobiologie des Rythmes, UMR7518 ULP/CNRS, Strasbourg, France; [2] School of Veterenary Medicine, Hannover, Germany

The Djungarian hamster shows distinct circadian and seasonal rhythms, which are entrained and synchronisized by the photoperiod. The circadian clock and the rhythmic secretion of melatonin by the pineal gland are part of the circadian system, conveying the photoperiodic message to the organism. In long photoperiod, 2 light-pulses given in 2 consecutive nights are known to induce an arhythmicity in certain animals. The trans-pineal microdialysis permits to study in vivo the sensibility to light during the dark phase and the effect of 2 light pulses given in 2 consecutive nights on the melatonin secretion. Male adult Djungarian hamsters were kept in an inversed long photoperiod (LD 16:8; D : 10h -18h). The microdialysis probe was perfused with Ringer's solution (2µl/min) and the dyalisates were sampled hourly for several days. The melatonin secretion shows a regular rhythm (ZT17 - ZT23,5) whereas the amplitude becomes stable only after the 3rd day of perfusion. In the experiments day 4 and 5 were defined as control days. In the course of the night a 15 min light pulse causes an immediate interruption at ZT18 and ZT20 and copletely stops the melatonin secretion at ZT 22. The following days the rhythm is modified: compression (ZT18), advance of the descending and reduction of the amplitude (ZT20) or advance of the descending (ZT 22). Two 15 min light pulses given in 2 subsessive night (ZT22 / ZT18) induce a diminution of the amplitude and the duration of the melatonin secretion with a progressive reinstallation of the normal rhythm. In the Djungarian hamster the stability of the melatonin rhythm by microdialysis is only obtained after 3 days of perfusion. The photosensibility during the dark phase is comparable to the rat. 2 light pulses given in 2 subsequent nights strongly modify the melatonin rhythm, although no long-term arhythmicity could be obtserved.

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The Role of rPER2 Nuclear Localization Sequence in Mammalian Circadian Clock

*Koyomi MIYAZAKI, Junko NARUKAWA, and Norio ISHIDA • National Institute of Advanced Industrial Science and Technology

Mammalian PERIOD2 protein (PER2) is the product of a clock gene that controls circadian rhythms, because PER2 deficient mice have an arrhythmic phenotype. The nuclear entry regulation of clock gene products is a key step in proper circadian rhythm formation in both Drosophila and mammals, because the periodic transcription of clock genes is controlled by an intracellular oscillating negative feedback loop. We used deletion mutants of rat PER2 (rPER2) to identify the functional nuclear localization signal (NLS) in rPER2. The elimination of putative nuclear localization signal (NLS, residue no. 778-794) from rPER2 fragment lost the nuclear entry activity. Intact rPER2 was preferentially translocated from the cytoplasm to the nucleus when co-expressed with mCRY1 or mCRY2, although the co-expression of nuclear localization domain (no. 512-794, NLD) lacking rPER2 and CRY changed the subcellular localization of CRY from the nucleus to the cytoplasm. These in vitro analysis data suggested that both rPER2 NLS is essential for nuclear entry of the rPER2 / CRY complex. To examine the role of PER2 nuclear localization regulation in generating circadian rhythm, we created transgenic mice overexpressing the NLD-deleted rPer2. The transgenic mice showed long periodicity as similar as Cry 2 KO mice. Further locomotor and biochemical analysis will be discussed at this presentation.

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24-hour Expression Profiles of Core Clock Components in an Equine Peripheral Tissue

Barbara A. Murphy* and B.P. Fitzgerald • University of Kentucky

Rhythmic regulation of Bmal1 transcription provides the positive driving force for the molecular clock, both in the brain and in peripheral tissues. Per2 is involved in the clock's post-translational feedback loop and initiates the subsequent cycle of Bmal1 gene expression. For this reason, Bmal1 and Per2 transcripts have circadian oscillations that are antiphase to each other. We report the successful isolation of Bmal1 and Per2 partial cDNA from a prepared equine lymphocyte library. Subsequent sequencing and NCBI Blast analysis has demonstrated high sequence homology between the equine and human genes. The deduced amino acid sequence of the equine BMAL1 protein shows 99% homology to that of the human. This sequence data has been used to design quantitative Real-Time RT-PCR assays for the detection of Bmal1 and Per2 levels in equine peripheral blood. Four mares were maintained under a 12:12 h light/dark cycle and blood samples collected via jugular venipuncture at three-hourly intervals over a 24h period. Total RNA was isolated from 1ml samples of whole blood stored in RNAlater using the RiboPure-Blood RNA isolation procedure (Ambion). Expression levels of Per2 and Bmal1 are currently being analyzed using the TaqMan system for quantitative Real-Time RT-PCR and results will be reported. The particularly high homology observed between equine and human clock genes provides incentive to consider a large mammal such as the horse as a molecular model for assessing human peripheral circadian systems.

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Appositions Between Orexin Fibers and Neuropeptide-Y cells in the Grass Rat Intergeniculate Leaflet

Joshua P. Nixon* and Laura Smale • Department of Zoology, Michigan State University, East Lansing, MI 48824

Neuropeptide-Y (NPY)-containing cells in the intergeniculate leaflet (IGL) convey non-photic information to the suprachiasmatic nucleus, but little is known about pathways through which cells in the IGL receive this information. This study evaluated the hypothesis that one route may involve cells in the lateral hypothalamus (LH) that contain orexin, a peptide associated with the arousal state of the animal. In the grass rat (Arvicanthis niloticus), the temporal pattern of wheel running is linked to that of nuclear c-Fos expression in both the orexin and NPY cell populations. Here we describe the anatomical relationship between the NPY cells in the IGL and orexin A (OXA) and orexin B (OXB) fibers in this region. Tissue from 6 male grass rats was immunohistochemically processed for the detection of OXA, OXB, OXA plus NPY, or OXB plus NPY. Both OXA and OXB fibers were present in the IGL of all grass rats examined. In double-labeled tissue, approximately half of the NPY cells in the IGL appear to be contacted by OXA or OXB fibers. OXA and OXB fibers form appositions with 55.90 ± 6.52% and 42.58 ± 6.47% of NPY cells, respectively. The nature of the spatial relationship between these cells and fibers clearly suggest the potential for functional synapses between NPY cells of the IGL and orexin fibers originating from the LH in the grass rat. These data provide further evidence that orexin may be involved in relaying feedback regarding the activity state of the animal to the circadian system.

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Effects of Simulated Microgravity on Circadian Rhythms of Cardiovascular Physiology and Clock Gene Expression in Laboratory Rats

Hillary M. Olin and Vincent M. Cassone • Department of Biology and Center for Biological Clocks Research, Texas A&M University, College Station, TX 77843

Astronauts experience cardiovascular system disruptions during and after space flight in response to microgravity. To determine whether microgravity has an effect on circadian rhythms of cardiovascular parameters, rats were subjected to simulated microgravity via tail suspension. Body temperature (BT) and heart rate (HR) were monitored using implated radiotelemeters, and analyzed in both light:dark (LD)12:12 and constant darkness (DD). Electrocardiographic recordings were taken for one day during suspension at one and two weeks, and immediately after suspension using a cross-control experimental regimen. Suspension abolished the circadian rhythm of HR without disrupting similar rhythms in BT or heart rate variability in both LD and DD. To determine whether disruption of circadian HR rhythms represented an effect on circadian output and/or core circadian regulation, rats were maintained as above but prepared for ex vivo analysis of mRNA derived from two clock genes, period2 and Brain Muscle Arnt-like 1. Tissue samples (brain, liver, heart and muscle) were removed in DD, following which quantitative real-time PCR was performed on all samples and analyzed using the €Ct method. Analysis showed that clock gene transcription was still rhythmic in all tissues concomitant with the loss of physiological rhythm in the heart. Thus, simulated microgravity affects the circadian modulation of HR in rats, most likely via direct mechanical effects on the heart itself. Furthermore, rhythmic cardiovascular function is separable from clock gene expression. The mechanisms of this separation may provide clues to the relationship between clock genes and their output(s). The National Aeronautics and Space Administration (NASA) supported this work through the NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute.

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Restoration of Rhythmicity in Per2/Cry1 Double Deficient Mice in Constant Light (LL) Conditions

Henrik Oster*[1], Robert Dallmann[2], Urs Albrecht[3], Gijsbertus T.J. van der Horst[4], Gregor Eichele[1] and Stephan Steinlechner[2] • [1] Max Planck Institute of Experimental Endocrinology Hannover, Germany; [2] School of Veterinary Medicine Hannover, Germany; [3] University of Fribourg, Switzerland; [4] Erasmus University Rotterdam, The Netherlands

The central circadian pacemaker of mammals located in the hypothalamic suprachiasmatic nuclei (SCN) is driven by cellular transcriptional/(post-)translational feedback-loops (TTLs). The period (Per1, Per2 and Per3) and cryptochrome (Cry1 and Cry2) genes play a critical role in this molecular oscillator conferring negative feedback on their own transcriptional activation and gating sensitivity to external time signals (Zeitgeber). For example, Per2 mutant mice eventually lose behavioral rhythmicity in constant darkness (DD) conditions. In contrast, Steinlechner et al. (2002) could show that constant light (LL) seems to stabilize rhythmicity in these animals. This study evaluates the effects of LL of various intensities on Per2/Cry1 double mutant mice, which ö like Per1/2 and Cry1/2 double mutants ö become immediately arrhythmic when released into DD. As expected, we could confirm that Per2-/-Cry1-/- mice are arrhythmic in DD and LL of low intensity (5 lx). At higher illumination (>300 lx), however, circadian rhythmicity was restored in these animals with a highly shortened period length (™) of 19.8 ± 0.1 hours.

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Circadian Locomotor Rhythms in Mice Are Not Altered by the Anti-tumor Drug, Cyclophosphamide

E. Todd Weber* and Rishikesh Gadhia • Rider University, Department of Biology, 2083 Lawrenceville Road, Lawrenceville, NJ 08648

Antitumor drugs such as cyclophosphamide (CYP) are effective in inhibiting tumor growth because they prevent cell division of rapidly dividing cells. Unfortunately for patients, these drugs can also have significant nervous system side-effects such as malaise, fatigue, and disrupted sleep patterns. To test the hypothesis that the non-antineoplastic effects of CYP might be mediated by the actions of CYP on the circadian timing system, we treated healthy young adult male C57BL/6 mice with CYP (at concentrations with known to mediate neurochemical and antitumor responses) or vehicle and measured locomotor activity patterns in constant darkness. Acute administration of CYP (0 or 50 mg/kg/day, i.p., for 3 consecutive days, n=10) did not significantly alter the free running period, the duration of activity, or the intensity of wheel running of mice under constant darkness, during administration, or in the 2-week period following treatment. Similarly, chronic administration of CYP to C57BL/6 mice (~15 mg/kg/day for 14 days via the drinking water, n=10) had no apparent affect on these parameters of circadian locomotor activity. These preliminary results in mice suggest that the side-effects of CYP related to sleep and fatigue in humans are not likely mediated through the same pathways that regulate the expression of circadian locomotor rhythms in mice.

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InterlockingLoops in the Molecular Mechanism Underlying Circadian Rhythms

*Emery Conrad, John Tyson • Departments of Mathematics & Biology, Virginia Polytechnic Institute & State University

In recent years, the molecular details of Drosophila melanogaster's circadian clock have become increasingly intricate, leading to a complex picture of the molecular network controlling the rhythm as a whole. This picture now includes several interconnected regulatory feedback loops and quite complicated phosphorylation behavior. The dynamics of this regulatory network in all its detail can no longer be understood by simple arguments, but requires the use of mathematical tools. Dynamical systems theory provides a means by which we can understand a large range of possible dynamic behaviors in complex models of the rhythm and can begin to understand how all the components of the network dynamically interoperate. Here we present a new candidate model of the rhythm in Drosophila and show how one can readily understand its dynamics through the use of such mathematics.

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Development of Two-dimension Manifolds for the Representation of High Dimension Mathematical Models of the Intra Cellular Mammalian Circadian Clock

Premananda P Indic, Katherine Gurdziel*, Richard E Kronauer, Elizabeth B Klerman • Brigham & Women's Hospital/Harvard Medical School, 221 Longwood Ave, Boston, MA 02115

Recent work by Leloup & Goldbeter (LG)(2002) and Forger & Peskin (FP)(2003) resulted in the development of two independent mathematical models of the intracellular mammalian circadian clock exhibiting ~24hr oscillation, entrainment by Light Dark (LD) cycles and phase and amplitude responses to light stimuli. Both models use sets of mathematical equations to represent the regulatory effects of protein products involved in the positive and negative regulatory mechanisms exerted on Per, Cry, Clock, Bmal1, and Reverb gene expression. Since the model development focused on detailing cellular mechanisms, a large number of dimensions were required to predict the biological processes: 19 dimensions for the LG model and 73 for the FP model. High dimension models require large computation time for simulation as well as analysis. To improve the computational capabilities, a two-dimensional manifold was developed for each model. Use of the Galerkin projection method (Sirovich)(1987) with two proper orthogonal functions derived from the time series of the model resulted in a transformation of the original model dimensions to two. The two-dimensional representation could predict the ~24 hr oscillations as well as entrainment to LD cycle. For future work, we propose to study the effect of light on the phase and amplitude of the pacemaker using this reduced two-dimensional model. This work was supported in part by grant F30602-01-2-0554 of Defense Advanced Research Projects Agency and National Aeronautics and Space Administration Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute.

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Theoretical Approach : Coupling Between Circadian Gene Activity and Other Cellular Process

Ivona Jakovljevic * and Friedemann Kaiser • TU Darmstadt

Circadian rhythms are investigated by theoretical approach, studding the minimal model, consists of two autonomous oscillating systems. One represents the intracellular circadian oscillator (Scheper at al., 1999), based on transcriptional/translational feedback loop (Dunlap, 1999 and Van Gelder, 2003) and other, simple, but biochemically plausible system, involving three molecular reactions (Schnackenberg, 1979). Analysing the behaviour of global system, i.e. coupled differential equations, representing two limit cycle oscillators, one could see that the main property of circadian rhythms, robustness of oscillations exists and that the period remains around 24 hours, but also that in certain region of coupling parameters, oscillatory behaviour could be complex and highly irregular. The model simulated the phase-response curves, and entrained oscillations due to light-dark cycles. Simulation was done, using deterministic as well as the stochastic approach. Dunlap J.C.(1999) Cell, 96, 271-;Van Gelder R.N., Herzog E.D., Schwartz W.J., Taghert P.H., (2003) Science, 300, 1534-;Scheper T., Klinkeneberg D., Pennartz C, Van Pelt J. (1999) Journal of Neuroscience 19(1), 40-;Schnackenebrg J., (1979) Journal of Theoretical Biology, 81, 389-;

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Characterization of the Nocturnin-Containing Deadenylase Complex

Julie E. Baggs* and Carla B. Green • Department of Biology, University of Virginia, Charlottesville, VA

Nocturnin mRNA and protein are rhythmically expressed in the clock-containing photoreceptor cells of the Xenopus laevis retina, with peak levels at night. In the mouse, nocturnin mRNA is expressed with high amplitude rhythmicity in peripheral tissues, including liver, kidney, and heart. Nocturnin functions as a deadenylase in vitro, trimming the poly(A) tail from a synthetic mRNA substrate. Deadenylation is the initial and rate-limiting step in mRNA turnover and mediates translational silencing in early development. We sought to look at the in vivo function of nocturnin to better understand its role in mRNA regulation of the circadian clock. Analysis of Xenopus retinal extracts using gel filtration chromatography reveals nocturnin in a complex approximately 2-3 times the size of the nocturnin monomer. Furthermore, nondenaturing gel electrophoresis of Xenopus embryo extracts shows nocturnin migrating at more than 150 kDa, a size much greater than the 43 kDa predicted size of nocturnin. We therefore set out to identify the proteins associating with nocturnin in this deadenylase complex. Using a bacterial two-hybrid screen, we identified 42 potential interacting proteins. In addition, using the recently published Drosophila protein interaction map, we identified three proteins that associate with the Drosophila version of nocturnin. We are currently testing the interaction of nocturnin with the Xenopus orthologues of these proteins in addition to confirming the results from our two-hybrid screen. Because this rhythmically expressed protein is part of a deadenylase complex, we hypothesize that nocturnin functions at night to deadenylate and therefore posttranscriptionally regulate specific clock-related messages.

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A C-Terminal Truncation Mutation in Zebrafish Cry1c Shortens the Period of Locomotor Rhythms

Hugo M. Borsetti*, Kimberly Mc Gee, Laura Gutierrez; Dan E. Wells and Gregory M. Cahill • Dept. of Biology and Biochemistry, University of Houston, Houston, Tx 77204

Zebrafish were mutagenized by N-ethyl-N-nitrosourea and screened for dominant mutations that alter the periods of larval activity rhythms. Among such mutations is g6, a semi-dominant mutation that shortens wild-type activity rhythm of ~25.3hr by ~1.5hr in homozygotes and ~0.5hr in heterozygotes. Systematic genome scanning with simple sequence length polymorphism (SSLP) markers localized the mutation to linkage group 8. Further meiotic mapping placed it between markers z14801 (50.5cM) and z26584 (54cM). Radiation hybrid mapping placed the cryptochrome 1c gene (cry1c) in this interval, making it a candidate. Zebrafish Cry1c has been shown to repress the transcriptional activation of Per gene expression by zebrafish Clock:Bmal1 heterodimer in transient transfection experiments. Systematic amplification and sequencing of the 15 exons revealed a mutation in exon 11. The mutation changed Gln521 (CAG) to a Stop codon (TAG), truncating the wild type 654 amino-acid protein. The truncated protein includes the N-terminal photolyase like domain (a.a. 1-488), a domain shared by all vertebrate Crys (a.a. 489-505) and a C-terminal similar to vertebrates Cry1s, but not Cry2s. The deleted segment has no similarity to any other Cry. In transient transfection assays the mutation causes a small but consistent decrease in repressor activity. We conclude that Cry1c, one of the six zebrafish Crys, has a role in the biological clock; that role depends on an intact C-terminus.

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Does Light Start or Synchronise Circadian Clocks in Culture?

Amanda-Jayne Carr*, Nicholas S. Foulkes & David Whitmore • University College London

Independent molecular clocks present within zebrafish peripheral tissues can be directly entrained by light. Cell lines derived from zebrafish embryos also display this property, and unlike mammalian cells which require non-photic stimuli to induce the circadian expression of various genes, rhythmicity can be set and sustained in these cells by exposure to a light:dark (LD) cycle. To permit the automated recording of clock gene expression in real-time, these cells have been stably transfected with a zfper4 promoter luciferase reporter construct. This provides us with an ideal tool with which to study the effects of light on clock gene expression at the cellular level. By examining the oscillations of bioluminescence in cells that have been cultured in constant darkness (DD) for over 2 months we have utilised this system to test whether light starts or synchronises circadian clocks in culture. Bioluminescence was recorded from cells cultured in DD, and from cells exposed to two 12:12 LD cycles prior to exposure to DD. Strong rhythmic oscillations were observed in cells entrained to the LD cycle, and on exposure to DD the amplitude of the rhythm decreased. Bioluminescence from cells cultured in DD was continuously high and did not exhibit equivalent circadian oscillations. Analysis of the temporal expression of zfper4 using a photon-counting camera revealed that oscillations were present within individual cells, however peak luminescence was not synchronised. These data suggest that in zebrafish the main action of light is to synchronise, rather than start, individual clocks within a population of cells.

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Perturbations Caused by Per Mutations: Phenotyping of Per1 and Per2 Deficient Mice

Robert Dallmann*[1], Urs Albrecht[2] and Stephan Steinlechner[1] • [1]School of Veterinary Medicine Hannover, Germany; [2]University Fribourg, Switzerland

The generation of transgenic animals is a very useful tool to dissect the molecular mechanisms underlying the mammalian circadian system. The phenotype of the transgenic can reveal the function of the gene of interest which is knocked-out. In case of the Period gene family, there are transgenic mice lacking Per1, Per2 and Per3. In contrast to the latter, Per1-/- and Per2-/- mice exhibit a severe circadian phenotype under constant conditions. Studies of these mutants suggested that Per1 and Per2 are essential for the proper function of the transcriptional/(post-)translational feed-back loops of the core clock. Despite the well studied effects on the circadian system, there are no data available on other aspects such as physiological and behavioral parameters. Here, we try to fill that gap by using a very broad approach including physiological (body weight, food and water intake, body temperature, corticosterone level), immunological (cytokine level, phagocytosis assay) and behavioral (open field, stress-induced hyperthermia, hot plate) parameters. We could show that Per1-/- and Per2-/- mice do differ in a variety of aspects compared to wildtype. Thus, our findings indicate that the lack of Per1 as well as Per2 perturbs not only the clock but has more consequences for the organism as a whole.

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New Regulatory Aspects of bZIP Transcription Factor E4BP4: Negative Regulation of E4BP4 by Casein Kinase 1 e

Masao Doi*(1)(2), Toshiyuki Okano(1), Irene Yujnovsky(2), Paolo Sassone-Corsi(2) and Yoshitaka Fukada(1) • (1) Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Tokyo, Japan, and (2) Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France

Light-dependent transcriptional regulation of clock genes is a crucial step in the entrainment of the circadian clock. E4bp4 is a light-inducible gene in the chick pineal gland, and it encodes a bZIP protein that represses transcription of cPer2, a chick pineal clock gene. Here we demonstrate that the prolonged light period-dependent accumulation of E4BP4 protein is temporally coordinated with a delay of the rising phase of cPer2 in the morning. E4BP4 protein was phosphorylated progressively and then disappeared in parallel with induced cPer2 expression. Characterization of E4BP4 protein revealed Ser182, a phosphoacceptor site located at the amino-terminal border of the Ser/Thr cluster forming the phosphorylation motifs for casein kinase 1e (CK1e). CK1e physically associated with E4BP4 and phosphorylated it. CK1e-catalyzed phosphorylation of E4BP4 in cultured LMH cells resulted in proteasomal proteolysis-dependent decrease of the E4BP4 protein level, and the nuclear accumulation of E4BP4 was attenuated by CK1e in a kinase activity-independent manner. The CK1e-mediated posttranslational regulation was accompanied by reduction of the transcriptional repression executed by E4BP4. These results not only demonstrate a phosphorylation-dependent regulatory mechanism for E4BP4 function, but also highlight the role of CK1e as a negative regulator for E4BP4-mediated repression of cPer2.

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Effects of Experimental Chronic Jet-lag on Clock and Cell Cycle Gene Expression

Pasquale Innominato[1,2], Elizabeth Filipski *[1], XiaoMei Li[1], and Francis L vi[1] • [1]INSERM E 0354 Ç Cancer chronotherapeutics È, H™p. P. Brousse, Villejuif, France and [2]Universitˆ G. D'Annunzio, Chieti, Italy

Disruption of the circadian coordination caused by chronic jet-lag accelerates tumor growth in mice. To understand the mechanism of this acceleration we investigated the consequence of experimental jet-lag on the expression of circadian genes and genes involved in carcinogenesis. Mice were randomly divided in 2 groups of 60 animals: one was kept in LD 12:12 and the other underwent experimental jet-lag produced by an 8-h advance of the light-dark cycle every 2 days. Ten days later mice were sacrified at six different circadian times every 4h over 24h and mRNA expression of several circadian genes was measured in the liver with quantitative RT-PCR. The 24-h rest-activity and temperature rhythms were markedly altered in mice subjected to experimental jet-lag as compared to those kept in LD12:12. Significant circadian rhythm in per2, cry1, bmal1 and reverbα mRNAs expression was demonstrated in controls. In jet-lagged mice the rhythm in cry1, bmal1 and reverbα expression was suppressed. It persisted in per2 expression, but with the peak advanced to ZT4 as compared to ZT12 in controls. There was no significant circadian variation in p53 and c-myc expression in controls. In jet-lagged mice, the level of p53 mRNA was decreased. C-myc mRNA level was increased and its expression showed significant circadian variation. This study demonstrates that experimental chronic jet-lag disturbs circadian genes expression and alters mRNA levels of genes involved in cell cycle progression (p53) and oncogenesis (c-myc), which could explain the acceleration of tumor growth. This emphasizes the role of circadian clock in malignant growth control.

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Drosophila's Second Clock Loop Is Essential for Temperature Compensation

Daniel Forger* & Justin Blau • NYU Department of Biology, 100 Washington Square East, New York

Most biochemical processes speed up as temperature increases. In contrast circadian clocks show a remarkably constant period over a wide range of temperatures (temperature compensation). Theoretical and computational work by Hastings & Sweeney and later Ruoff proposed that temperature compensation is achieved by balancing processes which speed up a clock as temperature increases with those that slow down a clock. A key prediction of this work is that when some part of the clock is removed, the rest of the clock will lose temperature compensation. The Drosophila circadian clock has two interlocked feedback loops. Here we explore, both theoretically and experimentally, how this two feedback loop structure can lead to temperature compensation. We have tested temperature compensation in flies with heterozygous mutations in either per or tim (loop 1) and either vrille or Pdp1 (loop2). We find that a clock with less vrille loses temperature compensation while a clock with less per, tim or Pdp1 is over-compensated for temperature. Therefore VRILLE may be the key clock component that slows down the clock at higher temperatures. Modeling work confirms this and shows how elements of the second feedback loop can introduce temperature compensation into a clock

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Photic Entrainment Mechanism of Per1 Rhythm

Akiko Fukuda*, Mariko Izumo, Shin Yamazaki, Hajime Tei, Carl H. Johnson • Vanderbilt University

Many biological and physiological activities show circadian rhythms. In mammals, circadian rhythms are governed by the central pacemaker in the SCN (suprachiasmatic nucleus of the hypothalamus) and are entrained by external light-dark cycles. Light pulses are known to be one of the most powerful factors that enable us to adjust to the environment. Light signals are delivered from the eye to the SCN via the RHT (retinohypothalamic tract). It is commonly accepted that glutamate is involved in the photic entrainment pathway. However, the light-signaling pathway is unclear, with multiple potential extracellular signals (e.g., glutamate, PACAP, etc.) and multiple intracellular pathways (e.g., Ca++, cAMP, MAP kinase) implicated in circadian phototransduction. The expression of most clock genes exhibits circadian oscillations. For example, the transcriptional level of the Per1 gene is high during the daytime and is low at night, and it is induced by a light pulse given during the night. Using a luminescence reporter system (Per1-luc), we found that luciferase activity driven by the Per1 (Period1) promoter shows robust oscillations in SCN slice culture in vitro. In this study, we examined the effect of various treatments on the Per1 rhythm in the SCN using the Per1-luc system.

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Role of p38 Mitogen-activated Protein Kinase in the Chick Pineal Circadian Clock System

Tsuyoshi Hirota*, Yuichiro Hayashi, Kamon Sanada, Fumiko Shimizu, and Yoshitaka Fukada • University of Tokyo

Extracellular signal-regulated kinase (ERK) and p38 are members of the mitogen-activated protein kinase (MAPK) family. ERK plays an important role for the regulation of the circadian clock system in many animal clock structures. On the other hand, p38 was originally identified as stress-activated protein kinase, and in some cases ERK and p38 serve for closely related cellular functions within a cell. Notably, it was reported that the circadian rhythm of the chick pineal cells was perturbed by various cellular stresses such as osmotic stress and temperature shifts. Therefore, we investigated the role of p38 in the chick pineal circadian clock system. Here we found that a constant amount of activated p38 was present over the day predominantly in the follicular and parafollicular pinealocytes that are potential clock-containing cells. Chronic application of SB203580, a selective and reversible inhibitor of p38, to the cultured chick pineal cells dramatically lengthened the free-running period of the melatonin secretion rhythm (up to 28.7 h). Importantly, in spite of no significant rhythmicity of activated p38 level, a 4-h pulse treatment with SB203580 delayed the phase of the rhythm only when delivered during the subjective day. These results indicate a time-of-day-specific role of continuously activated p38 in the period length regulation of the chick pineal clock, and suggest temporally separated regulation of the clock by two MAPKs, nighttime-activated ERK and daytime-working p38.

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A Role for Glycogen Synthase Kinase-3˚Ë in Mammalian Circadian Clock

*Chisato Iitaka, Koyomi Miyazaki and Norio Ishida • National Institute of Advanced Industrial Science and Technology

The Drosophila shaggy gene product is a mammalian glycogen synthase kinase-3˚Ë (GSK-3˚Ë) homologue that phosphorylates TIMELESS. We found that mammalian GSK-3˚Ë was expressed in the suprachiasmatic nucleus (SCN) and liver in mice and GSK-3˚Ë phosphorylation exhibited a circadian oscillation. Cyclic regulation of GSK-3˚Ë phosphorylation was also detected in serum-shocked NIH3T3 cells. Treatment of NIH3T3 cells with LiCl, the specific inhibitor of GSK-3˚Ë increased GSK-3˚Ë phosphorylation and delayed the phase of rhythmic clock gene expression in serum-shocked NIH3T3 cells. Over-expression of GSK-3˚Ë advanced the phase. The present data suggests that GSK-3˚Ë plays a role in the mammalian circadian clock.

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Real-time Monitoring System in Rat-1 Cell Culture: Toward the SDissection of the Mammalian Circadian Gene Expression

Mariko Izumo*, Shin Yamazaki and Carl H. Johnson • Department of Biological Sciences, Vanderbilt University, Nashville, TN, 37235

Since the first publication of rhythms of mRNA abundance in Rat-1 cells (Balsalobre et al., 1998), cultured mammalian cells have become a model system to study circadian gene expression at the cellular, molecular and biochemical levels. To facilitate studies on how cis- and trans-regulatory elements and factors are involved in the resetting, initiation, and maintenance of circadian gene expression, we have developed a cell culture-based real-time monitoring system using firefly luciferase as a reporter. We first generated a stable Rat-1 clone that has integrated a luciferase gene fused to the promoter region of the mper1 gene (promoter = 3.0 kb upstream of the transcription start site), and showed that this region contained the elements that are sufficient to drive circadian rhythmicity. The luminescence rhythms we observed are similar to the published reports of mper1 mRNA expression rhythms and the response of Rat-1 rhythms to "resetting reagents" such as serum, forskolin, et al (Izumo et al, 2003). In order to compare mper1 expression with the expression of other clock genes, we further extended the reporter assay to the promoters of mper2 (1.7 kb, a generous gift of Dr. Sassone-Corsi) and bmal1 (0.9 kb, a gift from Dr. Ikeda). These reporters not only showed circadian expression with the expected phase in Rat-1 cells, but also exhibited a robust amplitude in spite of having no canonical E-box (CANNTG) on the promoter region in these constructs. Partial analysis of the promoter structure and the comparison of the circadian expression will be reported.

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Genetic Analysis of Ectopic Clocks in Drosophila

Valerie L. Kilman* and Ravi Allada • Dept of Neurobiology and Physiology, and Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL

The Clock (Clk) gene in Drosophila and mammals lies at the center of two interdependent feedback loops. Previous work has shown (Zhao et al., Cell, 2003) that ectopic Clk expression in flies can induce rhythmic expression of cryptochrome and timeless transcripts in cells not known to harbor circadian function. Such ectopic rhythms were induced throughout the brain, suggesting that Clk expression alone is sufficient to induce a functioning clock. One prediction of this model is that Clk should be able to induce ectopic clocks, not only in adults, but in other developmental stages. To address whether Clk can induce ectopic clocks at an earlier developmental stage, we examined PERIOD (PER) protein rhythms in the brains of third instar larvae and find that Clk also induces ectopic rhythms here. These data show that ectopic clocks are also evident at the protein level. Previous work suggests that Clk is unique in its ability to induce ectopic rhythms. Thus, Clk may be a master control gene that organizes molecular clocks. To test this hypothesis, we will examine the requirements of other clock genes in ectopic clocks as well as ectopic clock induction by other pacemaker genes.

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Two Coupled Oscillators Control Morning and Evening Locomotor Behavior of Drosophila

Ying Peng, Dan Stoleru, JosŽ Agosto and Michael Rosbash • Howard Hughes Medical Institute and Department of Biology, Brandeis University, Waltham, MA 02454

Daily rhythms of physiology and behavior are precisely timed by an endogenous circadian clock. These include separate bouts of morning and evening activity, characteristic of Drosophila melanogaster and many other species including mammals. Whereas multiple oscillators have long been proposed to orchestrate such complex behavioral programs, their nature and interplay has remained elusive. By using cell-specific ablation, we show that morning and evening activity in Drosophila derive from two distinct groups of circadian neurons: the ventral lateral neurons (LNvs) and the dorsal lateral neurons (LNds), respectively. Although the two oscillators can function autonomously, cell-specific clock mutant rescue experiments indicate they are functionally coupled. The functional divergence of the two cell groups has been further examined by cell-specific gene expression profiling. Our results suggest that the two cell groups share similar and synchronous timekeeping mechanisms but that cell specific gene expression accounts for their different output specializations.

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Pas Gene Expression and Interactions Between the Molecular Pathways that Regulate Circadian Rhythms, Toxin Metabolism and Development

Xiaoyu Qu*, Gregg Allen, Yuhua Farnell, Richard Metz, Weston Porter, David Earnest • Texas A&M University

Basic-helix-loop-helix-PAS (bHLH-PAS) proteins comprise a family of transcription factors that play key roles in sensing and adapting to environmental changes. The bHLH-PAS proteins, CLOCK, BMAL and PER, are integral components of the mammalian circadian clockworks whereas the aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) are involved in development and responses to toxins (e.g., tetrachlorodibenzo-p-dioxin [TCDD]). Because many different bHLH-PAS proteins are expressed in the same cells, experiments were conducted to explore the possibility that AhR and ARNT may influence circadian clock function and that CLOCK, BMAL and PER may play some role in responses to environmental toxins. Our findings demonstrate that: the rat suprachiasmatic nucleus (SCN) contains many AhR-expressing neurons; in SCN2.2 cells, TCDD treatment affects genes mediating toxin metabolism in a manner similar to that observed in peripheral tissues, and alters clock gene rhythmicity; in an epithelial cell line (HC-11) derived from mammary gland which is a well-characterized model for toxin metabolism, clock gene expression is dependent on the state of cellular differentiation and is altered by TCDD treatment; and clock gene expression in human cancer cell lines varies as a function of their tumor invasiveness. These findings are suggestive of a role for PAS proteins in the interplay between the molecular pathways that regulate circadian rhythms, toxin metabolism and development. Supported by NIH P01-NS39546 (DE) and DOD BC01904 (WP).

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A Kinase-Phosphatase Balance in the Drosophila Circadian Clock

Sriram Sathyanarayanan*, Xiangzhong Zheng, Rui Xiao and Amita Sehgal • Howard Hughes Medical Institute, Univerity of Pennsylvania

The cycling of circadian clock proteins is controlled by transcriptional and post-transcriptional mechanisms. Since phosphorylation of Drosophila PERIOD (PER) cycles robustly over the course of a day, rhythmic phosphorylation is a likely mechanism for controlling cycling at a post-translational level. Although the kinases that phosphorylate PER have been identified, the mechanisms that drive cycling are not known. Here we demonstrate a role for Protein Phosphatase 2A (PP2A) in regulating PER protein stability and nuclear entry. Inhibitors of PP2A destabilize complexes containing PER and its partner clock protein, TIMELESS (TIM). We identified two regulatory subunits, TWINS (TWS/PR55) and WIDERBORST (WDB/B56-2), that target PP2A to the PER-TIM complex in S2 cells. In the adult fly head, expression of tws cycles robustly under control of the circadian clock. Hypomorphic tws mutants show delayed cytoplasmic accumulation and nuclear entry of PER, while over-expression of tws in clock neurons shortens circadian period and weakens the overt rhythm. Levels of PP2A are important for maintaining behavioral and molecular oscillations. Flies expressing a dominant negative PP2A catalytic subunit display long periods and arrhythmia, which is associated with a decrease in PER expression. In addition, over-expression of the PP2A catalytic subunit in clock neurons results in loss of behavioral rhythms and constitutive nuclear expression of PER. PP2A also affects PER phosphorylation in vitro and in vivo. These results suggest that the post-translational mechanisms which drive the cycling of PER levels require the rhythmic expression of PP2A. The effect of phosphatases on PER function is being investigated.

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Circadian Oscillation of Prokineticin 2 Promoter Activity after Serum Shock

Naoyuki Takashima*, Naoto Hayasaka, Atsuko Fujioka, Yasufumi Shigeyoshi • Kinki University School of Medicine

Previous studies have reported that the involvement of Prokineticin2 (PK2) in the formation of the circadian behavioral rhythm. The level of prokineticin 2 mRNA oscillates dynamically in a circadian manner in the suprachiasmatic nucleus (SCN), with a peak during the daytime and a trough during the night. In the present study, we investigated the site-dependent activity of PK2 promoter. We cloned the 3.5-k.b. fragment of 5Í-flanking region PK2, which contains 4-E-box (CACGTG) element. The fragment was subcloned into a promoterless firefly luciferase reporter plasmid. A rat embryonic fibroblast cell line, Rat-1, was transiently transfected with this plasmid. 48 hours after the transfection, we stimulated cells with 50% horse serum, which is supposed to drive circadian oscillation of clock genes in Rat-1 cells. Examination of luminescence with photomultiplier tubes (PMT) detectors system revealed a weak circadian rhythm, with peaks in phase with that of Per1: luc oscillation. Then, we prepared a 0.35-kb fragment of 5Í-flanking region of the PK2 gene containing only two CACGTG sequences, by deleting the upstream of the promoter. After the stimulation with serum, the amplitude of the oscillation was as high as that of the 3.5kb fragment. The finding suggests that the proximal 2 E-boxes mainly drive the circadian oscillation of PK2 transcription.

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Photolyase/cryptochrome Chimeras Reveal Differential Actions of N-terminal Regions in CRY1 and CRY2

Ellena A. van der Schalie and Carla B. Green • Department of Biology, University of Virginia, Charlottesville, VA 22903

Cryptochrome (CRY) plays an integral role in the negative feedback loop of the circadian clock by repressing the CLOCK:BMAL1 heterodimer, leading to down-regulation of period and cryptochrome genes. Although CRY1 and CRY2 are similar in sequence, CRY1-/- mice exhibit a short period locomotor phenotype, while Cry2-/- exhibit a long period (van der Horst et al 1999). In addition, Griffin et al 1998 suggests that CRY1 and CRY2 bind various clock proteins with different affinities. To investigate the relationship between the structure and function of the cryptochromes, we made chimeras of xcry1 and xcry2b (two repressor-type crys found in Xenopus laevis) and photolyase, a DNA repair enzyme that has high sequence similarity with CRY, but does not share its repressive function. Our goal was to test which regions of CRY are necessary for repression, without damaging the protein's structural integrity. We cloned two chimeras, one with the first half of xcry1 and the second half of photolyase and the other with the first half of xcry2b and the second half of photolyase. When these two chimeras were assayed for repression of CLOCK:BMAL1 heterodimer in a transient transfection luciferase assay, they displayed an intermediate level of repression and similar dose-response profiles. Despite these similarities, CRY1/Photolyase chimera localized primarily to the cytoplasm, while the analogous CRY2b chimera localized to the cytoplasm and nucleus evenly. This suggests that regions of CRY1 and CRY2b in the N-terminus of each protein may be differentially important for efficacy of nuclear localization, interaction with CLOCK:BMAL1, or repressive function.

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Tissue-specific Regulation of mPer Expression

Charlotte von Gall*[1], Choogon Lee[2], Jason P. DeBruyne, Steven M. Reppert, and David R. Weaver • Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, 01605 USA; [1]present address: Institute of Anatomy II, J.W Goethe-University, 60590 Frankfurt, Germany; [2]present address: Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL 32306

The suprachiasmatic nucleus (SCN) contains the central circadian pacemaker that regulates circadian rhythms and synchronizes peripheral oscillators in mammals. At the molecular level circadian oscillations are based on the cell-autonomous rhythmic expression of clock genes. Rhythmic clock gene expression can be found in the SCN and in peripheral tissues. Little is known about the similarities and differences in molecular mechanisms of circadian rhythms between the SCN circadian pacemaker and these peripheral oscillators. Therefore, we compared the impact of disruption of the Bmal1 (Mop3) gene on circadian clock gene and protein expression levels between the SCN, the forebrain and the liver. We found Clock mRNA levels to be unaffected in BMAL1-deficient mice, indicating that BMAL1 is dispensable for Clock transcription. Nevertheless, BMAL1 is crucial for the phosphorylation and nuclear accumulation of CLOCK protein in all tissues analyzed. However, the impact of BMAL1 deficiency on Period gene products was remarkably tissue-specific. Rhythmicity of mPer1 and mPer2 mRNA expression and protein levels in the SCN was strongly dependent upon BMAL1. In contrast, in the forebrain mPer2 and in the liver both mPer1 and mPer2 mRNAs and proteins were relatively unaffected by the absence of BMAL1.

These data reveal that mPer gene expression is differentially regulated in the SCN and in the periphery. This unexpected tissue specificity may have important implication for understanding the distinct properties of master and slave oscillators. The present results also suggest that the SCN master oscillator may be even more sensitive than other tissues to loss of rhythmicity following disruption of circadian genes.

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Attenuated per1 Expression in Zebrafish bmal1 Morphant Embryos

Han Wang*, Veronica Hochla, Andrew Abatiell, and Eric Lee • Department of Zoology, University of Oklahoma, Norman, OK 73019

Unlike the fruit fly, whose embryogenesis takes approximately one day, zebrafish embryonic development spans approximately 72 hours. Zebrafish eggs are fertilized outside the mother's body and the resulting embryos develop in the external environment. Mammalian embryos, in contrast, are kept inside the mother, and the mother is able to synchronize the circadian rhythms of the fetuses by rhythmically secreting melatonin. Zebrafish are a unique and important circadian model system. Using the whole-mount in situ hybridization, we show that zebrafish per1 is rhythmically expressed in the eyes and the brain in a robust fashion during days 3 and 4 of early development (48 to 92 HPF, hour postfertilization). The similar rhythmic expression pattern is maintained under constant darkness, and an 8:8H (ligh/dark) cycle induces an ultradian expression of per1. Furthermore, to examine whether a similar negative transcription/translation-based feedback loop also controls this zebrafish embryonic circadian clock, we perturb CLOCK:BMAL1 heterodimer by knocking down BMAL1 translation using antisense morpholino oligos. In comparison with that in uninjected control embryos, expression of per1 is significantly attenuated in bmal1 morphant embryos. Taken together, it appears that zebrafish have evolved an embryonic circadian clock, which is likely controlled by the conserved negative transcription/translation-based feedback loop. Supported by Whitehall Foundation Grant 2002-12-103-RES to HW.

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Long-period Rhythms Caused by Twins-Protein Phosphatase 2a

Meg A. Younger*, Shawn A. Cyran, and Justin Blau • New York University, 100 Washington Square East, New York, NY 10003

In one feedback loop of the Drosophila circadian clock, Period (Per) and Timeless (Tim) are required to repress transcription of their own genes. To maintain circadian oscillations in per and tim transcription it is necessary to separate the phases of transcription and repression. This is achieved in part by regulating accumulation of the Per/Tim complex in the cytoplasm, and by delaying Per/Tim nuclear entry. The participation of at least three kinases (Double-time, Shaggy, and CK2) in the Per/Tim cycle emphasizes the importance of phosphorylation in the molecular clock. It was recently shown that Protein Phosphatase 2a (PP2a) is also involved in the circadian clock, and that over-expressing one of its regulatory subunits, encoded by the gene twins (tws), initially shortens the period of the clock and then degenerates into arrhythmicity. However we identified an allele of tws in an over-expression screen that causes stable 26-hour behavioral rhythms. Over-expressing this allele in clock neurons delayed Per accumulation and nuclear entry, which was previously reported in loss-of-function tws mutants. Therefore we may have identified a dominant-negative tws allele. There are two different Tws isoforms which differ only in their N-termini. Therefore the N-terminus of Tws may direct the substrate specificity for PP2a, and we are currently testing this hypothesis.

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Light-activated IGL Neurons in Rats Are Not NPY-immunoreactive

Stephen Thankachan[1] and Benjamin Rusak[1,2]* • Depts. of [1]Psychology, [2]Psychiatry and Pharmacology, Dalhousie University, Halifax, Nova Scotia B3H 4J1 Canada

The intergeniculate leaflet (IGL) contributes to both photic and non-photic entrainment of mammalian circadian rhythms. IGL neurons contain a number of neurochemicals, including neuropeptide Y (NPY), GABA and enkephalin. Cells containing NPY that project to the suprachiasmatic nuclei have been implicated in mediating non-photic effects, but those mediating photic effects have not been characterized. We used juxtacellular recording/labeling methods in rats to investigate the neurochemical characteristics of different classes of IGL neurons. Photic responses of single neurons in and near the IGL were recorded extracellularly in urethane-anesthetized rats. Recorded cells were labeled with neurobiotin and the tissue was then immunostained for NPY. Confocal fluorescence microscopy was used to identify neurons containing neurobiotin, NPY or both. Among cells in the dorsal and ventral lateral geniculate nuclei and IGL that were successfully labeled with neurobiotin (76%: 63/82), 44 were activated and 10 suppressed by retinal illumination, while 9 were not responsive. Of 15 neurobiotin-labeled cells in the NPY-immunoreactive region defining the IGL, 12 were activated by retinal illumination, 1 was suppressed and 2 were unresponsive. None of these cells was double-labeled for NPY, although many adjacent, NPY-stained cells were visible in the same sections. The somata of three activated and two unresponsive IGL neurons contained a few small NPY-immunoreactive profiles, which appear to be NPY-containing terminals projecting to them. These results indicate that photically responsive IGL neurons are not NPY immunoreactive, but some receive NPY projections. Current studies are attempting to characterize photically responsive IGL neurons by double-labeling them for other neurochemicals. Supported by CIHR of Canada.

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Individual Differences in Rhythms of Sleep and Its Neural Substrates in Nile Grass Rats

Michael D. Schwartz* and Laura Smale • Neuroscience Program, Michigan State University, East Lansing, MI 48824

Circadian rhythms in sleep are governed by interactions between homeostatic and circadian mechanisms, but the neural substrates of this interaction are unknown. Here, we explore the relationship between ventrolateral preoptic area (VLPO) function and sleep behavior in Nile grass rats. Males were singly housed with running wheels in a 12:12 light/dark cycle, videotaped for 24 hours and then perfused at zeitgeber time (ZT) 4 or 16; brains were then processed for cFos-immunoreactivity (-ir). Animals were designated as "day-active" or "night-active" on the basis of wheel running behavior. Although day- and night-active animals differed with respect to levels of sleep during the light and dark portions of the LD cycle, respectively, they were the same with respect to total sleep in a 24 h period. In both groups sleep was more fragmented during daytime than nighttime sleep. In day-active animals cFos-ir in the VLPO was higher at ZT 16 than at ZT 4, and was positively correlated with levels of sleep in the 2 h preceding sacrifice. However, in night-active animals cFos-ir in the VLPO was the same at ZT 4, when sleep was high, as at ZT 16, when levels of sleep were very low. Furthermore, in these animals cFos-ir was not correlated with sleep in the 2 h preceding sacrifice. This clear dissociation between behavioral sleep and cFos-ir in the VLPO in night-active grass rats raises the possibility that the sleep-promoting role of cells in this area is compromised during the day. Supported by NIMH 53433

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Fast and Slow Resetting of Circadian Oscillations Within the Suprachiasmatic Nuclei

Henk Albus, Mariska J. Vansteensel*, Gene D. Block, Johanna H. Meijer • Leiden University Medical Center

Circadian rhythms are driven by the circadian pacemaker of the suprachiasmatic nucleus (SCN). We performed electrical impulse activity recordings in slices of the rat SCN that were prepared after a six-hour delay of the light-dark cycle, followed by constant darkness. On the first day after the shift, we found bimodal electrical activity patterns in 67% of our recordings. One component was shifted completely, while the other was not significantly shifted. On the third day in constant darkness, the bimodal electrical activity patterns were present in 42% of our recordings, with one component completely shifted and the other incompletely shifted. When recordings were made on day six in constant darkness, the components had resynchronized, resulting in a single peak that was completely shifted. A cut through the SCN slice revealed a single fast resetting component in the ventral SCN and a single slow resetting component in the dorsal SCN. The data indicate that fast and slow resetting oscillations in electrical activity are transmitted through the SCN in intact slices and play a role in regulating circadian behavior.

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Ectopic Expression of NaChBac Channels in Drosophila Melanogaster Pacemaker Neurons Alters Circadian Periodicity

Vasu Sheeba*, Michael Nitabach, Todd Holmes • New York University

Relatively little is known about the manner in which the information from core clocks in the pacemaker neurons is transmitted to other cells and tissues. Electrical activity of pacemaker neurons in mollusks, flies and mammals has been implicated in circadian regulation. Previous studies from our laboratory have shown that electrically silencing the pacemaker neurons of Drosophila by the expression of modified open rectifier potassium channels disrupts both the locomotor activity rhythm phenotype as well as the circadian molecular oscillations of core clock proteins PERIOD and TIMELESS (PER and TIM). We have now used an alternate method to alter the electrical properties of the pacemaker membrane, which is to cause hyperexcitability of neurons by expression of a bacterial sodium channel (NaChBac). NaChBac expression in Xenopus oocytes results in depolarization-activated large inward currents with slower activation and inactivation kinetics as compared to animal sodium channels. Transgenic fly lines that express the NaChBac channel were created. Expression of the NaChBac channel does not kill pacemaker neurons or alter gross pacemaker neuron morphology. The circadian locomotor activity periodicity of these lines is altered significantly. They also exhibit a phenomenon called "splitting", wherein the locomotor activity pattern shows both short and long period components. Preliminary studies indicate that the circadian molecular oscillations of PER do not differ between control and pacemaker neuron-expressing NaChBac flies after 24 hours of constant darkness. NaChBac expression in pacemaker neurons rescues rhythmic locomotor activity in lines where the expression of open rectifier potassium channels had caused arrhythmicity.

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Circadian Expression of Clock Genes in Cultured Chicken Granulosa Cells

Nobuhiro Nakao *[a], Shinobu Yasuo[a], Shizufumi Ebihara, Takashi Yoshimura[a] • Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601

Circadian rhythms in physiology and behavior are regulated by the circadian clock genes, which are expressed in most of the tissues. Although it is known that circadian clock is concerned with control of the ovulation-oviposition cycle in birds, the ovulatory clock that determines the ovulation-oviposition cycle is not clarified. In the previous study, we have reported the circadian expression of clock genes in the largest follicle (F1) of the Japanese quail ovary in relation with the ovulation-oviposition cycle. In the present study, clock gene expression was examined in the granulosa cells in vitro. Chicken (Gallus gallus) granulosa cells were isolated from the F1 and were cultured. Expression of clock genes was examined by real-time RT-PCR. Rhythmic expression of clock gene observed in cultured chicken granulosa cells. This result indicates the possibility of the presence of circadian clock in the largest follicle of bird. More noteworthy is that progesterone is produced rhythmically in the granulosa cells of the largest follicle and lead to ovulation, which coincides well with the present results. Together with previous reports, it appears that the circadian clock in the largest follicle is the pacemaker component which generates the ovulation-oviposition cycle. [a]This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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Protein Kinase G-type Ii Is Required for the Night-to-day Progression of the Mammalian Circadian Clock

Shelley A Tischkau; Jennifer W Mitchell; Julie S Pendergast; Laura A Pace*; Martha U Gillette • University of Illinois at Urbana–Champaign

Circadian rhythms are generated within the SCN by a complex transcriptional-translational feedback loop. Much like the checkpoints observed in the cell cycle, we propose that a checkpoint controls the transition of clock state from the night-time domain into the day-time domain. Previous work from our laboratory has shown that a window of sensitivity to cGMP/PKG activation exists at the end of the circadian nocturnal domain. Application of inhibitors to guanylyl cyclase or PKG activity during this time cause delays in the rhythms of neuronal activity and Per1 mRNA expression. In addition, intra-SCN injection of KT5823 in vivo causes phase delays in mouse circadian wheel-running activity. In this study, we show that inhibition of the cGMP/PKG activity during this window of sensitivity forces the clock to repeat the last 3.5-h of the circadian cycle. Furthermore, a 15-min pulse of KT5823 followed 1 h later by another 15-min pulse, resulted in a ~ 7-h phase delay. This is the sum of two 3.5-h delays, indicating that there exists a critical cGMP/PKG-mediated checkpoint at ~ CT 22 that requires the activity of cGMP/PKG in order for the clock to progress into the day-time domain. The SCN expresses two isoforms of PKG, I‰ and II. We have found that antisense oligodeoxynucleotides directed against the Type-II isoform and not the Type-I‰ isoform mimic the phase delays observed with the KT5823 treatments. Also, chronic inhibition of PKG-II disrupts electrical activity rhythms. This work indicates that the Type-II isoform of PKG mediates this essential checkpoint. Supported by NIH Grants NS22155 and Hl67007 (M.U.G.), NS10170 (S.A.T), and NS11158 (J.W.M).

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Time Memory in Mammals

Sean W. Cain, Caroline H. Ko, Jennifer A. Chalmers, Paula Schumacker, Thomas Tsai & Martin R. Ralph • University of Toronto

A variety of evidence suggests that animals are predisposed to anticipate the recurrence of significant events at 24-hour intervals. These events include the regular daily cycles tied to celestial motion, as well as those that could occur at any time of day or night. While one behavioral regulatory system involves a circadian hierarchy organized by the suprachiasmatic (SCN) clock, time memory can operate independently, but likely does so in concert with the SCN. We present a group of experiments demonstrating circadian modulation of performance of various tasks in three rodent species. In general, our results indicate that performance of learned tasks in hamsters, rats and mice varies according to the time of day that the tasks have been learned. Responses to cues that predict either reward or punishment are mitigated by the phase relationship between the time of training and the time of testing. As time of day is not a discriminative cue in our tasks, these animals appear to be predisposed to register the time of stimulus presentation, and to anticipate the recurrence of the event at 24-hour intervals. The results suggest the existence of a second circadian organization in rodents, substantiating the notion of non-SCN based time memory in animals. Learning of this type may be generalizable to a range of important stimuli, and may contribute to such phenomena as food entrainment. Supported by a grant from the Natural Sciences and Engineering Council of Canada to MRR.

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Scheduled Food Deprivation in Mice Reverses Patterns of Immediate-early Gene Expression Induced by Acute Deprivation

Marleen H.M. de Groot* and Benjamin Rusak • Dalhousie University

Mice exposed to regular fasting and refeeding cycles show increased activity and other changes in anticipation of the scheduled feeding time, while acute food deprivation of equivalent duration induces very different responses. We investigated the neural substrates underlying these differences by studying the patterns of immediate-early gene (IEG) products (c-Fos, Jun-B and NGFI-A) in the brains of mice in these two conditions. Male mice (C57BL/6J) were fed ad libitum, food deprived once for 19.5 h, or restricted to 4 h of food access daily for 12 days. Mice in all three groups were killed with a barbiturate overdose at the same time of day; namely, 19.5 h after food was removed for both the acutely and chronically fasted animals. Acute food deprivation caused a global increase in expression of one or all of the IEGs in most of the 13 brain structures examined. A similar duration of deprivation following adaptation to the fasting-feeding cycle did not cause similar increases in IEG expression. Only one structure responded with increased levels of NGFI-A in the chronically deprived mice. Adaptation to a restricted feeding schedule appears to involve suppressing the activations caused by acute food deprivation in most brain structures. These differences may reflect long-lasting functional changes in these structures that are induced by cycles of fasting-refeeding, and that do not require further IEG expression for their maintenance. Supported by NSERC and CIHR of Canada

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Activation of MT2 Melatonin Receptors Phase Shifts Circadian Rhythms of Neuronal Firing in the SCN Brain Slice But Did Not Affect the Circadian Phase of Running Wheel Activity in the MT1 Knockout Mice

Hudson, RL* and Dubocovich, ML • University of Illinois at Chicago

The goal of this study was to further explore the involvement of the MT2 melatonin receptor in melatonin-mediated phase shift of circadian rhythms in MT1 knockout (KO) C57BL/6 mice. C57BL/6 mice of either sex (4-8 months old) were placed in cages with running wheels in constant dark. In wild type mice, melatonin (90 ug/mouse, ip) administered at CT 10 significantly phase advanced the onset of running-wheel activity (0.6 + 0.09 h, n=41, $p < 0.05$) as compared to vehicle treated (-0.02+0.07 h, n=28) mice. In contrast, in C57BL/6 MT1 KO mice melatonin (-0.12+0.07 h, n=43) did not phase shift the onset of running wheel activity as compared with vehicle (-0.07+0.12 h, n=41) treated mice. In vivo results differ sharply from those obtained in vitro. Using single-unit recordings, melatonin (10 pM) phase advanced the peak (CT 6.4 + 0.3, n=3) of circadian rhythm of neuronal firing rate (6-10 Hz) by 3.6 + 0.3 h, (n=3) and 3.4 + 0.1 h, (n=3) in the SCN brain slice from wild-type and MT1 KO C57BL/6 mice, respectively. These phase advances are mediated through activation of the MT2 receptor in the SCN as they are blocked by the competitive MT2 melatonin receptor antagonist, 4P-PDOT (10 nM). Together, the results suggest that in vivo melatonin-mediated phase advances of running wheel activity onset may also require activation of the MT1 melatonin receptor. Supported by MH 52685 (MLD).

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Morphine Withdrawal in Per1 and Per2 Mutant Mice

Stephanie Perreau-Lenz*[1], Tarek Zghoul[1], Carles Sanchis-Segura[1], Brandon Cline[1], Urs Albrecht[2] and Rainer Spanagel[1] • [1] Department of Psychopharmacology, Central Institute of Mental Health, Mannheim, Germany; [2] Department of Biochemistry, University of Fribourg, Switzerland

Growing evidence confirms the involvement of the clock genes Per1 and Per2 in the modulation of drug-induced behavioral effects. Recently, Per2 has been shown to be one of three genes over-expressed in the frontal cortex of rats subjected to morphine withdrawal. These results strongly suggest that Per2 is specifically involved in neuronal changes observed after a chronic morphine treatment. Therefore, we successively studied morphine withdrawal and tolerance in Per1 and Per2 mutant mice as compared to wild-type mice. Tolerance to the analgesic effect of morphine was measured using the hot-plate and tail-flick tests. Dependence to morphine was induced via chronic injections of increasing doses, twice a day (ZT3 and ZT15). Withdrawal signs as well as locomotory activity were observed for 30 minutes after a single naloxone injection. Although acute effect of morphine did not vary between Per2 mutant and wild-type mice, Per2 mutant mice showed reduced degree of tolerance and attenuated withdrawal syndromes. Interestingly, preliminary results obtained in Per1 mutant mice seem to infer a decreased degree of tolerance as compared to wild-type mice, as well. Although the involvement of Per1 needs further investigations, the present results confirm the involvement of Per2 in neuronal adaptations with respect to repeated exposure to morphine.

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5-ht-induced Phase Advances of Scn Neuronal Firing in 5-ht7 Ko Mice: Mediation by 5-ht1a and Possibly 5-ht5a Receptors

J. Sprouse*, L. Reynolds, X. Li. • Pfizer Global Research & Development, Groton, CT

Peaks of neuronal firing in the SCN can be shifted by serotonergic agents, and yet selectivity issues with the available pharmacological tools have limited progress in establishing receptor mechanisms. As an alternative strategy, mice were bred (C57/BL6) lacking one 5-HT receptor, the 5-HT7. In hypothalamic slices prepared from KOs and wildtypes (WTs), the peak of activity occurred at nearly the same time, ZT4.3 ± 0.1 and 4.2 ± 0.6, respectively, where ZT0 marks the beginning of the light phase in a 12:12 LD cycle. When applied at ZT6 (10 uM, 10 mins), 8-OH-DPAT, a 5-HT1A/7 agonist, advanced neuronal firing to the same time in KO mice (ZT2.0 ± 0.1 h) as that observed in WTs (ZT2.1 ± 0.5 h). Co-application with WAY-100,635 (10 uM), a highly selective 5-HT1A antagonist, returned the peak toward control in both KO and WT animals. Such a phase advance, via 5-HT1A receptor activation, is clearly distinguished from that previously shown in rats to be mediated by 5-HT7 receptors. Bath application of 5-HT itself (1 uM) at ZT6 also yielded identical phase advances in KO and WT mice (ZT2.5 ± 0.4 vs. 2.1 ± 0.2 h) and was also sensitive to WAY-100,635. Unlike 8-OH-DPAT, 5-HT-induced phase advances, in both KO and WT mice, were also blocked by ritanserin, a 5-HT5A/7 antagonist. Taken together, these results highlight the importance of species dependence, and more provocatively, support the involvement of multiple 5-HT receptors in shifting the phase of circadian rhythms at ZT6.

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Effects of Chronic Methamphetamine Application on Circadian Wheel Running Activity in C57bl/6j, C3h and Per1-luc Mice

Ozgur Tataroglu* and Michael Menaker • University of Virginia

Methamphetamine (METH) is a psycho-stimulant which affects the circadian system of rodents in a unique way. Previous studies in rats have shown that when METH is given chronically via drinking water, animals' activity rhythm becomes desynchronized from the light/dark cycle and free runs in DD with a period length that is longer than 24 hours and is dose dependent. Furthermore, METH reversibly induces locomotor rhythmicity with a period longer than 24 hours in both suprachiasmatic nucleus lesioned arrhythmic rats and CLOCK mutant arrhythmic mice. Although there are many studies in rats and some in mice, there is no systematic study of the effects of METH on circadian wheel running rhythms in different strains of mice. We provided various doses of METH in drinking water and recorded circadian wheel running behavior in DD in C57BL/6J, C3H and Per1-Luc transgenic mice (per1-luc transgene on a C57BL/6J background). METH lengthened the period of the activity rhythm in all C57BL/6J and per1-luc mice, but changes in period length were inconsistent in C3H. Additionally, activity rhythms lengthened rapidly, after 5 days in all C57BL/6J and Per1-Luc mice. In contrast to previous literature on rats, our mice did not show multiple rhythm components. Optimal dose and the tolerance limits were strain dependent. Our results suggest that mice have advantages over rats as models in which to study circadian responses to METH. In particular, the availability of mutant and transgenic circadian mice should aid in the study of the mechanisms underlying the response of the circadian system to METH.

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Circadian Responses to Light in the Rpe65 Knockout Mouse

Susan Doyle[1]*, Ana Maria Castrucci[2,3], Justin Baynham[1], Daniel J. Bodmer[1], Ignacio Provencio[2], and Michael Menaker[1] • [1]Dept. Biology, University of Virginia, Charlottesville, VA, USA; [2]Dept.of Anatomy, Physiology & Genetics, Uniformed Services University, Bethesda, MD, USA; [3]Dept. of Physiology, University of S‹o Paulo, Brazil

In mammals, a subset of intrinsically photosensitive retinal ganglion cells express the putative photopigment melanopsin and, together with rod-cone systems, provide light information necessary for circadian photoreception. The biochemical steps of phototransduction and the visual cycle in these cells are unknown. We examined the effects of light on circadian locomotor activity in a line of mice with targeted disruption of RPE65, a protein of the retinal pigment epithelium involved in the generation of 11-cis-retinaldelhyde, the chromophore of most opsin-based photopigments. Rpe65 knockout (KO) mice almost completely lack rod and cone function; we found that in contrast to the unattenuated circadian photosensitivity reported for rodless/coneless mice (Freedman et al., 1999), Rpe65KO mice showed severe defects in circadian entrainment and photosensitivity. Activity onset in a 12-hour light:12-hour dark cycle occurred significantly earlier in knockouts (125±32 min. before lights off) than in age-matched wild type (WT, 10.7±8.6min.) or heterozygote (HET, 15.6±16.0min.) littermate controls. In addition, no phase shifting response distinguishable from handling controls could be detected from knockout mice in response to a 15 minute half-saturating (0.1μW/cm2) light pulse at either 480nm (KO, 13.0±8.0min., HET, 135.1±14min., WT, 156.4±11min.) or 515nm (KO, 11.3±9.5min., HET, 110.3±13.3min., WT 109.2±18min). The number of melanopsin-positive ganglion cells, estimated by immunocytochemistry in retinal flat-mounts, did not differ between Rpe65KO mice and controls. These results demonstrate that RPE65 is required for normal circadian responses to light and suggest that melanopsin-containing retinal ganglion cells, as well as rods and cones, are supplied with chromophore via a mechanism dependent on RPE65.

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Retinal Input to the SCN : An Electrophysiological Study in Normal and Retinal Degenereted Rats

Drouyer E.*, Rieux C., Hut R.A., Cooper H.M. • INSERM U-371, Cerveau et Vision, 69675 Bron, France, Institut fŽdŽratif des Neurosciences (IFNL), Lyon, France

In mammals, physiological and behavioral circadian rhythms are regulated by the suprachiasmatic nucleus (SCN), the main endogenous pacemaker. Photic information, conveyed from the retina to the SCN is essential for the animal to synchronize activity and internal physiology to the external day-night cycle. Recent studies show that circadian photoreception involves two distinct phototransduction systems: classical photoreceptors (rods and cones) and a population of intrinsically photosensitive melanopsin containing ganglion cells. The aim of this study is to characterize the roles of classic photoreceptors and melanopsin ganglion cells in the light encoding mechanism. The strategy involves the comparaison of normal control animals (rat) with an adult model of selective degeneration of rods and cones induced by a photoxic exposure to ultraviolet light. The response to monochromatic light stimulation is investigated using single unit microelectrode electrophysiological recordings in the SCN. The results show that SCN light-responsive neurons are characterized by a sustained response to light and by a long delay to recover the basal firing rate (post-stimulation persistence), the amplitude of which depends on light level. An

inexpected result is that the absence of rods and cones do not induce major modification in the response of SCN neurons to light. Slight alterations are observed in the amplitude, latency, post-stimulation persistence, and sensitivity of the response. Based on a comparison between the two groups, we have suggested a model to explain the possible interactions between melanopsin expressing retinal ganglion cells and classicals photoreceptors. Support : 5th PCRD (QLK6-CT-2002-02258), ACT INSERM, ACI MRT, Marie Curie (#QLK4CT199951420)

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Reduced Light Response of the Neuronal Firing Activity in the Suprachiasmatic Nucleus of Cryptocrome-deficient Mice

Takahiro J. Nakamura*, Shizufumi Ebihara and Kazuyuki Shinohara • Division of Neurobiology and Behavior, Department of Translational Medical Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8523, Japan; Division of Biomodeling, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.

To examine roles of Cryptocromes (Cry1 and Cry2) in circadian photoreception in mammals, we recorded the neuronal firing activity in the suprachiasmatic nucleus (SCN) and retinal ganglion cells in vivo after retinal illumination in Cry1 and Cry2 double-knockout (Cry-deficient) mice. In wild-type mice, the retinal illumination changed the firing rate in 96 % of the recorded SCN cells at night, whereas only 28 % of SCN cells changed their firing frequency during the daytime. In Cry-deficient mice, however, 45 % of SCN cells responded to light during the daytime, and 35 % of SCN cells responded at night. From these results, it is concluded that the proportion of light-responsible cells differs between the daytime and night in wild-type mice, but does not in Cry-deficient mice. Furthermore, the amplitude of photic response in SCN cells at night was significantly lower (1.3-fold of spontaneous firing) in Cry-deficient mice than that in wild-type mice (4.0-fold of spontaneous firing). In the retinal ganglion cell axon near the SCN, there was no difference in the proportion of light-responsive fibers between daytime and night in both mice. However, the response amplitude in retinal fibers was high at night and low during the daytime in wild-type mice, but no day-night difference was observed in Cry-deficient mice. These results suggest that Cryptocromes play some roles in the circadian photoreception in mice.

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Do M-cones and Rods Contribute to Circadian Photoreception?

Dkhissi-Benyahya O.*,[1,2], De Vanssay W.[1,2] , Flamant F.[3] and Cooper H.M.[1,2] • [1] INSERM U-371, Lab. Cerveau et Vision, Bron, France ; [2] IFR 19, Institut Federatif des Neurosciences ; [3] Lab. Biologie Moleculaire et Cellulaire, ENS, Lyon, France

The main circadian oscillator in the hypothalamic suprachiasmatic nucleus (SCN) is entrained to the day/night cycle by photic information transmitted from the retina. This photic entrainment is observed in the absence of classical rod and cone photoreceptors. Recent evidence suggests that melanopsin, expressed in a subset of intrinsically photosensitive ganglion cells that project to the SCN is a primary candidate for light mediated entrainment. Melanopsin knockout mice show a decrease in their ability to respond to changes in light intensity compared with normal mice, suggesting that multiple photopigments are involved in setting the circadian clock. In order to dissect out the role of different photoreceptors in clock mechanisms,

we used a knockout mouse (Trb1-/b2-) which is characterized by an absence of MW cones coupled with an over-expression of SW opsin. We examined entrainment to light and light-induced phase shifts by monitoring locomotor activity in groups of KO and normal mice. Photic entrainment was assayed using 3 levels of irradiances (1, 10 and 100 lux). To measure light-induced phase shift, mice were exposed to 15 min of monochromatic light at two wavelengths (360 and 530 nm) and 4 irradiance levels (1010 to 1014 photons/cm2/s). The results show that Trb1-/b2- knockout mice entrain to a light/dark cycle with reduced sensitivity compared to wild type mice. The KO mice show a robust phase-shift after a light pulse at 360 nm, however, the magnitude of the response at 530 nm is significantly reduced compared to wild type mice. These results demonstrate for the first time that M cones contribute significantly whereas rods play a minor role in constructing the photic signal that is transmitted to the circadian clock. Support : 5th PCRD (QLK6-CT-2002-02258), ACT INSERM, ACI MRT

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Classical Photoreceptors Are Required for the Daily and Circadian Expression of Melanopsin mRNA Expression in the Retinal Ganglion Cells of Rats

Katsuhiko Sakamoto*, Cuimei Liu, Gianluca Tosini • Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA 30310-1495, USA

Recent studies have demonstrated that melanopsin is the most probable candidate as a photopigment of the mammalian circadian system. This novel opsin-like is expressed in retinal ganglion cells (RGC) that are intrinsically sensitive to light, perhaps responding via a melanopsin-based signaling pathway. In this study we investigated the pattern of expression of melanopsin mRNA in light:dark (LD) and in constant darkness (DD), and the effect of photoreceptor degeneration on such expression pattern. Sixty days old tan-hooded RCS/N-rdy+ rats homozygous for the normal rdy allele and age-matched RCS/N-rdy homozygotes with retinal dystrophy were used in this study. At this age degeneration of the retina in RCS/N-rdy has advanced to the point where photoreceptors are both histologically and functionally undetectable. In RCS/N-rdy+ melanopsin mRNA levels were rhythmic in LD and DD ($P < 0.01$), whereas in RCS/N-rdy mRNA levels were greatly reduced (< 90%) and not rhythmic ($P > 0.1$). Photoreceptor degeneration did not affect the expression of Thy-1 (a marker for ganglion cells) mRNA ($P > 0.1$). In situ hybridization demonstrated that although melanopsin transcripts are still present in RGC of RCS/N-rdy rats the signal is much weaker than that observed in control (RCS/N-rdy+) animals. Our results suggest that classical photoreceptors (cones and rods) may control the expression of melanopsin mRNA in the rat. Supported by NIH grant NS43459 to G.T.

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Npy Microinjected into the Scn Suppresses Pineal Melatonin During the Late Night

K.L. Gamble[1,2]*; M.C. Karom[3]; G. Tosini[4]; H.E. Albers[1,2,3] • [1]Dept Psychol, Ga State Univ; [2]Ctr for Behav Neurosci, Ga State Univ; [3]Dept Biol, Ga State Univ; [4]Neurosci Inst, Morehouse Sch Med, Atlanta, GA, USA.

Light exposure at night phase shifts the primary mammalian circadian pacemaker, the suprachiasmatic nucleus of the hypothalamus (SCN), and suppresses melatonin levels in the pineal gland. Neuropeptide Y (NPY) released within the SCN appears to mediate the ability of nonphotic stimuli to phase shift circadian rhythms and inhibit the ability of light to phase shift rhythms. Because light suppresses pineal melatonin via a SCN-mediated pathway, we tested the hypothesis that NPY injected into the SCN region also reduces light-induced suppression of pineal melatonin. Male Syrian hamsters in constant darkness were microinjected (200nl) with 0.23mM NPY or vehicle into the SCN region followed by a 10-min, 50-lux light pulse or sham light pulse at CT 20. Radioimmunoassay of pineal melatonin levels revealed that animals exposed to light had significantly lower pineal melatonin levels than controls. NPY did not attenuate light's suppression of melatonin. Surprisingly, NPY alone significantly reduced pineal melatonin levels. This ability of NPY to mimic the effect of light in the suppression of pineal melatonin was unexpected because NPY inhibits the ability of light to induce phase shifts. In addition, intra-SCN injection of either an NPY Y2 or Y5 agonist did not suppress melatonin, even though injection of this same NPY Y5 agonist is sufficient to inhibit light-induced phase shifts at night. Taken together, these results suggest that NPY may have a dual function during the night, mimicking the effects of light on pineal melatonin production and inhibiting the effects of light on phase shifts of the circadian clock. Supported By: MH67420 (KLG), MH58789 (HEA), & NS043459 (GT)

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Functional Genomics of the in vitro Chick Pineal

Stephen Karaganis*, Vinod Kumar, and Vincent M. Cassone • TAMU

Cultured pineal cells exhibit all the characteristics of a complete circadian system, making the in vitro pineal a particularly useful model for exploring the relationship between inputs to the clock (both photic and noradrenergic entrainment cues) with expression of canonical clock genes and genes involved in melatonin biosynthesis. Here, we investigated the expression of nearly 8000 genes expressed within cultured pinealocytes subjected to both LD and DD cycles, using microarray analysis. We report that a subset of genes are rhythmically expressed in vitro compared to those in vivo (Bailey et al. 2003), and that gene expression rhythms are lower in amplitude. We also investigated the effects of 6-hour pulses of light or norepinephrine supplemented media on the expression of free-running cultures during both day and night. As expected, both light and norepinephrine inhibited melatonin production; however, the two treatments differentially upregulated or downregulated (at least 2-fold) specific sets of genes in a fashion which was dependent upon time of day. Among the most prominently affected genes were those involved in the melatonin biosynthesis pathway (Tr5H, SNAT, HIOMT), as well as a subset of other highly rhythmic genes with no known clock function, including chick purpurin and cystatin. These data suggest that light and norepinephrine differentially regulate pineal clock function, and that several candidate genes with no previously known clock function may play a role in the circadian physiology of the pineal. Supported by NIH PO1NS39546

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Investigating the Efficacy of Orange Lens Glasses that Could Be Use by Shift-workers to Block the Undesired Resynchronising Effect of Morning Light

A. Sasseville*, N. Paquet, J. Soevigny, M. Hoebert • CHUL

Objectives: It has been reported that the biological clock is most sensitive to blue light (460-480 nm). We investigated if Blue Blocker (orange lens glasses) that cut 100% of wavelengths below 530 nm could block the capacity of bright light to suppress nocturnal melatonin. Methods: Thirteen subjects (6F, 7M, age 23-27 y.o.) spent two consecutive nights in the laboratory from 22h00 to 03h00. On both nights, saliva samples were collected every hour until 00h00 and then, every-half hour until 03h00. Subjects were exposed to dim light (< 5 lux) with the exception of a 60-min light pulse of 3500 lux presented from 01h00 to 02h00 on night 2. All subjects did the experiment twice. In experiment 1, during the light pulse, subjects stared directly at the light source while wearing oranges lens glasses that transmitted 1300 lux (525ÏíW/cm2) whereas in experiment 2, they wore grey lens glasses that transmitted 1300 lux (489ÏíW/cm2). Results: When compared to baseline (night 1), the mean level of melatonin suppression achieved on night 2 was 1.2% (95% Cl [-15.29% to 17.78%]) with the Blue Blocker and 49.1% (95% Cl [38.02% to 60.13%]) with the grey lens (p=0.0007; paired t-test). Conclusions: Blue blockers can interfere substantially with the capacity of bright light to suppress melatonin. One other known fact about Blue Blocker is that they also increase substantially contrast sensitivity. Herefore, we believe that they could be use by shift-workers to drive home safely in the morning, when the biological effect of light is not favorable.

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Coordination of a Metabolic Pathway by a Circadian-Regulated bHLH

Michael Covington[1], Steve Kay[2], Stacey Harmer1 • [1]University of California-Davis, [2]TSRI, mfcovington@ucdavis.edu

Circadian clocks regulate many essential cellular processes in most organisms studied, controlling major metabolic pathways and developmental decisions. Our understanding of how the clock controls different output pathways to temporally-regulate physiological and developmental processes is incomplete. We are studying the strategies used by the clock to differentially control subsets of output genes and pathways. Circadian-regulated transcription factors appear to be a link between the central clock and its output pathways. A previous microarray experiment identifing circadian-regulated genes in Arabidopsis showed that the transcript levels of three members of the basic Helix-Loop-Helix family of transcription factors are circadian-regulated. The Circadian-Regulated bHLHs (CRBs) identified in Arabidopsis are expressed at different phases throughout the day and appear to be involved in the regulation of distinct physiological pathways. To study how the clock uses such transcription factors to control specific pathways, we have made use of Affymetrix's full genome Arabidopsis GeneChips to evaluate the effect of CRB2-overexpression at different times of day. Here we show that the entire flavonoid biosynthesis pathway is clock-regulated. Nearly all of the enzymes involved in the conversion of phenylalanine to flavonols and anthocyanins are positively regulated by CRB2. CRB2 also regulates the expression of transcriptional regulators of this pathway. To assess the physiological relevance of this circadian/CRB2-mediated regulation, we are assaying abundance of flavonoids and other related secondary metabolites for evidence of clock control.

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Conserved Expression Profiles of Circadian Clock-related Genes Between Two Lemna Plants Showing Long-day- and Short-day Photoperiodic Flowering Responses

Kumiko Miwa, Masayuki Serikawa, Takao Kondo, and *Tokitaka Oyama • Dept. Biol. Sci., Grad. Sch. Sci., Nagoya Univ. and CREST, JST, Furo-cho, Chikusa-ku, Nagoya, Aichi, 464-8602, Japan

Lemna plants (duckweeds, monocotyledonous plants) have suitable traits for analysis on photoperiodic responses. Two species in the same genus show opposite photoperiodic responses; L. gibba G3 and L. paucisostata 6746 are long-day- and short-day plants, respectively. Both show obligatory photoperiodic responses under certain conditions. Visible flowering responses quickly occur when plants are transferred from non-inductive to inductive photoperiods. A number of intriguing studies on photoperiodism, including analyses using night-break, skeletal photoperiods, and T-cycles, were conducted using these plants more than thirty years ago. Valuable characteristics of Lemna for general laboratory experiments include extremely small size, rapid growth, structural simplicity, and aseptic culture under strictly controlled conditions. We have launched molecular approaches to the photoperiodic flowering of Lemna plants, especially to the divergence of its mechanism. Since the system of photoperiodism involves the circadian clock in the measurement of photoperiod, we first focused on the clockworks of both Lemna species. From both Lemnas, we isolated a number of putative clock-related genes that were identified in Arabidopsis. They included homologues of CCA1/LHY, GI, and ELF3. Gene expression of them was examined with regard to day-night cycles. Gene expression profiles of corresponding homologues were similar between long-day and short-day Lemnas, suggesting the similarity of clockwork between them.

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Roles of the Pseudo Response Regulator Genes in the Arabidopsis Circadian Clock

Patrice A. Salomé; and C. Robertson McClung • Dartmouth College

Loss of function mutations of each of the five Arabidopsis Pseudo Response Regulator PRR genes affect either circadian period or phase. Mutations in *TOC1 Timing of CAB Expression* results in a strong period shortening in white light, and arrhythmicity in red light and in the dark. However, *toc1* mutants are entrained to temperature cycles, and retain clock function in blue light. In an attempt to define roles of the PRRs in the Arabidopsis clock, we are characterizing the circadian defects of *prr* mutants under a number of entraining and free-running conditions. For cotyledon movement during temperature entrainment, *prr3* and *prr5* mutants show no phenotype, although they display a slight short period after entrainment to light-dark or temperature cycles, implicating them in a light input pathway. In contrast, *prr7* and *prr9* mutants show a lagging phase in cotyledon movement during temperature entrainment, consistent with their phenotypes after entrainment of long period and lagging phase, respectively. This indicates possible roles in both light and temperature input pathways for PRR7 and PRR9, or directly in oscillator function. These analyses are being extended to examine effects of the *prr* mutations on the clock itself through analysis of luciferase fusions driven by promoters of the clock component genes CCA1, *LHY and TOC1/PRR1*. Double mutants are also being generated to test the degree of redundancy among PRRs. This work is supported by the NSF.

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Melatonin Synthesis in Dissociated Rat's Retina

J. Aguzzi* L. Graham; G. Tosini • Morehouse School of Medicine

Previous studies have demonstrated that the mammalian retina contains a circadian clock that controls melatonin synthesis. The cellular location of the retinal circadian is still unknown. In the present study, we tested if an intact retinal structure is required for the circadian regulation of melatonin synthesis. To that aim adult rat retinas were dissociated and then cultured at 37 oC in Dulbecco's Eagle Modified Medium supplemented with antibiotics (1%) and 5-hydroxitryptamine (0.1 mM). Dissociated retinal cultures (N = 14) were plated in a density of 4 - 4.5 x 103 cells/mm2, exposed to constant darkness (DD) or to 12light:12dark cycles (LD) and the culturing medium was collected twice a day (i.e., at 12 hours interval) for 5 consecutive days. Melatonin levels were measured by radioimmunoassay. Our data indicated that most of the dissociated retinal cultures synthesized melatonin. In LD 85.7% of the cultures were rhythmic on the 1st day of sampling, 42.9% on the 3rd, and only 14.2 on the 5th day. Similar results were obtained in DD (57% on the 1st day, 14.3% on the 3rd and 5th days respectively). Although the number of cells remained fairly constant over time, a decrease in the levels of melatonin production by the retinal cultures over the five days of culture was observed. Our data suggest that rhythmic melatonin synthesis may persist in dissociated retinal cultures.

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Melanopsin Is Expressed in PACAP Containing Retinal Ganglion Cells of the Human Retinohypothalamic Tract

Jens Hannibal[1]*, Peter Hindersson[1], Jens Ostergaard[1,2], Birgitte Georg[1], Steffen Heegaard[2], Philip Just Larsen[3], and Jan Fahrenkrug[1] • [1]Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Denmark, [2]Eye Pathology Institute, University of Copenhagen, Denmark, [3]Rheoscience A/S, Glerupvej 2, Rødovre, Denmark

The putative circadian photoreceptor melanopsin is in rodents found in a subpopulation of intrinsic light sensitive retinal ganglion cells (RGCs) constituting the retinohypothalamic tract (RHT). We examined if melanopsin is expressed in the human retina and co-stored with the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP), a marker for the RHT projecting to the suprachiasmatic nucleus (SCN). Furthermore, we examined if melanopsin expression is conserved in retinas from blind patients with severe retinal degeneration. Human eyes from a total of seventeen donors and two postmortem hypothalami containing the SCN were investigated by in situ hybridization and immunohistochemistry using melanopsin cRNA probes and an antiserum against human melanopsin in combination with a monoclonal PACAP antibody. Melanopsin expression was found in a subpopulation of RGCs located in the ganglion cell layer and displaced in the inner nuclear cell layer which represent approximately 0. 8 % of all RGCs. They had 2-4 dendritic processes with a receptive field of more than 800 ´"m forming a pan-retinal network. Melanopsin immunoreactivity was localized on the surface membrane of soma and processes. PACAP and melanopsin were co-localized in the RGCs and PACAP containing nerve fibres seem to innervate the retinorecipient part of SCN. Melanopsin expressing RGCs were conserved in retinae from blind patients with severe degeneration of the outer and/or inner layers. The expression of melanopsin in PACAP containing RGCs of the human RHT makes melanopsin a good candidate as a photoreceptor in light entrainment of normal and blind people.

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Identifying the Light-Activated Channel in Rat Retinal Ganglion Cells (RGCS) that Project to the Suprachiasmatic Nucleus (SCN)

E. Warren*, C.N. Allen, R.L. Brown and D.W. Robinson • Oregon Health & Science University, Portland, OR 97239

The purpose of the present study is to identify the ion channels mediating the light-activated current in rat RGCs projecting to the SCN. Stereotaxic injections of fluorescent microspheres into the SCN were utilized to identify SCN-projecting RGCs in retinal wholemounts. Our previous work revealed that SCN-projecting RGCs respond to light with a slowly activating inward cationic current. In this study, addition of cGMP or cAMP to the recording electrode in whole-cell voltage-clamp experiments did not activate an inward current and had no effect on the intrinsic light response. Perforated-patch recordings revealed that the intrinsic light response of SCN-projecting RGCs is inhibited by lanthanum, gadolinium, and ruthenium red, and potentiated by a diacylglycerol analogue and flufenamate. Such results are consistent with those previously reported for several transient receptor potential (TRP) channels, especially TRPC6. In a parallel set of studies, immunohistochemical experiments were performed to determine whether TRP channels and/or cyclic nucleotide gated (CNG) channels are expressed in SCN-projecting RGCs. We have found no evidence for olfactory, rod, or cone CNG channel expression in any RGCs, including those that project to the SCN. However, we have observed robust expression of TRPC1, TRPC3, TRPC4 and TRPC6 in RGCs. We have also shown that TRPC6 channels are present in SCN-projecting RGCs that express melanopsin.

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Effects of Food Availability and of Testosterone Administration on Compensatory Testicular Hypertrophy (CTH) in the Marsh Rice Rat Oryzomys Palustris

Kent E. Edmonds* • Indiana University Southeast

To examine whether food availability (Experiment 1) and the removal of one testis affect CTH during reproductive development, juvenile rice rats were left reproductively intact or unilaterally castrated (ULC) at 3 weeks of age and transferred to 14L:10D. Rice rats received either ad lib, 80%of ad lib, 60% of ad lib, or random amounts of one of the three food amounts listed on a daily basis and sacrificed at 8 weeks of age. Body mass, various organ masses, and testosterone were measured. In ULC animals, CTH was significantly affected by food availability (but still occurred within most groups) as were reproductive organ, Harderian gland (HG), spleen, and body masses. Periodic administration of ad lib amounts of food to the random group reversed some of the effects of mild food restriction. In most groups, testosterone was significantly elevated in ULC animals. Within a group, the number of testes did not significantly affect some of the parameters measured. The administration of testosterone via implants (Experiment 2) to ad lib fed ULC animals attenuated the magnitude of CTH, but stimulated reproductive organ and HG development. These results show that food availability and testosterone administration can modify the development of the remaining testis in ULC animals, as well as affect other reproductive and nonreproductive organs. (Supported by Indiana University Southeast and NSF Research Grant IBN-9812824 to KEE).

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ICV Neuropeptide Y Induces Torpor in Siberian Hamsters

David A. Freeman* (University of Memphis), Matthew J. Paul and John Dark (University of California)

Several species of small mammals abandon homeothermia for heterothermia and employ shallow, daily torpor as a tactic to increase overwinter survival. Body temperature (Tb) decreases during torpor to ~20¡C for several hours during the daily rest-phase and most likely involves a temporary suspension of brown adipose tissue non-shivering thermogenesis (BAT NST). The neural mechanisms of torpor are poorly understood. In laboratory rats, a species that fails to exhibit torpor, intracerebroventricular (ICV) and intrahypothalamic injections of neuropeptideY (NPY) reduce Tb by 2-3¡C, apparently via eliminating sympathetic nervous system input to BAT. The present experiment tests the role of NPY in Siberian hamsters, an animal that naturally employs bouts of torpor when exposed to a short photophase. Siberian hamsters housed in a long photophase (14 h light/day) at Ta = 10¡C received various doses of NPY (5.0, 7.5, 10.0, or 15.0 ug/ul) or saline injected ICV and monitored for the expression of torpor. ICV NPY, but not saline, injections produced torpor (Tb < 32¡C for at least 30 min) in 51.4% of the observations (pending histology). Torpor duration was 230, 107, 109, & 295 min in response to the successively increasing doses; several torpor bouts were unusually long, including 1 of ~20 h duration. Interestingly, animals responded with torpor to some trials but not others, suggesting that the decreased Tb is not due to a simple NPY-induced inhibition of BAT NST. These data suggest that NPY may participate in the neuroendocrine mechanism underlying the generation of torpor bouts.

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Seaonal Variations in Circadian Rhythms Characterize a Phase of Sensitivity to Short Photoperiods in the European Hamster, Cricetus Cricetus

Stefanie Monecke* & Franziska Wollnik • University of Stuttgart

European hamsters (Cricetus cricetus) show pronounced seasonal changes in their physiology and behavior. The present study provides the first detailed analysis of the temporal relationship between seasonal cycles of reproduction and body weight and seasonal changes of two circadian parameters, i.e., locomotor activity and 6-sulphatoxymelatonin (aMT6s) excretion, in individual animals kept under natural light conditions. Our results demonstrate a characteristic pattern of locomotor activity and aMT6s excretion observed around the summer solstice, i.e., from mid-May to mid-July. Locomotor activity was characterized by a high level of activity and an early onset of activity. Seasonal changes in aMT6s and the pattern of locomotor activity were only loosely related to changes in the reproductive status of the animals. Instead, they correlated well with a period of the annual cycle during which the animals are sensitive to short days and may thus reflect a specific state of the circadian pacemaker system within the SCN. The present finding of a behavioral and physiological marker for the annual phase of sensitivity to short photoperiods should thus be a valuable tool to further characterize molecular and physiological mechanisms of photoperiodic time measurements in European hamsters. Sponsored by the German Research Foundation (DFG).

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Circadian and Photoperiodic Responses of House Sparrow at 270N

Amit Kumar Trivedi, Sangeeta Rani* and Vinod Kumar • Department of Zoology, University of Lucknow, Lucknow, 226 007, India

Geographical variations in the biological clock traits have seldom been assessed in vertebrates. House sparrow (Passer domesticus) presents an ideal opportunity to investigate such variations as its breeding populations occur almost all over the world. Most previous clock studies on house sparrow pertain to its populations breeding in North America and Europe. Here, we report circadian and photoperiodic responses, measured as effects on general activity within a cage and on growth-regression of testes, respectively, of house sparrows breeding in the Indian subcontinent (270N). Long-term experiments showed seasonal changes in the responsiveness of sparrow's circadian and photoperiodic systems to both natural and artificial light-dark cycles. In the experiment, which investigated if exposure to twilight periods alone was sufficient to synchronize the circadian and photoperiodic responses, testicular regression was delayed in sparrows that received natural light only during twilight periods, compared to those that received natural light all day, and the effects of exposure to twilight on circadian activity rhythm was not consistently different from that of cage handling. Environmental factors other than day length seem contributing to temporal phasing of the seasonal cycles as evident by comparison of responses of sparrows held captives in outdoor aviary with that of wild birds. Clearly, the circadian and photoperiodic responses of house sparrows at 270N are regulated by daylight and seasons. (Supported by the DST research grant)

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Neural Tissues that Encode Photoperiod Histories in Siberian Hamsters

Brett J.W. Teubner* and David A. Freeman • Department of Biology, University of Memphis

Siberian hamsters exhibit seasonal rhythms of reproduction driven by changes in day length (DL). Long (16 hours of light/day; 16L) and short (10L) DL's stimulate and inhibit reproduction, respectively, whereas intermediate DL's (e.g., 14L) stimulate or inhibit reproduction, depending on whether prior DL's were shorter or longer, respectively (the so called-photoperiod history [PH] response). The neural tissues mediating PH responses remain unknown. DL is encoded by nocturnal release of melatonin from the pineal gland; the duration of which varies in proportion to the length of the night. Several neural melatonin target tissues mediate melatonin's inhibition of reproduction in Siberian hamsters (the suprachiasmatic nucleus [SCN], the paraventricular nucleus [PVt] and reuniens nucleus [NRe]). The present experiment is designed to determine the roles of these tissues in PH acquisition. Hamsters raised in 14L were exposed to 4 weeks of 16L in order to instate a new PH prior to transfer back to 14L. Testicular regression upon return to14L indicated that a new, 16L PH had been acquired. Constant-release melatonin cannulas were stereotaxically implanted into one of the three melatonin target tissues in order to obscure DL information at that tissue during 16L exposure, allowing the remaining tissues access to 16L information. Obscuring DL information at the SCN or PVt, but not NRe (pending histology), blocked the acquisition of a PH whereas vehicle implants allowed the formation of a new PH. The results indicate that the SCN and PVt, but not the NRe, mediate the acquisition of a PH.

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Non-photic Phase-shifting of Golden Hamsters to Induced Activity in Long and Short Photoperiod

Birgit Amann and Uwe Redlin* • University of Stuttgart, D-70550 Stuttgart, Germany.

Adjustment from a long summer photoperiod to a short winter photoperiod results in substantial reorganization of the circadian system. This is evident in circadian outputs (melatonin, locomotor acitvity) but can also be traced to the SCN and its molecular clock machinery (electrical firing rhythm, time course of c-fos activation, time course of key clock gene products). Also, phase-shifting to light is altered in golden hamsters when transferred from long to short photoperiod. This study tested whether golden hamsters respond to induced-activity pulses differentially dependent on photoperiod. Hamsters were either kept in LD 14:10 h or LD 10:14 h for a minimum of four weeks. Using the Aschoff Type-II method, phase shifts to 3-h confinement to novel wheels were tested at various times of the circadian cycle. Locomotor response of these hamsters was enhanced by 24-h food deprivation and a lowered temperature (ca. 15 ¡C) during the pulse. There were almost no differences in the locomotor response to confinement of the hamsters from the different photoperiods, indicating similar strength of the non-photic input. Maximal phase shifts of similar magnitude occurred at ZT 6 in both photoperiods. The only significant difference found concerns phase shifting at ZT 0: long day hamsters had small phase advances (with ZT 0 being in the light phase) and short day hamsters had small phase delays (with ZT 0 being in the dark phase). Non-photic phase shifting of hamsters in this study was phase-locked to activity onset. Moreover, the sensitive phase seemed to be shorter in a short photoperiod.

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Sleep-Wake Cycle of a Human Female and Seasonal Photoperiodic and Temperature Change in Warm-Temperate Zone

Tomoko Wakamura*, Sachiko Horita, Rieko Oshima, Keiko Chikata, Asako Miyajima • College of Nursing Art & Science, Hyogo, Japan

Light and Temperature have similar effects on circadian organization across a wide range of spices. Although light and temperature drive synergistically in nature, light is generally considered a more potent zeitgeber than temperature. However, temperature can be the dominant zeitgeber in Neurospora (Liu et al. 1998). The effectiveness of cycles of ambient temperature for entrainment of circadian rhythms is rather well established in poikilothemic and heterothermic species, whereas there is a paucity of data on homeothermic mammal including human. Actigraphy and sleep diary data from January to December in 2003, on healthy working woman (age 65) in Japan (Warm Temperate Zone), showed that hours in bed correlated closely with change in day length through the year ($r=-0.449$, $p<0.01$). The total daily sleep time correlated closely with both of day length($r=-0.497$, $p<0.01$) and mean daily ambient temperature ($r=-0.256$, $p<0.05$). The bedtime and the fall-in sleep timing did not significantly correlate with both of day length and ambient temperature, while awaking and get-up timings correlated with ambient temperate in a year ($r=-0.337$, $p<0.01$, $r=-0.323$, $p<0.01$, respectively). The results indicate that the duration of sleep synchronize to daily light cycle, whereas awaking time synchronize directly to daily ambient temperature in the environment. The room conditioning of temperature and lighting may affect sleep-duration and sleep-wake cycle drove by the circadian oscillators in humans all year around, as seasonal cues.

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Seasonal Morphological Changes of GnRH Nerve Terminals and Glial Endfeet in the Median Eminence of Japanese Quail

Takashi Yamamura[1]*, Kanjun Hirunagi[2], Shizufumi Ebihara[1], Takashi Yoshimura[1] • [1]Graduate School of Bioagricultural Sciences, Nagoya University, [2]The Nagoya University Museum, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

In a previous study, we clarified that photoperiodically generated triiodothyronine (T3) in the hypothalamus is critical for the photoperiodic response of gonads in Japanese quail (Coturnix coturnix japonica). Expression of thyroid hormone receptors in the median eminence (ME) suggested that photoperiodically generated T3 acts on the ME. Moreover, thyroid hormone is known to play a critical role in the development and plasticity of the central nervous system. These findings suggest thyroid hormones may induce the structural change of GnRH nerve terminals and glial processes in the ME. Immunoelectron microscopy revealed that GnRH nerve terminals are in close proximity to the basal lamina under long day conditions, and conventional transmission electron microscopy demonstrated the encasement of the nerve terminals by the endfeet of glia under short day conditions. These results suggest that morphological plasticity of glial processes may play a key role in accessing of nerve terminals of the external zone of the ME to the portal capillaries, and they may regulate gonadotropin secretion and gonadal growth. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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Photoperiodic Regulation of Phase Relationship Between Per2 and Cry1 Genes Expression in the Pars Tuberalis of Japanese Quail

[1]Shinobu Yasuo*, [1]Miwa Watanabe, [2]Akira Tsukada, [2]Kiyoshi Shimada, [1]Shizufumi Ebihara, and [1]Takashi Yoshimura • [1]Division of Biomodeling, [2]Division of Applied Genetics and Physiology, Graduate School of Bioagricultural Sciences, Nagoya University

In temperate-zone birds, prolactin (PRL) secretion is under photoperiodic control. In mammals, pars tuberalis (PT) of the pituitary is known to be the center of the photoperiodic PRL secretion. In birds, however, hypothalamic vasoactive intestinal peptide is implicated in PRL secretion and physiological roles of the avian PT remain unknown. In this study, we examined clock genes expression in the PT of Japanese quail under various light cycles. PRL secretion was increased under long days and short days with a night interruptive schedule, both of which also cause gonadal growth in Japanese quail. Among clock genes, Per2 and Cry1 were strongly expressed in the PT and both genes expression was rhythmic under long day, short day, and night interruptive lighting conditions. The peak of Per2 expression was seen during the early day under both long day and short day, while Cry1 peak was phase delayed under a lengthened photoperiod. Moreover, Cry1 gene expression was induced by a light pulse, but only when given during the photoinducible phase. Because the protein products of these genes are known to form complexes that regulate the transcription of target genes, our results suggest that different phase relationships between Per2 and Cry1 in the Japanese quail PT under different photoperiods may decode photoperiod and regulate PRL secretion in a manner similar to that of mammals. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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Circadian Gene Expression in Twilight and Square-wave Cycles: Is There Any Difference?

Comas M.[1]*, Rose J. [2], Spoelstra K. [1], Hut R. A. [1], Daan S. [1], Eichele G. [2] • [1] Zoological laboratory, Biological Center, University of Groningen, PO Box 14, 9750AA, Haren, The Netherlands; [2] Max Planck Institute for Experimental Endocrinology, Hannover, Germany

Like many other aspects of circadian organisation, circadian gene expression is normally studied using square-wave light-dark (LD) cycles with abrupt light transitions. Under natural conditions, animals are exposed to gradual changes in light intensity at dawn and dusk. Such light conditions may very well evoke patterns in circadian gene expression differing from those obtained under square-wave LD cycles. We studied whole brain expression of the genes Bmal1, Per1 and Avp in mice by in situ hybridization, with an emphasis on SCN and hippocampus both in square wave LD and in twilight. For this purpose, 30-day-old male mice of the strain C57BL/6 x 129ola were housed under different light regimes in cages equipped with a running wheel so that locomotor activity was recorded. Thirty-six of these mice were exposed to an LD 12:12 cycle for 30 days, another 36 to an LD 12:12 cycle for 27 days plus three days of DD, and a final group of 36 to a twilight LD 12:12 cycle for 30 days. At 60 days of age, the mice were sacrificed at six different time points (2, 6, 10, 14, 18, 22 ExT or InT). The brains were then removed, immediately frozen and stored at -80C. Serial sagittal sections 25 m thick were obtained from ML −2.40 mm to +0.14 mm. mRNA automated high-throughput in-situ hybridization was used to determine the spatiotemporal distribution of transcripts for those genes. Results for Bmal1, Per1 and Avp in the SCN and the hippocampus suggest that peak expressions under a square-wave cycle may be higher than under a twilight cycle, and that under twilight gene expression is more evenly distributed throughout the day. Supported by BRAINTIME (EC QLG3-CT-2002-01829)

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The Accurate Measurement of Plasma and Serum Melatonin Without Sample Extraction

Thomas M. Jermann[1], Irene Unger[1], Juerg C. Schwander[2] and Richard E. Conley[3]* • [1]Buehlmann Laboratories AG, 4123 Allschwil, Switzerland; [2]Clinic for Sleep Medicine, 5330 Zurzach, Switzerland; [3]ALPCO Diagnostics, Windham, NH 03087, USA

At Amelia Island in 1998, we announced an ultra sensitive direct salivary melatonin assay to accurately measure the Dim Light Melatonin Onset Point (DLMO). We now report a new direct plasma / serum RIA method to accurately measure the DLMO. By eliminating the extraction step, the new assay is faster to perform. An expanded dynamic standard range includes five ready to use calibrators (1 - 81 pg/ml) in a human serum matrix. This new method also utilizes the highly specific Kennaway G280 antibody with good analytical sensitivity (0.84 pg/ml). A 400 ul per tube sample size (plasma or serum) is suggested to achieve a 1.3 pg/ml functional least detectable dose (FLDD) observed at 10% CV. Intra-assay and inter-assay precision are 6.7% and 10.4%, respectively. Mean dilution / linearity-parallelism of plasma samples was observed as 103%. Mean spiking / recovery of samples was observed as 107 %. At 50% binding, there is < 0.03 % or no cross-reactivity to melatonin like substances as follows: 6-sulfatoxymelatonin, serotonin, 5-hydroxy-indolacetic acid, N-acetylserotonin, 5-methoxytryptamine, 5-methoxytryptophan and 5-methoxytryptophol. The original Buehlmann melatonin assay (with extraction) correlated significantly against GCMS (r = 0.99; slope = 0.96; y-intercept = +0.23). Split plasma samples were measured and

compared in the new direct method versus the original extraction method and significant correlation also resulted (r = 0.99; slope = 1.12; y-intercept = +0.34). We conclude that the DLMO and other melatonin measurements can be accurately determined in plasma and serum samples without extraction.

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Genes Affecting Sleep Regulation in Mice

John Hofstetter*, Mehdi Tafti and Aimee Mayeda • VA and Indiana University Sch of Medicine

Gene(s) on distal chromosome one of the mouse affect sleep patterns. C57BL/6J (B6) and DBA/2J (D2) mice have differences in total amount of sleep, and the timing and consolidation of naps during the normal waking period. Mice from a congenic line with D2 genes inserted onto a B6 background had more total sleep and more naps than either parent strain by EEG. We examined rest-activity records in LD 12:12 for timing and frequency of rest episodes during the waking period from interval-specific congenic mice derived from the original congenic strain. We narrowed the genetic area involved to approximately 1.5 cM. The same genomic area affects sensitivity to haloperidol-induced catalepsy. To identify candidate genes, we examined microarray data from B6 and D2 mice to identify polymorphic gene expression in the brain. We intend use pharmacologic challenges to test the hypothesis that the dopaminergic system is involved in sleep consolidation.

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Sleepless Mice Display Increased Levels of Daily Locomotor Activity

Ketema N. Paul*, Christine Dugovic, Susan Losee-Olson, Aaron D. Laposky, Fred W. Turek. • Northwestern University, Center for Sleep and Circadian Biology, Evanston, IL 60208

Wheel-running behavior was measured in a remarkable line of mice generated in an ENU mutagenesis program to screen for abnormal sleep/wake behavior in neonatal animals. We have labeled the line "sleepless" because adult males of this line display increased daily levels of wakefulness of one to two hours (h). In order to determine whether the locomotor activity of sleepless mice reflects their increased levels of wakefulness, we examined the amplitude of their wheel-running rhythms. Sleepless (n = 5) and wild-type (n = 9) C57BL/6J mice were placed in cages equipped with running wheels and housed in 14L:10D. Sleepless mutants displayed daily wheel-running activity (33,267 ± 1,237 counts) that was significantly greater ($p < .005$) than, and nearly double the daily activity recorded in wild-type mice (17,750 ± 2,729 counts). The increase in activity observed in sleepless mice occurred almost completely during the dark phase which is when mice typically display the highest levels of activity. During the dark phase, daily activity in sleepless mice (30,978 ± 2,325 counts) was significantly greater ($p < .005$) than, and again, nearly double the activity recorded in wild-type animals (15,898 ± 2,649 counts). These results demonstrate that sleepless mutants display robust increases in locomotor activity in 14L:10D which reflect their increased levels of wakefulness. Supported by DARPA Grant DAAD19-02-1-0038.

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Vasoactive Intestinal Polypeptide (VIP) Contacts on Orexin (ORX) Neurons in Diurnal but Not in Nocturnal Rodents

Gladys S. Martinez[1]*, Antonio Nunez[1, 2], and Laura Smale[1, 2, 3] • [1]Psychology Department, [2]Neuroscience Program and [3]Zoology Department, Michigan State University, East Lansing, MI 48824

The suprachiasmatic nucleus (SCN) regulates the sleep-wake cycle by acting on neuronal populations involved in the control of sleep and arousal. Some studies suggest that the SCN increases arousal rather than sleep, but the pathways involved in this interaction remain unknown. Both diurnal and nocturnal species show rhythms in areas involved in sleep and arousal and those rhythms correlate with their activity pattern. This is the case for brain areas containing neurons that express ORX (hypocretin), a peptide that is strongly associated with arousal: Fos expression in these neurons is high when the animals are awake and low when the animals sleep. Interactions between the SCN and areas controlling arousal may be responsible for differences between diurnal and nocturnal species. In this study we used dual immunocytochemistry to visualize projections from neurons containing arginine vasopressin (AVP) or VIP to neurons expressing ORX in rat and Arvicanthis niloticus (grass rat), a diurnal specie. We observed many contacts between VIP fibers and ORX neurons in grass rats but few contacts in rats. VP contacts were rare in both species. Currently, we are looking for projections from the SCN to another area involved in arousal, namely the tuberomammillary nucleus. These data suggest that circadian regulation of sleep and arousal may involve different pathways in diurnal and nocturnal species. Additional work is needed to confirm a functional relationship between VIP fibers and ORX neurons in grass rats. Supported by NIMH (MH053433)

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Spectral Analysis of Sleep EEG in Morning-type and Evening-type Individuals

Valerie Mongrain* and Marie Dumont • Chronobiology Laboratory, Sacre-Coeur Hospital & University of Montreal, Montreal (QC), Canada, H4J 1C5

Morning-type (M-type) individuals have earlier sleep schedules compared to evening-types (E-types). To investigate the possibility that this difference in sleep timing was associated with differences in sleep structure, M-type and E-type subjects were studied when sleeping according to their preferred sleep schedule. Two groups of healthy subjects (19-34 y.) were selected according to their score on the Morningness-Eveningness Questionnaire: 12 M-types and 12 E-types, 6 women in each group. All subjects had an habitual sleep duration between 7 and 9 hours. An 8-h sleep schedule was individually chosen to be as close as possible to the spontaneous schedule of the subject when free from obligations. This schedule was kept for one week prior to laboratory admission and for the polysomnography recordings. Sleep EEG recorded from C3/linked ears derivation was digitized at 256 Hz. Spectral analysis (FFT) was performed on 4-sec artifact-free sections of 24 1-Hz frequency bins. Spectral power (uV2/Hz) was averaged within the first 4 NREM/REM cycles and power spectra in each bin was analyzed using Group-by-Cycle ANOVAs. A significant Group-by-Cycle interaction was found for spectral power in the 2-3 Hz frequency bin ($p=0.05$), M-types having more power than E-types in the first two cycles. A similar interaction was found for 5-6 Hz ($p=0.04$). A significant group effect in 13-14 Hz revealed more power in M-types ($p=0.005$), with the same tendency found in adjacent 12-13 Hz bin ($p=0.06$). These results suggest that some aspects of sleep regulation may differ between M-types and E-types. Research supported by CIHR (MD) and NSERC (VM).

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Circadian Rhythmicity in a Subset of SCN Neurons and Synchrony Between SCN Neurons Depends on VIP/VPAC2 Signaling

Aton SJ[1]*, Colwell CS[2], Harmar AJ[3], Herzog ED[1] • Departments of [1]Biology, Washington University, St. Louis MO 63130; [2]Psychiatry and Biobehavioral Sciences, UCLA, Los Angeles CA 90024; [3]Neuroscience, University of Edinburgh, Edinburgh EH8 9JZ UK.

In mammals, a circadian pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus mediates daily rhythms in behavior and physiology. Individual SCN neurons fire rhythmically in vitro with near-24-hour periodicity. Little is known about how these neurons communicate to produce synchronous rhythms in vivo. Mice lacking vasoactive intestinal polypeptide (VIP-/-) or the VPAC2 receptor for VIP (VPAC2-/-) show similar phenotypes of behavioral and SCN arrhythmicity, suggesting that VIP may be required for the rhythmicity of or synchrony among SCN neurons. To distinguish between these possibilities, we recorded the firing rates of single SCN neurons from wild type, VIP-/-, and VPAC2-/- mice for several days in vitro. When cultured at high density on multielectrode arrays, 70% of wild-type neurons expressed circadian rhythms. Within a culture, these rhythms had indistinguishable periods and similar phases, indicating that the neurons synchronized their circadian activities to each other. In contrast, only 30% of recorded neurons were rhythmic in cultures from VIP-/- or VPAC2-/- mice. Rhythmic neurons from mutant SCN failed to synchronize to each other in vitro. Thus VIP/VPAC2 signaling appears to be required for rhythmic firing of some SCN neurons, and for coordinating synchronous firing among most, if not all, SCN neurons.

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Effects of 5HT1B Receptor Agonists on Miniature Ipscs in SCN Neurons

*J. R. Bramley, G. E. Pickard, F. E. Dudek • Colorado State University

The SCN receives a dense serotonergic innervation that arises from the median raphe nucleus. In this study we tested the hypothesis that 5HT inhibits GABA release in the SCN via 5HT1B presynaptic receptors on GABA terminals. The effects of mixed 5HT1B/1A/2C receptor agonist trifluoromethylphenylpiperazi ne (TFMPP) and the highly selective 5HT1B receptor agonist CP-93,129 on miniature IPSCs (mIPSCs) of SCN neurons were examined. Whole-cell patch-clamp recordings of mIPSCs were obtained from coronal hypothalamic slices (300 μm). Using CsCl-containing microelectrodes with QX314, and in the presence of DNQX (10 μM), APV (50 μM) and TTX (1 μM), we isolated mPSCs that were bicuculline sensitive. Bath application of TFMPP (100 μM) decreased the amplitude of mIPSCs by an average of 42% with no significant effect on frequency (n=6). However, application of CP-93,129 (1 μM) decreased the frequency of mIPSCs by an average of 57% with no clear effect on amplitude in 3 of 7 cells tested. If 5HT1B receptors are located on GABA terminals and mediate a presynaptic action of 5HT agonists, we would expect to see a decrease in mIPSC frequency. Since TFMPP is a mixed 5HT receptor agonist, it may have effects on other 5HT receptors (e.g. the 5HT2C subtype) which could mask the 5HT1B effect. However, CP-93,129 is highly selective for the 5HT1B receptor subtype, and these data suggest that 5HT1B receptors presynaptically inhibit GABA release in the SCN.

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Tract-tracing Analysis of Two Candidates for the Suprachiasmatic Nucleus of the Chicken, Gallus Domesticus

*Elizabeth L. Cantwell and Vincent M. Cassone • Center for Research on Biological Clocks and Department of Biology, Texas A&M University, College Station, Texas 77843-3258

Many studies have attempted to identify the avian homologue to the mammalian suprachiasmatic nucleus (SCN). Two hypothalamic structures, the visual SCN (vSCN) and the medial SCN (mSCN), are the most likely candidates. We have previously shown that the morphological mapping of antigens in the vSCN is more similar to the mammalian SCN than the mSCN. The vSCN receives retinohypothalamic tract (RHT) input and shows a daily and circadian rhythm in metabolic activity, similar to the mammalian SCN. The mSCN, which receives no RHT input, shows no daily rhythm, but does display a circadian rhythm in 2-deoxyglucose uptake. Clock gene expression is found in the mSCN, the vSCN or both depending on the species and/or study. We previously reported a direct astrocytic and neuronal link between the vSCN and mSCN of the chick. Iontophoretic injections of the retrograde tracer cholera toxin B subunit were directed at the mSCN and vSCN. The vSCN receives input from a wide variety of structures including the perirotundal area, the visual wulst, the hypothalamic paraventricular nucleus and the nucleus of the basal optic root. The mSCN receives afferents from the bed nucleus of the pallial commisure, the ventrolateral thalamic nucleus and the anterolateral thalamic nucleus. Interestingly, the vSCN and mSCN also project to each other, supporting our previous claim that the two may act together within circadian organization. This research was supported by NIH PO1 NS 39 546.

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GABA-A Receptor Activation by Muscimol Reduces Per1 but Not Per2 mRNA during the Mid-subjective Day in Free-running Syrian Hamsters

J.C. Ehlen[1]*; C.M. Novak[1,3]; K.L. Gamble[2,3] M.C. Karom[1]; H.E. Albers[1,2,3] • [1] Biology, [2] Psychology, Georgia State University, Atlanta, GA, USA; [3] Center for Behavioral Neuroscience, Atlanta, GA, USA

During the mid day, behavioral and neurochemical stimuli phase advance the circadian rhythms of nocturnal mammals and/or alter the mRNA levels of components in the SCN oscillatory mechanism. GABAA receptor activation is one of these neurochemical stimuli capable of inducing phase alterations, however its effect on mRNA levels in the SCN remains unknown. Evidence suggests that GABA may be involved in mediating the effect of some non-photic stimuli on clock cells. This hypothesis predicts that GABAA receptor activation by muscimol would reduce both Period 1 and Period 2 mRNA levels in a manner similar to daytime phase advancing stimuli. Syrian hamsters free-running in constant dark were microinjected in the SCN region at CT6 with 22mM muscimol or vehicle. At 1, 2, and 3 hours post injection animals were sacrificed. hPer1 and mPer2 mRNA levels were measured in the SCN region by in situ hybridization. Animals receiving muscimol showed a significant 30% reduction in hPer1 mRNA compared to vehicle treated controls 2 hours after injection ($p<0.05$). No differences in hPer1 or mPer2 mRNA were detected at any other time point. The observed decrease in Per1 mRNA without an associated reduction in Per2 mRNA is not consistent with changes reported by others in response to non-photic stimuli. These results indicate that the GABAA receptor does not exclusively mediate the effects of non-photic stimuli on SCN mRNA levels during the day. Supported By: MH58789 to HEA and IBN9876754

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Removal of Polysialylated Ncam from the Scn Abolishes the Sensitizing Effect of Brain-derived Neurotrophic Factor on Mouse Photic Phase-resetting

J. David Glass*[1], Gregory H. Grossman[1] and Rebecca A. Prosser[2] • [1]Department of Biological Sciences, Kent State University, Kent, OH; [2]Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, TN

Photic phase-resetting of the mammalian circadian clock is mediated by the stimulation of suprachiasmatic nucleus (SCN) retinorecipient cells by glutamate (Glu) from retinohypothalamic terminals. Sensitivity to this effect of Glu is dependent upon brain-derived neurotrophic factor (BDNF). We have demonstrated that polysialylated neural cell adhesion molecule (PSA-NCAM) is necessary for photic phase-resetting, and hypothesize that PSA-NCAM mediates BDNF sensitizing effects. This was tested in mice held under constant darkness receiving chronic intra-SCN perfusion with BDNF, vehicle, or BDNF + endoneuraminidase (endo N), which removes polysialic acid (PSA) from NCAM (n=3/group). After 2 wks a 1 h light pulse was delivered at CT6 (light PRC dead zone). BDNF-treated mice had phase-advances of 39+6 min vs. 7+7 and 0+0 for BDNF + endo N and vehicle groups, respectively (both $p<0.01$ vs. BDNF alone). In a second experiment, mouse SCN brain slices were treated with BDNF beginning at ZT 3, followed by 10 min Glu treatment at ZT 6. This induced a 3.2+0.3 h phase-advance in the circadian rhythm of single-unit neuronal activity. Co-application of endo N with BDNF abolished this response (0.15+0.2h advance); Glu alone had no effect (-0.18+0.1h; both $p<0.01$ vs. Glu + BDNF; n=3/group). This confirms that BDNF sensitizes the mouse clock to daytime photic phase-resetting, and demonstrates that PSA is necessary for daytime photic phase shifts. PSA may promote the sensitizing action of BDNF in the SCN photic signaling cascade.

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Localization of Serotonergic Receptors Mediating Photic-like Response of the Scn to Quipazine

*Caroline Graff[1,2], Paul Pevet[2], Franziska Wollnik[1] • [1]University of Stuttgart, Biological Institute, 70550 Stuttgart, Germany; [2]Universite Louis Pasteur, CNRS UMR 7518, 67000 Strasbourg, France

The mammalian circadian pacemaker in the suprachiasmatic nucleus (SCN) receives dense serotonergic projections both directly from the median raphe nucleus and indirectly from the dorsal raphe nucleus via the intergeniculate leaflet (IGL). In hamsters, serotonergic input to the SCN seems to be involved in the phase-resetting effects of non-photic stimuli. In contrast, recent studies performed in our lab suggest that in rats, serotonin (5-HT) and quipazine, a non-specific 5-HT agonist, have photic-like effects on the locomotor activity rhythm and on c-Fos expression in the ventrolateral area of the SCN. 5-HT may affect the SCN directly via postsynaptic receptors (e.g. 5-HT2C) or indirectly via presynaptic receptor (5-HT1B or 5-HT3) localized on synaptic terminals in the SCN. To further characterize the localization of the 5-HT receptors mediating the photic-like effects of quipazine, we first made IGL lesion (IGL-X) to induce the degeneration of the geniculohypothalamic tract (GHT), then we destroyed 5-HT fibers (5-HT-X) in the SCN. We also made enucleation to induce the degeneration of the retinohypothalamic tract (RHT). The results suggest that the 5-HT receptors mediating the photic-like effects of quipazine are most likely located on the RHT terminals in the SCN. Additional experiments are required to further characterize the 5-HT receptors subtype/s wich mediate the modulating effect of 5-HT on the circadian rhythms.

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Photic Regulation of the Gastrin-releasing Peptide Receptor in Mouse Suprachiasmatic Nucleus

Ilia N. Karatsoreos [1]*, Russell D. Romeo [4], Bruce S. McEwen [4], Rae Silver [1,2,3] • Departments of [1]Psychology and [2]Anatomy and Cell Biology, Columbia University, New York, NY; [3]Department of Psychology, Barnard College, Columbia University, New York, NY; [4]Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY

We have shown that gastrin-releasing peptide (GRP) containing cells of the mouse suprachiasmatic nuclei (SCN) are a component of the photic input pathway, but are not rhythmic in clock gene expression. Previous work indicates that GRP injections directed to the SCN produce photic-like phase-shifts of locomotor behavior in hamsters. Application of GRP to acute brain slices shifts the firing rate of SCN neurons of both rat and hamster in a phase-dependent manner. Finally, while GRP receptor (GRPr) knock-out mice display normal free-running rhythms, their photic phase-shifting is attenuated. The goal of the present study was to investigate the potential role of SCN GRP signalling in photic phase-shifting and entrainment by examining changes in the GRPr. Using receptor autoradiography with radiolabeled GRP we examined GRP binding in animals housed in a light:dark cycle (LD) or constant darkness (DD). Western blots were undertaken to examine rhythms in the peptide levels in SCN tissue dissected from animals housed in LD. GRP binding shows robust rhythms in LD, reaching peak levels by ZT12. No rhythm in GRP binding was detected in DD. These data indicate that in mouse, photic conditions can dynamically drive GRPr binding levels, and suggests that the GRPr is not a factor limiting responses to GRP (This work supported by grant NS-37919 to RS, and an NSERC grant to IK).

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Regulation of Glutamate Release from RHT Terminals by Distinct 5-HT Receptor Subtypes in the Rat SCN Slice

S.-H.Kim*; C.N.Allen • CROET, OHSU, Portland, OR, USA

The SCN receives glutamatergic input via the retinohypothalamic tract (RHT), which serves to entrain the SCN oscillator to the light-dark cycle. The SCN is also innervated by serotonergic fibers from the raphe nucleus. 5-HT and 5-HT agonists can alter the response of SCN neurons to light in vivo and phase shift circadian activity during the subjective day both in vivo and in vitro. The possibility that 5-HT has multiple effects on the circadian system is supported by reports of the presence, in the SCN, of six 5-HT receptor subtypes. The aim of this study is to identify which 5-HT receptor subtypes mediate the presynaptic modulation of the RHT-mediated glutamatergic transmission in the rat and elucidate what mechanisms underlie the modulation. For this study, the whole-cell voltage-clamp technique was used to record evoked excitatory postsynaptic currents (eEPSCs) and miniature excitatory postsynaptic currents (mEPSCs) in hypothalamic slices. For the mEPSC recordings, the slices were bathed in ACSF containing 20 mM [K+]. The activation of 5-HT2B and 2C receptors caused increases in mEPSC frequency but not amplitude or current kinetics. On the other hand, the activation of 5-HT1B and 2A receptors reduced the amplitude of eEPSCs and increased the paired pulse ratio in concentration dependent and partial reversible manner. 5-HT1A and 5-HT7 receptor activation had no effect on either the eEPSCs or mEPSCs.

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Differential Localization of Prokineticin 2-containing Neurons in the Rat Suprachiasmatic Nucleus

Koh-hei Masumoto*, Mamoru Nagano, Shin-Ichi, T. Inouye, and Yasufumi Shigeyoshi • Yamaguchi University

Prokineticin 2 (PK2) mRNA is strongly expressed in the SCN, and the involvement of Prokineticin 2 (PK2) in the formation of circadian locomotor activity has been reported. To investigate the circadian rhythmicity and the localization of PK2-expressing neurons in the SCN, We performed in situ hybridization using digoxigenin- and fluorescence-labeled PK2 probes. Under steady light: dark condition (L: D=12h: 12h), the number of mRNA-positive neurons began to increase in the morning, peaked at ZT4, then gradually decreased and formed a trough at ZT16-20. During the night, only a few mRNA-positive neurons were observed in the SCN. Further, we examined the localization of PK2 mRNA-expressing neurons. At ZT4, scattered PK2 mRNA-positive neurons existed mainly in the dorsomedial SCN (DMSCN), however, a few mRNA-positive neurons were localized in the ventrolateral SCN (VLSCN). In the DMSCN, arginine vasopressin (AVP) mRNA-positive neurons existed in the medial region of the DMSCN whereas PK2 mRNA-positive neurons occupied comparatively lateral and ventral region in the DMSCN. Double-labeling in situ hybridization using digoxigenin-labeled PK2 and fluorescence-labeled AVP probes demonstrated that a few number of neurons co-expressed both AVP and PK2 while most of mRNA-positive neurons contained either PK2 or AVP mRNA. Using PK2/VIP and PK2/GRP probes, we found that only a few PK2 mRNA positive neurons co-expressed VIP and GRP. These findings suggested that PK2 mRNA is differentially expressed in a population of neurons in the SCN.

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CGMP-dependent Protein Kinase-ibeta Mediates Glutamate Signaling in the Suprachiasmatic Circadian Clock

Jennifer W. Mitchell*, Shelley A. Tischkau, Sheue-Houy Tyan, Laura A. Pace and Martha U. Gillette • University of Illinois at Urbana-Champaign

In order to ensure that the circadian clock is attuned to its surrounding environment, the suprachiasmatic nucleus (SCN) exhibits temporally-gated, clock-controlled sensitivity to phase-adjustment of its circadian rhythm by light. During the late night, photic stimulation causes release of glutamate (GLU) onto the SCN resulting in a phase advance of the circadian clock. This phase-shifting stimulation by GLU during late night requires the activation of cGMP-dependent protein kinase (PKG) and the induction of the circadian clock gene, Per1, as well as the phosphorylation of the Ca2+/cAMP response binding protein (CREB) and consequent increase in CREB-mediated transcription. The rat SCN express two forms of the cGMP- dependent protein kinase (PKG), PKG-Ibeta and PKG-II. We found that the phase advance initiated by GLU applied directly to the SCN in vitro is blocked by pre-incubating the SCN with an oligodeoxynucleotide (ODN) against the PKG-I but not PKG-II. The late night stimulation by GLU resulted in an increase in the phosphorylation of PKGIbeta indicating an elevated level of its kinase activity. Furthermore, inhibiting PKG activity by incubating the SCN slice in the specific PKG inhibitor, KT5823, blocked the GLU-induced elevation of Per1 as well as the rise in the phosphorylation of CREB. Together, our data demonstrate that activation of PKGIbeta is an essential step in the phase-advancing response stimulated via GLU, and suggest that phosphorylation of CREB as well as induction of Per1 may be regulated by GLU through PKGIbeta. Supported by NIH grants NS22155, HL67007 (MUG), NS11158 (JWM), NS10170 (SAT)

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The Clock in Dorsal SCN Runs Faster Than that in Ventral SCN

Takako Noguchi*, Kazuto Watanabe1 and Sadao Yamaoka1 • Dokkyo

In mammals, circadian rhythms are driven by a pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The pacemaker is composed of an ensemble of multiple, single-cell oscillators in the SCN. We measured vasopressin (AVP) release in organotypic SCN slices. The SCN slice culture showed circadian oscillation of AVP release with a period length (a} S.E.M) of 23.83 a} 0.03 h. This period is very similar to the one we previously reported in dispersed SCN cultures and is also close to that of behavioral rhythms. When the ventral part was removed by surgical cut across the slice in the horizontal plane, however, the period became shorter (23.17 h a} 0.11 h). On the other hand, the removal of the dorsal part did not affect period length. These results suggest that the oscillators in ventral and dorsal cells contribute differently to period length and that the dorsal oscillators are regulated by ventral one to form a single integrated oscillator.

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Light and GABA Interact to Alter Period mRNA levels in the SCN of Diurnal Grass Rats

C.M. Novak*[1,3], J.C. Ehlen1, K.N. Paul[4], M.C. Karom[1], A. Piceli, M. Akpa[1], and H.E. Albers[1,2,3] • Departments of [1]Biology and [2]Psychology, [3]Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA 30303; [4]Center for Sleep and Circadian Biology, Northwestern University, Evanston, IL 60208

Environmental stimuli may alter the phase of the circadian clock by changing the expression of clock genes at particular times in the circadian cycle. Exposure to light during the night increases the expression of Period (Per) 1 and 2 in the SCN. First, we established that diurnal rodents (Nile grass rats, or Arvicanthis niloticus) have similar daily patterns of clock gene mRNA levels compared to nocturnal rodents. SCN Per mRNA levels were rhythmic in constant darkness (DD), with mPer1 mRNA levels highest between CT 4 and CT 8, and mPer2 mRNA levels highest at CT 8 and CT 12. Further, light exposure at CT 14 significantly increased Per mRNA levels. Nonphotic stimuli may also alter circadian phase by affecting SCN clock gene expression. GABA-A receptor activation in the SCN with muscimol decreases Per mRNA levels in both hamsters and diurnal grass rats during the subjective day. In the last experiment, the SCN region of grass rats was microinjected with muscimol or vehicle, followed by a light pulse during the subjective night. Muscimol significantly diminished the light-associated increase in Per mRNA levels. These data are consistent with the hypothesis that phase-shifting stimuli affect the clock by altering Per expression in the SCN, and the combined effects of GABA and light on circadian phase may be a result of their combined effects on Per gene expression. Supported by MH 58789 to HEA; MH12956 to CMN

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Role of P/Q-type Calcium Channels in the Regulation of Circadian Activity Patterns in Mice

Floor van Oosterhout*, Tom Deboer, Rob C.G. van de Ven, Arn M.J.M. van den Maagdenberg, Johanna H. Meijer • Leiden University Medical Center

Tottering mice (tg/tg) carry a spontaneously occurring mutation in the P/Q-type calcium channel ‡1A subunit gene. P/Q-type channels are highly voltage-dependent and can mediate the primary calcium influx in presynaptic axons, required to trigger transmitter release. P/Q-type channels are also present in the suprachiasmatic nuclei (SCN). In order to address the role of P/Q-type calcium channels in the circadian timing system, we investigated the phenotypic characteristics of circadian rhythmicity in Tottering mice. To this purpose, mice were individually housed in cages equipped with a running wheel to record the circadian activity pattern in LD (3 weeks), DD (3 weeks) and LL (3 weeks). Periodogram analysis was performed to determine circadian period and coherence of the circadian rhythmicity. All animals entrained to the LD cycle and freerunning periods in DD and LL conditions did not differ between wild-type and Tottering mice. However, Tottering mice were significantly less rhythmic as compared to wild-type mice under all lighting conditions (t-test: LD, $p<0.5$, wt n=9 tg/tg n=8; DD, $p<0.01$, wt n=9 tg/tg n=10; LL, $p< 0.01$ wt n=9 tg/tg n=10). These results imply that the decrease in rhythmicity cannot be explained by a change in light sensitivity or light responsiveness in Tottering mice. Instead the results point to a role for P/Q-type channels either within or downstream from the SCN. While future experiments are required to distinguish between these two possibilities, the present results indicate a critical role for P/Q-type calcium channels in the regulation of overt circadian rhythmicity.

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Attenuated Response to Light in the 5HT1B Receptor Knockout Mouse Results in Phase Delayed Entrainment to Winter-like Photoperiods

*Malcolm D. Ogilvie, Patricia J. Sollars, Gary E. Pickard • Colorado State University

The SCN receives a dense serotonergic innervation that arises from the median raphe. 5HT attenuates photic input to the SCN via 5HT1B presynaptic receptors on retinal terminals. It might be predicted that the loss of inhibitory 5HT1B presynaptic receptors on RHT terminals would produce an intensified SCN response to light. Indeed, 5HT1B receptor knockout (KO) mice exhibit enhanced responses to LL. In contrast, KO mice have reduced responses to acute light pulses resulting in attenuated light-induced phase shifts. However, the 80% reduction in light-induced phase advances at CT 23 is greater than the 42% reduction in light-induced phase delays at CT 16, resulting in an increased Delay/Advance ratio of the PRC and therefore the apparent enhanced response to LL. When KO mice are maintained in non-24 h photoperiods they: 1) fail to entrain to T=22 h; 2) have a delayed phase angle of entrainment in T=23 h; and 3) have an advanced phase angle of entrainment in T=26 h. When maintained in short-day T=23 h (winter-like) photoperiods (9.5L:13.5 D), the phase angle of entrainment in KO animals is 1.3 h more delayed than wild-type mice. Most people with seasonal affective disorder have similarly phase-delayed circadian rhythms during the short days of winter. The loss of 5HT1B presynaptic receptors on SCN GABA terminals may produce the attenuated responses of 5HT1B knockout mice to light.

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Circadian Rhythms in Mammalian Glia

Laura M. Prolo*[1], Joseph S. Takahashi[2], and Erik D. Herzog[1] • [1]Dept. of Biology, Washington University, St. Louis, MO 63130; [2]Dept. of Neurobiology and Physiology, Northwestern University, HHMI, Evanston, IL 60208

The role of glia in circadian regulation has been implicated, but understudied. We hypothesized that glia require signals from SCN neurons to express sustained circadian rhythms. We generated cultures of astrocytes from Period2::Luciferase knockin mice and Period1::Luciferase transgenic rats and recorded bioluminescence as a real-time reporter of gene transcription. We found that Per1::luc glia express damped circadian rhythms for 3.8 ± 0.2 cycles (mean ± SEM). Period2 expression peaked approximately 2 h later than that of Period1. The periods in murine and rat glia were significantly different. Oscillations in glia could be restarted by full-volume medium changes. To determine whether the SCN could influence rhythms in glia, we placed a bilateral SCN explant from a wild-type rat on a membrane above transgenic astroglia grown on a glass coverslip and monitored Period1 activity from the glia. We found that the SCN could sustain the near 24 h periodicity in glia for 5.8 ± 0.3 cycles. In 18% of these co-cultures, but none of the pure glia cultures, glia oscillated for 7 or more cycles. Our results indicate that glia can function as circadian oscillators, but that their clocks gradually stop or desynchronize from each other over the first 4 days in vitro. Importantly, SCN neurons appear able to sustain glial rhythms via an unknown, diffusible factor.

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Distribution of Immunoreactive PER1 Protein in the Suprachiasmatic Nucleus and Adjacent Hypothalamus of the Diurnal Grass Rat, Arvicanthis Niloticus

Chidambaram Ramanathan*[1], Gladys S. Martinez[1], Michael Schwartz[2], Antonio A. Nunez[1,2], David R. Weaver4 and Laura Smale[1,2,3] • [1]Psychology Department, [2]Neuroscience Program and [3]Zoology Department, Michigan State University, East Lansing, MI 48824 and [4]Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, 01605

The mammalian circadian clock residing in the suprachiasmatic nucleus (SCN) generates daily rhythms in physiology and behavior and synchronizes them with the light-dark cycle. The SCN of nocturnal and diurnal species is very similar, but the region immediately dorsal to it is very different with respect to rhythms in cFos expression. The molecular mechanisms of the circadian clock have been well studied in nocturnal animals, but data on molecular mechanisms of the circadian system in diurnal mammals are very limited. In the present study, we examined the distribution of a key component of the molecular clock, PER1 protein in and around the SCN of diurnal grass rats, Arvicanthis niloticus. Animals (n=42) were kept on a 12: 12 h LD cycle and perfused at different Zeitgeber times (ZT) and brains were processed for PER1 immunocytochemistry; quantitative analyses of labeled nuclei in and above the SCN are in progress. Preliminary observation reveals that animals perfused early in the day have high levels of PER1 protein in the lower subparaventricular zone (LSPV) and in a central region of the caudal half of the SCN. In the SCN this pattern is very similar that seen in nocturnal animals. More interestingly, the results provide further evidence of a dramatic difference between nocturnal and diurnal species with respect to LSPV function, and raise the possibility that in grass rats the endogenous rhythm in cFos expression seen in this region might be produced by a molecular oscillator intrinsic to it. This research was funded by NIMH grant 53433

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Light Pulses Induce Per1 and c-fos in the Auprachiasmatic Nucleus of Arrhythmic, but not Free-running, Siberian Hamsters

MT Barakat, V Cao, HC Heller, BF OâHara, and NF Ruby* • Department of Biologcial Sciences, Stanford University, Stanford CA 94305

Re-entrainment occurs spontaneously after a phase shift of the light-dark (LD) cycle in all animals except the Siberian hamster. Only 10% of these animals re-entrained to a 5-h phase delay of the photocycle (16 h light/day). Instead, 75% of these animals free ran with periods close to 25 h and 15% became arrhythmic in the LD cycle. Because current models of entrainment require light to phase shift molecular oscillators in the SCN, we investigated whether a light pulse (30 min) administered 2 h after activity onset would induce expression of per1 or c-fos mRNA in the SCN of hamsters that free ran after the phase delay. Arrhythmic animals were given light pulses 2 h after dark onset. mRNA expression was evaluated by in situ hybridization with probes made from cDNA clones of Siberian hamster per1 and c-fos genes. Light pulses had no effect on free-running animals, but induced robust expression of per1 and c-fos mRNA in the SCN of arrhythmic hamsters and in a control group of entrained animals. Thus, a modest phase shift of the LD cycle either disrupted transmission of photic information to molecular oscillators in the SCN or prevented those oscillators from responding to photic stimuli in free-running animals. Robust gene expression in arrhythmic hamsters may reflect arrhythmicity in individual SCN neurons in which photic gene induction is no longer dependent on circadian phase.

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Transient Dissociation in the Dorsomedial Region of the SCN

Y. Shigeyoshi* A. Adachi and M. Nagano • Kinki University

Under steady light-dark condition, the population of neurons in the SCN is supposed to be synchronized to generate a single robust rhythmicity. Previously we demonstrate the dissociation of dorsomedial region and ventrolateral region of the rat SCN after an acute shift of light-dark (LD) cycle (L: D=12h: 12h) by using clock genes as phase markers. After a rapid LD cycle shift, Per1/Per2/Cry1 gene expression in the ventrolateral region (VLSCN) showed a rapid shift whereas the Per1/Per2/Cry1 expression in dorsomedial region (DMSCN) shifted slowly. In the present study, we demonstrated that the transient intrinsic dissociation of the DMSCN occurred when animals experienced 10 hour advance of LD cycle. After the shift, rats showed arrhythmic locomotor activity for three to four days, followed by increased nighttime rest for some days. It took more than 7days to recover normal rest-activity patterns. in situ hybridization using a Digoxigenin-labeled Per1 and Per2 probes demonstrated rapid shifts of Per1/Per2 cycling in the ventrolateral region of the SCN. Dissociation occurred in the DMSCN in a manner that medial minor population in the DMSCN advances and lateral major population delays for the resynchronization. In the constant dark condition, Per1/Per2 expression begins at the most medial region of the DMSCN, then spreads laterally and ventrally. These findings together suggest that the phase differences among neurons in the DMSCN may affect the manner of resynchronization after the shift of LD cycle.

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Unequal Development of the Clock Gene Expression in the Rat Suprachiasmatic Nucleus

Alena Sumova • Institute of Physiology Academy of Sciences of the Czech Republic, Prague

Circadian clock within the suprachiasmatic nucleus (SCN) develops through the embryonic to postnatal periods. The underlying molecular core clock mechanism is based on the regulation of clock genes transcription and translation due to interlocked positive and negative feedback loops. The aim of the present work was to determine whether the parts of the molecular clockwork, namely Per1, Per2, Cry1, Bmal1 and Clock expression, develop equally within the rat SCN. On embryonic days E19 and E20 and postnatal days P1, P3 and P10, the profiles of Per1, Per2, Cry1, Bmal1 and Clock mRNA expression were examined at 2 h intervals throughout the circadian cycle by in situ hybridization. On P1, a 30-min light pulse was administered at CT15 or CT21 and expression of Per1 and Per2 mRNA was determined 30 min, 1h and 2 h after the light pulse. The results show an unequal development of the oscillation of the clock gene expression from the embryonic to early postnatal periods. Oscillation of Per1 and Bmal1 mRNA was significant already on P1, whereas Per2 mRNA on P3 and Cry1 mRNA only on P10. The expression of Clock mRNA was not rhythmic and did not change from the embryonic to postnatal periods. On P1, light pulses started to induce Per1 mRNA but not Per2 mRNA. Supported by Grants Nos. 309021241 and 30902D093 and the Research Project AVOZ5011922.

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Astrocytes and Calcium in the Suprachiasmatic Nucleus of Phodopus Sungorus

Ufer, N.*; Steinlechner, S. • School of Veterinary Medicine

The SCN is an endogenous oscillator composed of a heterogeneous population of neuronal and glial cells. Individual neurons in dissociated cell cultures have independently phased circadian firing rhythms but in the intact SCN the cells show one common rhythm. The multi-oscillator organization of the SCN suggests that the individual neural output must be synchronized into a coherent circadian signal in order to entrain the animal to a light dark cycle and to control behavior. The retinal input to the SCN comes via the retino-hypothalamic-tract. The retinal input signal can be translated into a calcium signal via glutamate by metabotropic glutamate receptors and inositoltriphosphate. Two relevant receptors are the mGluR5 (metabotropic glutamate receptor 5) and the IP3R3 (inositoltriphosphate receptor 3). Both receptors are present on astrocytes of the SCN. The receptors mGluR5 and IP3R3 were found in the Djungarian hamster (Phodopus sungorus) SCN and the levels of both receptors showed a reciprocal circadian rhythm. GFAP, as a marker for astrocytes, showed as well a reciprocal circadian protein rhythm. GFAP and mGluR5 immunoreactivity increased from the rostral to the caudal part of the SCN. The immunoreactivity against IP3R3, however, showed no differences in intensity throughout the whole SCN. The results in connection with investigations of glial cells in other brain areas suggest a new hypothesis for cell-to-cell synchronization in the SCN. Astrocytes appear to have a decisive influence on the neuronal network of the SCN and play a roll in the entrainment of the mammalian circadian clock by Ca2+ signaling.

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c-Fos expression in vlSCN of Common Voles Does Not Anticipate Light-dark Changes

D.R. van der Veen*, K. Jansen, M.P. Gerkema • University of Groningen

Light induced c-Fos expression in the SCN is often interpreted as a marker for transcriptional activation due to light input. When common voles (M.arvalis) are placed in 12:12 LD cycle, light-dark transition are preceeded by half an hour by a peak in c-fos expression in the vlSCN. When subjected to constant dim light conditions, no peaks were found in the vlSCN. This led to the hypothesis that this c-fos expression reflected a light-dark anticipating process. Whe then entrained voles to an 8:16 or 16:8 LD regime, onsets of activity were colocalized with lights off. In these L:D conditions, however, no c-Fos peaks were found preceeding the light dark changes in the vlSCN. This left us with two possible explanations. One hypothesis was that there was interference of c-Fos expression with ultradian activity patterns. Another explanation could be that c-Fos expression in the vlSCN is independent of L:D regimes, but is an intrinsic property, possibly coupled to the full length of an L:D cycle. When in L:D 16:8 c-Fos expression was analyzed, a peak in expression was found at 2 hours after lights onset, the location of the c-fos peak as it was found in LD 12:12. This has not yet been verified for 2 hour preedeeding lights off or in LD 8:16. Work on ultradian activity and c-fos expression is still ongoing

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Prokineticin-2 and Prokineticin Receptors in a Diurnal Rodent, Arvicanthis Niloticus

K. Machida*, C.M. Lambert, L. Smale and D.R. Weaver • Department of Neurobiology and Program in Neuroscience, UMass Med. School, Worcester, MA 01605 and Departments of Zoology, Psychology, and Program in Neuroscience, Michigan State Univ., E. Lansing, MI 48824

Prokineticin 2 (PK2) is a putative output molecule from the SCN. PK2 RNA levels are rhythmic in the mouse SCN, with high levels during the day, and PK2 inhibits nocturnal locomotor activity when delivered into rat brain (Cheng et al., Nature 2002). While a day-active (diurnal) activity pattern likely requires many molecular and physiological adjustments, one testable hypothesis is that diurnal activity is permitted by disruption of molecular mechanisms that suppress activity during the daytime in nocturnal species. We are thus examining the prokineticin system in a diurnal rodent, Arvicanthis niloticus. We found several alternatively spliced forms of A. niloticus PK2 (A.n. PK2). The amino acid sequence of the A.n.-PK2 splice variant most homologous to mouse PK2(1-106) consists of a highly hydrophobic 25-residue signal peptide followed by the presumed mature peptide of 81 residues. Polymorphic sequences and substitutions were observed within the Arvicanthis signal sequence, but the hydrophobic core of the signal sequence is preserved. Within the mature peptide, the sequence identity among Arvicanthis, rat, and mouse PK2's is 100%. PK Receptor1 and PK Receptor 2 (PKR2) cDNA sequences have also been isolated from Arvicanthis. A few amino acids differ among rodent species in each receptor. The distribution of PKR2 in Arvicanthis hypothalamus is similar to that in mouse, with high levels in the SCN. From the data currently available, there is no clear evidence indicating a molecular defect within the prokineticin output pathway in Arvicanthis niloticus. Supported by MH 53433 to L Smale.

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Circadian Oscillation and Light Induction of Immature Per2 mRNA in the Suprachiasmatic Nucleus

Akihito Adachi*, Mamoru Nagano, Yasufumi Shigeyoshi • Kinki University School of Medicine

Recent studies on the molecular mechanism of the circadian clock shed light on the degradation ratio of clock gen mRNAs as a factor regulating the circadian system. To examine the stability of mRNA in situ, we performed in situ hybridization by using 35S-labeled cRNA probe corresponding to Intron and Exon of mPer2 to examine the expression profile of precursor and mRNA of mPer2 gene in the central nervous system. In both LD (Light:Dark=12h:12h) and DD condition, strong signal was detected in the SCN, with peaks at ZT12 and CT8, in consistent with those of Per2 mRNA. However, significant differences lied in the localization of the signal. When Exon probes were hybridized, moderate signals were detected over the brain, especially high in piriform cortex and hippocampus. In contrast, using Intron probe, we detected no signal in the brain besides the SCN. Then we examined the effect of light pulse at CT16 on the amount of immature and mature mPer2 mRNA. Rapid induction of RNA signal occurred in the SCN with both probes. With the Intron probe, the signals peaked at 60 minutes after light pulse and then began to decrease whereas Exon probe showed a peak 90 minutes after the light pulse. The difference in the localization of signals shown by the Intron and Exon probes suggests the distinct splicing and/or degradation machinery of mPer2 transcripts between neurons in the SCN and other brain regions. The correspondence of precursor RNA expression to the increasing phase of mRNA suggests Intron probe would offer more precise profile of Per2 transcription in situ.

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The VPAC2 Receptor Is Required For Coordinated Rhythmic Activity of Mouse SCN Neurons in vitro

T.M.Brown* and H.D. Piggins • University of Manchester

Previously, we showed using discontinuous sampling that the suprachiasmatic nuclei (SCN) from mice lacking the VPAC2 receptor (Vipr2-/-) fail to manifest robust rhythms in electrical activity. However, we could not rule out the possibility that individual Vipr2-/- SCN neurons sustain low amplitude rhythms. Here, a novel recording technique was used to assess the long-term (>30 hr) activity patterns of SCN neurons in brain slices from wildtype and Vipr2-/- mice. Coronal hypothalamic slices (400 μm) from adult Vipr2-/- or wildtype (C57BL/6) mice, housed under 12h:12h light-dark cycle, were maintained in an interface style chamber continuously perfused with artificial cerebrospinal fluid. The activity of individual SCN neurons was discriminated (Spike2 software: CED, UK) from multiunit extracellular recordings, made using custom electrodes stabilised over the SCN via negative pressure. Most wildtype SCN neurons showed overt firing rate rhythms (period ~23.75h), with high discharge rates (~4 Hz) during the projected day and low firing rates over the projected night (~1.5 Hz). Arrhythmic neurons were very rarely observed (~5%). In contrast, neurons from Vipr2-/- slices were largely arrhythmic, showing low frequency firing (1.5-0.5 Hz) regardless of the time of day. A small proportion (~25%) of Vipr2-/- neurons manifested low amplitude rhythmic activity (~1 Hz peak to trough) with variable periods (~20-26h). These findings indicate that the VPAC2 receptor is not essential for rhythmic firing of SCN neurons per se but is required for normal cellular rhythmicity in the mouse SCN as a whole. Supported by the BBSRC.

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Circadian Activity of a Viral Gene Promoter in Tissues of Live Bioluminescent Mice

A.M. Collaco, S. Rahman, E.J. Dougherty, M.E. Geusz* • Bowling Green State University

The phase of rhythmic gene expression regulated by the circadian system varies between tissues. Clock-controlled genes have regulatory elements found in viral gene promoters. Transgenic mice containing the promoter and enhancer of the human cytomegalovirus immediate-early gene-1 upstream from the firefly luciferase gene luc (CMV::luc) display highest transgene expression in most body areas just after dusk when maintained in LD, according to bioluminescence imaging. When night is extended by 36 hrs, the paw, tail, and whole-body transgene expression increases during late projected night. A second transgenic mouse with the human c-fos promoter upstream from luc displays maximal expression at phases different from those of CMV::luc mice, indicating promoter-dependent timing of expression. CMV::luc mice crossed with hairless albino mice (HRS/J) were maintained in DD and then anesthetized and imaged at several locomotor rhythm phases. The brightest body areas displayed maximal expression near CT 18 in the 5 adult mice imaged. The highest signal was from the pancreas as shown by imaging after dissection. Pancreas from 3 CMV::luc mice were partially dissociated using collagenase digestion and imaged in vitro. Pancreatic acini and islets showed high transgene expression, but blood vessels and pancreatic ducts had no discernible bioluminescence signal. Insulin-producing pancreatic beta cells are sensitive to common viruses, and the circadian clock may modulate viral infections that lead to diabetes or pancreatitis. Supported by NIH grant 5R21RR012654-02.

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Title

Stacey L. Harmer1* and Steve A. Kay2 • 1Section of Plant Biology, University of California, Davis* slharmer@ucdavis.edu; 2The Scripps Research Institute, San Diego, California

Like many other organisms, plants possess internal biological clocks that help them coordinate internal events with the external environment. And like many model systems, a transcription/translation feedback loop has been proposed to lie at the heart of the plant circadian oscillator. The importance of transcriptional regulation in the plant circadian system is underscored by the fact that a large fraction of the plant transcriptome exhibits circadian regulation. Although these clock-regulated genes show peak expression at all phases of the subjective day and night, it is unknown how these diverse phases are generated. We have previously identified a motif found in the promoters of many evening-phased, clock-regulated genes and showed that this evening element (EE) is required for conferring circadian rhythmicity on a reporter gene (Harmer et al, Science, 2000 290:2110-3). The EE may also play an important role in the function of the central oscillator itself (Alabadi et al, Science, 2001 293:880-3). In work presented here, we demonstrate that the EE is sufficient to confer evening-phased expression on a reporter gene, and characterize trans-acting factors that mediate this clock-regulated expression. We also identify a new promoter motif sufficient to confer dawn-phased expression on a reporter gene. Through these studies, we hope to learn how the circadian clock regulates two distinct phases of gene expression, shedding light on how the clock allows plants to time physiological events most advantageously.

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Time-dependent Gene Regulation in Human Skeletal Muscle and Evidence of Exercise-induced Modulation

Erin L McDearmon, Alexander C Zambon, Kirsten L Johansen, Morris Schambelan, Bruce R Conklin and Joseph S Takahashi • Northwestern University/HHMI

Elucidating the circadian regulation of peripheral tissue pathways is necessary to understand holistic function under normal conditions. While most previous studies using peripheral tissues have focused on the rhythmic expression of the few known core clock genes, recent evidence suggests circadian output genes constitute 8-10% of all genes expressed in mouse tissues. Therefore, examination of tissue-wide gene expression changes with respect to time is important in determining circadian regulation of pathways and cellular functions. While some large-scale circadian gene expression studies in mouse peripheral tissues have been performed recently, the similarity of circadian regulated genes and pathways in human tissues was not previously known. We used oligonucleotide microarrays to determine the effects of time of day (8:00pm vs. 8:00am) on gene expression levels in control human quadriceps muscle. Comparison of our results with published circadian gene profiles in mice identified 44 putative genes that were regulated in a circadian fashion. Real time RT-PCR was performed to validate the circadian expression of selected gene orthologs in mouse skeletal muscle. In addition, we observed resistance exercise-induced regulation of human skeletal muscle core clock genes, suggesting that exercise may directly modulate circadian rhythms in skeletal muscle. These results, combined with ongoing studies in mouse skeletal muscle, will enable us to understand the relationship between circadian variation in gene expression and skeletal muscle function.

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The Role of Orphan Nuclear Receptor RORa in Clock Gene Transcriptions Demonstrated by a Novel Reporter Assay System

Y. Nakajima[1]*, M. Ikeda[2], T. Kimura[1], S. Honma[3], Y. Ohmiya[1] and K. Honma[3] • [1]Cell Dynamics Research Group, National Institute of Advanced Industrial Science and Technology (AIST), Osaka 5638577, Japan; [2]Molecular Clock Project, Research Center for Genomic Medicine, Saitama Medical School, 350-1241, Japan; [3]Department of Physiology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan

Circadian rhythms are generated by an extremely complicated transcription-translation feedback loop. To precisely analyze the molecular mechanisms of the circadian clock, it is critical to monitor multiple gene expressions and/or interactions with their transcription factors simultaneously. We have therefore developed a tricolor reporter assay system, in which two gene expressions can be monitor simultaneously from different luciferase emitters. The system consists of green- and red-emitting Phrixothrix luciferases as dual reporters and blue-emitting Renilla luciferase as an internal control. The activity of the green and red luciferases can be measured simultaneously using an optical filter. We have successfully employed this system in analyzing the effects of clock gene products on the enhancer elements of Bmal1 and Per1 promoters. The results indicate that the orphan nuclear receptor RORa regulates bidirectionally Bmal1 (positively) and Per1 (negatively) transcriptions simultaneously. Furthermore, the effects of RORa on the transcription of the enhancer element and the promoter fragment of Bmal1 were directly compared.

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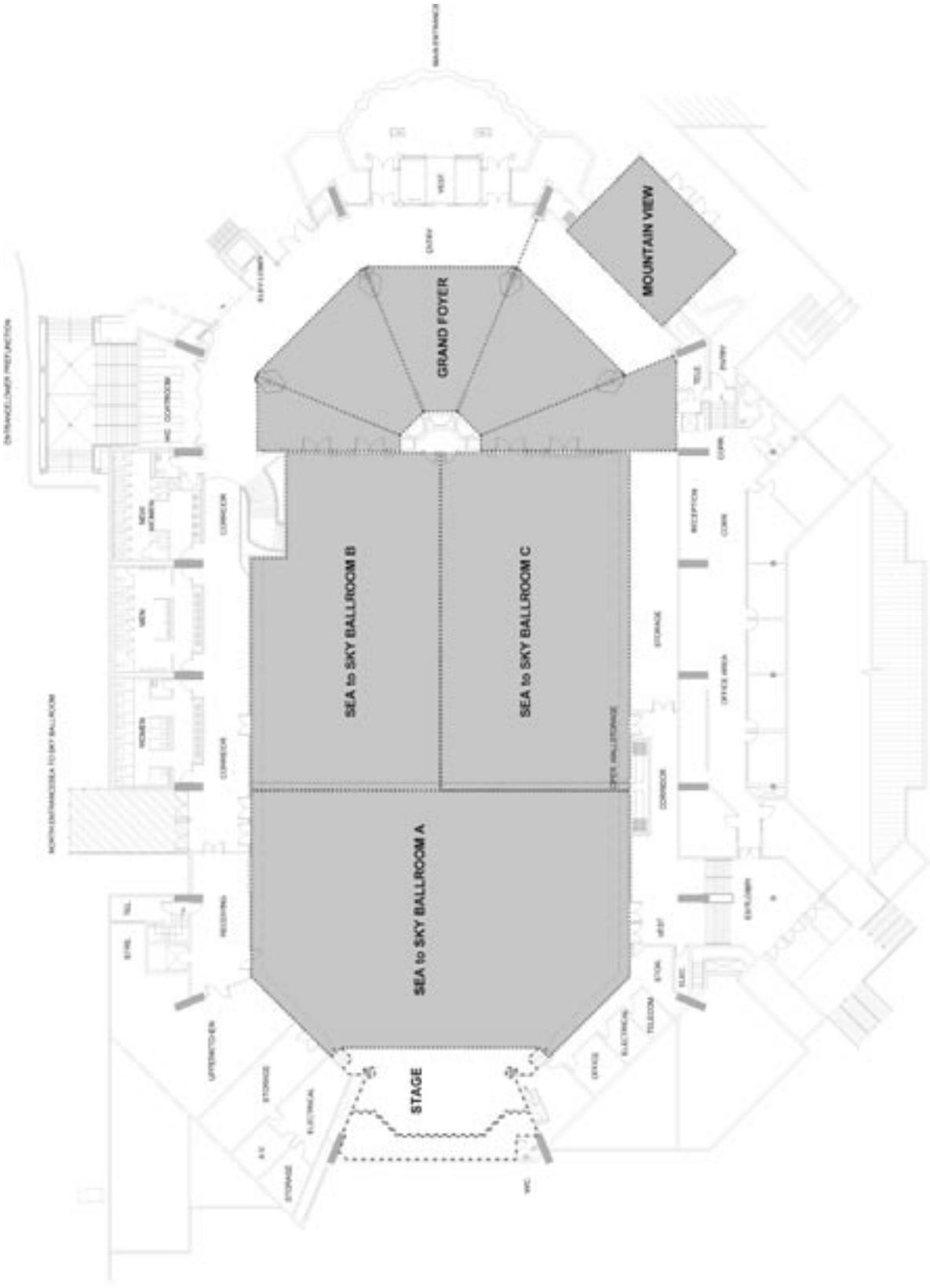
Maps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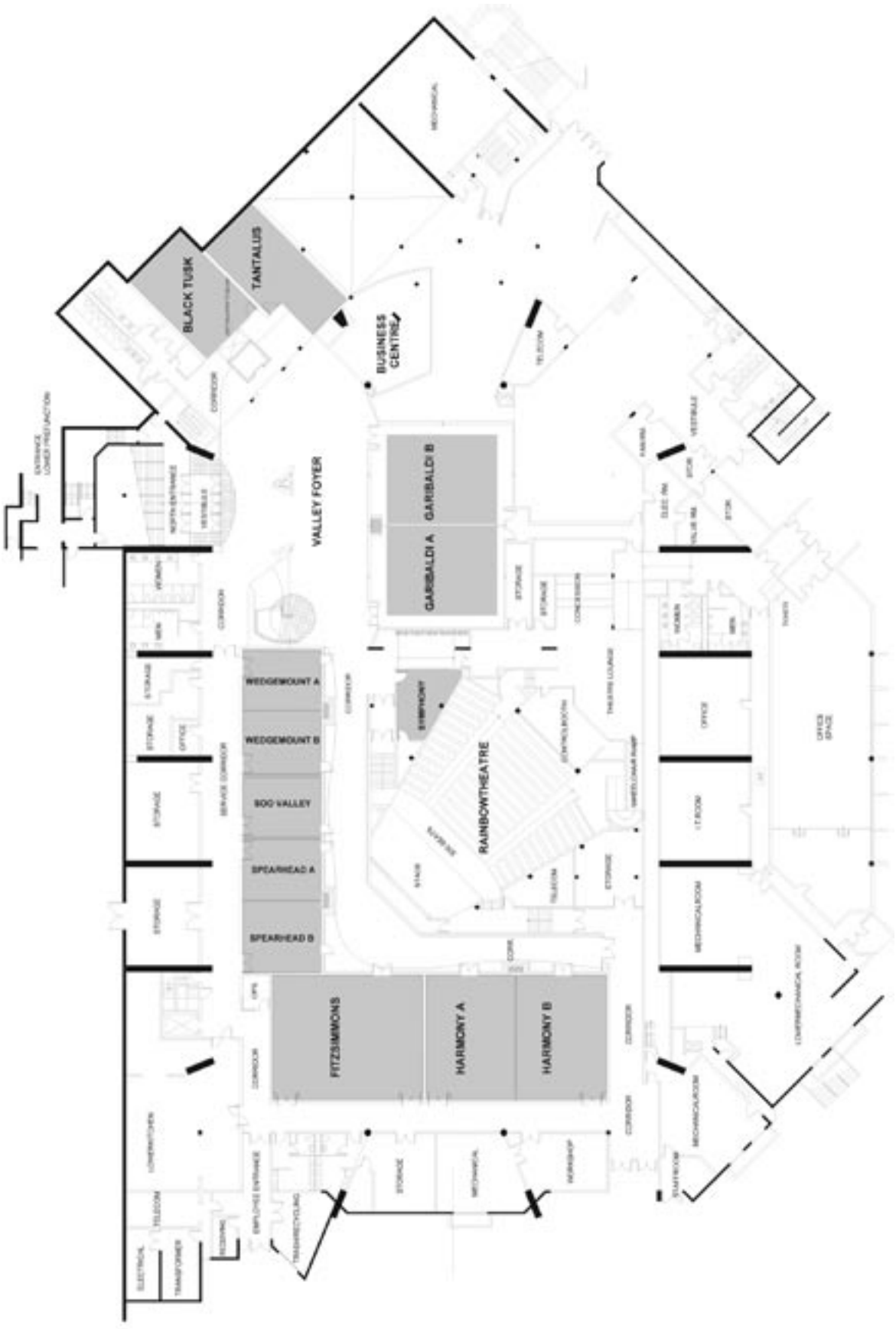
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