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Science has gotten such a good reputation for answering questions that just about everybody claims the adjective “scientific” for what they say. An impeccable scientific approach is, however, useless for most of life’s important questions like “Wherein lies the Good?” “Why me?” “Hold ‘em or fold ‘em?” “Shall we send troops?” Scientists are no better than anybody else at making most personal and political decisions and can be a real pain when it comes to providing clear answers to simple questions—especially if their defensive scientific cloaking device is turned on. The way we scientists deal with questions and answers often frustrates the people who consult us and support us.

There are two reasons for this. First, we frequently don’t accept the question. Many of the biggest, most urgent, or most important questions are concerned with what should be, and science addresses only what is. As Richard Feynman explained, “The question: ‘Should I do this?’—whether you want something to happen or not—must lie outside of science.” Second, our tendency, even when the questions posed are scientific, is to refuse to answer until we’re good and ready. Unlike policy makers or executives or police officers or editors, we need not (and often refuse to) come to a fast conclusion. We claim the privilege of uncertainty long after others have made up their minds. According to Feynman, a scientist is never certain. When a statement is made, the question is not whether it is true or false but rather how likely it is to be true or false. There is no certainty; even our best answers are at least a little provisional. This chronic hedging of ours is a remarkable trait and a precious privilege—rare across history and geography—the freedom to doubt and to declare “I don’t know” publicly.

Ever doubtful, wary of conclusions, even wary of facts, we parse the truth of statements ever so fine: How true is it? Is it the whole truth? Is it entirely true or just partially true? Is it strictly true, necessarily true, generally true, often true, true under certain circumstances? Is it conditionally true, likely true, possibly true? We thereby bypass some of the deeper, more intractable, issues of truth and causality and compensate with the benefits of open-mindedness, disinterest (not fooling ourselves), and small hard truths.

The ability to declare a question presently unanswerable, no matter how important, and to accept interim and partial truths without commitment, is perhaps the greatest strength of science and a hallmark of its different worldview. We have had the privilege, so far, of choosing our questions. Although everyone wants answers to big questions, we usually prefer to settle for results that clearly answer a small question over results that merely bear on a big question. What a peculiar way of getting answers normal science has: nibbling at a problem, not trying to swallow it whole. Yet, by an invisible hand, it seems to end up giving us a better grasp of truth and causality after all.

Isaac Newton said, “I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.” This pretty scene embodies several of the ideals of science: modesty, curiosity, and wonder. We have found treasures on the beach: shiny shells and pebbles—what stars and people and firefly flashes are made of. The shells and pebbles add up and tell us about the sea. Each of us gets to place some on the pile.

Martin Zatz
Editor
Clockless Yeast and the Gears of the Clock: How Do They Mesh?

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Abstract In spite of its apparent weakness as a clock model, the budding yeast has spawned a technique that has revolutionized our ability to study specific protein-protein interactions like those at the core of the molecular timekeeping mechanisms. Here, the author will summarize the evolution, power, and limitations of this technique and highlight its potential and actual contributions to the field of chronobiology.

Key words yeast two hybrid, circadian interactome, clock genes

Specific interactions between proteins form the basis of almost every aspect in a cell’s life. These interactions create a dynamic and tightly regulated communications network and weave a complex connectivity map from which cell phenotypes emerge. It is hardly surprising that the identification, characterization, modification, and exploitation of all these specific contacts would constitute such major focal points in modern biological research.

The study of protein-protein interactions was largely confined, until 1989, to biochemical techniques such as crosslinking, co-precipitation, and fractionation. However, these techniques are limited by the fact that the interaction of interest is either initiated or measured outside of a living cell. This situation was dramatically changed with the introduction of a genetics-based strategy (Fields and Song, 1989), referred to as the yeast two-hybrid system (YTH), that made it possible, for the first time, to investigate protein-protein interactions inside a living cell.

The continued application of the YTH and related protocols led to the description of thousands of binding partners. This new information contributed greatly to the identification of novel genes (Boulton et al., 2001), the dissection of complex signal transduction networks (Hartwell et al., 1999), and the assignment of likely roles to functionally uncharacterized proteins (Brent and Finley, 1997; Uetz et al., 2000).

THE YTH SYSTEM: A CATALYST OF DISCOVERY AND SELF-IMPROVEMENT

YTH relies on the modular nature of transcription activating factors (Sadowski et al., 1988), which tend to bind to the control region of a gene with one domain while activating the transcriptional machinery with another (Fig. 1A). The strategy that makes YTH possible is based on the separation of the DNA binding and the activation domains of a known transcription factor, which are then fused to putative binding partners (Fig. 1B). The two resulting fusion proteins are not expected to activate transcription on their own. However, after their simultaneous expression into a suitable yeast reporter strain, a successful interaction will reestablish the physical link between the DNA binding and activation domains (Fig. 1C). This event, even if transient or of low affinity, can be recorded if the yeast strain carries an easily detectable (e.g., colori-

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metric, such as lacZ) or selectable (e.g., prototrophic, such as his3) reporter gene. Typically, one of the binding partners is a known entity and referred to as the “bait” (b) because it is used to “catch” an unknown “prey” (p), via more or less stable interactions.

The remarkably high sensitivity of YTH screens, however, comes at the expense of a relatively high number of false positives, a persistent concern for users of this technique. Another disturbing fact relates to the predicted interactions that are not detected by this technique, the so-called false negatives. Indeed, less than 15% of previously established binding partners in yeast have been rediscovered by supposedly comprehensive screens (Hazbun and Fields, 2001). As a consequence, researchers are constantly looking for the perfect balance between increased stringency and maximal coverage. At the present time, YTH techniques can suggest potential binding partners, but independent confirmation by additional methods is required to demonstrate that the interaction is specific and physiologically relevant.

These clear disadvantages notwithstanding, the YTH strategy enjoys a high level of success and acceptance, possibly due to its remarkable ability to evolve rapidly in response to the frequently encountered technical obstacles (Finley and Brent, 1996; Brachmann and Boeke, 1997; Fashena et al., 2000). For example, as already mentioned, a significant fraction of YTH-derived findings represent biologically irrelevant interactions. Commonly isolated interactors, such as heat shock, ribosomal, or proteasome-related proteins, can interact with approximately one-third of randomly chosen baits.

Hence, much of the initial efforts to improve the YTH protocol focused on assessing and increasing the specificity of the observed interaction. An early step forward in this direction was the swapping of the bait and prey moieties between the DNA binding and transactivation domains of the reporting factor (Du et al., 1996). This domain swap was initially designed to bypass the false positives that result from the fusion of transcriptional activators onto the DNA binding domain, but it quickly became a standard specificity
control. Later, the use of carefully designed double bait systems significantly increased specificity through the introduction of a second bait vector carrying a different DNA binding domain fused to a nonspecific interactor (Serebriiskii et al., 1999). The extent to which this alternative pathway also becomes activated by the same prey provides a good indication of the level of nonspecific interaction.

The most significant advance in the specificity front, however, has been the inclusion of multiple and independent reporter systems. Thus, true binding partners have to activate several different genes before being considered for further analysis. This strategy dramatically increased the stringency of the screen and, as a result, the capacity of YTH to expose candidates worth pursuing.

Another limitation of the original YTH method lies in the nature of the exclusively transcription-based readout. This feature excludes a significant number of potential interactors that are either membrane bound, unable to access the nuclear compartment in an active form, or independently active on transcription. Over time, several variations were developed, either in mammalian or yeast cells, that address these problems by probing interactions at their natural site in the cell (Johnsson and Varshavsky, 1994), in the cytosol (Rossi et al., 2000), or in association with the plasma membrane (Johnsson and Varshavsky, 1994; Aronheim et al., 1997; Isakoff et al., 1998; Medici et al., 1997).

In addition, a given interaction might occur only as a consequence of unique posttranslational modifications. Absence of a differentially expressed modifying system would prevent the detection of such an interaction, a limitation of significant consequences when we consider a recent report regarding the disproportionate representation of proteins involved in signal transduction among different species (Chervitz et al., 1998). If the missing link is known, it is possible to co-express it during the interaction screen (Kochan et al., 2000); this approach appears to be particularly useful when tyrosine kinases are involved (Chervitz et al., 1998). Similarly, some interactions that involve ternary complexes are unstable if one of the components is missing. After the arrival of the three-hybrid system (Zhang and Lautar, 1996; Kochan et al., 2000; Brachmann and Boeke, 1997), formation of three-component complexes has been achieved repeatedly, thus allowing us to address the issue of how larger protein structures (or even scaffolds) are put together. Predictably, the development of reliable yeast hybrid (YH) screens that target quaternary complexes is just around the corner (Pause et al., 1999).

Finally, YH screens have not remained confined to the realm of interactions between proteins. In the so-called RNA three-hybrid system (Putz et al., 1996), interactions between proteins and RNA molecules can also be investigated. Meanwhile, a one-hybrid protocol (Inouye et al., 1994) has been developed to identify DNA binding proteins using specific cis-acting elements as interaction targets. On the other hand, reverse two- and one-hybrid systems (Vidal et al., 1996) offer the possibility to screen for mutations or small ligands that disrupt a particular interaction, adding a new dimension to the analysis of structure-function relationships and the high-throughput search for compounds with potentially useful pharmacological properties.

**BEYOND YTH**

Most classic biochemical techniques have been used to corroborate an interaction first suggested by a yeast hybrid search. In the context of new technologies, however, a group of methods referred to as resonance energy transfer (RET; Li et al., 2001) is emerging as one of the most powerful tools for confirming a suspected interaction in vivo. RET is based on the interaction of two energetically linked luminescent (LRET), or fluorescent (FRET) probes (fused to interacting proteins). When these moieties are brought into proximity, energy resonance causes either quenching or excitation and the concomitant change in emission spectra that can be monitored (see De Angelis, 1999, for a comprehensive review on the major applications of FRET). The demonstration that the cyanobacteria circadian protein KaiB can form homodimers, using bioluminescence RET (BRET; Xu et al., 1999), was among the earliest successful applications of this approach. The study of conformational changes in proteins (Wang et al., 2001) or the kinetics of proteasome targeted degradation (Tung et al., 2000) are just two additional examples of the exciting possibilities that these techniques offer for the real-time monitoring of interactive events.

The arrival of the proteomics age has brought along the intriguing possibility of a genome-wide application of the YTH and derived approaches (Uetz et al., 2000; Legrain and Selig, 2000; Ito et al., 2001). Several consortia are combining the power of robotics, multicloning, and high throughput screening to lay out
(more or less) comprehensive protein interaction maps. It is hoped that the painstaking construction of these “interactomes” will usher in a new era of proteomic databases. By “mining” these resources, we might be able to generate sound hypotheses regarding the possible role of uncharacterized proteins, once their likely location within such a map is identified. It is important to bear in mind, however, that two early attempts at defining a comprehensive catalogue of all possible interactions in the yeast system yielded disappointingly few overlapping hits (Uetz et al., 2000; Ito et al., 2001). This result indicates that current techniques have not yet reached the level of saturation required to detect every possible interaction (Hazbun and Fields, 2001) and that the type of data obtained depends strongly on the details of a particular screen design.

DISSECTION OF GENERAL AND CIRCADIAN INTERACTION NETWORKS

Most signal transduction pathways use a standard approach to deliver information into the nucleus. Although different pathways display a wide range of variations in the details, they operate in three conceptually distinct domains designed to control the all-important crossing of the nuclear/cytoplasmic barrier by factors that affect transcription. In the first domain (domain I = input), the primary information, from channel traffic, receptor activation, intracellular variables, and so on, is received by the proper sensor and conveyed through second messengers onto regulatory proteins that control the fate of a nucleus-bound device (domain II = processing). The translocation, final destination, activity, and partners of this nuclear device(s) determine the nature of the genetic response (domain III = output). Predictably, a dynamic balance between the rescue and degradation of these nucleus-bound proteins, and their regulators, is a recurring theme in a large number of signaling pathways. This control mechanism is particularly common in the second domain of this simplified model, where the stability and fate of key factors is determined, to a large extent, by complex phosphorylation/dephosphorylation cascades and selective interactions.

The levels of organization defined above can be easily recognized in many well-characterized pathways, a few of which are outlined in Table 1. The first point of this exercise is to emphasize that while information in a cell is handled through “traditional” channels, distinguishing features within individual pathways are conferred by a rich tapestry of highly specific protein-protein contacts. A real understanding of any particular pathway (or circuit) will depend on a comprehensive description of the sequence, location, and timing of every specific interaction.

The second point, of course, is that the biological clock (see Lowrey and Takahashi, 2000; Chang and Reppert, 2001, for recent reviews) can be construed as the signal transduction pathway that keeps track of internal time. As such, the underlying transcriptional/translational mechanism that supports circadian rhythmicity can be fitted onto a similar model, once we recognize that it represents a special case in which, in addition to outside cues, key variables along the (path) way can feed into itself to generate a self-sustaining loop (Shearman et al., 2000).

Consequently, the explosive progress in molecular circadian biology in recent years can be largely accredited to the discovery of clock-relevant interactions (many through YTH screens) and the tacit effort to describe a “circadian interactome.”

Consider the heterodimerization of PER (Period) and TIM (Timeless) in Drosophila (Sehgal et al., 1994; Vosshall et al., 1994; Gekakis et al., 1995); the light-dependent sequestration of dTIM by dCRY (Ceriani et al., 1999); the combinatorial capacity of the three mammalian PER proteins to interact among themselves (Zylka et al., 1998) and with the CRYs (Cryptochromes) (Kume et al., 1999); the interaction, discovered through a YTH screen, between the apparently clock gene–specific transcription factors BMAL1 and CLOCK (Gekakis et al., 1998) and their transcriptional repression by a PER/CRY complex (Kume et al., 1999). Each example turned out to be a pivotal association for the establishment of a circadian clock. More recently, we learned of the ability of Casein Kinase Iε/δ to recognize PER 1 and 2 as phosphorylation substrates, thereby regulating their susceptibility to proteosomal degradation (Suri et al., 2000; Camacho et al., 2001; Vielhaber et al., 2000). This finding retraced the previously reported light-induced tyrosine phosphorylation of dTIM by DOUBLETIME, an event that targets TIM for ubiquitination and proteosomal degradation (Naidoo et al., 1999).

Similarly, the key scaffolding role of the nonrhythmic protein WC-2 is of particular interest in this context because it seems to control the formation of a ternary complex as part of the timing mechanism.
in *Neurospora* (Collett et al., 2001). This hypothesis has now been confirmed by independent biochemical methods (Denault et al., 2001).

By the same token, it can be argued that the pieces still missing in the circadian puzzle represent unique opportunities to find novel interactions that might affect the phase and output of the circadian clock. It seems reasonable to predict that these unknown interactions are still many and likely to play important roles in the compartments described above.

First, we do not yet fully understand the nature of the interactions underlying circadian photoreceptive signaling. ZEITLUPE (ZTL), for example, is an *Arabidopsis* protein that exerts profound effects on clock-controlled processes consistent with a role in light input to the clock (Somers et al., 2000), possibly just downstream of converging photoreceptive pathways (Millar, 2000). The role of ZTL within or around the clock is far from clear; however, the presence of F-box and PAS domains suggests likely classes of ZTL binding partners belonging to the degradation and transcription control pathways, respectively.

Second, we still have a far from complete description of the components responsible for the specific modification, degradation, and translocation events that control the level and subcellular localization of all known clock proteins in different species. One would assume that the turnover of specific clock components should be a carefully monitored parameter, an assumption amply supported by the arrhythmicity of DOUBLETIME mutant flies in which the hypophosphorylation of TIM (Naidoo et al., 1999) and PER (Price et al., 1998) results in their increased stability. Similarly, the tightly regulated nucleocytoplasmic shuttling of proteins like PER, TIM, and CRY is likely to involve a number of specific (and not so specific) auxiliary factors that need to be identified. A similar case can be made regarding the pathways and factors involved in controlling the intranuclear trafficking and subnuclear localization of circadian transcription factors. There is a growing number of examples in which these processes play an important regulatory role in determining the outcome of a specific transcription program (Stein et al., 2000). Similar mechanisms are likely to play a role in circadian molecular routines.

Third, we are quite in the dark regarding the specific interactions that must occur around circadian DNA consensus elements, such as E-boxes (or GATA-boxes in *Neurospora*), to induce selective transcription of clock-controlled genes. As a matter of fact, we do not even know (although we might suspect this to be the case) whether clock components do contribute to other pathways, as part of different protein constellations, in either oscillatory or linearly responsive networks. The one-hybrid system and its variations

Table 1. Many different signal transduction pathways utilize common themes of protein interactions to process information and reprogram gene expression. The mechanisms involved operate in three domains to ultimately control the activity of nucleus-bound factors. Circadian clocks are no exception.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Domain I (Input)</th>
<th>Domain II (Processing)</th>
<th>Domain III (Output)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat shock (HS)/ stress response</td>
<td>Abnormal protein production</td>
<td>HS factor trimerization and nuclear translocation. Modulated by calcium-activated kinases</td>
<td>HS gene activation, protection from stress</td>
<td>Baler et al., 1996; Soncin et al., 2000</td>
</tr>
<tr>
<td>Nuclear hormone receptors</td>
<td>Ligand-mediated activation</td>
<td>Receptor release from HSPr09 and nuclear translocation. Modulated by Tyr and CKII kinases</td>
<td>Hormone-responsive gene activation, regulation of reproductive (and other) tissues</td>
<td>Miyata et al., 1997</td>
</tr>
<tr>
<td>Immune, anti cell-death. (NFκB)</td>
<td>Extracellular stimulation through IL-1, TNF, and other cytokines</td>
<td>Inactive NFκB subfamily members released from IκB cytoplasmic inhibitors, nuclear translocation. Modulated by IKK kinases</td>
<td>Activation of anti-apoptotic and immune response genes such as IL-2, TNFα, GM-CSF</td>
<td>Zandi et al., 1997</td>
</tr>
<tr>
<td>Tumor suppressor protein (p53)</td>
<td>Cell cycle, DNA damage, apoptosis</td>
<td>Reduced affinity for Mdm2 (a p53 E3 ligase), nuclear translocation. Modulated by ATM kinase</td>
<td>Regulation of genes involved in cell cycle arrest, apoptosis, DNA damage repair</td>
<td>Maya et al., 2001</td>
</tr>
<tr>
<td>Circadian clock</td>
<td>Photic input, phase of the cellular clock (i.e., relative level of state variables)</td>
<td>Per/Tim, Per/Cry heterodimerization, nuclear translocation. Modulated by CKIδ/ε</td>
<td>Activation/suppression of clock and clock-controlled genes</td>
<td>Shearman et al., 2000; Camacho et al., 2001</td>
</tr>
</tbody>
</table>
might prove useful in this context, since they provide a means for the identification of transacting factors and cis-acting regulatory element pairs.

It is interesting to consider that the apparent absence of a typical circadian clock mechanism in the yeast might have resulted in a particularly well-suited environment for the search of clock-associated molecules through the various YH systems described. We can expect that the wider and deeper use of these techniques will illuminate new areas of the molecular chronobiology field, as the mapping of all possible interactions yields ever-denser networks of interacting proteins. I like to think that this illumination has in store for us many advances, few delays, and one or two paradigm shifts.

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rescent proteins (GFP) two-fusion fluorescence resonance energy transfer (FRET). J Cell Biochem 80:293-303.


Abstract

The authors sought to investigate the role of phytochromes A and B (phyA and phyB) and cryptochromes 1 and 2 (cry1 and cry2) in the synchronization of the leaf position rhythm in Arabidopsis thaliana. The seedlings were transferred from white light–dark cycles to free-running conditions with or without exposure to a light treatment during the final hours of the last dark period. The phase advance caused by a far-red light treatment was absent in the phyA mutant, deficient in the fhy1 and fhy3 mutants involved in phyA signaling, and normal in the cry1 and cry1 cry2 mutants. The phase shift caused by blue light was normal in the cry2 mutant; reduced in the phyA, cry1, phyA cry1, and cry1 cry2 mutants; and abolished in the phyA cry1 cry2 triple mutant. The phase shift caused by red light was partially retained by the phyA phyB double mutant. The authors conclude that cry1 and cry2 participate as photoreceptors in the blue light input to the clock but are not required for the phyA-mediated effects on the phase of the circadian rhythm of leaf position. The signaling proteins FHY1 and FHY3 are shared by phyA-mediated photomorphogenesis and phyA input to the clock.

Key words Arabidopsis, circadian rhythms, cryptochrome, leaf movement, light input, phytochrome

Light is acknowledged as the most important environmental cue involved in resetting the circadian clocks. Our understanding of the light input to the clock has improved very significantly in recent years. The discovery of cryptochromes first in plants (cry1, cry2; Ahmad and Cashmore, 1993; Guo et al., 1999), then in mammals (mCRY1, mCRY2; Hsu et al., 1996; Todo et al., 1996, Kobayashi et al., 1998), insects (Stanewsky et al., 1998), and so on, accounts for a significant deal of this advance. Cryptochromes have sequence homology to distinct types of photolyases in plants and animals and, for this reason, are believed to be the product of independent evolution events (Cashmore et al., 1999). Cryptochromes lack photolyase activity and possess a distinctive C-terminal extension. In Drosophila, cryptochrome appears to be the only photoreceptor involved in light input to the clock, and residual effects of light on rhythmic behavior are considered to be the indirect consequence of vision effects on behavioral rhythms (Emery et al., 2000). In Drosophila, cryptochrome physically interacts with clock components (Ceriani et al., 1999). In mammals, the situation has been more controversial. Cryptochromes were first proposed as photoreceptors involved in the light input to the circadian clock (Miyamoto and Sancar, 1998). Although the observation that the mCRY2 mutant of mice shows reduced sensitivity to induction of mPER1 (Thresher et al., 1998) is in favor of the latter
view, the idea lost support because mcry2 displayed increased and not reduced sensitivity to light resetting of the clock (Thresher et al., 1998). The observation that mcry1 mcry2 double mutants were arrhythmic in darkness gave credit to a role of cryptochromes as central components of the mammalian clock (Van der Horst et al., 1999). More recently, experiments with mutant mice lacking mcry1, mcry2 as well as rods and most cones have suggested a redundant role of cryptochromes and opsins in the light input to the clock (Selby et al., 2000). Thus, cryptochromes in mammals would have a dual role as photoreceptors and as central components of the clock. In plants, cry1 and cry2 are clearly important for normal control of growth and development by blue light (Lin, 2000). Mutations on these genes increase the period of rhythmic expression of a photosynthetic gene under certain fluence rates of blue light (Devlin and Kay, 2000; Somers et al., 1998). The double cry1 cry2 mutant retains robust rhythmicity in Arabidopsis, indicating that in contrast to the situation in mammals, cryptochromes are not essential components of the clock (Yanovsky et al., 2000b; Devlin and Kay, 2000). In plants, phytochromes A, B, D, and E (phyA, phyB, phyD, and phyE) are also involved in the input to the clock (Somers et al., 1998; Yanovsky et al., 2000a; Devlin and Kay, 2000). phyA is a light-modulated kinase (Fankhauser et al., 1999; Yeh and Lagarias, 1998). phyA and phyB are present in the cytoplasm in dark-grown seedlings and migrate to the nucleus upon light activation (Kircher et al., 1999; Yamaguchi et al., 1999), where they interact with factors that bind to DNA (Martinez-Garcia et al., 2000). A bacteriophytochrome is involved in resetting the circadian clock in Synechococcus elongatus (Schmitz et al., 2000).

The identification of cryptochromes as circadian photopigments requires a demonstration that the function of cryptochromes is directly related to their light-absorbing properties (Lucas and Foster, 1999). Selective spectral effects on biological rhythms have not been demonstrated for cryptochromes. In plants, the effects of cry1 and cry2 on photomorphogenesis are observed under UV-A/blue light but do not extend to the red/far-red region of the spectrum. Phytochromes, in contrast, operate predominantly in the red/far-red wavebands and to a lesser degree in the blue region (Casal and Mazzella, 1998; Neff and Chory, 1998), where the pigment shows a secondary peak of absorption. Devlin and Kay (2000) recently observed that the absence of both cry1 and cry2 causes an increase in the length of the period not only under blue light but also under red light, indicating that cryptochromes are necessary for phyA signaling to the clock but not for phyA signaling during photomorphogenesis. In the present work, we use a different protocol (Yanovsky et al., 2000a) to quantify the phase shift of the circadian rhythm of Arabidopsis leaf position in response to different light qualities. The results show that phyA-mediated resetting of the circadian clock by far-red light is unaffected by the cry1 and cry2 mutations and impaired by the photomorphogenic mutants fhy1 and fhy3.

**MATERIALS AND METHODS**

Plants of Arabidopsis thaliana of the ecotype Landsberg erecta or of the phyA-201 (Reed et al., 1994), fhy1, fhy3 (Whitelam et al., 1993), cry1-1 (Ahmad and Cashmore, 1993), phyA-201 phyB-1 (Mazzella et al., 1997), cry1-1 cry2 (where cry2 is the fha-1 allele; Guo et al., 1999), phyA-201 cry1-1, phyA-201 cry1-1 cry2 (Mazzella and Casal, 2001) were grown in 5 cm² pots containing a soil/sand medium. After sowing, the pots were incubated for 3 days in darkness at 7 °C, exposed to a saturating pulse of red light (30 min, 30 µmol.m⁻².s⁻¹) to induce germination, and allowed to germinate for 24 h in darkness at 25 °C. The seedlings were grown at 20 °C under a 12 h white light/12 h dark photoperiod provided by fluorescent lamps (80 µmol. m⁻².s⁻¹).

After 2 weeks under 12-h photoperiods, during the last 5 h of the dark period prior to transfer to free-running conditions, the seedlings were either exposed to the light treatment (far-red, red, or blue light) or remained as dark controls. The far-red light treatment (100 µmol.m⁻².s⁻¹) was provided by 60-Watt incandescent lamps in combination with a water filter, six blue acrylic filters (Paolini 2031), and two red acetate filters (La Casa del Acetato, Buenos Aires, Argentina). Blue light (20 µmol.m⁻².s⁻¹) was provided by fluorescent lamps (Philips, TLF 40W/54) in combination with a blue acetate filter. Red light (40 µmol.m⁻².s⁻¹) was provided by red fluorescent tubes (Philips, 40/15).

After transfer to free running conditions (20 °C, continuous white light 10 µmol.m⁻².s⁻¹), the position of the first pair of leaves was recorded every 2 h with a digital video camera (Quick Cam, Connectix Corporation, San Mateo, CA, USA), and the angle between the petioles was measured using the Scion Image analysis program (Scion Corporation, Frederick, MD, USA).
Data were fitted, according to Millar et al. (1995), to the equation: \( L(t) = (c_0 + c_1 t) + (a_0 - a_1 t) \sin \left( \frac{2\pi T}{T} (t - \phi) \right) \), where \( L \) is the leaf angle, \( c_0 \) is the estimated value of the leaf angle at \( t = 0 \), \( c_1 \) is an estimate of the linear rate of change in leaf angle, \( a_0 \) is the estimated value of the amplitude at \( t = 0 \), \( a_1 \) is an estimate of the linear change in cycling amplitude, \( t \) is time, \( T \) is the period estimate, and \( \phi \) is the estimate of the phase at \( t = 0 \). The first 6 h of measurements were not included among the data used to estimate the parameters of the above equation to avoid effects produced by shifting the plants from colored to dim white light. The phases were calculated by linear regression through the peaks in the days following the light pulse, extrapolated back to the day of the pulse (Kondo et al., 1991; Roenneberg and Deng, 1997). Phase shifts were calculated as the difference between the phases of light-treated and dark-control plants. Data are means of at least six different plants, from three independent experiments. Period length and phase shift data are presented with their standard errors.

**RESULTS**

Seedlings of *Arabidopsis* were grown under daily white light–dark cycles. Five hours before the end of the last dark period, different groups were exposed to far-red, red, or blue light while control seedlings remained in darkness. At the end of the treatments (or objective night in the controls), all the seedlings were transferred to free-running conditions, that is, constant low fluence white light and constant temperature (Fig. 1A). In control seedlings, the leaf tips moved upward, reaching the most erect position at the beginning of the subjective night. This was followed by the opposite movement leading to the least erect position at the beginning of the subjective day (Fig. 1B). This rhythm continued for several days under free-running conditions. The period of the rhythm was 25.6 h (Table 1). Exposure to 5 h of far-red, red, or blue light had no effects on the length of the period but caused a persistent phase advance of the circadian rhythm, that is, each phase of the rhythm occurred at an earlier time point (Fig. 1B, Table 1).

The *phyA*, *cry1*, and *cry1 cry2* mutants showed normal rhythmicity when transferred to free-running conditions without a selective light treatment (Fig. 2, Table 1). The *phyA* mutant showed no phase advance in response to the far-red light treatment and only partial phase advance (compared to the wild type) in response to blue light. The *cry1* and *cry1 cry2* mutants showed a normal phase advance in response to far-red light and partial phase advance by blue light (Fig. 2, Table 1). This indicates that under the present experimental conditions, the effects of *cry1* and *cry2* were...
Table 1. Period of the leaf movement rhythm in *Arabidopsis thaliana* under free-running conditions and phase shift caused by 5 h of far-red (100 μmol·m⁻²·s⁻¹), red (40 μmol·m⁻²·s⁻¹), or blue light (20 μmol·m⁻²·s⁻¹) compared with the controls that remained in darkness. The seedlings were grown under day-night cycles and given the light treatments during the last 5 h of the last night before transfer to free-running conditions.

<table>
<thead>
<tr>
<th>Light Pulse</th>
<th>Genotype</th>
<th>Period (h)</th>
<th>Phase-Shift (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>WT</td>
<td>25.6 ± 0.4</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>phyA</em></td>
<td>26.1 ± 0.4</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><em>cry1</em></td>
<td>25.6 ± 0.5</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>cry2</em></td>
<td>25.4 ± 0.5</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>fhy1</em></td>
<td>25.6 ± 0.4</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td><em>fhy3</em></td>
<td>24.7 ± 0.4</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>Far-red</td>
<td>WT</td>
<td>24.8 ± 0.4</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>phyA</em></td>
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<td>6.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><em>cry1</em></td>
<td>24.4 ± 0.5</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>cry2</em></td>
<td>26.3 ± 0.5</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
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<td>25.1 ± 0.5</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>fhy3</em></td>
<td>24.6 ± 0.6</td>
<td>6.0 ± 0.4</td>
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<tr>
<td>Blue</td>
<td>WT</td>
<td>25.8 ± 0.5</td>
<td>8.6 ± 0.6</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td><em>cry1</em></td>
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<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>cry2</em></td>
<td>24.6 ± 0.6</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td><em>cry1 cry2</em></td>
<td>28.1 ± 0.6</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td><em>phyA cry2</em></td>
<td>24.9 ± 0.5</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td></td>
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<td>3.7 ± 0.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>Red</td>
<td>WT</td>
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<td>3.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td><em>phyA phyB</em></td>
<td>24.8 ± 0.4</td>
<td>2.2 ± 0.5</td>
</tr>
</tbody>
</table>

Period of the leaf movement rhythm in *Arabidopsis thaliana* under free-running conditions and phase shift caused by 5 h of far-red (100 μmol·m⁻²·s⁻¹), red (40 μmol·m⁻²·s⁻¹), or blue light (20 μmol·m⁻²·s⁻¹) compared with the controls that remained in darkness. The seedlings were grown under day-night cycles and given the light treatments during the last 5 h of the last night before transfer to free-running conditions.

The residual phase advance observed in the *cry1* and *phyA* mutants in response to blue light suggested that different photoreceptors could be redundantly involved in the resetting of the circadian clock under these conditions. To investigate this issue in further detail, the phase shift was analyzed in multiple mutants. The dark controls of the *cry2*, *cry1 cry2*, *phyA cry1*, *phyA cry2*, and *phyA cry1 cry2* mutants retained normal rhythmicity (Fig. 3, Table 1). The phase shift induced by blue light in the *cry1 cry2* and *phyA cry1* double mutants was reduced compared with the wild type and similar to that observed in the *cry1* single mutant. Although the *cry2* mutation did not reduce the phase shift produced in response to blue light in the wild-type background (cf. *cry2* and wild type) or *cry1* mutant background (cf. *cry1 cry2* and *cry1*), it did eliminate the residual phase shift observed in the *cry1 phyA* mutant (cf. *cry1 cry2 phyA* and *cry1 phyA*) (Table 1). This indicates that *phyA*, *cry1*, and *cry2* participate as partially redundant components of the blue light input to the clock.

The *fhy1* and *fhy3* mutants show impaired seedling de-etiolation under continuous far-red light and have been implicated in selective branches of *phyA* signaling in photomorphogenesis (Barnes et al., 1996; Cerdán et al., 1999; Yanovsky et al., 2000c). To investigate whether these mutants also affect *phyA* signaling to the clock, the seedlings were transferred to free-running conditions with or without exposure to far-red light during the final 5 h of the night. Both *fhy1* and *fhy3* showed normal rhythmicity but no phase advance of the circadian clock in response to far-red light (Fig. 4, Table 1).

The effect of red light on the phase of the circadian clock was only partially reduced by the absence of *phyA* and *phyB* (Fig. 5, Table 1). The analysis of seedling morphology (hypocotyl length, cotyledon unfolding) in response to different wavebands compared with darkness indicates that the *phyA* mutant is blind to far-red light (Whitelam et al., 1993), the *phyA cry1 cry2* mutant is blind to blue light (data not shown), and the *phyA phyB* mutant is blind to red light (Mazzella et al., 1997). Thus, additional red light photoreceptors control the phase of the circadian clock compared with seedling morphology during de-etiolation.

**DISCUSSION**
rhythmic bioluminiscence driven by the luciferase gene under the control of a light-harvesting complex gene promoter (CAB:LUC) is extended by the phyA mutation (Somers et al., 1998). A similar effect is caused by the cry1 mutation (Devlin and Kay, 2000).

Since phyA and cry1 mutants also cause extended periods over the same range of fluence rates of white light and the phyA cry1 double mutant shows a deficiency that is not larger than that observed in any of the single mutant parental lines, cry1 appears to be necessary for...
phyA signaling to the clock (Devlin and Kay, 2000). Our data show that cry1 is not universally required for phyA signaling to the clock.

Both sets of data can be reconciled by considering that phyA signals via two different pathways that can be dissected photobiologically (Casal et al., 2000), genetically (Yanovsky et al., 1997; Yanovsky et al., 2000c), and molecularly (Cerdán et al., 2000). The so-called very-low-fluence response (VLFR) pathway operates under red or far-red light and saturates with infrequent (hourly) light pulses. The high-irradiance response (HIR) pathway requires sustained (continuous or very frequently pulsed) far-red light. phyA requires cry1 under red light (Devlin and Kay, 2000), that is, under conditions where phyA operates via the VLFR pathway. Hourly pulses of far-red light (instead of continuous far-red light) were fully ineffective to reset the circadian rhythm of leaf movement in Arabidopsis (data not shown), indicating that phyA operated via the HIR pathway. This is consistent with the dramatic failure to reset the clock in response to far-red light observed in fhy3, a mutant impaired in HIR but retaining VLFR (Yanovsky et al., 2000c). In conclusion, we propose that cry1 is required for phyA signaling to the clock in the VLFR but not in the HIR mode. This working hypothesis should be tested in a system where both VLFR and HIR operate.

Little is known about the mechanisms by which phytochromes and cryptochromes signal to the clock. In Drosophila, for instance, cryptochrome shows direct interaction with clock components (Ceriani et al., 1999). The observation that phyA requires cry1 for signaling to the clock but not for photomorphogenesis (Devlin and Kay, 2000) suggests that some elements could be different between both processes. The failure in photomorphogenesis and far-red-mediated phase
shifting observed in fhy1 and fhy3 demonstrates that, at least in the HIR mode, some signaling elements are shared between the two processes. fhy1 and fhy3 are predicted to operate downstream phyA in the light-signaling cascade. This argues against a direct action of phyA on clock components as observed for cryptochrome in Drosophila. The effects of fhy1 and fhy3 are consistent with the earlier conclusion that phyA, although affecting the response to several wavebands, is not an essential component of the clock itself.

The phyA phyB cry1 cry2 quadruple mutant is virtually blind to white light for de-etiolation but retains robust circadian rhythmicity implicating other photoreceptor(s) involved in the entrainment of the circadian clock (Yanovsky et al., 2000b). The observations that the phyA cry1 cry2 triple mutant shows no phase shift in response to blue light whereas the phyA phyB double mutant retains a small but significant phase shift under red light points toward a red-light photoreceptor as the pigment involved in changing the phase of the rhythm in response to white light in the quadruple mutant. The small effect of red light could accumulate over several days. Since the period of the circadian rhythm of CAB::LUC bioluminescence is extended in the phyA phyB phyD and phyA phyB phyE triple mutants compared with the phyA phyB double mutant (Devlin and Kay, 2000), phyD and phyE are good candidates. phyD and phyE have very weak effects during de-etiolation (Aukerman et al., 1997; Devlin et al., 1998), and this favors the idea that like flies and mammals, plants could also have photoreceptors predominantly or exclusively involved in circadian rhythms (Yanovsky et al., 2000a).

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Distinct Pharmacological Mechanisms Leading to c-fos Gene Expression in the Fetal Suprachiasmatic Nucleus

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Abstract

Maternal treatment with cocaine or a D₁-dopamine receptor agonist induces c-fos gene expression in the fetal suprachiasmatic nuclei (SCN). Other treatments that induce c-fos expression in the fetal SCN include caffeine and nicotine. In the current article, the authors assessed whether these different pharmacological treatments activate c-fos expression by a common neurochemical mechanism. The results indicate the presence of at least two distinct pharmacological pathways to c-fos expression in the fetal rat SCN. Previous studies demonstrate that prenatal activation of dopamine receptors affects the developing circadian system. The present work shows that stimulant drugs influence the fetal brain through multiple transmitter systems and further suggests that there may be multiple pathways leading to entrainment of the fetal biological clock. Key words circadian rhythms, D₁-dopamine receptor, gene expression, entrainment, SKF 38393, caffeine, nicotine

The suprachiasmatic nuclei (SCN) contain a biological clock that regulates circadian rhythmicity in mammals (Klein et al., 1991; Weaver, 1998). The biological clock within the SCN is oscillating prior to birth in rodents, and the timing of the fetal clock is set (entrained) prior to birth (Davis, 1997; Reppert and Weaver, 1991). It appears that redundant signals from the mother are normally involved in entraining the fetal SCN (Reppert and Weaver, 1991; Viswanathan et al., 1994; Viswanathan and Davis, 1997).

Prenatal administration of melatonin and the D₁-dopamine receptor agonist, SKF 38393, can set the phase of the fetal biological clock (Davis and Mannion, 1988; Viswanathan et al., 1994; Viswanathan and Davis, 1997). Both D₁ receptors and melatonin receptors are expressed in the developing rodent SCN (Bender et al., 1997; Carlson et al., 1991; Duffield et al., 1999; Shearman et al., 1997; Strother et al., 1998a; Weaver et al., 1992). Activation of these receptor types, which have opposite effects on cyclic AMP levels, sets the phase of the fetal clock to opposite phases in hamsters (Viswanathan and Davis, 1997). Prenatal entrainment by these pharmacological treatments demonstrates that functional receptors are present in the fetal brain. The correlation of entrainment with c-fos gene induction in the fetal SCN following dopaminergic drug treatment suggests that induction of c-fos gene expression is a useful marker for functionally relevant stimulation of the SCN. It is important to note, however, that failure of a treatment to induce c-fos gene expression does not necessarily indicate insensitivity to this input. For example, melatonin entrains the fetal

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SCN without induction of c-fos expression (Viswanathan and Davis, 1997).

Nicotine also has been reported to induce c-fos gene expression in the fetal SCN (Clegg et al., 1995; O’Hara et al., 1998; see O’Hara et al., 1999, for review). The effects of nicotine on c-fos mRNA levels in the rat SCN are restricted to the perinatal period (O’Hara et al., 1999). Prenatal nicotine treatment leads to increased expression of other immediate early genes, including junB (O’Hara et al., 1999). In these respects, prenatal treatment with nicotine is similar to prenatal treatment with SKF 38393, which has a transient, perinatal period of efficacy and leads to induction of several immediate early genes (Weaver and Reppert, 1995; Weaver et al., 1995). The possibility that prenatal nicotine treatment alters entrainment or other aspects of developing circadian function has not been examined. In adult rodents, however, nicotine treatment can cause phase shifts (Ferguson et al., 1999; O’Hara et al., 1999; Trachsel et al., 1995), and there are nicotinic components to light-induced c-fos expression and light-induced phase shifts (Keefe et al., 1987; O’Hara et al., 1998; Zhang et al., 1993).

We are interested in identifying agents that induce c-fos gene expression in the fetal SCN, as these agents may reveal receptor-mediated mechanisms for influencing the SCN relevant to prenatal entrainment. Caffeine treatment induces c-fos gene expression in adult rodents that is limited to the striatum (Nakajima et al., 1989). Our interest in the functional development of adenosine receptors (Shearman and Weaver, 1997; Weaver, 1993, 1996) led us to perform preliminary studies on the effects of caffeine on c-fos gene expression in fetal brain. To our surprise, maternal caffeine treatment caused a widespread increase in c-fos expression in the fetal brain, most notably in the SCN. The anatomical pattern of c-fos expression in the fetal brain after maternal caffeine treatment did not closely match the distribution of expression of either A1 or A2A adenosine receptor subtypes, but it did resemble the pattern observed after maternal treatment with SKF 38393 (Shearman et al., 1997; Fig. 1). The patterns of c-fos gene expression after SKF 38393 and caffeine, while similar, are distinct from the restricted pattern of c-fos gene expression in the fetal brain after maternal treatment with nicotine (Clegg et al., 1995). These observations suggested to us that caffeine and SKF 38393 may share a convergent neurochemical mechanism for induction of c-fos expression, while the effects of nicotine may be independent. In the present report, a pharmacological approach was used to identify the receptor types leading to c-fos gene expression in the fetal SCN. The results indicate that there are multiple and distinct pharmacological mechanisms leading to c-fos gene expression in the fetal SCN.

MATERIALS AND METHODS

Animals and Drug Treatments

Timed-pregnant Sprague-Dawley rats (MBM:VAF, Zivic Miller Laboratories, Zelienople, PA, USA) were housed in a centralized vivarium and were exposed to a light-dark cycle consisting of 12 h light:12 h dark, with lights on at 0600 h EST (LD). During the dark phase of the cycle, animals were exposed to red light. Gestational day (GD) 0 is defined as beginning the morning after overnight pairing resulting in a sperm plug. Rats were studied in the afternoon, 6 to 10 h after lights-on, on GD 20.

Each pregnant rat received two intraperitoneal injections, 30 min apart. The first injection was a potential antagonist or vehicle. The antagonists used were the D1-dopamine receptor antagonist SCH 23390 (0.5 mg/kg), the nicotinic cholinergic antagonist mecamylamine (Meca) (5 mg/kg), and the NMDA receptor antagonist MK 801 (0.5 mg/kg). The second injection was an agent to induce c-fos gene expression, SKF 38393 (10 mg/kg), caffeine (100 mg/kg), nicotine (free base, 1 mg/kg), or vehicle. Limitations on the number of animals that could be treated on 1 day and the number of sections that could be processed together for in situ hybridization dictated that three separate experiments be conducted, with each experiment consisting of all four pretreatments in combination with two posttreatments (one inducing drug and its vehicle). Doses were selected on the basis of preliminary dose-response studies (caffeine; see also Nakajima et al., 1989) or on the basis of responses reported in the literature (Clegg et al., 1995; Weaver and Clemens, 1987; Weaver et al., 1992). In preliminary studies, we noted that the noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, MK 801, was not well tolerated by fetal rats at the doses most frequently used in the literature (1.0-3.0 mg/kg; Kennaway and Moyer, 1999; Nakazato et al., 1998; Svenningsson et al., 1996), and so we used a lower dose (0.5 mg/kg). Injections were given at a volume of 2 mL/kg. Dams were killed by decapitation 30 min after the second injection. Fetuses were then removed from the uterus and decapitated. Fetal heads were
frozen in cooled 2-methylbutane (–20°C) and stored at –80°C. Animal studies were reviewed and approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

**In Situ Hybridization**

Sections (15 microns) were cut on a Bright-Hacker cryostat at –20°C and thaw-mounted onto slides coated with Vectabond (Vector Labs, Burlingame, CA, USA). In situ hybridization was used to detect c-fos mRNA. Antisense and sense (control) cRNA probes were produced from linearized plasmid DNA by in vitro transcription in the presence of 35S-alpha-thio-UTP (1100-1300 Ci/mmol, NEN). Probes were purified by extraction and ethanol precipitation prior to use. The coding region of the mouse c-fos cDNA in pGEM3 (plasmid MUSFOS3, kindly provided by Dr. Michael E. Greenberg) was used as the template in the transcription reaction, as previously described (Weaver et al., 1992). Prehybridization, hybridization, and wash conditions were as previously described (Weaver, 1993). Film autoradiograms were generated by apposition of slides to Kodak SB-5 film for 10 to 15 days. Radioactive standards (14C, 20-micron thickness; American Radiolabeled Chemical, St. Louis, MO, USA) were included on each film.

**Data Analysis**

Optical density measurements were performed using a computer-based image analysis system as previously described (Weaver, 1993). In situ hybridization results are presented as SCN relative optical density (OD) values, defined as the OD of the SCN divided by the OD of the adjacent hypothalamus in the same section. Relative OD values from the two to three sections having the most intense SCN hybridization signal were averaged to give the relative OD value for each fetus. To determine whether

Figure 1. c-fos gene expression in fetal rat brain after administration of (A) SKF 38393, (B) caffeine, or (C) vehicle. Sections shown in panels A, B, and C were hybridized with an antisense probe to detect c-fos mRNA. Panel D represents a section adjacent to the section shown in B but hybridized with a sense strand (control) probe to illustrate nonspecific hybridization. Abbreviations: CP = caudate-putamen (striatum); DE = dorsal endopiriform nucleus; SCN = suprachiasmatic nuclei.
pretreatments had a statistically significant impact on c-fos gene expression, data (OD values) were analyzed by one-way analysis of variance and Dunnett’s test using Statview version 1.03 on a Macintosh computer. Pairwise comparisons were performed using Student’s t test. Statistical significance was set at p < 0.05.

For production of the figures, images were captured using a Polaroid DMCIE digital camera and imported into Photoshop 5.0 for assembly of composite images.

Chemicals and Drugs

SKF 38393, SCH 23390, MK 801, and Meca were obtained from Research Biochemicals, Inc., division of Sigma-Aldrich, Inc. (Natick, MA, USA). Caffeine and nicotine (free base) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). General lab chemicals were purchased from Sigma or Fisher Scientific (Springfield, NJ, USA).

RESULTS

Maternal treatment with caffeine (100 mg/kg) or SKF 38393 (10 mg/kg) significantly increased c-fos gene expression in the fetal SCN (Fig. 1). The two drug treatments produced a grossly similar, widespread pattern of c-fos expression in the fetal brain, extending beyond the SCN and including the striatum and other regions (Fig. 1 and data not shown). The similarity in pattern between fetuses exposed to the dopaminergic agonist and to caffeine suggested that there might be a similar neurochemical mechanisms, for example, that the effects of caffeine might be exerted through indirect effects on dopamine systems in the fetal brain (Ferre et al., 1992; Fredholm, 1995; Garrett and Holtzman, 1994). To assess this possibility, pregnant dams were pretreated with antagonists prior to receiving a stimulant drug.

The antagonists used had differential effects on stimulant-induced c-fos expression in the SCN (Figs. 2, 3). Pretreatment with the D_1-dopamine receptor antagonist, SCH 23390, prevented the response to SKF 38393 (Figs. 2E, 3A). Pretreatment with the nicotinic cholinergic receptor antagonist Meca or the NMDA receptor antagonist MK 801 did not affect the SCN response to SKF 38393 (Figs. 2H, 2K, 3A). MK 801 pretreatment did, however, markedly reduce the induction of c-fos expression in the lateral portion of the fetal striatum (Fig. 2K).

Caffeine-induced c-fos expression in the SCN (Fig. 2C) was not prevented by pretreatment with SCH 23390 (Figs. 2F, 3B). In fact, SCN c-fos mRNA levels were actually higher following treatment with SCH 23390 plus caffeine than after treatment with vehicle plus caffeine (Fig. 3A; p < 0.05, Dunnett’s test). This increase may be due in part to an increase in c-fos expression due to treatment with SCH 23390. Remarkably, however, the induction of c-fos in the striatum was completely prevented by the D_1 receptor antagonist (compare Figs. 2C and 2F). Both Meca and MK 801 reduced the amplitude of the SCN response to caffeine (Fig. 2I, 2L; Fig. 3). MK 801 also reduced the striatal response to caffeine (Fig. 2L).

Experiments were also conducted to assess the pharmacological characteristics of c-fos induction in response to nicotine treatment (Figs. 4, 5). Preliminary experiments demonstrated that nicotine bitartrate did not produce a substantial c-fos response in the fetal SCN when administered at 2.9 mg/kg (equivalent to 1 mg/kg nicotine). In the free-base form, however, nicotine did cause a modest increase in c-fos expression in the fetal SCN, consistent with previous reports (Clegg et al., 1995; O’Hara et al., 1998, O’Hara et al., 1999). Nicotine also induced c-fos expression in the supraoptic and paraventricular nuclei of the hypothalamus (Fig. 4 and data not shown; see also O’Hara et al., 1999), but not in the striatum, giving an anatomical pattern clearly distinct from that seen following maternal treatment with caffeine or SKF 38393. Pretreatment with each of the antagonists, SCH 23390, Meca, or MK 801, significantly reduced the effect of nicotine on SCN c-fos mRNA levels (Fig. 5; Dunnett’s test). SCH 23390 and mecamylamine each blocked the effect of nicotine, while MK 801 reduced the amplitude of response but did not prevent it (Fig. 5; t tests).

DISCUSSION

The results of these experiments indicate the existence of multiple and distinct pharmacological mechanisms for regulating c-fos gene expression in the fetal SCN. In the adult SCN, major regulators of c-fos include glutamate and serotonergic agonists (Ebling, 1996; Kennaway and Moyer, 1999), while in the fetal SCN, major regulators of c-fos expression include dopamine receptor activation and an unidentified pathway activated by caffeine. Remarkably, none of the treatments used to induce c-fos expression in the fetal SCN in this study causes a detectable increase in
c-fos expression in the adult SCN (Clegg et al., 1995; Nakajima et al., 1989, unpublished data; Weaver and Reppert, 1995; Weaver et al., 1992). The mechanisms regulating c-fos expression in the brain appear to vary depending on the pharmacological stimulus, the region, and the developmental stage examined, and coincident stimulation of multiple neurotransmitter systems may play a more important role in some regions (e.g., striatum) than in others (e.g., SCN, see below).

The dopaminergic agonist, SKF 38393, acts through D1 receptors to induce c-fos gene expression in the fetal SCN. The D1-receptor antagonist, SCH 23390, blocked the induction of c-fos by SKF 38393. The present results are consistent with previous results in mice with targeted disruption of the D1-dopamine receptor gene and previous pharmacological data, indicating that dopaminergic agents induce c-fos expression in the SCN via activation of D1-dopamine receptors (Bender et al., 1997; Weaver et al., 1992). In contrast, induction of c-fos expression in the fetal SCN by caffeine was not disrupted by SCH 23390, indicating the existence of an additional, D1-receptor-independent pathway lead-
The mechanism by which caffeine induced $c$-$fos$ expression in the fetal SCN is unclear. Caffeine is an adenosine receptor antagonist (Fredholm, 1995; Nehlig et al., 1992), but $A_1$ and $A_{2A}$ adenosine receptor mRNAs are not expressed at detectable levels in the fetal SCN (Weaver, 1993, 1996). Furthermore, the induction of $c$-$fos$ expression in the adult striatum occurs only at pharmacological levels of caffeine and does not appear to be due to antagonism of specific adenosine receptor subtypes, suggesting a more complicated mechanism (Fredholm, 1995; Johanssen et al., 1992; Nakajima et al., 1989). Notably, the mechanism by which caffeine stimulates $c$-$fos$ gene expression in the fetal striatum is pharmacologically distinct from the mechanism by which caffeine leads to $c$-$fos$ in the fetal SCN. Pretreatment with the $D_1$-dopamine receptor antagonist, SCH 23390, prevented induction of $c$-$fos$ expression in the striatum but not in the SCN. $D_1$-dopamine receptors are present in both the SCN and striatum, and dopaminergic innervation to both areas has been demonstrated during late fetal life (Bender et al., 1997; Duffield et al., 1999; Shearman et al., 1997; Strother et al., 1998a). The presence of $D_2$-dopamine receptors in the fetal striatum and their absence in the SCN (Schambra et al., 1994; Weaver et al., 1992) raises the possibility that synergistic interactions between the $D_1$ and $D_2$ receptor subtypes occur in the striatum and that these interactions may contribute to regulation of $c$-$fos$ expression in fetal striatum. In the fetal SCN, the effects of caffeine on $c$-$fos$ expression appear to be independent of $D_1$-dopamine receptor stimulation.

In the adult brain, glutamate receptor activation plays a major role in the regulation of $c$-$fos$ expression (Chaudhuri, 1997). Glutamate regulates $c$-$fos$ expression in the SCN and is a principal mediator conveying photic information from the retina to the SCN for entrainment (Ebling, 1996; Mintz et al., 1999). In the adult striatum, induction of $c$-$fos$ expression by $D_1$-dopamine-receptor activation appears to require coactivation of NMDA receptors (Nakazato et al., 1998). Similarly, in the present study, the NMDA receptor antagonist MK801 prevented SKF 38393−induced $c$-$fos$ expression in the lateral striatum. In contrast, the induction of $c$-$fos$ expression in the fetal SCN by SKF 38393 treatment was remarkably unaffected by pretreatment with MK 801. Thus, synergism between $D_1$-dopamine and NMDA receptors is apparently required in the adult and fetal striatum, but not in the fetal SCN.
Dopaminergic, cholinergic, and glutaminergic mechanisms each appear to contribute to the fetal response to nicotine. There may be serial activation of several receptor types in generating the response to nicotine, making it susceptible to disruption at each of several points, or responsive cells may require coincident stimulation by several receptor subtypes. Nicotine may influence c-fos expression by acting as an indirect agonist, stimulating release of monoamines and/or glutamate through a presynaptic mechanism. Indeed, nicotinic induction of Fos in the adult brain is disrupted by NMDA receptor antagonists and D₁ dopamine receptor antagonists (Kiba and Jayaraman, 1994). Data from adult SCN slices indicate that nicotine affects the SCN by action directly within the nucleus, however (Trachsel et al., 1995). Furthermore, nicotinic receptor subunit gene expression has been documented in the fetal SCN (O’Hara et al., 1999), providing a substrate for direct action of nicotine in the fetal SCN. These lines of evidence indicate that while...
The mechanism of action of nicotine is pharmacologically complex, this complexity may occur within the anatomical confines of the SCN.

Our studies were conducted during the subjective day on GD 20. Previous studies indicate that the fetal SCN expresses detectable but nonrhythmic (“basal”) levels of c-fos mRNA (Viswanathan et al., 1994; Weaver et al., 1992). Nevertheless, there is abundant evidence that the circadian oscillator in the SCN is functioning at this age (Reppert and Weaver, 1991), raising the possibility that the responses observed were influenced by the time of day of study. In the fetal SCN, c-fos gene expression is induced equally well following administration of dopaminergic drugs during the subjective day and subjective night (Viswanathan et al., 1994; Weaver et al., 1992). This is in contrast to the pronounced “gating” of photic induction of c-fos, junB, and mPer gene expression in the adult, in which responses are restricted to subjective night. While it is possible that the basal neurochemical tone to the fetal SCN varies over the course of the day, it seems unlikely that the pharmacological mechanisms leading to drug-induced c-fos expression would vary as a function of time of day. Thus, we would not expect different results had the studies been conducted at night. The results might have been different, however, had we conducted them at a different developmental age. Rhythmicity of basal c-fos levels appears in the dorsomedial SCN shortly after birth. Detection of drug-induced c-fos during the daytime would have been more difficult during the postnatal period. Furthermore, the loss of the c-fos responses to drugs during the early postnatal period limits the developmental window during which these studies could be conducted (Weaver and Reppert, 1995).

Pharmacological activation of dopamine receptors in the fetal SCN can influence the development and function of the circadian timing system. A single injection of SKF 38393 late in gestation is sufficient to entrain fetal Syrian hamsters (Viswanathan and Davis, 1997). Sensitivity to SKF 38393 begins during the prenatal period and continues into the neonatal period (Grosse and Davis, 1999; Viswanathan et al., 1994). Entrainment to SKF 38393 may result from induction of Per1 gene expression; Per1 gene expression is increased in the fetal rat SCN by maternal treatment with SKF 38393 (Spearman and Weaver, unpublished data). Prenatal stimulation of dopamine receptors also alters the development of SCN responsiveness to light at night in both rats and hamsters (Ferguson & Kennaway, 2000; Ferguson et al., 2000; Strother et al., 1998b).

Prenatal exposure to caffeine or nicotine may also alter the circadian timing system. There is limited evidence to suggest that theophylline, a methylxanthine with pharmacological effects similar to caffeine, can influence the circadian timing system in adult rodents (Ehret et al., 1975). While much of the current research on caffeine relates to its ability to promote arousal and reduce fatigue (Wright et al., 1997), the possibility that caffeine may have effects mediated by the SCN should be considered. The absence of detectable c-fos expression in the adult SCN after high-dose caffeine treatment (Nakajima et al., 1989; our unpublished data) does not rule out an effect of caffeine on the circadian timing system, as c-fos gene expression is not necessarily induced by phase-shifting stimuli (Colwell et al., 1993; Kumar et al., 1997; Rea et al., 1993; Viswanathan and Davis, 1997).

The present results support the concept that multiple, functional neurotransmitter systems are active in the fetal brain, and suggest that exposure to widely used stimulant drugs during pregnancy may influ-
ence brain development and function. Indeed, ingestion of caffeine and nicotine during development have been shown to alter neurochemical development and can have long-lasting neurobehavioral effects (Aden et al., 2000; Etzel and Guillet, 1994; Fisher and Guillet, 1997; Grimm and Frieder, 1988; Nehlig and Deby, 1994; Ribary and Lichtensteiger, 1989; Slotkin, 1998; Sobotka, 1989). Among the effects of prenatal exposure to common neuromodulatory drugs may be altered development of the circadian timing system.

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Daily Novel Wheel Running Reorganizes and Splits Hamster Circadian Activity Rhythms

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Abstract  The phenomenon of splitting of locomotor activity rhythms in constant light has implied that the mammalian circadian pacemaker is composed of multiple interacting circadian oscillators. Exposure of male Syrian hamsters to novel running wheels also induces splitting in some reports, although novel wheel running (NWR) is better known for its effects on altering circadian phase and the length of the free-running period. In three experiments, the authors confirm and extend earlier reports of split rhythms induced by NWR. Male Syrian hamsters, entrained to LD 14:10, were transferred for 6 to 11 consecutive days to darkened novel Wahmann wheels at ZT 4 and were returned to their home cages at ZT 9. All hamsters ran robustly in the novel wheels. NWR caused a marked reorganization of home cage wheel-running behavior: Activity onsets delayed progressively with each additional day of NWR. After 11 days, activity onset in the nighttime scotophase was delayed by 7 h and disappeared completely in 2 hamsters (Experiment 1). After 6 to 7 days of NWR (Experiment 2), activity onset delayed by 5 h. Transfer of hamsters to constant darkness (DD) after 7 days of NWR revealed clearly split activity rhythms: The delayed nighttime activity bout was clearly identifiable and characterized by a short duration. A second bout associated with the former time of NWR was equally distinct and exhibited a similarly short duration. These components rejoined after 3 to 5 days in DD accomplished via delays and advances of the nighttime and afternoon components, respectively. The final experiment established that rejoining of activity components could be prevented by perpetuating the light-dark:light-dark cycle used to induce split rhythms. The data suggest that NWR causes selective phase shifting of some circadian oscillators and that component oscillators interact strongly in constant darkness.

Key words  splitting, oscillator interaction, coupling, nonphotic

A multioscillator basis for mammalian circadian rhythms has been adduced through studies of photoperiodic control of activity duration (α), internal desynchronization, splitting, and most recently, in vitro electrical recordings of single SCN cells (Aschoff, 1965; Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova, 1991; Liu et al., 1997; Pittendrigh and Daan, 1976). Each set of studies reinforces the idea that coherent circadian rhythms are generated from the interaction of coupled constituent oscillators with a range of free-running periods, τ. Although significant advances have been made in clarifying the neuroana-
tomycal and physiological substrates for rhythm generation and entrainment, the formal properties of oscillator interaction have received less sustained attention.

A major exception to this generalization is the study of split locomotor activity rhythms first reported in the arctic ground squirrel, *Spermophilus undulatus* (Pittendrigh, 1960) and elaborated further in studies of Syrian hamsters, *Mesocricetus auratus* (Pittendrigh and Daan, 1976). After prolonged (e.g., 60 days) exposure to constant light (LL), locomotor activity rhythms of some individuals dissociate into two components that free-run initially with different frequencies. When the two split activity components adopt an antiphase relationship (180 degrees apart), they free-run with a common frequency greater than that measured just prior to splitting. A comparable phenomenon is obtained in a day-active species exposed to low levels of light intensity (Hoffmann, 1971).

Exposure to constant lighting conditions is not the only manipulation capable of splitting mammalian circadian rhythms. Although not discussed in the text, Bruce’s (1960) study of frequency demultiplication includes a single actogram of a hamster maintained in short cycles of 2 h light, 4.5 h dark (LD 2:4.5). In this record, two activity components 180 degrees apart were apparent for approximately 7 days before one of these components disappeared. Mrosovsky and Janik (1993) reorganized the activity rhythms of hamsters maintained in LD 14:10 by exposing them each afternoon to 3-h pulses of novel wheel running (NWR) in the dark (beginning 7 h before normal lights-off). When NWR was discontinued and hamsters were left in their home cages in constant darkness (DD), locomotor activity rhythms were split into two components that rejoined after 3 to 5 days, although this pattern was not equally clear in all records shown (e.g., #3802 in their Fig. 2). Nighttime activity onsets in LD 14:10 were also phase-delayed by several hours during NWR. Sinclair and Mistlberger (1997), using a different strain of hamster and a slightly modified protocol, found less compelling evidence of splitting after 17 days of NWR, although nighttime activity onset was delayed in some animals. Using the hamster strain employed by Mrosovsky and Janik (1993) and a modification of their experimental protocol, we here describe marked reorganizations of locomotor activity rhythms induced by three regimens of daily NWR.

**MATERIALS AND METHODS**

**Animals and Husbandry**

For all experiments, a subset of the same 24 male Syrian hamsters that were used in a separately reported study published in this issue (HsdHan: AURA; Harlan, Indianapolis, IN, USA) (Gorman et al., 2001 [this issue]), 5 to 6 weeks of age at acquisition, were housed with Sani-Chip bedding in polypropylene cages (48 × 27 × 20 cm) equipped with Nalgene (d = 34 cm) running wheels (Fisher Scientific, Pittsburgh, PA, USA). Food (Purina Rodent Chow #5001, St. Louis, MO, USA) and water were available ad libitum. Syrian hamsters were entrained to LD 14:10 (lights on 0500-1900 h; approximately 100 lux) for 3 weeks before an initial regimen of daily NWR was initiated.

**Novel Wheel Running**

Following entrainment to LD 14:10 (lights off = ZT 12), hamsters were transferred within the same room to Wahlmann wheels (d = 34 cm) 0 to 15 min before lights were extinguished at 1100 h (ZT 4) EST. At 1600 h (ZT 9), the lights were turned on and hamsters were returned to home cages in the light over the next 15 min. Thus, during NWR, animals were exposed to an LDLD 6:3:5:10 light schedule. On one day, the darkened hamster room was entered through a light lock at hourly intervals from 1200 to 1600 h to record the number of novel wheel revolutions with the aid of a dim red light.

**Analysis**

Wheel-running activity in the home cage was monitored by Dataquest III software (Mini-mitter, Sun River, OR, USA) and compiled into 10-min bins. While in the novel wheels, activity patterns were not monitored, but the total number of wheel revolutions after the 5-h interval was recorded manually. Data analyses were carried out with Excel (Microsoft, Bellevue, WA, USA) and ClockLab software (Actimetrics, Evanston, IL, USA).

Activity onset was defined as the first time point in a scotophase in which a hamster ran more than 20 revolutions in a 10-min interval followed immediately by
an additional 10-min interval with more than 20 wheel revolutions. Activity offset was defined as the last time point in a scotophase that the animal ran more than 20 revolutions and that was preceded immediately by a similar 10-min interval of activity. Activity duration (α) was calculated as the interval between activity onset and activity offset. An interval of inactivity was calculated as the difference between activity offset and the subsequent activity onset. The circadian period of activity onsets either in constant conditions (τ) or while exposed to a light-dark cycle (τ*) was estimated with linear regression by determining slope of activity onsets over 4- to 7-day intervals. The phase angle of entrainment was determined from the average value predicted by the regression line and was expressed in relation either to the time of lights-off (ψlights-off) or lights-on (ψlights-on). When activity components were split, circadian parameters were calculated separately for activity bouts corresponding to the original 10-h dark period (i.e., the nighttime, n, activity bout) and to the 5-h interval of NWR (i.e., the afternoon, a, activity bout). The phase angle between components was defined as the difference between their respective activity onsets (ψn–a).

Experiment 1

Hamsters, 8 to 9 weeks of age, previously entrained to LD 14:10, were exposed to NWR in LDLD 6:3:5:10 (n = 20). After 11 days of these treatments, hamsters remained undisturbed in their home cages for 2 additional days under the same light conditions.

Experiment 2

Because 11 days of NWR in Experiment 1 phase-delayed nighttime activity onset more than expected on the basis of published studies, we next assessed whether more evenly split activity would be obtained after fewer days of NWR. Hamsters from Experiment 1, 12 to 13 weeks of age, were re-entrained to LD 14:10 for 14 days and exposed to NWR under LDLD 6:3:5:10 for 7 days (n = 19). Four additional hamsters, with identical light histories but no previous running-wheel exposure, were equipped with Nalgene wheels. In this experiment, these control hamsters were exposed to LDLD 6:5:3:10 without NWR. All hamsters were minimally disturbed during days of NWR except for a single retro-orbital bleeding conducted on the final day as part of another study. After the final day of NWR, 9 of the 19 hamsters, randomly selected, remained in this study and were exposed to constant darkness (DD) initiated during the subsequent 10-h scotophase (i.e., the lights remained off at 0500 h). Data from the remaining 10 hamsters are reported here only through the final day of NWR, after which they received a different light treatment described in a separate study (Gorman et al., 2001). Periods of the free-running rhythms of activity onset were calculated for each of the 9 hamsters during days 1 to 4 and 8 to 11 of DD.

Experiment 3

Because a distinctly and evenly split home cage running rhythm was obtained in Experiment 2, we asked whether these hamsters could be entrained to the LDLD cycle in effect during NWR. The same hamsters (n = 20 including 1 former control hamster from Experiment 2), 30 to 31 weeks of age, were re-entrained to LD 14:10 and treated as described in Experiment 2 except that they were not bled. After 6 days of NWR in LDLD 6:5:3:10, hamsters remained in their home cages for 11 days on the same LDLD cycle described above. Two hamsters with no prior NWR exposure (controls from Experiment 2) were exposed to identical light conditions but were not transferred to novel wheels.

Analyses of activity onsets were performed using data from the last 7 days of exposure to LD 14:10 prior to NWR and the first 7 days of continuous home cage exposure to LDLD 6:5:3:10 after NWR.

Statistical tests (all two-tailed where applicable) were performed with Statview 5.0 software (SAS Institute, Cary, NC, USA).

RESULTS

Experiment 1

Hamsters transferred to novel wheels exhibited robust wheel-running (mean = 8186 ± 160 revolutions/5 h, range = 6817-9210, n = 20), with no significant change in amount of wheel running over the 11 days of the experiment (p > 0.70, repeated measures ANOVA). When measured on Day 2 of NWR, the number of wheel revolutions varied over time (p < 0.001), with significant monotonic increases (p < 0.05) over the first 4 h and a decrease from the 4th to the 5th hour (p < 0.05).

All 20 hamsters showed a marked reorganization of nighttime activity during NWR characterized by progressive delays in the onset of home cage activity
(Figs. 1, 2). In 2 hamsters (e.g., Fig. 1B), activity onsets delayed so far as to eliminate all nighttime activity on the last 1 to 2 days of NWR. When left in the home cage in the LDLD cycle, 19 out of 20 hamsters showed spontaneous activity in the afternoon dark period, and 17 out of 20 hamsters showed activity in both the afternoon and nighttime scotophases (Fig. 1). The 1 hamster that did not run in the afternoon dark phase was exceptional in having the smallest delay of nighttime activity onset. On each of the final 2 days when hamsters remained in the home cage on the LDLD cycle, a disproportionate amount of running activity occurred in the afternoon scotophase (65% ± 5%, 72% ± 5%, respectively, \( n = 20 \)).

**Experiment 2**

As in Experiment 1, NWR was observed to be robust in the entire cohort of animals tested (mean = 8153 ± 220 revolutions/5 h, range = 6,127-10,044, \( n = 19 \)). After 7 days in novel wheels, nighttime activity onset was delayed approximately midway through the scotophase (Figs. 2, 3).

Upon release into DD, 8 out of 9 hamsters showed two distinct (i.e., split) activity components roughly coincident with the prior time of nighttime running and novel wheel exposure, respectively (Fig. 3). In DD, nighttime activity onsets occurred progressively later in the first 3 days of DD (Table 1), whereas the afternoon component was neither markedly advanced nor delayed. Thus, the phase angle between components (\( \Psi_{n-a} \)) and interval of inactivity initially separating the nighttime and afternoon bouts in DD rapidly diminished. In the first 3 days of DD, the two activity bouts contained comparable amounts of activity, and bouts were characterized by short \( \alpha \)s. A redistribution of activity from the afternoon activity component to the nighttime component was commonly seen on the 3rd to 5th day in DD (Fig. 3). The free-running rhythm derived from onsets of the nighttime component shortened significantly after 7 days in DD (Table 1, Fig. 3). A comparable analysis of the afternoon component was not undertaken, because it seldom remained distinct for more than 3 days in DD. Abimodal activity pattern persisted beyond the fifth day of DD, after the disappearance of a robust, clearly distinguishable afternoon component. However, this bimodality appeared to be indistinguishable from that characteristic of unsplit hamsters with comparable \( \alpha \) in DD. In other words, by this time the bimodal pattern does not suggest persistent splitting. A quantitative description of nighttime and afternoon activity components in DD is presented in Table 1.

Control animals exposed to identical LDLD conditions showed no reorganization of nighttime locomotor activity patterns or splitting in DD (data not shown).

**Experiment 3**

As in Experiments 1 and 2, transfer to novel wheels elicited running in the entire sample of experimental hamsters (mean = 7631 ± 268 revolutions per 5 h; range = 4036-9197, \( n = 20 \)), with no significant changes in activity over the 6 days of NWR. Running induced progressive delays in nighttime activity onset. After 6 days of NWR, nighttime activity onset occurred approximately 6 h after lights-out. Compared to substantial further delays observed in identical light conditions in Experiment 1, discontinuation of NWR after 6 days largely prevented further delays in nighttime activity onset (Figs. 2, 4).

Hamsters (18 out of 20) exposed to NWR adopted similar novel entrainment patterns in the home cage under LDLD: Locomotor activity was distributed into two roughly equal components corresponding to the two daily scotophases (i.e., splitting occurred; Fig. 4). Of the 2 nonsplitters, 1 showed the least amount of activity in the novel wheels (4036 rev/5 h) whereas the other showed typical activity levels (7820 rev/5 h). Neither of the 2 control hamsters exposed to this same LDLD cycle, without NWR, adopted this entrainment pattern (data not shown). This split pattern of locomotor activity was sustained for a minimum of 7 days in all 18 animals and for the duration of the experiment (11 days) for 15 of these hamsters.

Quality of entrainment was assessed by examining whether the slope of the best-fitting regression line through activity onsets differed significantly from 24 h (\( p < 0.05 \)). Under baseline entrainment conditions, all but 2 animals yielded regression lines not significantly different from 24 h, indicating that they were well entrained by the 24-h LD cycle. Slopes of these 2 hamsters, moreover, deviated only slightly from 24 h (0.04 and 0.05 h/day, respectively). Likewise, after NWR the afternoon activity component of split hamsters was well entrained under LDLD with only 3 out of 18 split hamsters producing activity onsets with slopes significantly different from 24 h. Activity onsets were less well entrained for the nighttime component of the split rhythms. The majority of hamsters exhibited \( \tau \)'s significantly greater than 24 h. Only 3 hamsters had...
quality of entrainment was further assessed by quantifying the sum of squared residuals of actual onsets from the best-fit regression line. Variability of both split components was greater than for the baseline condition (Table 2; *p* < 0.001), but the two components did not differ from one another (*p* > 0.05).

Phase angle to lights-off (ψ_{lights-off}) differed significantly from the unsplit to the split state and between the two split components (Table 2). ψ_{lights-off} was significantly less negative in the baseline condition prior to NWR than in either the afternoon (*p* < 0.05) or nighttime (*p* < 0.001) activity components in LDLD. In the split condition, ψ_{lights-off} was far more negative for the nighttime activity component than for the afternoon component. Relative to lights-on (ψ_{lights-on}), the phase angle of the afternoon activity component was greater than that of the nighttime activity component (*p* < 0.05; Table 2). Phase angle of the two split components relative to each other (ψ_{n-a}) varied from 8.40 to 12.24 h (mean = 10.5 ± 0.22 h). Total activity was nearly equally distributed between nighttime (55% ± 2%) and afternoon (45% ± 2%) scotophases.

**DISCUSSION**

In three separate experiments, daily NWR markedly reorganized the locomotor activity rhythms of male Syrian hamsters maintained in an LD cycle. Nighttime activity onset was progressively delayed with subsequent days of NWR: Whereas nighttime
activity disappeared entirely in some hamsters after 11 days of NWR, more modest delays were observed after 6 to 7 days of NWR. This latter condition was associated with distinctly split activity rhythms that rejoined after several days of DD. Perpetuation of the LDLD cycle, however, allowed the split rhythms to be sustained for at least an additional 11 days in the home cage. In the absence of NWR, exposure to the LDLD cycle had no marked effect on nighttime locomotor activity rhythms and yielded no evidence of splitting. As suggested previously (Mrosovsky and Janik, 1993), afternoon NWR phase-shifts some component circadian oscillators, which thereafter give rise to the expression of a new activity bout in the afternoon dark period. Subsequently, when the system is released into DD, the two bouts fuse or rejoin under the influence of strong oscillator interactions, but alternatively may be effectively entrained by an LDLD cycle. NWR can therefore override typical entrainment patterns established in an LDLD cycle and reorganize activity into a second stable configuration.

It is not clear why others have failed to replicate the induction of splitting with afternoon NWR (Sinclair and Mistlberger, 1997) and why NWR induced larger phase-delays of home cage activity onset in this study than in others (Mrosovsky and Janik, 1993). We used the same hamster supplier as the original report, in contrast to the study with largely negative effects.
Minor differences in intrinsic periods, propensity to run in novel wheels, or oscillator coupling may distinguish splitting and nonsplitting strains. We also used longer exposures to NWR (5 h vs. 3 h) than used in previous studies. Notably, running in novel wheels was most intense during the 4th hour of exposure. Regardless of differences between studies, the significance of this experimental paradigm is as a probe of specific aspects of oscillator function, which likely differ quantitatively rather than qualitatively among different hamster strains and experimental conditions.

Various formal mechanisms may account for the progressive delays in nighttime activity onset during successive days of NWR. A more negative phase angle of entrainment as was obtained in each experiment can result from a lengthening of $\tau$. Alternatively, each day of NWR may induce a single phase-delay in activity onset without lengthening $\tau$. In prior studies employing a single bout of NWR in early subjective afternoon of hamsters in DD, however, activity onset was advanced rather than delayed, and $\tau$ was lengthened (Mrosovsky, 1993; Weisgerber et al., 1997). Moreover, at the conclusion of NWR in the present study, the nighttime activity component free-ran in DD with a long $\tau$. Together, these results suggest an enduring effect on $\tau$ as opposed to a transient (e.g., phase shift) effect of NWR on the circadian pacemaker.

Two factors may dictate the pattern of rejoining observed in DD, which in all cases was achieved via reduction of the inactive interval following nighttime activity and preceding afternoon activity. First, independent of any oscillator interactions, the two components may have different intrinsic free-running periods, which would favor rejoining. The large negative phase angle of the nighttime component and the relatively small negative value for the afternoon component suggest free-running periods, which are $>24$ and $<24$ h for the nighttime and afternoon components, respectively. Alternatively, coupling interactions between oscillator components may favor the observed pattern of rejoining regardless of the periods of the free-running rhythms. That is, the split state may be intrinsically unstable, and oscillators may interact in DD to establish a limited range of phase angles with respect to each other. Oscillator interactions have been invoked to understand the limited decompression of $\alpha$, which may be obtained in DD in unsplit hamsters. The pattern exhibited in this experiment is consistent with oscillators recoupling by the shortest possible route (i.e., reducing the shorter of the two respective phase angles, $\Psi_{n-a}$ versus $\Psi_{a-n}$ between them), although this proposition cannot be evaluated.
against alternatives with the present data set. A role of oscillator interactions is further suggested by the marked change in $\tau$ measured from the nighttime component after several days in DD, when splitting is presumably ended.

The results of Experiment 3 complement those of Boulos and Morin (1985) who, with daily dark pulses, entrained activity rhythms split by LL. In that study, one component roughly coincided with a daily 2-h dark phase, while the second activity component persisted 8 to 12 h out of phase with the dark-entrained component. In the current study, it appears that the split nighttime activity component, which free-runs in DD with $\tau > 24$ h, may be entrained solely by the phase-advancing effects of light onset at ZT 22. In contrast, the split afternoon activity component may be entrained by either phase-delaying effects of light prior to lights-off at ZT 4, by phase-advancing effects of lights-on at ZT 9, or by both. In hamsters split by LL, each component of the activity rhythm expresses a PRC to dark pulses with defined regions of delays and advances (Boulos and Rusak, 1982).

How does NWR split circadian activity rhythms? Convergent evidence from cellular, physiological, behavioral, and mathematical paradigms (e.g., Illnerova, 1991; Liu et al., 1997; Pittendrigh and Daan, 1976; Enright, 1980) points to the following model of the circadian pacemaker: Overt circadian rhythms reflect the output of multiple circadian oscillators that constitute a coupled dual oscillatory system, which may be functionally described in terms of evening and morning oscillators (Fig. 5A). A functional evening oscillator results from the coupling of oscillators with relatively short $\tau$s, and as such, its overall $\tau$ is $< 24$ h, whereas a functional morning oscillator is derived from coupling of longer period constituent oscillators with $\tau > 24$ h. As one effect of NWR at ZT 4 appears to be a marked lengthening of the period of the nighttime activity component (Mrosovsky, 1993; Weisgerber et al., 1997), and because NWR delayed nighttime activity onset (present studies), we hypothesize that early afternoon NWR preferentially lengthens the period of the oscillators underlying nighttime activity, perhaps by uncoupling some of the short-period component “evening” oscillators from the coupled oscillator network that generates normal nighttime activity (Fig. 5A). In DD, the larger coupled system might therefore free-run under the influence of its remaining coupled constituent oscillators, of which those with longer intrinsic $\tau$s predominate. Under entraining LD conditions, one would expect a more negative phase angle as a result of the oscillator’s lengthened $\tau$. Whether NWR selectively uncouples short-period oscillators because of a particular anatomical relationship (e.g., such oscillators receive neuropeptide Y projections) or a temporal relationship (e.g., a particular phase angle between NWR and short-period oscillators) is entirely unknown.

Additionally, NWR apparently phase-shifts constituent oscillators just as it phase-shifts without overtly splitting the pacemaker in two other paradigms: After an 8-h phase-advance of the LD cycle, hamsters running in novel wheels during the new ZT 13-16 re-entrained within one to two cycles, whereas nonrunning controls required several days (Mrosovsky and Salmon, 1987). NWR of sufficient duration beginning at ZT 5, moreover, induced rapid phase shifts in excess of 8 h in some hamsters transferred to DD (Gannon and Rea, 1995). The present paradigm likewise induces phase shifts, albeit of only a fraction of the oscillators formerly generating the nighttime activity. The present data strongly suggest that successive days of NWR recruit cohorts of oscillators to express their subjective night in the afternoon scotophase. After 6 to 7 days of NWR, activity is nearly equally divided between nighttime and afternoon scotophases, whereas the afternoon scotophase contains disproportionate activity (and in some cases all) after 11 days of NWR. We suggest that a single day of NWR produces a large phase shift of a small fraction of component oscillators. With additional days of NWR, a threshold fraction of oscillators may be phase-shifted to generate an activity component in the afternoon scotophase. The progressive delays in nighttime activity onset are consistent with this model.

In contrast to other paradigms used (Gannon and Rea, 1995; Mrosovsky and Salmon, 1987), the presence of an LDLD cycle with a short (5 h) second scotophase may prevent the entire complement of oscillators from being phase-shifted to express subjective night in the afternoon. Moreover, in the absence of further NWR, the light pulses that bracket the afternoon scotophase impede the recoupling of constituent oscillators back into the unsplit state (Experiment 3), which so readily occurs in DD. Notably, when the intervening light intervals were very short as in skeleton photoperiods, daily NWR from ZT 5 to ZT 8 induced complete inversion of activity rhythms to what was previously subjective day (Sinclair and Mistlberger, 1997). Similarly, 11 days of NWR apparently also shifted the entire oscillatory system in a few hamsters of Experiment 1. Thus, these two factors—a titratable shifting of oscilla-
tors and countering of oscillator tendencies to rejoin—may facilitate induction and maintenance of stable, split activity rhythms under an LDLD cycle and 1 or more days of NWR.

Beyond the significance of NWR-induced splitting noted previously (Mrosovsky and Janik, 1993), the demonstration that some oscillatory components can be dissociated from others and rephased with respect to the nighttime activity component may provide insight into the mechanism of phase-shifting effects of NWR, or indeed, of any photic or nonphotic zeitgeber. For example, a single bout of NWR around ZT 5 produces large phase advances and lengthening of τ in subsequent DD (Mrosovsky, 1991; Reebs and Mrosovsky, 1989a, 1989b; Weisgerber et al., 1997). A single bout of NWR is not sufficient to induce measurable splitting under LDLD conditions (MR Gorman, unpublished observations), but if it selectively phase-shifts a small fraction of circadian oscillators (Fig. 5B), overt circadian rhythms might be altered as a consequence of recoupling dynamics among constituent oscillators. Unfortunately, little is known about these processes, except that darkness favors recoupling in LL-splitted hamsters (Earnest and Turek, 1982) and intervening light pulses appear to minimize recoupling in NWR-induced splitting (Experiment 3). Actograms of NWR-split hamsters suggest that recoupling may be accompanied by abrupt changes in phase, with the rejoining activity bout typically expressed in an inter-

Figure 5. Formal model of multioscillator basis of novel wheel running (NWR) effects on circadian rhythms. In all panels, the inverted U shape represents subjective night of hypothetical individual circadian oscillators. For clarity, the remaining phases of each oscillator are not depicted. In A, the integrated rhythm reflects a distribution through the scotophase of the subjective nights of individual oscillators. Those with short and long periods express subjective night early and late in the scotophase, respectively. The oscillator denoted with a dashed line will be phase-shifted by NWR. After a single day of NWR from ZT 4 to ZT 9, oscillators with short periods undergo large phase shifts to roughly the same circadian phase as NWR. Light pulses bracketing NWR preclude oscillator recoupling for reasons that are not yet clear. Subsequent activity onset is delayed, and τ is lengthened (τ > τ). Additional days of NWR phase-shift additional cohorts of oscillators such that approximately half are phase-shifted after 6 to 7 days but all or nearly all are shifted after 11 or more days. B. Application of the splitting model to understand effect of a single day of NWR applied in DD. In the absence of a light pulse following NWR, the phase-shifted oscillator pulls (advances) the nighttime component that follows. Until full recoupling is achieved, the nighttime activity component may continue to express a lengthened τ.
mediate phase between the former two components (Mrosovsky and Janik, 1993). If no light follows a bout of NWR, strong oscillator interactions may result in rapid recoupling via reduction of the shorter phase angle between component oscillators. This would advance rather than delay the main activity onset (Fig. 5B). Consistent with this general model, light pulses shortly after a single day of NWR, such as those that impeded oscillator recoupling in the present study, greatly attenuated the phase-advancing effects of NWR (Mrosovsky, 1991).

An understanding of oscillator-oscillator interactions has lagged behind our knowledge of other features of circadian rhythms, although physiological data suggest that such interactions must be central to an understanding of the pacemaker. Only a fraction of SCN cells, for instance, receive direct retinal or IGL projections, and yet both of these pathways are capable of phase-shifting, presumably, the entire SCN. Any zeitgeber, therefore, first shifts a subpopulation of SCN cells that receives direct projections from the time-giving entrainment mechanism. These cells in turn interact with the greater complement of SCN cells to arrive at a steady-state phase shift. Under routine conditions, this selective shifting and oscillator interaction may happen in a cycle or even more rapidly. The use of LDLD cycles in the present paradigm, in contrast, facilitates a temporal dissociation of these processes by impeding the recoupling process.

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Temporal Reorganization of the Suprachiasmatic Nuclei in Hamsters with Split Circadian Rhythms

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Abstract  A dual oscillator basis for mammalian circadian rhythms is suggested by the splitting of activity rhythms into two components in constant light and by the photoperiodic control of pineal melatonin secretion and phase-resetting effects of light. Because splitting and photoperiodism depend on incompatible environmental conditions, however, these literatures have remained distinct. The refinement of a procedure for splitting hamster rhythms in a 24-h light-dark:light-dark cycle has enabled the authors to assess the ability of each of two circadian oscillators to initiate melatonin secretion and to respond to light pulses with behavioral phase shifting and induction of Fos-immunoreactivity in the suprachiasmatic nuclei (SCN). Hamsters exposed to a regimen of afternoon novel wheel running (NWR) split their circadian rhythms into two distinct components, dividing their activity between the latter half of the night and the afternoon dark period previously associated with NWR. Plasma melatonin concentrations were elevated during both activity bouts of split hamsters but were not elevated during the afternoon period in unsplit controls. Light pulses delivered during either the nighttime or afternoon activity bout caused that activity component to phase-delay on subsequent days and induced robust expression of Fos-immunoreactivity in the SCN. Light pulses during intervening periods of locomotor inactivity were ineffective. The authors propose that NWR splits the circadian pacemaker into two distinct oscillatory components separated by approximately 180 degrees, with each expressing a short subjective night.

Key words  novel wheel running, melatonin, c-Fos, phase-shift, oscillator interaction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the principal circadian pacemaker in mammals (Weaver, 1998). Although individual cultured SCN cells express circadian rhythms with varying free-running periods and phases (Herzog et al., 1997; Liu et al., 1997; Welsh et al., 1995), in vivo cellular activity from the SCN is largely synchronized (Bouskila and Dudek, 1993). Thus, the question of oscillator-oscillator interactions would appear to be a central issue in understanding the mammalian pacemaker. A dual-oscillator basis for mammalian circadian rhythms was posited following the observation that prolonged exposure to constant light (LL) induced locomotor activity patterns of hamsters and other species to diverge into two components (Pittendrigh and Daan, 1976). The SCN of split ham-
sters express comparable split rhythms in multiunit electrical activity, suggesting that splitting is intrinsic to the principal circadian pacemaker (Mason, 1991). Studies of pineal melatonin synthesis and photo-periodic regulation of activity duration (α) likewise point to a dual oscillator model of circadian rhythms (Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova, 1991; Pittendrigh and Daan, 1976). An evening oscillator (E) entrained by evening light, is purported to initiate nocturnal melatonin secretion and locomotor activity, whereas a morning oscillator (M), entrained by morning light, mediates the expression of these functions near the end of night. In longer day lengths of spring and summer, E and M are entrained in a phase relation that generates a short subjective night, while short day lengths of fall and winter allow the phase angle between E and M to increase to generate a long subjective night. Parallel photoperiodic modulation of light-induced phase-resetting of locomotor rhythms, as well as of expression of the immediate early gene c-Fos in the SCN, suggests that component oscillators are localized within the SCN (Elliott and Kripke, 1998; Pittendrigh et al., 1984; Sumová et al., 1995), although identification of two distinct oscillators has been lacking. Unfortunately, the clear temporal resolution of distinct oscillators achieved in LL-induced splitting is not seen in the photoperiodism literature, where, with rare exceptions (Jagota et al., 2000), evening and morning oscillator functions may greatly overlap. Conversely, notwithstanding the theoretical milestone it represents, the splitting paradigm has been of limited utility for probing the nature of underlying oscillators largely because its requirement for LL complicates application of the bulk of analytical techniques fruitfully employed in photoperiodism research. Specifically, LL masks locomotor activity, inhibits melatonin secretion, and precludes assessment of acute effects of light pulses on pacemaker function. Thus, these two theoretically important literatures have remained largely separate.

Mrosovsky and Janik (1993) suggested that LL is not the only stimulus capable of splitting rhythms in the hamster: Repeated afternoon exposures to 3 h of novel wheel running (NWR) delayed the onset of nighttime wheel running and, in some cases, led to a transient splitting of locomotor activity rhythms when hamsters were later transferred to constant darkness (DD). Modifying this protocol, Gorman and Lee (2001 [this issue]) recently demonstrated that activity rhythms could be reliably split and that components of the split rhythms could be entrained to a light-dark:light-dark (LDLD) cycle.

Entrainment of NWR-induced split activity rhythms to an LDLD cycle indirectly suggests that each of the two oscillators mediating the split rhythms can be separately phase-shifted by light. To assess this directly, and to rule out the possibility that extra-SCN oscillators are recruited to mediate one or both activity components (Honma et al., 1989; Stephan et al., 1979), we characterized the effects of light pulses on locomotor activity rhythms and on induction of Fos immunoreactivity in the SCN during each of the two split activity bouts induced by NWR. Additionally, we presented light pulses during the inactive periods between the activity components to exclude the possibility that α was simply lengthened to incorporate both activity components. Finally, models of photoperiodic control of melatonin secretion have posited the existence of distinct evening and morning oscillators timing the initiation and termination, respectively, of the nightly pattern of elevated pineal melatonin secretion (Illnerova, 1991). Because light acutely suppresses melatonin secretion, it has not been possible to evaluate the role of component oscillators split in LL. We therefore assessed whether each of the two split components was capable of initiating melatonin secretion.

**MATERIALS AND METHODS**

**Animals and Husbandry**

For all experiments except one, the same 24 male Syrian hamsters (*Mesocricetus auratus*, HsdHan: AURA, Harlan, Indianapolis, IN, USA), 5 to 6 weeks of age at acquisition, were housed with Sani-Chip bedding in polypropylene cages (48 × 27 × 20 cm) equipped with Nalgene (d = 34 cm) running wheels (Fisher Scientific, Pittsburgh, PA, USA). Food (Purina Rodent Chow #5001, St. Louis, MO, USA) and water were available ad libitum. These hamsters are the same as used in another separately reported study published in this issue (Gorman and Lee, 2001). Eighteen additional hamsters from the same supplier and housed similarly were used only for the final study of Fos-immunoreactivity in the SCN. Hamsters were housed for 2 to 3 weeks in a 14 h light, 10 h dark condition (LD 14:10; lights on 0500-1900 h) before each regi-
men of daily NWR was used to split activity rhythms. Room illumination at the level of the cage lid varied from 100 to 300 lux.

Split activity rhythms were induced by scheduled exposures to NWR as previously described (Gorman and Lee, 2001). Briefly, after entrainment to LD 14:10, 20 hamsters were transferred daily for 6 to 10 days within the same room to Wahmann wheels 0 to 15 min before lights were extinguished at 1100 h (ZT 4) EST. At 1600 h (ZT 9), the lights were turned on and hamsters were returned to home cages in the light over the next 15 min. With additional days of NWR, onset of nighttime locomotor activity in the home cage is progressively delayed. The number of days of NWR within each experimental run was adjusted so that nighttime activity onset occurred approximately midway through the 10-h nighttime scotophase. After the final day of NWR, hamsters were left in their home cages and exposed to DD by leaving lights off after the subsequent 10-h nighttime scotophase. Nonsplitting control hamsters (n = 4) housed in the same room experienced identical lighting conditions but were not exposed to NWR and thus never split their activity rhythms.

Wheel-running activity in the home cage was monitored by Dataquest III software (Mini-mitter, Sun River, OR, USA) and compiled into 10-min bins. Activity onset (CT 12) was defined as the first bin in each activity bout with wheel revolutions of more than 20, and that was immediately followed by a second interval exceeding this threshold. While in the novel wheels, activity patterns were not monitored, but the total number of wheel revolutions after the 5-h interval was recorded manually. Data analyses were carried out with Excel (Microsoft, Seattle, WA, USA) and ClockLab software (Actimetrics, Evanston, IL, USA).

Light Pulse–Induced Phase Shifts of Activity

Changes in the phase response to light following NWR-induced splitting were assessed in three sequential experimental iterations (begun at approximately 12, 18, and 24 weeks of age, respectively). The same cohort of hamsters was repeatedly first entrained to LD 14:10, then exposed to a regimen of NWR to split activity, and then transferred to DD (Aschoff Type II methodology) where they received either a 15-min light pulse or a sham pulse (~450 lux at the level of the cage lid).

Afternoon Light Pulse (CT 13-a)

In the first run, lights remained off after the nighttime scotophase following the final day of NWR (Fig. 1A). Activity rhythms were monitored remotely for onset of wheel running the following afternoon. One hour after onset of this afternoon component of the split activity rhythm, 9 randomly selected hamsters were moved in their home cages to an adjoining room where they experienced the 15-min light pulse. Unpulsed split control animals (n = 10) were similarly jostled in their home cages but remained in the same room in darkness. Following the light pulse, animals were returned to the housing room and remained undisturbed for 2 weeks.

Evening Light Pulse between Split Activity Components

Hamsters were next re-entrained to LD 14:10 for 2 weeks and then exposed to an additional regimen of NWR. Following the final day of NWR, lights were turned off 2 h prematurely (1700 EST) to minimize potential phase-shifting effects of the entraining photoperiod and to unmask activity onset in unsplit control hamsters. Of the split hamsters, 10 were randomly selected and pulsed with light for 15 min beginning approximately 1 h after unsplit animals began their normal nighttime activity (n = 4). The remaining split hamsters (n = 9) served as unpulsed controls: they were similarly jostled at the designated time but not exposed to light.

Nighttime Light Pulse (CT 13-n)

Hamsters were again re-entrained to LD 14:10 for 2 weeks and then induced to split their rhythms with NWR. Following the final day of NWR, hamsters remained in their home cages, and lights remained off after the subsequent nighttime scotophase. Activity was monitored to verify that hamsters exhibited a nighttime activity bout followed by an interval of inactivity and then an afternoon activity bout. Hamsters were pulsed with light for 15 min (n = 9) or sham pulsed (n = 8) as described above beginning 1 h after the next nighttime activity onset.

Analyses of Phase-Shift Data

Because split circadian rhythms are unstable in DD (i.e., components re-fuse rapidly and $\tau$ and phase are
influx), establishment of a prepulse baseline and calculation of a light-induced phase shift in an individual animal are highly problematic. Therefore, phase shifts were assessed by comparing light-pulsed and sham-pulsed animals in a between-subjects design. For each animal, nighttime and afternoon activity onset of the day preceding the light-pulse or sham-pulse were compared with the corresponding activity onsets on the 2 days following the stimulus (see Fig. 2). Timing of the light pulse was such that we could not obtain a prepulse afternoon activity onset to use as a phase reference in the second experimental run. Hence, phase shifts of this component were not determined. Moreover, activity onsets of the nighttime component were always unambiguous, but as the experiment progressed, fragmentation of the activity rhythms occasionally precluded identification of a single clear afternoon activity component. In such cases, this component was not analyzed. Last, the free-running period of the nocturnal activity component was computed using least squares regression over the 4 days following the pulse or sham pulse. Because the afternoon component typically rejoined with the nighttime component within this interval, a comparable analysis of the free-running period of the afternoon component was not attempted.

Melatonin in Circulation

To assess whether melatonin secretion patterns were altered by NWR-induced splitting, hamsters were lightly anesthetized with methoxyflurane vapors (Metofane) and retro-orbitally bled in darkness with the aid of a dim red light (< 1 lux). Blood samples were collected into ethylene diamine tetraacetic acid–treated tubes. Plasma was harvested after centrifugation at 5000 rpm for 20 min and was stored at −70 °C until assay. Plasma samples were thawed and extracted with dichloromethane, and melatonin concentrations were determined in a single assay as previously described (Bae et al., 1999). The intra-assay coefficient of variation was 14%, and assay sensitivity was 17 pg/ml. Samples below the limit of detectability were assigned values of 17 pg/ml for purposes of graphing and statistics. One to 3 days prior to each of the three runs of NWR described above, blood samples were collected at ZT 16, ZT 20, and ZT 21 from pseudo-randomly selected unsplit hamsters (n = 3-5/timepoint). After the 6th day of NWR of each experimental run, additional samples were collected at these same times or at ZT 9 from hamsters that were then split (n = 3-6/time point; Fig. 1B). Among unsplit control animals that remained at home, sampling was performed at ZT 9 on the 6th day of the afternoon dark pulse of each run (Fig. 1B). No hamster contributed more than one sample at any given time point, except non-NWR controls at ZT 9. As melatonin concentrations were always undetectable at ZT 9 for these 4 hamsters, only one determination from each hamster was considered in statistical analyses.

SCN Fos-Immunoreactivity

Finally, we assessed whether the temporal pattern of light-induced Fos expression in the SCN was split following exposure to daily NWR. Following the
behavioral phase-shifting experiment, hamsters (now 38 weeks of age) were re-entrained to LD 14:10 and exposed to 6 days of NWR. A replicate experiment was subsequently performed with 18 additional hamsters at 12 weeks of age. For both cohorts, hamsters remained at home and lights remained off after the night that followed the 6th day of NWR (Fig. 1C). Hamsters with split activity patterns were randomly assigned to receive a 15-min light pulse or sham pulse at one of three time points (Fig. 1C; n = 3-6/group): 1 h after the subsequent nighttime activity onset (CT 13-n), during the morning inactive period (break) following nighttime activity offset, and 1 h after the subsequent afternoon activity onset (CT 13-a). Unsplit control hamsters were also left in DD and were exposed to light or sham-
pulsed the next afternoon at the same time when split hamsters were given their light pulses. Additional unsplit control hamsters received light pulses or sham pulses 1 h after the following nighttime activity onset.

Hamsters were injected with a lethal dose of Nembutal 55 min after the light pulse or sham pulse and perfused intracardially with 40 to 60 ml 0.1M phosphate buffered saline (PBS; pH 7.5) followed by 100 to 150 ml 4% paraformaldehyde in PBS. Brains were postfixed in paraformaldehyde at room temperature for 2 h and transferred to 20% sucrose/PBS. Serial coronal slices were cut at 40 µm and every fourth section processed for immunocytochemistry. Free-floating sections were incubated successively in 0.1% hydrogen peroxide; 1:1000 anti-fos rabbit IgG (sc-52, Santa Cruz, CA, USA) in PBS with 0.4% Triton-X (Fisher) and 4% normal goat serum for 24 h at 4 °C; 1:200 biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA, USA) for 1 h at room temperature; ABC reagent (Vector) for 1 h; and 0.1% diaminobenzidine (DAB) with 0.02% peroxide for 2 min. Sections were mounted on gelatin-coated slides. The most densely stained section was selected by an observer blind to experimental treatment, and the number of Fos-positive cells in the SCN was counted.

Statistical tests (all two-tailed where applicable) were performed with Statview 5.0 software (SAS Institute, Cary, NC, USA).

RESULTS

NWR and Splitting

In each induction of splitting by NWR, virtually the entire sample (>85%) of hamsters ran robustly in the novel wheels. A subset of the activity data during NWR has been analyzed in detail (Gorman and Lee, 2001) and thus is not reported here. Figure 2 depicts hamsters split by NWR, released into DD, and pulsed with light (A) or sham-pulsed (B).

LIGHT-PULSE INDUCED PHASE SHIFTS OF ACTIVITY

Afternoon Light Pulse (CT 13-a)

The afternoon activity component of pulsed hamsters was phase-delayed relative to that of unpulsed controls on Day 1 (p < 0.05; Figs. 2A, 3A), with a trend in the same direction on Day 2 (p < 0.10). The nighttime activity component, which was not itself pulsed, was significantly phase-advanced by this afternoon light pulse (p < 0.05 for both days, Fig. 3A). The free-running period of the nighttime component was unaffected by the light pulse (mean \(\tau\pm SE: 25.08 \pm 0.12\) vs. 24.80 \(\pm 0.18\) h for unpulsed and pulsed animals, respectively; p > 0.20).

Evening Light Pulse between Split Activity Components

Early evening light pulses (matched to CT 13 of unsplit control hamsters) had no significant effect on the nighttime activity component of split hamsters (Fig. 3B). The free-running period of this component also was not affected by the light pulse (25.11 ± 0.14 vs. 25.37 ± 0.20 h for unpulsed and pulsed animals, respectively; p > 0.30).

Nighttime Light Pulse (CT 13-n)

On Days 1 and 2, the nighttime activity component of pulsed hamsters was significantly phase-delayed by the 15-min light pulse (p < 0.005, p < 0.01, respectively, Fig. 3C), compared with sham light pulse controls. On Days 1 and 2, afternoon activity in the split rhythm component was not significantly advanced by light pulses (p > 0.25; Fig. 3C). The free-running period of the nighttime activity component was unaffected by the light pulse (mean \(\tau\pm SE: 24.68 \pm 0.04\) vs. 24.55 ± 0.11 h for unpulsed and pulsed hamsters, respectively; p > 0.28).

Melatonin in Circulation

Prior to NWR, plasma melatonin concentrations were detectable in only a minority (4/12) of unsplit hamsters at ZT 16 but had increased above threshold in 9 of 10 animals by ZT 20 (Fig. 4A). One hour later (ZT 21), only 2 out of 12 remained above detectable limits. In contrast, plasma melatonin concentrations of hamsters with split activity patterns in no case (0 out of 10) exceeded the minimum detectable level at ZT 16, a significantly smaller proportion than among controls at this time (\(\chi^2 = 4.1; p < 0.05\); Fig. 4B). By ZT 21, however, plasma melatonin had increased in 6 out of 10 hamsters, a proportion significantly greater than among hamsters not exposed to NWR (\(\chi^2 = 4.4; p <

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Figure 3. Phase-shifting responses to 15-min light pulses delivered at three phases of the circadian cycle of split hamsters. In each panel, the mean (±SE) change in nighttime and afternoon activity onset relative to the first value in DD is depicted for light-pulsed (open bars) and unpulsed (sham) hamsters (filled bars). The net phase shift is indicated above each pair. Sample size is represented below or above bars (pulsed/unpulsed). Asterisks denote significant differences between pulsed and unpulsed animals (p < 0.05; Mann-Whitney U; two-tailed). (A) Day 1 and Day 2 phase shifts after light pulse or sham pulse delivered 1 h after onset of the afternoon activity component (CT 13-a). (B) Phase shifts after pulse or sham pulse during the evening inactive period (see text). Design limitations prevented collection of a prepulse reference value for the afternoon activity component. (C) Phase shifts after pulse or sham pulse delivered 1 h after the nighttime activity onset (CT 13-n).
0.05), but not different from controls at ZT 20. Moreover, after 6 days of NWR, nearly all NWR-split hamsters (17 out of 18) had elevated concentrations of melatonin in circulation at ZT 9. This proportion was significantly greater than in non-NWR controls exposed to similar light conditions where none yielded detectable melatonin titers at ZT 9 ($\chi^2 = 16.6; p < 0.001$).

SCN Fos-Immunoreactivity

In hamsters with unsplit activity rhythms, the 15-min light pulse after nighttime activity onset induced significant Fos expression, compared with that in unpulsed controls ($p < 0.05$; Mann-Whitney U; Figs. 5A, 6A). A light pulse coinciding with the previous afternoon dark period had no stimulatory effect on the number of Fos+ cells in these unsplit hamsters ($p > 0.50$; Figs. 5B, 6A). Hamsters split by NWR, in contrast, showed two periods of increased Fos expression (Figs. 5C, 5E, 6B): Light pulses increased Fos-ir after the nighttime activity onset ($p < 0.05$) and after afternoon activity onset ($p < 0.05$). Moreover, light administered between these two time points had no effect on the number of Fos+ cells ($p > 0.40$; Figs. 5D, 6B). Unpulsed controls at every time point tested—whether split or unsplit—showed minimal Fos expression (Figs. 5F, 6). In groups where Fos was robustly induced by light (split hamsters in the night and the afternoon and unsplit hamsters in the night), there were no differences in the number of Fos+ cells counted ($p > 0.05$). Fos expression was symmetric with respect to the left and right SCN and was concentrated in the ventrolateral SCN in all groups.

DISCUSSION

As in previous experiments, daily exposure to NWR in an LDLD cycle induced split activity rhythms that free-ran and recoupled in DD (Mrosovsky and Janik, 1993). Circadian mechanisms underlying each bout responded similarly to timed light pulses as measured by behavioral phase-shifts or induction of Fos-ir in the SCN, and both activity components were accompanied by elevated plasma melatonin concentrations. The results suggest that NWR temporally reorganizes circadian oscillators within the SCN into two distinct components. We propose that each component oscillator or group of oscillators contains a relatively short subjective night characterized by locomotor activity, elevated melatonin secretion, and light responsiveness.

Conceivably, NWR might have re-entrained the circadian system to generate a long subjective night that spanned the original night and the afternoon scotophase paired with NWR. If one of the two
photophases separating dark periods masked locomotor activity, the rhythm might only appear split. In the present study, light pulses had marked effects when delivered during either activity component but did not induce Fos expression in the SCN during the morning inactive period or shift activity rhythms in the early evening inactive interval. Limited resources precluded assessment of possible light-induced phase shifts during the morning rest period or of SCN Fos-ir expression in the early evening. In unsplit hamsters, however, Fos induction by light is restricted to subjective night and temporally correlates closely with periods of behavioral phase shifting (Kornhauser et al., 1990; Sumová et al., 1995; Travnickova et al., 1996). This close relationship, moreover, is maintained in various photoperiods that alter \( \alpha \). Despite assessment by different methodologies, each inactive period thus appears to represent a dead zone with respect to light responsiveness. These dead zones separate intervals of light responsiveness as measured jointly by behavioral phase-shifting and Fos-ir induction.

Elevated melatonin in circulation during both the subjective afternoon and nighttime bouts of activity in hamsters with split activity rhythms supports the hypothesis that the principal circadian pacemaker is split by NWR. Among unsplit hamsters, in contrast, plasma melatonin concentrations were elevated in a monophasic pattern. Four hours into the night (ZT 16), 4 out of 12 hamsters had detectable concentrations of melatonin in circulation, consistent with onset of the rising phase in pineal melatonin production in this species (Elliott and Tamarkin, 1994; Goldman et al., 1981). A nighttime elevation in plasma melatonin at ZT 20 was followed by decreases below detectable levels that were manifest by some hamsters at ZT 21 and all hamsters at ZT 9. If NWR delayed the circadian pacemaker as suggested by nighttime activity onsets (Gorman and Lee, 2001) in split hamsters, no elevation of melatonin concentrations at ZT 16 would be expected; importantly, none was found. Increased melatonin secretion clearly occurred later during the nighttime bout of activity. However, the morning decline in circadian melatonin production, evident in the majority of unsplit hamsters at ZT 21, was not observed in split animals, providing further evidence of a delayed nighttime oscillator. Most important, plasma melatonin concentrations were elevated at the end of the afternoon scotophase paired with NWR. Among hamsters exposed to light-dark cycles, darkness in the afternoon to our knowledge has never been
reported to induce melatonin secretion, and no elevation was apparent in nonrunning controls. Thus, these data suggest that the pattern of melatonin, like that of activity, is split by NWR into two components. The ability of both nighttime and afternoon light pulses to induce Fos expression in the SCN discounts the possibility that split rhythms reflect recruitment of extra-SCN oscillators that might mediate one of the two activity components. Although their neural substrates are unknown, extra-SCN oscillators are sufficient to generate circadian rhythms in a variety of experimental paradigms (Honma et al., 1989; Stephan et al., 1979). It is not feasible to observe SCN Fos expression in a single hamster during each activity bout, but the uniformity of expression of Fos in the SCN following the afternoon or nighttime light pulse establishes that the principal circadian pacemaker itself responds to light during both activity components. In LL-induced splitting, rhythms of Fos and clock gene expression are out of phase in the left and right SCN (de la Iglesia et al., 2000), although other investigators discerned no left/right differences in electrophysiological activity in the SCN of LL-induced split hamsters (Zlomanczuk et al., 1991). The present Fos data do not suggest an anatomical or physiological distinction between the two oscillators. In the present study, all sections revealed symmetrical induction of Fos in the two SCN, indicating that both left and right are expressing a subjective night simultaneously rather than alternately. Within each SCN, the pattern of Fos induction was also similar between the two bouts, with expression concentrated in the ventrolateral SCN. How each SCN becomes Fos-inducible twice daily is not clear. At the tissue level of organization, the oscillators may be two distinct, but spatially intermixed, cell populations, each responsive to light during either the afternoon or nighttime dark period, but not both. Alternatively, a single population of SCN cells may be induced to express Fos twice daily, perhaps because individual cells in this population contain two oscillators within them, or because they are simultaneously clock-controlled by two other cell populations with rhythms out of phase.

Between-group comparisons demonstrated robust behavioral phase shifts after light pulses. Phase shifts calculated in this fashion might result either from discrete phase shifts or from changes in free-running period (e.g., a lengthening of $\tau$ after a light pulse would appear as Day 1 and Day 2 phase-delays by this method). The latter possibility is discounted, however, by the analysis of free-running period in the days following the light pulse. After all pulses, the free-running period was not significantly altered; and in the case of the nighttime pulses, the trend was toward a shortening of $\tau$, which would act counter to the reported phase delays. Thus, phase shifts of the activity components were not secondary to changes in $\tau$ but instead reflect discrete phase shifts.
As in LL-induced split animals probed with dark pulses (Boulos and Rusak, 1982; Lees et al., 1983), both NWR-split activity components responded with similar phase shifts to light pulses 1 h after their respective activity onsets. Afternoon light pulses additionally induced phase advances of the unpulsed nighttime component, but pulses in the early nighttime scotophase did not shift rhythms; nor did light pulses in the morning inactive period lead to induction of Fos-ir in the SCN, as is generally a prerequisite for light-induced phase shifts. How is this pattern of responsiveness to be understood? One possibility is that the light-pulse PRC for the nighttime component does indeed contain two light-responsive zones separated by two dead zones. Alternatively, each component may be directly light responsive only during a single short subjective night associated with only one of the two scotophases. The greater fraction of each oscillation may be a dead zone. The observed phase advances of the nighttime activity component following light at CT 13-a, however, could be a secondary consequence of interactions with the afternoon component, which is directly phase-delayed by light. When light phase-delays the afternoon activity component by 90 min as in the current study, the phase angle between the nighttime and afternoon components is altered. If the interaction between the two oscillators depends on their phase relation as commonly presumed, the phase shift of the unpulsed component might reflect this altered interaction with the pulsed component rather than a direct action of light, per se. Future work can develop and test this model of oscillator interactions.

Multioscillatory models of circadian rhythms commonly distinguish between E and M oscillators (Daan and Berde, 1978; Elliott and Tamarkin, 1994; Gorman et al., 1997; Illnerova and Vanacek, 1985; Pittendrigh and Daan, 1976; Sumová et al., 1995). It is not possible to attribute the respective activity components generated here to hypothesized evening and morning oscillators. Regardless of the relation to E and M, however, the circadian timekeeping system can be resolved into two seemingly similar oscillators, both of which can be phase-delayed by light and both of which can drive melatonin secretion within a single 24-h period. Further probing of these two oscillators will complement in vitro work to characterize the suboscillatory basis of the mammalian pacemaker.

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Light-Induced Resetting of the Circadian Pacemaker: Quantitative Analysis of Transient versus Steady-State Phase Shifts

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Abstract  The suprachiasmatic nuclei of the hypothalamus contain the major circadian pacemaker in mammals, driving circadian rhythms in behavioral and physiological functions. This circadian pacemaker’s responsiveness to light allows synchronization to the light-dark cycle. Phase shifting by light often involves several transient cycles in which the behavioral activity rhythm gradually shifts to its steady-state position. In this article, the authors investigate in Syrian hamsters whether a phase-advancing light pulse results in immediate shifts of the PRC at the next circadian cycle. In a first series of experiments, the authors aimed a light pulse at CT 19 to induce a phase advance. It appeared that the steady-state phase advances were highly correlated with activity onset in the first and second transient cycle. This enabled them to make a reliable estimate of the steady-state phase shift induced by a phase-advancing light pulse on the basis of activity onset in the first transient cycle. In the next series of experiments, they presented a light pulse at CT 19, which was followed by a second light pulse aimed at the delay zone of the PRC on the next circadian cycle. The immediate and steady-state phase delays induced by the second light pulse were compared with data from a third experiment in which animals received a phase-delaying light pulse only. The authors observed that the waveform of the phase-delay part of the PRC (CT 12-16) obtained in Experiment 2 was virtually identical to the phase-delay part of the PRC for a single light pulse (obtained in Experiment 3). This finding allowed for a quantitative assessment of the data. The analysis indicates that the delay part of the PRC—between CT 12 and CT 16—is rapidly reset following a light pulse at CT 19. These findings complement earlier findings in the hamster showing that after a light pulse at CT 19, the phase-advancing part of the PRC is immediately shifted. Together, the data indicate that the basis for phase advancing involves rapid resetting of both advance and delay components of the PRC.

Key words  circadian rhythms, suprachiasmatic nucleus, entrainment, phase shift, transients, phase response curve, evening/morning oscillators

An endogenous circadian pacemaker in the suprachiasmatic nuclei (SCN) determines the timing of many behavioral and physiological functions (Meijer and Rietveld, 1989). For proper timing, this
pacemaker is responsive to the environmental light-dark cycle. Light presented during the beginning of the subjective dark period induces phase delays of the circadian activity rhythm, whereas light presented toward the end of the subjective dark period produces phase advances. The effects of light on the circadian activity rhythm can be described by a phase response curve (PRC). In a PRC, the magnitude and direction of phase shifts in activity onset are plotted as a function of the circadian time of pulse application (Daan and Pittendrigh, 1976).

After a light pulse, a phase shift is often not completed within the first circadian cycle but instead grows over the course of several days. Such circadian cycles are called transient cycles. They are most pronounced following phase-advancing light pulses, but they occur after delaying pulses as well (Pittendrigh et al., 1958). It is not clear whether activity onset during the transient cycles reflects the true position of the underlying pacemaker. One possibility is that the pacemaker itself requires several days to complete the phase shift. Alternatively, the pacemaker may shift immediately to its final position but the activity rhythm requires several days to become fully synchronized with the new phase of the pacemaker.

On the molecular level, it has been shown that the expression of the mammalian homologs of the insect period gene, mper1 and mper2, peak 1 h and 2 h after application of a light pulse, respectively (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). It is clear that resetting mechanisms are induced very quickly and increases in the putative protein products of mper may be the cause of resetting to a new phase. The finding that inhibition of mper1 expression blocks the phase shifts induced by light and glutamate pulses supports the notion that mper is required for phase shifting by light (Akiyama et al., 1999). It appears therefore that resetting of the clock is established within a few hours. To understand the discrepancy between the molecular biology and the behavior of the overt rhythm, it is important to get insight into the position of the pacemaker during the transient cycles in vivo. For this purpose, it is necessary to determine the position of the PRC during the transient cycle, assuming that the position of the PRC provides a true reflection of the phase of the pacemaker.

One way to investigate the position of the PRC is by applying a second light pulse during the first transient cycle after a light pulse and to investigate the phase-shifting effect of the two light pulses on the final phase of the activity rhythm. Two-pulse experiments have been performed in invertebrates such as Drosophila (Pittendrigh, 1979) and Neurospora (Crosthwaite et al., 1995), in mice (Sharma and Chandrashekaran, 2000), and in Syrian hamsters (Elliott and Pittendrigh 1996; Best et al., 1999). In the hamster, it has been established that the phase-advance part of the PRC shifts within a few hours after a light pulse at CT 18 by applying a second light pulse 1 to 2 h after the first pulse (Best et al., 1999). It was also shown that the phase-delay part shifts within a few hours after a light pulse at CT 13 by applying a second pulse 1 to 2 h after this pulse (Best et al., 1999). However, it has not been investigated whether the phase-delay part of the PRC shifts immediately after a phase-advancing light pulse. This matter is of great importance in view of recent findings, indicating that the pacemaker is composed of distinct genetic components that exhibit different responsiveness to phase-advancing and phase-delaying light pulses (see Daan et al., 2001).

We investigated the responsiveness of the phase-delay part of the PRC during a transient cycle that was induced by a phase-advancing pulse. To this purpose, we applied a second light pulse shortly after activity onset at the first transient cycle. By comparing the effect of a single light pulse with the effect of the double light pulse, we could investigate the phase-shifting effect of the second light pulse. From the magnitude of the phase shift, it can be estimated what the position of the circadian clock was at the time point of application of the second light pulse. To optimize this estimate, we determined the relation between activity onset during the first transient cycles and the final steady-state phase after application of a single phase-advancing light pulse. This enabled us to make a reliable estimate of the steady-state phase shift after the first activity onset.

**METHODS**

This study was performed on 60 male Syrian hamsters (Mesocricetus auratus, Harlan/CPB, Zeist, the Netherlands) aged 2 months at the start of the experiment. The animals were individually housed in cages (36.5 × 25.0 × 16.0 cm) with a running wheel (diameter 26 cm) and were kept in a sound-attenuating, ventilated room at a temperature of 23 °C. Food (Hope farms B.V. the Netherlands) and water were continuously available. Running-wheel activity was recorded per minute to determine the animals‘ circadian activity rhythm. In all the experiments, light pulses (15
min) were presented to groups of animals, and the protocol was repeated three times. Positioning of the light sources on the wall behind every single cage ensured similar light levels (100-120 lux) for all animals. During presentation of the light pulses, the animals remained in their home cages. All animals received all three treatments, and treatments were presented in a randomized order. At least 1 month elapsed between application of subsequent light pulses. Data were excluded from the analysis when the actogram of running-wheel activity did not allow for unambiguous determination of activity onsets.

Experiment 1

The animals were entrained to LD 14:10 for at least 7 days before they were released into constant darkness (DD). After 7 days in DD (Day 0), the animals received a light pulse between CT 17.77 and CT 21.90. After the light pulse, the animals were kept in DD for 14 days. Lines were eye-fitted through activity onsets before and after the light pulse. The first transient cycles after the light pulse were excluded from the fit. Steady-state phase shifts were determined by measuring the difference between the fitted lines extrapolated to the first cycle after the light pulse (Day 1, Fig. 1A). In addition, the immediate phase shift on the first (Day 1) and second circadian cycle (Day 2) after the light pulse were determined. The immediate phase shift was defined as the difference between the time of observed activity onset and the time as predicted by the line through activity onsets before the light pulse.

A strong correlation between the immediate phase shift on Day 1 and the steady-state phase shift was found. Moreover, we found a strong correlation between the immediate shift on Days 1 and 2 (for further details, see Results and Fig. 3 B,C). These correlations were used for the analysis of Experiment 2.

Experiment 2

A light pulse was applied between CT 17.75 and CT 21.71 according to the protocol as in Experiment 1. In this experiment, the light pulse was followed by a second light pulse given 0.01 to 4.31 h after activity onset on the first transient cycle (Day 1, Fig. 1B). After the second light pulse, the animals were kept in DD for 14 days. The immediate phase shift induced after the first and second light pulses and the steady-state phase shift induced by the two light pulses were determined.

We considered that the steady-state phase shift induced by the second light pulse is equal to the difference between the steady-state phase shift induced by the first light pulse and the steady-state phase shift induced by the two pulses together. To estimate the steady-state phase shift induced by the first light
pulse, the strong correlation between the immediate phase shift and the steady-state phase shift obtained in Experiment 1 was used. In other words, the immediate phase shift on Day 1 was determined and the steady-state phase shift \((A_1 - C_1)\) for that particular animal was estimated on the basis of the regression line obtained in Experiment 1 (Fig. 3B). To determine the steady-state phase shift induced by the two light pulses, the difference between the steady-state activity onset lines before and after the two light pulses was calculated \((A_1 - E_1)\). The steady-state phase shift induced by the second light pulse only is then \((A_1 - E_1) - (A_1 - C_1) = C_1 - E_1\).

Similarly, the immediate phase shift induced by the second light pulse was estimated by measuring the difference between the time of observed activity onset on Day 2 \((B_2)\) and the time of activity onset on Day 2 when the second light pulse would not have been given \((D_2)\). This latter value was predicted from the strong correlation between immediate shifts on Day 1 and Day 2 obtained in Experiment 1.

**Experiment 3**

A light pulse was presented according to the protocol as in Experiment 1. The light pulse was applied between CT 10.80 and 15.43 on Day 1. After the light pulse, the animals were kept in DD for 14 days. The steady-state phase shift on the day of the light pulse (Day 1) was determined by measuring the difference between the fitted lines before and after the light pulse (Fig. 1C). The immediate phase shift on the first cycle after the light pulse (Day 2) was measured as the difference between the time of observed activity onset and the time predicted by the line through activity onsets preceding the light pulse. ANOVA and post hoc t tests served to compare Experiment 2 and Experiment 3.

**RESULTS**

**Experiment 1**

In Experiment 1, 93 phase advances were analyzed (Fig. 2A). These light pulses were presented between CT 17.77 and CT 21.90 (mean = 19.07). The effect of the light pulse on the steady-state phase shift, \(\Delta \phi_{st}\), and on the immediate phase shift on the first cycle after the light pulse, \(\Delta \phi_{im(1)}\), are summarized in Figure 3A. The magnitudes of the steady-state phase shift and the immediate phase shift were \(1.42 \pm 0.44\) h and \(0.64 \pm 0.24\) h, respectively (mean ± SD). Although the same animals received light pulses several times, the difference between interindividual and intraindividual variations was not significant for the magnitudes of both steady-state and immediate phase shifts. Therefore, phase shifts obtained from the same animal were treated as independent values in all of the experiments.

The results of this experiment are important for the protocol of the next experiment. As is evident from Figure 3A, a rather large range of phase shifts can be obtained at each circadian time. However, when plotting the steady-state phase shift, \(\Delta \phi_{st}\), as a function of the magnitude of the immediate shift on Day 1, \(\Delta \phi_{im(1)}\) (Fig. 3B), a strong relation is observed between immediate and steady-state phase shift, which can be described as follows:

\[
\Delta \phi_{st} = 1.462 \times \Delta \phi_{im(1)} + 0.482 \quad (r = 0.79, \quad p < 1 \times 10^{-20}).
\]

This regression line was used to predict for each animal the steady-state phase shift from the immediate shift on Day 1 in Experiment 2.

Moreover, a strong correlation between the magnitude of the immediate shift on Day 1, \(\Delta \phi_{im(1)}\), and the immediate shift on Day 2, \(\Delta \phi_{im(2)}\), was found (Fig. 3C). This correlation \((r = 0.89, \quad p < 1 \times 10^{-20})\) can be described by the following function:
$\Delta \phi_{im(2)} = 1.325 \times \Delta \phi_{im(1)} + 0.150$.

This regression line was used in Experiment 2 to predict for each animal the time of activity onset on Day 2 in the case that only the first light pulse would have been applied and to measure the difference with the activity onset on Day 2.

**Experiment 2**

In the double pulse experiment, 85 activity records were obtained that allowed for an unambiguous analysis (Fig. 2B). The first light pulse fell between CT 17.75 and CT 21.71 (mean = 19.48), inducing a phase advance on Day 1 in all cases (range from 0.24 to 1.26 h; mean = 0.76 h). The second pulse was given 0.01 to 4.31 h (mean = 1.61 h) after activity onset on Day 1. Those cases where the second light pulse fell before activity onset were excluded from the analysis since no estimate of activity onset on Day 1 could be made. In most cases, the second light pulse induced a phase delay but a few advances were also observed.

**Experiment 3**

Eighty-five clear phase-shifts were obtained from light pulses that fell between CT 10.80 and CT 15.43 (mean = 12.95) and were used to describe the phase-delay part of the PRC (Fig. 2C). The maximum delay was obtained around CT 13 (Fig. 4). Mean hourly values for the immediate and steady-state phase shift were calculated between CT 11 and CT 16 and were compared with the data from Experiment 2.

**Comparison of Experiments 2 and 3**

In Experiment 2, the second light pulse was given at the beginning of the subjective night. The phase shift induced by this second light pulse was compared with the shift induced by a single light pulse applied at comparable phases (CT) in Experiment 3. The circadian time of application of the second light pulse is unknown. However, there are two predictions for the circadian time of the second light pulse. (1) If the overt rhythm is the manifestation of the underlying oscillator during transient cycles, the time of the activity onset on Day 1 is equal to CT 12. (2) If the steady-state phase shift reflects the real position of the oscillator, the extrapolated steady-state phase shift on Day 1 is equal to CT 12.
The two possible phase delays obtained in Experiment 2 with the second light pulse were calculated and compared with the data obtained in Experiment 3 (Fig. 4). The analysis indicates that the steady-state phase shift in Experiment 2 did not differ significantly from the PRC obtained in Experiment 3 when supposing that the pacemaker shifted to its steady-state position within one circadian cycle (Fig. 4, Prediction 2). In contrast, significant differences were obtained at CT 13 and CT 14 between the steady-state phase shifts in Experiment 2 and Experiment 3 when supposing that the first transient indicates the phase of the pacemaker (Prediction 1). Also, the two predicted PRCs differed significantly at CT 13 and CT 14 (Fig. 4).

**DISCUSSION**

The question posed in this article is whether transient cycles accompanying a light-induced phase advance reflect the circadian time of the pacemaker or whether the pacemaker shifts immediately to its steady-state position. We specifically addressed the question of whether the phase-delay part of the PRC shifts immediately after application of a phase-advancing light pulse. To answer this question, we determined the position of the PRC at the time of application of the second light pulse (Experiment 2) with the delay part of the PRC induced by a single light pulse (Experiment 3). The results are in accordance with previous studies (Best et al., 1999) and add to this that (a) the phase-delay part of the pacemaker shifts within one circadian cycle to its new steady-state position after a phase-advancing light pulse, (b) the magnitude of a transient shift predicts the phase of the new steady-state position, and (c) the waveform and amplitude of the phase-delay part of the PRC do not change after a phase-advancing light pulse.

**PRC Amplitude and Waveform Stability**

**Amplitude**

The question arises whether the properties of the pacemaker change during transient cycles. In fact, the data can only be interpreted on the premise that the pacemaker responds in its usual way to light during the transient cycles. Only then do the results from Experiment 3 form a reliable prediction for the pacemaker’s responsiveness to the second light pulse.

Previous double-pulse experiments in invertebrates (Drosophila, Pittendrigh, 1979; Neurospora, Crosthwaite et al., 1995), and the Syrian hamster (Best et al., 1999) were performed to investigate if there is a refractory period in the pacemaker after application of a light pulse and to determine how long this refractory period lasts (i.e., how fast the pacemaker can react to a second light pulse). These data show that the pacemaker is capable of reacting to a new light pulse within a few hours. In other studies, however, it was shown that mice and hamsters are significantly less responsive to a second light pulse when applied within 4 h after the first light pulse (Khammanivong and Nelson, 2000; Nelson and Takahashi, 1999). Moreover, Khammanivong and Nelson (2000) indicated that responses are not even back to normal in the next circadian cycle (about 70% of expected shift). Our experiments show that the amplitude of the phase delay is back to normal 17 to 21 h after a phase-advancing light pulse. This raises the possibility that responsiveness to light is decreased 24 h after a light pulse but is unchanged 17 to 21 h after a light pulse. In other words, the part of the PRC that received the first light pulse is still affected by it after 24 h, whereas other parts of the PRC may be unaffected.
Waveform

The waveform of the PRC appeared unaltered on the first cycle after the light pulse between CT 12 and CT 16. We have no knowledge of the phase response properties at other circadian times. Our data are consistent with unpublished data of Elliot and Pittendrigh (1996; Pittendrigh, 1981, Fig. 9). With respect to the phase delay part of the PRC, they showed that between CT 12 and CT 16, the PRC had shifted to the steady-state position within one circadian cycle. However, they showed that the onset of the phase-delay part of the PRC (about CT 11) had not shifted. As a consequence, the phase-delay part of their PRC was compressed. We did not investigate responsiveness before CT 12 because we administered our second light pulse after activity onset of the animal to obtain an accurate prediction for the induced phase shifts.

Predicting Steady-State Phase Shift from the First Transient Cycle

The present analysis shows that there is a strong relationship between the position of the activity onset during the first and second transient cycles and the steady-state phase shift of activity onset (Fig. 3). This enabled us to make a very reliable estimate of the steady-state phase shift induced by the first light pulse in Experiment 2 on the basis of the first activity onset. The strong correlation indicates that the daily shifts in activity onsets during transient cycles are regulated with great precision and reflect the magnitude of the steady-state shift. Thus, the immediate shift is a fixed fraction of the steady-state shift. This indicates by itself that during the first transient cycle the steady-state phase shift is already determined. This is consistent with our conclusion that the pacemaker has reached its steady-state position on the first transient cycle.

This result is suggestive for an interaction between the endogenous pacemaker and a secondary downstream oscillatory system, either inside or outside the SCN, of which the kinetics can be described with great mathematical precision. We will discuss possible secondary systems in the next section.

Immediate Resetting

We showed that after a phase-advancing light pulse, the phase-delay part of the PRC shifts within one circadian cycle to the new steady-state position while the overt rhythm displays transients. If the pacemaker shifts immediately, the question arises, What process induces transient behavior? Transients in Drosophila have been attributed to the existence of two coupled oscillators of which one is light sensitive, whereas the other is temperature sensitive (Pittendrigh et al., 1958). In mammals, two mutually coupled circadian oscillators within the central pacemaker have been proposed (Pittendrigh and Daan, 1976). One oscillator is thought to lock onto the evening light (the E oscillator) and controls activity onset in nocturnal animals, while the other locks onto the morning light (the M oscillator) and controls activity offset. It has been suggested that the differential shift in onset and offset after a phase-advancing light pulse causes different shifts of E and M, with the M oscillator shifting immediately to its new steady-state position and E shifting more slowly (Honma et al., 1985; Meijer and Devries, 1995; Elliot and Tamarkin, 1994; Pittendrigh and Daan, 1976).

Accumulating evidence at a number of research levels supports the proposition, initially put forward by Pittendrigh and Daan (1976), that the circadian pacemaker is composed of different oscillators. Daan et al. (2001) summarized these results elegantly in a conceptual framework. The E and M components are thought to result from a double set of circadian genes. The M oscillator consists of per1 and cry1 and is accelerated by light, the E oscillator of cry2 and per2 and is decelerated by light. Evidence in favor comes, for instance, from Albrecht et al. (2001), who demonstrated that per1 knockout mice lost the capacity to respond with advances to a light pulse, whereas per2 knockouts no longer respond with phase delays (see Daan et al., 2001, for more details). An alternative model was proposed by Hastings (commentary to Daan et al., 2001). E and M could reflect per1/per2 expression (M) versus cry1/cry2 expression (E). This model is supported by differential peak times of the pers and crys under different photoperiods.

The transients that are observed after a phase-advancing light pulse are attributed to an immediate shift of M (the oscillator that responds with advances to light) and a delayed shift of E, as a consequence of coupling forces. This explanation would also be consistent with Jagota et al. (2000), who demonstrated two peaks in multiunit activity in the SCN slice preparation. One peak occurred at the onset of dawn (possibly the M oscillator) and responded to glutamate with an immediate advance, while the other component did not respond. The evening rise and morning decline of melatonin show similar differences in their
responsiveness to light. A phase-advancing light pulse results in an immediate advance of the melatonin decline and in a delayed advancing shift in melatonin rise (Elliot and Tamarkin, 1994; Illnerova, 1991). We have summarized the responses to phase-advancing light pulses in Table 1.

Best et al. (1999) demonstrated that the phase-advancing part of the PRC is reset within a few hours by light at CT 19. Our results demonstrate that within one circadian cycle light presentation at CT 19 also results in advances of the delay part of the PRC, at least of the delay part between CT 12 and CT 16. If the delay part of the PRC shows rapid resetting, similar to the advance part of the PRC, transients in the onset of activity cannot readily be explained on the basis of differential shifts of the E and M component when it is assumed that E is represented by the delay and M by the advance portion of the PRC.

Although our results do not follow directly from the proposed model of Daan et al. (2001), they are not necessarily in conflict with it either. An immediate shift of the M component \((\text{per1/cry1})\) in response to light at CT 19 may result in an immediate shift of the E component \((\text{per2/cry2})\) as a consequence of strong coupling forces between the two. The same reasoning is applicable to the model of Hastings (2001), but now with a different set of genes; an immediate shift of M \((\text{per1/per2})\) may result in a shift of E \((\text{cry1/cry2})\) within one cycle as a consequence of coupling. However, irrespective of the set of genes that may underlie E and M, our results suggest rapid resetting of both the delay and advance portions of the PRC.

A different viewpoint arises when data from intact animals are compared with data obtained in slice preparations. Phase resetting of the pacemaker has been studied in vitro by recording circadian rhythms in sampled single unit activity from SCN brain slice preparations. This activity displays a circadian rhythm, and the time of peak activity is used as a phase marker (Prosser and Gillette, 1989; Prosser, 1998). SCN brain slices can be kept in good condition and recorded from for two or three cycles. Phase shifts are commonly induced between the first and second cycle. Phase shift experiments in vitro have indicated that phase shifts in the SCN slice are immediate and stable (McArthure et al., 1991; Prosser, 1998; Watanabe et al., 2000) also when glutamate is administered at CT 19 (Ding et al., 1994). In these preparations, most downstream processes are eliminated because the slice is disconnected from most of its input and output. This indicates that transients could be the result of secondary processes, which are downstream from the pacemaker, outside the SCN.

The latter offers an alternative explanation for the occurrence of transients. It has been proposed that there might be secondary oscillatory systems (slave oscillators) regulating the coupling of the activity rhythm with the circadian pacemaker (Pittendrigh and Daan, 1976; Takamura et al., 1991; Yamazaki et al., 2000). These secondary oscillators may require more than one circadian cycle to achieve complete re-entrainment. This hypothesis is supported by the finding that the velocity of re-entrainment of different circadian rhythms (urinary ion secretion, adrenocortical levels, core body temperature) after crossing several time zones is not the same (Klein et al., 1972; Moore-Ede et al., 1982; Van Cauter and Turek, 1986). Recently it was proposed that resetting of circadian

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**Table 1.** Phase shifts induced by a phase-advancing light pulse at CT 19. The table illustrates clear asymmetry in the effect of light on behavioral activity and melatonin (NAT) rhythms and on multiunit activity (MUA). Immediate shifts were obtained in activity offset, melatonin offset, and morning component of MUA. No immediate shifts were obtained in activity onset, melatonin onset, and in the evening component of MUA. No asymmetry exists for the shifts in advancing and delaying parts of the PRC, as both shift immediately. The shift of the rhythm in \(m\text{Per}1\) is investigated in response to a 6-h advance of the light-dark cycle (and not in response to a short light pulse at CT 19) \(m\text{Per}1\) shifts immediately in response to this shifted cycle. It is unknown whether \(m\text{Per}2\) rhythms are immediately reset by phase-advancing stimuli.

<table>
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<tr>
<th>Response to Advancing Light Pulse at CT 19</th>
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<td>Behavioral activity rhythm offset</td>
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<td>Multiunit activity peak dusk (E)</td>
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<td>Advance part PRC</td>
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<td>(m\text{Per}2) rhythms</td>
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time in peripheral tissue occurs via glucocorticoid signaling (Balsalobre et al., 2000), indicating that glucocorticoid is one of the possible candidates entraining secondary oscillators to the circadian pacemaker. Together the data indicate the existence of various time lags from the circadian pacemaker to each secondary oscillatory system.

The present experiments were not aimed to indicate where transients originate and leave open the possibility that behavioral transients are attributable to downstream processes, outside the SCN. The results have demonstrated that at least part of the pacemaker has shifted fully and within one circadian cycle in response to light. It is possible that the first part of the delaying area of the PRC (before CT 12) shows compression in response to a phase-advancing light pulse (see Pittendrigh, 1981) and that this accounts for transients in activity onset. This would lead to the conclusion that part of the delay curve shifted immediately, and part of it did not. The question of whether the pacemaker shows immediate resetting in response to light pulses should then be reformulated into a more specified question: Which parts of the pacemaker show immediate resetting and which parts do not? It may appear necessary to not only distinguish between the advance and delay areas of the PRC but even within these areas.

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Temperature Cycles Induce a Bimodal Activity Pattern in Ruin Lizards: Masking or Clock-Controlled Event? A Seasonal Problem

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Abstract The daily locomotor activity pattern of Ruin lizards in the field is mainly unimodal, except for summer months when soil temperatures exceed 40 °C to 42 °C around midday. In such a situation, lizards reduce their locomotor activity around midday to avoid overheating, and thus their daily activity pattern becomes bimodal. The bimodal pattern expressed in the field is usually retained in the free-running rhythm under constant temperature and DD for a couple of weeks, after which the bimodal pattern changes into a unimodal pattern. In the present study, the authors examined whether 24-h temperature cycles (TCs) would change lizard activity from a unimodal to a bimodal pattern. Administration of TCs to unimodal lizards free-running in DD is able to entrain locomotor rhythms and to induce a bimodal pattern both in summer and autumn-winter. There are, however, striking seasonal differences in the effectiveness with which TCs achieve bimodality: (a) Numbers of lizards rendered bimodal are significantly higher in summer than in autumn-winter; (b) TCs require less time to achieve bimodality in summer than autumn-winter; (c) bimodality is retained as an aftereffect in the postentrainment free-run in summer, but not in autumn-winter; (d) TCs change activity duration in summer, but not in autumn-winter. All this demonstrates the existence of seasonal changes in responsiveness of the circadian oscillators controlling activity to the external factors inducing bimodality. Oscillators’ responsiveness is high in summer, when bimodality is the survival strategy of Ruin lizards to avoid overheating around midday in open fields, and low in autumn-winter, when bimodality has no recognizable adaptive significance.

Key words circadian rhythms, locomotor activity, entrainment, temperature cycles, bimodal activity pattern, lizards

Seasonal changes in the daily pattern of locomotor activity in the field were reported in most diurnal Lacertid lizards from southern Europe (Bowker, 1986; Foà et al., 1992; Henle, 1988; Pough and Busack, 1978; Tosini et al., 1992; Van Damme et al., 1990). Generally, the daily pattern of locomotor activity changes from unimodal in spring (with only one activity peak per day) to bimodal in summer (with two well-separated activity peaks per day), becoming unimodal again in autumn. Since lizards are ectotherms, which can be active only within a limited range of body temperatures, seasonal changes in locomotor patterns have
been regarded more or less explicitly as a direct behavioral response of these animals to related changes in solar radiation and ambient temperature (Avery, 1980; Ouboter, 1981). Recent studies in our model animal, the Ruin lizard (Podarcis sicula), showed that a great deal of these activity changes are controlled by endogenous temporal programs (Bertolucci et al., 1999; Foà et al., 1994). Short-term experiments (20-30 days), in which Ruin lizards collected in different months were tested immediately after capture under constant temperature (29 °C) and darkness (DD), showed that the activity pattern typical of each season (bimodal/unimodal) is retained in the lizard circadian locomotor rhythm (Foà et al., 1994). Furthermore, while the bimodal locomotor pattern expressed by the lizards in constant conditions between June and August is typically associated with a short free-running period (τ) and a long circadian activity (α), the unimodal locomotor pattern expressed in the remaining months is typically associated with long τ and short α (Foà et al., 1994). To test whether such seasonal changes in circadian locomotor rhythms were driven by a circannual clock, the locomotor activity of Ruin lizards was recorded in long-term experiments (over 12-15 months) under constant temperature (29 °C) and DD. The results demonstrated the existence of circannual cycles of both τ and α and, at the same time, the absence of a circannual cycle of activity pattern from bimodal to unimodal and vice versa (Bertolucci et al., 1999). The longest τ along its circannual cycle was associated with short α, and the shortest τ in the same cycle with long α, so that the pattern of mutual association between τ and α was found to be the same as in short-term experiments (Bertolucci et al., 1999; Foà et al., 1994). Most lizards, however, stayed unimodal all the time, showing that seasonally changing environmental factors (such as, for instance, photothermoperiodic conditions) are involved in the induction of a bimodal activity pattern (Bertolucci et al., 1999). In other reptiles, such as the Namib desert dune lizard (Aporosaura anchietae) and the garter snake (Thamnophis radix), the locomotor activity pattern was shown to be switched between unimodal and bimodal patterns, respectively, by lowering or raising the ambient temperature (Heckrotte, 1962, 1975; Holm, 1973; Underwood, 1992). This suggests that the expected seasonal changes of locomotor activity pattern (from unimodal to bimodal and vice versa) can be achieved in lizards by means of appropriate manipulations of ambient temperature levels and/or administration of temperature cycles in the laboratory. Hoffmann (1968) showed that 24-h temperature cycles (TCs) of low amplitude (0.9-3 °C) are capable of entraining circadian locomotor rhythms of Ruin lizards kept in constant lighting conditions.

The present study carried out completely in DD examined whether the administration of 24-h TCs is capable of changing a unimodal activity pattern into a bimodal activity pattern. When Ruin lizards were tested in summer, TCs were found to induce a bimodal pattern. To establish whether effectiveness of TCs in achieving bimodality changes depending on season, we compared the locomotor behavior of lizards collected and exposed to the same TCs in different seasons. We expected effectiveness to be maximal in the summer, as Ruin lizards usually express a bimodal activity pattern in summer but not at other times of the year (Foà et al., 1992, 1994). Studies focused on the role of the pineal and other clock components in establishing and/or maintaining a bimodal activity pattern in Ruin lizards have been reported previously (Innocenti et al., 1994, 1996; Tosini et al., 2001).

MATERIALS AND METHODS

Animals and Locomotor Recording

Ruin lizards, Podarcis sicula campestris De Betta 1857 (adult males only, 6.5-8 cm snout-vent length) from the area of Ferrara (Italy) were used. Lizards were collected in the field in groups of 8 to 14 individuals in June and November 1999 and June 2000. After capture, each seasonal group of lizards was carried to the lab and immediately put into individual tilt-cages (30 × 15 × 11 cm) for locomotor recording. Tilt-cages were placed inside thermally programmable environmental chambers and connected to a computer-based data acquisition system (DataQuest III, MiniMitter, Sunriver, OR, USA) for monitoring locomotor activity. Food (Tenebrio molitor larvae) and water were supplied twice a week. During experiments, lizards were kept under DD and either constant temperature (30 ± 0.2 °C) or 14:10-h TCs. Experiments used three different TCs. TC1: 30:27 °C (30 °C from 0800 to 2200 h; 27 °C from 2200 to 0800 h); TC2: 30:28.3 °C (30 °C from 0800 to 2200 h; 28.3 °C from 2200 to 0800 h); and TC3: 30:28.3 °C (30 °C from 1400 to 0400 h; 28.3 °C from 0400 to 1400 h).
Experimental Design

Summer 1999 (June 4 to July 27):
Pilot Experiment

Lizards \((n = 8)\) were allowed to free-run in DD and constant temperature \((29 \, ^\circ\mathrm{C})\) for 3 weeks and then were subjected to the TC\(_1\) for 25 days.

Autumn-Winter 1999 to 2000
(November 17 to March 8)

Lizards \((n = 14)\) were allowed to free-run in DD and constant temperature \((29 \, ^\circ\mathrm{C})\) for 2 weeks and then were subjected to three TCs: TC\(_1\) for 28 days, TC\(_2\) for 22 days, and TC\(_3\) for 22 days. After these periods of time, locomotor activity in constant temperature \((29 \, ^\circ\mathrm{C})\) was recorded for a further 2 to 3 weeks.

Summer 2000 (June 19 to September 9)

Lizards \((n = 14)\) were allowed to free-run in DD and constant temperature \((29 \, ^\circ\mathrm{C})\) for 2 weeks and then were subjected to two TCs: TC\(_1\) for 23 days and TC\(_2\) for 17 days. After these periods of time, locomotor activity in constant temperature \((29 \, ^\circ\mathrm{C})\) was recorded for a further 4 weeks.

Data Evaluations

For locomotor rhythms free-running in constant temperature and DD, \(\tau\) and \(\alpha\) were estimated by the eye-fitting method (Pittendrigh and Daan, 1976a). \(\tau\) was also measured by means of \(\chi^2\) periodogram analysis (Sokolove and Bushell, 1978). Bimodality and unimodality of the locomotor pattern were established by visual inspection: The bimodal pattern is characterized by the existence of two daily peaks of locomotor activity regularly repeated in subsequent days, while the unimodal pattern is characterized by the existence of one peak of activity each day. In presence of a bimodal activity pattern, either duration of circadian \((\alpha)\) or duration of entrained activity includes duration of the pause between the two daily activity peaks.

RESULTS

Before administration of TCs, lizards were free-running in constant temperature and DD. The mean value of \(\tau\) in summer was significantly shorter than the mean value of \(\tau\) in autumn-winter \((p < 0.05)\), and the mean value of \(\alpha\) in summer was significantly longer than the mean value of \(\alpha\) in autumn-winter \((p < 0.001)\) (Fig. 1).

Locomotor rhythms of all lizards kept in DD entrained to the TC\(_1\) of 3 °C in amplitude (Figs. 2-5). Entrainment continued after reduction of the TC amplitude to 1.7 °C (TC\(_2\) and TC\(_3\); Figs. 2-3, 5) in all cases. The entrained activity phase always fell within the 30 °C portion of the TC. When, in the final part of experiments, lizards were exposed again to constant temperature, activity onsets in the postentrainment free-running rhythm always extrapolated back to the time of activity onsets during entrainment. In winter, a 6-h shift from TC\(_3\) to TC\(_1\) induced shifts of the activity onsets, which resulted, after several transient cycles, in entrainment to the new schedule (Fig. 5). Hence, 14:10-h TCs actually entrained the activity rhythm and did not merely cause masking of the underlying oscillation. No seasonal differences in all these aspects of entrainment were found. In most lizards, activity onsets delayed about 1 h the daily onsets of high \((30 \, ^\circ\mathrm{C})\) temperature phase of TC. No differences in \(\psi\) either among TCs or among seasons were found.

Administration of the TC\(_1\) to lizards expressing a unimodal circadian locomotor activity pattern under constant temperature and DD was capable of inducing the appearance of a bimodal pattern in all seasons (Table 1, Figs. 2-3, 5). Bimodality always persisted after reduction of amplitude of the TC from 3 °C to 1.7 °C (TC\(_2\) and TC\(_3\) cycle; representative examples in Figs. 2-3, 5). However, the percentage of lizards rendered bimodal by TC\(_1\) is significantly higher in summer than in autumn-winter (Fisher exact test, \(p < 0.04\); Fig. 6A). Furthermore, the time elapsed between administration of TC\(_1\) and appearance of bimodality is significantly shorter in summer than in autumn-winter (Kruskal-Wallis one-way ANOVA, \(H = 4.47, p < 0.04\); Fig. 6B). Administration of TC\(_1\) also increases significantly the duration of daily activity in summer (paired Student \(t\) test, \(p < 0.01\)) but leaves the duration of daily activity unchanged in autumn-winter (paired Student \(t\) test, \(p > 0.10\)). As expected, from the very beginning of locomotor recording in summer, several lizards already expressed a bimodal pattern in constant temperature. In all cases, the administration of TC\(_1\) lengthened the rest interval between the two daily peaks of activity that characterize the bimodal state from 2 to 3.1 h, enhancing in that way bimodality (paired Student \(t\) test, \(p < 0.002\); representative examples in Fig. 4).
Administration of constant temperature led all lizards to free-run (Figs. 3, 5). While in autumn-winter no bimodal lizard maintains bimodality after release in constant temperature, in summer several lizards do so (Table 1 and Figs. 3, 5). The seasonal difference is statistically significant ($p < 0.006$, Fisher exact test). Furthermore, the time elapsed between administration of constant temperature and reappearance of unimodality is significantly shorter in autumn-winter than in summer (Kruskal-Wallis one-way ANOVA, $H = 7.17$, $p < 0.02$; Fig. 6B). Several summer lizards were still bimodal 3 to 4 weeks after release in constant temperature (Table 1 and Fig. 3).

**DISCUSSION**

Behavioral observations of Ruin lizards in their natural environment showed that the daily locomotor pattern of focal animals in the field is mainly unimodal, except for those summer months in which soil temperatures exceed 40 to 42 °C around the middle of day. In such situations, lizards reduce their locomotor activity dramatically around midday, retreating into shade or burrows to avoid overheating, and thus exhibiting the markedly bimodal activity pattern so typical of summer (Foà et al., 1992, 1994; Tosini et al., 1992). Once carried to the laboratory and placed in constant temperature (29 °C) and DD, summer lizards mainly retained their endogenous, free-running rhythm under constant conditions, the bimodal pattern previously expressed in the field (Foà al., 1994). This demonstrates that bimodality is not merely a direct behavioral reaction of these ectotherm animals to the extremely high levels of soil temperatures around midday in summer but is a season-dependent state of the circadian pacemaker that has evolved as an adaptation to high temperatures predictably occurring at that time of day. Long-term experiments further revealed that the retained bimodal pattern disappears—that is, the pattern becomes unimodal—in the course of the first 2 months of locomotor recording under constant temperature (29 °C) and DD, and bimodality never reappears (Bertolucci et al., 1999). Hence, bimodality of Ruin lizards typically shows all recognized properties of aftereffects on the circadian pacemaker: (a) Its appearance in constant conditions is dictated by previous exposure to specific environmental stimuli, such as, for instance, summer photo-thermoperiodic conditions; (b) once established, it persists for several weeks in constant conditions, after which, (c) it decays to a different state: unimodality. The present study examined whether changes in ambient temperature—which were administered in the form of 24-h TCs—were the specific environmental stimuli capable of inducing bimodal activity patterns at the expected time of the year. First of all, our results show that circadian locomotor rhythms of Ruin lizards become entrained to 24-h TCs of low (3-1.7 °C) amplitude. This confirms the results...
of the pioneering work done by Hoffmann in Ruin lizards, which for the first time indicated that low-amplitude TCs are capable of entraining circadian behavioral rhythms in ectotherm vertebrates (Hoffmann, 1968). More important, administration of 24-h TCs in the laboratory to unimodal lizards collected in the
Figure 3. Behavioral effects of temperature cycles (TCs) in summer. Brackets (on the right) delimit sections of records where the activity pattern was bimodal. Records are representative examples of the fact that TC administration entrained circadian locomotor rhythms and induced a fast change of locomotor activity pattern from unimodal to bimodal. Furthermore, after final release in constant temperature, lizards maintained bimodality. Further information in Figure 2.
field during late spring to early summer was found to induce rapid appearance of a bimodal activity pattern in most animals (Table 1 and Figs. 2-3).

As expected, in summer several lizards were already bimodal in constant temperature before starting with TCs administration (example in Fig. 4). TCs further enhanced bimodality by lengthening the pause between the two daily peaks of the bimodal activity pattern of these lizards. Most summer lizards were still bimodal 3 to 4 weeks after cessation of the TCs (after final release in constant temperature), and after this period of time, bimodality disappeared in about 50% of the animals. These aspects of the data confirm our assumption that bimodality is retained as an aftereffect in the postentrainment free-running rhythm in constant conditions. To our surprise, 24-h TCs were capable of inducing bimodality even in autumn-winter, seasons in which changing from unimodal to bimodal activity pattern is not of apparent adaptive significance (Foà et al., 1994). It must be pointed out, however, that all lizards that became bimodal in autumn-winter returned to being unimodal immediately after cessation of TCs (Fig. 5).

Thus, differently from summer, in autumn-winter bimodality is not retained in the postentrainment free-running rhythm in constant conditions: It is not an aftereffect. Instead of resulting from TCs affecting the circadian oscillators that control activity, the transition from a unimodal to a bimodal pattern in autumn-winter seems to result from TCs directly affecting activity, therefore bypassing the circadian clock. In other words, bimodality achieved in autumn-winter looks like a phenomenon of masking (Underwood, 1992, p. 249). For the sake of clarity, it seems useful to underline that the case of masking proposed here exclusively concerns achievement of bimodality in autumn-winter. In fact, “true” entrainment (and not masking) of circadian locomotor rhythms of lizards to the administered 24-h TCs has occurred in all seasons, as postentrainment free-runs in constant temperature have verified.

Changes in locomotor pattern in response to ambient temperature manipulations in the laboratory have been reported previously in other reptiles. For instance, in the Namib desert dune lizard (Aporosaura anchietae) kept under constant conditions and the garter snake (Thamnophis radix) exposed to an LD cycle, the locomotor activity pattern was shown to be switched between unimodal and bimodal patterns, respectively, by lowering or raising the ambient temperature. It is unclear, however, whether the reported effects were season-dependent (Heckrotte, 1962, 1975; Holm, 1973). Noteworthy, Underwood (1992) proposed that each activity peak of the bimodal pattern of lizards and snakes may be the overt expression of one of the two (or sets of) circadian oscillators described in Pittendrigh and Daan’s (1976b) model. Underwood further stated that since a rise in ambient temperature switches activity from a unimodal to a bimodal pattern and increases the duration of daily activity, temperature as well as photoperiod may be able to change phase relationship between the two oscillators controlling activity. Because the model proposes that the time interval between activity onset and activity offset each day would be a measure of the phase relationship between the two oscillators, changes in that phase relationship would be expected to cause a change in duration of activity. According to this view, it is important that in Ruin lizards the bimodal pattern achieved in response to TCs is associated with a prominent increase in duration of daily activity in summer—thus
Figure 5. Effects of temperature cycles (TCs) in autumn-winter. Records are representative examples of the fact that TC administration entrained circadian locomotor rhythms. However, it took several days for the TC to induce a bimodal pattern. Furthermore, differently from summer, autumn-winter lizards returned unimodal immediately after release in constant temperature. Other information in Figure 3. Finally, 50% of autumn-winter lizards remained unimodal after TC administration, as it is shown in Figure 2.
potentially reflecting a change in phase relationship between circadian oscillators controlling activity—while the bimodal pattern achieved in autumn-winter is not associated with a change in activity duration.

Furthermore, there are striking seasonal differences in the effectiveness with which TCs achieve bimodality: (a) The percentage of lizards that TCs have rendered bimodal is significantly higher in summer than in autumn-winter; (b) the time required for the first administered TC to achieve a bimodal pattern is significantly less in summer than in autumn-winter. According to Pittendrigh and Daan’s (1976b) model, such differences in effectiveness may depend on seasonal differences in the mutual phase relationship between the two oscillators, that is reflected in the seasonally different associated values of $\tau$ and $\alpha$ of the overt rhythm. With regard to the present results, such seasonal differences are expressed in the free-running rhythm before TC administration: in summer a short $\tau$ associated with a long $\alpha$, and in autumn-winter, a long $\tau$ associated with a short $\alpha$ (Fig. 1). Because previous experiments in constant conditions made it clear that a short $\tau$ together with a long $\alpha$ typically brings about a bimodal pattern, the summer effectiveness of TCs in achieving bimodality shown here is likely to depend on the expression in the free-running rhythm of a short $\tau$ together with a long $\alpha$, facilitating a change in pattern from unimodal to bimodal as soon as Ruin lizards perceived the TC (Foà et al., 1994). The long $\tau$ together with the short $\alpha$ of autumn-winter reflect a phase relationship between constituent oscillators that presumably hampers a change in pattern from unimodal to bimodal, thus reducing autumn-winter effectiveness of TCs in achieving bimodality. When, in spite of all that, TCs administered in autumn-winter were successful in changing the pattern from unimodal to bimodal, they clearly did it by bypassing the circadian clock, as bimodality did not entail a change in activity duration and, moreover, was not retained as an after-effect in the postentrainment free-run.

Unlike bimodality, effectiveness of 24-h TCs in entrainment of locomotor rhythms does not change with season. This was somehow expected. In fact, Ruin lizards are diurnal, ectotherm animals whose locomotor activity is necessarily limited by temperature. In such a situation, the circadian clock of lizards has to entrain to the TC of the external day, to force the locomotor activity it drives into the range of the ambient temperatures that are most suitable for locomotor performances, thus optimizing daily time-budget for biologically significant behaviors through the entire year. Again, no seasonal differences in any aspect of entrainment were found. Both summer and autumn-winter entrained activity fell in the 30 °C phase and (entrained) rest in the 27 °C (or 28.3 °C) phase of TCs. This confirms previous work indicating that Ruin lizards that are held on a thermal gradient in DD spontaneously select warm temperature zones of the gradient during activity and cooler temperature zones during rest (Innocenti et al., 1993). On the other hand, field studies in Ruin lizards showed the existence of seasonal differences in body temperatures recorded during activity phase, with a mean temperature of 29 °C in autumn-winter and a mean of 32.7 °C in late spring–summer (Tosini et al., 1992). It looks like the temperature of 30 °C we chose in the present experi-
ments is a reasonable compromise between those measured in the field and therefore suitable for activity in each season, while 27 °C seems less suitable for activity.

**CONCLUSIONS**

Although long-term records in constant conditions did not find circannual changes between bimodal and unimodal patterns, the same experiments did reveal the existence of circannual changes of both $\tau$ and $\alpha$ of locomotor rhythms with, moreover, some phases of the circannual cycle characterized by long $\tau$ associated with short $\alpha$ (potentially conducive to unimodality) and other phases characterized by short $\tau$ associated with long $\alpha$ (potentially conducive to bimodality) (Bertolucci et al., 1999). The circannual data made it clear, on one hand, that environmental factors are necessary to obtain a change from a unimodal to a bimodal pattern, and, on the other hand, that there are months in which the state of the system, expressed in the association between short $\tau$ and long $\alpha$ of its overt rhythm, facilitates achievement of a bimodal pattern. The present results show that a change in ambient temperature—in the form of a 24-h TC—is the appropriate stimulus to induce bimodality, and they confirm the existence of pronounced changes in responsiveness of the circadian oscillators controlling activity to the external factors inducing bimodality, with high responsiveness in summer and low responsiveness in autumn-winter. The data further suggest the possibility that a circannual rhythm in responsiveness of the circadian system to the bimodality-stimulating effects of TCs parallel the previously demonstrated circannual rhythms of $\tau$ and $\alpha$ or, alternatively, the circannual rhythm in responsiveness may be merely a consequence of those of $\tau$ and $\alpha$.

**ACKNOWLEDGMENTS**

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| Table 1. Summary of the results. All numbers of unimodal versus bimodal lizards in each stage of experiments in different seasons. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Season**                      | **n**                           | **T Cost**                      | **Temperature Cycles**          | **T Cost**                      |
|                                 | **Unimodal** | **Bimodal** | **Unimodal** | **Bimodal** | **Unimodal** | **Bimodal** |
| Summer 1999                     | 8            | 6           | 2*           | 1           | 7           | —           | —           |
| Autumn-Winter 1999-2000         | 14           | 14          | 0            | 7           | 7           | 14          | 0           |
| Summer 2000                     | 14           | 9           | 5*           | 1           | 13          | 7           | 7           |

*Summer lizards that already expressed a bimodal pattern in constant temperature.*


Persistence of Masking Responses to Light in Mice Lacking Rods and Cones

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Key words cones, irradiance, masking, phase shifting, retinal degeneration, rods

Overt rhythms of locomotor behavior are a result of two complementary processes. The first mechanism is the synchronization of an endogenous clock, which in turn directs the animal to be active in the day or the night; this is called entrainment. The second mechanism involves an acute response to light, which inhibits or promotes activity, depending on whether the species is nocturnal or diurnal; this is called masking (Aschoff, 1960; review in Mrosovsky, 1999). The fact that masking can occur in hamsters with lesions of the SCN (Redlin and Mrosovsky, 1999) and in mice lacking cryptochromes (van der Horst et al., 1999; Vitaterna et al., 1999), that is, in animals lacking any persistent circadian rhythm to be entrained, is further evidence that entrainment and masking are behaviorally and physiologically different responses to light. Although masking may be of considerable importance in influencing the amplitude of overt rhythms, it has received much less study than has entrainment. Indeed, the main concern of rhythms researchers has been to exclude masking as much as possible from their experiments.

At the receptor level at least, however, masking and entrainment may have considerable commonalities, in that they are both spared in retinally degenerate (rd/rd) mutant mice (Foster et al., 1991; Mrosovsky, 1994). Eventually, rd/rd mice come to lose all their rods, and many cones degenerate secondarily (Carter-Dawson et al., 1978; Foster et al., 1991). Nevertheless, a few cones remain, and it is possible that such residual cones might be enough to mediate responses to light. This has been ruled out for phase shifting, which has been shown to persist even in mice genetically modified to lack both rods and cones (Freedman et al., 1999). The current experiment was undertaken to determine if masking was also spared in such rodless coneless mice.

METHODS

C3H/He wild-type and rodless coneless (rd/rd cl) mice were bred in London as previously described (Lucas et al., 1999) and sent to Toronto for testing. Assessment of inhibition of locomotion by light followed previous procedures (Mrosovsky et al., 1999). Tests began when the mice were approximately 4 months old. Male mice of both genotypes (wild-types n = 8, rd/rd cl n = 5) were housed individually in cages 44 × 23 ×20 cm fitted with running wheels (17.5 cm d). Revolutions were monitored with Dataquest III hardware and software (Sunriver, OR, USA). Prior to the start of the masking tests, the animals were entrained to a 16:8 h light-dark (LD) cycle for 17 days; the entraining light provided approximately 1300 lux in the cages, as measured with a ISO-TECH ILM350 meter.

To examine the acute effects of light on wheel running, an additional set of lights were used. These were fluorescent tubes (Sylvania Octron 32 watt 4100 K) fitted with a broad-spectrum green filter (Rosco Supergel filter #89, maximum transmission around 520 nm, half bandwidth approximately 60 nm; Rosco,
Irradiance, as measured with a Hagner E2X luxmeter, was ca 120 lux in the cages below. To reduce this illumination for subsequent tests, neutral density filters (Rosco Cinegel), calibrated in photographic stops, were added as needed; illumination at three, six, and nine stops, respectively, was approximately 12, 1.5, and 0.25 lux. Tests with 1-h light pulses, starting 2 h after dark onset, were spaced not less than 3 days apart and were conducted in the following order for all animals: 0, 3, 6, 9, 7.5, 4.5, 1.5, 1, 2, 0.5 stops. The number of wheel revolutions made during a light pulse was expressed as a percentage of the number made by the same animal during the same hour on the previous day when there was no light. For additional details, see Mrosovsky et al. (1999).

RESULTS

Both wild-type and rd/rd cl mice inhibited their wheel running on exposure to the broad-spectrum green light presented in the early night (Fig. 1). At the highest irradiance studied here, wheel running was reduced to about 10% of normal levels in both genotypes (means ± SEMs: 12.8 ± 4.6% wild types, 10.7 ± 9.3% rd/rd cl). In both genotypes, the effects were irradiance dependent but there were no significant differences between the genotypes (two-way ANOVA p > 0.1).

DISCUSSION

It is evident that, as well as phase shifting, rodless coneless mice retain an acute masking response to light and that no impairment of this acute response is present.

Both masking and entrainment serve the function of confining an animal’s activity to a daytime or nighttime niche. Both masking and entrainment require only detection of overall illumination, not of spatio-temporal differences in light (Mrosovsky, 1994). Indeed, to determine whether it is night or day, a system that integrates illumination over space and time is desirable. For it to be day, it should be light in all directions and this input should be maintained. Both masking and entrainment are spared in mice lacking rods and cones and so presumably depend on some novel photoreceptor type elsewhere in the eye.

Given these similarities, it would be most parsimonious to suppose there is a single novel receptor type that projects to various brain areas involved in different responses. If there is a single receptor type for these different responses to light, whether or not it had been called a circadian receptor in the past, it should be conceptualized more as a general irradiance detector. It has been demonstrated that besides unimpaired phase shifting and masking, rd/rd mice spend more time in the dark side of a differentially illuminated box (Mrosovsky and Hampton, 1997). It has long been known that they retain pupillary responses (Keeler, 1927) and suppression of melatonin by light pulses (Goto and Ebihara, 1990). Recently it has been shown that pupillary contraction and melatonin suppression are also present in rd/rd cl mice (Lucas et al., 1999, 2001). Thus, there are a variety of responses to light that do not require rods or cones.

Of course it remains possible that there are several different receptor types subserving phase shifting, masking, dark preferences, melatonin suppression, and pupillary responses, perhaps with some functional redundancy (Selby et al., 2000). While being open-minded on this possibility, it seems more likely that photoreception for resetting circadian rhythms is
an aspect of a more general irradiance detection system and that the hunt for the circadian receptor will turn up a receptor type with a much wider functional role.

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The Eighth Meeting of the Society for Research on Biological Rhythms (SRBR) will take place on May 22-26, 2002 at Amelia Island Plantation, Jacksonville, FL.

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