Genetic Analysis of the Circadian System in *Drosophila melanogaster* and Mammals

Ralf Stanewsky

Universität Regensburg, Institut für Zoologie, Lehrstuhl für Entwicklungsbiologie, Universitätsstraße 31, 93040 Regensburg, Germany

ABSTRACT: The fruit fly, *Drosophila melanogaster*, has been a grateful object for circadian rhythm researchers over several decades. Behavioral, genetic, and molecular studies helped to reveal the genetic bases of circadian time keeping and rhythmic behaviors. Contrary, mammalian rhythm research until recently was mainly restricted to descriptive and physiologic approaches. As in many other areas of research, the surprising similarity of basic biologic principles between the little fly and our own species, boosted the progress of unraveling the genetic foundation of mammalian clock mechanisms. Once more, not only the basic mechanisms, but also the molecules involved in establishing our circadian system are taken or adapted from the fly. This review will try to give a comparative overview about the two systems, highlighting similarities as well as specifics of both insect and murine clocks. © 2003 Wiley Periodicals, Inc. J Neurobiol 54: 111–147, 2003

***Keywords:* *Drosophila melanogaster*; circadian time deeping; genetic analysis**

INTRODUCTION

In many ways, circadian clocks are a powerful example to exemplify the link between genes and behavior. Moreover, they reveal that the environment influences gene expression, demonstrating that the interplay between genetic and external factors determine the overt behavior of an organism.

Circadian clocks are inherent to many organisms, ranging from bacteria to humans, and probably evolved as a consequence of their endless exposition to the daily light and dark cycles caused by the earth's rotation around its own axis. Although one could assume that a given organism merely reacts to the daily changes between day and night (e.g., by starting to look for food after nightfall, in case you are a nocturnal animal, or by starting to sing after sunrise, if you are a songbird) this is not what nature invented. Instead, endogenous timekeepers evolved, able to drive rhythmic behaviors with ca. 24-h periodicities (hence: circadian rhythms) in the absence of any rhythmic environmental fluctuations (e.g., DeMairan, 1729). These timekeepers can be synchronized to match the environmental cycles, a feature used to optimize the timing of the daily occurrence of a certain behavioral or physiologic process in a given species. Considering the broad distribution of circadian clocks among the organisms living on our planet, this adaptive value must be high, and it has been demonstrated that clocks indeed positively influence fitness (e.g., Ouyang et al., 1998; Beaver et al., 2002).

A genetic basis for circadian clocks was first demonstrated for plants by Bünning (1935) and for insects by Pittendrigh (1967). The studies involved the selection and breeding of individuals based on the length of their free-running period, or the timing of their rhythms with respect to an external light–dark (LD) cycle. In both cases the temporal characteristics were transmitted to the next generations, indicating a genetic component of circadian timekeeping. Systematic genetic screens aimed to identify "clock genes," were first conducted in fruit flies (*Drosophila melanogaster*; Konopka and Benzer, 1971), algae (*Chlamydomonas reinhardtii*; Bruce, 1972), fungi (*Neurospora crassa*; Feldman and Hoyle, 1973), and much later, in mammals (mouse; Vitaterna et al., 1994).

The immediate success of isolating genetic rhythm variants in *Drosophila* (Konopka and Benzer, 1971)

Correspondence to: R. Stanewsky (ralf.stanewsky@biologie.uni-regensburg.de).

Contract grant sponsor: the Deutsche Forschungsgemeinschaft.

© 2003 Wiley Periodicals, Inc.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/neu.10164

prompted numerous additional genetic as well as molecular approaches aimed to isolate clock factors. In total, they led the way to uncover the myth once connected with circadian timekeeping, a—still ongoing—process by some researchers referred to as “Clockwork Explosion” (Reppert, 1998). The core of circadian clocks consists of molecular feedback loops, where transcriptional activation of clock genes is inhibited by proteins that are encoded by the same genes, resulting in rhythmic gene expression. In *Drosophila*, by now seven genes—“the Magnificent Seven” —which contribute to central clock function are known: *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), *cycle* (*cyc*), *vrille* (*vri*), *double-time* (*dbt*), and *shaggy* (*sgg*). Details of their characteristics and interactions will be outlined below, but here the principles of molecular timekeeping will be briefly summarized. The proteins encoded by the *Clk* and *cyc* loci (CLK and CYC, respectively) belong to the family of bHLH-PAS transcription factors (Crews and Fan, 1999). A CLK–CYC heterodimer binds to specific sequences within the promoters of the *per*, *tim,* and *vri* genes to activate their transcription (Fig. 1). Cytoplasmic accumulation and nuclear translocation of the PERIOD (PER) and TIMELESS (TIM) proteins are actively delayed by the function of the *dbt* and *sgg* encoded protein kinases (DBT and SGG), respectively. As a result, CLK and CYC can continue to activate transcription of the *per* and *tim* genes, while the PER and TIM repressor proteins accumulate in the cytoplasm. After nuclear entry of PER and TIM, transcription is inhibited by direct binding of the PER–TIM heterodimer to the CLK–CYC dimer (Fig. 1). This shutoff lasts until the PER and TIM proteins are degraded (again involving the function of the above-mentioned kinase activities) allowing a new round of transcription to occur. This oscillatory loop is enforced by a second one, in which an as-yet unidentified transcriptional activator of *Clk* is rhythmically repressed by the product of a gene that is coregulated with *per* and *tim*, probably the *vri* encoded basic-Zipper (bZip) transcription factor (Blau and Young 1999; Fig. 1).

In mammals, the basic principle and even some of the genes involved are the same. In a crucial clock structure of the mouse brain, the suprachiasmatic nuclei (SCN), at least eight genes contribute to clock function: *mPer1, mPer2, mPer3,* the mouse *cryptochrome* genes *mCry1* and *mCry2*, *mClk, Bmal1* (or *Mop3* which is the homolog of fly *cyc*), as well as the gene encoding mammalian homolog of the DBT kinase CKIϵ. So far, the true ortholog of *tim* has not been identified in mammals. The gene referred to as *mTim* in the literature shows highest homology to the *Drosophila* gene *timeout* (or *tim2*) to which no function has been assigned (reviewed in Reppert and Weaver 2001). Instead, the mCRY proteins seem to fulfill the repressor function of fly TIM: as in *Drosophila, mClk* and *Bmal1* activate transcription from the *mPer* promoters, but in contrast to the fly also from the *mCry* promoters (Fig. 1). CKIϵ function likely destabilizes mPER proteins, thereby delaying the accumulation and probably also nuclear localization of the repressor proteins. CKIϵ function seems to be augmented by that of the related kinase CKIδ and other, yet unknown protein kinases (Lee et al., 2001; Reppert and Weaver 2001). After nuclear entry of a mCRY–mPER–CKIϵ–CKIδ protein complex, transcription is blocked by direct binding of this complex to mCLK–BMAL1 heterodimers, most likely mediated by direct interactions between mCLK and mCRY (Lee et al., 2001; Fig. 1). As in flies, a second enhancing feedback loop exists, in which mPER2 positively influences *Bmal1* transcription by an unknown mechanism (Fig. 1). There is also a *vri* homolog in mammals: like VRI, the murine E4BP4 protein is a basic leucine zipper transcription factor. Other, positively acting transcription factors of this class contain also a PAR activation domain, which is missing from both VRI and E4BP4 (Mitsui et al., 2001). In fact, E4BP4 seems to be part of yet another transcriptional feedback loop involved at least in *mPer1* transcriptional regulation. The rhythmically expressed PAR transcription factor DBP (Albumin D-element-binding protein) activates *mPer1*, whereas E4BP4 (also rhythmically expressed but with the opposite phase) competes for the DBP binding site to inhibit *mPer* transcription (Fig. 2).

In the following I will try to give a more detailed view of the clock mechanisms in flies versus the murine system, focussing on those studies that involved genetic strategies. Because the little fly, once again, set the stage for similar breakthroughs concerning the mammalian timekeeping system, parallels and differences between the two systems will be highlighted.

CLOCK- AND CLOCK-RELATED GENES IDENTIFIED BY GENETIC APPROACHES

Rhythm Mutations Affecting the Circadian System of *Drosophila*

The initial genetic screens were based on a rhythm displayed by *Drosophila* populations: the emergence

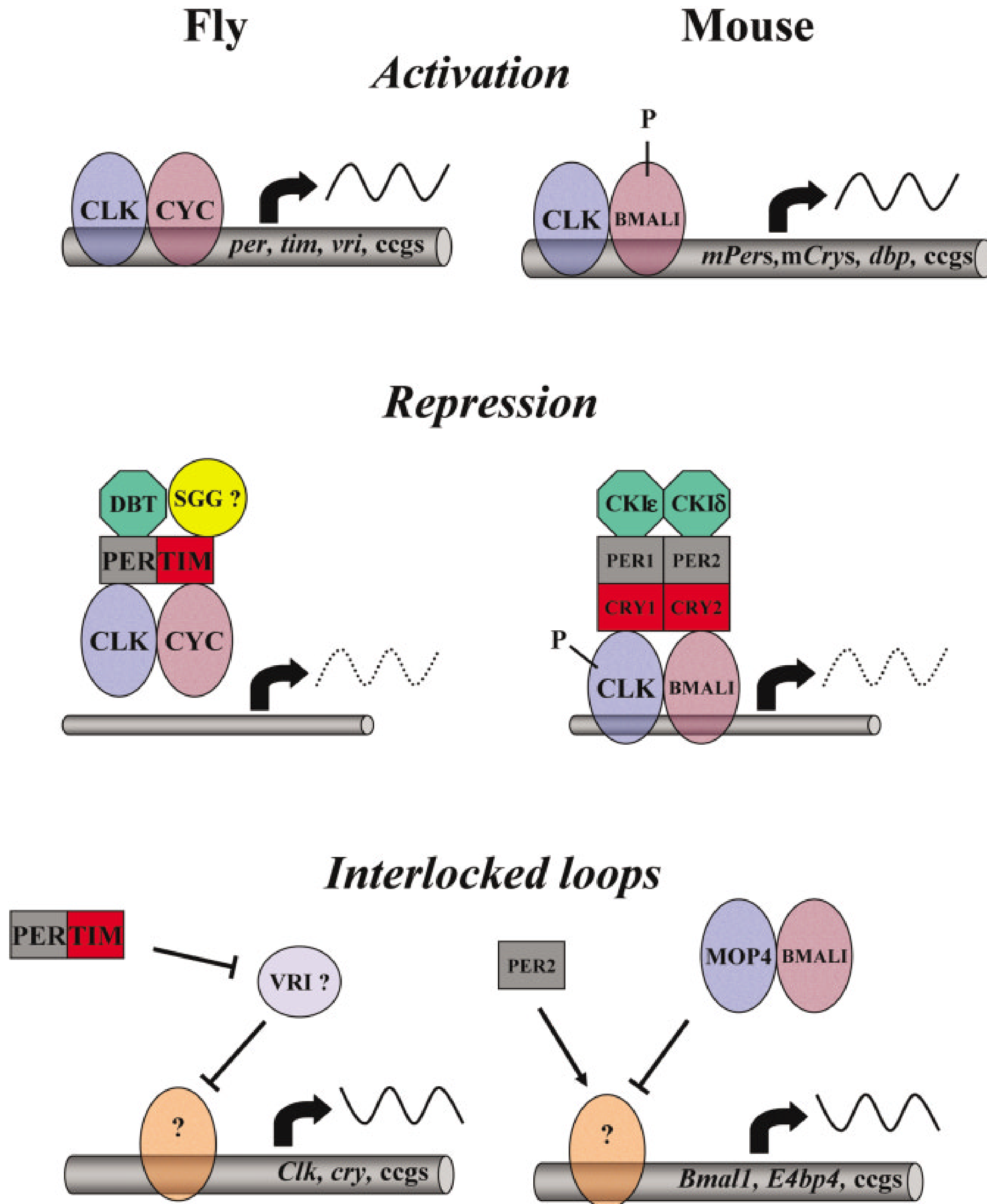

Figure 1 Comparative view of the clock protein interactions in flies and mammals. The simplified models are inferred from *in vitro* and *in vivo* experiments discussed in the text. Clock genes under regulation are represented as gray tubes. For abbreviations of gene and protein names see text. Active transcription is indicated by solid waved lines, repression by dotted such lines. *P*s indicate the phosphorylation status of CLK and BMAL1, which seems relevant for transcriptional activation or repression (see text). For the *Interlocked loops* activating and repressing factors are shown together. Straight arrows indicate positive influence on transcription, blunt ends repression. Note that the negative influence of the NPAS2/MOP4–BMAL1 dimer on *Bmal1* expression was not shown for the CLK–BMAL1 dimer. (?): nuclear SGG function and repressor activity of VRI are hypothetical; also, the factor activating clock genes and ccgs not directly regulated by the CLK–CYC/BMAL1 dimer is unknown.

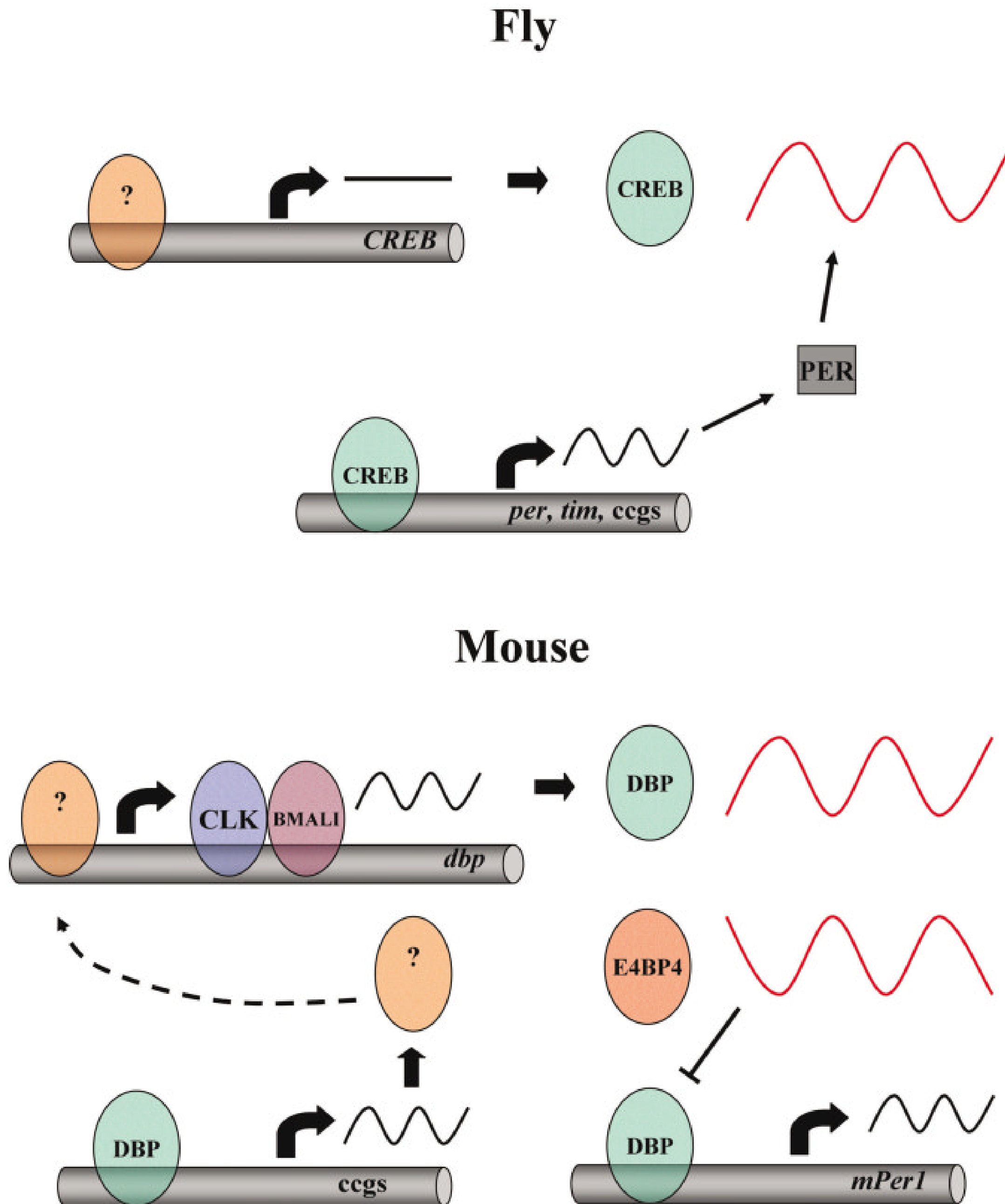

Figure 2 Two bZip-transcription factors regulating pacemaker- and output-genes. Fly *CREB* gene expression is not circadianly regulated, yet the CREB protein is rhythmically active in a *per*-dependent manner. CREB likely binds to CRE elements in the promoters of *per*, *tim*, and ccgs, contributing to their rhythmic expression. Among other (unknown) factors, mouse *dbp* gene expression is activated by CLK (and probably BMAL1) binding to intronic E-box sequences. Rhythmically expressed DBP activates *mPer1*, by binding to DBP recognition sequences within the *mPer1* promoter. The same sequences are competitively bound by the E4BP4 repressor, which is expressed in an opposite phase compared to DBP. DBP also regulates several ccgs in liver. Some of these gene products could theoretically feed back on the *dbp* promoter, thereby indirectly regulating *mPer1* expression. Red waved lines indicate rhythmic DBP and E4BP4 expression, respectively. Note that in addition to CREB and DBP, clock-gene expression is also regulated by the CLK–CYC/BMAL1 transcription factors (Fig. 1). Other symbols, see Fig. 1. For details see text.

of adult flies from the pupal case referred to as "eclosion." Wild-type populations of fruit flies show eclosion peaks that are separated by ca. 24 h in constant darkness (DD). Konopka treated flies with a chemical mutagen, generated lines with a single mutagenized chromosome, and tested them for their periodic eclosion pattern. He found two different variants with free-running periods of 19 h and 29 h, respectively, and one showing no rhythmicity at all. All three mutations mapped to the same locus on the *X*-chromosome and were dubbed $period^{Short}$ (per^{S}), per^{Long} (per^{L}), and per^{01}, respectively (Konopka and Benzer, 1971). When the rest–activity pattern of individual flies from those strains were tested in DD, the same period alterations were effected by the various *per* alleles (Konopka and Benzer, 1971). The fact that different clock outputs were influenced by the same gene indicated that *per* is an integral part of the central clock, and the success of Konopka's screen prompted the search for other such factors with similar strategies (Table 1).

The initial "eclosion screens" were later replaced by "locomotor screens," with the crucial advantage that large numbers of individual flies could be tested for impaired behavioral activity rhythms (e.g., Allada et al., 1998). Because a clock output is measured in both eclosion and locomotor screens, it should be possible to isolate not only mutations in clock genes, but also those specifically affecting only one of the two biological rhythms. In fact, several mutations specifically affecting eclosion rhythms have been isolated (Jackson, 1983; Newby and Jackson, 1993; Table 1).

Many clock-gene mutations are semidominant in that one copy of a period-altering allele combined with a normal allele produces periodicities intermediate between the homozygous mutant and the wild-type case. This feature allowed to screen for novel clock mutations in so-called "F1" screens, where flies carrying only one copy of a mutagenized chromosome are phenotypically analyzed for rhythm defects. The benefits of such a strategy are plentyfold: (1) the throughput of mutagenized lines is much higher because no homozygous stocks need to be generated. (2) During chemical mutagenesis often several hits are induced on a particular chromosome. If one of them occurs in a vital gene, no homozygous flies (with a potential hit in a rhythm-related gene) could be analyzed. (3) The mutated clock gene itself could be essential for the survival of the fly, again militating against the analysis of homozygous mutant animals. Conducting such F1 screens resulted in isolation of several novel period-altering *timeless* and *double-time* mutations; the latter gene indeed turned out to be essential for the survival of the fly (Kloss et al., 1998; Rothenfluh et al., 2000a, 2000b, 2000c; Table 1).

Other forward genetic screens were performed using transposon-mobilization as mutagen. Here, one hopes that the transposable element in question (usually a *P*-element) integrates close to or within a rhythm-related gene, thereby negatively impinging on gene function. Although several clock-relevant mutations have been isolated this way (the most prominent being the original *timeless* mutation tim^{01}) transposon mutagenesis was clearly less efficient for isolating new rhythm-variants compared to chemical treatments in that ca. 10 times more lines needed to be analyzed to find a new mutation (Table 1). In addition, it turned out that even in the case of tim^{01} the *P*-element insertion in this strain was not related to the induced mutation (Sehgal et al., 1994). A different, also *P*-element–based screen was more efficient compared to the previous transposon screens: Martinek et al. (2001) used an expression system, where a large number of fly genes can be driven in a desired cell type of the organism. They made use of a "library" of 2300 so-called "EP" lines, a collection of *P*-element insertion lines carrying UAS-repeats on their transposon (Rørth, 1996; Rørth et al., 1998). This allows activation of the genes close to the insertion site, if the fly line is crossed to another *P*-element line expressing the yeast GAL4 transcription factor, whose targets are the UAS repeats (Brand and Perrimon 1993). Martinek et al. (2001) crossed the EP collection to a "driver-line" where GAL4 overexpression was controlled by the *timeless* promoter so that genes next to the EP insertions were over- or misexpressed in all clock gene expressing cells. The resulting double transgenics were subsequently tested behaviorally, and two arrhythmic, one short period, and four long period strains could be isolated. One of these strains was analyzed in more detail and helped to establish a circadian role for the protein kinase encoded by *shaggy*.

Yet a different strategy involves both chemical mutagenesis and transposable elements. Here, a fly line stably transformed with a *period-luciferase* reporter gene was chemically mutagenized. Normally, such transgenic luciferase flies exhibit bioluminescence oscillations, nicely reflecting those of endogenous *per* mRNA (Stanewsky et al., 1997). From the progeny of the mutagen-treated *per-luc* flies 5137 strains with mutagenized second or third chromosomes were screened for altered luminescence rhythms (Stanewsky et al., 1998). Two mutations—both of them causing arrhythmic expression of the

Table 1 Mutations in Known Genes of *Drosophila* Identified by Forward Genetic Screening for Rhythm Abnormalities

Locus	Allele	Type of Screen	Mutagen	Molecular Lesion	Phenotype	Reference
period						
PAS protein	per^{01} (amorph)	eclosion	EMS	nonsense mutation in exon 4	eclosion and behavior AR	Konopka and Benzer 1971; Baylies et al., 1987; Yu et al., 1987
	per^{04} (strong hypomorph)	locomotor	EMS	faulty splice-acceptor site caused by a single base pair substitution	7–23% AR when tested over a *per* deletion. Rhythmic flies exhibit either ultradian or extremely long periods (>30 h). LD behavior almost normal. No PER protein detectable	Hamblen-Coyle et al., 1989; Hamblen et al., 1998
	per^{S} (*Short*) (hypomorph)	eclosion	EMS	missense mutation ($Ser^{589} \rightarrow Asn$)	19-h rhythms of eclosion and behavior	Konopka and Benzer 1971; Baylies et al., 1987; Yu et al., 1987
	per^{L} (*Long*) (hypomorph)	eclosion	EMS	missense mutation ($Val^{243} \rightarrow Asp$)	29-h rhythms of eclosion and behavior	Konopka and Benzer 1971; Baylies et al., 1987
	per^{Clk} (*Clock*) (hypomorph)	locomotor	EMS	missense mutation ($Ala^{969} \rightarrow Val$)	22.5-h rhythms of eclosion and behavior	Dushay et al., 1992; Hamblen et al., 1998
	per^{T} (hypomorph)	locomotor	EMS	missense mutation ($Gly^{593} \rightarrow Asp$)	17-h rhythms of eclosion and behavior	Konopka et al., 1994; Hamblen et al., 1998
	per^{SLIH} (*SomeLikeItHot*) (hypomorph)	locomotor	spontaneous	missense mutation ($Ser^{45} \rightarrow Tyr$)	28-h or 27-h rhythms of eclosion and behavior, respectively. Period becomes shorter at higher temperatures	Hamblen et al., 1998
timeless						
PER interacting protein	tim^{01} (amorph)	eclosion	*P*-element	internal deletion	eclosion and behavior AR	Sehgal et al., 1994
	tim^{03} (amorph)	gene-expression	EMS	n.d.	behavior AR, no TIM protein detectable	Stempfl et al., 2002
	tim^{04} (amorph)	gene-expression	EMS	n.d.	behavior AR, no TIM protein detectable	Stanewsky and Hall (unpublished)
	tim^{SL} (*Suppressor of* per^{L})	locomotor	EMS	missense mutation ($Thr^{494} \rightarrow Ile$)	behavior normal. Isolated because it decreases period-length of eclosion and behavioral rhythms of per^{L}- to 24.5 and 25.5 h, respectively	Rutila et al., 1996
	tim^{S1} (hypomorph)	locomotor (F1)	EMS	n.d.	21-h behavioral rhythms	Rothenfluh et al., 2000a
	tim^{S2} (hypomorph)	locomotor (F1)	EMS	n.d.	22-h behavioral rhythms	Rothenfluh et al., 2000a
	tim^{L1} (hypomorph)	locomotor	EMS	missense mutation ($Lys^{586} \rightarrow Met$)	28-h eclosion and behavioral rhythms	Rothenfluh et al., 2000a

	tim^{L2} (hypomorph)	locomotor (FI)	EMS	missense mutation (Phe^{799} → Ile)	26-h eclosion and behavioral rhythms	Rothenfluh et al., 2000a
	tim^{L3} (hypomorph)	locomotor (F1)	EMS	n.d.	27.5-h behavioral rhythms when tested over *tim*^{01}	Rothenfluh et al., 2000a
	tim^{L4} (hypomorph)	locomotor (F1)	EMS	n.d.	28-h behavioral rhythms	Rothenfluh et al., 2000a
	tim^{UL} (*UltraLong*) (hypomorph)	locomotor (F1)	EMS	missense mutation (Glu^{260} → Lys)	33-h behavioral rhythms	Rothenfluh et al., 2000c
	tim^{rit} (*ritsu* = jap. for rhythm) (hypomorph)	locomotor	spontaneous	missense mutation (Pro^{1093} → Ala)	27-h behavioral rhythms	Murata et al., 1995; Matsumoto et al., 1999
double-time						
Casein kinase Iε homolog.	*dbt*^{S} (hypomorph)	locomotor (F1)	EMS	missense mutation (Pro^{47} → Ser)	20-h and 18-h eclosion and behavioral rhythms, respectively	Kloss et al., 1998; Price et al., 1998
	dbt^{L} (hypomorph)	locomotor (F1)	EMS	missense mutation (Met^{80} → Ile)	27-h eclosion and behavioral rhythms, respectively	Kloss et al., 1998; Price et al., 1998
	dbt^{P}-(strong hypomorph, homozygous lethal)		*P*-element	*P*-element insertion in intron 2 disrupting gene function	behavior rhythms normal over a wild-type copy of *dbt.* High accumulation of PER in homozygous *dbt*^{P}-larvae	Kloss et al., 1998; Price et al., 1998
	dbt^{ar} (strong hypomorph, homozygous viable)	locomotor	EMS	missense mutation (His^{126} → Tyr)	behavior AR. Combination with *per*^{S} or *per*^{T} results in extremely long, but robust behavioral rhythmicity	Rothenfluh et al., 2000b
	Dbt^{g} (strong hypomorph)	locomotor	EMS	missense mutation (Arg^{127} → His)	Behavior AR when combined with *dbt* deletion or *dbt*^{P}	Suri et al., 2000
	dbt^{h} (hypomorph)	locomotor	EMS	missense mutation (Thr^{44} → Ile)	29-h behavioral rhythms	Suri et al., 2000
Clock						
bHLH-PAS protein	*Clk*^{Jrk} (dominant negative)	locomotor	EMS	nonsense mutation at Glu^{776}	behavior and eclosion AR. Ca. 50% of *Clk*^{Jrk}/+ are behaviorally AR (the truncated protein misses the transcriptional activation domain, but not the DNA binding one)	Allada et al., 1998
	Clk^{ar} (recessive)	locomotor	EMS	missense mutation destroying a splicing site	behavior AR	Allada and Rosbash (unpublished)
cycle						
bHLH-PAS protein	*cyc*^{01}	locomotor	EMS	missense mutation (nonsense mutation at Lys^{159}	eclosion and behavior AR	Rutila et al., 1998
	cyc^{02}	locomotor	EMS	nonsense mutation at Gln^{113}	behavior AR	Park et al., 2000

Table 1 (*Continued*)

Locus	Allele	Type of Screen	Mutagen	Molecular Lesion	Phenotype	Reference
shaggy						
glycogen synthase kinase-3 ortholog	EP(X)1576	locomotor; misexpression	*P*-element	*P*-insertion upstream of the *sgg zw3-C* transcript	20.5-h behavioral rhythms when crossed to *tim*-GAL4 (resulting in overexpression of *zw3-C* in all *tim* expressing clock-cells, see text)	Martinek et al., 2001
	sgg^{M11} (amorph, homozygous lethal)	locomotor[a]	spontaneous	n.d.	25.5-h behavioral rhythms when rescued to adult survival by a *heat shock-sgg* transgene	Martinek et al., 2001
	sgg^{D127} (amorph, homozygous lethal)	locomotor[a]	n.d.	n.d.	26-h behavioral rhythms when rescued to adult survival by a *heat shock-sgg* transgene	Martinek et al., 2001
cryptochrome						
Flavoprotein with homology to photolyase	cry^b (*baby*) (strong hypomorph or amorph)	gene expression	EMS	missense mutation ($Asp^{410} \rightarrow Asn$)	abolishes clock-gene cyclings in peripheral clocks. Behavioral response to light-pulses impaired	Stanewsky et al., 1998
lark						
RNA-binding protein	$lark^a$ (amorph, homozygous lethal)	eclosion	*P*-element	*P*-insertion in exon 1	early eclosion in LD and Temperature cycles	Newby and Jackson, 1993
dusky						
no homologies	$dy^{Andante}$	eclosion	EMS	n.d.	26-h eclosion and behavioral rhythms	Konopka et al., 1991
	dy^{n1}	locomotor[b]	γ-ray	n.d.	25–26-h behavioral rhythms	Newby et al., 1991; van Swinderen and Hall, 1995
	dy^{n3}	locomotor[b]	γ-ray	n.d.	26-h behavioral rhythms	Newby et al., 1991; van Swinderen and Hall, 1995
	dy^{n4}	locomotor[b]	γ-ray	n.d.	25–26-h behavioral rhythms	Newby et al., 1991; van Swinderen and Hall, 1995

Overview of the nine loci, where mutations have been induced by forward genetic screens for rhythms variants. Most mutations have been isolated in screens for altered eclosion or behavioral rhythms. A different screening method (gene expression) relied on alterations of *luciferase* reported *period* RNA rhythms. Full allele designations are given only once, in case they are used for mutations of different genes (e.g., *S* for *Short*). Semidominance of a mutation is usually indicated by a capital letter of the allele designator (e.g., per^L), strongly dominant or dominant-negative alleles by upper designation of the gene name (e.g., Clk^{Jrk}). Note that all of the period-altering alleles of the five major clock genes—listed at the beginning of the table—are semidominant, even if not indicated by the nomenclature (e.g., dbt^h). The phenotype is specified for the hemizygous or homozygous mutant situation, unless started otherwise.

[a] These two *sgg* alleles originate from screens designed to identify developmental mutants. They were analyzed for their effects on locomotor rhythms after a circadian role for *sgg* had been inferred from overexpressing EP(X)1576.

[b] Mutations at the *dy* locus cause a wing defect in addition to the rhythm phenotype. Only the $dy^{Andante}$ allele was isolated after directly screening for an altered circadian rhythm. The other *dy* alleles were found after screening for the wing defect. Not all *dy* mutations showing the wing defect also show a rhythm phenotype. Rhythm variants isolated in forward genetic screens for which the nature of the mutated gene is unknown ($n = 13$) are not listed. AR = arrhythmic.

per-luc gene—turned out to be novel loss-of-function *tim* alleles (tim^{03} and tim^{04}: Stempfl et al., 2002; Stanewsky and Hall, unpublished). Additionally, a mutation of a dedicated circadian photoreceptor encoded by the fly *cryptochrome* gene was induced in that screen, also eliminating the usual luminescence oscillations (Stanewsky et al., 1998). Eleven other lines, with more subtle, yet reproducible effects on *per-luc* rhythms, still await a more detailed characterization and complementation analysis with other known clock mutations (Hall and Stanewsky, unpublished). It should be noted that the strategy just described is inherently different from the ones previously described. Whereas in the classical approaches different fly output behaviors serve as screening phenotype, the *per-luc* screen involves measuring of rhythmic clock-gene activity of a central oscillator component. Therefore, this strategy is aimed to isolate central clock factors, as well as input components (in case the latter are mutated in a way that abolishes synchronization among the many clock-gene expressing tissues in the fly: cf. Plautz et al., 1997).

In addition to forward genetic screens, naturally occurring rhythm variants have been isolated (e.g., the period-lengthening tim^{ritsu} mutation, Murata et al. 1995; Matsumoto et al., 1999). Those cases and all clock mutations that resulted from forward genetic screens are listed in Table 1. In addition, a number of other rhythm mutations exist (Table 2). They include cases where the circadian function of a particular gene was inferred from molecular findings (e.g., a gene was found to be rhythmically expressed, and previously isolated mutant forms of this gene were then inspected for rhythm phenotypes), and those that were expected to play a role in the rhythm system based on their spatial expression pattern (e.g., in case it overlaps with that of a known clock gene). Moreover, mutations affecting specific signal transduction pathways and second-messenger systems were probed for a potential role in the circadian system and are also listed in Table 2, in case such a function was revealed.

Rhythm Mutations Affecting the Mammalian Circadian System

The only mammalian rhythm mutation that emerged from a forward genetic screen is *mClock (Clk)* (Vitaterna et al., 1994; Table 3). In fact, this mutation and the gene linked to it were found and described (Antoch et al., 1997; King et al., 1997) before the corresponding *Drosophila* locus was mutated and cloned (Allada et al., 1998, Darlington et al., 1998, see above). Homozygous *Clk* mice exhibit lengthened (28 h) free-running behavioral rhythms that eventually deteriorate to arrhythmicity (Table 3). Consistent with this behavioral arrhythmicity, *mPer1*, *mPer2, mCry1*, *mCry2*, and *Bmal1* RNA expression in *Clk* mutant mice is blunted and arrhythmic (Jin et al., 1999, Kume et al., 1999; Shearman et al., 2000b).

Moreover, several spontaneous mammalian rhythm mutations are known, the most prominent being *tau* (Ralph and Menaker, 1988; Table 3). Homozygous *tau* mutant hamsters have short free-running locomotor rhythms (20 h), and they were used to establish crucial characteristics of the mammalian timing system (Young, 2000). Cloning of the *tau* gene revealed that it encodes the mammalian *dbt* homolog Casein kinase Iϵ (CKIϵ), and although able to bind to mPER, the mutant protein has a reduced capacity to phosphorylate this clock protein *in vitro*, suggesting a similar role for both proteins in flies and mammals (Lowrey et al., 2000). Other spontaneous mutations include three human variants: Familial Advanced Sleep Phase Syndrome (FASPS) is associated with a mutation of human *Per2* (Toh et al., 2001; Table 3). FASPS patients possess a fast running clock (23-h free-running period), leading to a daily 4-h advance of awakening (0430 h) and sleep onset (1930 h), which makes it difficult for them to adjust their activities to those of healthy people (Jones et al., 1999). The mutation occurred at a CKIϵ phosphorylation site, again indicating the crucial function of this enzyme in establishing circadian period (Toh et al., 2001). The second one involves human *Per3*. Analyzing this gene for polymorphisms revealed a significant association of a certain haplotype with Delayed Sleep Phase Syndrome (DSPS), interestingly also in a region of the hPER3 protein that could involve phosphorylation by CKIϵ (Ebisawa et al., 2001; Table 3). But the connection of this *hPer3* polymorphism to DSPS was not very tight: 85% of the DSPS patients under study (48) did not have the particular polymorphism, suggesting that additional genetic factors contribute to the susceptibility to develop DSPS (Ebisawa et al., 2001). A similar poor correlation was found between a single nucleotide polymorphism in the 3′-untranslated region of *hClock* and delayed morningness-eveningness tendencies of activity or sleep onset, respectively (Katzenberg, et al., 1998; Table 3).

All other mutations in rhythm related genes were induced by reverse genetics involving targeted gene-knockouts in mice (Table 3). In summary, these studies revealed that the *mPer* genes have distinct functions within the murine circadian system. Two independently generated *mPer1* mutants show subtle shortenings of free-running behavioral periods (Cer-

Table 2 ***Drosophila*** **Rhythm Genes and Their Mutant Versions Isolated on other Criteria**

Gene	Criteria for Analysis	Allele	Mutagen	Molecular Lesion	Phenotype	Reference
vrille bZIP transcription factor	*ccg*					
		vri[1] (antimorph; homozygous lethal)	EMS	nonsense mutation upstream of bZIP domain	22.9-h behavioral rhythms when tested over wild type	Blau and Young, 1999
		vri[5] (antimorph; homozygous lethal)	*P*-element	*P*-insertion within intron 1	22.7-h behavioral rhythms when tested over wild type	Blau and Young, 1999
		V1	*P*-element (UAS-*vri*)	*vri* overexpression	25.5-h behavioral rhythms after overexpression of UAS-*V1* using *tim*-GAL4	Blau and Young, 1999
		V2	*P*-element (UAS-*vri*)	*vri* overexpression (stronger compared to *V1*)	27.8-h behavioral rhythms (induced as for *V1*). Ca. 80% of *tim*-GAL4/UAS-*V2* behavioral AR	Blau and Young, 1999
		V3	*P*-element (UAS-*vri*)	*vri* overexpression (stronger compared to *V2*)	28-h behavioral rhythms (induced as for *V1*). Ca. 90% of *tim*-GAL4/UAS-*V3* behavioral AR	Blau and Young, 1999
takeout ligand binding protein	*ccg*	*ry*[506]	X-ray[a]	partial deletion of to 3′ UTR	aberrant behavior under starvation conditions	Sarov-Blat et al., 2000
numb phosphotyrosine binding protein	*ccg*					
		numb[nuts] (amorph; homozygous lethal)	*P*-element	*P*-insertion in the 5′ regulatory region of *numb*	24.5-h behavioral rhythms when tested over wild type	Stempfl et al., 2002
		numb[1] (amorph; homozygous lethal)	*P*-element	*P*-insertion in exon 2	24.5-h behavioral rhythms when tested over wild type	Stempfl et al., 2002; Wülbeck and Stanewsky, unpublished
		numb[SW] (hypomorph)	*P*-element excision	imprecise excision of *numb*[1] associated *P*-insertion	25-h behavioral rhythms	Stempfl et al., 2002; Wülbeck and Stanewsky, unpublished
		numb[3] (amorph; homozygous lethal)	DEB[c]	n.d.	24.5-h behavioral rhythms when tested over wild type	Stempfl et al., 2002

Gene / product	Notes	Allele	Mutagen	Molecular lesion	Phenotype	Reference
pigment dispersing factor neuropeptide	spatial expression partially overlaps with *per*					
		pdf^{01} (amorph)	spontaneous	nonsense mutation at Tyr^{21} of the precursor peptide	23-h behavioral rhythms. Progressive loss of overall rhythmicity	Renn et al., 1999
dCREB2 bZIP transcription factor	suggested function of *CREB* in the mammalian circadian system					
		dCREB2^{S162}	EMS	n.d.	38% behaviorally AR. Rhythmic flies have 22.8-h period	Belvin et al., 1999
dunce cAMP phosphodieste rase	suggested circadian role of cAMP signaling in other organisms					
		dnc^{1} (hypomorph)	EMS	n.d.	23-h behavioral rhythms, increased phase delays after light pulses	Levine et al., 1994
		dnc^{ML} (amorph)	EMS	n.d.	see *dnc*^{1}	Levine et al., 1994
Pka-C1 (a.k.a. *DC0*) catalytic submit of PKA	suggested circadian role of cAMP signaling in other organisms					
		Pka-C1^{581} (hypomorph, homozygous lethal)[b]	*P*-element	*P*-insertion in exon 1	see *Pka-C1*^{B10}	Levine et al., 1994
		Pka-C1^{B10} (hypomorph, homozygous lethal)[b]	EMS	missense mutation (Asn^{219} → Ile)	*Pka-C1*^{B10}/*Pka-C1*^{581} 75–80% behaviorally AR. Poor synchronization to LD cycles. Eclosion normal	Levine et al., 1994; Majercak et al., 1997

Table 2 (*Continued*)

Gene	Criteria for Analysis	Allele	Mutagen	Molecular Lesion	Phenotype	Reference
		Pka-C1^{T4} (cold sensitive, homozygous lethal)[b]	n.d.	n.d.	see *Pka-C1*^{B3}	Levine et al., 1994
		Pka-C1^{B3} (amorph, n.d. homozygous lethal)[b]	n.d.	nonsense mutation at Trp^{299}	*Pka-C1*^{T4}/*Pka-C1*^{B3} 65% behaviorally AR. Poor synchronization to LD cycles	Levine et al., 1994
		Pka-C1^{X4} (cold sensitive)	n.d.	n.d.	80% behaviorally AR. Eclosion normal	Majercak et al., 1997
Pka-R2 regulatory subunit of PKA	see *Pka-C1*					
		Pka-R2^{EP2162}	*P*-element	*P*-insertion 89-bp upstream of transcription start	ca. 60% behaviorally AR. Rhythmic flies have normal period	Park et al., 2000b
Neurofibromatosis-1 GTPase activating protein	suggested circadian role of cAMP/PKA signaling in flies					
		Df(3R)Nfl^{P1} (amorph)	*P*-element excision	15 kb deletion removing *Nf-1* and at least two *E(spl)* transcripts	behavior AR	Williams et al., 2001; The et al., 1997
		Nf1^{P2} (amorph)	*P*-element	*P*-insertion in intron 1	behavior AR	Williams et al., 2001; The et al., 1997

Overview of the nine loci (listed along with the nature of the encoded protein/peptide in the first column), where previously isolated mutations were analyzed for behavioral phenotypes. The rationale for analyzing circadian behaviors of these mutants is given in the second column. *ccg*: *clock regulated gene;* meaning it was found in a screen for (clock-dependent) rhythmically expressed RNAs. All listed mutations are homozygous viable and were analyzed behaviorally as homozygotes, unless specified otherwise.

[a] *to* is unrelated to *ry,* yet maps to the same chromosome. Therefore the *to* deletion was probably coinduced with the *ry*^{506} mutation.

[b] Although homozygous lethal, some of these *Pka-C1* alleles produce viable flies in transheterozygous condition that was analyzed behaviorally.

[c] Diepoxybutane. AR = arrhythmic.

makian et al., 2001; Zheng et al., 2001). In addition, mice of one variant frequently exhibit a total loss of rhythmicity after 10–14 days in DD (Bae et al., 2001). Interestingly, rhythmic transcription of the *mPer1, mPer2, mCry1,* and *Bmal1* genes is not disrupted in *mPer1* mutants, indicating that *mPer1* is dispensable for transcriptional feedback regulation (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). Instead, it turned out that the mPER1 protein is contributing to posttranscriptional regulation of at least the mPER2 and mCRY1 proteins, because their expression rhythms are blunted in *mPer1* mutants (Bae et al., 2001; Zheng et al., 2001).

For *mPer2*, two mutant mouse lines were generated (Zheng et al., 1999; Bae et al., 2001; Table 3). Both show more severe behavioral defects compared to the *mPer1* mutants, in that they exhibit arrhythmic behavior after free running with short periods for variable amounts of time (Bae et al., 2001; Zheng et al., 1999, 2001). Consistent with the behavioral phenotype, consequences for the expression of other clock genes are also more drastic: *mPer1, mPer2, mCry1*, and *Bmal1* RNA levels and rhythms are severely reduced or blunted, respectively, indicating a positive role of mPER2 in the transcriptional regulation of these clock genes (Zheng et al., 1999, 2001; Shearman et al., 2000b; Bae et al., 2001). In contrast, rhythms of *mPer3* and levels of *Clock* RNA are not affected by *mPer2* mutants (Zheng et al., 1999).

Disruption of *mPer3* had the mildest effects on behavioral rhythms: the free-running period of robustly rhythmic *mPer3* mutant mice is only 0.5 h shorter compared to wild type, and no changes in the rhythmic RNA expression pattern of the three *mPer*s, *mCry1,* and *Bmal1* could be observed (Shearman et al., 2000a).

None of the *mPer* mutations resulted in total loss of circadian clock function, indicating a partial redundancy of their functions. Given the phenotypical consequences of *mPer1* and *mPer2* mutations, double-mutant animals were generated. Simultaneously abolishing the transcriptional and posttranscriptional functions of *mPer2* and *mPer1* resulted in an immediate loss of behavioral rhythmicity in free-running conditions, demonstrating that these two *per* genes are indeed crucial components of the mammalian clock (Bae et al., 2001; Zheng et al., 2001). Consistent with this, no RNA cycling of either *mPer1* or *mPer2* mRNA could be observed in the double-mutants (Zheng et al., 2001). A potential redundancy for *mPer3* was ruled out by a similar double-mutant analysis: if *mPer3* plays any role in the central circadian clock, eliminating *mPer3* along with *mPer1* or *mPer2* should result in a more severe phenotype compared to that of the single mutants. But the behavioral phenotypes of these double-mutant combinations were indistinguishable from those of the single *mPer1* and *mPer2* mutants (Table 3), suggesting that *mPer3* plays only a minor, if any, role in the central clockworks (Bae et al., 2001).

A similar situation was revealed for the two *mCry* genes: disruption of *mCry2* results in 1-h longer free-running periods, whereas *mCry1* mutants show the opposite period-altering phenotype, and *mCry1/mCry2* double-mutants are completely arrhythmic (Thresher et al. 1998; van der Horst et al., 1999; Vitaterna et al., 1999; Table 3). Moreover, circadian RNA rhythms of *mPer1* and *mPer2* are abolished in the double-mutants and expression occurs at intermediate to high levels, consistent with a role for the mCRYs as negative regulators of clock-gene expression (Vitaterna et al., 1999; Okamura et al., 1999). In addition, *Bmal1* levels are low and noncycling in the *mCry* double knockouts, probably due to the low levels of mPER2 in these mutants, further supporting the hypothesis that mPER2 positively influences *Bmal1* (Shearman et al., 2000b).

THE MOLECULAR CLOCK-WORKS IN FLIES AND MAMMALS

In this article the current knowledge about the molecular details of clock-gene interactions in the two systems will be presented. Because the emerging models are often based on experiments conducted *in vitro*, or on nonpacemaker tissues, the validity of the models will be judged by the behavioral and molecular phenotypes of the various clock mutants described above.

CLK and CYC as Positive Regulators in Flies

The genes *Clk* and *cyc* encode transcription factors containing a PAS protein dimerization domain (for **P**ER-**A**RNT-**S**IM; the founding members of the PAS-protein family) and a basic helix-loop-helix (bHLH) domain involved in DNA binding. Mutations in either gene cause arrhythmic eclosion and locomotor rhythms, demonstrating the importance of their function for the circadian system (Table 1). Expression levels of other clock genes (e.g., *per*, *tim*, and *vri*) is severely reduced and arrhythmic in the face of *Clk* and *cyc* mutants, indicating that CLK and CYC proteins positively influence transcription of other genes (Allada et al., 1998, Rutila et al., 1998; Blau and

Young, 1999). Consistent with these findings it was shown that a CLK–CYC heterodimer is able to bind to E-box sequences—a stretch of six consensus nucleotides that are the target for bHLH transcription factors (reviewed by Kyriacou and Rosato 2000)—in the promoters of *per* and *tim*, thereby activating transcription (Darlington et al., 1998; Lee et al., 1999; Fig. 1). This mode of action can also account for the dominant-negative phenotype of the Clk^{Jrk} mutation (Table 2). This allele hypothetically produces a truncated CLK protein, able to bind DNA and to dimerize with CYC (Allada et al., 1998). But because CLK^{JRK} lacks the transcriptional activation domain the dimer is not active, and competes with functional CLK–CYC complexes.

Although *cyc* is constitutively expressed and the CYC protein highly abundant throughout the day, *Clk* is circadianly regulated resulting in cycling *Clk* RNA and protein levels. Both exhibit peak levels from late in the night until early morning, and trough levels at the end of the day and early evening (Bae et al., 1998; Lee et al., 1998; Rutila et al., 1998; Bae et al., 2000; Fig. 1). Because CLK seems to be the limiting factor for constituting the CLK–CYC dimer, CLK dictates the amount of functional dimers resulting in rhythmic expression of its target clock-genes (Bae et al., 2000). A problem with this view is that *per* and *tim* transcription is initiated in the morning while CLK levels decrease during this time. Thus, it appears that some factors negatively influence CLK–CYC activity while the dimer is present at peak levels.

CLK and BMAL1 (MOP3) as Positive Regulators in Mammals

mClk and *Bmal1* are the homologs of *Clk* and *cyc* and have similar functions (Fig. 1). As in flies, the mouse *Clk* mutation has a dominant-negative effect, with *Clk*/+ animals showing significant period lengthenings and unstable periods including gradual drift into arrhythmicity (Vitaterna et al., 1994; Table 3). Interestingly, the nature of the *Clk* mutation likely affects only the activation domain of CLK, leaving the PAS and DNA binding domain intact (King et al., 1997; Gekakis et al., 1998). As in the case of Clk^{Jrk}, this can nicely account for the dominant feature of the murine mutation, and indicates the importance of the CLK–BMAL1 dimer in activating transcription of the *mPer* and *mCry* genes. Further *in vivo* evidence that *mPer1* rhythms are indeed mediated transcriptionally stems from the analysis of *mPer1*-reporter transgenics. Several studies show that *mPer1* promoter sequences drive robust rhythmic GFP or luciferase expression in SCN slices or live animals, closely reflecting that of endogenous *mPer1* RNA (Kuhlman et al., 2000; Yamazaki et al., 2000; Yamaguchi et al., 2000a, 2001; Wilsbacher et al., 2002). Yet, it remains to be shown if this activation is mediated by binding of the CLK–BMAL1 dimer to E-boxes *in vivo*.

The role of *Bmal1* was convincingly demonstrated after induction of a targeted gene knockout (Table 3). *Bmal1*-deficient mice are the only single mutant animals so far exhibiting complete arrhythmic behavior immediately after transfer to DD (Bunger et al., 2000). Moreover, *mPer1* and *mPer2* expression is flat and occurs at trough levels in the mutant mice, demonstrating the positive function of BMAL1 for *mPer* gene expression (Bunger et al., 2000).

Contrary to flies, *Clk* is expressed constitutively in mice (at least in the SCN and fibroblasts), while *Bmal1* shows rhythmic expression, both on the RNA and protein level in all tissues examined, including the SCN (Shearman et al., 2000b; Tamaru et al., 2000; Lee et al., 2001; Yagita et al., 2001). In liver, both *Clk* and *Bmal1* mRNAs cycle with similar phase as *Clk* RNA in the fly (i.e., high levels late at night until early in the morning), and BMAL1 protein exhibits circadian oscillations in both abundance and phosphorylation status with a similar phase compared to its encoding RNA (Lee et al., 2001). Moreover, BMAL1 seems to be rate limiting for CLK–BMAL1 heterodimer formation as is CLK in flies (Lee et al., 2001). This means that—as in flies—low levels of a transcriptional activator coincide with the time of maximal transcriptional enhancement of *mPer1*, *mPer2*, and *mCry*s. In turn, high levels of the activating proteins are correlated with low levels of target gene expression, suggesting that repression by negative elements of the feedback loop is dominant over activation by positive elements. CLK protein does not show clear circadian fluctuations in abundance in liver, yet its phosphorylation status clearly changes as a function of time exhibiting a complex pattern with at least four different protein forms, probably reflecting two CLK isoforms each in a nonphosphorylated and phosphorylated form (Lee et al., 2001).

In liver cell nuclei, both CLK and CYC can be found bound to DNA throughout the day (Lee et al., 2001). This means that although BMAL1 levels are fluctuating, dynamic interactions between the repressor proteins and the CLK–BMAL1 dimer substantially contribute to rhythmic transcriptionally activity discussed below.

PER, TIM, DBT, and SGG Regulate Onset and Duration of Transcriptional Repression in Flies

Like CLK and CYC, PER belongs to the family of PAS proteins, yet it lacks the bHLH DNA-binding domain. It uses its PAS domain to dimerize with TIM (e.g., Gekakis et al., 1995), and the heterodimer functions as a repressor of *per* and *tim* expression by interfering with the CLK–CYC heterodimer (Lee et al., 1998, 1999; Bae et al., 2000; Fig. 1).

After initial activation of *per* expression in the afternoon, PER protein cannot accumulate in the cytoplasm, due to the action of the DBT kinase (Kloss et al., 1998; Price et al., 1998). Only after sufficient amounts of TIM protein are present, PER–TIM heterodimer formation protects PER from degradation. Genetic evidence for this aspect of DBT function is derived from experiments involving the dbt^{P} mutation (Table 1): in homozygous mutant larvae hypophosphorylated PER accumulates to high levels, even though only very little TIM protein is detectable in this mutant (Price et al., 1998).

In addition, both the SGG and DBT kinases seem to determine the time of nuclear entry of PER and TIM. SGG influences the temporal pattern of TIM phosphorylation, and overexpression of SGG in all *tim* expressing cells results in an advanced nuclear entry of both PER and TIM, which probably accounts for the shorter free-running periods observed in these flies (Martinek et al., 2001; Table 1). In the case of DBT, analysis of the dbt^{S} allele indicates a role for this kinase in the timing of PER nuclear localization: although cytoplasmic accumulation and nuclear turnover are accelerated, nuclear entry of PER is delayed by several hours (Bao et al., 2001).

In the middle of the night at least three proteins—PER, TIM, and DBT—enter the nucleus, probably as a complex (Curtin et al., 1995; Kloss et al., 2001). Both PER and TIM are progressively phosphorylated in the nucleus in a DBT and SGG dependent manner (Edery et al., 1994; Zeng et al., 1996; Price et al., 1998; Martinek et al., 2001; Fig. 1). Although not directly shown, this suggests that SGG might also be part of the nuclear complex. After nuclear entry this complex likely inhibits CLK–CYC induced transcription of *per* and *tim*. However, transcription of both genes has already substantially decreased *before* nuclear translocation of the repressors (So and Rosbash, 1997). A likely explanation for this could be that the extremely low CLK levels around subjective dusk dictate the initial drop in *per* and *tim* transcription (Lee et al., 1998; Bae et al., 2000). Thus, the repressor complex would rather prevent the newly accumulating CLK–CYC dimers from reinitiating transcription in the second half of the night.

The actual repression is thought to be mediated by direct interactions of the PER–TIM dimer with the CLK–CYC dimer, resulting in a loss of the DNA-binding ability of the transcription factors (Lee et al., 1998, 1999; Bae et al., 2000; Fig. 1). This is substantially different from the mammalian mechanism, where CLK and BMAL1 remain bound to DNA throughout the circadian cycle (Lee et al., 2001; Fig. 1).

But how is repression terminated? The initial step could involve nuclear DBT function. Evidence for this was revealed by applying the tim^{UL} mutation which causes 33-h behavioral periods (Table 1; Rothenfluh et al., 2000c). The nuclear TIM^{UL}-PER dimer is very stable, and PER is hypophosphorylated in tim^{UL} mutants. Clearing the mutant TIM protein by exposure to light. Results in rapid phosphorylation of PER and subsequent degradation (Rothenfluh et al., 2000c). These findings suggest that TIM also inhibits nuclear DBT function, and that the dissociation of TIM from the repressor complex is crucial for determining the period length of a molecular cycle. The question remains what triggers the disappearance of TIM, and here SGG comes into play again: mutants with reduced *sgg* function do not show the characteristic phosphorylation induced mobility shift of TIM in the middle of the night, which probably contributes to the prolonged molecular and behavioral cycles observed in these flies (Martinek et al., 2001; Table 1). This event could therefore be the signal that triggers TIM's dissociation and subsequent degradation (note that TIM levels start to fall already during the night, even in LD cycles; e.g., Zeng et al., 1996; Martinek et al., 2001).

In agreement with this hypothesis, it has been shown that monomeric PER can act as a potent repressor in the complete absence of TIM. This was again shown with help of tim^{UL} flies: after removal of the mutant TIM protein, PER exhibited robust repression of CLK–CYC-mediated transcription in fly heads (Rothenfluh et al., 2000c). This also explains why in wild-type flies *per* and *tim* transcription remains (PER-) depressed several hours after the light-induced degradation of TIM under LD conditions (e.g., Zeng et al., 1996; So and Rosbash, 1997).

A whole set of period-lengthening or arrhythmia inducing *dbt* alleles support the nuclear function of DBT on PER stability (Table 1). Under LD conditions, all mutations causing a lengthened behavioral period also show a slower decay of PER in the morn-

ing hours (Price et al., 1998; Suri et al., 2000). Because peak levels are reached at normal times in these mutants this finding is consistent with increased nuclear stability of PER. Surprisingly, the PER molecules in the various period-lengthening *dbt* alleles and in the arrhythmia inducing dbt^{ar} mutation are hyperphosphorylated, indicating that other kinases also contribute to PER phosphorylation (similar to mammals, see below) (Price et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000). Nevertheless, dbt^{ar} also highlights the specificity of the DBT–PER interaction: Combining dbt^{ar} with per^{S} or per^{T} suppressed the arrhythmic phenotype and resulted in flies exhibiting extremely long, yet stable behavioral periods (Table 1; Rothenfluh et al., 2000b). Most likely the faster nuclear PER turnover in the per^{S} (Curtin et al., 1995) and per^{T} alleles partially compensated for the increased PER stability induced by dbt^{ar} (Rothenfluh et al., 2000b).

per, tim, and Maybe *vri* Are Involved in Rhythmic Regulation of *Clk* Expression

Rhythmic expression of *Clk* RNA and protein occurs with a phase opposite to that of *per* and *tim* RNA, suggesting the existence of an additional feedback loop (see above). Most likely this additional loop amplifies and helps to sustain molecular oscillations of *per* and *tim* by increasing the amplitude of transcriptional rhythms. How these *Clk* oscillations are generated is largely unknown. Genetic evidence supports a positive role for *per* and *tim* on *Clk* expression, because in per^{01} and tim^{01} mutant animals *Clk* RNA and protein levels are low (Bae et al., 1998; Lee et al., 1998). Furthermore, in per^{01} Clk^{Jrk} and per^{01} cyc^{01} double-mutants, *Clk* expression is at peak levels, suggesting that CLK and CYC normally repress *Clk* and that *per* and *tim* derepress this function (Glossop et al., 1999). Because CLK and CYC are positively acting transcription factors it is unlikely that they simultaneously act as repressors. Instead, they may indirectly regulate a *Clk* repressor, and there is some circumstantial evidence that the VRI b-ZIP transcription factor could serve this function.

vri RNA is expressed with a similar phase as *per* and *tim*, suggesting that it is regulated by the same mechanism (Blau and Young, 1999). In fact, *vri* RNA levels are low in Clk^{Jrk} and cyc^{01} flies, and CLK has been shown to activate *vri* expression *in vitro* in an E-box–dependent manner (Blau and Young, 1999). Low VRI levels in Clk^{Jrk} and cyc^{01} flies are consistent with repressor function, and would explain the high levels of *Clk* RNA in the face of these mutants (Glossop et al., 1999). Second, *vri* levels are intermediate in per^{01} and tim^{01} flies (Blau and Young, 1999). This probably results in the accumulation of fairly high and constant amounts of VRI, which would explain the low *Clk* RNA amounts in these mutant backgrounds. Finally, *vri* overexpression results in a reduction of *per* and *tim* RNA levels (Blau and Young, 1999). Although this could be a direct effect, it is also possible that the low *per* and *tim* levels are a consequence of low CLK levels—caused by VRI-mediated repression of *Clk*. If true, some prediction can be made about the phase relationship between *vri* RNA and protein. Because *vri* RNA rises while both *Clk* RNA and protein are falling (midday to dusk), it is likely that VRI protein production and nuclear entry closely follows the phase of its encoding RNA to immediately repress *Clk* transcription. After the delayed nuclear entry of PER and TIM in the middle of the night, VRI function would be abrogated, allowing a new cycle of *Clk* transcriptional activation late in the night (which indeed occurs around this time; Lee et al., 1998). In this scenario of rhythmically repressing *Clk* activity, no additional complicated assumptions about the so far unknown activator of *Clk* transcription need to be made. Any constitutively available transcription factor that is temporally repressed by VRI would serve the purpose (Fig. 1).

In addition to *Clk*, many other clock-controlled genes (ccgs) seem to be regulated by this second loop (Fig. 1). This was inferred to be the case when antiphase oscillations of a particular RNA (relative to *per* and *tim*) was correlated with high RNA levels in Clk^{Jrk} and low levels in per^{01} and tim^{01} mutant animals, respectively, as, for example, in the case of *cry* (e.g., Emery et al., 1998). In fact, regulating the phase of certain ccgs rather than that of *Clk* might be the main function of the second loop. This is because the phase of *Clk* RNA oscillations can be switched to match that of *per* and *tim* without dramatically altering the CLK protein cycling (Young Kim et al., 2002). Thus, although in the case of *Clk* posttranscriptional regulation seems to overrule transcriptional regulation, the latter might be important to set the phase of ccgs.

mPERs, mCRYs, CK1ϵ, and CK1δ Regulate Onset and Duration of Transcriptional Repression in Mammals

Pacemaker Function of Mouse Cryptochromes. The *mCry1* and *mCry2* genes are rhythmically expressed in the SCN with a phase similar to the *mPer*s and opposite to that of *Bmal1* (Miyamoto and Samcar

1998; Kume et al., 1999; Fig. 1). Moreover, mCRY1 and mCRY2 protein levels in the SCN are high during maximal inhibition of BMAL1–CLK-mediated transcription (Kume et al., 1999). Consistent with this negative role of mCRYs in the feedback-loop *mCry* RNA levels are severely reduced and noncycling in the SCN of *Clk* mutant mice (Kume et al., 1999) and in *mCry*-deficient mice, *mPer1* and *mPer2* RNA levels are at medium to high levels (Okamura et al., 1999; Vitaterna et al., 1999). Moreover, each of the mCRYs alone and without help of any mPER protein is able to potently inhibit CLK–BMAL1-induced transcription *in vitro*, suggesting that it is the direct interaction between mCRY and the dimer that represses its activity (Kume et al., 1999; Shearman et al., 2000b).

If the mCRYs are such potent repressors, then what might the mPERs be needed for? The fact that *mPer1* and *mPer2* fulfill crucial functions in the circadian clock was demonstrated by the behavioral phenotypes of mice deficient for the two genes (Table 3). Insight into their potential biochemical role within the pacemaker came from a study involving a peripheral pacemaker, the liver (Lee et al., 2001).

***Pacemaker Function of Mouse* Per1 *and* Per2.** When nuclear complexes of liver cells were analyzed at times of transcriptional repression, mPER1 and mPER2 were part of a complex with mCRY1, mCRY2, CLK, and BMAL1 suggesting a functional role for mPERs in this process (Lee et al., 2001; Fig. 1). The mCRYs are mainly located in the cytoplasm throughout the day, and a potential role of the lower abundant mPERs could be to help translocating the mCRYs into the nucleus. In fact, distribution of mCRY1 and mCRY2 in $mPer1^{ldc}/mPer2^{ldc}$ double knockouts (Table 3) was almost exclusively cytoplasmic, strongly supporting the nuclear shuttle function of mPER1 and mPER2 (Lee et al., 2001). Thus, the daily increase of cytoplasmic mPER molecules likely dictates mPER–mCRY dimerization and the onset of repression by determining the moment of nuclear entry. Conversely, in *mCry* double knockouts mPER1 and mPER2 localized mainly to the cytoplasm indicating that mCRYs are necessary for efficient nuclear translocation of mPER1 and mPER2 (cf. Kume et al., 1999). Consistent with this, nuclear entry of ratPER2 was shown to depend on a nuclear localization signal and interaction with hCRY1 *in vitro* (Miyazaki et al., 2001). However, nuclear localization of mPER1 can also occur without help of the mCRYs in the SCN, likely mediated via direct interactions of mPER1 with mPER3 (Yagita et al., 2000).

In addition, overall amounts of mPER2 protein (but not RNA!) are severely reduced in mCRY deficient mice in SCN and liver, suggesting that the mCRY proteins stabilize mPER2 (Shearman et al., 2000b; Yagita et al., 2000; Lee et al., 2001). Therefore, mCRYs exert distinct effects on the different PER proteins in that they are required for nuclear localization of mPER1 (at least in liver) and mPER2, and for stabilization of mPER2.

Although mPER2 is part of the protein complex thought to repress CLK–BMAL1 mediated transcription, it also positively influences *Bmal1* expression (Fig. 1). Low *Bmal1* RNA levels were found in mice deficient for *mPer2* (Bae et al., 2001), as well as in mutations that reduce *mPer2* or mPER2 levels like $Clk^{-/-}$, or *mCry* double-knockouts (Shearman et al., 2000b). This dual function of *mPer2* could explain the more severe behavioral phenotype of mice deficient for *mPer2* compared to *mPer1* mutants (Table 3).

Function of Mammalian CK1ϵ, and CK1δ and Temporally Regulated Clock-Protein Phosphorylation. Genetic evidence for the importance of kinase-function within the circadian clock is twofold: first, the syrian hamster *tau* mutation was cloned, and found to be a point mutation in the *CKIϵ* gene, the homolog of fly *dbt* (see above and Table 3). The mutant kinase is still able to bind mPER1 and mPER2 *in vitro*, but shows reduced efficiency in phosphorylating both proteins compared with the wild-type protein (Lowrey et al., 2000). This feature likely accounts for the dominant-negative nature of the *tau* mutation, whereby the mutant enzyme competes with the fully functional CKIϵ (and possibly CKIδ, see below) molecules. Moreover, a mutation in *hPer2* occurring at a phosphorylation site of CKIϵ is associated with the sleep disorder FASPS (Table 3). These *in vivo* findings are bolstered by several *in vitro* studies, highlighting biochemical aspects of the role CKIϵ and its close relative CKIδ play in the circadian clock.

As discussed above, the mPERs are important for nuclear translocation of the mCRYs. The proposed role for the CKI enzymes in nuclear translocation of the mPER proteins is at least ambiguous. Vielhaber et al. (2000) elegantly demonstrated that in the human cell line HEK293 phosphorylation of mPER1 by CKIϵ is crucial for cytoplasmic *retention* of both proteins. Using various deletion constructs they showed that CKIϵ binds close to a nuclear localization region (NLS), which is responsible for translocating mPER1 into the nucleus in the absence of CKIϵ or other mPER proteins. Binding and phosphorylation of mPER1 masks a crucial region adjacent to the NLS, resulting in cytoplasmic localization of the kinase and

Table 3 Mammalian Rhythm Mutations

Gene	Allele or Polymorphism	Source	Molecular Lesion	Phenotype	Reference
mClock bHLH-PAS protein	*Clk* (antimorph, semi-dominant)	ENU[a]	missense mutation in a splice donor site resulting in exon skipping and 51 AS deletion	26- to 29-h locomotor periods followed by complete loss of circadian rhythmicity after ca. 14 days in DD	Vitaterna et al., 1994; King et al., 1997
humanClock	3111C (polymorphism)[b]	spontaneous	C→T substitution at position 3111 in the 3′-VTR of the *Clk* cDNA	delayed morningness-eveningness tendencies of activity- or sleep-onset	Katzenberg et al., 1998
hamsterCKIε Casein Kinasele Iε	*tau* (hypomorph, semi-dominant)	spontaneous	missense mutatio n ($Arg^{178} \rightarrow Cys$)	20-h behavioral rhythms	Ralph and Menaker, 1988; Lowrey et al., 2000
humanPer2 PAS-protein	$hPer2^{S662G}$ (hypomorph)	spontaneous	missense mutation in CKIε binding site ($Ser^{662} \rightarrow Gly$)	Familial Advanced Sleep Phase Syndrome (FASPS)	Toh et al., 2001
humanPer3 PAS-protein	G647 (polymorphism)[b]	spontanous	missense mutation ($Val^{647} \rightarrow Gly$)	loosely linked to Delayed Sleep Phase Syndrome (DSPS)	Ebisawa et al., 2001
mPer1 PAS-protein					
	$mPer1^{null}$ (amorph)	targeted k.o.	replacement of exons 4–10	0.7- to 0.8-h shortening of free-running period	Cermakian et al., 2001
	$mPer1^{ldc}$ (amorph)	targeted k.o.	replacement of exons 2–12	0.6 shortening of free running period (not significant). Behavior AR after 10–14 days in DD	Bae et al., 2001
	$mPer1^{Brdm1}$ (amorph)	targeted k.o.	replacement of exons 3–18	1-h shortening of behavioral period	Zheng et al., 2001
mPer2 PAS protein					
	$mPer2^{ldc}$ (amorph)	targeted k.o.	replacement of exon 5 and parts of exon 6	Behavior AR after variable amounts of time spent in DD (ranging from 0 to 21 days). $mPer1^{ldc}/mPer2^{ldc}$ double-mutants immediately AR in DD	Bae et al., 2001
	$mPer2^{Brdm1}$ (amorph)	targeted k.o.	2.1 kb internal deletion removing the C-terminal half of the PAS-B domain	1.6-h shortening of free running period. Behavior AR after 2–18 days in DD. $mPer1^{Brdm1}/mPer2^{Brdm1}$ double-mutants immediately AR in DD	Zheng et al., 1999, 2001
mPer3 PAS protein	*mPer3* (amorph)	targeted k.o.	1.6 kb internal deletion removing exon 3 and parts of exon 4	0.5-h shortening of free-running period	Shearman et al., 2000a

Bmal1 (Mop3) bHLH-PAS protein	*mMop3*$^{-/-}$ (amorph)	targeted k.o.	replacement of bHLH-domain in exon 4 and complete exon 5	Behavior AR in DD	Bunger et al., 2000
mCry1 Flavoprotein with homology to photolyase					
	Cry1$^{-/-}$	targeted k.o.	13-kb deletion containing exon sequences encoding the FAD binding domain	0.8-h shortening of free-running period	Vitaterna et al., 1999
	cry1$^{-/-}$	targeted k.o.	deletion of coding sequences corresponding to bp 730–1479 of the *Cry1* cDNA	1.3-h shortening of free-running period	van der Horst et al., 1999
mCry2 Flavoprotein with homology to photolyase					
	Cry2$^{-/-}$	targeted k.o.	1.1-kb deletion containing exon sequences encoding the FAD binding domain	0.6-h lengthening of free-running period. *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ double mutants behaviorally AR.	Thresher et al., 1998; Vitaterna et al., 1999
	cry2$^{-/-}$	targeted k.o.	deletion of coding sequences corresponding to bp 397–810 of the *Cry2* cDNA	0.9-h lengthening of free-running period. *cry1*$^{-/-}$/*cry2*$^{-/-}$ double mutants behaviorally AR.	van der Horst et al., 1999
dbp bZip-PAR transcription factor					
	Dpb^{null} (amorph)	targeted k.o.	replacement of all but 9bp of *dbp* coding sequences with *lacZ* reporter gene sequences	0.5-h shortening of free running period	Lopez-Molina et al., 1997
	Dbp^{dim}	targeted k.o.	insertion of reporter gene into exon 4. Unstable, truncated protein lacking dimerization domain	n.d.	Lopez-Molina et al., 1997
Tgf-α growth factor	*waved-2* (hypomorph: kinase activity reduced by >90%)	spontanous	missense mutation, changing a Val to Gly in the N-terminus of the tyrosine kinase domain	DD behavior normal; increased activity during the light portion of LD cycles; no masking response	Luetteke et al., 1994; Kramer et al., 2001.

Overview of rhythm genes and mutations in mammals (listed along with the nature of the encoded protein/peptide in the first column). All listed mutations are homozygous viable and were analyzed behaviorally as homozygotes.

[a] *N*-Ethyl-*N*-Nitrosourea.

[b] The polymorphisms associated with the particular gene are not tightly linked with the phenotype (e.g., 85% of DSPS patients did not carry the G647 polymorphism in *hPer3:* Ebisawa et al., 2001).

mPER1 (Vielhaber et al., 2000). Although in itself conclusive, it is difficult to imagine that such a mechanism indeed works *in vivo*, given that a complex consisting of mPER1 and CKIϵ can be found in liver nuclei at times of transcriptional repression (Lee et al., 2001).

Moreover, a different *in vitro* study showed that mPER1 is mainly cytoplasmic in the primate COS-7 cell line, and nuclear translocation depends on binding and phosphorylation of CKIϵ (Takano et al., 2000), just the opposite of what was found in HEK293 cells. Yet another study showed no influence of CKIϵ or CKIδ on the localization of mPER1 or mPER2, although the same COS-7 cells were used (Akashi et al., 2002). Instead, this article reported a kinase-dependent nuclear entry of mPER3. These apparent discrepancies demonstrate the difficulties associated with such cell culture studies, at least in terms of studying nuclear translocation mechanisms. A particular problem arises from the fact that different cell lines show endogenous expression of certain clock genes in varying degrees, which can easily create conflicting results. Moreover artifacts could be created by dramatically overexpressing mRNAs and proteins using certain expression plasmids in the cotransfection experiments described above.

To this date, there is no *in vivo* evidence that the CKI kinases are involved in the regulation of mPER nuclear translocation, so what else could be their function? Both kinases are constitutively expressed in the SCN, displaying mainly nuclear localization (Takano et al., 2000; Camacho et al., 2001; Ishida et al., 2001). Several studies show that CKIϵ and CKIδ are able to bind and phosphorylate mPER1, mPER2, and mPER3 *in vitro*, and that this is connected with protein destabilization (Keesler et al., 2000; Lowrey et al., 2000; Takano et al., 2000; Vielhaber et al., 2000; Camacho et al., 2001; Toh et al., 2001; Akashi et al., 2002). In this respect, the function of the mammalian CKI enzymes might be similar to that of cytoplasmic fly DBT, and could contribute to the observed delay between *mPer* RNA and protein accumulation, as in flies (see above). Interestingly, and in contrast to flies, mPER degradation seems to be mediated by the ubiquitin–proteasome pathway, whereby CKIϵ and CKIδ-mediated phosphorylation of mPER1 and mPER3 promotes their ubiquitination (Akashi et al., 2002). In flies, this degradation pathway seems to be specific for the TIM and CRY proteins and not for PER (Naidoo et al., 1999; Lin et al., 2001).

Lee et al. (2001) could show that a complex consisting of mPER1, mPER2, mCRY1, mCRY2, CKIϵ, and CKIδ enters the nucleus (of liver cells) coinciding with the time of inhibiting transcriptional activity mediated by the CLK–BMAL1 dimer (Fig. 1). This results in a circadian rhythm of CKI subcellular distribution, because it only accumulates in the nucleus during times of transcriptional inhibition. It raises the possibility that the kinases could also function in the nucleus, and indeed, that same study showed prominent circadian variations in the phosphorylation status of nuclear mPER1, mPER2, CLK, and BMAL1. These changes might, in fact, be crucial for switching between transcriptionally active and silent periods. During transcriptional activation only phosphorylated forms of BMAL1 were present in the nucleus together with both phosphorylated and unphosphorylated forms of CLK. During repression both forms of BMAL1, yet only the two phosphorylated forms of CLK (likely created by alternative splicing) were in the nucleus, suggesting that phosphorylated BMAL1 and nonphosphorylated CLK are the active transcription factors (Lee et al., 2001; Fig. 1).

As in flies, mPER1 and mPER2 were progressively phosphorylated throughout the circadian cycle, reaching maximum levels during times of transcriptional repression in the middle of the night. Unfortunately, none of these *in vivo* phosphorylation events can be linked to the function of CKIϵ or CKIδ. In *tau* mutant hamsters—and in contrast to the *in vitro* results alluded to above—the phosphorylation patterns of hamster CLK, PER1, and PER2 proteins were normal, suggesting that these kinases are either not responsible for the phosphorylation of clock proteins, or that CKIδ can compensate for the impaired *tau* kinase (Lee et al., 2001). However, there where mPER-specific differences observable between wild-type and *tau* mutant animals: the appearance of hyperphosphorylated forms of mPER1 and mPER2 was delayed in the mutants, probably because the mutant kinase bound to the mPERs is less active (Lee et al., 2001). Moreover, the amounts of mPER1 and mPER2 that are bound to either CKIϵ or CKIδ were severely reduced in *tau* hamsters, suggesting a decreased binding affinity of the mutant kinase to the mPER proteins, again opposing the results obtained from *in vitro* studies (Lowrey et al., 2000; Lee et al., 2001).

In summary, it is unquestionable that at least CKIϵ has an important role in the circadian system (Lowrey et al., 2000). In addition, it seems likely that both CKIϵ and CKIδ are involved in temporal phosphorylation of mPER1 and mPER2, probably regulating their stability in the cytoplasm and nucleus. Moreover, other kinases must exist that give rise to the prominent circadian phosphorylation patterns of CLK and BMAL1, which almost certainly determine sta-

bility and activity of these transcription factors. As a last word of caution, one should note that the *in vivo* study that led to most of these conclusions (Lee et al., 2001) was performed on a peripheral oscillator, the mouse liver. It is known that these oscillators, in contrast to the SCN, are not able to maintain circadian oscillations for more than a few cycles in a SCN independent manner (Yamazaki et al., 2000), as previously shown for flies (Plautz et al., 1997). It follows that something is missing from the molecular clock works in the periphery, or that the mechanisms differ at least in some details. Therefore one hopes that a similarly elegant study will also be conducted for SCN tissue.

Other Transcriptional Loops Contributing to Overall Clock Gene Cyclings

Besides the transcriptional and posttranslational mechanisms discussed above, there are other known modes of regulation that contribute to overall clock gene cyclings. In the fly case, this includes posttranscriptional regulation at the *per* RNA level (as reviewed in Stanewsky, 2002). In addition, other transcriptional forms of clock-gene regulation in both flies and mammals were discovered, which will now be discussed.

Role of CREB in the* Drosophila *Circadian Clock. Based on its role in gating the light-input into the mammalian SCN (reviewed by Reppert and Weaver, 2001), the cAMP response element binding protein (CREB) has been studied with respect to a potential role in the fly circadian system. Using a reporter strain where *luciferase* expression is under the control of CREB binding sites, Belvin et al. (1999) were able to show that CREB activity is regulated by the circadian clock (Fig. 2). Moreover, a mutant in *dCREB* (Table 2) displayed altered locomotor activity rhythms and severely blunted rhythms of *per* transcription as reported by a *per-luciferase* transgene. This indicates that CREB is part of the regulatory feedback loop comprising the circadian clock (Fig. 2). CREB-mediated transcription of target genes occurs in response to many environmental stimuli. Therefore, the additional circadian regulation of CREB activity suggests that there are optimal times during the 24-h day for the CREB-regulated processes to occur. The speculation that CREB could play a more general role in the circadian system is supported by the presence of CREB binding sites in the *per* and *tim* promoters (Belvin et al., 1999; Okada et al., 2001) as well as in the regulatory regions of many newly isolated rhythmically expressed genes (Claridge-Chang et al., 2001; Stempfl et al., 2002; Fig. 2).

Role of DBP and E4BP4 for Regulation of* mPER *Genes. DBP (albumin **D**-element **B**inding **P**rotein) belongs to the PAR domain class of transcription factors. They contain the PAR activation domain (**p**roline and **a**cidic amino acid **r**ich), as well as a basic leucine Zipper (bZip) domain, necessary for DNA binding and protein dimerization. In addition to DBP two other PAR protein encoding genes HLF (**h**epatic **l**eukemia **f**actor) and TEF (**t**hyroid **e**mbryonic **f**actor) are expressed in a circadian fashion in both liver and SCN (Wuarin and Schibler, 1990; Falvey et al., 1995; Fonjallaz et al., 1996; Lopez-Molina et al., 1997; Ripperger et al., 2000; Mitsui et al., 2001). The subtle behavioral phenotype of *dbp* mutant mice did not reveal if this gene functions in the central pacemaking mechanisms or only in the clock output (Lopez-Molina et al., 1997; Table 3). Circadian expression of clock-output genes in the liver is directly regulated by DBP and severely blunted in *dbp* mutant mice, demonstrating a prominent role for DBP in regulating output genes (Lavery et al., 1999; Fig. 2). It was also shown that CLK directly binds to E-box sequences within intronic regions of *dbp* to activate its transcription, demonstrating that the core transcriptional mechanism of the clock also regulates output genes (Ripperger et al, 2000; Yamaguchi et al., 2000b; Fig. 2).

Surprisingly, DBP seems also to be involved in regulating *mPer1* gene expression by activating its expression through direct binding of DBP target sequences within the *mPer1* promoter, thereby enhancing the CLK–BMAL1 mediated activation (Yamaguchi et al., 2000b; Fig. 2). This positive action of DBP is counteracted by that of another b-Zip transcription factor that lacks the PAR domain, called E4BP4 (adenovirus **E4** promoter **B**inding **P**rotein **4**). E4BP4 is able to compete efficiently for the target sequence of all three PAR proteins *in vitro*, and represses basal expression from the *mPer1* promoter, as well as PAR protein activated *mPer1* expression (Mitsui et al., 2001; Fig. 2). That this type of additional *mPer1* regulation might indeed occur *in vivo* is indicated by the antiphase expression pattern of *e4bp4* RNA and protein compared to that of the other PAR proteins in SCN and liver: the latter are expressed at peak levels during times of maximal transcriptional activation of *mPer1*, whereas *e4bp4* reaches high RNA and protein levels during times of maximal *mPer1* repression (Mitsui et al., 2001). Consistent with this model *dbp* and *e4bp4* are differentially affected in *mCry* deficient

mice, by showing high or low constitutive levels of expression, respectively (Mitsui et al., 2001).

Role of NPAS2 (MOP4) and MOP9 in the Circadian System of Mammals. Several other bHLH PAS proteins were found to be expressed in the SCN or in other brain regions (Shearman et al., 1999a; Hogenesch et al., 2000). The *Bmal1* homolog *Mop9* is expressed in the SCN and able to activate transcription from E-boxes *in vitro* when coexpressed with *Clk* (Hogenesch et al., 2000). Whether *Mop9* indeed plays a role in the circadian clock awaits the analysis of a mouse strain deficient for this gene.

Similarly, the CLK analog NPAS2 (MOP4) is able to activate transcription when coexpressed with BMAL1 (Hogenesch et al., 1998; Reick et al., 2001; Rutter et al., 2001), and this activation can be repressed by mCRY1 and mCRY2 (Kume et al., 1999; Reick et al., 2001). *NPAS2* is not expressed in the SCN, and mice deficient for this gene are not impaired in their behavioral locomotor rhythms (Shearman et al., 1999; Reick et al., 2001; S. McKnight, personal communication). A prominent region of *NPAS2* expression is the forebrain—a structure involved in acquisition of specific types of memory (Garcia et al., 2000)—and there is evidence that *NPAS2* plays a role in the circadian clock operating in this brain area: *Bmal1* is rhythmically expressed in the forebrain, in a phase opposite to that of *mPer1*, *mPer2,* and *mCry1*, similar to the situation in the SCN (Reick et al., 2001). This opposite phase relationship is likely to be established by a second feedback-loop in which the BMAL1–NPAS2 dimer represses transcription of the *Bdrm1* gene (Reick et al., 2001; Fig. 1). Strikingly, rhythmic expression of *mPer2* was abolished in the forebrain of *NPAS2* deficient mice, but only in brain areas that normally express this gene, strongly suggesting that *NPAS2* functions as a *Clk* analog in certain tissues (Reick et al., 2001).

In addition, *NPAS2* is expressed rhythmically in the vasculature, consistent with its proposed role to support core clock feedback oscillations in this tissue (McNamara et al., 2001). The same study shows that MOP4–BMAL1-mediated transcription is repressed by binding of nuclear hormone receptors to the dimer, resulting in a reduction of its ability to bind the E-box target sequences. Because hormonal signals are thought to be involved in the synchronization of peripheral clocks by the SCN, the NPAS2/hormone receptor interaction can serve as a powerful working model for studying the mechanism of such "entrainment" (McNamara et al., 2001).

MECHANISMS OF CLOCK-OUTPUT IN FLIES AND MAMMALS

In the following, I will try to outline the principles of how the clock gene oscillations described above are used and translated into rhythmic biological processes. Only selected examples will be presented, and for a detailed description of clock-output mechanisms, the reader is referred to more specialized reviews (Jackson et al., 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001). As we will see, both flies and mammals can use the same molecules and mechanisms that contribute to molecular oscillations in the core-clock to pass along temporal information to downstream genes by regulating their expression in a dynamic fashion. In addition, it is clear that other mechanisms exist that are only indirectly connected to the core clock molecules.

Known Entities Involved in Regulating Locomotor Rhythms in Flies

PDF, a Neuropeptide Influencing Locomotor Rhythms. The spatial expression pattern of the Pigment Dispersing Factor (PDF) suggested that this neuropeptide might play a role in the fly circadian system. PDF is expressed in pacemaker neurons in larvae and adult flies that control eclosion or locomotor rhythms, respectively (reviewed by Helfrich-Förster, 2002). A mutation in the *pdf* gene (*pdf*^{01}; Table 2) results in short behavioral periods, progressively turning into arrhythmicity (Renn et al., 1999). Formally, this phenotype does not distinguish if this peptide functions in the clock-input, the output, or even in the pacemaker itself. Yet, there is good evidence for *pdf* functioning in the output pathway. First, *pdf* is expressed in only a subset of clock-gene expressing pacemaker neurons in the adult brain, which probably explains the remaining rhythmicity in *pdf*^{01} mutant animals (Renn et al., 1999). Second, there is a circadian rhythm of PDF accumulation in the nerve terminals of the pacemaker neurons (Fig. 3). This rhythm is disrupted in *per*^{01} and *tim*^{01} mutant animals, and PDF levels are severely reduced in VRI overexpressing flies, demonstrating that PDF accumulation is clock controlled (Blau and Young, 1999; Helfrich-Förster et al., 2000; Fig. 3). The fact that neither of the three genes have any effect on the *pdf* RNA levels argues for a purely posttranscriptional PDF regulation (Blau and Young, 1999; Park et al., 2000a). Consistent with this, *pdf* RNA is constitutively expressed in wild-type flies (Park and Hall, 1998). However, at least in a subset of the PDF

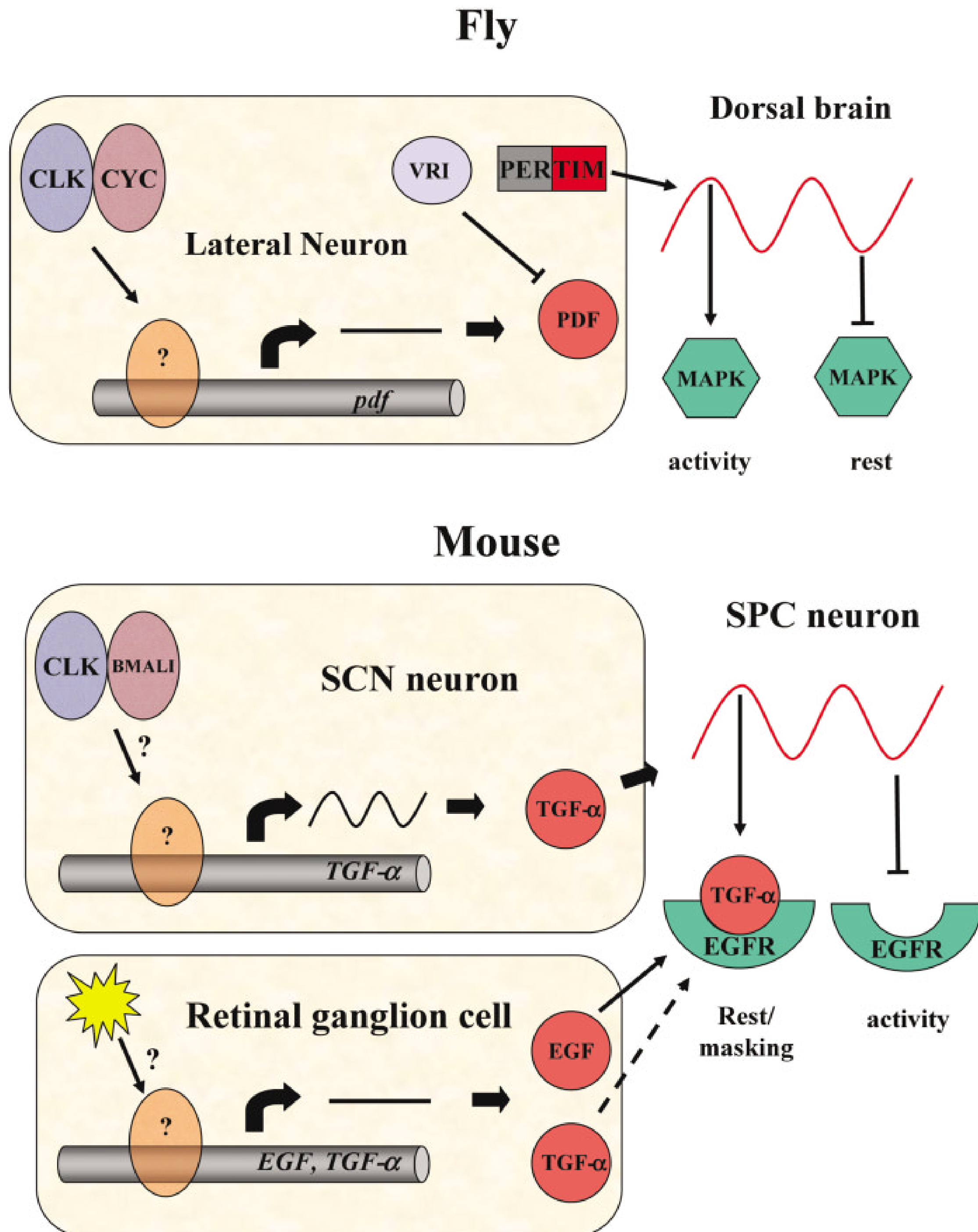

Figure 3 Model of output mechanisms regulating locomotor behavior in flies and mammals. Symbols and abbreviations as in Figure 1. The straight black line above the *pdf* gene indicates that the RNA is expressed constitutively. Expression depends on CLK and CYC, but most likely through an indirect mechanism. The red waved line indicates rhythmical PDF release from LNv terminals into the dorsal brain. PER and TIM are required for this rhythm. PDF probably activates the MAPK kinase pathway, resulting in behavioral activity of the fly. In mammals, rhythmic release of TGF-α from the SCN (indicated by the red waved line) regulates behavioral activity via binding the EGFR in SPC neurons. Light has also a direct effect on locomotor behavior, probably mediated by EGF and TGF-α expressed in retinal ganglion cells. Somehow light activates EGF/TGF-α signaling via the RHT directly to the SPC. This could involve indirect or direct transcriptional activation by light, but this is purely hypothetical. It is also unknown if rhythmic expression of *TGF-α* within the SCN is directly or indirectly regulated by known clock factors. See text for details.

positive pacemaker neurons, *pdf* RNA levels are transcriptionally regulated by clock genes: in Clk^{Jrk} and cyc^{01} flies, *pdf* RNA does not accumulate (Blau and Young, 1999; Park et al., 2000a). Interestingly, this regulation does not involve the usual CLK–CYC-mediated activation by binding to an E-box in the *pdf* promoter, because these sequences can be deleted without affecting *pdf* expression (Park et al., 2000a; Fig. 3). Therefore, *pdf* regulation is a first example of indirect transcriptional regulation by clock genes, which seems to be a common theme in regulation of fly output genes.

The Ras/MAP-Kinase Pathway, a Potential Target of PDF Signaling. PDF is likely to be released rhythmically in a brain region called the dorsal protocerebrum, a place where most of the pacemaker neurons (including the non-PDF expressing ones) send their projections (e.g., Helfrich-Förster, 2002). Ultimately, this temporal information has to be transmitted further towards the locomotor centers in the thorax, a process that is poorly understood. A potential downstream pathway, dependent on *pdf* and clock gene function is the Ras/MAP-kinase pathway. This was revealed by studying the involvement of *Nf1* in circadian rhythms. *Nf1* encodes a Ras-GTPase activating protein and mutations cause an overactivation of MAP-kinase signaling (Williams et al., 2001). The same mutants show arrhythmic locomotor behavior (Table 2), although TIM cycling in the larval pacemaker neurons—which are precursors of the adult PDF expressing neurons—appears to be normal. Together with the observation that mutations that downregulate MAP-kinase signaling suppress the behavioral *Nf1* mutant phenotypes, this firmly places the Ras/MAP-kinase pathway in the clock output (Williams et al., 2001). But where is the link to *pdf*? In the same study it was shown that in pdf^{01} mutations the levels of activated MAP-kinase are downregulated, suggesting that this pathway is indeed a downstream target of the clock and activated by PDF (Fig. 3).

cAMP-Dependent Protein Kinase A (PKA), Another Regulator of Behavioral Rhythms. Mutations in the catalytic and regulatory subunit of PKA result in arrhythmic behavior (Levine et al., 1994; Majercak et al., 1997; Park et al., 2000b). Moreover mutations in *dunce*—a gene encoding a cAMP specific phosphodiesterase—affect both light entrainment and behavioral output (Table 2), probably due to an altered cAMP metabolism (Levine et al., 1994). Yet it is curious that the two *dunce* alleles analyzed (Table 2) *increase* the amplitude of circadian cAMP fluctuations in fly heads compared to wild-type controls (Levine et al., 1994). Analysis of these mutations was motivated by the known role of cAMP signaling in the input and output pathways of neuronal pacemakers in other organisms, including mammals (Reppert and Weaver, 2001). That PKA function likely does not affect the central pacemaking mechanism was demonstrated by the normal rhythmic eclosion pattern of PKA mutants, which contrasts the severely disrupted behavioral rhythms (Majercak et al., 1997). Hence, PKA signaling seems specific for light input into the clock and locomotor output.

takeout (to) ***Provides a Link between Feeding Behavior and the Circadian Clock.*** *to* was identified as a rhythmically expressed gene using a subtractive hybridization approach (So et al., 2000). It encodes a ligand binding protein that also cycles in abundance, and is the founding member of a whole family of genes that are circadianly regulated (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001). Interestingly, although the *to* promoter contains a *per* like E-box, and *to* expression depends on CLK and CYC, this gene serves as yet another example of indirect regulation by clock genes. Elegant *in vivo* experiments show that the *to* E-box is neither necessary nor sufficient for rhythmic expression, suggesting that other transcription factors, regulated by CLK and CYC, mediate *to* transcriptional regulation (So et al., 2000).

A hint about TO function came from the analysis of a *to* mutation lurking on the $rosy^{506}$ chromosome, which was probably coinduced with the *ry* mutation (Table 2). This mutation deletes parts of the *to* 3′-UTR, probably creating an unstable transcript resulting in low TO protein levels (Sarov-Blat et al., 2000). When analyzed under starvation conditions, these mutants show aberrant locomotor rhythms and die much earlier than wild-type flies. Moreover, *to* expression is induced by starvation, and this induction is dependent on a functional clock, suggesting that the clock copes with this stress via regulation of *to*. TO levels could also serve as a daily indicator for the metabolic state of the individual, adjusting its locomotor activity accordingly. For example, starvation or the daily peaks of TO levels could elicit increased locomotor behavior to stimulate the search for food (Sarov-Blat et al., 2000).

Mutations Affecting Eclosion Rhythms

Rhythmic emergence from the pupal case is gated by the clock, and this output has been successfully used

to isolate mutations of central clock components (Table 1). In addition, mutations specifically affecting eclosion were also found, similar to the PKA and *Nf1* cases for locomotor behavior (e.g., Jackson, 1983). A prominent example is the *lark* mutation, which results in an early phase of eclosion (Newby and Jackson, 1993). LARK is an RNA binding protein that is rhythmically expressed in certain neurons of the pupal brain thought to be involved in regulating rhythmic eclosion (McNeil et al., 1998; Zhang et al., 2000). Interestingly, this form of regulation seems to involve only posttranscriptional mechanisms, because *lark* RNA levels are temporally flat (Newby and Jackson, 1996). Although this is consistent with a function in controlling timed eclosion, the mechanism or downstream targets of LARK are unknown.

The Sensitivity of the Olfactory System Is Controlled by the Circadian Clock

Searching for new biologic rhythms controlled by the circadian clock, Krishnan et al. (1999) reported that the fly's electrophysiologic responses to food odors and to those causing behavioral avoidance, vary throughout the day. This rhythm is dependent on clock gene expression in cells other than those controlling locomotor behavior. Given that rhythmic gene expression can be observed in cultured antennae (the "nose" of the fly), this suggests that clock gene activity in peripheral tissues is necessary for regulating rhythmic biologic processes (Plautz et al., 1997; Krishnan et al., 2001). Moreover, molecular and sensitivity rhythms in fly antennae seem to depend on an additional clock-factor, not required for the pacemaker mechanism regulating locomotor rhythms. This factor would be CRY, for which a clock function in peripheral clocks was originally suggested by Stanewsky et al. (1998). The cry^b mutation was isolated based on its effects on rhythmic *per-luc* expression: reporter-gene expression in individual flies became arrhythmic in the face of the mutant, suggesting that it is needed for oscillator function in many clock-gene expressing tissues. Moreover, PER and TIM oscillations in individual photoreceptor cells were pegged at an intermediate constant level, demonstrating that the clock had stopped in these cells under LD conditions (that is, the whole-animal loss of reporter-rhythms is likely not caused by desynchronization of individual cells, but rather by stopping the clock; Stanewsky et al., 1998). Nevertheless, PER and TIM still cycled in head extracts of cry^b mutant flies during and after temperature entrainment, suggesting that *cry* is not a true clock factor in this tissue (Stanewsky et al., 1998). In contrast, antennal rhythms were abolished in cry^b flies after light and temperature entrainment, demonstrating a clock function for this gene similar to *mCry* function in mammals (Krishnan et al., 2001).

The Molecular Clock Operating in Malpighian Tubules (MT) Depends on CRY

MT (the fly's kidney) contain a brain-independent, light-entrainable molecular clock with as yet unknown biologic function (reviewed by Giebultowicz, 2001). Although CRY fulfills photoreceptive function in this tissue, it is also required for maintaining PER and TIM oscillations under constant conditions, similar to the situation in antennae (Ivanchenko et al., 2001). Strikingly, this study also showed convincingly that this is not the case for larval pacemaker neurons: here PER and TIM cycle robustly in cry^b mutant flies, both in LD (cf. Kaneko et al., 2000) and DD conditions, similar to what has been shown for the adult behavioral pacemaker neurons (Stanewsky et al., 1998; Helfrich-Förster et al., 2001).

Microarray and Genetic Studies Point to "Circadian Orchestration" of Fly Biology

Although there are examples of posttranscriptional regulation of clock-controlled genes (ccgs), most ccgs seem to be controlled at the transcriptional level, directly or indirectly influenced by clock genes. This prompted the search for novel rhythmically expressed genes with molecular tools, such as subtractive hybridization (e.g., So et al., 2000) or differential display (Blau and Young, 1999). With the advent of gene-chip analysis and the completion of the *Drosophila* genome sequence (Adams et al., 2000), several groups analyzed genome-wide circadian regulation of gene expression in flies (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002). Although the sets of cycling genes identified in the three studies did not overlap exactly, the fact that each group reidentified the known oscillating loci *per, tim, Clk, vri, cry* (except Claridge-Chang et al., 2001), and *to* demonstrates the validity of this approach. The combined studies suggest that at least ca. 140 genes are rhythmically expressed in the fly head under LD and DD conditions, involved in functions like vision, olfaction, locomotion, detoxification, immuneresponse, learning and memory, ion-channel activity, and areas of metabolism. This demonstrates the widespread transcriptional control of circadian gene expression in

flies, which is—also revealed by the above studies—mainly under indirect control of the central clock genes.

Moreover, a genetic approach was performed to identify rhythmically regulated enhancers and genes using the real-time *luciferase* reporter gene in conjunction with *P*-element mutagenesis (Stempfl et al., 2002). Together the molecular and genetic approaches suggest that between 1 and 6% of the fly genome is circadianly regulated, similar to what has been observed for plants (Harmer et al., 2000; Schaffer et al., 2001). Only one of the four ccgs found in the genetic study—coding for an NAD-kinase—was also identified in the gene chip studies (Claridge-Chang et al., 2001; Ueda et al., 2002). Also, two previously identified ccgs, *Dreg5* (Van Gelder and Krasnow, 1996) and *crg1* (Rouyer et al., 1997), were not identified in any of the chip studies, suggesting that not all rhythmically expressed genes can be isolated by the chip approach. It is likely that low-abundant cycling RNAs are difficult to detect on chips, making it necessary to also apply other methods. This is especially important considering the few neurons that are responsible for the control of locomotor rhythms. Because the molecular clock-work within these cells is certainly different from the one operating in peripheral clocks (see above), it is possible that RNAs specifically cycling within these neurons make the difference.

Peripheral Oscillators and Output Mechanisms in Mammals

One general difference between the mammalian and fly clock-output has to do with the principally different organization of the two circadian systems. Peripheral oscillators in flies function partially brain-independent, because they are light entrainable and show (dampening) molecular oscillations for a limited amount of time in DD (e.g., Plautz et al., 1997; Giebultowicz, 2001). In mammals, sustained molecular rhythms and light-synchronization of most peripheral clocks depend on the SCN (Sakamato et al., 1998; Yamazaki et al., 2000). In other words, one clock *output* from the SCN serves as *input* for the peripheral clocks, most likely in form of humoral signals (e.g., Silver et al., 1996). Consistent with this, rhythmic expression of clock-genes in cultured Rat-1 fibroblasts can be induced by high concentrations of serum (Balsalobre et al., 1998). Further analysis revealed that this induction in several peripheral clock cells is accomplished by components of multiple signaling pathways present in the serum, including glucocorticoid hormones (Akashi and Nishida, 2000; Balsalobre et al; 2000a, 2000b; Yagita and Okamura, 2000).

Role of DBP as Output Factor. One of the genes rhythmically expressed in peripheral oscillators is *dbp* (Wuarin and Schibler, 1990; see above). Like all cycling genes in the periphery, the phase of expression lags behind several hours compared to the SCN, suggesting regulation by the master brain clock (Balsalobre et al., 1998; Zylka et al., 1998). Indeed, transcription of *dbp* is regulated by the core clock genes (Ripperger et al., 2000; Yamaguchi et al., 2000b; Fig. 2). In the liver DBP regulates the rhythmic activity of genes involved in cholesterol and sex-hormone metabolism (Lavery et al., 1999; Fig. 2). It has also been shown that regulation of *dbp* transcription likely involves additional proteins, not necessarily belonging to the known clock factors (Ripperger et al., 2000). Because DBP also influences *mPer1* expression, it is conceivable that DBP feeds back temporal information about the metabolic state of the cell to the liver oscillator (Fig. 2).

Entrainment of Peripheral Oscillators by Feeding. That the metabolic state can be a powerful Zeitgeber is demonstrated by experiments involving daily restricted feeding schedules under LD and DD conditions. Under these conditions peripheral oscillators can be synchronized without contribution of and without influence on clock gene rhythms in the SCN (Balsalobre et al., 2000a; Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). In fact, this peculiar property of the liver oscillator makes the hierarchic view of the organization of the mammalian circadian system (i.e., SCN entrains periphery) questionable. Rather, the liver behaves like a fly peripheral oscillator, because both are brain-independently entrainable by external cues. Because the SCN nevertheless clearly influences peripheral oscillators (see above), this means that food *can* override the synchronizing signals from the SCN, although itself immune to such entrainment. Under normal conditions peripheral oscillators are entrained by the SCN. But if food supply for some reason gets out of phase with locomotor activity, this system would guarantee that the liver is still able to anticipate food uptake by adjusting its temporal activity accordingly.

Another output gene, which might be crucial for transmitting the temporal metabolic state to the clock, is *lactate dehydrogenase A* (Rutter et al., 2001). LDH reversibly catalyzes the reaction from pyruvat to lactate, a reaction oxidizing the cofactor NADH to NAD. This led to the speculation that the redox state of the

cell (a hallmark for the state of cellular metabolism) could somehow feed back on the clock as a Zeitgeber for entrainment by feeding (Rutter et al., 2001). Indeed, these authors could show that *in vitro* an excess of NADH stimulates DNA binding of the NPAS2/MOP4–BMAL1 and CLK–BMAL1 dimers, whereas the oxidized NAD favors homodimerization of BMAL1, which cannot induce transcription. Because the redox changes between NAD and NADH involve electron transfer, this also provoked speculations about the mechanism of mCRY function. Upon interaction of NPAS2 with CRY, its cofactor Flavin Adenin Dinucleotide (FAD) could receive an electron from the NADH bound to NPAS2, thereby abolishing NPAS2:BMAL1 binding (Schibler et al., 2001). Although a tempting model, a different study questioned that the concentrations of NAD and NADH used in the Rutter study were within the physiologic range (Zhang et al., 2002). Moreover, conserved mCRY aminoacids thought to be involved in electron transfer mechanisms can be replaced without affecting the repressor function of CRY proteins *in vitro* (Froy et al., 2002). Therefore, one has to wait for *in vivo* data, for example, involving mutations in the putative NAD binding sites of NPAS2/MOP4 and CLOCK, to validate this kind of regulation. It would also be interesting to test if restricted feeding is able to entrain molecular rhythms in NPAS2-deficient mice.

Neuropeptides and Growth Factors Rhythmically Released from the SCN. The output gene *vasopressin* is rhythmically expressed in the SCN (Uhl and Reppert, 1986). The encoded neuropeptide is also released in a rhythmic fashion from SCN neurons, suggesting a role in transmitting temporal information from the central clock to peripheral oscillators (Reppert et al., 1987), similar to PDF in flies. This gene seems to be a direct target of the CLK–BMAL1 dimer (Jin et al., 1999; Fig. 1), and is not involved in the core molecular feedback loop: a mutant rat line with an internal deletion in the *vasopressin* gene still exhibits oscillations of *vasopressin* RNA (Uhl and Reppert, 1986). Moreover, *vasopressin* is not required for the expression of several circadian rhythms (reviewed by Reppert and Weaver, 2001). Hence, the precise function of this neuropeptide within the circadian system is still elusive.

The last example involves a factor that has been elegantly demonstrated to build a link between molecular rhythms in the SCN and locomotor rhythmicity. In a straightforward approach Kramer et al. (2001) tested the effects of constantly supplying factors that are normally rhythmically released from the SCN. They focussed their attention on the main projection area of the SCN, called the SPZ (subparaventricular zone), flanking the third ventricle of the hypothalamus. Constant infusion of TGF-α (transforming growth factor-α) into this region of hamster brains led to prominent repression of locomotor activity. TGF-α RNA expression is under circadian control in the SCN, with a peak phase during the day, suggesting that release of this peptide normally suppresses activity during the day in nocturnal mammals (Kramer et al., 2001; Fig. 3). Because TGF-α was known to signal through the EGF–receptor (EGFR) pathway, and the EGFR is also expressed in the SPZ, the authors also tested the behavior of an EGFR mutant mouse strain. A hypomorphic mutant needed to be analyzed (Table 3), because the null mutation results in lethality. This probably explains why the mutant mice entrained nicely to LD cycles and were rhythmic in DD. Nevertheless, they showed a behavioral phenotype because daytime activity was significantly increased compared with control animals. This light-specific phenotype prompted the authors to check if the EGFR might be involved in a phenomenon known as masking response to light. Light induces an immediate halt of locomotor activity, regardless of the time of exposure and independent of a functional clock and the SCN (e.g., it is still observed in *mCry* double knockouts). This masking response is severely impaired in EGFR mutant mice, suggesting that circadian light entrainment (which is normal in the same animals) and masking differentially affect the SPZ (Fig. 3). This implies that the masking-relevant ligands originate from outside the SCN, and in fact, the authors showed that both EGF and TGF-α are expressed in cells of the inner retina. The cells in question likely correspond to a type of glia cells that give rise to the retinohypothalamic tract (RHT), which is the relevant eye-SCN connection for circadian entrainment. In addition, the RHT projects to the SPC (Johnson et al., 1988), leading to the attractive speculation that masking is mediated by direct light-signaling via TGF-α or EGF to the EGFR in the SPC (Kramer et al., 2001). This is a very nice model, explaining both clock-regulated control of behavior via TGF-α release in the SCN and direct regulation by light through a connection from the eye to the SPC (Fig. 3).

In summary, aspects of TGF-α function are reminiscent of PDF signaling in flies. In both cases the disruption of rhythmic peptide release has drastic effects on locomotor behavior. It is likely that completely abolishing TGF-α signaling would have similarly severe consequences on free-running behavior

as the *pdf*^{01} mutation (Table 2). In this regard, it is interesting that panneural overexpression of *pdf* also impairs locomotor rhythms in flies (Helfrich-Förster et al., 2000). This treatment resulted in flies that showed more night activity compared to wild-type animals in LD cycles, and led to a general increase of activity and loss of rhythmicity in DD. In contrast to TGF-α, PDF seems to be an activator of locomotor activity (Fig. 3). However, activity was not generally lowered in *pdf*^{01} animals (Renn et al., 1999), probably because other neuropeptides involved in this process also activate behavior (Taghert et al., 2001).

LIGHT-INPUT MECHANISMS IN FLIES AND MAMMALS

Although many Zeitgebers like temperature, food (see above), and social cues are able to entrain circadian clocks, light is the most important one, probably because in earth's history organisms have always been exposed to the daily light–dark changes. Several excellent reviews about how light reaches the clock structures and influences clock molecules have been published, also including ones that compare insects with mammals (e.g., Hall, 2000; Devlin and Kay, 2001; Lucas et al., 2001b; Foster and Helfrich-Förster, 2001; Zordan et al., 2001). Therefore, I will present only a brief summary of the (*my*) current view of photic entrainment in both systems.

Multiple Pathways Contribute to Light Entrainment in Flies

Isolation of the *cry*^{b} mutation revealed that *cryptochrome* is a crucial element for photic entrainment in flies and most likely even a photoreceptor (Emery et al., 1998; Stanewsky et al., 1998; Ceriani et al., 1999). That CRY proteins could function as photoreceptors is not surprising, given their homology to bacterial photolyases, which harvest light energy to repair DNA damage (reviewed by Sancar, 2000). Photolyases contain two cofactors—FAD and a pterin (in most species). The latter serves as photoantennae, whereas FAD receives the energy from the excited pterin, and donates an electron to the DNA, to repair the damage. Interestingly, *cry*^{b} encodes an amino acid change in one of the highly conserved residues thought to contact the FAD cofactor, suggesting that CRY could receive light energy similar to photolyase (Stanewsky et al., 1998). Favoring this hypothesis, Froy et al. (2002) demonstrated that FAD binding residues as well as other conserved CRY amino acids known to play a role in electron transfer in photolyase, are crucial for CRY-mediated light responses *in vitro*.

Mutant *cry*^{b} flies are unable to adjust their behavior to brief light pulses, and behave rhythmically under constant illumination, which causes arrhythmicity in wild type flies (Stanewsky et al., 1998; Emery et al., 2000). Although these findings demonstrate the importance of CRY for the light input, *cry*^{b} flies behave fairly normal under more natural LD entrainment conditions, and even when performing artificial jet-lag experiments (by applying shifted LD cycles) (Stanewsky et al., 1998). This pointed to the existence of additional photoreceptors, and indeed, it could be shown that retinal and other extraretinal photoreceptors contribute to photic entrainment (Stanewsky et al., 1998; Helfrich-Förster et al., 2001; reviewed by Shafer, 2001).

Photic Resetting in Flies Is Mediated by TIM Degradation

CRY and TIM probably directly interact in a light-dependent manner. This causes TIM degradation that resets the molecular pacemaker (e.g., Suri et al., 1998; Yang et al., 1998; Ceriani et al., 1999; Lin et al., 2001). Consistent with this, TIM protein is constitutively expressed in most cells of *cry*^{b} flies, including a subset of the pacemaker neurons in the brain (Stanewsky et al., 1998; Helfrich-Förster et al., 2001). In fact, *cry* is coexpressed with other clock genes and *pdf* within these pacemaker neurons, suggesting that these important cells contain a circadian photoreceptor, the central clock-works, and output functions (Emery et al., 2000). Yet, in another subset of these pacemaker neurons—in fact, the ones projecting to the dorsal brain area, where rhythmic PDF release is thought to control locomotor behavior—TIM cycling and its degradation by light is not impaired by *cry*^{b} pointing to a contribution of other pigments (Stanewsky et al., 1998; Kaneko et al., 2000; Helfrich-Förster et al., 2001; Ivanchenko et al., 2001). Only after removal of all other retinal and extraretinal photoreceptors can *cry*^{b} flies no longer entrain to LD cycles. Consistent with this behavioral phenotype, TIM cycling in all pacemaker neurons of these doubly defective flies can no longer be synchronized by light (Helfrich-Förster et al., 2001). This clearly suggests that, in addition to CRY yet another molecule is able to transmit light sensitivity to TIM, and future research will certainly focus on the identification of this factor.

Multiple Pathways Contribute to Light-Entrainment in Mammals

The principle route how light-information from the eye reaches the SCN is via the RHT. Although the eyes are required for photic entrainment of circadian behavior and melatonin production, the rods and cones in the mammalian retina are not, suggesting that crucial circadian photoreceptors are located in the inner retina (Freedman et al., 1999; Lucas et al., 1999). Pupillary constriction in response to light exposure also occurs independent of rods and cones (Lucas et al., 2001a). An action spectrum of this response indicates that it is mediated by an opsin-type photoreceptor with maximum sensitivity around 479 nm, suggesting that the circadian responses could also be mediated by this pigment (Lucas et al., 2001a). A promising candidate is melanopsin, which was found to be expressed in the mammalian inner retina (Provencio et al., 2000). In addition, several studies show that the melanopsin pigment is expressed in a subset of retinal ganglion cells that give rise to the RHT in the inner retina (Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2002) and probably also express TGF-α and EGF. Interestingly, this subset of cells was also shown to be intrinsically light sensitive, with the spectral sensitivity and other properties of their light-response matching those for photic entrainment (Berson et al., 2002; Hattar et al., 2002). Although not a final proof, this strongly suggests that melanopsin is a circadian photoreceptive pigment in retinal ganglion cells, and one eagerly awaits whether this opsin is able to form a functional photopigment.

Other candidates discussed as circadian photopigments are the mCRYs. Although firmly established as clock factors in the SCN and peripheral clocks, there is evidence that they could as well function as light sensors in the retina. Like melanopsin, *mCry1* and *mCry2* are expressed in the ganglion cell layer and inner nuclear layer of the retina, regions that remain intact in rodless and coneless mice (Miyamoto and Sancar, 1998). Although *mCry* double-knockouts are behaviorally arrhythmic in free running conditions, they show a diurnal pattern of activity under LD conditions (van der Horst et al., 1999). This behavior was shown to be due to a masking response to light (van der Horst et al., 1999; Mrosovsky 2001). Interestingly, this masking response is lost when the *mCry* double mutant is combined with a mutation that removes the rods (Selby et al., 2000). Because rodless mice entrain nicely to LD cycles, this means that both mCRYs and rods contribute to masking behavior, and principally could also influence photic entrainment. Therefore, similar as in flies, it seems that multiple photoreceptive pigments share the task of light detection in order to synchronize the SCN clock.

Light Induces *mPer1* and *mPer2* Expression in the SCN

Induction of the *mPer1* and *mPer2* genes in the SCN after light exposure has been suggested to mediate molecular and behavioral resetting of the circadian rhythms in mammals (reviewed by Reppert and Weaver, 2001). Evidence for this stems from experiments where *mPer1* antisense RNA was injected into the mouse brain (Akiyama et al., 1999). Normally, light pulses given in the early night elicit phase delays of activity onset, but this behavioral response was inhibited in the injected animals. Although this suggested a specific role of *mPer1* in mediating phase *delays*, mice deficient for this gene ($mPer1^{Brdm1}$) are not able to perform phase *advances* after exposure to light pulses in the late night (Albrecht et al., 2001). Although *mPer1* is also light-induced in the early night, $mPer1^{Brdm1}$ mice show normal behavioral phase shifts (=delays) after such pulses (Albrecht et al., 2001). Even more puzzling, a different *mPer1* mutant ($mPer1^{null}$) was not impaired in either phase delays or advances (Cermakian et al., 2001). The opposing results are probably explained by the different approaches applied (antisense vs. knockout) and by the different length of the light pulses applied (15 min for $mPer1^{Brdm1}$ and 30 min for $mPer1^{null}$ mice). Moreover, the light intensity could have varied between the two studies, and the different nature of the two alleles, or their various genetic backgrounds could also contribute to the observed behavioral differences.

Although contradictory, the results indicate that *mPer1* induction is linked to light-induced behavioral responses. The question remains whether *mPer1* plays a role in both phase advances and delays, which would be more consistent with *mPer1* light induction occurring in the advance and the delay zone (Reppert and Weaver, 2001). The picture seems clearer for *mPer2*. This gene is only induced after light pulses administered in the early night, and consistently behavioral phase delays are abolished in *mPer2* mutant mice (Albrecht et al., 2001; Reppert and Weaver, 2001). Moreover, patients with a mutation in *hPer2* develop FASPS (Table 3), very likely because they are not able to perform a daily resetting (delay) of their fast running clock.

Light-induction of the *mPer* genes was also challenged to prove if mCRYs are involved in the light-

input pathway. Again, the results obtained are contradictory, and further studies are necessary to prove the point. Although one study did not see any decrement or alteration of *mPer1* and *mPer2* induction in the SCN of *cry* double knockout mice (Okamura et al., 1999), two other reports (Thresher et al. 1998; Vitaterna et al.; 1999) reported effects of the single mutants as well as of the *mCry* double mutants on light-induced *mPer1* expression. Both single *mCry* mutants exhibited a *decreased* sensitivity of *mPer1* induction, which was in the case of $mCry2^{-/-}$ correlated with *increased* behavioral phase delays after light pulses in the early night (Thresher et al., 1998, Vitaterna et al., 1999). Interestingly, a similar phenotype as for $mCry2^{-/-}$ mice was observed in animals mutant for a receptor of the neuropeptide PACAP, which is expressed in retinal ganglion cells and thought to be one transmitter of the light signal via the RHT to the SCN (Hannibal et al., 2000). After application of light pulses in the early night, *mPer1* induction in the PACAP receptor mutants was blunted and behavioral phase delays were increased, suggesting that *mCry2* function in the light-input pathway could be linked to PACAP signaling. Although *mPer1* induction was also blunted in the *mCry* double knockouts, *mPer2* induction was not affected (Vitaterna et al., 1999). Thus, it seems reasonably safe to assume that at least *mPer2* induction does not depend on *mCry* function (Okamura et al., 1999; Vitaterna et al., 1999). Likely, the contradictory results with respect to *mPer1* induction are again caused by subtle differences between the experimental protocols or genetic backgrounds of the animals applied. There is, however, one additional argument in favor of *mCry*s being involved in the light induction of *mPer1*. Retinal-depleted mice—which should be devoid of functional opsins—show normal *mPer1* and *mPer2* induction in the SCN, suggesting the existence of nonopsin photoreceptors in the eye (Thompson et al., 2001). Yet it is still possible that residual retinal leads to some functional opsins in these mice, which is also suggested by the normal *mPer2* induction in the retinal-depleted mice.

Light Induces Rapid Degradation of BMAL1

A different line of evidence implicates the BMAL1 protein in light resetting. The amount of this protein (not RNA, see Abe et al., 1998) in the SCN is significantly reduced in response to light pulses during the night, especially in the early night, suggesting a role in mediating phase delays (Tamaru et al., 2000). Consistent with this hypothesis, substances that mimic photic phase shifts when applied to the SCN (glutamate and NMDA) also result in a reduction of BMAL1 levels. Because the kinetics of *mPer1* and *mPer2* induction is roughly similar to that of BMAL1 disappearance, it is difficult to predict whether the two events are causally linked to each other. Moreover, not the absolute BMAL1 levels, but rather its phosphorylation status seems to be correlated with transcriptional activity of the CLK–BMAL1 dimer (Fig. 1). So it is formally possible that the reduction of BMAL1 somehow induces *mPer* expression. If true, the light resetting mechanism of circadian clock molecules in flies and mammals would be remarkably similar after all, both involving rapid disappearance of a central clock protein.

In summary, it seems very likely that both induction of *mPer1* and *mPer2* and the reduction of BMAL1 levels are intimately tied to light pulse-induced behavioral resetting (but see Hannibal et al., 2000). The anatomical location of the circadian photoreceptors almost certainly includes the retinal ganglion cells which express melanopsin and *mCry*s. Given the contradictory results found in the literature it is possible that both, opsin- and CRY-mediated light reception contributes to synchronization of the SCN pacemaker. This would also be similar to the situation in flies, where multiple distinct photoreceptors (both in terms of anatomical location and pigment type) mediate the task of circadian photoreception (Stanewsky, et al., 1998; Helfrich-Förster et al., 2001). This complexity is probably necessary to ensure the detection of twilight, which is characterized by drastic changes in both light-quality and quantity, and has been shown to be most effective in entraining circadian clocks (Zordan et al., 2001).

I thank the members of my lab and Alois Hofbauer for comments on the manuscript.

REFERENCES

Abe H, Honma S, Namihira M, Tanahashi Y, Ikeda M, Honma K. 1998. Circadian rhythm and light responsiveness of *BMAL1* expression, a partner of mammalian clock gene *Clock*, in the suprachiasmatic nucleus of rats. Neurosci Lett 258:93–96.

Adams MD, et al. 2000. The genome sequence of *Drosophila melanogaster*. Science 287:2185–2195.

Akashi M, Nishida E. 2000. Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev 14:645–649.

Akashi M, Tsuchiya Y, Yoshino T, Nishida E. 2002. Control of intracellular dynamics of mammalian period pro-

teins by casein kinase 1ϵ (CKIϵ) and CKIδ in cultured cells. Mol Cell Biol 22:1693–1703.

Akiyama M, Kouzu Y, Takahashi S, Wakamatsu H, Moriya T, Maetani M, Watanabe S, Tei H, Sakaki Y, Shibata S. 1999. Inhibition of light- or glutamate-induced *mPer1* expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. J Neurosci 19:1115–1121.

Albrecht U, Zheng B, Larkin D, Sun ZS, Lee CC. 2001. *mPer1* and *mPer2* are essential for normal resetting of the circadian clock. J Biol Rhythms 16:100–104.

Allada R, White NE, So WV, Hall JC, Rosbash M. 1998. A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93:791–804.

Antoch M, Soog E, Chang A, Vitaterna M, Zhao Y, Wilsbacher L, Sangoram A, King D, Pinto L, Takahashi J. 1997. Functional identification of the mouse circadian *CLOCK* gene by transgenic BAC rescue. Cell 89:655–667.

Bae K, Lee C, Sidote D, Chang K-Y, Edery I. 1998. Circadian regulation of a *Drosophila* homolog of the mammalian *Clock* gene: PER and TIM function as positive regulators. Mol Cell Biol 18:6142–6151.

Bae K, Lee C, Hardin PE, Edery I. 2000. dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. J Neurosci 20:1746–1753.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. 2001. Differential functions of *mPer1, mPer2,* and *mPer3* in the SCN circadian clock. Neuron 30:525–536.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93:929–937.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, Schibler U. 2000a. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289:2344–2347.

Balsalobre A, Marcacci L, Schibler U. 2000b. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. Curr Biol 10:1291–1294.

Bao S, Rihel J, Bjes E, Fan J-Y, Price JL. 2001. The *Drosophila double-time^S* mutation delays the nuclear accumulation of *period* protein and affects the feedback regulation of *period* mRNA. J Neurosci 21:7117–7126.

Baylies MK, Bargiello TA, Young MW 1987. Changes in abundance and structure of the *per* gene product can alter periodicity of the *Drosophila* clock. Nature 326:390–392.

Beaver LM, Gvakharia BO, Vollintine TS, Hege DM, Stanewsky R, Giebultowicz JM. 2002. Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. Proc Natl Acad Sci USA 99: 2134–2139.

Belvin MP, Zhou H, Yin JCP. 1999. The *Drosophila* dCREB2 gene affects the circadian clock. Neuron 22: 777–787.

Berson DM, Dunn FA, Takao M. 2002. Phototransduction by retinal ganglion cells that set the circadian clock. Science 295:1070–1073.

Blau J, Young MW. 1999. Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell 99:661–671.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Bruce VG. 1972. Mutants of the biological clock in *Chlamydomonas reinhardtii.* Genetics 70:537–548.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA. 2000. *Mop3* is an essential component of the master circadian pacemaker in mammals. Cell 103: 1009–1017.

Bünning E. 1935. Zur Kenntnis der erblichen Tagesperiodizität bei den Primärblättern von *Phaseolus multiflorus.* Jb wiss Bot 81:411–418.

Camacho F, Cilio M, Guo Y, Virshup DM, Patel K, Khorkova O, Styren S, Morse B, Yao Z, Keesler GA. 2001. Human casein kinase Iδ phosphorylation of human circadian clock proteins period 1 and 2. FEBS Lett 489: 159–165.

Ceriani MF, Darlington TK, Staknis D, Más P, Petti AA, Weitz CJ, Kay SA. 1999. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553–556.

Cermakian N, Monaco L, Pando MP, Dierich A, Sassone-Corsi P. 2001. Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. EMBO J 20:3967–3974.

Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW. 2001. Circadian regulation of gene expression systems in the *Drosophila* head. Neuron 32:657η671.

Crews ST, Fan C-M. 1999. Remembrance of things PAS: regulation of development by bHLH-PAS proteins. Curr Opin Genet Dev 9:580–587.

Curtin KD, Huang ZJ, Rosbash M. 1995. Temporally regulated nuclear entry of the *Drosophila* period protein contributes to the circadian clock. Neuron 14:363–372.

Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U. 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14:2950–2961.

Darlington T, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves T, Weitz C, Takahashi J, Kay SA. 1998. Closing the circadian loop: CLOCK-induced transcription of its own inhibitors, *per* and *tim.* Science 280:1599–1603.

DeMairan J. 1729. Observation botanique. Hist Acad R Sci 35–36.

Devlin PF, Kay SA. 2001. Circadian photoreception. Annu Rev Physiol 63:677–694.

Dushay MS, Rosbash M, Hall JC. 1992. Mapping the *Clock* mutation rhythm mutation to the *period* locus of *Drosophila melanogaster* by germline transformation. J Neurogenet 8:173–179.

Ebisawa T, Uchiyama M, Kajimura N, Mishima K, Kamei Y, Katoh M, Watanabe T, Sekimoto M, Shibui K, Kim K, Kudo Y, Ozeki Y, Sugishita M, Toyoshima R, Inoue Y, Yamada N, Nagase T, Ozaki N, Ohara O, Ishida N, Okawa M, Takahashi K, Yamauchi T. 2001. Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. EMBO Rep 2:342–346.

Edery I, Zwiebel LJ, Dembinska ME, Rosbash M. 1994. Temporal phosphorylation of the *Drosophila period* protein. Proc Natl Acad Sci USA 91:2260–2264.

Emery P, So WV, Kaneko M, Hall JC, Rosbash M. 1998. CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679.

Emery P, Stanewsky R, Helfrich-Förster C, Emery-Le M, Hall JC, Rosbash M. 2000. *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron 26:493–504.

Falvey E, Fleury-Olela F, Schibler U. 1995. The rat hepatic leukemia factor (HLF) gene encodes two transcriptional activators with distinct circadian rhythms, tissue distributions and target preferences. EMBO J 14:4307–4317.

Feldman JF, Hoyle MN. 1973. Isolation of circadian clock mutants of *Neurospora crassa.* Genetics 75:605–613.

Fonjallaz P, Ossipow V, Wanner G, Schibler U. 1996. The two PAR leucine zipper proteins, TEF and DBP, display similar circadian and tissue-specific expression, but have different target promoter preferences. EMBO J 15:351–362.

Foster RG, Helfrich-Förster C. 2001. The regulation of circadian clocks by light in fruitflies and mice. Philos Trans R Soc Lond B 356:1779–1789.

Freedman MS, Lucas RJ, Soni B, von Schanz M, Munoz M, David-Gray Z, Foster RG. 1999. Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 284:502–504.

Froy O, Chang DC, Reppert SM. Redox potential. 2002. Differential roles in dCRY and mCRY1 functions. Curr Biol 12:147–152.

Garcia JA, Zhang D, Estill SJ, Michnoff C, Rutter J, Reick M, Scott K, Diaz-Arrastia R, McKnight SL. 2000. Impaired cued and contextual memory in NPAS2-deficient mice. Science 288:2226–2230.

Gekakis N, Saez L, Delahaye-Brown A-M, Myers MP, Sehgal A, Young MW, Weitz CJ. 1995. Isolation of timeless by PER protein interaction: defective interaction between TIMELESS protein and long-period mutant PER^{L}. Science 270:811–815.

Gekakis N, Staknis, D, Nguyen HB, Davis FC, Wilsbacher DP, King DP, Takahashi JS, Weitz CJ. 1998. Role of the Clock protein in the mammalian circadian mechanism. Science 280:1564–1569.

Giebultowicz JM. 2001. Peripheral clocks and their role in circadian timing: insights from insects. Philos Trans R Soc Lond B 356:1791–1799.

Glossop NRJ, Lyons LC, Hardin PE. 1999. Interlocked feedback loops within the *Drosophila* circadian oscillator. Science 286:766–768.

Hall JC. 2000. Cryptochromes: sensory reception, transduction, and clock functions subserving circadian systems. Curr Opin Neurobiol 10:456–466.

Hamblen MJ, White NE, Emery PTJ, Kaiser K, Hall JC. 1998. Molecular and behavioral analysis of four *period* mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 149:165–178.

Hamblen-Coyle M, Konopka RJ, Zwiebel LJ, Colot HV, Dowse HB, Rosbash M, Hall JC. 1989. A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. J Neurogenet 5:229–256.

Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug J. 2002. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J Neurosci 22:RC191.

Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, Fahrenkrug J. 2000. Dissociation between light-induced phase shift of the circadian rhythm and clock gene expression in mice lacking the pituitary adenylate cyclase activating polypeptide type 1 receptor. J Neurosci 21: 4883–4890.

Hara R, Wan K, Wakamatsu H, Aida R, Moriya T, Akiyama M, Shibata S. 2001. Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells 6:269–278.

Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA. 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110–2113.

Hattar S, Liao H-W, Takao M, Berson DM, Yau K-W. 2002. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295:1065–1070.

Helfrich-Förster C. 2003. The neuroarchitecture of the circadian clock in the *Drosophila* brain. Microsc Res Technol (in press).

Helfrich-Förster C, Täuber M, Park JH, Mühlig-Versen M, Schneuwly S, Hofbauer A. 2000. Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. J Neurosci 20:3339–3353.

Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R. 2001. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261.

Hogenesch JB, Gu YZ, Jain S, Bradfield CA. 1998. The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc Natl Acad Sci USA 95:5474–5479.

Hogenesch JB, Gu YZ, Moran SM, Shimomura K, Radcliffe LA, Takahashi JS, Bradfield CA. 2000. The basic helix-loop-helix-PAS protein MOP9 is a brain-specific heterodimeric partner of circadian and hypoxia factors. J Neurosci 20:RC83.

Ishida Y, Yagita K, Fukuyama T, Nishimura M, Nagano M, Shigeyoshi Y, Yamaguchi S, Komori T, Okamura H. 2001. Constitutive expression and delayed light response of casein kinase Iϵ and Iδ mRNAs in the mouse suprachiasmatic nucleus. J Neurosci Res 64:612–616.

Ivanchenko M, Stanewsky R, Giebultowicz JM. 2001. Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central clocks. J Biol Rhythms 16:205–215.

Jackson, FR. 1983. The isolation of biological rhythm mutations on the autosomes of *Drosophila melanogaster*. J Neurogenet 1:3–15.

Jackson FR, Schroeder AJ, Roberts MA, McNeil GP, Kume K, Akten B. 2001. Cellular and molecular mechanisms of circadian control in insects. J Insect Physiol 47:833–842.

Jin X, Shearman LP, Weaver DR, Zylka MJ, De Vries GJ, Reppert SM. 1999. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96:57–68.

Johnson RF, Morin LP, Moore RY. 1988. Retinohypothalamic projections in the hamster and rat demonstrated using cholera toxin. Brain Res 462:301–312.

Jones CR, Campbell SS, Zone SE, Cooper F, DeSano A, Murphy PJ, Jones B, Czajkowski L, Ptacek LJ. 1999. Familial advanced sleep-phase syndrome: a short-period circadian rhythm variant in humans. Nat Med 5:1062–1065.

Kaneko M, Hamblen MJ, Hall JC. 2000. Involvement of the *period* gene in developmental time-memory: effect of the per^{Short} mutation on phase shifts induced by light pulses delivered to *Drosophila* larvae. J Biol Rhythms 15:13–30.

Katzenberg D, Young T, Finn L, Lin L, King D, Takahashi JS, Mignot E. 1998. A CLOCK polymorphism associated with human diurnal preference. Sleep 21:569–576.

Keesler GA, Camacho F, Guo Y, Virshup D, Mondadori C, Yao Z. 2000. Phosphorylation and destabilization of human *period* I clock protein by human casein kinase I epsilon. Neuroreport 11:951–955.

King D, Zhao Y, Sangoram A, Wilsbacher, L, Tanaka M, Antoch M, Steeves T, Vitaterna M, Kornhauser J, Lowrey P, Turek F, Takahashi J. 1997. Positional cloning of the mouse circadian *CLOCK* gene. Cell 89:641–653.

Kloss B, Price JL, Saez L, Blau J, Rothenflluh A, Young MW. 1998. The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iϵ. Cell 94:97–107.

Kloss B, Rothenfluh A, Young MW, Saez L. 2001. Phosphorylation of PERIOD is influenced by cycling physical associations of DOUBLE-TIME, PERIOD, and TIMELESS in the *Drosophila* clock. Neuron 30:699–706.

Konopka RJ, Benzer S. 1971. Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116.

Konopka RJ, Smith RF, Orr D. 1991. Characterization of *Andante*, a new *Drosophila* clock mutant, and its interactions with other clock mutants. J Neurogenet 7:103–114.

Konopka RJ, Hamblen-Coyle MJ, Jamison CF, Hall JC. 1994. An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. J Biol Rhythms 9:189–216.

Kramer A, Yang F-C, Snodgrass P, Li X, Scammell TE, Davis FC, Weitz CJ. 2001. Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. Science 294:2511–2515.

Krishnan B, Dryer SE, Hardin PE. 1999. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. Nature 400:375–378.

Krishnan B, Levine JD, Lynch KS, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE. 2001. A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature 411:313–317.

Kuhlman SJ, Quintero JE, McMahon DG. 2000. GFP fluorescence reports *Period1* circadian gene regulation in the mammalian biological clock. Neuroreport 11:1479–1482.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM. 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian feedback loop. Cell 98: 193–205.

Kyriacou CP, Rosato E. 2000. Squaring up the E-box. J Biol Rhythms 15:483–490.

Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, Schibler U, Bonfils C. 1999. Circadian expression of the steroid 15 α-hydroxylase (*Cyp2a4*) and coumarin 7-hydroxylase (*Cyp2a5*) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. Mol Cell Biol 19:6488–6499.

Lee C, Bae K, Edery I. 1998. The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation and interactions with the PER-TIM complex. Neuron 21:857–867.

Lee C, Bae K, Edery I. 1999. PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. Mol Cell Biol 19:5316–5325.

Lee C, Etchegaray J-P, Cagampang FRA, Loudon ASI, Reppert SM. 2001. Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855–867.

Levine JD, Casey CI, Kalderon DD, Jackson FR. 1994. Altered circadian pacemaker functions and cyclic AMP rhythms in the *Drosophila* learning mutant *dunce*. Neuron 13:967–974.

Lin F-J, Song W, Meyer-Bernstein E, Naidoo N, Sehgal A. 2001. Photic signaling by cryptochrome in the *Drosophila* circadian system. Mol Cell Biol 21:7287–7294.

Lopez-Molina L, Conquet F, Dubois-Dauphin M, Schibler U. 1997. The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. EMBO J 16:6762–6771.

Lowrey PL, Shimomura Z, Antoch MP, Yamazaki, S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. 2000. Positional syntenic cloning and functional characterization of a mammalian circadian mutation *tau*. Science 288:483–491.

Lucas RJ, Douglas RH, Foster RG. 2001a. Characterization of an ocular photopigment capable of driving pupillary constriction in mice. Nat Neurosci 4:621–626.

Lucas RJ, Freedman MS, Lupi D, Munoz M, David-Gray ZK, Foster RG. 2001b. Identifying the photoreceptive inputs to the mammalian circadian system using transgenic and retinally degenerate mice. Behav Brain Res 125:97–102.

Lucas RJ, Freedman MS, Munoz M, Garcia-Fernandez J-M, Foster RG. 1999. Regulation of the mammalian pineal by non-rod, non cone, ocular photoreceptors. Science 284: 505–507.

Luetteke NC, Phillips HK, Qiu TH, Copeland NG, Earp HS, Jenkins NA, Lee DC. 1994. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev 8:399–413.

Majercak J, Kalderon D, Edery I. 1997. *Drosophila melanogaster* deficient in protein kinase A manifests behavior-specific arrhythmia but normal clock function. Mol Cell Biol 17:5915–5922.

Martinek S, Inonog S, Manoukian AS, Young MW. 2001. A role for the segment polarity gene *shaggy/*GSK-3 in the *Drosophila* circadian clock. Cell 105:769–779.

Matsumoto A, Tomioka K, Chiba Y, Tanimura T. 1999. tim^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343–4354.

McDonald MJ, Rosbash M. 2001. Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107:567–578.

McNamara P, Seo S-b, Rudic RD, Sehgal A, Chakravarti D, FitzGerald GA. 2001. Regulation of CLOCCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell 105:877–889.

McNeil GP, Zhang X, Genova G, Jackson FR. 1998. A molecular rhythm mediating circadian clock output in *Drosophila*. Neuron 20:297–303.

Mitsui S, Yamaguchi S, Matsuo T, Ishida Y, Okamura H. 2001. Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. Genes Dev 15:995–1006.

Miyamoto Y, Sancar A. 1998. Vitamin B_2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc Natl Acad Sci USA 95:6097–6102.

Miyazaki K, Mesaki M, Ishida N. 2001. Nuclear entry mechanism of rat PER (rPER2): role of rPER2 in nuclear localization of CRY protein. Mol Cell Biol 21:6651–6659.

Mrosovsky N. 2001. Further characterization of the phenotype of *mCry1/mCry2*-deficient mice. Chronobiol Int 18: 613–625.

Murata T, Matsumoto A, Tomioka K, Chiba Y. 1995. *Ritsu*: a rhythm mutant from a natural population of *Drosophila melanogaster.* J Neurogenet 9:239–249.

Naidoo N, Song W, Hunter-Ensor M, Sehgal A. 1999. A role for the proteasome in the light response of the timeless clock protein. Science 285:1737–1741.

Newby LM, Jackson FR. 1993. A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. Genetics 135:1077–1090.

Newby LM, Jackson FR. 1996. Regulation of a specific circadian clock output pathway by Lark, a putative RNA-binding protein with repressor activity. J Neurobiol 31: 117–128.

Newby LM, White L, Dibartolomeis SM, Walker BJ, Dowse HB, Ringo JM, Khuda N Jackson FR. 1991. Mutational analysis of the *Drosophila miniature-dusky* (*m-dy*) locus: effects on cell size and circadian rhythms. Genetics 128:571–582.

Okada T, Sakai T, Murata T, Kako K, Sakamoto K, Ohtomi M, Katsura T, Ishida N. 2001. Promoter analysis for daily expression of *Drosophila timeless* gene. Biochem Biophys Res Commun 283:577–582.

Okamura H, Miyake S, Sumi Y, Yamaguchi S, Yasui A, Muijtjens M, Hoeijmakers JH, van der Horst GT. 1999. Photic induction of *mPer1* and *mPer2* in cry-deficient mice lacking a biological clock. Science 286:2531–2534.

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH. 1998. Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci USA 95:8660–8664.

Park JH, Hall JC. 1998. Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster.* J Biol Rhythms 13:219–228.

Park JH, Helfrich-Förster C, Lee G, Liu L, Rosbash M, Hall JC. 2000a. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila.* Proc Natl Acad Sci USA 97:3608–3613.

Park SK, Sedore SA, Cronmiller C, Hirsh J. 2000b. Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. J Biol Chem 275:20588–20596.

Pittendrigh CS. 1967. Circadian systems. 1. The driving oscillation and its assay in *Drosophila pseudoobscura*. Proc Natl Acad Sci USA 58:1762–1767.

Plautz JD, Kaneko M, Hall JC, Kay SA. 1997. Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278:1632–1635.

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW. 1998. *double-time* is a new *Drosophila*

clock gene that regulates PERIOD protein accumulation. Cell 94:83–95.

Provencio I, Rodriguez IR, Jiang G, Pär Hayes W, Moreira EF, Rollag MD. 2000. A novel human opsin in the inner retina. J Neurosci 20:600–605.

Provencio I, Rollag MD, Castrucci AM. 2002. Photoreceptive net in the mammalian retina. Nature 415:493.

Ralph MR, Menaker M. 1988. A mutation of the circadian system in golden hamsters. Science 241:1225–1227.

Reick M, Garcia JA, Dudley C, McKnight SL. 2001. NPAS2: an analog of Clock operative in the mammalian forebrain. Science 293:506–509.

Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH. 1999. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802.

Reppert SM. 1998. A clockwork explosion! Neuron 21:1–4.

Reppert SM, Schwartz WJ, Uhl GR. 1987. Arginine vasopressin: a novel peptide rhythm in cerebrospinal fluid. Trends Neurosci 10:76–80.

Reppert SM, Weaver DR. 2001. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63: 647–676.

Ripperger JA, Shearman LP, Reppert SM, Schibler U. 1999. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Genes Dev 14:679–689.

Rørth P. 1996. A modular misimpression screen in *Drosophila* detecting tissue-specific phenotypes. Proc Natl Acad Sci USA 93:12418–12422.

Rørth P, Szabo K, Bailey A, Laverty T, Rehm J, Rubin GM, Weigmann K, Milan M, Benes V, Ansorge W, Cohen SM. 1998. Systematic gain-of-function genetics in *Drosophila*. Development 125:1049–1057.

Rothenfluh A, Abodeely M, Price JL, Young MW. 2000a. Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. Genetics 156:665–675.

Rothenfluh A, Abodeely M, Young MW. 2000b. Short-period mutations of *per* affect a *double-time*-dependent step in the *Drosophila* circadian clock. Curr Biol 10: 1399–1402.

Rothenfluh A, Young MW, Saez L. 2000c. A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron 26:505–514.

Rouyer F, Rachidi M, Pikielny C, Rosbash M. 1997. A new gene encoding a putative transcription factor regulated by the *Drosophila* circadian clock. EMBO J 16:3944–3954.

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC. 1998. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless.* Cell 93:805–814.

Rutila JE, Zeng H, Le M, Curtin KD, Hall JC, Rosbash M. 1996. The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron 17:921–929.

Rutter J, Reick M, Wu LC, McKnight SL. 2001. Regulation of Clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 293:510–514.

Sancar A. 2000. Cryptochrome: the second photoactive pigment in the eye and its role in circadian photoreception. Annu Rev Biochem 69:31–67.

Sakamoto K, Nagase T, Fukui H, Horikawa K, Okada T, Tanaka H, Sato K, Miyake Y, Ohara O, Kako K, Ishida N. 1998. Multitissue circadian expression of rat *period* homolog (*rPer2*) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. J Biol Chem 273:27039–27042.

Sarov-Blat L, So WV, Liu L, Rosbash M. 2000. The *Drosophila takeout* gene is a novel link between circadian rhythms and feeding behavior. Cell 101:647–656.

Schaffer R, Landgraf J, Accerbi M, Simon VV, Larson M, Wisman E. 2001. Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. Plant Cell 13: 113–123.

Schibler U, Ripperger JA, Brown SA. 2001. Chronobiology—reducing time. Science 293:437–438.

Sehgal A, Price JL, Man B, Young MW. 1994. Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless.* Science 263:1603–1606.

Selby CP, Thompson C, Schmitz TM, Van Gelder RN, Sancar A. 2000. Functional redundancy of cryptochromes and classical photoreceptors for nonvisual ocular photoreception in mice. Proc Natl Acad Sci USA 97:14697–14702.

Shafer OT. 2001. Blind clocks reveal elusive light input pathway in *Drosophila*. Trends Neurosci 24:627–628.

Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR. 2000a. Targeted disruption of the *mPer3* gene: subtle effects on circadian clock function. Mol Cell Biol 20: 6269–6275.

Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GTJ, Hastings MH, Reppert SM. 2000b. Interacting molecular loops in the mammalian circadian clock. Science 288: 1013–1019.

Shearman LP, Zylka MJ, Reppert SM, Weaver DR. 1999. Expression of basic helix-loop-helix/PAS genes in the mouse suprachiasmatic nucleus. Neuroscience 89:387–397.

Silver R, LeSauter J, Tresco PA, Lehman MN. 1996. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 382:810–813.

So WV, Rosbash M. 1997. Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J. 16:7146–7155.

So WV, Sarov-Blat L, Kotarski CK, McDonald MJ, Allada R, Rosbash M. 2000. *takeout,* a novel *Drosophila* gene under circadian clock transcriptional regulation. Mol Cell Biol 20:6935–6944.

Stanewsky R. 2002. Clock mechanisms in *Drosophila.* Cell Tissue Res 309:11–26.

Stanewsky R, Jamison CF, Plautz JD, Kay SA, Hall JC.

1997. Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila.* EMBO J 16:5006–5018.

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC. 1998. The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila.* Cell 95:681–692.

Stempfl T, Vogel M, Szabo G., Wülbeck C, Liu J, Hall JC, Stanewsky R. 2002. Identification of circadian-clock-regulated enhancers and genes of *Drosophila melanogaster* by transposon mobilization and luciferase reporting of cyclical gene expression. Genetics 160:571–593.

Stokkan K-A, Yamazaki S, Tei H, Sakaki Y, Menaker M. 2001. Entrainment of the circadian clock in the liver by feeding. Science 291:490–493.

Suri V, Hall JC, Rosbash M. 2000. Two novel *doubletime* mutants alter circadian properties and eliminate the delay between RNA and protein in *Drosophila*. J Neurosci 20:7547–7555.

Suri V, Qian Z, Hall JC, Rosbash M. 1998. Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster.* Neuron 21:225–234.

Taghert PT, Hewes RS, Park JH, O'Brien MA, Han M, Peck ME. 2001. Multiple amidated neuropeptides are required for normal circadian locomotor rhythms in *Drosophila*. J Neurosci 21:6673–6686.

Takano A, Shimizu K, Kani S, Buijs RM, Okada M, Nagai K. 2000. Cloning and characterization of rat kinase 1ϵ. FEBS Lett 477:106–112.

Tamaru T, Isojima Y, Yamada T., Okada M, Nagai K, Takamatsu K. 2000. Light and glutamate-induced degradation of the circadian oscillating protein BMAL1 during the mammalian clock resetting. J Neurosci 20:7525–7530.

The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, Hariharan IK, Bernards A. 1997. Rescue of a *Drosophila NF1* mutant phenotype by protein kinase A. Science 276:791–794.

Thompson CL, Blaner WS, Van Gelder RN, Lai K, Quadro L, Colantuoni V, Gottesman ME, Sancar A. 2001. Preservation of light signaling to the suprachiasmatic nucleus in vitamin A-deficient mice. Proc Natl Acad Sci USA 98:11708–11713.

Thresher RJ, Vitaterna MH, Miyamoto Y, Kazantsev A, Hsu DS, Petit C, Selby CP, Dawut L, Smithies O, Takahashi JS, Sancar A. 1998. Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. Science 282:1490–1494.

Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM, Ptacek LJ, Fu Y-H. 2001. An *hPer2* phosphorylation site mutation in familial advanced sleep phase syndrome. Science 291:1040–1043.

Ueda HR, Matsumoto A, Kawamura M, Iino M, Tanimura T, Hashimoto S. 2002. Genome-wide transcriptional orchestration of circadian rhythms in *Drosophila*. J Biol Chem [epub ahead of print].

Uhl GR, Reppert SM. 1986. Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattleboro rats. Science. 232:390–393.

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A. 1999. Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms. Nature 398:627–630.

Van Gelder RN, Krasnow MA. 1996. A novel circadianly expressed *Drosophila melanogaster* gene dependent on the *period* gene for its rhythmic expression. EMBO J 15:1625–1631.

Van Swinderen B, Hall JC. 1995. Analysis of conditioned courtship in *dusky-Andante* rhythm mutants of *Drosophila.* Learn Mem 2:49–61.

Vielhaber E, Eide E, Rivers A, Gao Z-H, Virshup DM. 2000. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase Iϵ. Mol Cell Biol 20:4888–4899.

Vitaterna MH, King DP, Chang A-M, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS. 1994. Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science 264:719–725.

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, Sancar A. 1999. Differential regulation of mammalian *period* genes and circadian rhythmicity by *cryptochromes 1* and *2*. Proc Natl Acad Sci USA 96:12114–12119.

Williams JA, Sehgal A. 2001. Molecular components of the circadian system in *Drosophila*. Annu Rev Physiol 63: 729–755.

Williams JA, Su HS, Bernards A, Field J, Sehgal A. 2001. A circadian output in *Drosophila* mediated by *neurofibromatosis-1* and Ras/MAPK. Science 293:2251–2256.

Wilsbacher LD, Yamazaki S, Herzog ED, Song EJ, Radcliffe LA, Abe M, Block G, Spitznagel E, Menaker M, Takahashi JS. 2002. Photic and circadian expression of luciferase in *mPeriod1-luc* transgenic mice *in vivo*. Proc Natl Acad Sci USA 99:489–494.

Wuarin J, Schibler U, 1990. Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm. Cell 63:1257–1266.

Yagita K, Okamura H. 2000. Forskolin induces circadian gene expression of *rPer1*, *rPer2* and *dbp* in mammalian rat-1 fibroblasts. FEBS Lett 465:79–82.

Yagita K, Tamanini F, van der Horst GTJ, Okamura H. 2001. Molecular mechanisms of the biological clock in cultured fibroblasts. Science 292:278–281.

Yagita K, Yamaguchi S, Tamanini F, van Der Horst GT, Hoeijmakers JH, Yasui A, Loros JJ, Dunlap JC, Okamura H. 2000. Dimerization and nuclear entry of mPER proteins in mammalian cells. Genes Dev 14:1353–1363.

Yamaguchi S, Kobayashi M, Mitsui S, Ishida Y, van der Horst GT, Suzuki M, Shibata S, Okamura H. 2001. View of a mouse clock gene ticking. Nature 409:684.

Yamaguchi S, Mitsui S, Miyake S, Yan L, Onishi H, Yagita

K, Suzuki M, Shibata S, Kobayashi M, Okamura H. 2000a. The 5′ upstream region of *mPer1* gene contains two promoters and is responsible for circadian oscillation. Curr Biol 10:873–876.

Yamaguchi S, Mitsui S, Yan L, Yagita K, Miyake S, Okamura H. 2000b. Role of DBP in the circadian oscillatory mechanism. Mol Cell Biol 20:4773–4781.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. Science 288:682–685.

Yang Z, Emerson M, Su HS, Sehgal A. 1998. Response of the timeless protein to light correlates with behavioral entrainment and suggests separate pathways for visual and circadian photoreception. Neuron 21:215–223.

Young M. 2000. Marking time for a kingdom. Science 288:451.

Young Kim E, Bae K, Ng, FS, Glossop NRJ, Hardin PE, Edery I. 2002. *Drosophila* CLOCK protein is under post-transcriptional control and influences light-induced activity. Neuron 34:69–81.

Yu Q, Jacquier AC, Citri Y, Hamblen M, Hall JC, Rosbash M. 1987. Molecular mapping of point mutations in the *period* gene that stop or speed up biological clocks in *Drosophila melanogaster*. Proc Natl Acad Sci USA 84: 784–788.

Zeng H, Qian Z, Myers MP, Rosbash M. 1996. A light-entrainment mechanism for the *Drosophila* circadian clock. Nature 380:129–135.

Zhang Q, Piston DW, Goodman RH. 2002. Regulation of corepressor function by nuclear NADH. Science 295: 1895–1897.

Zhang X, McNeil GP, Hilderbrand-Chae MJ, Franklin TM, Schroeder AJ, Jackson FR. 2000. Circadian regulation of the Lark RNA-binding protein within identifiable neurosecretory cells. J Neurobiol 45:14–29.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC. 2001. Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. Cell 105:683–694.

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A. 1999. The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400:169–173.

Zordan MA, Rosato E, Piccin A, Foster R. 2001. Photic entrainment of the circadian clock: from *Drosophila* to mammals. Semin Cell Dev Biol 12:317–328.

Zylka MJ, Shearman LP, Weaver DR, Reppert SM. 1998. Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20:1103–1110.