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Genetic Studies on the Circadian Clock in the Model Organism Drosophila Melanogaster

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GENETIC STUDIES ON THE CIRCADIAN CLOCK IN THE MODEL ORGANISM *DROSOPHILA MELANOGASTER*

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by

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Abbreviations and Units

°C	degrees Celsius
β-Gal	β-Galactosidase
µl	10^{-6} l
µM	10^{-6} Molar
BDGP	Berkeley Drosophila Genome Project
bp	base pair
CLD	cytoplasmic localization domain
CT	circadian time in hours after onset of subjective day
DD	constant darkness
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EMS	ethyl methanesulfonate
EST	expressed sequence tag
GFP	green fluorescent protein
h	hour
kDa	kilo Dalton
l	liter
LD	Light:Dark cycle, 12h:12h
LNs	lateral neurons
M	Molar, mole per liter
min	minute
ml	10^{-3} l
mM	10^{-3} Molar
ON	over night
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SCN	suprachiasmatic nucleus
STS	sequence tagged site
Tris	tris (hydroxymethyl) aminomethane
UAS	upstream activating sequence
ZT	time in hours in an entrainment cycle, ZT0 is onset of light in an LD cycle

Abstract

Although the basic neural mechanism that regulates circadian behavior in *Drosophila* is known in quite some detail, several aspects of this biological process still remain to be elucidated. In this study a variety of genetic and molecular approaches was used to identify and characterize genes that are involved in circadian rhythms and that may shed light on the gaps in the model.

In an F_1 screen for altered period length of the circadian locomotor activity cycle the new mutation 2ob9 was isolated. Cloning and sequencing identified the affected gene as a *Drosophila* homolog of the yeast RNA helicase and splicing factor Prp43. Consistently, splicing efficiency of the *timeless* gene is reduced in the presence of a dominant negative form of DPRP43. The period-lengthening phenotype is most likely a reflection of the daily cycle of transcription of several clock genes that is delayed in the 2ob9 mutant. The results suggest that splicing may be a rate determining factor in the circadian cycle.

Levels of expression as well as temporal and spatial expression patterns are in many cases an integral part of gene function. Thus, over-expression of a particular gene often interferes with its wild type function. Based on this notion, a screening method has previously been developed that allows for tissue specific over-expression of random genes throughout the *Drosophila* genome. Using this approach, the segment polarity gene *shaggy* has been identified as an integral regulator of period length of the circadian oscillator in *Drosophila*; over-expression of *shaggy* shortens the circadian cycle whereas reduction of *shaggy* function appears to lengthen to locomotor activity cycle. These behavioral

phenotypes coincide with increased and decreased TIMELESS phosphorylation respectively, suggesting that SHAGGY controls period length through post-translational modification. Furthermore, gain of *shaggy* function concurs with an advanced nuclear entry of the PERIOD/TIMELESS heterodimer. A model is proposed, whereby SHAGGY determines the rate of PERIOD/TIMELESS nuclear translocation through its effect on the phosphorylation pattern of TIMELESS.

This screen also resulted in the identification of the transcription factor ADF-1 as a potential circadian regulator. Flies over-expressing *Adf-1* fail to display any circadian rhythmicity of locomotor activity. Increased *Adf-1* function dampens the amplitude of PERIOD and TIMELESS oscillation and reduces PDF levels. Thus, molecular oscillator as well as output mechanism of the clock are possible targets of ADF-1. However, mostly due to the lack of loss of function data, a circadian function of *Adf-1* could not be ascertained.

In an effort to interfere with gene function in a tissue specific manner, inverted repeat sequences specific to the *period* gene were expressed in neurons responsible for circadian locomotor activity. A consistent lengthening of the circadian period was found. In accordance with a double stranded RNA mediated mechanism, the inverted repeats caused a decrease of endogenous *period* RNA levels. The spatial selectivity of this approach may provide a tool to investigate behavioral functions of genes that are essential for viability.

Using a molecular approach, a correlation between MAP kinase activation and phase responses to photic stimuli was found. Furthermore, light stimulated MAP kinase activation appeared to be dependent on proper functioning of the phosphodiesterase DUNCE. Since *dunce* had previously been implicated in the

entrainment pathway, cAMP metabolism and MAP kinase activity may act together in an input pathway of the circadian clock in *Drosophila*.

The implications of each of these findings for the general model of the circadian clock in *Drosophila* are discussed at the end of the respective chapters.

Chapter 1: Overview and Background

All organisms interact constantly with their environment, a process which is observed as behavior. Whether an amoeba is engulfing a bacterium, a bee is searching for a food source, or a pigeon is homing from an unfamiliar location, the animal has to collect information and act accordingly. Because of its omnipresence, its complexity as well as its relevance for the social sciences, animal behavior is a very fascinating field of biology.

The development of new techniques over the last few decades has enabled investigators to study the neural mechanisms that underlie different aspects of behavior on a molecular level. Biochemical approaches on neural tissue extracts revealed the presence and plasticity of protein phosphorylation in the nervous system (reviewed in Nestler and Greengard 1984). This work provided the framework for the understanding of signaling events within neurons that underlie phenomenon like long term potentiation and synaptic plasticity. Electrophysiological methods allow the investigation of the principal element of neuronal communication, the electrical membrane potential. Following neural activation upon a particular stimulus provides a systemic understanding of neural circuits. Developmental neurobiology has provided markers for anatomical and probably also functional units of the nervous system. An intriguing example are the cadherins, a family of cell adhesion molecules, which are thought to mediate the specificity of neuronal interactions during development, in particular during synaptogenesis. Consequently, areas of the nervous system that are physically and presumably functionally connected express the same type of cadherin which therefore can serve as an excellent

marker to identify such circuits (Iwai *et al.* 1997; Martinek and Gaul 1997; Suzuki *et al.* 1997). Through genetic approaches the identification of key regulators of behavior was made possible. Examples include the obese mutant mice, in which a mutation of a gene encoding leptin interferes with proper control of food uptake (Zhang *et al.* 1994; Elmquist *et al.* 1999). Great advances have also been made by studying genes involved in learning and memory. The isolation and characterization of the *dunce* and *rutabaga* mutations in flies linked cAMP metabolism with these qualities of the nervous system (Nighorn *et al.* 1991; Levin *et al.* 1992). Although the application of any of these techniques has been more or less successful in different fields, most neurobiological questions are being addressed by a combination of these approaches.

One of the fastest developments in any field of neurobiology has been witnessed by the followers of chronobiology over the past ten years or so--a process that has been described aptly as a 'clockwork explosion' (Reppert 1998). Within a relatively short period of time, a plethora of speculations has been replaced by a group of proteins that, at least on paper, is sufficient to account for the core mechanism of the biological clocks, which drive behavioral and physiological rhythmicity.

Biological Timing

It probably is part of everybody's experience that we and our domesticated animals possess a sense of time. Some people can wake up in the morning without having to set an alarm clock, a dog can get impatient when its master is late, and people are 'jet lagged' after traveling over several time-zones. However, this sense of time is much more widely distributed. One of the earliest

documentations of this phenomenon was made by the French geologist Jean Baptiste Dortous de Mairan. He described in 1729 that the periodic leaf movement of *Mimosa* is maintained once the plant is kept free of any daily cycle of light or temperature (quoted from Pittendrigh 1965). By now it is known that almost every form of life displays a pattern of physiological and behavioral activity over the course of a day. This ranges from oscillating bioluminescence of the dinoflagellate *Gonyaulax* over rhythmic growth pattern of the fungus *Neurospora* to periodic changes in body temperature of mammals. Interestingly, these rhythms are maintained under constant environmental conditions, providing evidence for the existence of an endogenous time-keeping mechanism, or a biological clock, as opposed to it being a mere response to changing environmental stimuli. Since the period length of these rhythms under constant conditions is approximately one day or 24 hours, they are known as circadian rhythms (L. *circa, dies*).

Ecological Advantage of Biological Clocks

Behavior, just like the anatomy or physiology of an organism, is subject to natural selection. Implicit in this notion is that the presence of a biological clock is also advantageous for a given species. In general, a biological clock enables the organism to synchronize all its physiological processes and anticipate environmental changes. An example is the eclosion rhythm, which is the emergence of the adult animal from the pupal case, in *Drosophila* (Pittendrigh 1966). The animals eclose in the early morning when humidity is usually highest thereby improving the chance of survival. Also sleep-wake cycles illustrate the importance of orchestrating physiological parameters with cycles of

environmental changes. Abnormalities in the sleep-wake cycle can cause social stress as illustrated by the advanced sleep-phase onset in patients suffering from Advanced Sleep Phase Syndrome (ASPS). The circadian period of one ASPS patient was investigated and found to be shorter than previously reported circadian periods in humans indicating that the early sleep-phase onset is caused by a faster running biological clock (Jones *et al.* 1999). Accordingly, it was found only very recently that the genetic basis for ASPS is a mutation of a gene known to be essential for biological timing (Toh *et al.* 2001). Thus, a defective biological clock has an adverse effect on the interaction of an organism with its environment.

Furthermore, a biological clock can provide information essential for the orientation of an organism in its environment. In an elegant way G. Kramer demonstrated that birds can employ their biological clock as a compass by integrating time of day information with the position of the sun. He trained starlings to use the position of the sun to find their way to a food source. Subsequently, the sun was substituted in a laboratory setting with a stationary light. The starlings responded as if the light were moving, *i.e.* the bearing taken by the birds shifted 15 degrees per hour (quoted from Keeton 1974).

Despite the variety of apparent ecological advantages provided by biological clocks, there are only very few well documented examples of their effects on the fitness of an organism. In one study on cyanobacteria, Ouyang and colleagues were able to show that reproductive fitness was optimal if the endogenous biological clock was in resonance with the imposed light:dark regimen (Ouyang *et al.* 1998). Another study focused on nitrogen-fixing, unicellular cyanobacteria (Mitsui *et al.* 1986). Interestingly, the metabolism of

these organisms includes two incompatible reactions, oxygen-evolving photosynthesis and oxygen-labile nitrogen fixation. Only the temporal separation of these two reactions allows this organism to grow. This phenomenon is maintained under constant lighting conditions, supporting the idea that it is driven by an endogenous time-keeping mechanism. Evidently, interfering with this temporal organization would be detrimental to the metabolism of this organism.

As exemplified by hibernation, cycles of reproductive activity, and migration of birds, seasonal changes in physiology and behavior are just as widespread as circadian rhythms. In many cases, these seasonal changes are regulated by day length, or photoperiod. W. M. Hamner showed in a series of elegant studies that coupling between the circadian clock and photoperiod is essential for seasonal patterns of reproductive activity in finches (Hamner 1963; Hamner 1964). Finches were kept in LD cycles of different periods with 6 hours of light per cycle. If the period was a multiple of 24 hours (24, 48, 72), this regime caused gonadal regression. However, periods of 12, 36, or 60 hours inhibited gonadal regression. Clearly, while the duration of light *per se* is insufficient to regulate reproductive activity, the timing of the light portion relative to circadian time is relevant. Accordingly, experiments on mutant hamsters with shortened circadian period show that measurement of the photoperiod is dependent on the endogenous circadian clock (Menaker and Refinetti 1992; Shimomura *et al.* 1997).

Kenneth C. Fisher and Eric T. Pengelley demonstrated that the timing of hibernation of the golden-mantled ground squirrel is maintained under constant laboratory conditions suggesting that annual rhythms may also be regulated by an endogenous mechanism (reviewed in Pengelley 1974). Similarly, migration of

some birds can be regulated by an endogenous clock as first shown by E. Gwinner. These examples illustrate that there are clocks with period lengths of about one year, therefore called circannual. Just like circadian clocks, circannual clocks tune physiology and behavior of an organism to rhythmically changing environmental conditions.

Characteristics and Conceptual Components of Biological Clocks

A biological clock has to fulfill several criteria to be functional and to provide a selective advantage. A) Because it appears to run without continual environmental cues, it must be self-sustained. B) Because it has to be in synchrony with the environment, there must be a setting mechanism or input. C) There must be a relay of information from the clock to behavior or output. D) The rate of most biological processes is temperature dependent. Since a biological clock in a cold blooded animal should measure time and not heat, the period length of the clock should be insensitive to temperature, *i.e.* it has to be temperature compensated.

In the following, observations on a macroscopic and microscopic level concerning each of those four points will be discussed before it will be described how a combination of genetic and biochemical studies shed light on the molecular basis of biological clocks.

A) For a long time after daily rhythms had become the concern of plant physiologists and zoologists, the question whether these rhythms are driven by an exogenous or an endogenous source was subject of controversy. As mentioned above, rhythmic behavior is maintained under constant conditions, *i.e.* constant light and temperature. Since there could be environmental stimuli

beyond the control of the investigator (*e.g.* daily changes in an electromagnetic field or a 'FactorX'), this observation by itself does not exclude an exogenous mechanism. But the period length of rhythms under constant laboratory conditions deviates from 24 hours dependent on the species and the genetic make-up of the organism under investigation. If the driving force were a geo- or astrophysical event, one would expect an identical period length for all investigated organisms. As this is not the case, an endogenous biological clock is very likely to exist.

A crucial step towards the understanding of biological clocks was the identification of their anatomical bases. Removal of distinct regions of the brain in different species and transplantation experiments demonstrated that the nervous system contains an essential regulator of circadian rhythmicity. In particular, surgical removal of the suprachiasmatic nucleus (SCN) in mammals caused arrhythmic behavior (Stephan and Zucker 1972; reviewed in Klein *et al.* 1991). Interestingly, electrophysiological activity of individual SCN neurons displays a circadian pattern *in vitro*, indicating that the basic mechanism is cell-autonomous (Gillette 1991; Welsh *et al.* 1995). However, an intact nervous system is essential for other aspects of circadian regulation, such as entrainment, synchronization and relay of circadian information.

Insects also appear to require an intact nervous system for circadian rhythmicity as severing the neural connection between optic lobes and thoracic ganglia in cockroaches results in arrhythmia (Nishiitsutsuji-Uwo and Pittendrigh 1968). Likewise surgical experiments on the brain of silkmoths showed that the nervous system is essential for the regulation of their circadian flight rhythm (Truman 1974). These observations suggest that in higher metazoa circadian

rhythmicity is a neurally regulated function which has a defined anatomical basis. However, phenomenon like circadian leaf movements in plants and rhythmic growth pattern of the fungus *Neurospora* demonstrate that biological clocks are not a privilege of nervous systems.

B) As the period length of the endogenous rhythm is not precisely 24 h, the biological clock needs to be continuously re-synchronized with the cycle of an environmental stimulus. Under laboratory conditions the effects of isolated environmental factors, like light or temperature, on the phase of the biological clock are studied. It has been shown for a wide variety of organisms that the phase of an imposed 12 hour:12 hour light:dark regimen sets the phase of the endogenous rhythm. Furthermore, brief exposures to light during the subjective night can change the phase of the biological clock. Magnitude and polarity of the ensuing phase shift are dependent on the time of day when the light pulse is administered indicating that the input is gated by a circadian mechanism. Temperature cycles and heat pulses were found to have similar effects on the biological clock. These observations clearly demonstrate that the phase of a biological clock can be adjusted in accordance to external stimuli. In a natural environment an organism is exposed to a combination of environmental stimuli. Possibly, the factor of greatest ecological importance for a given organism will dominate in its ability to set the clock of the given organism.

The precise nature of the circadian photoreceptor in mammals is still an unresolved issue. Nevertheless, the existence of a direct neural connection between retina and SCN, the retino-hypothalamic tract (RHT) and other afferents (Meijer 1991), suggests that the photic input originates in the retina. As mice that are completely lacking both rod and cone photoreceptors are still capable of

entraining to photic stimuli (Lucas and Foster 1999), additional non-rod, non-cone retinal photoreceptors are likely to exist. The finding that light administered to the back of the knee in humans is sufficient to mediate circadian entrainment suggests even the existence of extraocular photoreceptors (Campbell and Murphy 1988). Similarly, *eyeless* mutant flies exhibit a fairly normal entrainment behavior. It is possible that redundancy between different entrainment pathways complicates the clarification of this process.

C) In an elegant study to elucidate the neural substrate of clock output, Inouye and Kawamura showed that neural isolation of SCNs abolishes at least one aspect of circadian rhythms (Inouye and Kawamura 1979). While the rhythmic pattern of neural activity persisted within the neurally isolated SCN, circadian patterns of neural activity outside this island were eliminated. Evidently, at least one first order output from the SCN is neural. Consistently, several efferents do exist. One of the best documented is the neural connection between the SCN and the melatonin-producing pineal gland. Suggestively, melatonin abundance oscillates over the course of a day. Amplitude and duration of the peak appear to encode day length thereby regulating seasonal changes such as reproductive activity (Tamarkin *et al.* 1985; Karsch *et al.* 1991). The SCN and neural connection between the SCN and pineal are essential for the circadian pattern of melatonin production demonstrating that the pineal melatonin rhythm is under neural control of the SCN (Illnerová 1991).

Not unlike many other signaling molecules, melatonin is produced in a few enzymatic reactions from a ubiquitous metabolite, in this case tryptophan. The key regulatory enzyme in this process is thought to be the acetyltransferase NAT (reviewed in Li *et al.* 1998). The presence of melatonin receptors in the SCN

and the fact that injection of melatonin shifts the phase of the circadian oscillator in some organisms suggest the existence of feedback of melatonin output on the clock. This finding illustrates the complexity of the circadian regulatory system. It should be pointed out in this context that circadian regulation is not an on-off switch, but that it instead increases or lowers thresholds for physiological and behavioral events.

Another aspect of SCN output appears to be mediated through diffusible signals. Silver *et al.* isolated an SCN graft within a polymeric capsule preventing neural outgrowth but allowing diffusion of humoral signals. This isolated SCN tissue was sufficient to restore behavioral rhythms in hamsters whose own SCNs had been ablated (Silver *et al.* 1996). A candidate for such a diffusible signal is the antidiuretic and vasoactive hormone vasopressin (from Majzoub *et al.* 1991). Vasopressin was first implicated in the regulation of physiological and/or behavioral rhythms by the circadian oscillations of its levels in the cerebrospinal fluid. Although surgical removal of the SCN abolished the vasopressin rhythm, vasopressin oscillation was maintained if the SCN was neurally isolated identifying the SCN as source of rhythmic vasopressin production. Later it was found that vasopressin abundance is regulated on an mRNA level in the SCN. This observation was among the first examples pointing towards the importance of temporally controlled gene expression in chronobiology.

D) If one imagines a biological clockwork as a series of biochemical reactions, one would expect a correlation of period length with temperature. As this is not the case, there has to be a mechanism that compensates for fluctuations in temperature. For example one could envision a scenario where competing reactions produce an inhibitor(s) of the clock reactions. Once the temperature

increases, more inhibitor is produced compensating for the increase of the rate of the clock reactions. Alternatively, temperature compensation could be a new feature of the entire system of biochemical reactions that make up the clock and cannot be attributed to a distinct process. If this were the case temperature compensation could only be understood once the kinetics of all those biochemical reactions is known. A few indications of how the clock might cope with temperature fluctuations only came with the identification of the molecular components of biological clocks.

Biochemical Approaches

Initial biochemical approaches to a molecular understanding of behavioral rhythms fall into one of two general categories: assays for oscillating enzymatic activities or metabolite abundance (A), and identification of drugs that interfere with circadian rhythmicity (B, reviewed in Feldman 1982). (A) Examples include the aforementioned melatonin and vasopressin rhythms but also glucose utilization appears to oscillate in the SCN during the course of a day (reviewed in Schwartz 1991). However, an oscillating activity fails to provide a causal relationship between circadian behavior and the rhythm of a given molecule. It still needs to be established what effect the absence of this molecular rhythm has on behavior/physiology. (B) Although it could for example be shown that protein synthesis is a requirement for circadian rhythmicity by the application of cycloheximide, most experiments of this kind fail to identify a specific protein component as many inhibitors are specific for a particular catalytic activity and not for a specific protein (reviewed in Feldman 1982). But even if a given drug is known to inhibit a given protein selectively, the existence of additional,

unknown targets cannot be excluded. Because of these difficulties many researches have turned to genetics as the tool of choice for the investigation of behavioral rhythms. The advantage of a genetic approach is that a single point mutation will impair only one protein component thereby allowing a conclusive connection between cause (mutation) and result (circadian abnormalities) to be drawn.

Genetic Dissection of Behavior

The complexity of behavior, its adaptibility, and its individuality suggest an equally elaborate underlying mechanism. Nevertheless, the building blocks of behavior are genes, as evidenced by the fact that certain aspects of behavior can be selected for by breeding and can be maintained within a population. However, this observation does not provide an entry point into genetic dissection of behavior since such a selection depends on multiple genetic changes that will be difficult to disentangle.

In the 1960s, a conceptual breakthrough led to a new focus on the effects of single nucleotide polymorphisms on behavior. It was shown that in some strains a significant behavioral change can be caused by a mutation in a single gene (Benzer 1967; Konopka and Benzer 1971). One gene encodes an individual protein component or in some cases a restricted group of proteins with similar properties; a mutation of a gene either causes an amino acid substitution or a shortened version of the protein. The observation that such a structural change can affect specific aspects of animal behavior clearly shows that a single protein component can be an essential part of the regulatory network that governs behavior.

The amenability of behavior to a genetic approach provides a plurality of research tools. An example is the combination of different mutations to investigate interactions between protein components. A complex network of interacting protein components underlies the regulation of behavior. If two protein components are essential for the same step of a signaling pathway, eliminating their respective genes individually will cause the same phenotype as combining the two mutations. Alternatively, if the effect of the single mutations differs from the combination of both, the protein components may act in separate signaling events. Using this method, protein components can be placed in distinct signaling pathways. Another example that illustrates the utility of mutations for investigating the regulation of animal behavior are transplantation studies. Grafts from the mutant donor can be transplanted in a wild type host. This approach enables the researcher to determine whether a given structure is sufficient to support the trait of interest. As described below, this approach was successfully employed to define the SCN as the location of an autonomous oscillator.

Model Systems and First Clock Mutants

In order to identify mutants with abnormal behavioral rhythms, Konopka and Benzer screened EMS-mutagenized *Drosophila melanogaster* for abnormal circadian eclosion rhythms (Konopka and Benzer 1971). They isolated three mutations, which all mapped to the same locus, *period*. Two of the mutations alter the period length of the eclosion rhythm and one abolishes it. Interestingly, all three mutations influence the circadian pattern of locomotor activity cycles in the same way they affect eclosion rhythms suggesting a shared regulatory

mechanism. Genetic mosaics revealed that proper function of the *period* gene is required in the nervous system consistent with a neural basis for circadian rhythms in *Drosophila* (Handler and Konopka 1979; Konopka *et al.* 1983). These observations are in accordance with prior conclusions drawn from surgical experiments that uncovered the essential nature of an intact nervous system for circadian rhythmicity in insects (Nishiitsutsuji-Uwo and Pittendrigh 1968; Truman 1974).

Another model organism used for genetic dissection of circadian rhythms is the fungus *Neurospora*. Conidial banding patterns (asexual spores) reflect circadian growth rhythms of *Neurospora* and provide an assay for aberrations in circadian regulation. Mutations in the *frequency* gene were found to shorten or lengthen the periodicity of the conidial banding pattern (Feldman and Hoyle 1973; Feldman 1982).

The first identified mutation to alter circadian rhythm in a mammal was the *tau* mutation in the hamster (Ralph and Menaker 1988). The *tau* mutation shortens the period of wheel running activity by approximately 2 hours. Using a similar assay, the mouse gene *Clock* was identified (Vitaterna *et al.* 1994). A mutation in *Clock* lengthens the circadian cycle if heterozygous and abolishes rhythm if homozygous. The identification of these mutants enabled researchers to perform transplantation experiments that provided proof that the SCN is not only necessary but also sufficient for circadian rhythmicity in mammals. By surgically substituting the SCN of one hamster with SCN tissue of a different genotype, Ralph *et al.* generated chimeras, which always exhibited the period of the donor (Ralph *et al.* 1990).

Clock mutations were identified in more organisms, and many characteristics appeared to be conserved between phyla. In all model organisms mutations could be obtained that would affect specifically circadian rhythm but not development, metabolism, locomotion or any other aspect of life. This indicates that at least at some level of circadian regulation a distinct molecular process exists. Interestingly, mutations in clock genes appear to be equally likely to either lengthen or shorten the circadian cycle (Rothenfluh *et al.* 2000a). This is remarkable as mutations caused by conventional mutagens are more likely to cause a reduction in gene activity. Most alleles of one gene should therefore have similar, *i.e.* either short or long period, but not opposite phenotypes. This discrepancy could be explained by invoking the presence of multiple regulatory domains in each clock gene. Each regulatory domain could have a positive or a negative effect, so depending on whether a mutation is in a positive or negative regulatory domain, the phenotype would be either short or long. Although this says little about the molecular machinery of biological clocks, it suggests a certain degree of mechanistic similarity among the investigated organisms.

The *Drosophila* clockwork

The first clock gene to be cloned and sequenced was the *period* gene in *Drosophila* (Bargiello and Young 1984; Reddy *et al.* 1984). Except for some similarities to proteoglycans, sequence analysis and comparison did not reveal any biochemical function of the gene product of *period* (Jackson *et al.* 1986, Citri *et al.* 1987). However, as available sequence information increased, it became apparent that one domain of PERIOD is also found in other proteins. After the first three identified proteins with this domain, PERIOD, ARNT, and SINGLE-

MINDED, this portion of the protein was termed the PAS domain. By now many more PAS domain containing proteins have been reported, and a protein-binding/dimerization function has been ascribed to the PAS domain (Ponting and Aravind 1997).

The generation of antibodies against the *period* gene product allowed for the spatial and temporal localization of PERIOD. In support of data obtained by genetic mosaics, PERIOD immuno-reactivity within the central nervous system pointed to a neural function of *period* (Saez and Young 1988; Siwicki *et al.* 1988). Interestingly, PERIOD levels appeared to cycle over the course of a day, both in light:dark as well as in constant darkness (Siwicki *et al.* 1988; Zerr *et al.* 1990). One of the first insights into the molecular mechanism of the clockwork was the finding that *period* mRNA levels undergo circadian fluctuations in fly heads (Hardin *et al.* 1990). The period of this oscillation was found to be shortened in $period^{short}$, lengthened in $period^{long}$, and abolished in $period^{0}$ demonstrating that PERIOD somehow feeds back on its own abundance.

Two different approaches provided evidence that the abundance of *period* RNA is regulated on a transcriptional level. First, *period* precursor RNA oscillation parallels the oscillation of the mature RNA. Secondly, a reporter gene construct containing regulatory sequences of the *period* locus resulted in oscillating levels of the reporter gene RNA (Hardin *et al.* 1992; Zwiebel *et al.* 1991). However, oscillating transcription alone is not sufficient to mediate protein oscillation (Dembinska *et al.* 1997; Stanewsky *et al.* 1997a). This was demonstrated by the observation that protein cycling of PERIOD-β-Gal fusion proteins depends on crucial portions of PERIOD. It is thought that those sequences contribute to regulated protein degradation.

The oscillation of *period* was reminiscent of the circadian changes in vasopressin abundance. However, a fundamental difference is that Vasopressin is not essential for its own oscillation (Uhl and Reppert 1986). Evidently, *period* is a part of the self-sustained time-keeping mechanism itself, whereas vasopressin may be an output component.

The functional importance of *period* oscillation was shown by artificially elevating PERIOD levels in tissues with cycling *period* activity (Zeng *et al.* 1994). Expression of *period* under control of a constitutive eye specific promoter stalled the molecular oscillator in the eye (Zeng *et al.* 1994). In a conceptually similar experiment Edery *et al.* could show that a transient increase in *period* expression can phase shift the circadian clock in *Drosophila* (Edery *et al.* 1994a). In summary, the rhythm phenotype caused by mutations in *period,* the feedback loop that regulates the oscillation of *period,* its expression in the central nervous system, and the effect of elevated PERIOD levels on rhythmicity clearly demonstrate that *period* is a component of the self-sustained biological clock that drives circadian behavior.

Consistent with a function of *period* in a negative transcriptional feedback loop it was found that PERIOD enters the nucleus with a circadian rhythm (Vosshall *et al.* 1994; Curtin *et al.* 1995). While PERIOD is cytoplasmic during protein accumulation, it appears to enter the nucleus once a threshold concentration is reached. In the nucleus PERIOD might exert direct influence on its own transcription.

The molecular characterization of *period* raised several new questions and most of them were pointing to the existence of more clock components. What regulates the delay between RNA and protein accumulation? How is nuclear

entry of PERIOD regulated? How is PERIOD involved in the down-regulation of its own transcription? How is the degradation of PERIOD regulated? How are input and output coupled to this oscillator? As the peak of PERIOD coincides with *period* RNA trough levels, PERIOD most likely serves a negative regulatory role. The existence of additional regulators of *period* transcription became evident with the finding that *period* levels are intermediate in a $period^0$ background. If PERIOD were the only regulator then *period* transcription should be at its peak in the absence of its repressor PERIOD. Several genetic screens in various laboratories led to the identification of new clock genes whose molecular characterization shed light on most of these problems.

Sehgal *et al.* screened a collection of P-element induced mutants for aberrant eclosion rhythms (Sehgal *et al.* 1994). This screen yielded one mutation, *timeless*, that abolished both eclosion rhythm and locomotor activity rhythm. The lack of *period* oscillation in *timeless* mutant flies suggested that *timeless* is also involved in the self-sustained circadian oscillator rather than its output.

Cloning and sequence comparison identified *timeless* as another novel gene without known biochemical function (Myers *et al.* 1995). Abundance of RNA and gene product of the *timeless* gene undergo circadian oscillations, which are synchronous to the oscillation in *period* gene activity (Sehgal *et al.* 1995; Hunter-Ensor *et al.* 1996; Myers *et al.* 1996). Oscillations of *timeless* and *period* are mutually dependent on each others proper gene function. These observations suggested that *period* and *timeless* are components of the same transcriptional feedback loop. A molecular role for TIMELESS was revealed when Vosshall *et al.* tested *timeless* mutant flies for the subcellular localization of the PERIOD-β-Gal fusion protein SG (Vosshall *et al.* 1994). In a wild type background, SG is

constitutively nuclear. Interestingly, at no time was the fusion protein SG detectable in the nucleus in *timeless*^{0}, indicating that TIMELESS is essential for nuclear transport of PERIOD. This notion could be confirmed using a cell culture assay (Saez and Young 1996). While *period* or *timeless* transfected individually into Schneider cells stayed cytoplasmic, cotransfection of both genes resulted in translocation of PERIOD and TIMELESS into the nucleus. Additionally, it was found that deletion of a domain in *period* or *timeless*, called cytoplasmic localization domain (CLD), resulted in constitutive nuclear localization of the respective proteins.

Since PERIOD abundance in *timeless* mutants was low despite intermediate *period* RNA levels, TIMELESS was thought to affect the stability of PERIOD (Vosshall *et al.* 1994; Price *et al.* 1995). Conversely, TIMELESS abundance in *period* mutant flies appears to be fairly high suggesting that TIMELESS stability is not dependent on PERIOD (Myers *et al.* 1996; Zeng *et al.* 1996). The functional significance of the genetic connection between *period* and *timeless* was supported by the finding that TIMELESS and PERIOD physically interact with each other (Gekakis *et al.* 1995; Zeng *et al.* 1996). Thus, heterodimerization of PERIOD with TIMELESS may prevent PERIOD degradation directly.

Most interestingly, TIMELESS is degraded rapidly in response to light implicating TIMELESS in the light-entrainment pathway (Hunter-Ensor *et al.* 1996; Myers *et al.* 1996; Young *et al.* 1996; Zeng *et al.* 1996; Yang *et al.* 1998). The molecular behavior of TIMELESS after a light pulse during the night correlates well with the phase response of locomotor activity rhythms. A light pulse administered in the early night causes the degradation of cytoplasmic TIMELESS

followed by re-accumulation of protein. The phase of the locomotor activity cycle is consistently delayed. A light pulse in the late night, on the other hand, causes the degradation of nuclear TIMELESS and an advanced start of the subsequent molecular and behavioral cycle. The behavioral and molecular responses to light are maintained in flies that lack eyes (Yang *et al.* 1998) consistent with the existence of extra-retinal photoreceptors as was suggested already for the mammalian circadian system.

Screening of a collection of chemically mutagenized flies for aberrant locomotor activity rhythms yielded several more candidate genes (Allada *et al.* 1998; Rutila *et al.* 1998). Closer inspection of some of the mutant lines revealed constitutively low *period* RNA levels in two arrhythmic mutants suggesting that the underlying genes encode positive regulators of *period* transcription. Cloning and sequence analysis of the two genes, called *dclock* and *dbmal*, identified them as two PAS domain containing bHLH transcription factors. Interestingly, a target sequence for bHLH transcription factors, the so-called E box, had previously been identified as a key element of oscillating *period* transcription (Hao *et al.* 1997). Using a cell culture assay, Darlington *et al.* "closed the circadian loop" (Darlington *et al.* 1998). They were able to demonstrate that the dCLOCK/dBMAL heterodimer induces the transcription of *period* and *timeless* in an E box dependent manner. Furthermore, Darlington *et al.* reported that cotransfection of *period* and *timeless* inhibits dCLOCK mediated transcription. This inhibition is probably mediated through a reduction of the DNA binding activity of the dCLOCK/dBMAL heterodimer (Lee *et al.* 1999). If the cytoplasmic localization domain (CLD) of *period* is deleted, transfection of *period* alone is sufficient to inhibit dCLOCK induced transcription (Rothenfluh *et al.* 2000b).

Taken together, the genetic and biochemical interactions of all four identified clock components strongly support a negative transcriptional feedback loop as model for the core mechanism of the circadian clock in *Drosophila*. Activation of *period* and *timeless* transcription through dCLOCK/dBMAL gives rise to an increase in RNA levels followed by an increase in protein abundance. Once a threshold level of PERIOD/TIMELESS is reached, the heterodimer enters the nucleus where PERIOD and/or TIMELESS are involved in the down-regulation of their own transcription. Subsequently, PERIOD/TIMELESS are degraded releasing the repression on their own transcription and the cycle starts anew.

An additional level of complexity was added, when it was found that *dclock* RNA also undergoes circadian oscillations in its abundance (Bae *et al.* 1998; Darlington *et al.* 1998; Glossop *et al.* 1999). Surprisingly, *dclock* RNA levels were constitutively high in *dclock* and *dBmal* mutants suggesting a negative regulatory role for dCLOCK/dBMAL in *dclock* transcription. On the other hand *dclock* RNA levels are low in *period* or *timeless* mutants suggesting that PERIOD/TIMELESS activate *dclock* transcription (Bae *et al.* 1998; Glossop *et al.* 1999). Since *dclock* RNA levels are also high in a *period* and *dBmal* double mutants, PERIOD/TIMELESS act probably upstream of dCLOCK/dBMAL. It was therefore suggested that dCLOCK/dBMAL repress *dclock* transcription, and that PERIOD/TIMELESS alleviate this repression possibly by a mechanism similar to the deactivation of dCLOCK mediated *period* transcription (Glossop *et al.* 1999). Thus, *period/timeless* oscillation and *dclock* oscillation form two interlocked feedback loops. As discussed below, interlocked transcriptional feedback loops may provide features like high accuracy of frequency and temperature compensation.

In addition to mutations of *dClock* and *dBmal*, the screening of chemically mutagenized flies for altered circadian period length yielded also mutations of the *Drosophila* casein kinase 1ε homolog *double-time* (Kloss *et al.* 1998; Price *et al.* 1998). The catalytic activity of this casein kinase 1ε homolog has immediate implications for its function in the oscillator, since PERIOD and TIMELESS are increasingly phosphorylated during the course of a circadian day (Edery *et al.* 1994b; Myers *et al.* 1996; Zeng *et al.* 1996). Although *double-time* is essential for viability, the hypomorphic allele *double-time*^{P} confers lethality only by the onset of pupal development. As third instar larvae contain already neurons with a molecular oscillator (Kaneko *et al.* 1997), homozygous mutant third instar larvae provide a close to loss of function system to investigate the role of *double-time* in the molecular oscillator. It was found that the circadian oscillation of *timeless* is stalled in those mutants. Additionally, it was found that PERIOD accumulates to unusually high levels indicating a role for *double-time* in regulating accumulation and degradation of PERIOD. Consistently, it was found that *double-time*^{P} uncouples PERIOD abundance from the presence of TIMELESS (Price *et al.* 1998) establishing PERIOD stability as a target of DOUBLE-TIME function.

In another screen, investigators took advantage of the oscillating luciferase activity of a PERIOD-LUCIFERASE fusion protein, which is under transcriptional control of the *period* promoter (Brandes *et al.* 1996). They isolated a mutation that completely abolished *period* and *timeless* oscillation in peripheral tissues but did not affect the period of locomotor activity rhythm (Emery *et al.* 1998; Stanewsky *et al.* 1998; Emery *et al.* 2000b). However, the mutant flies displayed abnormal responsiveness to light both on a behavioral and molecular

level indicating a role of the underlying gene in the input pathway of the clock. Consistently, cloning and sequencing identified the gene as encoding a cryptochrome. Cryptochromes are pterin/flavin-containing proteins, first identified in plants in which they are involved in blue light dependent entrainment of circadian functions (reviewed in Cashmore *et al.* 1999). A series of *in vitro* experiments shed some light on the mechanism by which CRYPTOCHROME sets the clock (Ceriani *et al.* 1999). CRYPTOCHROME and TIMELESS were found to physically interact in a yeast two hybrid assay in a light dependent manner. The functional significance of this interaction was demonstrated by the observation that light in combination with CRYPTOCHROME is sufficient to prevent PERIOD/TIMELESS from inhibiting dCLOCK induced transcription. It is therefore conceivable that light induced changes in CRYPTOCHROME are directly transmitted to TIMELESS resulting in a disruption of its ability to influence transcriptional activity.

Homologies of CRYPTOCHROME to a family of DNA repair enzymes, the photolyases, invites to some speculation regarding the evolutionary origin of circadian clocks. Because of its DNA binding activity and its light responsiveness it is conceivable that an ancestor molecule of CRYPTOCHROME was a light dependent transcription factor. Thus, an ancestral biological clock might have been under direct control of an exogenous light:dark cycle.

The genetic identification and the molecular characterization of these clock components yielded a detailed picture of input into the biological clock and into the molecular oscillator itself. However, relatively little is known about the mechanism of clock output. One interesting clue came from the pigment-dispersing hormones (PDH), neuropeptides that are known to translocate retinal

as well as epithelial pigments in crustaceans (Rao and Riehm 1993). Two different lines of evidence implicated PDH in the regulation of circadian rhythms. First, injection of PDH in cockroaches caused a phase shift of their locomotor activity rhythm (Petri and Stengl 1997). Second, PDH-immunoreactive neurons have been identified in the CNS of *Drosophila* (Helfrich-Förster and Homberg 1993). Double-labeling experiments showed that PDH- and PERIOD-immuno-reactivity colocalize in these neurons (Helfrich-Förster 1995). Interestingly, these neurons, the so-called lateral neurons (LNs), had previously been suggested to be the pacemakers of circadian locomotor activity rhythm in flies (Ewer *et al.* 1992; Frisch *et al.* 1994).

PIGMENT-DISPERSING FACTOR (PDF), the *Drosophila* homolog of PDH, undergoes a circadian oscillation in abundance specifically at the terminals of some LNs. As levels of *pdf* RNA are constant in LNs, processing or transport of PDF is probably under circadian control (Park *et al.* 2000). Isolation of an insect pigment-dispersing factor gene enabled investigators to address its function using reverse genetics in flies (Helfrich-Förster *et al.* 2000). Although over-expression of *pdf* exclusively in LNs did not interfere with behavioral rhythm, over-expression in all neurons did result in arrhythmia or splitting of locomotor activity rhythms. Using different promoters, Helfrich-Förster *et al.* found an effect on circadian rhythms only if *pdf* was expressed ectopically in neurons that project into the same area of the brain where the terminals of the LNs are located. Consistently, the daily fluctuation of PDF immuno-reactivity was only abolished if *pdf* was ectopically expressed in those neurons but it was retained if *pdf* was over expressed in LNs. Regulation of PDF transport or processing is evidently absent in most neurons other than the LNs. All these data implied an important

role for *pdf* in the control of circadian behavior. However, only the demonstration that loss of *pdf* function abolishes circadian locomotor activity rhythm provided unambiguous evidence that *pdf* is an essential component of this process (Renn *et al.* 1999). The biochemical nature of PDF and its conspicuous temporal expression at the nerve terminals of some LNs suggest a function as an output signal, probably connecting the oscillator with locomotor activity. An output component might be expected to leave the core oscillator unaffected while disrupting one or more aspects of rhythmic behavior. It will therefore be very interesting to see whether PERIOD/TIMELESS oscillations are affected in the LNs of *pdf* mutant flies.

In an approach to isolate clock-controlled genes, Blau and Young combined the high-throughput of a PCR based differential display screen with the powerful genetics of *Drosophila* (Blau and Young 1999). They found that levels of the basic zipper transcription factor *vrille* (George and Terracol 1997) oscillate in a *period*- and therefore clock-dependent manner. Interestingly, the mammalian basic zipper transcription factor DBP shows homologies over the basic zipper region to VRILLE and displays a circadian rhythm in its abundance (Wuarin and Schibler 1990). Using the GAL4/UAS binary expression system (Brand and Perrimon 1993), Blau and Young eliminated cycling expression of *vrille* and rhythmic behavior. Strikingly, over-expression of *vrille* abolished PDF immuno-reactivity in LNs while leaving *pdf* transcript unaffected. In the light of PDF's rhythm at the terminals of the LNs, this observation would suggest that target gene(s) of VRILLE are involved in the circadian pattern of PDF processing and/or stability.

In a similar approach So *et al.* identified *takeout* as a novel, clock-controlled gene (So *et al.* 2000). Interestingly, *takeout* expression is induced upon starvation in a clock-dependent manner implicating TAKEOUT in a molecular link between feeding behavior/metabolism and the circadian clock (Sarov-Blat *et al.* 2000).

From the above a picture emerges in which a cell-autonomous transcriptional feedback loop is the core time keeping device and on the same time regulator of cycling activity of output genes like *pdf, vrille* and *takeout*, which may or may not partake in the core oscillator. It will be interesting in the future to learn more about the mechanism by which PDF levels at nerve terminals are regulated. Also, many aspects of the core oscillator remain to be elucidated. Several lines of evidence point to a post-transcriptional contribution to *period* RNA cycling by an unknown mechanism (Frisch *et al.* 1994; So and Rosbash 1997; Stanewsky *et al.* 1997b). The delay between RNA and protein accumulation is only partially elucidated. Nuclear entry of PERIOD/TIMELESS is probably gated in a circadian fashion not merely dependent on protein abundance. The precise nature of the underlying mechanism is still to be elucidated. In spite of the existing gaps it seems that the principle of circadian regulation on a single neuron level is becoming apparent (Figure 1).

Despite the progress in understanding the molecular basis of the biological clock, the neuronal network that regulates circadian activity in *Drosophila* remains to be identified. The LNs project in a region of the brain where the mushroom bodies, centers of higher brain functions like learning and memory (Davis 1993), are located. However, constitutive over-expression of *pdf* in the mushroom bodies had no effect on locomotor activity rhythm (Marcus Täuber, personal communication) suggesting that the LNs do not directly

innervate this brain structure. Identification of target neurons of the LNs might shed light on the mechanism whereby a biological clock orchestrates circadian rhythms of physiological and behavioral activities.

The mammalian clockwork

More than ten years after the *Drosophila period* gene had been sequenced, Takahashi and colleagues reported the cloning of the first mammalian clock gene, aptly named *clock,* in the mouse (Vitaterna *et al.* 1994; Antoch *et al.* 1997; King *et al.* 1997). The *clock* gene had been identified by a forward genetic approach (Reppert and Weaver 1997) and differs in that respect from most other mammalian clock genes, which were first isolated based on homologies. The mouse genetic model system allows investigators to generate loss of function mutations by site specific recombination which facilitates a reverse genetic approach (Thompson *et al.* 1989). The *Drosophila* model, in contrast, benefits more from its accessibility to forward genetic approaches as the generation of loss of function alleles by homologous recombination has been successful only very recently in this model organism (Rong and Golic 2000)

Sequence analysis revealed that *clock* encodes a PAS domain containing bHLH transcription factor. FASTA alignment of *Drosophila* dCLOCK and the mouse CLOCK revealed 35% identity over the entire overlap (Allada *et al.* 1998). The strong homology between components of the molecular oscillators in two species suggested that the molecular mechanism of the two systems is similar. This idea was further bolstered when several mammalian *period* homologs (*Period1, Period2* and *Period3*) were identified (Sun *et al.* 1997; Tei *et al.* 1997; Takumi *et al.* 1998). RNA levels of all three *period* homologs undergo circadian

cycling (Shearman *et al.* 1997). However, only *mPeriod2* appears to be essential for circadian rhythms as shown by loss of the circadian locomotor activity pattern and loss of *mPeriod1* and *mPeriod3* RNA cycling in a mouse model with nonfunctional *mPeriod2* (Zheng *et al.* 1999). As in *Drosophila*, mammalian CLOCK physically interacts with a second bHLH-PAS transcription factor BMAL1, the mammalian homolog of dBMAL (Gekakis *et al.* 1998; Hogenesch *et al.* 1998). The CLOCK/BMAL heterodimer has also been found to activate the transcription of the mammalian *period* homologs (Gekakis *et al.* 1998; Jin *et al.* 1999).

Several salient features of the molecular oscillator in flies, *e.g.* nuclear entry and response to light, are supported by TIMELESS. However, neither of these aspects of TIMELESS function is conserved in a recently cloned mammalian homolog of *timeless*: mammalian *Timeless* does not cycle in any tissue, it appears to be constitutively located in nuclei of the SCN, and physical interactions with other clock components are controversial (Sangoram *et al.* 1998; Zylka *et al.* 1998). Database searches of the completed *Drosophila* genome (Celera) showed that *mTimeless* is not the true ortholog of *timeless*, but it is more closely related to the newly discovered *timeout* gene in *Drosophila* (Gotter *et al.* 2000; Reppert and Weaver 2000). A function for *timeout* as well as the existence of a true *timeless* ortholog in mammals has yet to be established.

Closer inspection of the three mammalian *Period* genes suggested that the evolution of three different *period* homologs has partially supplanted a *Drosophila*-like function of *Timeless* in mammals. Resetting of the oscillator by light is correlated with an induction of *mPeriod1* and *mPeriod2* (Shearman *et al.* 1997) suggesting that a transcriptional activation is the primary response to light stimuli in the SCN. Accordingly, administration of antisense oligonucleotides

against *mPeriod1* inhibited light induced phase delays (Akiyama *et al.* 1999). However, light affects not only gene expression of *mPeriod* but light pulses also induce immediate-early genes such as *junB* and *c-fos*. Preventing the expression of these genes through antisense oligonucleotides inhibits light-induced phase shifts of the mammalian circadian clock demonstrating the relevance of these genes for the molecular oscillator in the SCN (Wollnik *et al.* 1995). Several studies implicated other signaling molecules, like CREB, glutamate, NMDA receptor, and nitric oxide, in the clock resetting process (Ginty *et al.* 1993; Ding *et al.* 1994; Watanabe *et al.* 1994; Ding *et al.* 1997). And a very recent report implicated the light induced degradation of BMAL1 in the resetting of the circadian clock (Tamaru *et al.* 2000). These observations suggest that a complex network of signaling events including the induction of *mPeriod1* and *mPeriod2* is involved in the resetting of the molecular oscillator in the SCN.

Another aspect of the circadian oscillator in flies is regulated nuclear transport of PERIOD. Yagita *et al.* showed that mPERIOD3 contains a functional nuclear localization sequence as well as a cytoplasmic localization domain (Yagita *et al.* 2000). They also demonstrated that mPERIOD3 coexpression with either mPERIOD2 or mPERIOD1 promoted nuclear entry probably through heterodimerization again supporting the idea, that the evolution of three homologs of *period* has at least partially supplanted the function of *timeless* in mammals (Zylka *et al.* 1998; Kume *et al.* 1999; Yagita *et al.* 2000).

It is probably not too surprising that cryptochrome (CRY) photoreceptors are also present in mammals. However, one might find it remarkable that the two mammalian blue light photoreceptors , CRY1 and CRY2, are expressed in a deep brain structure like the SCN (Miyamoto and Sancar 1998). This finding

pointed to a light-independent role for *CRY1* and *CRY2*. Consistent with this idea, double knockout mice displayed a 24 hour locomotor activity rhythm in LD demonstrating that mammalian CRYs are dispensable for light entrainment (van der Horst *et al.* 1999). Accordingly, *mPeriod1* and *mPeriod2* are still light inducible in those mice (Okamura *et al.* 1999). In contrast to the normal behavior in an LD regimen, however, the double mutant mice are arrhythmic under constant environmental conditions demonstrating that the CRYs are essential for the self-sustained circadian oscillation in mammals (van der Horst *et al.* 1999). Direct evidence confirming this notion was provided by the observation that expression of *mPeriod1* and *mPeriod2* are constitutively high in the SCN of double mutant mice (Okamura *et al.* 1999). An intrinsic role for CRYs in the mammalian oscillator is further suggested by their cycling expression levels in the SCN. Several groups were able to show that CRYs are potent inhibitors of CLOCK/BMAL1 mediated transcription implicating their function in the "negative limb of the circadian clock feedback loop" (Griffin *et al.* 1999; Kume *et al.* 1999; Shearman *et al.* 2000).

In addition to its negative regulatory role in transcription, the CRYs have been found to promote nuclear translocation of the PERIOD proteins in cell culture (Kume *et al.* 1999). In support of the idea that the CRYs take part in the nuclear translocation of the PERIOD proteins, Shearman and colleagues found cytoplasmic PERIOD immunoreactivity in the SCN of *cry1* and *cry2* double mutant mice. In contrast PERIOD in the wild type control was entirely nuclear (Shearman *et al.* 2000). Coimmunoprecipitation experiments demonstrated *in vivo* associations among CRY proteins and PERIOD proteins (Griffin *et al.* 1999; Kume *et al.* 1999). This physical interaction may provide a mechanism by which

CRY shuttles PERIOD into the nucleus. However, the observation that PERIOD can enter the nucleus in the absence of both CRYs argues that redundant mechanisms ensure proper nuclear translocation of the PERIOD proteins in mammals (Shearman *et al.* 2000; Yagita *et al.* 2000).

Lowrey and colleagues cloned the gene affected by the *tau* mutation and uncovered that the underlying gene encodes a mammalian ortholog of *double-time* (Lowrey *et al.* 2000). This mammalian casein kinase 1ε binds and phosphorylates mammalian PERIOD (Keesler *et al.* 2000; Vielhaber *et al.* 2000). Furthermore, PERIOD stability and nuclear translocation appear to involve casein kinase 1ε function (Keesler *et al.* 2000; Vielhaber *et al.* 2000). Thus, a substantial aspect of PERIOD cycling appears to be conserved among species.

In contrast to cycling *dclock* expression in *Drosophila*, in mice the activity of the CLOCK/BMAL1 heterodimer appears to be regulated by a circadian change in BMAL1 levels (Shearman *et al.* 2000). Furthermore, trough levels of *Bmal1* RNA are detected throughout the circadian day in *Clock* mutant mice implying a positive regulatory role for CLOCK, *i.e.* CLOCK/BMAL1 heterodimer, in *Bmal1* expression. This is unlikely to be a direct effect since the decline in *mPeriod* RNA levels (indicative for low CLOCK/BMAL1 activity) coincides with the rise of *Bmal1* RNA levels. Because *Bmal1* RNA oscillation is blunted in *mPeriod2* mutant mice, Shearman *et al.* proposed an indirect mechanism whereby CLOCK/BMAL1 activates *mPeriod* and subsequently mPERIOD2 activates *Bmal1*. Accordingly, mPERIOD2 immuno-reactivity is high in SCN nuclei when *Bmal1* expression is activated.

Taken together, a model of two interlocked transcriptional feedback loops similar to that in *Drosophila* emerges (Figure 2). The CLOCK/BMAL1 heterodimer activates the transcription of the *mPeriod* genes and of the *Cryptochrome* genes. While the precise nature of the nuclear translocation is still controversial, CRYPTOCHROME may promote the transport of PERIOD to the nucleus through complex formation. Once in the nucleus the CRYPTOCHROME1 and 2 inhibit CLOCK/BMAL1 mediated transcription, which is followed by a decline in *Period* RNA and eventually protein levels. This process may or may not involve any of the three mPERIODs. mPERIOD2 probably is involved in the up-regulation of *Bmal1* expression. Once a threshold level of BMAL1 is reached, the CLOCK/BMAL1 heterodimer activates the transcription of *mPeriod* as well as *cryptochrome1* and 2 and the cycle starts anew.

The mouse model provided detailed mechanistic insight into the relay of information from the molecular oscillator to rhythmic output. It was mentioned earlier that the antidiuretic and vasoactive hormone vasopressin is expressed in and released from the SCN with a circadian cycle. Jin *et al.* demonstrated that an E box enhancer, the target sequence for the CLOCK/BMAL1 heterodimer, resides in the promoter region of the *vasopressin* gene suggesting that the cycling expression of *vasopressin* might be regulated in the same way like *mPeriod* (Jin *et al.* 1999). As predicted by this hypothesis, *vasopressin* RNA levels are constantly low in *Clock* mutant mice. Using a reporter gene construct with the cis regulatory elements of the *vasopressin* gene, the authors could show that CLOCK/BMAL1 are sufficient and necessary for activation of transcription from the *vasopressin* promoter.

Similarly, the expression of the transcription factor DBP is regulated by CLOCK/BMAL1, and DBP in turn controls the circadian activity of metabolic target genes (Lopez-Molina *et al.* 1997; Lavery *et al.* 1999; Ripperger *et al.* 2000). It is therefore conceivable that the circadian expression pattern of other output genes is similarly interlocked with the transcriptional feedback loop of the core oscillator.

The *Neurospora* clockwork

As mentioned earlier, the conidial banding pattern of *Neurospora* provides an attractive assay for circadian rhythms in this organism. Single gene mutations that alter or abolish this rhythm were isolated. Many of them mapped to the *frequency* locus. Cloning and sequence analysis of *frequency* revealed a novel gene product without any known enzymatic activities (McClung *et al.* 1989). However, a series of elegant experiments demonstrated unambiguously that *frequency* encodes a *bona fide* component of a molecular feedback loop (Aronson *et al.* 1994). First, the authors showed that the period of *frequency* RNA oscillation is equal to the period of the overt circadian rhythm. Additionally, in a mutant without an overt rhythm of the banding pattern, *frequency* RNA oscillation was also undetectable. This correlation was suggestive and prompted the authors to test a causal relationship between *frequency* RNA oscillation and overt rhythm. The authors argued that constitutive expression of *frequency* should stall the circadian oscillator if cycling *frequency* is an integral part of this system. Indeed, constitutive expression of *frequency* from an inducible promoter abolished rhythmic conidial banding. In another set of experiments the authors showed that temporary over-expression of *frequency* results in a reduction of endogenous

frequency RNA demonstrating that FREQUENCY is indeed capable of negatively regulating its own expression. Consistent with this molecular phenotype the authors also described a phase resetting of the conidial banding rhythm after temporary *frequency* over-expression.

Interestingly, the *frequency* locus encodes two different forms of FREQUENCY, which arise through alternative in-frame initiation of translation (Garceau *et al.* 1997; Liu *et al.* 1997). The preference for one or the other initiation codon is temperature dependent. Consequently, temperature determines the ratio between the two forms of FREQUENCY. One may speculate that temperature compensation is attained by this mechanism.

Further insight into the molecular clockwork of *Neurospora* was provided by the analysis of two other loci, *wc1* and *wc2*. Strains bearing lesions in either gene are arrhythmic and affect all known light responses, including photo-induction of the *frequency* gene (Crosthwaite *et al.* 1995; Crosthwaite *et al.* 1997). Cloning and sequence analysis revealed that both genes encode zinc finger transcription factors (Ballario *et al.* 1996; Linden and Macino 1997). Interestingly, both proteins appear to contain a PAS protein dimerization domain and WC-1 exhibits extended similarity to mammalian BMAL1 (Lee *et al.* 2000). In order to test whether overt arrhythmia in the *wc* mutants is caused by a failure to entrain to light or by a stalled molecular oscillator, Crosthwaite *et al.* investigated *frequency* RNA and protein cycles in those mutants (Crosthwaite *et al.* 1997). They found continuously low *frequency* RNA levels in both mutants suggesting that WC-1 and WC-2 function as positive regulators of *frequency* expression analogous to the role of CLOCK/BMAL in respectively *Drosophila* and the mouse. This analogy is further bolstered by the observation that WC-1 protein

levels undergo circadian cycling and that WC-1 and WC-2 form a complex (WCC; Talora *et al.* 1999). In contrast to BMAL1, however, *wc-1* RNA levels are constant suggesting post-transcriptional control of circadian changes in protein abundance (Lee *et al.* 2000). Low levels of WC1 in a *frequency* null mutant implicated FREQUENCY as a positive regulator of this process. Accordingly, the authors were able to stimulate WC1 production through temporary induction of *frequency* in this mutant. Moreover, in the presence of cycloheximide to block *de novo* protein synthesis, FREQUENCY had no effect on WC1 levels, suggesting that FREQUENCY up-regulates translation of WC1 as opposed to its stability (Lee *et al.* 2000). These data are consistent with a dual role of FREQUENCY in the transcriptional feedback loop; it depresses its own synthesis and it activates expression of WC1, the transcriptional co-activator of *frequency* (Figure 3A). It should be pointed out that there is precedent for circadian control of translation in another model system, the circadian expression of the luciferin-binding protein in the dinoflagellate *Gonyaulax* (Mittag *et al.* 1994; Mittag 1996).

Similar to the PERIOD protein, FREQUENCY is increasingly phosphorylated during the day. In a recent study, it was shown that FREQUENCY phosphorylation is an integral part of the circadian oscillator in *Neurospora* and that it regulates the temporal degradation pattern of FREQUENCY (Liu *et al.* 2000). These observations exemplify the significance of post-translational modification in circadian oscillators of different species.

General Considerations

As illustrated in Figure 3B, the circuitry of the self-sustained oscillators of different phyla highly resemble each other. However, the constituent molecules

of these oscillators are not always conserved. It also appears that the function of some genes has been supplanted by others during the course of evolution. The lack of homology between clock genes in *Neurospora* on one side and *Drosophila* and mammalian system on the other (with the exception of *wc-1* and *Bmal1*) raises the possibility of independent evolutionary origins of these clocks. If this were the case then the almost identical underlying circuitry is an intriguing example of convergent evolution. Since there are various ways in which to assemble an oscillating system, as illustrated by the "repressilator" (Elowitz and Leibler 2000), this strong similarity is striking. It is possible that this system provides *per se* features that are not intrinsic to all oscillatory systems. For example, the existence of two interlocked feed back loops invites the conjecture that this system may be temperature compensated. Additionally, one might be tempted to speculate that this system provides a more stable oscillation, or in other words a more precise watch. These speculations may be tested by future quantitative modeling.

Open Questions and Scope of this Thesis

Some questions concerning the molecular clock-work in *Drosophila* remain unanswered despite the vast amount of available data. A post-transcriptional contribution to *period* RNA cycling seems to exist in addition to oscillating transcription (Frisch *et al.* 1994; So and Rosbash 1997). But neither the mechanism nor the components responsible for it is known. Nuclear entry of the PERIOD/TIMELESS heterodimer is probably a controlled event, but no protein component has been implicated in this process yet. Although the *vrille* and *pdf* genes are likely to function as output genes, the precise mechanism by which the

information from the oscillator is relayed to behavior is still unknown. The influence of CRYPTOCHROME on TIMELESS stability and possibly other factors of the molecular clock has yet to be elucidated. In this thesis several genetic and molecular approaches were employed to identify new components that may shed light on some of these questions.

Figure 1: Schematic model of the molecular oscillator in *Drosophila melanogaster.*

Interlocked feedback loops, as highlighted by the coloring of arrows and molecule names, and compartmentalization of individual steps are represented. Names of proteins are in uppercase, names of genes and RNAs are in lowercase. Arrows represent a positive regulation, bars at the end of lines represent a negative regulation, and the two concentric circles in the middle of the diagram represent the nuclear envelop. In brief, dCLOCK/dBMAL heterodimer activates the transcription of *period* and *timeless*. With a delay of about 6 hours relative to their RNAs, PERIOD and TIMELESS proteins accumulate under the control of DOUBLE-TIME. PERIOD/TIMELESS heterodimer is translocated into the nucleus, where PERIOD is involved in the repression of dCLOCK/dBMAL mediated transcription of *period* and *timeless* resulting in a decline of *period* and *timeless* RNA levels. At the same time dCLOCK/dBMAL down-regulation effects a derepression of *clock* expression. As dCLOCK levels increase, PERIOD is being degraded under the influence of DOUBLE-TIME. Both events have a positive effect on *period* and *timeless* expression and the cycle recommences. The dCLOCK/dBMAL heterodimer also activates the transcription of *vrille* and *pdf*. The oscillating activity of *vrille* has been implicated in the regulation of PDF protein accumulation. Abbreviations are: per - *period*; tim - *timeless*; dbt - *double-time*; clk - *dclock*; dbmal - *dBmal*; *vri –vrille*; *pdf – pigment dispersing factor*;

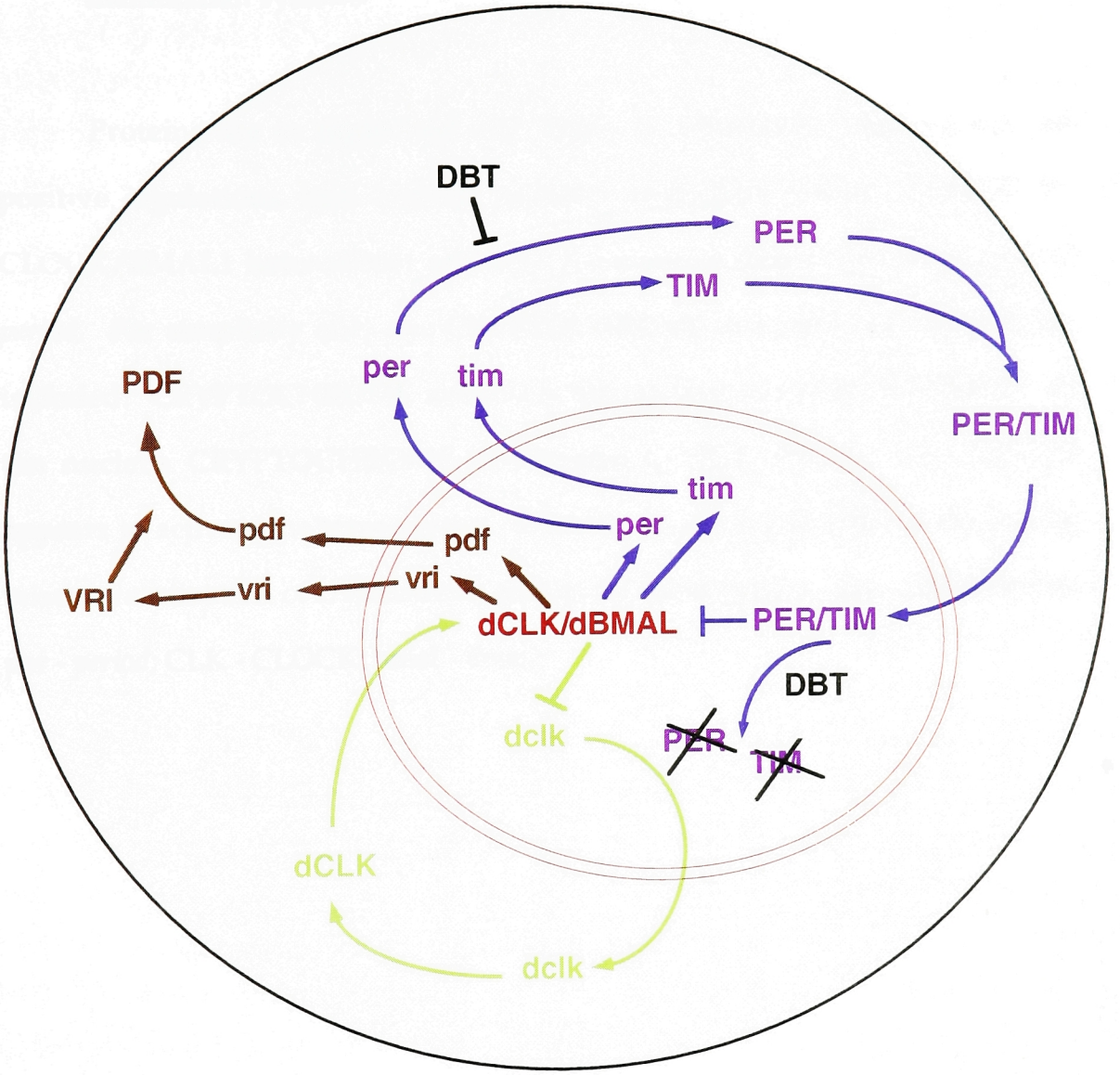

DBT
PER
TIM
per
tim
PDF
PER/TIM
tim
per
pdf
pdf
VRI
vri
vri
dCLK/dBMAL
PER/TIM
DBT
dclk
PER
TIM
dCLK
dclk

Figure 2: Schematic representation of the self-sustained oscillator in mammalian systems.

Proteins are in uppercase and RNAs in lowercase. Arrows indicate positive regulations, bars indicate negative regulatory events. In brief, the CLOCK/BMAL1 heterodimer activates the transcription of *cryptochrome* and *period*. For simplicity only one CRYPTOCHROME and only one PERIOD are included. CRYPTOCHROME influences the nuclear transport of PERIOD. In the nucleus CRYPTOCHROME deactivates CLOCK/BMAL1 and PERIOD appears to activate the transcription of *Bmal1*. Increasing BMAL1 activity gives raise to reactivation of *cryptochrome* and *period* transcription. cry - *cryptochrome;* per - *period;* CLK - CLOCK; bmal - *Bmal1;*

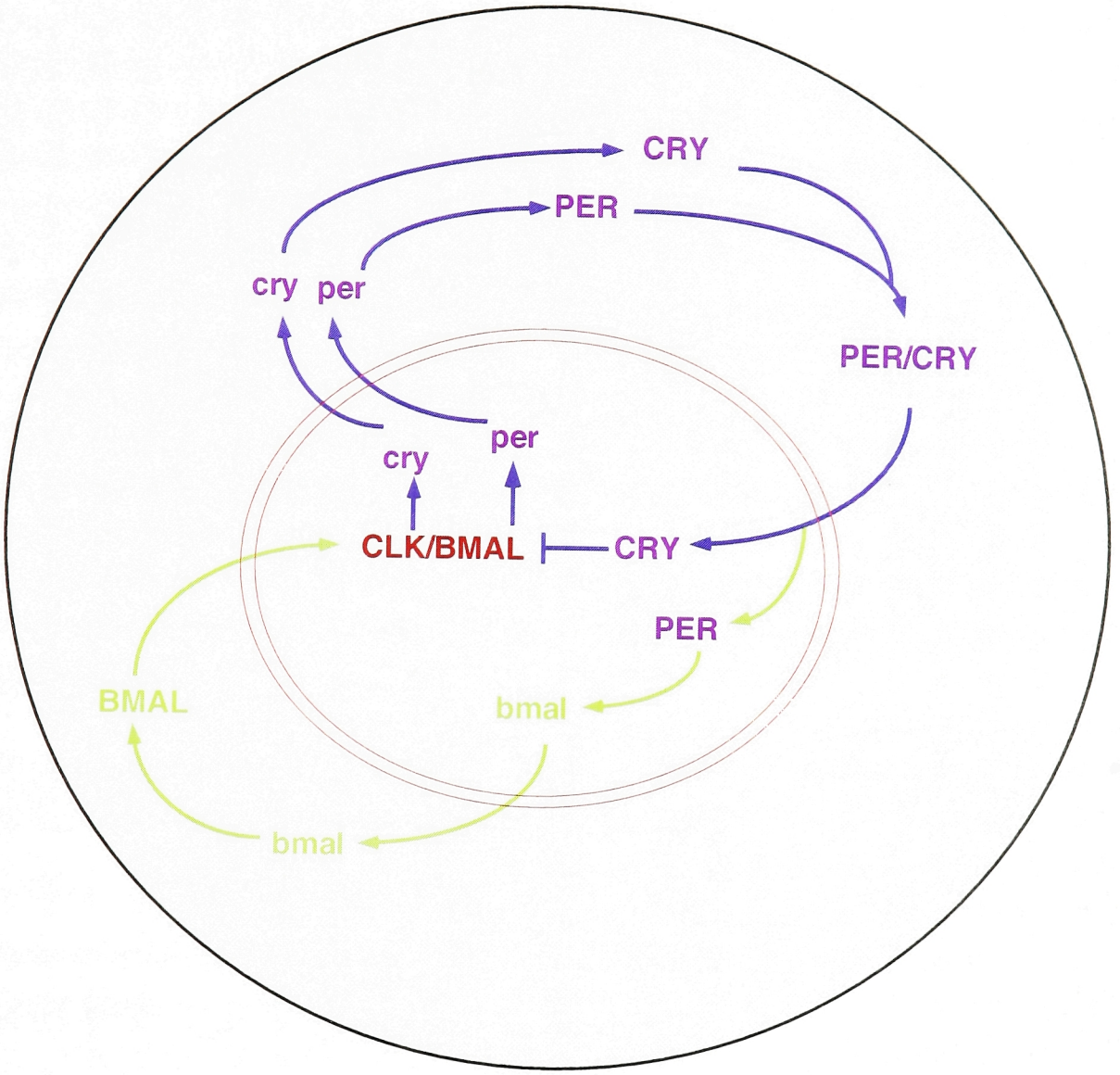
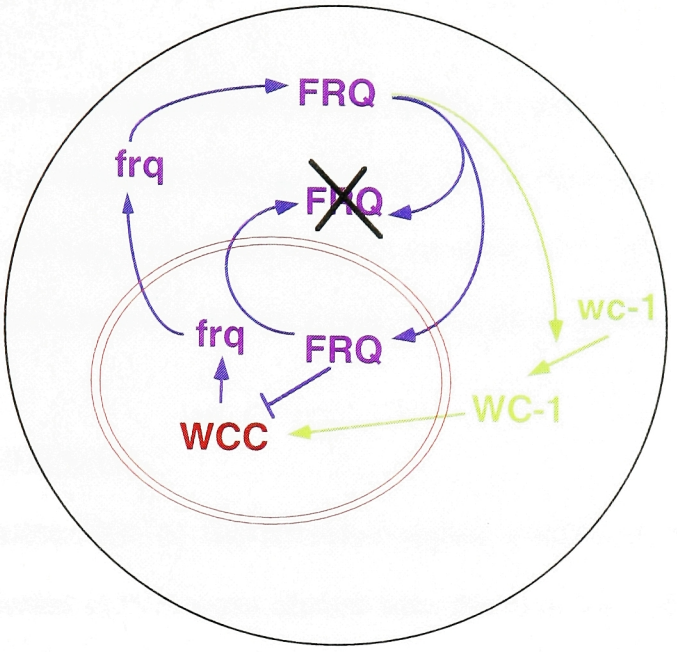

CRY
PER
cry
per
PER/CRY
per
cry
CLK/BMAL
CRY
PER
BMAL
bmal
bmal

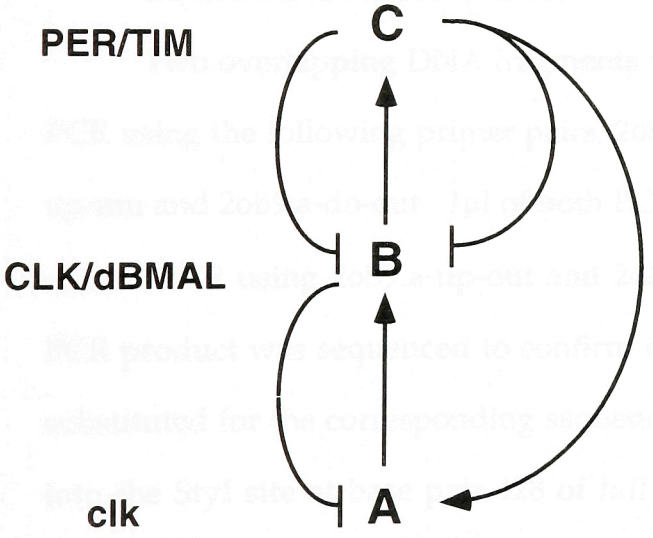
Figure 3: Schematic model of the *Neurospora* circadian oscillator (A) and comparison between general features of the *Drosophila, Neurospora,* and mammalian clocks (B).

A) Interlocked feedback loops are depicted by different coloring. Names of proteins are in uppercase and names of genes and RNAs are in lowercase. Briefly, WCC activates the expression of *frequency* giving raise to increasing levels of FREQUENCY, which in turn deactivates its own expression by inhibiting WCC. Simultaneously, FREQUENCY positively regulates the translation of *white collar-1* RNA. Consequently, WHITE COLLAR-1 levels rise resulting in an increased *frequency* transcription. Once the FREQUENCY pool is depleted by degradation, the inhibition on WCC is alleviated, *frequency* expression resumes, and the cycle starts again. Abbreviations are: frq – *frequency;* WCC – heterodimer of WHITE COLLAR-1 and WHITE COLLAR -2; wc-1 – *white collar-1*; B) Comparisons of interactions between components in the *Drosophila, Neurospora* and in the mammalian oscillator. Arrows and bars represent positive and negative effects of one component on abundance or activity of the another component. These effects correspond to genetic interactions and are not intended to imply a biochemical process. Note the high degree of similarity between the systems. Also, if the two negative interactions in *Drosophila* were replaced with one positive, which should have the same qualitative effect, the oscillators are principally identical.

A



B



Drosophila	Neurospora	Mammals
PER/TIM	FRQ	mPER/CRY
CLK/dBMAL	WCC	CLK/BMAL
clk	WC-1	bmal

Chapter 2: Materials and Methods

Molecular cloning of transgenes and transgenic fly strains

For all cloning steps standard procedures were employed (Ausubel *et al.* 1999). Unless otherwise stated enzymes were obtained from New England Biolabs. The constructs were injected into *Drosophila* embryos by standard methods.

INVERTED REPEAT

For the construction of the inverted-repeat construct, PCR amplified fragments of the *period* cDNA were cloned into the cloning vector pBluescript (Stratagene). Using different restriction sites of the vector pBluescript the respective fragments were first cloned in antisense orientation into pUASt and subsequently in sense orientation 3prime of the first insert.

DOMINANT NEGATIVE 2ob9 CONSTRUCT

Two overlapping DNA fragments were amplified from the *tuII* cDNA by PCR using the following primer pairs, 2ob9.a-up-out and 2ob9.a-do-mu; 2ob9.a-up-mu and 2ob9.a-do-out. 1µl of both PCR products were used as template in a second PCR using 2ob9.a-up-out and 2ob9.a-do-out as primers. The resulting PCR product was sequenced to confirm the presence of the 2ob9 mutation and substituted for the corresponding sequence in the wild type cDNA by cloning it into the StyI site at base pair 428 of *tuII* and BglII at 842. The mutant cDNA (2ob9.a) was cloned into the the *Drosophila* transformation vector pUASt.

tuII-GFP CONSTRUCT

Green fluorescent protein cDNA (Prasher *et al.* 1992) was amplified by PCR using the primers gfp-upper with NarI site and gfp-lower with ApaI site. The PCR product was subsequently cloned at the end of the *tuII* cDNA and the fusion was cloned into AKSII (pBluescript containing *actin* cis regulatory sequences; Simon Kidd, personal communication).

timeless-(UAS)-GAL4 CONSTRUCT

A pentamer of the UAS was amplified by PCR using the vector pUASt as template. The PCR product was flanked by an EcoRI and a MunI site and cloned into the EcoRI site 333 bp upstream of the transcription start for *timeless* into a genomic DNA fragment from the *timeless* locus. This genomic DNA fragment contains the sequence from the BamHI site 4015 bp upstream of the transcription start to the SalI site at 2191 bp downstream of the transcription start. The genomic DNA fragment including the UAS was cloned in front of the cDNA for GAL4 and subsequently ligated into the *Drosophila* transformation vector pUASt.

Sequencing of 2ob9 allele

cDNA fragments were amplified by RT-PCR from heterozygous mutant flies using *tuII* specific primers. The RT-PCR products were subsequently sequenced (Sequenase™ kit; USB) according to manufacturer's guidelines.

Genomic Southern blotting and cDNA screening

Restriction digests, gel electrophoresis and blotting onto nitrocellulose membranes were performed by standard techniques (Ausubel *et al.* 1999). Also plating of cDNA libraries and transfer onto membranes were performed by

standard methods (Ausubel *et al.* 1999). Membranes were pre-hybridized in Church's hybridization buffer (0.5M $NaPO_4$, 1mM EDTA, 1% BSA, 7% SDS, pH7.2). ^{32}P labeled DNA probes were prepared according to manufacturers instructions (Prime-It II®, Stratagene). Hybridization was performed in Church's over night at 65°C. Membranes were washed in wash mix for a hour with several changes of the wash mix (40mM $NaPO_4$, 1mM EDTA, 1% SDS, pH 7.2). Signals were detected by exposing the membranes to x-ray films for the appropriate length of time.

***in situ* hybridization on adult head sections**

Heads were sectioned in O.C.T. using a cryostat from Bright Instruments Company. Sections were dried at room temperature for 1 hour, fixed for 20min in 4% paraformaldehyde in PBS, washed 3 times in PBS, acetylated for 10min at room temperature (1ml triethanolamine, 131µl HCl, and 187.5µl acetic anhydride in 73.5ml H_2O, and washed again 3 times in PBS. Sections were prehybridized (50% formamide, 5xSSC, 5xDenharts, 250µg/ml bakers yeast RNA, 100µg/ml herring sperm DNA) for 2 hours at room temperature. DIG-labeles RNA probes were prepared according to the manufacturers instructions (Roche) and boiled for 2min prior to hybridization. Probes were hybridized in hybridization solution at different concentrations over night at 55°C. Sections were washed for 2 hours at 55°C in 0.2xSSC, equilibrated in B1 (100mM Tris, pH7.5, 150mM NaCl), blocked in B1 with 10% heat inactivated normal goat or sheep serum, and incubated with alkaline phosphatase conjugated anti-DIG antibodies over night

at 4°C. Sections were washed the next day in B1. Signal detection was performed as described for the *in situ* hybridization on embryos.

RNAse protection assay

Total RNA was isolated from heads as described in the manufacturers manual (TEL-TEST, INC.). The RNAse protection assay was performed as described in the manufacturers manual for the RPAII kit (Ambion). 10 μg of total RNA were used for each sample. The riboprobe for *period* (Sehgal *et al.* 1994), *timeless*, and tubulin (Sehgal *et al.* 1995) have been described previously. As riboprobe for *rhodopsin1*, an RT-PCR fragment (608-878) was used. The intron-exon boundary spanning riboprobe for *timeless* contained the sequence from the AvaI site at base pair 1842 of the genomic DNA (base pair 0 is transcription start) to SalI site at base pair 2192.

Fly culture and locomotor analysis

Drosophila melanogaster were reared on standard medium at 25°C. Monitoring and analysis of locomotor activity of individual flies was performed at 25°C using the *Drosophila* Activity Monitoring System IV (TriKinetics).

Immuno-histochemistry on third instar larvae and adult brains

Third instar larvae were dissected in PBS, collagenase treated, fixed in 4% paraformaldehyde, washed (PBS, 0.5% TritonX100), and blocked in blocking solution (PBS, 0.5% TritonX100, 10% donkey serum). Primary antibody incubation was done overnight at 4°C, samples were subsequently washed,

incubated for 1 hour at room temperature in secondary antibody (Jackson ImmunoResearch), washed, and mounted in 50% glycerol.

Immuno-histochemistry on embryos

Egg collections of the required ages were washed, dechorionized and fixed in Formaldehyde (Fisher) by standard procedures. Embryos were subsequently washed in BBT (10mM Tris, 55mM NaCl, 40mM KCl, 7mM $MgCl_2$, 5mM $CaCl_2$, 50mM sucrose, 0.1% BSA, 0.1% Tween20, and 20mM glucose, pH 6.95) and incubated in primary antibodies in BBT at 4°C over night. After the primary antibody incubation embryos were washed in PBT (PBS, 0.1% Tween20), subsequently incubated in secondary antibodies in PBT for 1 h at room temperature and washed again in PBT. The DAB reaction was performed in PBT with 0.2mg/ml DAB (Sigma) and 0.003% H_2O_2.

***in situ* hybridization on embryos**

DIG labeled RNA was prepared with the DIG RNA labeling kit following the manufacturers instruction (Roche). Embryos were fixed as for immuno-histochemistry, washed in PBT (PBS, 0.1% Tween20), Proteinase K digested (0.05mg/ml) for 3 minutes, incubated in glycine (2mg/ml in PBT) for 3 minutes, washed in PBT, and postfixed in 4% Formaldehyde in PBS. Embryos were incubated in PBT:hybridization solution, 1:1, and subsequently prehybridized in hybridization solution (50% Formamide, 5xSSC, 50µg/ml heparine, 0.1% Tween20, and 0.1 mg/ml hering's sperm). The probe was hybridized at different concentrations to embryos at 55°C in hybridization solution over night. The

following day embryos were washed in hybridization solution, PBS:hybridization solution, 1:1, and in PBT. Subsequently, embryos were incubated in phosphatase conjugated anti-DIG antibodies (Jackson ImmunoResearch) for 1 hour at room temperature and again washed in PBT. The phosphatase reaction was performed in staining solution (0.1M Tris pH9.5, 0.1M NaCl, 50mM $MgCl_2$, 0.1% Tween20) supplemented with 4.5µl/ml NBT (75mg/ml) and 3.5µl/ml BCIP (50mg/ml).

Preparation of genomic DNA

Approximately 100 frozen flies were ground and subsequently mixed with 0.5ml homogenization buffer (30mM Tris, pH 8.0, 100mM NaCl, 10mM EDTA, 0.5ml TritonX100/100ml, 140µl β-mercaptoethanol/100ml). Samples were spun in a microcentrifuge at 15k rpm for 1 min, the supernatant was discarded and the pellet resuspended in 0.5ml extraction buffer (100mM Tris, pH 8.0, 100mM NaCl, 20mMEDTA). Samples were spun again, supernatants discarded, and the pellet resuspended in 0.3ml extraction buffer, supplemented with 30µl proteinase K (Roche, 1mg/ml) and 30µl 10% Sarkosyl, and incubated at 50°C for 4 hours. Subsequently the suspension was subjected to a phenol extraction and the DNA was ethanol precipitated from the supernatant.

Western blot analysis

Proteins were extracted from ~50µl heads (~200 heads) in 100µl head extraction buffer (100mM KCl, 20mM HEPES, 5% glycerol, 10mM EDTA, 0.1% TritonX100, 1mMDTT, pH 7.5) with a handheld homogenizer (Kontes). Samples

were subsequently centrifuged at 15K for 10 minutes at 4°C in an Eppendorf microcentrifuge. 10μg of total protein were loaded per lane on a 5% SDS polyacrylamide gel. Gels were run and blotted on nitrocellulose membrane (Schleicher & Schuell) using the Mini-Vertical Electrophoresis System from Bio-Rad. Membranes were blocked (PBS, 0.5% Tween20, 5% non-fat dry milk) at room temperature and subsequently incubated with the primary antibodies (1:2,000 in blocking solution for anti-TIMELESS) at 4°C over night. Membranes were washed three times for 10 minutes in PBT (PBS, 0.5%Tween20), incubated with secondary antibodies (1:1,000 in PBT, Jackson ImmunoResearch), and washed again three times for 10 minutes. Immuno-complexes were detected using the ECL immunoassay signal reagent (Amersham Pharmacia Biotech).

Kinase Assay

The fusion proteins were expressed in the bacterial strain BL21 and purified using Glutathione Sepharose®4B according to manufacturer's instructions (Pharmacia Biotech). After the last wash step, 10μl beads with the attached fusion proteins were incubated with 2 units of GSK-3β (NEB) and approximately 1μCi (γ–^{32}P) ATP (NEN) in phosphorylation buffer for 30min at 30°C. Following the kinase reaction, the beads were washed to remove free radio-nucleotide and boiled in 2xSDS gel loading buffer. The beads were precipitated by centrifugation and the samples were subjected to SDS PAGE. After the Electrophoresis the proteins were either transferred to a nitrocellulose membrane (Protran®, Schleicher and Schuell) for autoradiography or stained with coomassie blue.

Phosphorylation site prediction

The TIMELESS sequence was submitted to the on-line protein substrate motif searching program Motif Scanner at http://cansite.bidmc.harvard.edu/motifscanner/motifscan1.phtml?database=_SWS_#. The analysis was conducted at medium stringency.

Primers

project	name	sequence (5'->3')
Inverted Repeat	perup1894	TGATAAGCsTTAGATCTCCACATGTAAGCTG AAGATATCG
	perdo2889	TAGGAATTCATTCGCATCTGTTCCCAGAGTTAG
	perup1065	TGATAAGCTTAGATCTAGCGGGTGAAGGAGGACAG
	perdo1887	TAGGAATTCATCCTCGAAGACGTTGCACTG
rhodopsin RPA probe	up608	ATGGAATTCACTTGGAACGCGACTGGAAC
	do878	ATGAGATCTAGGTATGGTGTCCACGCCATGAAC
Adf-1	up498	GAAGAGTCTGCGCGACAAGTTC
	do1157	TGGGTCTGTTCTCGTTTGGTCTCAG
2ob9 dominant negative	2ob9.a-up-out	ACAGAATTCCAGTTATCCAAGGGGCGCAAG
	2ob9.a-up-mu	TGGTCATGTTCGCCACACTG
	2ob9.a-do-mu	AGTGTGGCGAACATGACCAC
	2ob9.a-do-out	ACTAAGCTTTGTAGAAGATCTCCACGGGATG
tuII-GFP	gfp-upper	TAAGGCGCCCATGAGTAAAGGAGAAGAAC
	gfp-lower	ATTGGGCCCTTATTTGTATAGTTCATCC
tuII and 2ob9.a sequencing	tuII43	TGAGAATTAAGTGCAGTAATCACC
	tuIIup-1	ATCGAAGTGGGGGAGACCTACG
	tuIIup-2	ATGAACCCGCTGACCAACACGA
	up-428	AAGGGGCGCAAGGGAGTC
	up-560	ACGGCCAAGACGCTGCTCAAGTAC
	up-827	ATCTCTACACGCCCGAACC
	up-942	AGGAGGAAGATTGAGGAGGCTTGC
	up-1077	CCCGCCGCCAAATGCTAATGG
	up-1295	TCCTGGCAAGTGTTTCCGTCTG
	up-1468	TTGATGCGTGCCCTGGAG
	up-1552	AGTTTCCATTGGATCCGCAG
	up-1778	ATCCAAACTGGTGCTACGAGAGC
	up-3	TGCTGACTACAAAGAACTACATTCG
	do-4	TTACAAACAATAAAAAGCAAACGGG

	do-3	CCAGGCAGCACAACCATTCAGG
	do-1839	ACGTTATCCGCACTCTTTAGC
	do-1511	TCCAGGGCGGCCAGATAGTTGAGC
	do-1441	ATCGAAATGCACCAGATCATC
	do-1359	AGGTGTTGTCCTGCATCTCG
	do-1161	TCCGTCAATGGTCAGCGAGGTTTC
	do-881	TCGCACATGTGTATCTGGATTAC
	do-848	AGCCTCTAGGTAATCTCTTTC
	do-701	TCCTTGAGCACGCCCATTAGAATG
	do-499	TTTTACGTCCATCTCTCC
	do-2	TCGCCCACCAGCACAATACAC
timeless-(UAS)-GAL4	up	ATGAATTCTCCGGCGCTCGCTAGA
	do	CACAATTGCATGCCTGCAGGTCG

Chapter 3: The splicing factor DPRP43 is required for proper circadian clock function in *Drosophila melanogaster*

Introduction

Several studies showed that mutations in genes encoding clock components can alter the period of the locomotor activity rhythm in a semidominant manner (Konopka and Benzer 1971; Rothenfluh *et al.* 2000a). This finding sets the biological clock in *Drosophila* apart from most developmental systems in which heterozygous mutations are usually without detectable effects. The reasons for this difference may be twofold. First, the availability of a precise quantitative assay for circadian behavior allows detection relatively small deviations from wild type. The absence of such an assay in other systems may prevent the investigator from identifying equally small changes. Secondly, measuring the period of locomotor activity patterns extends over several days. The iteration of the phenotype each day renders this system more tractable to a statistical analysis. Only few developmental systems offer a similar feature. One example is the compound eye of *Drosophila* which is composed of ~800 identical units, the ommatidia. Even a mutation with low penetrance will result in a phenotype in some ommatidia, thus lending itself to a statistical analysis.

Taking advantage of the semidominant, period-altering effects of many mutations in clock related genes, an F_1 screen of EMS induced mutations was conducted in our laboratory. This screen yielded mutations in the *timeless* and *double-time* genes as well as in several new loci (Rothenfluh 1999). In this chapter the characterization of a new period-lengthening mutation is described. Cloning

identified the corresponding gene as an RNA helicase of the DEAH box family (formerly known as DEAD box; Wassarman and Steitz 1991).

On the basis of 78% overall homology of this gene to the yeast splicing factor Prp43 (Arenas and Abelson 1997), it was termed *dprp43*. This gene is highly conserved from yeast to humans and appears to be an essential component of the general splicing machinery (Ono *et al.* 1994; Arenas and Abelson 1997; Gee *et al.* 1997). Accordingly, we were able to demonstrate an increase in the ratio of unspliced/spliced *timeless* RNA and an overall reduction of *period* and *timeless* RNA in flies over-expressing a dominant negative form of *dprp43*. The period-lengthening effect of the mutation that was isolated in our screen can be explained by virtue of its effect on splicing. Throughout the day splicing efficiency and/or accuracy is reduced in the mutant, thereby delaying the accumulation of clock components which in turn results in an overall lengthening of the circadian cycle.

Results

Cloning of a *dprp43*

The 2ob9 mutation, which lengthens the circadian period by approximately 2 hours in a dominant manner, was isolated in a genetic screen for defects in circadian locomotor activity (Figure 4). In addition, the isolated chromosome carries a recessive lethal mutation. Both lethality and period-lengthening mutation were mapped by meiotic recombination to chromosome band 43B. A complementation analysis of mutations in this region showed that the lethality conferring mutation is allelic to a previously isolated

complementation group, *l(2)43Bd* (Heitzler *et al.* 1993). Two other alleles of this complementation group, $l(2)43Bd^{P}$ and $l(2)43Bd^{4}$, were tested for their locomotor activity rhythm, but no significant deviation from wild type was observed in heterozygotes. A possible explanation for this observation is that $l(2)43Bd^{P}$ and $l(2)43Bd^{4}$ are hypomorphic or null alleles but a reduction of gene function by 50% does not result in a phenotype. In contrast, the 2ob9 mutation could be a dominant negative allele, thus reducing gene function by more than 50%. However, an alternative explanation is that period-lengthening phenotype and lethality are caused by mutations in two independent loci. In order to distinguish between these possibilities, we tested whether the phenotypes could be separated through recombination. 200 recombined chromosomes were tested for locomotor activity rhythm and for complementation of the lethality of *l(2)43Bd*. As shown in Figure 4, failure to complement *l(2)43Bd* segregated with the long period phenotype. Since no recombination between lethality and period-lengthening phenotype was found among 200 recombined chromosomes, it was assumed that lethality and rhythm phenotype are caused by the same mutation.

As an entry point into the molecular cloning of the gene, a P-element induced allele of *l(2)43Bd*, $l(2)43Bd^{P}$, was used. Hybridization of the plasmid rescue fragment to an annotated P1-filter (Berkeley *Drosophila* Genome Project; Genome Systems, Inc.) showed that the P-element does not map to chromosomal band 43B. It is therefore likely that the P-element was inserted first in *l(2)43Bd* and was subsequently excised and reinserted in another location thereby creating a chromosomal rearrangement in *l(2)43Bd*. The cloning strategy was based on locating this chromosomal rearrangement.

Previous genetic mapping of the region had shown that *l(2)43Bd* resides between the chromosomal deficiencies Def(2R)cos2 and Def(2R)NCX11 (Heitzler *et al.* 1993). In order to restrict the region which contains *l(2)43Bd* on a physical map, breakpoints of these complementing deficiencies were mapped by restriction fragment length polymorphisms (Figure 5A). DNA fragments of the 25 kb between Def(2R)cos2 and Def(2R)NCX11 were used as probes on genomic Southern blots made from wild type and $l(2)43Bd^{p}/+$ genomic DNA. Restriction fragment length polymorphisms were detected only with fragment X/S3.5 (Figure 5A, B). This fragment contains part of a previously identified transcription unit, *tuII*, and borders at *costal2* (Sisson *et al.* 1997). Since *costal2* complements lethality of *l(2)43Bd* as well as the rhythm phenotype of 2ob9, *l(2)43Bd* most likely is encoded by *tuII*. In order to test this, *tuII* RNA levels in homozygous mutant $l(2)43Bd^{p}$ embryos were measured. Placing $l(2)43Bd^{p}$ over a balancer chromosome that is marked with lacZ expression under control of the *wingless* promoter allowed to identify homozygous mutant embryos. As shown in Figure 5D, *tuII* RNA levels are substantially reduced in homozygous mutant $l(2)43Bd^{p}$ embryos. Both observations, the chomosomal rearrangement and the reduction of *tuII* RNA levels, argue strongly for *tuII* being identical with *l(2)43Bd*.

Screening of an adult head cDNA library using DNA fragment X/S3.5 (Figure 5A) as probe yielded a 2.5 kb long cDNA. This is in accordance with the size of the *tuII* mRNA on a Northern blot (data not shown). Sequencing analysis revealed *l(2)43Bd/tuII* as a member of the DEAH box family of RNA helicases. With 64% identity and 78% homology, *l(2)43Bd* appears to be most closely related to the yeast splicing factor Prp43 (Figure 6; Arenas and Abelson 1997). The high degree of homology prompted us to term *l(2)43Bd/tuII dprp43*.

In order to test whether DPRP43 is indeed a nuclear protein as expected from its biochemical function, a construct encoding a DPRP43-GFP fusion protein was generated. Transfection of cultured cells from *Drosophila* with DNA of this construct revealed that most if not all of the fusion protein is localized in the nucleus (data not shown).

The 2ob9 mutation is a dominant negative allele of *dprp43*

In order to elucidate the molecular nature of the 2ob9 mutation, *dprp43* was sequenced from heterozygous mutants. As shown in Figure 7A, a missense mutation was detected at position 740 of the cDNA. Conceptual translation of the mutated cDNA sequence results in a serine to phenylalanine exchange at amino acid position 225 (Figure 7B). This region has been implicated in coupling of NTPase and RNA helicase activity (Plumpton *et al.* 1994). A mutation in the very same position of the yeast homolog shows dominant negative effects. The mutant protein is presumably still incorporated into the spliceosome, but the failure to exert its catalytic function stalls the splicing-reaction. The 2ob9 mutation could in a similar fashion interfere with splicing. Since the molecular oscillator is based on the alternation of activation and deactivation of *timeless* and *period* gene expression, a decrease in the efficiency/accuracy of RNA processing could account for the lengthening of the locomotor activity period.

Broad *dprp43* expression in adult heads

The effect of a mutation in *dprp43* on circadian rhythmicity requires its presence in tissues relevant for circadian behaviour. In order to test whether *dprp43* is present in LNs, *in situ* hybridizations on adult head sections with a

dprp43 specific probe were performed. As shown in Figure 8B, *dprp43* RNA is widely expressed in the adult head. Regions without signal consist mostly of axons, whereas staining was observed in all regions containing cell bodies. It was therefore concluded that *dprp43* is expressed ubiquitously but its RNA is not transported into axons. Ubiquitous expression of *dprp43* was also observed in embryos (data not shown). Thus, *dprp43* expression includes the LNs consistent with a function in the molecular clock. The TIMELESS antibody staining in Figure 8A shows the location of clock relevant cells in the adult head for comparison.

The molecular defect in 2ob9 occurs prior to nuclear entry

In order to test in what step of the circadian cycle 2ob9 is acting, the phase shift response to photic stimuli at different times of the day under otherwise constant darkness, *i.e.* a phase response curve (PRC; Aschoff 1965), was measured. When nuclear TIM is degraded by light, nuclear processes are accelerated, and the clock is advanced. In contrast, when cytoplasmic TIM is degraded in response to light, time has to elapse until TIM reaccumulates, and thus the clock is delayed (reviewed in Young *et al.* 1996). Depending on the part of the cycle affected by the 2ob9 mutation the advance or the delay part of the PRC should deviate from wildtype.

As shown in Figure 9A, the transition point between the delay and advance portions of the PRC is delayed by approximately 2 hours in 2ob9 mutant flies. This corresponds to the 2 hour period-lengthening effect of 2ob9 suggesting that the molecular defect in 2ob9 mutant flies occurs prior to nuclear entry. Accordingly, TIM protein accumulation and phosphorylation are slightly

delayed in an LD cycle (Figure 9B). Both of these observations are consistent with the idea that the lengthened period in 2ob9 mutant flies results from a splicing defect resulting in delayed RNA and protein accumulation.

Over-expression of the mutant cDNA has dominant negative effects

The mild phenotype in the original mutant made it impossible to investigate the molecular defect more closely. In order to generate a stronger phenotype, the 2ob9 mutation (Figure 7) was introduced into the *dprp43* cDNA by *in vitro* mutagenesis. Using the UAS/GAL4 binary expression system, the mutant protein can be expressed in any tissue (Brand and Perrimon 1993; Chapter 4). Flies over-expressing 2ob9 under control of the *period* promoter showed arrhythmic locomotor behaviour. To test whether the lateral neurons are differentiated properly in those flies, we performed immuno-histochemistry using an antibody against PDF which is a well documented marker for these cells (Helfrich-Förster 1995). In all cases no staining or a very reduced signal in fewer cells than in wild type controls was observed (data not shown). The same result was obtained with other markers for the lateral neurons suggesting that over-expression of 2ob9 interferes with differentiation and/or cell viability. This notion is supported by the observation that ubiquitous expression of 2ob9 under control of the *daughterless* promoter is lethal. Accordingly, expression of a dominant negative form of Prp43 in yeast interferes with viability. As Prp43 is an essential splicing factor, the lethality associated with reduction of Prp43 function is a reflection of global reduction in splicing and consequently gene expression.

In contrast to the lateral neurons, the eyes of flies over-expressing 2ob9 under control of *period*-GAL4 appeared normal, possibly because of the late onset of *period* promoter conferred transcription in the retina. Since the adult fly retina contains a functioning molecular oscillator and approximately 75% of *period* and *timeless* RNA is expressed there (Zeng *et al.* 1994), the effect of 2ob9 over-expression on the molecular oscillator could be tested in the eyes.

In order to assay the molecular oscillator in flies over-expressing 2ob9, total head RNA from two different time points in constant darkness was subjected to an RNAse protection assay. The two time points, CT2 and CT14 (CT0 is the beginning of the subjective day), were chosen because at these times RNA levels of *period* and *timeless* are at their peak or trough, respectively. As shown in Figure 10A, *period* RNA levels are reduced in the mutant. Over-expression of 2ob9 probably decreases splicing efficiency which may cause the degradation of unprocessed RNA thereby reducing overall RNA levels.

Because reduction of total RNA levels is only an indirect way of estimating splicing efficiency, testing the levels of unprocessed RNA was desirable. For this purpose a *timeless* specific RPA probe containing intron and exon sequences was constructed (see Materials and Methods). To test the specificity of this probe transcribed genomic sequence as well as the transcribed *timeless* cDNA were tested with this probe. In both cases a protected fragment of the expected size was observed (Figure 10B). As shown in Figure 10C, the unspliced/spliced RNA ratio for this intron is increased by a factor of 2 in flies over-expressing 2ob9, confirming a role of *dprp43* in RNA processing. Since the RPA probe used in this experiment detects only one out of 14 introns, the total amount of not completely spliced RNA is likely to be higher.

Discussion

The period of the molecular oscillator that regulates circadian behavior is susceptible to changes in function of its constituent components. Here it is shown that a dominant negative form of the splicing factor *dprp43* interferes with the function of this oscillator.

In a behavioral screen the mutant 2ob9, which lengthens the circadian period by approximately 2 hours, was isolated. The isolated chromosome also carried a recessive lethal mutation. Both traits, lethality and rhythm phenotype, were tightly linked indicating that lethality and rhythm phenotype are caused by the same mutation.

The mutation *l(2)43Bd*^{P} failed to complement the lethality of 2ob9 and served as an entry point in the cloning of the mutated gene. A chromosomal rearrangement in *l(2)43Bd*^{P} near the previously identified transcript *tuII* implied that this transcript is identical with *l(2)43Bd*/2ob9. Accordingly, *tuII* levels are reduced in *l(2)43Bd*^{P}. Sequence comparison of the *tuII* cDNA revealed *l(2)43Bd*/2ob9 as a member of the DEAH box family of RNA helicases. The most closely related homolog appeared to be the yeast splicing factor Prp43, the gene *l(2)43Bd* was therefore termed *dprp43*.

Sequencing of *dprp43* RNA from 2ob9 revealed that the serine at amino acid position 225 had been substituted with a phenylalanine in the mutant. A mutation at the corresponding amino acid position in a yeast homolog was found previously to exhibit dominant negative effects on splicing (Plumpton *et al.* 1994). Furthermore, this and other dominant negative mutations of several very closely related RNA helicases/splicing factors appear to interfere with the

coupling between NTPase and RNA helicase activities (Edwalds-Gilbert *et al.* 2000; Schwer and Meszaros 2001). Introduction of this mutation into the wild type cDNA and ubiquitous over-expression of this mutant cDNA resulted in lethality. This supports the idea that 2ob9 is responsible for the lethality in the originally isolated mutant. Unfortunately, over-expression of the mutant cDNA in cells relevant for circadian rhythmicity interfered with differentiation and viability of these cells, thereby preventing a behavioral analysis. However, over-expression in the retina caused no morphological abnormalities in this tissue, probably due to the late onset of the applied promoter. A reduction of *period* and *timeless* RNA in this tissue of flies over-expressing the mutant cDNA was observed. This observation is in accordance with a lengthening of the period of the molecular oscillator as a previous gene dosage study has established a correlation between *period* RNA levels and period length (Baylies *et al.* 1987). The tight linkage between period phenotype and lethality as well as the data obtained by over-expression of the dominant negative construct strongly suggest that the mutation in *dprp43* is responsible for the period phenotype in the original mutant.

Yeast Prp43 is thought to be primarily required for the disassembly of the spliceosome (Arenas and Abelson 1997). Since the splicing factors are recycled (reviewed in Staley and Guthrie 1998), a failure to disassemble the spliceosome will eventually result in a slowing down of the splicing reaction. A negative transcriptional feedback loop forms the core component of the circadian clock in *Drosophila* (Young 1998; Dunlap 1999; Scully and Kay 2000). Consequently, the rate of expression of the clock genes will partially be responsible for the period of the molecular oscillator. It is therefore a conceivable scenario for the molecular

defect in the 2ob9 mutant that a splicing defect decelerates the accumulation of clock components during the circadian cycle, which manifests itself in a long period phenotype. Accordingly, TIM protein accumulation is delayed in the mutant. Also compatible with a splicing defect is the observation that the transition from the delay to advance part of the PRC occurs 2 hours later in 2ob9 compared to wild type suggesting a molecular defect prior to nuclear entry of the PER/TIM heterodimer.

Over-expression of the 20b9 mutant cDNA under control of the *period* promoter caused an increase of the ratio of unspliced to spliced *timeless* RNA. This supports the idea that *dprp43* has an affect on the processing of RNA like that of its homolog in yeast.

It was suggested previously that RNA helicases of the DEAH box family act as kinetic proof reading devices in order to increase accuracy of the splicing reaction (Burgess and Guthrie 1993). It is therefore also conceivable that a decrease in splicing accuracy is responsible for the delay in protein accumulation and consequently for the long period phenotype.

Flies heterozygous for the original mutation show no obvious defects in their morphology, sex ratio, or developmental timing, yet their circadian behavior is abnormal. Although subtle phenotypes in other systems could have remained undetected, the specificity of the phenotype indicates that the molecular clock is more sensitive to splicing defects than other systems. This may be a reflection of the daily oscillation of transcriptional activity whose frequency determines the period of circadian behaviour.

Figure 4: The mutation 2ob9 causes a long period phenotype in a dominant manner and is allelic to *l(2)43Bd*.

The chromosome carrying the period-lengthening mutation 2ob9 was recombined for one generation and flies carrying this chromosome were subsequently subjected to a locomotor analysis and to a complementation analysis with *l(2)43Bd*. Note that the average period length of flies carrying the recessive lethality is 25.4h and the average of the flies without this mutation is 23.8h. Stocks were established from all flies carrying the lethality with periods less than 25h and their locomotor activity was tested again. The average period of all these stocks was over 25h. Similarly, stocks from all flies without the lethality and with periods longer than 24h had average rhythms of less than 24h.

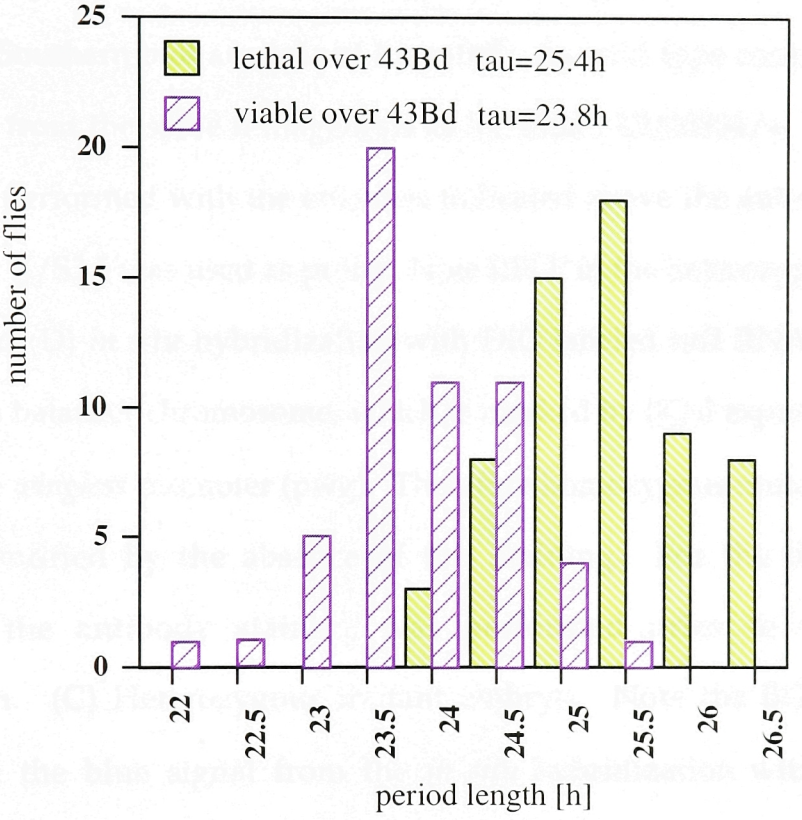
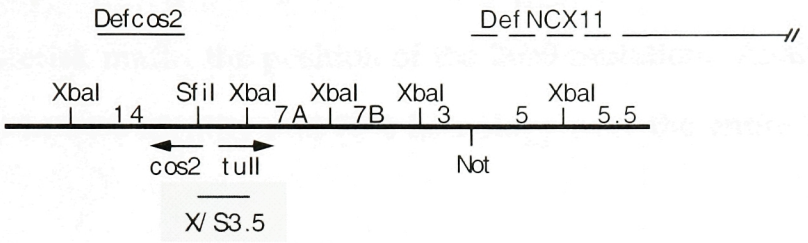

lethal over 43Bd tau=25.4h
viable over 43Bd tau=23.8h
number of flies
0
5
10
15
20
25
22
22.5
23
23.5
24
24.5
25
25.5
26
26.5
period length [h]

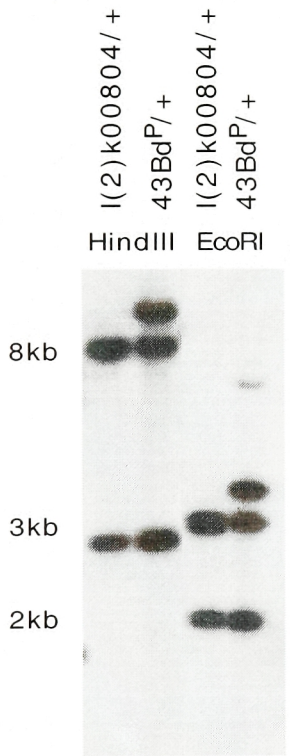
Figure 5: *l(2)43Bd* is encoded by transcription unit *tuII*.

(A) Restriction map of locus *l(2)43Bd*. Approximate position of closest complementing deficiencies is indicated above the map. Below, positions of *costal2*, transcript *tuII* (arrows), and the genomic fragment X/S3.5 are shown. (B) Genomic Southern blot analysis of $l(2)43Bd^{P}$. As wild type control served a chromosome from the same mutagenesis as $l(2)43Bd^{P}$, l(2)00804/+. Restriction digests were performed with the enzymes indicated above the autoradiograph. The fragment X/S3.5 was used as probe. Note RFLP in the heterozygous mutant BdP. (C) and (D) *in situ* hybridization with DIG labeled *tuII* RNA. BdP was placed over a balancer chromosome, which is marked by βGal expression under control of the *wingless* promoter (pwg). Therefore homozygous mutant embryos could be identified by the absence of this staining. For the double-label experiment the antibody staining was performed prior to the *in situ* hybridization. (C) Heterozygous mutant embryo. Note the βGal staining (brown) and the blue signal from the *in situ* hybridization with *tuII*. (D) Homozygous mutant embryo. Note the absence of both, the βGal staining and the *in situ* hybridization signal.

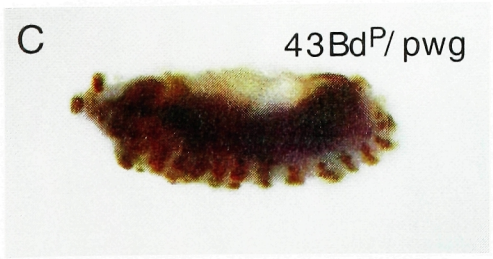
A


Defcos2
Def NCX11
Xbal
Sfil
Xbal
Xbal
Xbal
Xbal
14
7A
7B
3
5
5.5
cos2
tull
Not
X/S3.5

B


I(2)k00804/+
43Bd^P/+
I(2)k00804/+
43Bd^P/+
HindIII
EcoRI
8kb
3kb
2kb

C


43Bd^P/pwg

D


43Bd^P/43Bd^P

Figure 6: *tuII* encodes a homolog of the yeast splicing factor Prp43.

Alignment of amino acid sequences of conceptually translated *tuII* cDNA with Prp43. Asterisk marks the position of the 2ob9 mutation. Analysis with BLAST resulted in 64% identity and 78% homology over the entire length of dPRP43.

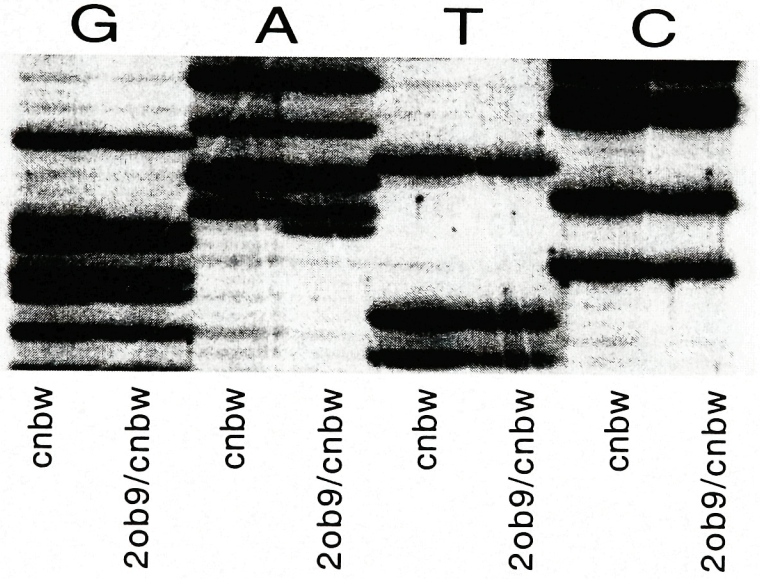
```
tuII/dPRP43   MSKRRIEVGETYGSKAKKDPEASSSSAAAAAGTVAQILGGFVPNKQPPTMNPLTNTPYSQRYQNLYKKRIALPVFEYQAD   80
                :    E  ..  : :..:P .  .::.  :    :. :. ..: :  .:NP:T.  ::.:Y :: K R .LPV . :.:
PRP43         QAAEIAEELSKQHPLPSEEPLVHHDAGEFKGLQRHHTSAEEAQKLEDGKINPFTGREFTPKYVDILKIRRELPVHAQRDE   101

tuII/dPRP43   FMRLLSLHQCIVLVGETGSGKTTQIPQWCV-DFAVSKGRKGVSCTQPRRVAAMSVAQRVSEEMDVKLGEEVGYSIRFEDC   159
              F::L   :Q :V:VGETGSGKTTQIPQ. : D     ... V:CTQPRRVAAMSVAQRV:EEMDVKLGEEVGYSIRFE:
PRP43         FLKLYQNNQIMVFVGETGSGKTTQIPQFVLFDEMPHLENTQVACTQPRRVAAMSVAQRVAEEMDVKLGEEVGYSIRFENK   181

                                                                          *
tuII/dPRP43   STAKTLLKYMTDGMLLREAMSDPMLDQYQVILLDEAHERTLATDILMGVLKEVIRQRSDLKLVVMSATLDAGKFQQYFDN   239
              ::.KT:LKYMTDGMLLREAM.D. L.:Y  I:LDEAHERTLATDILMG:LK:V:::R:DLK:::MSATLDA.KFQ:YF::
PRP43         TSNKTILKYMTDGMLLREAMEDHDLSRYSCIILDEAHERTLATDILMGLLKQVVKRRPDLKIIIMSATLDAEKFQRYFND   261

tuII/dPRP43   APLMKVPGRTHPVEIFYTPEPERDYLEAAIRTVIQIHMCEEIEGDILMFLTGQEEIEEACKRIKREIDNLGSEI--GELK   317
              APL: VPGRT.PVE::YTPE :RDYL::AIRTV:QIH  EE .GDIL:FLTG::EIE:A ::I. E D:L .E  G L.
PRP43         APLLAVPGRTYPVELYYTPEFQRDYLDSAIRTVLQIHATEE-AGDILLFLTGEDEIEDAVRKISLEGDQLVREEGCGPLS   340

tuII/dPRP43   CIPLYSTLPPNLQQRIFEPAPPPNANGAIGRKVVVSTNIAETSLTIDGVVFVIDPGFAKQKVYNPRIRVESLLVSPISKA   397
                PLY::LPP: QQRIFEPAP :: NG  GRKVV:STNIAETSLTIDG:V:V:DPGF:KQKVYNPRIRVESLLVSPISKA
PRP43         VYPLYGSLPPHQQQRIFEPAPESH-NGRPGRKVVISTNIAETSLTIDGIVYVVDPGFSKQKVYNPRIRVESLLVSPISKA   419

tuII/dPRP43   SAQQRSGRAGRTRPGKCFRLYTEKAFKNEMQDNTYPEILRSNLGTVVLQLKKLGIDDLVHFDFMDPPAPETLMRALELLN   477
              SAQQR:GRAGRTRPGKCFRLYTE.AF::E: :::YPEILRSNL:::VL:LKKLGIDDLVHFDFMDPPAPET:MRALE LN
PRP43         SAQQRAGRAGRTRPGKCFRLYTEEAFQKELIEQSYPEILRSNLSSTVLELKKLGIDDLVHFDFMDPPAPETMMRALEELN   499

tuII/dPRP43   YLAALDDDGNLTDLGAVMSEFPLDPQLAKMLIASCQHNCSNEILSITAMLSVPQCFVRPNEAKKAADEAKMRFAHIDGDH   557
              YLA LDD:GNLT LG : S:FPLDP LA MLI:S : :CS:EIL:I.AMLSVP: F:RP...KK AD:AK  FAH DGDH
PRP43         YLACLDDEGNLTPLGRLASQFPLDPMLAVMLIGSFEFQCSQEILTIVAMLSVPNVFIRPTKDKKRADDAKNIFAHPDGDH   579

tuII/dPRP43   LTLLNVYHAFK--QSSE--DPNWCYENFINFRSLKSADNVRQQLARIMDRFNLRRTSTEFTSKDYYVNIRKALVQGFFMQ   633
              :TLLNVYHAFK  :: E   .:WC ::::N:RSL.:ADN:R QL.R:M:R:NL  .:T::.S .Y: NIRKAL. GFFMQ
PRP43         ITLLNVYHAFKSDEAYEYGIHKWCRDHYLNYRSLSAADNIRSQLERLMNRYNLELNTTDYESPKYFDNIRKALASGFFMQ   659

tuII/dPRP43   VAHLERTGY-YLTIKDNQNVQLHPSTCLDHKPDWVIYNEFVLTTKNYIRTVTDVKPEWLCCLAPQYYDLNNFPQCEAKRQ   712
              VA.  ..:  Y:T:KDNQ:V :HPST L:H.::WVIYNEFVLT:KNYIRTVT.V:PEWL  :AP.YYDL:NF.: :.K
PRP43         VAKKRSGAKGYITVKDNQDVLIHPSTVLGHDAEWVIYNEFVLTSKNYIRTVTSVRPEWLIEIAPAYYDLSNFQKGDVKLS   739

tuII/dPRP43   LELLQQRLETKQYQKGF   729
              LE ::::::  :  K
PRP43         LERIKEKVDRLNELKQG   756
```

Figure 7: The 2ob9 mutation results in a serine -> phenylalanine exchange in *dprp43*.

(A) *dprp43* specific primers were used to amplify DNA by RT PCR from wild type and heterozygous 2ob9 total RNA. Sequencing of these RT PCR products revealed a missense mutation in 2ob9/+ at position 740 of the cDNA. As wild type control a chromosome carrying the phenotypic markers cinnabar and brown (cnbw) was used. (B) This mutation causes a serine to phenylalanine change at position 225. For the position of this mutation in the context of the whole protein see asterisk in Figure 6.

A

reverse strand



B

forward strand

```
     ValValMetSerAlaThr
cnbw GTGGTCATGTCCGCCACA
2ob9 GTGGTCATGTTCGCCACA
     ValValMetPheAlaThr
                  225
```

Figure 8: Expression pattern of *dprp43* includes that of the clock gene *timeless* in the fly head.

(A) Head section stained with anti-TIMELESS antibodies. Note the staining in the lateral neurons (LNd, LNv), which are essential for circadian locomotor behaviour. (B) *In situ* hybridization on head section with DIG labeled *dprp43* RNA. Most if not all cell bodies are labeled. White areas consist mostly of axons. Note that this staining includes the cells seen with anti-TIMELESS antibody staining.

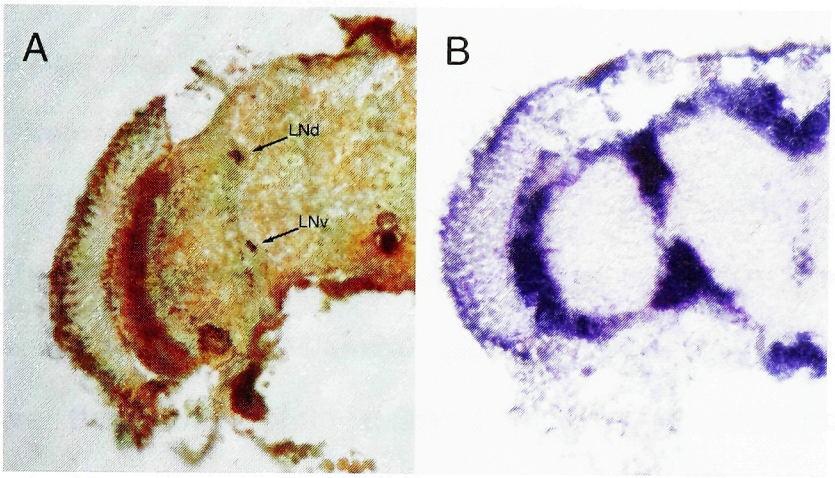
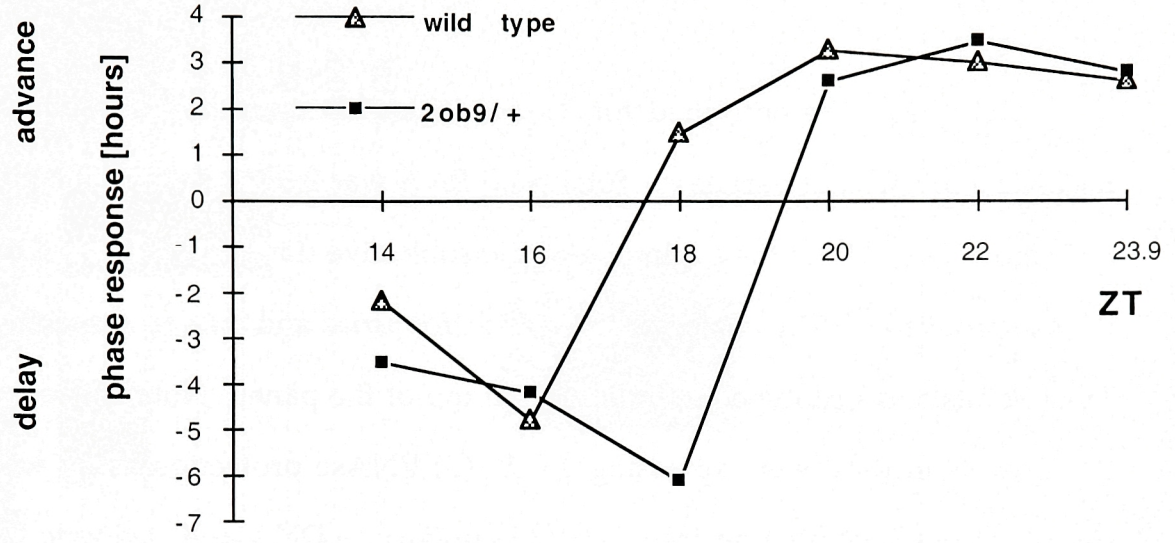

A
LNd
LNv
B

Figure 9: Molecular defect conferred by the 2ob9 mutation occurs prior to nuclear entry.

(A) Phase Response Curve (PRC). Time of day of the onset of a 1 hour light pulse is indicated on the x-axis. The resulting phase shift is shown for wild type and 2ob9/+. Note that the transition point between delay and advance part of the PRC is shifted by approximately 2 hours in the mutant. (B) Western blot of head extracts probed with anti-TIM antibodies. Timepoints and genotype are indicated above the blot. Note delayed protein accumulation and phosphorylation in the mutant.

A


advance
delay
phase response [hours]
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
wild type
2ob9/ +
14
16
18
20
22
23.9
ZT

B

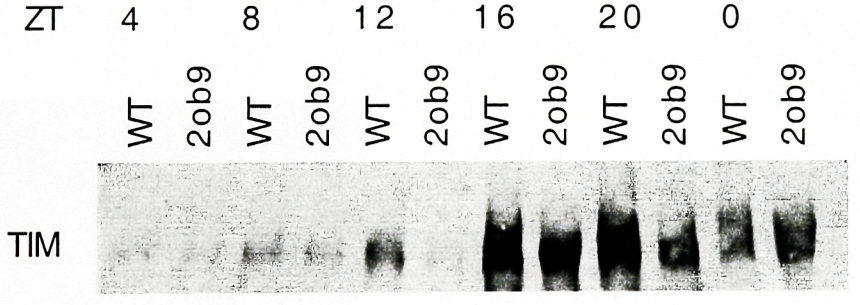

ZT
4
8
12
16
20
0
WT
2ob9
TIM

Figure 10: Effects of over-expression of mutant *dprp43* cDNA (2ob9) on *period* and *timeless* RNA.

(A) Flies were entrained for 3 days in an LD cycle and subsequently released into constant darkness. Total head RNA was isolated from time points CT2 and CT14 (CT0 is the beginning of the subjective day) and subjected to an RNAse protection assay using probes specific for *period* and *timeless* respectively. Time points and genotype are indicated on top of the panel. Note the reduced RNA levels in flies over-expressing 2ob9. (B) RNAse protection assay with a *timeless* probe spanning an intron-exon boundary. cDNA and genomic DNA (gDNA) were transcribed and probed in order to confirm the specificity of the probe. As negative control total yeast RNA was used. Note the increase of unspliced RNA in 2ob9 over-expressing flies. (C) Histogram showing the unspliced RNA/mRNA ratios for wild type (100%) and 2ob9 over-expressing flies.

A
period
timeless
tubulin
CT2
CT14
per-GAL4/+
CT2
CT14
UAS-2ob9/
per-GAL4
B
unspliced
tim
spliced
tim
cDNA
gDNA
yeast RNA
UAS-2ob9/per-GAL4
per-GAL4/+
UAS-2ob9/per-GAL4
per-GAL4/+
ZT2
ZT14
C
unspliced/ spliced
0
50
100
150
200
250
300
350
2ob9/perGAL4 ZT14
perGALA4/+

Chapter 4: An over-expression screen for aberrant circadian behavior

Introduction

Many genes have pleiotropic effects; they often partake in a variety of different processes rather than in a single biological event . For example *Notch* is a pivotal regulator of lateral inhibition during the cell fate decision between neuronal and epidermal lineage, and it is a crucial factor in boundary formation (Pourquié 2000). Another example *Krüppel* is involved in the early segmentation of the *Drosophila* embryo, and later in development *Krüppel* partakes in the differentiation of the larval photoreceptive organ, the Bolwig organ (Schmucker *et al.* 1992; Schmucker 1995). Consequently, the effect of a mutation on the process of interest can be masked by an earlier defect caused by the same mutation. Loss of *Krüppel* function, for example, causes abnormal development of the Bolwig organ. However, this defect alone is not sufficient to demonstrate a role for *Krüppel* in Bolwig development since the observed phenotype could also be accounted for by the defective segmentation prior to Bolwig's differentiation. Therefore, tissue/time specific gene interference is a desirable approach to resolve such questions. Temperature sensitive mutations are the tool of choice to address such problems but are rarely available. In the case of *Krüppel*$_L$ a cis-acting regulatory element in the *Krüppel* promoter had been identified, which allowed for tissue specific gene interference and thereby for the clarification of *Krüppel*'s role in Bolwig development (Schmucker *et al.* 1992). Obviously, a more dramatic problem exists if lethality associated with a mutation prevents analysis of the process under investigation.

During the last decade several approaches have been developed to interfere tissue and/or time specifically with gene function. Conceptually, there are two different ways to interfere with gene function: (A) increase or (B) decrease of gene activity. (A) Ectopic expression of a given gene usually results in elevated activity and/or broader distribution. As all cellular processes have to be regulated and orchestrated with each other, abnormally high/localized protein activities can clash with wild type signaling events. This is very graphically illustrated by molecules that are involved in the guidance of axonal growth. Essential to the function of such a guidance molecule is its spatial distribution. Ubiquitous expression of a guidance molecule would abolish this spatial information thereby preventing proper axonal growth. (B) For the tissue and time specific reduction of gene function, on the other hand, dominant negative alleles, antisense RNA, or double stranded RNA can be expressed. The bases for all controlled expression experiments is the P-element mediated gene transfer (Rubin and Spradling 1982; Spradling and Rubin 1982; Karess and Rubin 1984). P-elements are naturally occurring transposons in *Drosophila*, and they have been genetically modified to allow the introduction of recombinant DNA, transgenes, into the genome. Expression from the introduced transgenes can be controlled in different ways. Heat inducible promoters enable the researcher to induce transcription ubiquitously at any given time (for example Schneuwly *et al.* 1987). An analogous approach replaces the heatshock promoter with tissue specific cis-acting regulatory elements thereby providing control over spatial distribution. A more recently developed expression system, the UAS/GAL4 binary expression system, permits the combination of a series of existing driver lines that confer tissue specificity with any reporter line (Brand and Perrimon

1993). Each driver line contains one transgene, which expresses the yeast transcription factor GAL4 under control of a particular set of cis-acting regulatory elements. The reporter line contains a transgene with the GAL4 target sequence directly upstream of the sequence that is to be transcribed. Combining the two transgenes in one genome will result in the expression of the protein, antisense-RNA, inverted repeat, or any other sequence of interest in the tissue where GAL4 is expressed.

A combination of these tools provides the means to elucidate gene function once a cDNA has been identified based on other criteria like sequence homology or expression pattern. However, none of these methods allowed for tissue specific screening of larger numbers of random genes for defects in the biological process of interest until the introduction of the EP-element by Rørth *et al.* (Rørth 1996). The EP-element system is based on the UAS/GAL4 binary expression method. Whereas the reporter for the UAS/GAL4 method contains a defined cDNA, the EP-element contains the UAS in conjunction with a basal promoter thereby permitting GAL4-dependent transcription of flanking sequences (Rørth 1996). Because of a tendency of this element to insert itself in the 5prime region of a gene, the activation of a particular EP-element causes in many cases ectopic expression of this gene. Alternatively, EP-element activation may reduce gene function through the synthesis of antisense RNA.

Several studies have provided evidence that the molecular clock is sensitive to the dosage of some of its components. For *period* an inverse relationship between gene copy number and period length was found (Smith and Konopka 1982; Baylies *et al.* 1987; this study, Chapter 7). Consistently, over-expression of *period* was found to interfere with proper oscillations of the

molecular clock (Zeng *et al.* 1994) and with the phase of the circadian locomotor activity cycle (Edery *et al.* 1994a). In another study the importance of the clock relevant gene *vrille* could be demonstrated by interfering with *vrille* cycling using the UAS/GAL4 binary expression system (Blau and Young 1999). These findings clearly demonstrated that over-expression can reveal a clock-related function, and that screening a collection of EP-elements may yield new genes involved in circadian clocks.

Results

Construction of the *timeless*-(UAS)-GAL4 line

To activate the EP-elements in a tissue specific manner, a transgenic fly line was constructed that would express GAL4 in the neurons required for circadian locomotor activity, the lateral neurons (LNs). When this study was initiated, only the *timeless* and *period* genes were well enough characterized to provide the necessary cis-acting regulatory elements. Between the two, the *timeless* promoter was chosen because its pattern of expression is more restricted than that of *period*. This should minimize the risk of lethality, which may be associated with the activation of some EP-elements in vital tissues. One additional concern was the oscillating activity inherent to the *timeless* promoter. Cycling GAL4 transcription from a *timeless*-GAL4 transgene may lead to oscillating activation of the EP-element under investigation. If this oscillation were in synchrony with a wild type oscillation of the associated gene, a possible function may remain concealed. To ensure constitutive levels of GAL4, the target sequence for GAL4, called upstream activating sequence (UAS), was inserted into the *timeless* promoter. A genomic DNA fragment that encompasses

4 kb upstream and 2 kb downstream of the *timeless* transcription start is known to confer proper *timeless* expression and seemed therefore suitable for our purposes. Five repeats of the UAS were cloned 333 bp upstream of the transcription start of *timeless*. The resulting DNA fragment was cloned in front of the cDNA encoding GAL4 in a P-element derived vector that allowed for subsequent insertion of the construct into the genome of *Drosophila*.

To test the tissue specificity of GAL4 expression, the *timeless*-(UAS)-GAL4 line was crossed with a reporter line that expresses tau-β-Gal fusion protein under control of the UAS. The tau domain of the fusion protein confers active transport of the protein into axons thereby allowing the visualization of whole neurons. As shown in Figure 11, immuno-histochemistry using anti-β-Gal antibodies on larval brains expressing tau-β-Gal under control of *timeless*-(UAS)-GAL4 revealed a distinct neuronal network. Double label experiments with anti-β-Gal antibodies and antibodies against TIMELESS demonstrated that all neurons positive for TIMELESS were stained also with the anti-β-Gal antibodies (see Chapter 7; Figure 25C). The anti-β-Gal antibodies seemed to label more neurons than the antibodies against TIMELESS at a given time of day. However, it has previously been reported for some larval neurons that *timeless* is expressed with a phase opposite to the phase of *timeless* and *period* expression in the PDF positive neurons (Kaneko *et al.* 1997). At a given time, antibodies against TIMELESS will therefore reveal only a subset of neurons while tau-β-Gal reaches a steady state in all neurons where the *timeless* promoter has been active.

A neuronal connection between the larval visual system and *timeless* expressing cells

The nerve of the larval visual organ, the Bolwig nerve, projects very closely to TIMELESS positive cells that presumably are the lateral neurons (see also Figure 25D). Neural input from the Bolwig organ could account for light entrainment of the oscillator in larval lateral neurons. Consistently, it has been shown for the adult oscillator that the lack of the visual transduction cascade attenuates the light response of the molecular oscillator suggesting a supporting role for the visual system in entrainment (Yang *et al.* 1998). However, as anti-TIMELESS antibodies stain only the cell body, it was not possible to decide whether the Bolwig nerve terminates in close vicinity of the TIMELESS expressing cells or innervates them. To address this question, a double label experiment on larval brains expressing tau-β-Gal under control of *timeless*-(UAS)-GAL4 was performed. The larval visual system was visualized using the monoclonal antibody 24B10 (Zipursky *et al.* 1984), and the network of neurons with *timeless* expression was visualized using antibodies against β-Gal were. As shown in Figure 12, the Bolwig nerve defasciculates in close vicinity of a β-Gal signal, and the single axons appear to contact a *timeless* expressing cell. At this level of resolution it cannot be decided whether synapses between the neurons are formed, but a physical contact seems very likely. This observation suggests that light reception of the larval visual system contributes to the entrainment of the molecular oscillator in larval brains. Disconnecting the larval visual system from the lateral neurons should allow investigation of this notion. Unfortunately, the only viable mutant that disrupts this neuronal connection,

disconnected, has been found to impair differentiation of lateral neurons (Helfrich-Förster 1998).

Constitutive over-expression of *timeless* confers arrhythmia

To test whether over-expression driven by *timeless*-(UAS)-GAL4 is sufficient to cause reproducible changes of circadian locomotor activity, over-expression of *timeless* was chosen as a positive control. As shown in Figure 13, flies carrying the transgene UAS-*timeless* in combination with *timeless*-(UAS)-GAL4 fail to show circadian rhythms of locomotor activity. Based on what is known about TIMELESS function, this phenotype can be explained in the following way. The constitutive high TIMELESS levels would stabilize PERIOD and promote nuclear transport at all times resulting in a constitutive presence of PERIOD and TIMELESS in the nucleus thereby continuously suppressing oscillation. This observation demonstrates that *timeless*-(UAS)-GAL4 driven over-expression is strong enough to reveal a clock related function. Encouraged by this result, an existing collection of EP-elements was screened for aberrant locomotor activity cycles (BDGP).

Screening of EP-collection reveals several new loci with clock function

Flies carrying the *timeless*-(UAS)-GAL4 transgene were crossed to individual lines of the EP-collection. 3 single males of the F_1 generation were tested for their locomotor activity pattern. Not a single one of the 2300 lines tested caused lethality when activated with *timeless*-(UAS)-GAL4 although many of the affected genes are known to be essential for viability. Besides altered period length and arrhythmia, entrainment to light:dark cycles was another

criterion in this screen. To achieve this, F_1 flies were entrained in 3 consecutive light:dark cycles, subjected to a 2 min light pulse at ZT16 of the 4th day and subsequently released into constant darkness. This regimen usually results in a 4 to 5 hour delay of the phase of the locomotor activity rhythm. All lines that were arrhythmic, had periods of 23 hours and shorter, or 25 hours and longer were kept as positives. None of the lines displayed an abnormality with regard to its light-responsiveness. Table 1 summarizes the EP-elements found through our screening procedure. For many of the EP-elements sequence of neighboring genomic DNA and cytological location are available from the BDGP. This information in combination with the completed genome sequence of *Drosophila* allowed in some cases for a rapid identification of a candidate for the affected gene/EST (Table 1).

Several controls were performed on all lines that were positive in the primary screen. To exclude the possibility that the observed phenotype is caused by a mutation other than the activated EP-element, an outcross of the EP-line with wild type was tested. Unless the phenotype was dependent on the presence of *timeless*-(UAS)-GAL4, the line was discarded. Since the cytological location of most of the EP-elements is known, mutations and other genetic aberrations in the respective regions could be tested for circadian behavior. This is important since the majority of activated EP-elements cause a gain of gene function. Yet a gain of function phenotype reveals only the potential of a gene but not its function under physiological conditions. Therefore, reduction of the gene's function is essential for establishing a role in the regulation of circadian rhythms. Other existing mutations may reduce the gene's function thereby revealing its role in circadian rhythms. In some cases the EP-element was viable in homozygosity. As the EP-

element insertion itself may interfere with proper gene function, those flies were also tested for circadian locomotor activity (Table 1).

Discussion

The isolation of several EP-elements in this screen demonstrated that over-expression is a feasible assay for the identification of new genes involved in circadian rhythmicity. 11 EP-elements were uncovered out of 2300 lines tested, that is ~0.5%. In a previous screen of this EP-element collection for developmental defects, 4% positives were obtained with *sevenless*-GAL4 as a driver (Rørth 1996). In another screen 7,000 EP-element insertions were tested for genetic modification of polyglutamine toxicity in the eye (Kazemi-Esfarjani and Benzer 2000). Approximately 1% of the these lines were found to either enhance or suppress the phenotype with *gmr*-GAL4 as driver. Using a modified EP–element that has a UAS on both ends, Toba et. al. obtained numbers of up to 50% when they activated these elements ubiquitously in imaginal tissue and screened for developmental abnormalities (Toba *et al.* 1999). The lower number obtained in the screen described here is probably indicative for a smaller subset of genes involved in circadian rhythmicity compared to development. However, the yield is high enough to make the screen of EP-elements for behavioral defects feasible. Consequently, the generation of more EP-element insertions has been started in our laboratory.

One rational for conducting this screen was that tissue specific gene-interference may allow behavioral testing of genes that are essential for viability. As mentioned above, none of the EP-elements caused lethality when activated with *timeless*-(UAS)-GAL4 although a substantial fraction of the affected genes is

known to be necessary for development or cell viability. Additionally, several of the EP-elements are lethal when activated by a driver with broader pattern of expression. Taken together, these data show that tissue specific gene interference may in some cases be the only feasible way to implicate a gene in the regulation of circadian behavior.

Table 1. EP-lines with abnormal circadian rhythmicity when activated in trans with *timeless*-(UAS)-GAL4.

EP-line	Phenotype Activated [hours]	Phenotype w/o driver	Cytological Location	EST/ candidate gene	Remarks
EP(3)3576	25.5	27 hours, homozygous	3rd chr.		
EP(2)2583 EP(2)2486	AR	23 hours, homozygous	21B4-21B6		
EP(2)2371	25-26	-	57D/E	3 ESTs, no homologies	homo-zygous lethal
EP(2)2326	26	-	30B	1 EST, no homologies	
EP(X)1149	AR/26	26 hours, homozygous and EP(X)1149 over EP(X)0950, EP(X)1410, B952	8F8/10	-	
EP(2)0815	AR	-	42C1/2	Adf-1	homo-zygous lethal

EP-line	Phenotype Activated [hours]	Phenotype w/o driver	Cytological Location	EST/ candidate gene	Remarks
EP(2)2039	AR/long and short component	-	35B1-35	Zn-finger?	-
EP(3)3015	AR	26, heterozygous	3rd chr.	-	homo-zygous lethal
EP(3)0473	AR		85D1-85D2	chromosomal protein D, HMG	
EP(3)0637	25-26	26, heterozygous	-	-	-
EP(X)1576	20.5	-	3A8/9	shaggy	see chapter 5

Figure 11: A neuronal network uncovered by active *timeless* promoter.

Flies carrying the *timeless*-(UAS)-GAL4 transgene were crossed to flies carrying UAS-*tau-lacZ*. β-Gal expression in larvae of the F_1 generation was detected by immuno-histochemistry using anti-β-Gal specific antibodies. Since fusion of β-Gal to Tau causes active transport of the protein into the axons, the entire neurons are visualized.

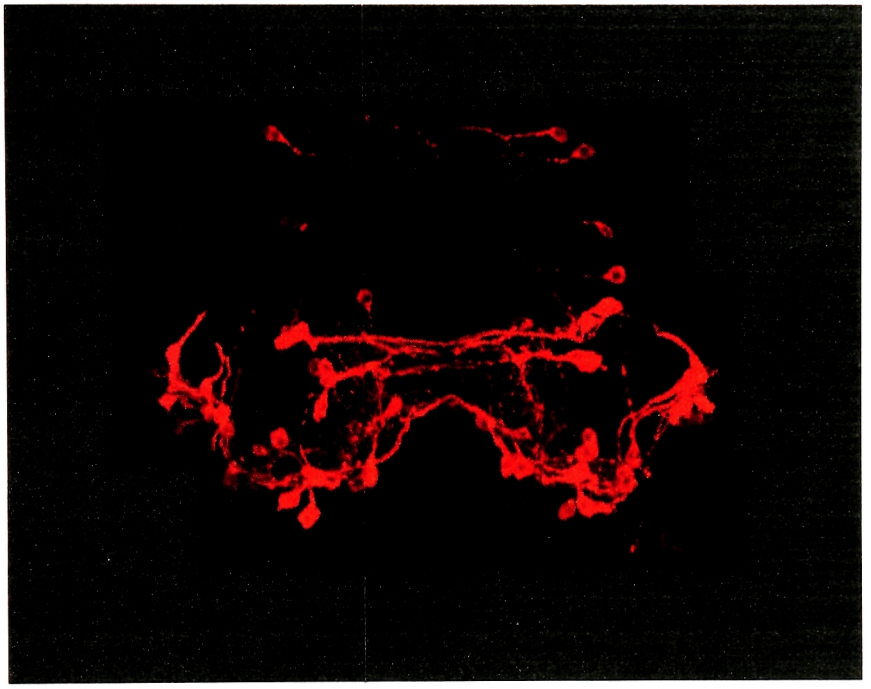


Figure 12: Neuronal connection between larval visual system and *timeless* expressing cells.

The cross was performed as in Figure 11. Larvae were subjected to immuno-histochemistry using the 24B10 antibody (green) and anti-β-Gal specific antibodies (red). 24B10 labels the neurons of the larval visual system. Note the close contact between the neurons of the larval visual system and the neurons with an active *timeless* promoter.

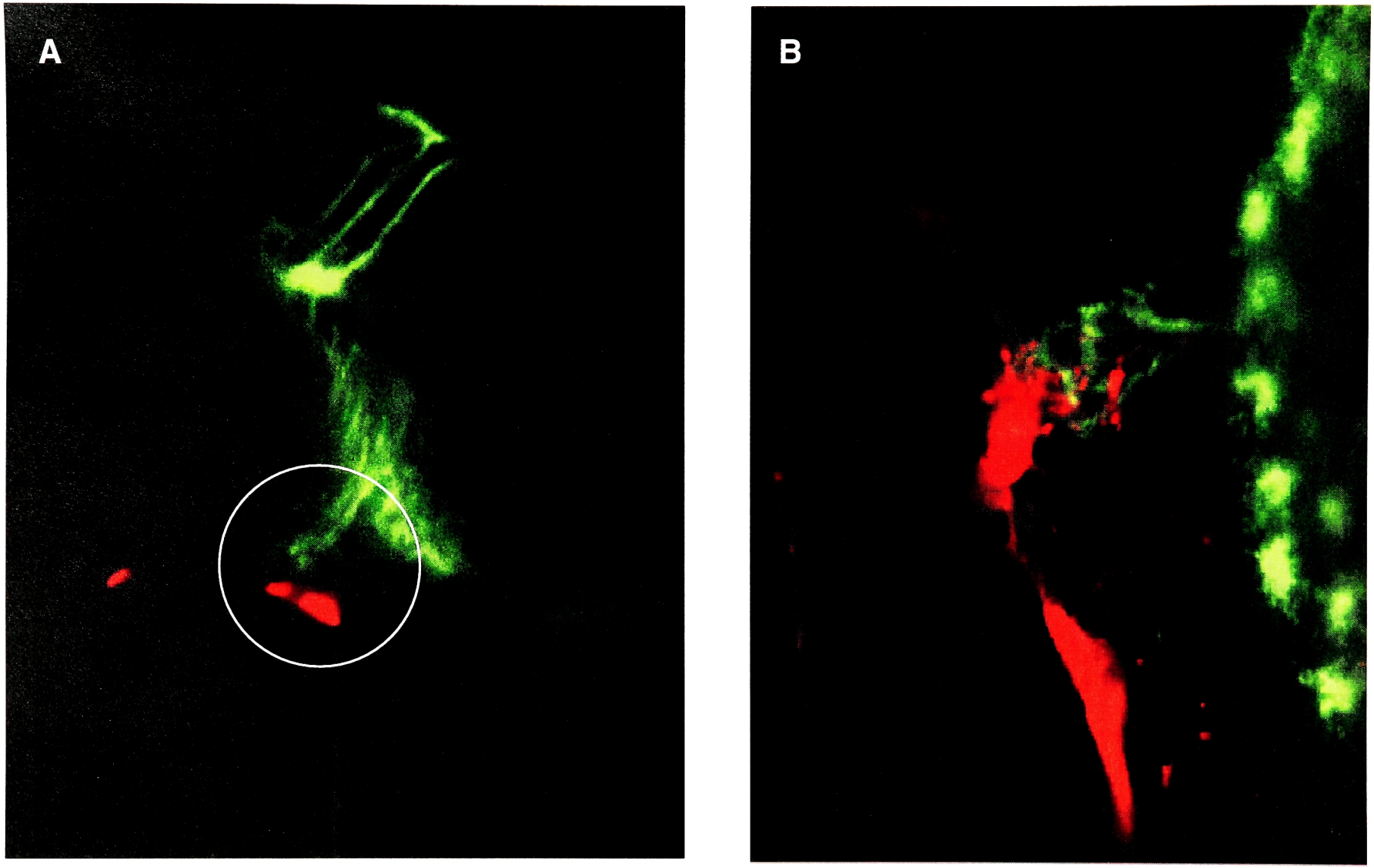
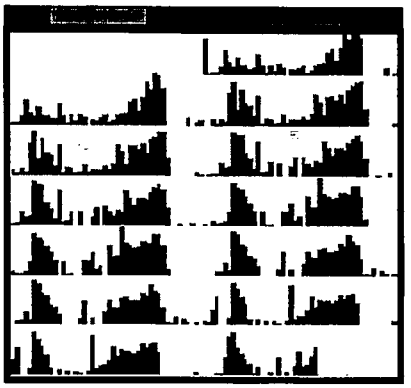

A
B

Figure 13: Constitutive over-expression of *timeless* causes arrhythmic locomotor activity rhythm.

Flies of the indicated genotypes were entrained to 3 consecutive LD cycles and consequently released in constant darkness. The locomotor activity of an individual fly is depicted in each panel.

timeless-(UAS)-GAL4/+



timeless-(UAS)-GAL4/
UAS-*timeless*

Chapter 5: A role for the segment polarity gene *shaggy* in the *Drosophila* circadian clock

Introduction

Unlike the *period, timeless, dClk,* and *cyc* genes, whose sole function may be the regulation of circadian rhythms, the clock genes *dbt* and *vri* are required for viability (Kloss *et al.* 1998; Price *et al.* 1998; Blau and Young 1999). Certain genes organizing rhythmic behavior in response to the clock have also been found to perform vital functions (*e.g. lark;* Newby and Jackson 1993; McNeil *et al.* 1998). Because classical genetic screening might fail to identify all clock-associated genes with vital functions, a series of alternative studies to extend the search for genes influencing circadian behavior in *Drosophila* has been begun. Prior work has shown that constitutive over-expression of either *period* or *vri* is sufficient to stall the molecular oscillator (Blau and Young 1999; Zeng *et al.* 1994). Therefore, a screen involving pacemaker-cell-specific over-expression of individual genes throughout much of the *Drosophila* genome has been initiated (see Chapter 4). In this chapter the characterization of *shaggy* (*sgg*), which encodes the *Drosophila* ortholog of Glycogen Synthase Kinase-3 (GSK-3; Bourouis *et al.* 1990; Siegfried *et al.* 1990) as a component of the *Drosophila* clock is described. The results demonstrate a role for SGG in the regulation of TIMELESS phosphorylation, which probably influences the timing of PERIOD/TIMELESS nuclear translocation.

Results

Gain of *sgg* function shortens the period of the circadian oscillator

In order to generate tissue specific gain of function alleles, an existing collection of fly strains each of which carried a random insertion of the binding sequence for the yeast transcription factor GAL4 in conjunction with a basal promoter, the EP-element (Brand and Perrimon 1993; Rørth 1996), was used. By providing GAL4, genes residing downstream of the EP-element are transcribed ectopically. To regulate EP-element activity specifically in cells controlling circadian rhythms, a strain of *Drosophila* that would express GAL4 under control of the *timeless* promoter was constructed (see Chapter 4). To overcome the endogenous oscillating activity of the *timeless* promoter, the target sequence of GAL4 was cloned 333 base pairs upstream of the transcription start site (*timeless*-(UAS)-GAL4; Blau and Young, 1999). The resulting positive feedback should ensure constant levels of GAL4.

Screening a collection of 2300 EP-lines (Berkeley Drosophila Genome Project) for effects on locomotor activity rhythms yielded 2 arrhythmic, 4 long period, and 1 short period strain. Here, the molecular and phenotypic characterization of the short period strain is reported.

The X-chromosomal genetic element EP(X)1576 in combination with *timeless*–(UAS)-GAL4 as a driver decreased the locomotor activity period by approximately 3 hours. Similar results were obtained when *timeless*-GAL4 (Emery *et al.* 1998) was employed as a driver (Figure 14A, Table 2).

By means of the plasmid rescue technique (Mlodzik *et al.* 1990) genomic DNA downstream of EP(X)1576 was isolated and sequenced. A BLAST search with this sequence failed to detect homology to a known gene at the time of

initial comparison. A 4 kb genomic DNA fragment directly downstream of the EP-element was subsequently used to probe an adult head cDNA library, yielding several positive clones. Sequencing identified each clone as derived from the segment polarity gene *shaggy* (*sgg*), the *Drosophila* ortholog of Glycogen Synthase Kinase 3 (GSK3; Bourouis *et al.* 1990; Siegfried *et al.* 1990). The *sgg* gene is essential for development of the fly and is probably best known for its role in *wingless* signaling where it regulates the subcellular distribution of ARMADILLO (Siegfried *et al.* 1992; Peifer *et al.* 1994a; Peifer *et al.* 1994b).

The *sgg* locus produces multiple transcripts that encode proteins with identical kinase domains but different N- and C-termini (Siegfried *et al.* 1992; Ruel *et al.* 1993a; Ruel *et al.* 1993b). Since lethality associated with mutations of *sgg* can be rescued by expressing the SHAGGY 10 isoform alone, the different forms of SHAGGY (SGG) must have redundant functions (Ruel *et al.* 1993a). Closer investigation of genomic DNA surrounding EP(X)1576 revealed that the ORF for *sgg10* begins 693 bp upstream of the EP-element insertion site (Figure 14B). However, translation start sites of other forms of SGG are located downstream of the insertion site of EP(X)1576. To examine whether a form(s) of SGG is affected by EP(X)1576, extracts of flies activating EP(X)1576 by means of the *glass*-responsive element (*gmr*-GAL4) were subjected to Western blot analysis. As shown in Figure 14C, only the SGG Y isoform was more abundant than in wild type. It has been suggested that SGG Y is translated from a transcript termed *zw3-C* (Siegfried *et al.* 1992; Ruel *et al.* 1993b). Consistent with activation of *zw3-C* by EP(X)1576, the translation start of SGG Y is located 1022 bp downstream of EP(X)1576.

In order to test whether activation of EP(X)1576 and consequently over-expression of *shaggy* is responsible for the shortening of the circadian period, EP(X)1576 was mobilized and resulting lines were screened for the absence of EP(X)1576 (Figure 15). Two strains in which EP(X)1576 was mobilized are shown in Figure 15A. The circadian behavior of the line Excission#7 is reverted to wild type in the presence of *timeless*-(UAS)-GAL4 and, as shown in Figure 15B, there is no sequence of the EP-element left in the genome. Consistently, SHAGGY levels are not elevated in those flies (Figure 15C). In contrast, SHAGGY appears to be reduced compared to wild type. This could be due to a chromosomal rearrangement caused by the mobilization of the EP-element. However, the line Excission#10 displays still a GAL4 dependent short period phenotype. Consistently, a portion of the EP-element is still present in the genome and SHAGGY Y levels are elevated in those flies (Figure 15B, C). This results demonstrate a strict correlation between the presence of EP(X)1576, SHAGGY over-expression, and the behavioral phenotype.

To confirm that over-expression of *sgg* is responsible for the short period phenotype, a *sgg10* cDNA was expressed under control of the *timeless* promoter. As shown in Figure 14 and Table 2, these flies have a period length of 21 - 22 hours depending on the driver demonstrating that over-expression of *sgg* can shorten the circadian cycle of locomotor activity rhythm and that a proper level of *sgg* activity is required for wild type circadian behavior. Over-expression of *sgg10* by means of *timeless*-(UAS)-GAL4 appeared to also affect longevity preventing the analysis of data obtained from approximately 50% of these flies (Table 2). The slightly weaker phenotype of *sgg10* compared to activated EP(X)1576 could be attributed to the difference in the N-terminus. Only SGG10

contains a serine at a position homologous to serine 9 in GSK3-β. This residue is known to negatively regulate the kinase activity of GSK3-β upon phosphorylation (Sutherland *et al.* 1993; Stambolic and Woodgett 1994). As SGG Y lacks this serine, and therefore a potential negative regulator, its potentially higher kinase activity could explain the stronger phenotype.

Over-expression of *sgg* alters TIMELESS oscillation

To determine whether *sgg* over-expression affects molecular oscillations in pacemaker cells, the pattern of TIMELESS accumulation in third instar larval LNs was followed immunocytochemically. Prior studies have demonstrated that period-altering mutations of *period*, *timeless*, and *double-time* (*dbt*) produce corresponding effects on these molecular cycles and on behavioral rhythms (Kaneko *et al.* 1997; Price *et al.* 1998). It has also been shown that the molecular oscillations can be entrained with light:dark (LD) cycles by the beginning of the first larval instar (Sehgal *et al.* 1992). Because *Drosophila*'s circadian rhythms are temperature-compensated (Konopka *et al.*, 1989) wild type and *sgg* over-expressing larvae for this experiment were reared at 20°C instead of 25°C. This lengthened developmental time and allowed an extended analysis encompassing several days of constant darkness (DD).

As shown in Figure 16, with the exception of a single time-point (CT10 of the third day), TIMELESS was not detectable in wild type lateral neurons at either CT4 or CT10 during any of 4 consecutive days in constant darkness (CT0 is subjective dawn and CT12 is subjective dusk in DD). Conversely, at CT16 TIMELESS was largely cytoplasmic and at CT22 predominantly nuclear in wild

type LNs, during all 4 days of the analysis. This confirmed that molecular oscillations in wild type lateral neurons had been entrained by a prior LD cycle (Figure 16, legend) and persisted with a period length of ~24 hours in constant darkness. In contrast, over-expression of *sgg* caused a drift in the appearance of TIMELESS immuno-reactivity and nuclear subcellular localization towards earlier time points with each consecutive day (Figure 16). A total shift of ~12 hours was observed over 4 days, showing that the period-shortening effect of *sgg* over-expression on molecular cycling was correlated with the new period of the circadian locomotor rhythm.

Reduction of *sgg* function lengthens period

A gain of function phenotype alone would not be sufficient to demonstrate a role for *sgg* in the organization of circadian rhythmicity in wild type *Drosophila*. The effects of reducing *sgg* gene expression on circadian behavior were therefore tested. Since it had previously been shown that expression of *sgg10* under control of a heat shock promoter can rescue the lethality of *sgg* mutants (Ruel *et al.* 1993a), it was attempted to rescue the development of otherwise inviable *sgg* mutants by supplying heat-shock-controlled *sgg10* during the larval and pupal stages. Two strains of *Drosophila*, each carrying a different hypomorphic *sgg* allele and a heat-shock-controlled *sgg10* transgene, were heat-shocked twice a day for 1 hour at 37°C beginning after hatching of the first larval instar. Heat-shocks were discontinued at the mid-pupal stage, and flies were subsequently maintained at a temperature well below that necessary to activate the heat shock promoter (25^{0}C) to determine the contribution of each hypomorphic *sgg* allele to circadian rhythmicity. Adults

were entrained for 3 days to a 12h:12h LD cycle and then subjected to locomotor activity analysis in constant darkness. As shown in Table 3, *Drosophila* bearing hypomorphic alleles of *sgg* produce uniform, long-period phenotypes.

The observations that reduced *sgg* function lengthens the period while *sgg* over-expression produces a contrasting short-period phenotype demonstrate that SGG is an intrinsic regulator of the period length of the circadian clock in *Drosophila*. However, interference with *shaggy* function failed to cause arrhythmicity suggesting that SHAGGY may not be essential for the molecular oscillation *per se* but rather for the adjustment of its frequency. Alternatively, remaining SHAGGY activity in the rescued mutants may prevent us from revealing the complete loss of *shaggy* phenotype. In order to test this possibility, protein was extracted from rescued adult sgg^{D127} mutant flies approximately one week after the last heat-shock. Protein extract was subsequently subjected to a Western blot analysis using SGG specific antibodies. As shown in Figure 19D, SGG10, which presumably is derived from the HS-*sgg* transgene, is the only detectable form in these flies. Due to the persisting SGG10 protein, the behavioral phenotype of the rescued *sgg* mutant does not represent a complete loss of *sgg* function. Thus, removing *sgg* function entirely will most likely effect a stronger phenotype, possibly even arrhythmicity.

Advanced nuclear entry of PERIOD/TIMELESS by *sgg* over-expression

In order to investigate the basis for *sgg* mediated changes in the clock, behavioral responses to photic stimuli were measured to construct a Phase Response Curve (PRC; Aschoff 1965). A light pulse administered in the early subjective night, when PERIOD and TIMELESS are cytoplasmic, produces a

phase delay in the molecular oscillator, whereas a similar pulse administered late at night eliminates TIMELESS from nuclear PERIOD/TIMELESS complexes to advance the molecular mechanism. The transition from behavioral delays to advances in the PRC has been correlated with the timing of nuclear entry of PERIOD/TIMELESS complexes (Rothenfluh *et al.* 2000b).

As shown in Figure 17A, over-expression of *sgg* alters the PRC. A premature transition (~3 h) is observed from the phase delay to the phase advance portion of the curve. This shift indicated that over-expression of *sgg* might produce an advanced nuclear entry of the PERIOD/TIMELESS heterodimer corresponding to the short period molecular and behavioral cycles.

To test this possibility, subcellular localizations of PERIOD were followed at time points that would reveal the timing of nuclear translocation in larval LNs. As shown in Figure 17B and Table 4, PERIOD in wild type larvae begins to enter nuclei at ~ZT20 (ZT0 is lights on and ZT12 is lights off in an LD 12:12 cycle) and is predominantly nuclear at ZT21. In contrast, larval lateral neurons over-expressing *sgg* begin to show nuclear PERIOD immuno-reactivity at ZT17.5, and by ~ZT19 PERIOD is mostly nuclear. The premature nuclear entry of PERIOD proteins in *sgg* over-producing larvae should thus account for most if not all of the period-shortening phenotype.

Advanced nuclear entry is not associated with increased RNA accumulation

Because advanced nuclear translocation of PERIOD/TIMELESS could reflect higher levels of *period* and/or *timeless* expression, the time course of *period* RNA accumulation in constant darkness was determined for *Drosophila* over-expressing *sgg*. Although the phase of *period* RNA accumulation was slightly

advanced in these flies, there was no detectable change in the amplitude of the RNA accumulation cycle (Figure 18). Identical results were obtained in studies of the *timeless* RNA cycle (data not shown). While the slightly altered phases of *period* and *timeless* RNA accumulation could contribute to the short-period phenotype, such shifts have been observed previously for period-altering alleles of *period*, *timeless*, and *dbt* whose primary defects involved changes in the encoded proteins. Those phase differences were viewed as secondary responses to a fast- or slow-running clock (Price *et al.*, 1998; Rothenfluh *et al.*, 2000b).

Hypomorphic mutations of *sgg* reduce TIMELESS phosphorylation and increase levels of both the TIMELESS and PERIOD proteins

A comparison of rescued sgg^{D127} mutants and wild type flies also failed to indicate a difference in *period* and *timeless* RNA levels (data not shown), further suggesting that altered *sgg* function might affect nuclear entry of PERIOD/TIMELESS through a post-transcriptional mechanism. To test this possibility, PERIOD abundance was investigated in our rescued sgg^{D127} flies. As shown in Figure 19A, levels of PERIOD protein were significantly elevated by hypomorphic *sgg* function.

TIMELESS accumulation, like that of PERIOD, was substantially increased in the mutant flies (data not shown). However, these studies also revealed a difference in the pattern of TIMELESS electrophoretic mobility (Figure 19B). During the late subjective night and the early subjective day, TIMELESS is increasingly phosphorylated in wild type flies (Myers *et al.* 1996; Zeng *et al.* 1996). This is associated with the conversion of half or more of the accumulated TIMELESS protein to a slowly migrating species near the end of each molecular

cycle. In rescued sgg^{D127} flies, TIMELESS is predominantly found as a rapidly migrating protein at all times (compare wild type versus sgg^{D127} CT2 and CT4 of Fig. 5B). Such a difference was not observed for PERIOD (Figure 19A), suggesting that *sgg* function specifically influences the phosphorylation and therefore mobility of TIMELESS.

Since the long-period rhythms produced by sgg^{D127} could elicit rather than result from the novel pattern of TIMELESS migration, cycles of TIMELESS mobility in another long period mutant, *double-time*^{L} were examined (dbt^{L}; Price *et al.*, 1998). The analysis focussed on time-points corresponding to early subjective day when highest levels of phosphorylated TIMELESS are observed in wild type flies. Figure 19C shows that even with respect to dbt^{L}, TIMELESS phosphorylation in sgg^{D127} is not only delayed, but at all times TIMELESS migrates predominantly as the hypophosphorylated protein. These observations suggest that *sgg* function is required for proper phosphorylation of TIMELESS.

Over-expression of *sgg* elevates phosphorylation of TIMELESS

The reduced phosphorylation of TIMELESS in rescued sgg^{D127} flies prompted an investigation of the pattern of TIMELESS phosphorylation in *Drosophila* over-expressing *sgg*. As the cycling accumulation of TIMELESS would complicate the analysis, TIMELESS phosphorylation was studied in an arrhythmic ($period^{0}$) genetic background (Konopka and Benzer 1971). Although TIMELESS is largely hypophosphorylated in $period^{0}$ flies, over-expression of *sgg* in $period^{0}$ converted much of this protein to a slowly migrating form of TIMELESS (Figure 20A): Instead of the single immunoreactive band that is readily seen in

period^0 flies, two equally represented forms of TIMELESS were observed in extracts from *sgg*-over-producing *period*^{0} flies.

To test whether the slowly migrating TIMELESS protein observed in flies over-expressing *sgg* is indeed caused by phosphorylation, protein extracts were subjected to treatment with alkaline phosphatase. As shown in Figure 20B, the slower migrating forms of TIMELESS from wild type at CT4 and from *period*^{0} flies over-expressing *sgg* are equally sensitive to dephosphorylation.
This demonstrates that the slowly migrating form of TIMELESS is generated mostly or entirely through phosphorylation.

The similar electrophoretic migrations and phosphatase sensitivities of the low mobility forms of TIMELESS in wild type and *sgg* over-expressing flies suggested that these phosphorylated TIMELESS proteins may be identical, and that over-production of SGG accelerates production of a wild type, hyperphosphorylated form of TIMELESS. This conclusion was also supported by an examination of the differential light sensitivity of low versus high mobility forms of TIMELESS in wild type and *sgg* over-expressing flies. Earlier studies of TIMELESS accumulation in wild type flies have shown that hyperphosphorylated forms of TIMELESS are preferentially lost with the onset of light in LD cycles (Rothenfluh *et al.* 2000b). As shown in Figure 20C, this acute light-sensitivity of hyperphosphorylated TIMELESS can be seen by comparing proteins isolated from wild type flies collected at CT2 (2 hours after subjective dawn in constant darkness) to those from wild type flies collected at ZT2 (2 hours after lights on in LD). The lower mobility form of TIMELESS is abundant at CT2, but is absent in extracts from flies collected at ZT2. Figure 20C also demonstrates that when *sgg* over-expressing *period*^{0} flies are exposed to constant

light, only the hypophosphorylated form of TIMELESS is detected. Thus, the specific pattern of TIMELESS phosphorylation produced by sgg over-expression fosters selective degradation of TIMELESS in response to light, as in wild type Drosophila.

In order to test whether a subtle increase in the abundance of TIMELESS in sgg over-expressing flies might be responsible for the change in phosphorylation pattern, *timeless* (rather than *sgg*) was over-expressed in *period*^{0} flies. As shown in Figure 20D (protein loading has been adjusted, see legend), most if not all of the TIMELESS in those flies is hypophosphorylated indicating that SGG activity directly influences the phosphorylation pattern of TIMELESS.

In wild type flies, phosphorylation of TIMELESS can be detected around the middle of the subjective night whereas in period0 the lower mobility form is weakly detected at all times. This raised the possibility that phosphorylation of TIMELESS is influenced by physical association with PERIOD, and that over-expression of sgg might bypass this regulation. To test this the phosphorylation pattern of TIMELESS in flies that express the PERIOD-βGal fusion protein PER-SG was investigated. PER-SG consists of the amino-terminal half of PERIOD (amino acids 1 to 636) fused to β-Gal (Liu *et al.* 1988; Vosshall *et al.* 1994; Stanewsky *et al.* 1997a). These fusion proteins physically associate with TIMELESS, and PER-SG/TIMELESS complexes are constitutively translocated to the nucleus in a *period*^{0} background. PER-SG is defective for other PERIOD functions, and is not sufficient to rescue molecular or behavioral rhythms in a *period*^{0} background (Vosshall *et al.*, 1994; Stanewsky *et al.*, 1997). As shown in Figure 20D, TIMELESS proteins in *period*^{0}; per-SG flies appear to be mostly

hypophosphorylated. This result demonstrates that heterodimerization and nuclear transport are not sufficient for the hyperphosphorylation of TIMELESS, nor is hyperphosphorylation essential for nuclear transport.

Mammalian GSK-3β phosphorylates TIMELESS in an in vitro assay

SGG's influence on the phosphorylation state of TIMELESS in vivo suggested to investigate whether TIMELESS is a direct substrate of SGG. Major aspects of SGG function can be substituted for by GSK-3β as exemplified by the rescue of sgg mutant flies with transgene-derived GSK-3β (Ruel *et al.* 1993). Indeed, the degree of amino acid identity between SGG and GSK-3β across the entire kinase domain is 85%. SGG's mammalian ortholog, whose emzymatic activity is well characterized, was therefore used for the kinase assay. Different fragments of the TIMELESS protein were synthesized as GST fusion proteins (Lino Saez, unpublished data; Myers *et al.* 1996; Saez and Young 1996) and subsequently incubated with GSK-3β. As shown in Figure 20E, the fragments TIM 1-1159 and TIM 222-577 were phosphorylated. However, the carboxy-terminal fragment 504-1121 did not appear to be phosphorylated by GSK-3β under the applied conditions. Likewise, dBMAL protein fused to GST was not phosphorylated at a detectable level. Using the Motif Scanner protein phosphorylation prediction software, two potential GSK-3 sites were found in the sequence encompassed by fragment TIM 222-577 (see Experimental Procedures), and no sites were found in the sequence encompassed by fragment 504-1121; both predictions are in agreement with the *in vitro* phosphorylation

data. Taken together, these observations argue that GSK-3β phosphorylates TIMELESS specifically.

Discussion

In this study it was shown that the segment polarity gene *sgg* contributes to *Drosophila*'s circadian clock. Increased *sgg* function results in period-shortening whereas decreased function causes period-lengthening of the molecular oscillator. In contrast to previously described clock components, even the strongest hypomorphic alleles of *sgg* failed to abolish rhythmicity. However, persisting SGG proteins, induced by heatshock during development in our experiments, presumably account for the remaining oscillation. Thus, a hypothetical complete removal of SGG function may cause arrhythmicity. Due to the possibly essential nature of *sgg* for cell viability, it is questionable whether such an analysis can be realized.

It has been shown in *Xenopus* that kinase deficient GSK-3 can interfere with wild type GSK-3 function in a dominant manner (Dominguez *et al.* 1995; He *et al.* 1995). Based on this observation, a transgene was constructed that encodes a dominant negative SHAGGY molecule. Expression of dominant negative SHAGGY in the eye resulted only in a very mild rough eye phenotype suggesting that this approach is not very effective. Consistently, expression of the dominant negative form of SHAGGY in lateral neurons did not interfere with circadian behavior.

Two independent lines of evidence suggest that *sgg* regulates the period of molecular cycling primarily through effects on nuclear translocation of the PERIOD/TIMELESS heterodimer. First, the transition point between delays and

advances of the Phase Response Curve, an indicator for nuclear entry of PERIOD/TIMELESS complexes (Rothenfluh *et al.* 2000b), is advanced by 3 hours in flies over-expressing *sgg*. Second, nuclear PERIOD was detected ~2 hours earlier in the lateral neurons of larvae over-expressing *sgg*, than in wild type LNs.

sgg-induced shifts in the timing of nuclear translocation are likely to reflect changes in TIMELESS phosphorylation that are in turn connected to altered levels of PERIOD and TIMELESS. Because PERIOD and TIMELESS are over-produced when *sgg* activity is low, *sgg*-dependent TIMELESS phosphorylation may accelerate PERIOD/TIMELESS heterodimerization or directly promote nuclear translocation of PERIOD/TIMELESS complexes in wild type flies. In this view, decreased TIMELESS phosphorylation in *sgg* mutants would tend to retard nuclear transfer, and so require higher concentrations of the PERIOD and TIMELESS proteins at times of nuclear entry.

The hyperphosphorylated form of TIMELESS produced by *sgg* over-expression is as sensitive to light as the co-migrating form of TIMELESS found late at night in wild type flies. Since lowered *sgg* function decreases accumulation of light-sensitive, phosphorylated TIMELESS proteins, *sgg* appears to promote a normal physiological process as opposed to generating a novel form of TIMELESS. The finding that such phosphorylated TIMELESS proteins can be generated by SGG activity in *period*^{0} flies indicates that the kinase can contribute to a cytoplasmic pathway since TIMELESS fails to enter the nucleus without functional PERIOD protein (Saez and Young 1996). SGG dependent TIMELESS phosphorylation does not bypass this requirement for nuclear entry (data not shown).

Hyperphosphorylated TIMELESS begins to emerge during the late night in wild type flies. This corresponds to the timing of PERIOD/TIMELESS nuclear translocation. However, analysis of the PERIOD fusion protein PER–SG showed that a physical association of PERIOD and TIMELESS that permits nuclear translocation will not restore TIMELESS phosphorylation. The insufficiency could be due to a physical requirement for the carboxy terminus of PERIOD which is missing in PER-SG, or sequences composing β-Gal in the PER-SG fusion protein might interfere with TIMELESS phosphorylation. In the absence of PERIOD, TIMELESS is predominantly hypophosphorylated, and over-expression of TIMELESS is not sufficient to enhance TIMELESS phosphorylation in PERIOD-deficient flies. Accordingly, wild type PERIOD might influence the rate of TIMELESS phosphorylation, or it might stabilize hyperphosphorylated TIMELESS. It is also possible that an unidentified factor regulated by the circadian clock influences *sgg*-dependent TIMELESS phosphorylation in *period*^{0} flies.

Although SHAGGY activity appears to determine the period of *Drosophila*'s circadian clock by influencing the rate of PERIOD/TIMELESS nuclear transfer, two observations suggest that TIMELESS phosphorylation is not essential for all nuclear localization of these proteins. First, expression of the fusion protein PER-SG in a *period*^{0} background allows nuclear translocation of hypophosphorylated TIMELESS (Vosshall *et al.*, 1994; L. Vosshall and M. W. Young, unpublished observation). Second, even though a substantial portion of TIMELESS is hypophosphorylated at the end of the night, most TIMELESS immuno-reactivity is nuclear (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996). It should also be pointed out that a tyrosine-linked phosphorylation of TIMELESS

has been implicated in the degradation of TIMELESS by the proteasome (Naidoo *et al.* 1999). Because SHAGGY should not promote tyrosine phosphorylation, a single kinase is unlikely to regulate all aspects of TIMELESS function.

Here it is shown that TIMELESS can be directly phosphorylated by SGG's mammalian ortholog, GSK-3β, *in vitro*. Based on the high degree of identity between the kinase domains of SGG and GSK-3β, it can be concluded that TIMELESS probably also serves as a substrate for SGG. Moreover, the observation that SGG can be substituted for by GSK-3β in many assays further strengthens this conclusion. However, such experiments do not rule out indirect regulation of TIMELESS phosphorylation by SGG *in vivo*. The *in vitro* kinase assays did not result in any mobility shifts of the phosphorylated TIMELESS fragment compared to the unphosphorylated fragment. In contrast, hyperphosphorylated TIMELESS *in vivo* runs at a higher molecular weight in an SDS PAGE than that of hypophosphorylated TIMELESS further supporting the notion that SGG is not the only kinase that phosphorylates TIMELESS.

As monomeric PERIOD and TIMELESS proteins are actively retained in the cytoplasm (Saez and Young, 1996), *sgg* may participate in this regulation. The microtubule-associated protein TAU is a well-documented substrate for mammalian GSK3. Phosphorylation of TAU determines its affinity for microtubules (Bramblett *et al.* 1993; Hong and Lee 1997). Assuming a homologous function in *Drosophila*, effects of SGG on the composition of the cytoskeleton could influence the cytoplasmic retention of PERIOD and TIMELESS. As a consequence of altered cytoskeletal binding, differences in the kinetics of PERIOD/TIMELESS heterodimerization, nuclear transport, and/or

TIMELESS phosphorylation, might be generated. However, as described above, regardless of the level of SHAGGY activity, TIMELESS's movement to the nucleus must remain dependent on PERIOD.

GSK-3β/SGG is well known for its central role in Wnt/WINGLESS signaling. Surprisingly, recent work has indicated that the vertebrate ortholog of DOUBLE-TIME, casein kinase Iε, may also participate in this developmental pathway. For example, in *Xenopus* inhibition of casein kinase Iε produced developmental abnormalities closely corresponding to a loss of Wnt function. Casein kinase Iε was also found to stabilize β-catenin and to bind and phosphorylate DISHEVELLED, both established components of the Wnt signal transduction pathway (Peters *et al.* 1999). It is remarkable that two kinases that function together to provide specific developmental regulation may both act as controlling elements in a patently unrelated behavioral process. This could reflect an underlying synergism between GSK-3β and casein kinase 1ε. Certainly the activities of both kinases must be integrated at some level for coherent transduction of Wnt signals. Because DBT and SGG appear to produce opposing effects on PERIOD/TIMELESS nuclear transfer, with DBT retarding transfer (Kloss *et al.*, 1998; Price *et al.*, 1998) and SGG accelerating the process (this study), the relative activities of these kinases could establish an important focus for stabilizing the period of *Drosophila*'s circadian rhythms. For example a control point composed of offsetting kinase activities might contribute to such homeostatic mechanisms as temperature compensation of the clock.

In conclusion, a novel genetic screen has uncovered a role for the segment polarity gene *sgg* in the organization of the *Drosophila* circadian oscillator. This

approach allowed the detection of *sgg*'s involvement in circadian rhythmicity in spite of a lethal, loss of function phenotype. *sgg* is thus added to a growing list of vital genes affecting the operation of biological clocks in the fly (Yin *et al.* 1994; McNeil *et al.* 1998; Belvin *et al.* 1999; Blau and Young 1999). The strong evolutionary conservation of SHAGGY-like proteins will surely facilitate a search for related function in vertebrate behavioral rhythms.

Table 2: Period-shortening of locomotor activity rhythms by *sgg* over-expression

genotype	tau [hours]	S.D. [hours]	number rhythmic	total number
EP(X)1576/Y; timeless-(UAS)-GAL4/+	20.3	0.4	20	20
EP(X)1576/+; timeless-(UAS)-GAL4/+	21	0.4	13	13
EP(X)1576/+; timeless-GAL4	21.1	0.3	12	14
UAS-sgg/ timeless-(UAS)-GAL4	20.9	0.4	5	10
UAS-sgg/ timeless-(UAS)-GAL4	20.8	0.3	5	10
UAS-sgg/ timeless-GAL4	22.2	0.3	16	16
UAS-sgg/ timeless-GAL4	21.8	0.6	10	10
timeless(UAS)-GAL4/+	23.6	0.4	20	20
timeless-GAL4/+	23.9	0.5	14	16
EP(X)1576/+	23.5	0.4	17	17
UAS-sgg/+	23.3	0.6	12	16

Flies of indicated genotypes were entrained for three 12h:12h light:dark cycles and subsequently subjected to a locomotor activity analysis in constant darkness. tau: period length in hours; S.D.: standard deviation.

Table 3: Locomotor activity rhythms of *sgg* hypomorphic alleles

genotype	tau [hours]	S.D. [hours]	number rhythmic	total number
sgg^{M11}/Y;hs-sgg10	25.4	0.9	14	15
sgg^{D127}/Y; hs-sgg10	26.2	1.0	12	18
sgg^{M11}/sgg^{D127}; hs-sgg10	25.2	1.0	10	10
FM/+; hs-sgg10	24.0	0.5	26	30
sgg^{D127}/ Dp(1:Y)2D1-2;3D3-4;Y	23.7	0.5	20	30

Females carrying the respective *sgg* allele were crossed to males carrying a heat-shock-*sgg* transgene (hs-*sgg10*). The progeny were heat-shocked twice a day for 1 hour at 37°C beginning after hatching until approximately the mid-pupal stage. After eclosion, flies were entrained for three 12h:12h light:dark cycles and locomotor activity was assayed in constant darkness. tau: period length; S.D.: standard deviation.

Table 4: Quantification of data obtained in Figure 17B

	timeless(UAS)-GAL4;+			EP(X)1576;timeless(UAS)-GAL4		
	C	C/N	N	C	C/N	N
ZT17.5	6	-			10	1
ZT18	9	-	-	-	12	2
ZT19	10	-	-	-	1	8
ZT20	3	3	-		-	4
ZT21	-	7	5			10

The number of brain hemispheres found with the indicated subcellular distribution of PERIOD at a given time point is listed. C, cytoplasmic; C/N, cytoplasmic and nuclear; N, nuclear.

Figure 14: Over-expression of *sgg* shortens locomotor activity period in *Drosophila*.

(A) Each panel represents the locomotor activity of an individual fly over time. For visual continuity two days are plotted per row. On top of the panels the subjective light and dark phases, genotypes, and average period length are indicated. Flies were entrained for three days in an 12h:12h light:dark (LD 12:12) cycle and subsequently maintained in constant darkness (DD) for the locomotor analysis. (B) Genomic organization of the *sgg* locus. Exons of transcripts are represented by boxes, filled areas represent translated regions. Shared sequences between transcripts are drawn on top of each other. The position of EP(X)1576 is represented as a triangle with an arrow pointing in the direction of GAL4 induced transcription. Nomenclature for the transcripts was taken from Ruel *et al.*, 1993. However, not all known transcripts were included in the diagram. The position for the first exon of *sgg y* was determined by sequence alignment of the unique N-terminus encoded by *zw3-C* (Siegfried *et al.*, 1992) with genomic sequence. (C) SGG Y over-expression in EP(X)1576. Western blot analysis of extracts from indicated genotypes with an antibody against SGG. Arrows on the side of the gel point out the different SGG isoforms. For comparison, over-expression of SGG 10 is shown, *gmr*-GAL4/+ serves as wild type control.

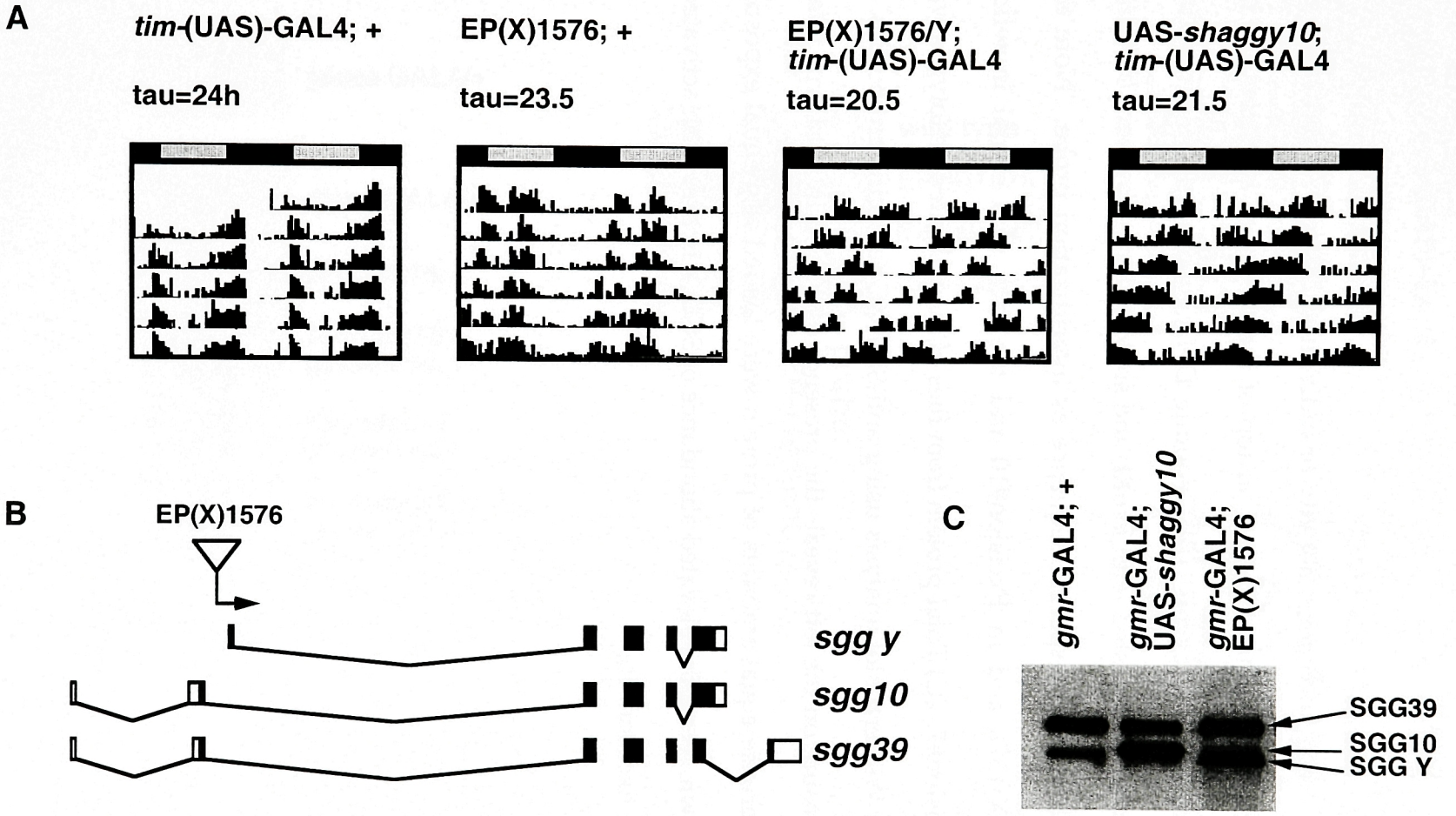
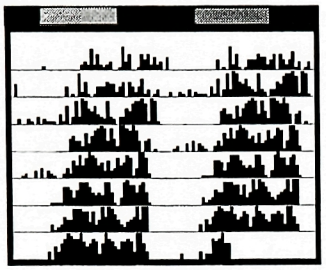

A
tim-(UAS)-GAL4; +
tau=24h
EP(X)1576; +
tau=23.5
EP(X)1576/Y;
tim-(UAS)-GAL4
tau=20.5
UAS-shaggy10;
tim-(UAS)-GAL4
tau=21.5
B
EP(X)1576
sgg y
sgg10
sgg39
C
gmr-GAL4; +
gmr-GAL4;
UAS-shaggy10
gmr-GAL4;
EP(X)1576
SGG39
SGG10
SGG Y

Figure 15: Precise excision of EP(X)1576 reverts short period rhythm to wild type.

(A) Locomotor activity of individual flies of the indicated genotypes is plotter over time. The bar on top of the actogram indicates the phase of the preceding LD cycle. (B) Genomic DNA was prepared from the indicated genotypes, digested with EcoRI, and subjected to a Southern blot analysis using an EP-element specific sequence as hybridization probe. Note the signal in EP(X)1576 and in Excision#10 and the lack of a signal in wild type and Excision#7. (C) Total protein from flies of the indicated genotypes was subjected to a Western blot analysis using anti-SHAGGY specific antibodies. The longer exposure on the left reveals the presence of SGG Y in wild type (*glass*-GAL4/+) otherwise equal amounts of protein were loaded and equal exposure times are shown. Note the elevated abundance of SGG Y in flies with activated EP(X)1576 and Excision#10.

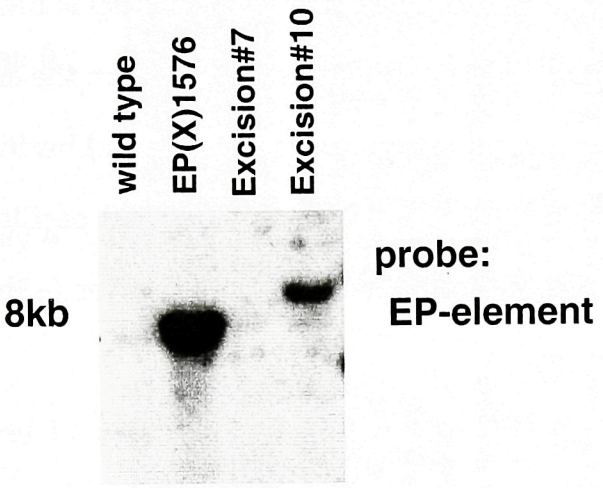
A

Excision#7;
timeless-(UAS)-GAL4



Excision#10;
timeless-(UAS)-GAL4

B



C

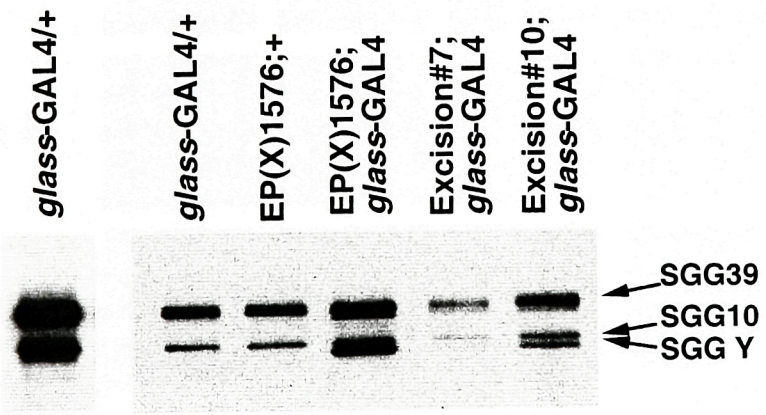


Figure 16: Over-expression of *sgg* alters TIMELESS oscillations.

Eggs of indicated genotypes were collected at 20°C in consecutive one-day bins and entrained for at least 3 LD 12:12 cycles. Subsequently, larvae of different developmental ages were transferred into constant darkness (CT0 is onset of subjective day). Third instar larvae were subjected to immuno-histochemistry every 6 hours using TIMELESS specific antibodies. 3 larval brain hemispheres were analyzed for each time point. Time in DD is indicated on top of the diagram, the day of the collection in DD is indicated on the left. The (L) in some panels denotes lower magnification views of the larval brain hemisphere. Note that wild type (*timeless*-(UAS)-GAL4/+) TIMELESS oscillations have a period of ~24 h, whereas the period is substantially shorter (~3h/day) in *sgg* over-expressing lateral neurons.

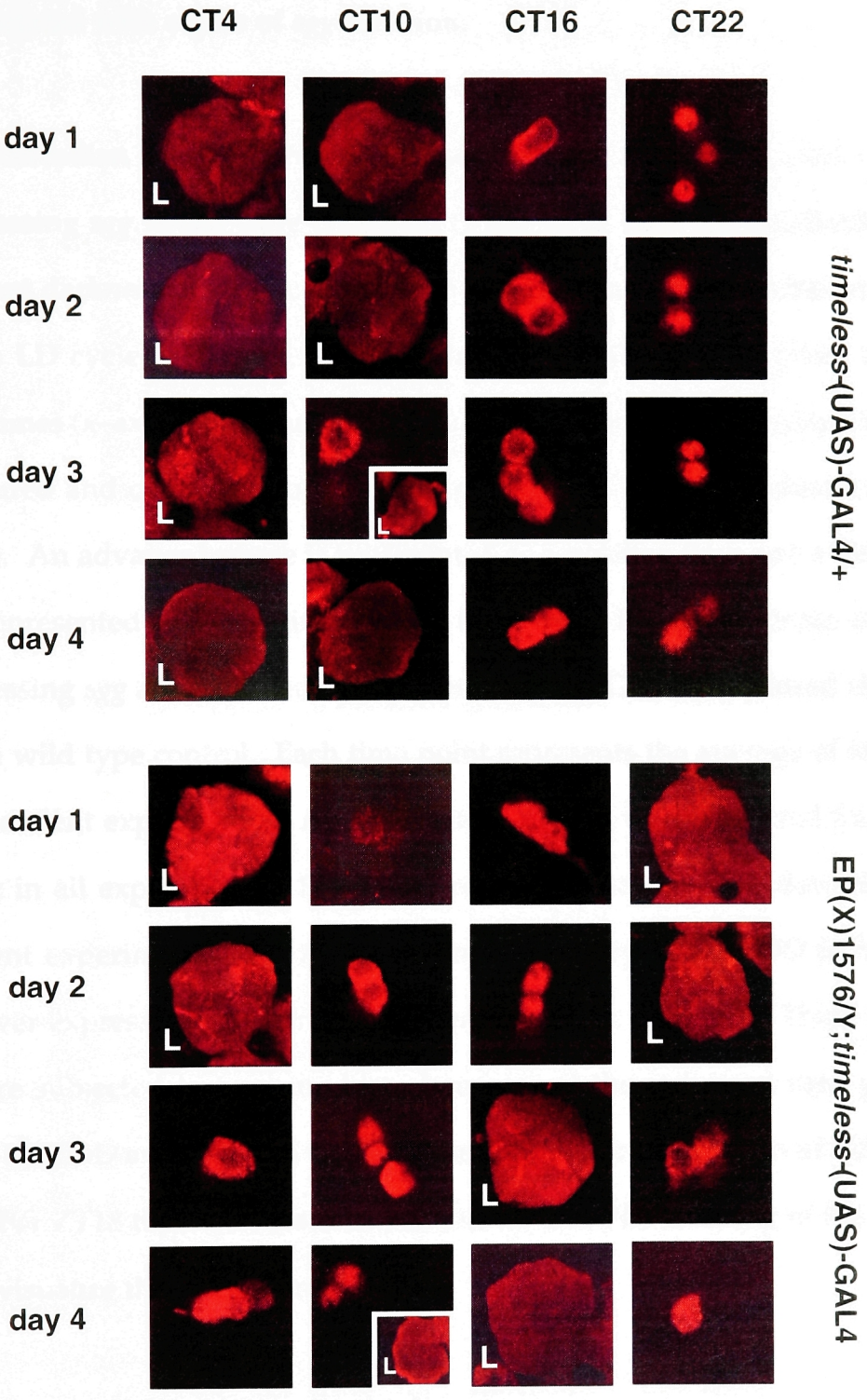
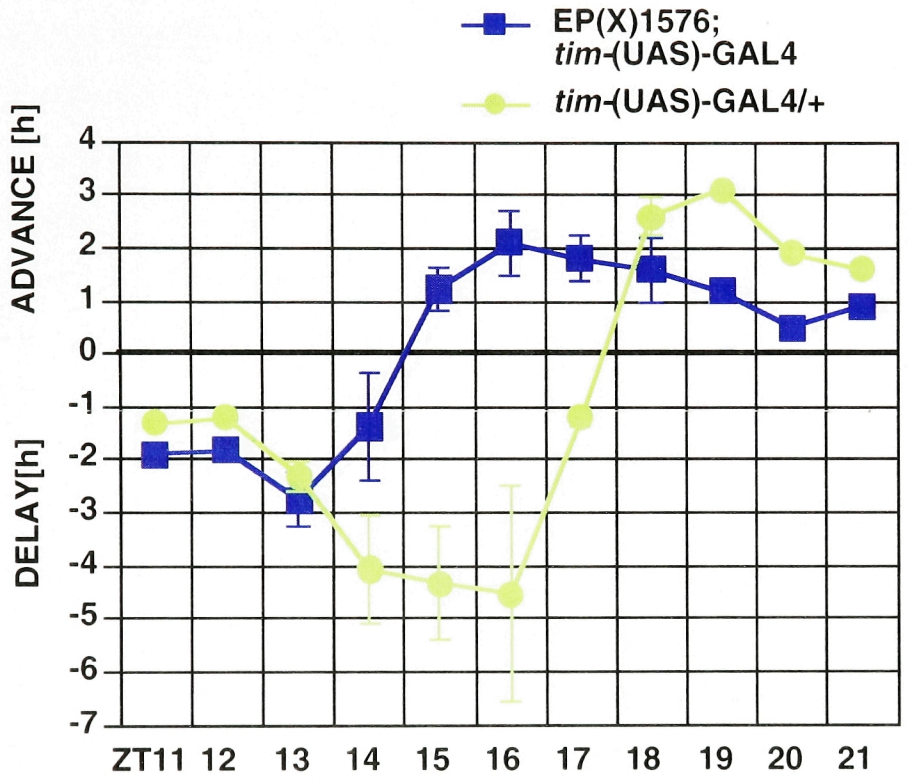

CT4
CT10
CT16
CT22
day 1
day 2
day 3
day 4
L
timeless-(UAS)-GAL4/+
EP(X)1576/Y;timeless-(UAS)-GAL4

Figure 17: Advanced nuclear entry of the PERIOD/TIMELESS heterodimer is associated with a gain of *sgg* function.

(A) Circadian gating of phase responses to photic stimuli is altered in flies over-expressing *sgg*. Flies were entrained to LD 12:12 for 3 days and released into constant darkness at ZT10 of the fourth day (ZT0 is defined as the onset of light in an LD cycle). 10 minute light pulses of ~2000 lux were given at the indicated times (x–axis). The phase of the subsequent locomotor activity rhythm was measured and compared to the phase of flies that were not subjected to a light pulse. An advanced phase is represented as a positive time and a delayed phase is represented as a negative time on the y-axis. Phase responses of flies over-expressing *sgg* are indicated by squares. *timeless*(GAL4)/+ (closed circles) serves as a wild type control. Each time point represents the average of at least two independent experiments. Approximately 10 flies were analyzed for each time point in all experiments. Error bars represent the standard deviation of independent experiments. (B) Advanced nuclear entry of PERIOD in lateral neurons over-expressing *sgg*. *Drosophila* were reared in LD 12:12. Third instar larvae were subjected to immuno-histochemistry at the indicated time points using anti-PERIOD antibodies to visualize the subcellular localization of PERIOD proteins. For ZT18 the insert (asterisk) shows an anti-PDF staining of the same neuron to visualize the cytoplasm.

A


EP(X)1576;
tim-(UAS)-GAL4
tim-(UAS)-GAL4/+
ADVANCE [h]
DELAY[h]
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
ZT11 12 13 14 15 16 17 18 19 20 21

B

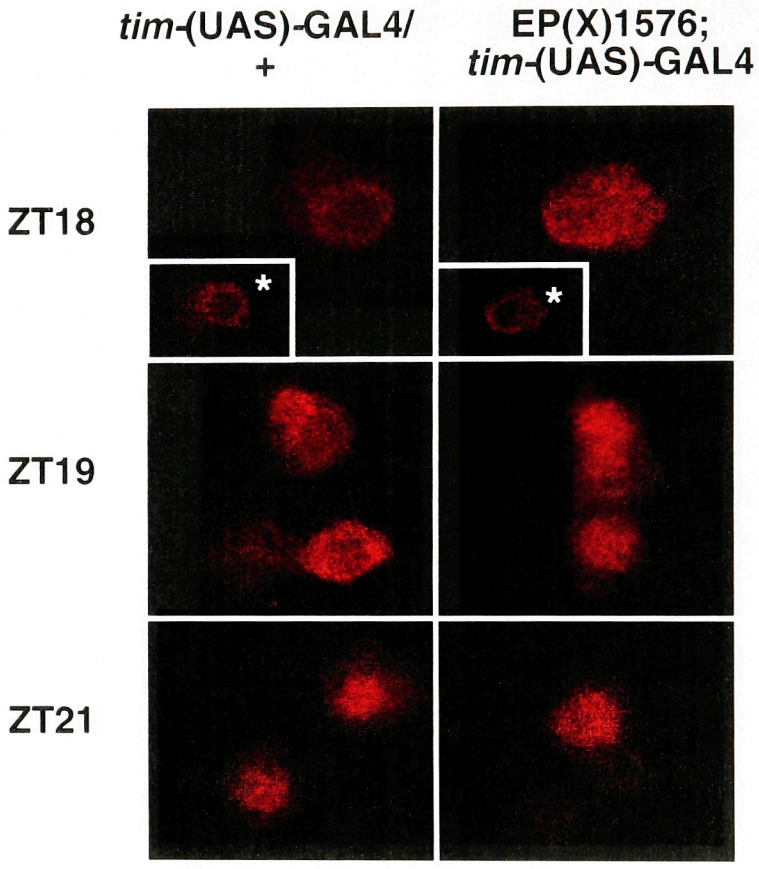

tim-(UAS)-GAL4/
+
EP(X)1576;
tim-(UAS)-GAL4
ZT18
ZT19
ZT21
*
*

Figure 18: *period* RNA levels are not elevated by over-expression of *sgg*.

Flies were entrained for three days in LD 12:12 and subsequently released into DD (CT0 is defined as subjective dawn in DD). Time points were taken every two hours during the first day in constant darkness. Total head RNA was subjected to an RNAse protection assay using a *period* specific probe. Genotypes are indicated on the left of the gel and the time point for each lane is shown on top of the gel. A *tubulin* specific probe was used as loading control.

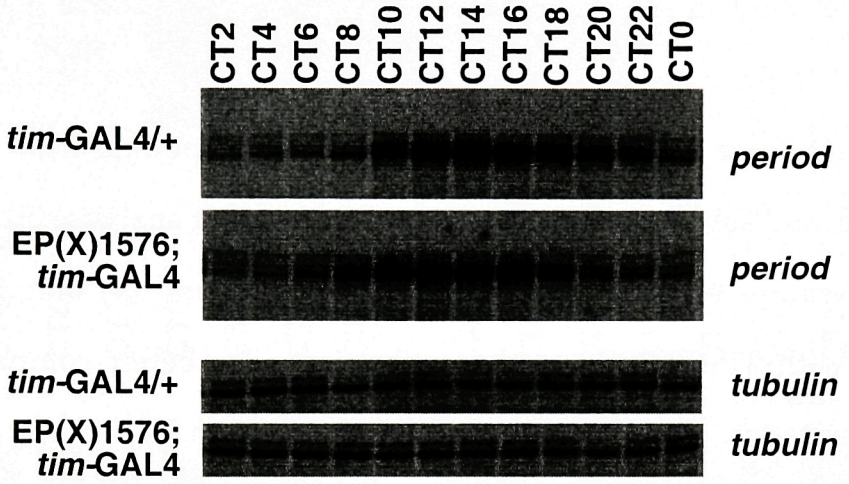

CT2
CT4
CT6
CT8
CT10
CT12
CT14
CT16
CT18
CT20
CT22
CT0
tim-GAL4/+
period
EP(X)1576;
tim-GAL4
period
tim-GAL4/+
tubulin
EP(X)1576;
tim-GAL4
tubulin

Figure 19: Changes in PERIOD and TIMELESS proteins due to reduced *sgg* function.

(A) Western blot analysis showing increased levels of PERIOD during the subjective night in *sgg* mutant flies. Flies of the respective genotypes were entrained to three consecutive LD 12:12 cycles and subsequently released into DD. Flies were collected at the indicated time points, head protein was extracted, and subsequently subjected to Western blot analysis. (B-C) Loss of *sgg* function causes reduced TIMELESS phosphorylation. (B) Blot as in (A) was probed with TIMELESS specific antibodies. For better comparison of the phosphorylation pattern exposure time and protein loaded were adjusted to result in similar signal intensities. Consequently, the quantitative difference is not apparent. Note that at CT2 and CT4, TIMELESS in the mutant is less phosphorylated than TIMELESS in wild type (sgg^{D127}/Dp(1:Y)2D1-2;3D3-4;Y). (C) Flies homozygous for *double-time*^{L} (*dbt*^{L}) or hemizygous for sgg^{D127} were collected at indicated time points. Head extracts were subjected to a Western blot analysis using antibodies specific for TIMELESS. Note that the low mobility form(s) of TIMELESS are always less abundant in the *sgg* mutant. (D) Protein extracts from flies of the indicated genotypes were subjected to a Western blot analysis using SGG specific antibodies. Heat-shocks, where indicated, were administered one hour before protein purification. The rescued *sgg* mutants were kept for approximately one week at 24°C following the last heat-shock. Please note that residual SGG10 persists in the *sgg* mutant.

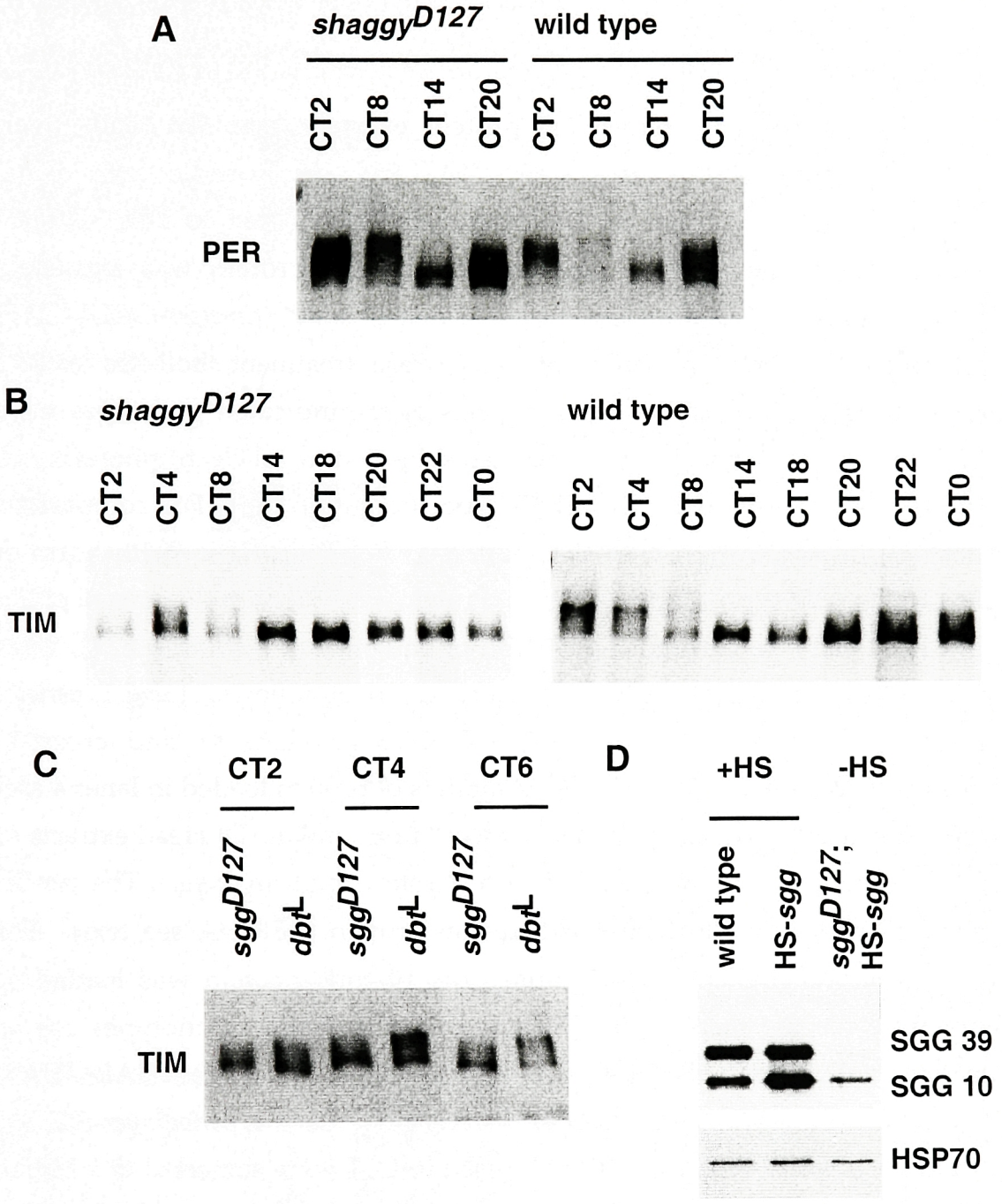
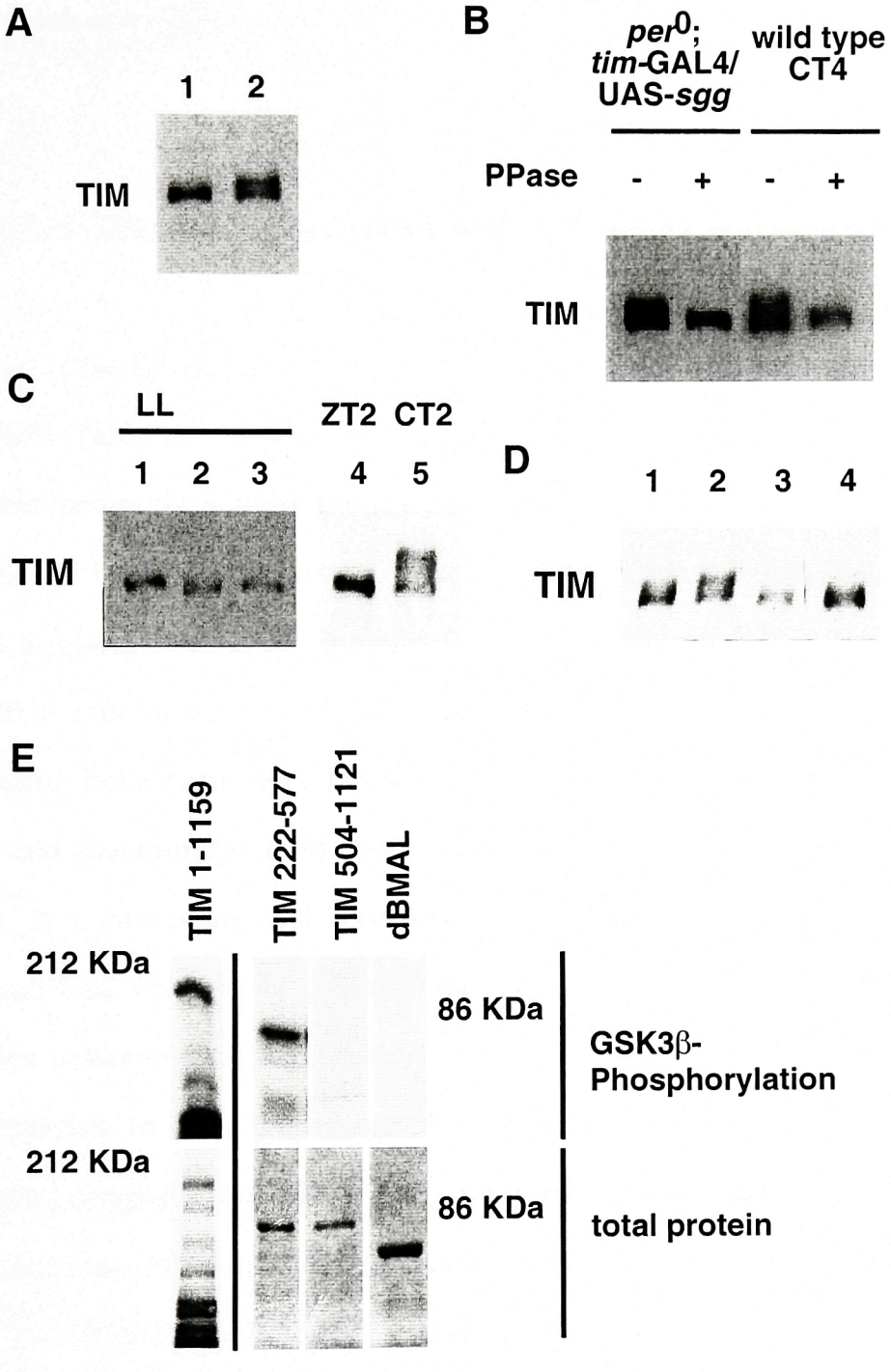

A
shaggy^D127
wild type
CT2
CT8
CT14
CT20
CT2
CT8
CT14
CT20
PER
B
shaggy^D127
wild type
CT2
CT4
CT8
CT14
CT18
CT20
CT22
CT0
CT2
CT4
CT8
CT14
CT18
CT20
CT22
CT0
TIM
C
CT2
CT4
CT6
sgg^D127
dbt^L
sgg^D127
dbt^L
sgg^D127
dbt^L
TIM
D
+HS
-HS
wild type
HS-sgg
sgg^D127; HS-sgg
SGG 39
SGG 10
HSP70

Figure 20: Over-expression of SHAGGY promotes the phosphorylation of TIMELESS.

(A) Western blot analysis of protein extracts from fly heads over-expressing *sgg*with antibodies against TIMELESS. Flies were entrained in 3 consecutive LD 12:12 cycles and subsequently transferred to DD. After 3 subsequent days in constant darkness, total head protein was extracted. Genotypes are as follows: Lane 1, *period*^{0}: Lane 2, *period*^{0}; *timeless*-GAL4/UAS-*shaggy* cross#1 and cross#2 (B) Phosphatase (PPase) treatment abolishes the low mobility form of TIMELESS. Head extracts of the indicated genotypes were treated with Alkaline Phosphatase and then subjected to gel electrophoresis and Western blot analysis using TIMELESS specific antibodies. For comparison untreated samples were run on the same gel. (C) The low mobility form of TIMELESS in wild type is light sensitive. Protein extracts from flies raised either in LD 12:12 (ZT), in constant light (LL), or in constant darkness (CT), were subjected to Western blot analysis. Genotypes are as follows: Lane 1, *period*^{0}: Lane 2 and 3, *period*^{0}; *timeless*-GAL4/UAS-*shaggy* cross#1 and cross#3, respectively: Lane 4 and 5, wild type. Amounts of protein loaded in lanes 4 and 5 were adjusted so that signal intensities would be similar. (D) Head extracts of indicated genotypes were subjected to a Western blot analysis. The *per-SG* transgene encodes a PERIOD-βGAL fusion protein (PER-SG, see text). For *period*^{0}; *timeless*-GAL4/UAS-*timeless*, only 1μg of total protein was loaded to compensate for the increase in TIM protein abundance. Genotypes are as follows: Lane 1, *period*^{0}; *timeless*-GAL4/+: Lane 2, *period*^{0}; *timeless*-GAL4/UAS-*shaggy*: Lane 3, *period*^{0}; *timeless*-GAL4/UAS-*timeless*: Lane 4, *period*^{0}; *per-SG*. (E) Indicated fragments of TIM or dBMAL fused to GST were subjected to a kinase assay with GSK-3β and (γ-^{32}P) ATP (top). Because of considerable size differences, the fusion proteins were run on different gels (seperated by vertical line). As a loading control, the same amount of protein was loaded on a second set of gels and stained with coomassie blue (bottom).


A
1
2
TIM
B
per0; tim-GAL4/ UAS-sgg
wild type CT4
PPase
-
+
-
+
TIM
C
LL
ZT2
CT2
1
2
3
4
5
TIM
D
1
2
3
4
TIM
E
TIM 1-1159
TIM 222-577
TIM 504-1121
dBMAL
212 KDa
212 KDa
86 KDa
86 KDa
GSK3β-Phosphorylation
total protein

Chapter 6: Identification and functional characterization of ADF-1 as a putative clock component

Results

Activation of EP(2)0815 is associated with arrhythmia and elevated ADF-1 levels

One of the EP-elements that led to rapid identification of the affected gene is EP(2)0815 (Table 1). Activation of EP(2)0815 with *timeless*-(UAS)-GAL4 causes arrhythmic locomotor activity (Figure 21A). As shown in Figure 21B, EP(2)0815 is inserted 313 base pairs upstream of the translational start of *Adh* Distal Factor-1 (*Adf-1*; England *et al.* 1990; England *et al.* 1992) suggesting that activation of EP(2)0815 affects the activity of *Adf-1*. ADF-1 contains structural similarities to the putative helix-turn-helix DNA binding motif of Myb and Myb-related proteins and was found to bind and activate the *Adh* distal promoter (England *et al.* 1992). It is interesting in this context that cycling transcription for *Adh* has been described previously (Van Gelder *et al.* 1995) suggesting a possible connection between circadian transcription and ADF-1 function. Additionally, ADF-1 appears to bind to promoters of a number of genes including *dopa decarboxylase* (England *et al.* 1990). The sum of ADF-1 target genes or at least one of them must be vital as loss of *Adf-1* function results in lethality.

The orientation of EP(2)0815 suggested that its activation results in over-expression of *Adf-1*. To test whether this is the case, *Adf–1* RNA abundance was measured by RT-PCR. As shown in Figure 21C, *Adf-1* RNA levels are increased when EP(2)0815 is activated by *timeless*-(UAS)-GAL4. Additionally, the phenotype caused by EP(2)0815 activation could be reverted by excision of the

EP-element (data not shown) indicating that EP(2)0815 is indeed responsible for the observed phenotype. Taken together, these data indicate that over-expression of *Adf-1* through activation of EP(2)0815 can result in arrhythmic locomotor activity.

At this point it cannot be excluded that EP(2)0815 affects gene(s) in addition to EP(2)0815 which may or may not contribute to the behavioral phenotype. However, there are no obvious additional ORFs in close vicinity of EP(2)0815 that could be influenced by activation of EP(2)0815. It therefore seems reasonable to assume that *Adf-1* is the only gene affected by EP(2)0815 activation.

Adf-1 is expressed ubiquitously

Expression of *Adf-1* in the lateral neurons is a prerequisite for its function in the molecular oscillator. To investigate the expression pattern of *Adf-1*, wild type third instar larvae were subjected to immuno-histochemistry using ADF-1 specific antibodies (Figure 22). The observed ubiquitous nuclear staining suggests a broader role for *Adf-1* function consistent with the wide variety of promoters to which ADF-1 can bind. Nevertheless, *Adf-1*'s expression pattern includes the lateral neurons in agreement with a circadian function of *Adf-1*.

Reduced PDF abundance caused by *Adf-1* over-expression

As shown previously, failure of the lateral neurons to differentiate properly causes severe abnormalities of circadian locomotor activity rhythms (Renn *et al.* 1999). To test whether differentiation of the lateral neurons is affected by activation of EP(2)0815, the neuropeptide PDF was chosen as a marker for the lateral neurons. As shown in Figure 23, PDF expressing cells are

clearly present in *Adf-1* over-expressing tissue. Additionally, their axonal projections are indistinguishable from wild type at this level of resolution indicating that the lateral neurons develop properly despite *Adf-1* over-expression. However, PDF immuno-reactivity appears to be decreased in lateral neurons with elevated ADF-1 levels (Figure 23). The reduction of immuno-reactivity is more pronounced in adult flies suggesting that PDF abundance may be a target of ADF-1 function (Figure 23). This phenotype is reminiscent of VRILLE over-expression (Blau and Young 1999). Constitutively elevated abundance of VRILLE is associated with lengthened period and reduction of PDF protein abundance. Interestingly, *pdf* RNA is unaffected by *vrille* over-expression indicating a role for VRILLE in regulating PDF protein translation or stability. To test whether over-expression of *Adf-1* is associated with an analogous effect on *pdf*, an *in situ* hybridization with a *pdf* specific probe on adult brains over-expressing *Adf-1* was performed. The abundance of *pdf* RNA appeared to be strongly reduced (data not shown) suggesting that ADF-1 affects PDF levels by a mechanism different from *vrille*. It should be pointed out in this context that developmental defects and lethality are associated with ubiquitous activation of EP(2)0815. This suggests that the reduction in PDF immuno-reactivity caused by *Adf-1* over-expression may be a consequence of a general defect of the lateral neurons. Thus, the available data are not sufficient to decide unambiguously whether the behavioral phenotype associated with *Adf-1* over-expression is a consequence of interference with an *Adf-1* wild type function in the molecular clock or a consequence of a global cellular defect.

Activation of EP(2)0815 with *timeless*-(UAS)-GAL4 does not abolish molecular oscillation

The next issue to be addressed was whether the arrhythmia associated with over-expression of *Adf-1* also stalls the molecular oscillator. As shown in Figure 24A, *period* RNA oscillations in *Adf-1* over-expressing flies are indistinguishable from wild type in DD. Since cycling of all known clock components are interdependent on each other, it can be inferred from this observation that other clock genes remain also cycling despite increased ADF-1 activity. Consequently, increased ADF-1 levels may interfere with the output mechanism of the circadian clock to confer arrhythmia. This notion is consistent with the abnormally low levels of the neuropeptide PDF in lateral neurons with elevated ADF-1 levels as *pdf* function has been implicated in this step of the biological clock in *Drosophila melanogaster* (Helfrich-Förster *et al.* 2000; Park *et al.* 2000).

It is also possible, however, that the binary expression system employed here does not work equally well in all *period* expressing tissues of the head. If the main source of *period* RNA, the eye, were less responsive to GAL4, or *timeless*-(UAS)-GAL4 were less active in this tissue, *period* RNA from total head RNA would still cycle even if the LNs lacked *period* RNA oscillation. In order to investigate this possibility, the molecular oscillator in larval lateral neurons over-expressing *Adf-1* was tested by immuno-histochemistry. As shown in Figure 24B, TIMELESS appears to oscillate even though with reduced amplitude. Similar data were obtained for PERIOD (data not shown). It is possible that the primary clock defect in *Adf-1* over-expressing flies is the reduced amplitude of cycling molecules and attenuation of PDF expression may ensue. However,

because of the decreased abundance of all tested markers, a global effect on gene expression cannot be excluded.

Construction of dominant-negative *Adf-1* transgenes

As discussed above, a gain of function phenotype is not sufficient to demonstrate the function of a gene in a given process. Since complete and ubiquitous loss of *Adf-1* function results in lethality and all heterozygous mutations of *Adf-1* display wild type locomotor rhythms, tissue specific reduction of *Adf-1* function may be the only way to uncover a role of *Adf-1* in circadian rhythmicity. ADF-1 has several biochemical characteristics that in their sum support its function as a transcription factor. The main biochemical functions are homodimerization, sequence-specific DNA binding, and transcriptional activation through interaction with $TAF_{II}110$ and $TAF_{II}250$ (Cutler *et al.* 1998). Since these different functions have distinct structural bases, one can imagine that a mutation may interfere specifically only with one of these biochemical functions. The resulting molecule may, for example, still bind to DNA but lack its ability to interact with the TAFs. This will result in interference with wild type ADF-1 molecules by occupying a substantial fraction of the target sequences for ADF-1 without activating transcription. Several mutant cDNAs that are known to encode such dominant negative proteins were provided by Dr. Cutler (Cutler *et al.* 1998). Transgenic fly lines were generated that would express these mutant cDNAs under control of the UAS/GAL4 binary expression system. Unfortunately over-expression of these mutant cDNAs under control of *timeless*-GAL4 caused lethality preventing us from a behavioral analysis. As loss of *Adf-1* function is lethal, expression of the dominant negative forms of ADF-1 in

essential tissues should cause lethality. Thus, the observed lethality may stem from *timeless*-GAL4 driven expression of the dominant negative forms of ADF-1 in vital organs of the fly, such as the Malpighian tubules (Giebultowicz and Hege 1997).

Discussion

The behavioral arrhythmia associated with over-expressing *Adf-1* is suggestive but not conclusive mainly for two reasons. First, the vital function of *Adf-1* prevents us from investigating the effects of greatly reduced *Adf-1* function on circadian rhythms. Mild reduction of *Adf-1* function does not interfere with circadian locomotor activity rhythms since all heterozygous *Adf-1* mutants tested were wild type. Thus, at this point we do not know whether *Adf-1* is required for proper circadian rhythmicity. Secondly, although lateral neurons appear to be differentiated in larval brains over-expressing *Adf-1*, lethality associated with ubiquitous increase of ADF-1 suggests that defects caused by elevated ADF-1 abundance in lateral neurons may not be specific to circadian rhythmicity. Also, all markers for the molecular oscillator that have been tested in lateral neurons over-expressing *Adf-1* (PERIOD, TIMELESS, PDF, and *pdf*) showed a decrease in their abundance. This could be a reflection of a general interference with cell viability rather than a reflection of a stalled oscillator. Alternatively, ADF-1 could be a transcriptional cofactor that is involved in the expression of several oscillating genes. This idea is supported by the fact that at least one of ADF-1's known targets, *Adh*, displays a circadian oscillation (Van Gelder *et al.* 1995).

Despite the doubts cast on ADF-1's role as a specific regulator of a behavioral process, a recent report on ADF-1's function in learning and memory

demonstrates that a very specific phenotype can result from interference with ADF-1 function (DeZazzo *et al.* 2000). DeZazzo *et al.* found that a viable, hypomorphic mutation in *Adf-1*, *nalyot*^{P1}, causes reduced performance in an olfactory conditioning experiment. Similar defects were observed with elevated levels of ADF-1. The authors were able to correlate this behavioral phenotype with a defect in synapse formation at the larval neuromuscular junction. Thus, some of the target genes of ADF-1 are involved in the maturation of synapses. The failure of the synapses to form properly could readily explain the observed defective memory of *nalyot*^{P1} flies.

A study on the Neural Cell Adhesion Molecule has implicated proper neuronal contacts in the maintenance of circadian rhythm in mice (Shen *et al.* 1997). It is therefore possible that defective maturation of synapses caused by over-expression of *Adf-1* is responsible for the behavioral arrhythmia. As the lateral neurons appear to be neurally connected to each other (Figure 11) and to target neurons that may transduce circadian information from the lateral neurons to the output organs this is a viable hypothesis. However, it is not clear how impaired neural communication would result in a reduced amplitude of components of the molecular oscillator.

As mentioned above, ADF-1 binds to the promoters of a number of genes indicating that ADF-1 influences the transcriptional activity of those genes. It is possible that a set of those genes is involved in cell viability whereas others are required for behavioral processes, possibly through their function in synapse maturation. Therefore, a genetic dissection of the different functions of *Adf-1* may be difficult as any change of *Adf-1* activity will affect all its target genes and elicit phenotypes associated with those genes. An alternative approach would be

the isolation of *Adf-1*'s target genes. This could for be achieved by microarray analysis of the transcriptome in *Adf-1* over-expressing flies (Spellman *et al.* 1998; White *et al.* 1999). Clock related genes among the targets of *Adf-1* could be identified by reverse genetic approaches as dsRNA and over-expression if no mutation exists.

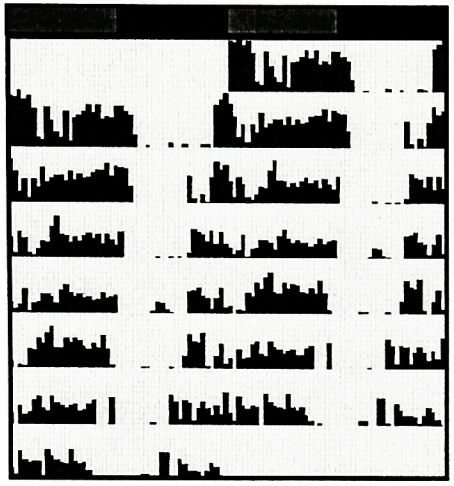
Figure 21: Over-expression of *Adf-1* results in arrhythmia.

Flies of indicated genotypes were entrained in 3 consecutive LD cycles and subsequently released into DD. The locomotor activity patterns of individual flies are shown. (B) The genomic organization around EP(2)0815 is shown. The open triangle represents the EP-element insertion, the arrow represents the translational start site of *Adf-1*, and the bar represents the sequence available from the BDGP data base (STS, sequence tagged site). The orientation of EP(2)0815 is such that activation would result in over-expression of *Adf-1*. (C) Increased *Adf-1* RNA levels by activation of EP(2)0815 with *tim*-(UAS)-GAL4. 1μg total head RNA from indicated genotypes were subjected to an RT-PCR reaction with primers specific for *Adf–1* (up498 and do1157, see Materials and Methods).

A

EP(2)0815; +

tau=23.5h



EP(2)0815; timeless Gal4

B

C

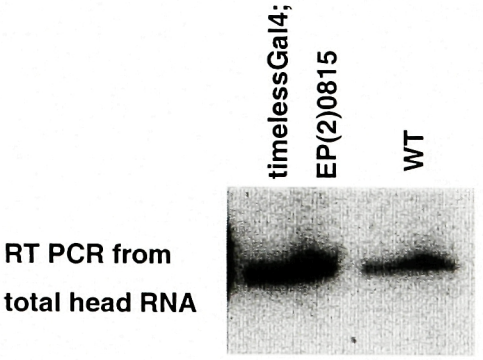


Figure 22: Ubiquitous expression of *Adf-1* in larval cephalic complexes.

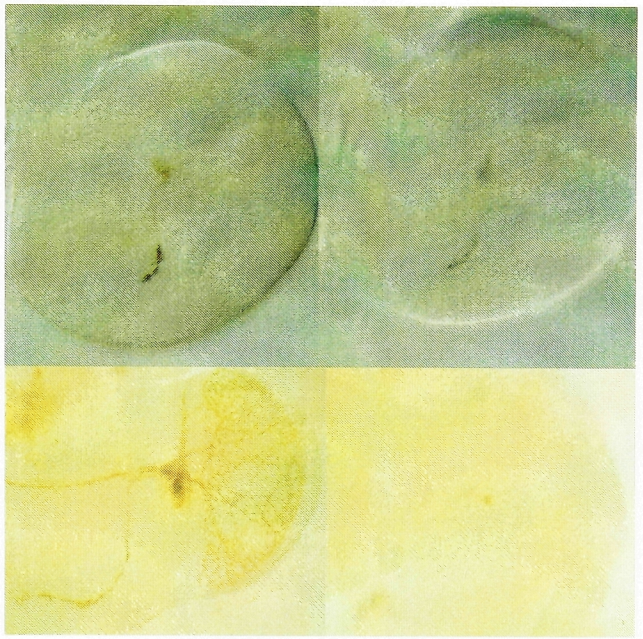
Larval cephalic complexes were subjected to immuno-histochemistry using antibodies specific for ADF-1. Note the ubiquitous nuclear immuno-reactivity.

Figure 23: Reduced PDF immuno-reactivity associated with *Adf-1* over-expression.

Larval cephalic complexes and adult brains, as indicated, were subjected to immuno-histochemistry using antibodies specific to PDF.

timGal4;ST
anti-PDF/ larval

timGal4;EP(2)0815
anti-PDF/ larval



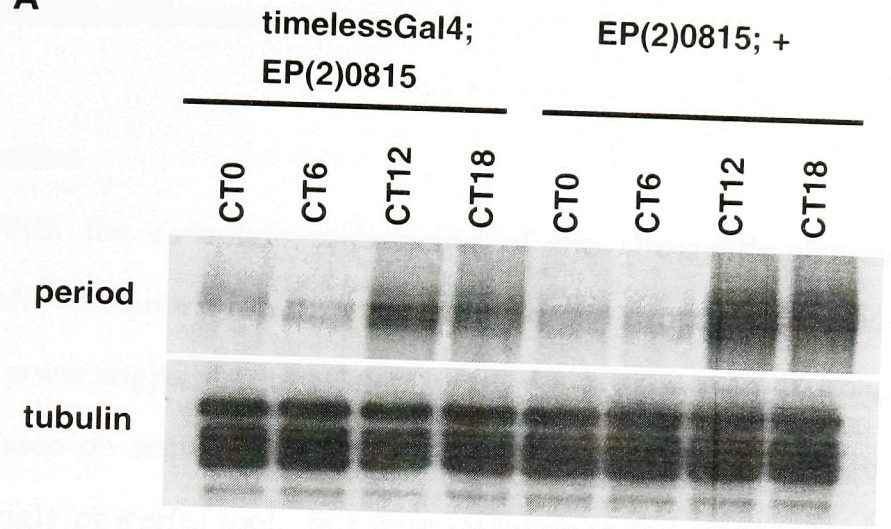
timGal4;ST
anti-PDF/ adult

timGal4;EP(2)0815
anti-PDF/ adult

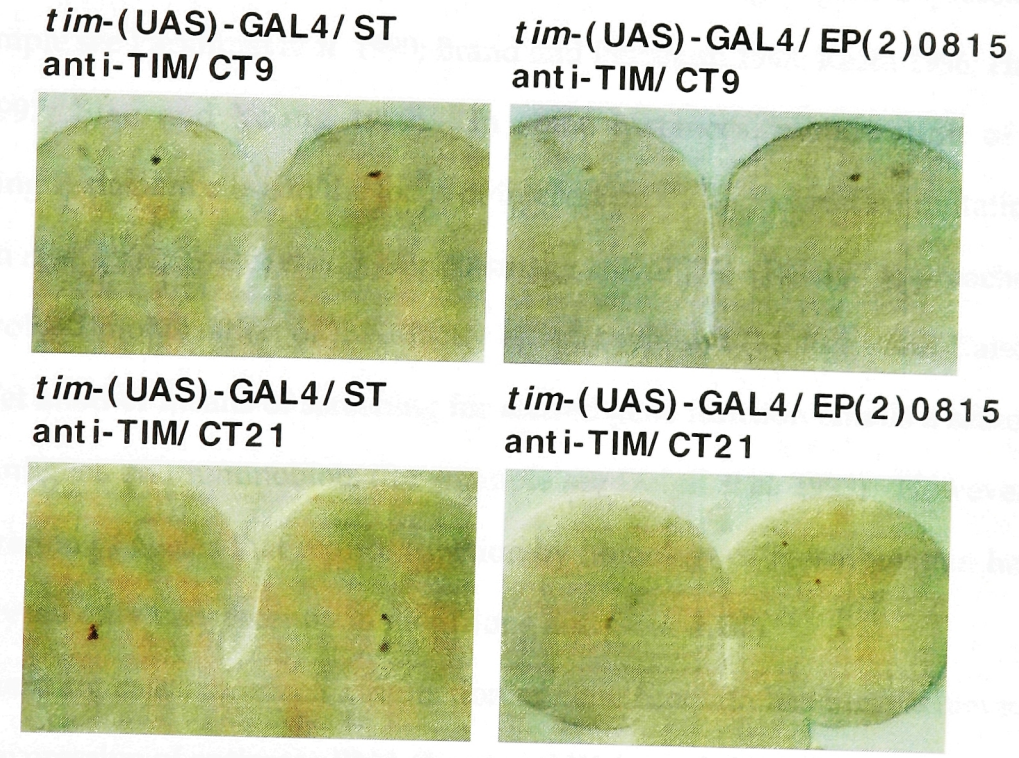
Figure 24: Molecular oscillation in flies over-expressing *Adf-1*.

(A) Total head *period* RNA oscillation is unaffected by activation of EP(2)0815 with *timeless*-(UAS)-GAL4. Flies of the indicated genotypes were entrained in 3 consecutive LD cycles and subsequently released into DD. Flies were collected at the indicated time points. Total head RNA from these time points was subjected to an RNAse protection assay using *period* and tubulin specific probes. (B) Larvae of the indicated genotypes were entrained to 3 consecutive LD cycles and subsequently released into DD. Larval brains were subjected to immuno-histochemistry at indicated times of the subjective day using TIMELESS specific antibodies. Please note that despite reduced immuno-reactivity TIMELESS protein abundance still cycles in a circadian manner.

A


timelessGal4;
EP(2)0815
EP(2)0815; +
CT0
CT6
CT12
CT18
CT0
CT6
CT12
CT18
period
tubulin

B


tim-(UAS)-GAL4/ ST
anti-TIM/ CT9
tim-(UAS)-GAL4/ EP(2)0815
anti-TIM/ CT9
tim-(UAS)-GAL4/ ST
anti-TIM/ CT21
tim-(UAS)-GAL4/ EP(2)0815
anti-TIM/ CT21

Chapter 7: Specific genetic interference with behavioral rhythms in *Drosophila* by expression of inverted repeats

Introduction

With the complete sequencing of the *Drosophila* genome (Berkeley *Drosophila* Genome Project, www.fruitfly.org; European *Drosophila* Genome Project, www.edgp.ebi.ac.uk; Celera, www.celera.com) the identification of new genes based on sequence composition and comparison is likely to become an increasingly powerful tool. But if no mutation in the given gene exists, *in vivo* studies are often restricted to reverse genetics. P-element mediated gene transfer allows gene interference by altering pattern, level or timing of gene expression (for example see Parkhurst *et al.* 1990; Brand and Perrimon 1993; Rørth 1996; Hay *et al.* 1997; Blau and Young 1999). In some instances, mobilization of a preexisting P-element close to the locus of interest provides the needed mutation (Littleton *et al.* 1993; Tower *et al.* 1993; Zinsmaier *et al.* 1994). Related approaches have involved mobilization of the I factor retrotransposon (Milligan and Kaiser 1993). Yet another means of screening for altered gene function entails a search for lost antigens on immunoblots (for example see Dolph *et al.* 1993). However, the generation of alleles that reduce function by homologous recombination has been reported only very recently in flies (Rong and Golic 2000).

There are cases in which a reduction of gene function has been achieved through expression of antisense RNA (Izant and Weintraub 1984; Rosenberg *et al.* 1985) or ribozymes (Zhao and Pick 1993). But as demonstrated by Fire *et al.*, double-stranded RNA is substantially more effective at blocking gene function than antisense RNA in *Caenorhabditis elegans* (Fire *et al.* 1998). Subsequent studies

established that double-stranded RNA (dsRNA) is also a potent and specific inhibitor of gene function in zebrafish (Wargelius *et al.* 1999), *Trypanosoma brucei* (Ngô *et al.* 1998), planarians (Sánchez Alvarado and Newmark 1999) and in *Drosophila* (Kennerdell and Carthew 1998). Although the mechanism of dsRNA is still controversial, a loss of the endogenous mRNA constitutes an important step (Montgomery *et al.* 1998; Sharp 1999; reviewed in Fire 1999).

We were interested in applying dsRNA-mediated gene interference to the investigation of behavioral rhythms in adult *Drosophila*. In contrast to previous applications of dsRNA-mediated gene interference in *Drosophila*, where dsRNA had been injected into embryos (Kennerdell and Carthew 1998; Misquitta and Paterson 1999), we wished to apply this technique to adult animals. It seemed unlikely that dsRNA, once injected into embryos, would persist through several days of development and then still be effective in promoting gene interference for another 1-2 weeks to allow adult behavioral analysis. Although Misquitta and Paterson (1999) observed that injection of dsRNA of the *white* gene into embryos was effective at blocking *white* gene function in adult flies, the low penetrance obtained in those studies would make a behavioral analysis almost impossible (Misquitta and Paterson 1999). In order to bypass this problem, we wished to produce dsRNA endogenously.

The rationale for our experiment is that an inverted-repeat sequence could, once transcribed, fold back and form a double-stranded RNA molecule. This idea was supported by the observation that inverted-repeat sequences could induce gene silencing in plants (Waterhouse *et al.* 1998) and in *Caenorhabditis elegans* (Tavernarakis *et al.* 2000). In contrast to the ubiquitous expression of the inverted-repeat sequences in *Caenorhabditis elegans* under the control of a heat

shock-inducible promoter (Tavernarakis *et al.* 2000), we employed the GAL4/UAS binary expression system (Brand and Perrimon 1993) to ensure tissue specificity. This should allow the analysis of functions of the adult nervous system, such as circadian behavior, by permanent interference with endogenous gene function. Furthermore, tissue specific reduction of gene function potentially allows investigations not possible with ubiquitous gene interference or gene loss: A) Genes which play an essential role not only in behavior but also in development may not be accessible to a behavioral analysis via traditional mutations. However, a tissue specific reduction of gene function might yield viable, yet behaviorally abnormal flies. B) Taking advantage of the large number of existing GAL4 driver lines (for example see Yao Yang *et al.* 1995), this technique would allow the researcher to decide in which cells the gene product acts for a given aspect of behavior. In contrast to over-expression methods, reduced expression can only elicit responses in cell types in which the target gene is normally expressed.

In order to test this approach, we chose the circadian clock gene *period* as a model system. Mutations in the *period* ORF lengthen or shorten the period of the circadian rhythm and a complete loss of *period* function causes arrhythmia (Konopka and Benzer 1971). Additionally, reduction of *period* dosage lengthens the period of *Drosophila* behavioral rhythms (Smith and Konopka 1982; Baylies *et al.* 1987). The dosage sensitivity of *period* can be explained by its crucial function in the oscillator: A reduction of *period* dosage delays production of PERIOD protein sufficient for interaction with TIMELESS and nuclear transport. Consequently, the deactivation of *period* and *timeless* transcription is delayed, resulting in an overall lengthening of this molecular cycle. The specificity of the

phenotype, its dosage sensitivity, as well as the availability of a quantitative assay, made *period* an especially well suited candidate for our study.

Here we demonstrate that the expression of inverted-repeats of sequences composing the *period* gene results in a reduction of endogenous *period* RNA levels. As well, endogenous production of the same inverted-repeats in cells that are important for the circadian locomotor activity of the fly produce a long period behavioral phenotype.

Results

Expression of inverted-repeat sequences of the *period* gene interferes with circadian clock function

Sequences from two different regions of the *period* open reading frame were used for the generation of inverted-repeats (Figure 25A). *perCt* contains a carboxy terminal fragment without any known homologies. *perPAS* contains a sequence encoding a putative protein dimerization domain, the PAS domain (Huang *et al.* 1993). The PAS domain is found in several other genes (reviewed in Ponting and Aravind 1997), but since the similarity on a nucleic acid level is weak, we did not expect cross hybridization with other mRNAs. The length of a single repeat was in both cases close to 1 kb in accordance with the average size of dsRNA used in Fire *et al.* 1998. The location of the inverted-repeat sequence and the length of the gap between the repeats of 67 bp were chosen to facilitate cloning.

We wished to express the inverted-repeats using the GAL4/UAS binary expression system (Brand and Perrimon 1993) in cells relevant for rhythmic locomotor activity. To this end, a transgenic line that expresses GAL4 under the

control of the *timeless* promoter, *timeless*-GAL4, was employed (Figure 25B, C; Emery *et al.* 1998). As described in the introduction to this chapter, the *timeless* gene is another essential clock component, and therefore expressed in all cells with a functioning clock (reviewed in Hardin 1998; Young 1998; Scully and Kay 2000). These cells include the Lateral Neurons (LNs), which are believed to be the location of the regulator of rhythmic locomotor activity (Ewer *et al.* 1992; Frisch *et al.* 1994; Renn *et al.* 1999). As shown in Figure 25C *timeless*-GAL4 is active in cells expressing *timeless*.

Expression of *perCt-inverted-repeat* (*perCt-IR*) and *perPAS-inverted-repeat* (*perPAS-IR*) resulted in a lengthening of the average period of the circadian locomotor activity cycle by approximately 2 hours compared to *timeless*-GAL4/+ (Figure 26, Table 5). Almost identical phenotypes were observed for several independent transgenic lines (Table 5). The phenotype is nearly fully penetrant. Only 10% of the flies expressing *perCt-IR* and only 3% of the flies expressing *perPAS-IR* showed a period of less than 25 h compared to 9% of wild type controls with periods of more than 24 h.

Since there is an inverse correlation between *period* dosage and period length (Smith and Konopka 1982; Baylies *et al.* 1987), the described phenotype is in good agreement with a decrease in *period* RNA levels caused by expression of the inverted-repeats. Baylies *et al.* (1987) also noted that very long period rhythms of over 35 h can be caused by an extremely strong reduction of *period* RNA abundance (approximately 20 fold). However in our study the maximal increase in period length was 3 h. This suggests that our experiments reduce, but do not eliminate expression of *period*.

Neither sense nor antisense RNA expression is able to interfere with clock function

In order to test whether a behavioral phenotype would be caused by the sense or antisense RNA alone, we generated transgenic lines that express either the sense RNA (*perCt-sense*) or the antisense RNA (*perCt-antisense*) of *perCt*. Independent expression of each construct under the control of *timeless*-GAL4 resulted in wild type circadian behavior (Table 6). Therefore, expression of the entire inverted-repeat sequence is responsible for the observed interference with *period* function.

Photoreceptor-specific expression of inverted-repeat does not affect circadian rhythm

We also wanted to know whether expression of the inverted-repeat outside the LNs would result in behaviorally normal flies. To this end, expression of the inverted-repeat was driven with *gmr*-GAL4, which expresses GAL4 under control of the *glass* responsive element (Freeman 1997). As *glass* is expressed predominantly in photoreceptors (Moses *et al.* 1989; Moses and Rubin 1991; Ellis *et al.* 1993; Vosshall and Young 1995), flies carrying *gmr*-GAL4 and *perCt-IR* or *perPAS-IR* should express the inverted-repeat primarily in that cell type. Consistently, as shown in Figure 25D, *timeless*-expressing LNs in larval brains monitored at ZT23 to optimize TIMELESS expression failed to express *glass*. The circadian behavior of flies expressing the inverted-repeats under the control of *gmr*-GAL4 was indistinguishable from wild type (Table 5).

It had previously been reported that expression of *period* under control of the *glass* promoter can rescue behavioral arrhythmia in a *period* loss of function

mutant (Vosshall and Young 1995). In that study *glass* expression was observed in cells of the brain other than the LNs. Thus, it seems likely that expression of *period* in such alternative cells can restore behavioral rhythmicity. Since *period* RNA interference in *glass*-expressing cells did not significantly alter behavioral rhythmicity in the current study but would not affect LNs, correct function of a clock in LNs may be sufficient to establish wild type behavioral rhythmicity. Evidence for a dominant, but not exclusive role for LNs in maintenance of behavioral rhythms was previously set forth by Renn *et al.* 1999 (Renn *et al.* 1999). Taken together, these data indicate that the expression of an inverted-repeat can result in tissue specific and permanent interference with gene function.

Specific reduction of *period* RNA by inverted-repeat

Furthermore, we wondered whether presence of the inverted-repeat would result in a decrease of endogenous RNA levels as is the case for dsRNA (Fire *et al.* 1998). Roughly 75% of the *period* RNA in the head is produced in the eye, and *period* RNA levels oscillate with a circadian rhythm in this tissue (Zeng *et al.* 1994). For this reason, we used *gmr*-GAL4 as driver to examine the effect that expression of *perCt-inverted-repeat* would have on *period* RNA abundance. Flies were entrained to a 24 hour light dark cycle (LD; 12/12) and collected at times when *period* RNA abundance is usually at its trough (ZT2) and when it is at its peak (ZT14; ZT0 is lights on). Total RNA was isolated from heads and subjected to an RNAse protection assay. At ZT 14 abundance of *period* RNA was found to be reduced to approximately 50% that of wild type in flies expressing *perCt-IR* (Figure 27). In addition, a subtle increase in *period* RNA levels at ZT2 was observed (Figure 27). This may be accounted for by the inhibiting effect

PERIOD has on its own transcription. Due to the reduction of *period* RNA levels at ZT14 in the transformed flies, less PERIOD protein is translated. Consequently, less PERIOD should be available to inhibit its own transcription. This experiment was repeated for several independent transgenic lines (see error bars in Figure 27). A decrease of *period* message at ZT14 to roughly 50% of that seen in wild type was also observed for *perPAS-IR* (Figure 27C, E, Figure 28).

The described reduction of *period* RNA levels at ZT14 might be due to an effect of the inverted-repeat on the phase of *period* oscillation. In order to test this possibility, *period* RNA levels were measured at 12 time points during an LD cycle (Figure 27C-E). Although *period* RNA abundance stays at its peak in the inverted-repeat over-expressing flies from ZT14-ZT18 it never exceeds 50% of wild type peak levels. In contrast, *period* RNA at its trough is slightly increased in the mutant compared to wild type. The observed damping of the *period* RNA oscillation is in accordance with an inhibiting effect of the inverted-repeats on *period* RNA accumulation. This indicates that expression of an inverted-repeat might indeed follow a mechanism similar to that reported for dsRNA.

Consistent with our results, Baylies *et al.* (1987) observed that a threefold decrease in *period* RNA levels led to periods of about 27 hours (Baylies *et al.* 1987). Although this former study did not take *period* RNA oscillation into account, the pooled RNA of an unsynchronized population should nevertheless reflect differences in *period* RNA levels between genotypes. The current study extends that of Baylies *et al.* (1987) by clarifying the role of *period* RNA level in determining the amplitude of the RNA oscillation and in setting the period length of the molecular and behavioral rhythm.

We also investigated *timeless* RNA levels, which normally oscillate in synchrony with *period*. As shown in Figure 28, in flies expressing *perCt-IR* or *perPAS-IR*, *timeless* RNA abundance at ZT14 is at 75% of the wild type control. This observation supports the idea that the reduction of *period* RNA by expression of an inverted-repeat is the primary cause of the lengthening of the circadian cycle. Since oscillations of *period* and *timeless* expression are coupled to each other (reviewed in Dunlap 1999), a change in the abundance of one gene's product might be expected to affect the other gene's activity. For example, a long period mutation of *double-time* (dbt^L) affects the period and amplitude of both *period* and *timeless* RNA cycles, even though PERIOD protein appears to be the primary target of the altered DBT kinase (Price *et al.* 1998). We suggest that the modest change in *timeless* RNA abundance observed in the present study may be a secondary consequence of the change in *period* function in the transformed flies.

A very remarkable feature of gene interference by dsRNA is its specificity. Since we did not observe any morphological defects in the eyes of flies that expressed the inverted-repeats under the control of *gmr*-GAL4 or *eyeless*-GAL4 (data not shown), it seemed unlikely that the expression of the inverted-repeats causes a general reduction of RNA levels. In order to further test this notion, we examined the RNA levels of *rhodopsin1* (O'Tousa *et al.* 1985; Zuker *et al.* 1985; Mismer and Rubin 1987) in flies which express the *period* inverted-repeats under control of *gmr*-GAL4. As shown in Figure 28C, expression of the inverted-repeats does not affect *rhodopsin1* RNA levels. This result argues also in favor of a specific interference of the inverted-repeats with *period* gene function in the same cells.

Discussion

Whereas in most cases of injected dsRNA loss of function phenotypes were observed (for example see Fire *et al.* 1998; Kennerdell and Carthew 1998), in our study a hypomorphic phenotype was usually obtained. Several factors may be responsible for this difference. Since Kennerdell and Carthew (1998) injected early embryos, it is possible that additional factors, which are present more abundantly in the germline and early embryos than they are in adults, are required for efficient gene interference by dsRNA. This idea is supported by the observation that the progeny of injected worms showed a stronger phenotype than the injected animal itself (Fire *et al.* 1998). Alternatively, nuclear RNA binding proteins might prevent the majority of the synthesized inverted-repeat RNA molecules from forming a hairpin loop or from being transported into the cytoplasm. Consequently the number of double-stranded RNA molecules per cell would be lower and the interference weaker. Since it was reported that very low concentrations of dsRNA are effective at interfering with gene function (Fire *et al.* 1998), the latter possibility seems less likely.

In this study we were able to lower *period* gene function specifically through the expression of inverted-repeat sequences. We achieved permanent and tissue specific gene interference in *Drosophila*. Although the example we have explored involved modification of gene expression in the adult nervous system, in principle this approach should be applicable to the study of previously uncharacterized gene function within any tissue and at any stage of development.

Table 5: Tissue specific expression of *perCt-IR* or *perPAS-IR* causes lengthening of behavioral rhythm

Genotype	tau	±S. D.	N	%R
timeless-GAL4/+	23.8	0.4	24	100
perCt-IR I1/timeless-GAL4	25.9	0.3	21	100
perCt-IR F1/timeless-GAL4	25.7	1.6	15	88
perCt-IR B1/timeless-GAL4	25.5	1	18	85
perCt-IR B1/gmr-GAL4	23.5	0.9	11	100
perCt-IR F1/gmr-GAL4	23.1	0.3	16	100
perCt-IR I1/gmr-GAL4	23.6	0.5	15	100
perPAS-IR M1/timeless-GAL4	26	0.5	21	100
perPAS-IR K1/timeless-GAL4	26.3	0.6	22	89
perPAS-IR F2/timeless-GAL4	25.6	0.6	16	100
perPAS-IR G2/timeless-GAL4	26.2	1.3	22	93
perPAS-IR G2/gmr-GAL4	23.2	0.3	10	94
perPAS-IR H2/gmr-GAL4	23.1	0.3	10	100

Lines carrying either *perCt-IR* or *perPAS-IR* were crossed to flies carrying *timeless*-GAL4 or *gmr*-GAL4. Locomotor activity rhythms of individual males of the F1 generation were monitored in constant darkness. The average period length (tau, in hours) for each genotype is indicated. The results for several independent transgenic lines are shown. N - total number of flies assayed; %R - percent rhythmicity; S. D. - standard deviation;

Table 6: Expression of sense or antisense RNA alone does not affect behavioral rhythm

Genotype	tau	±S. D.	N	%R
perCt-sense G1/timeless-GAL4	23.6	0.4	17	100
perCt-sense C1/timeless-GAL4	23.8	0.4	11	100
perCt-antisense G1/timeless-GAL4	23.5	0.4	20	100
perCt-antisense I1/timeless-GAL4	23.4	0.4	14	100
perCt-antisense D1/timeless-GAL4	23.1	0.3	9	100

Lines carrying either *perCt-sense* or *perCt-antisense* were crossed to flies carrying *timeless*-GAL4. Locomotor activity rhythms of individual males of the F1 generation was monitored in constant darkness. The results for several independent transgenic lines are shown. N - total number of flies assayed; %R - percent rhythmicity; S. D. - standard deviation;

Figure 25: Experimental strategy for the expression of the inverted repeat sequences *perCt-IR* and *perPAS-IR* in clock-relevant tissues.

(A) The DNA fragments *perCt* and *perPAS* were amplified by PCR from *period* cDNA. Subsequently, each PCR fragment was cloned as inverted-repeat into the vector pUASt (Brand and Perrimon 1993). Nucleotide positions are indicated above the cDNA. (B) Schematic diagram of the GAL4/UAS binary expression system (Brand and Perrimon 1993). The *timeless* promoter region controls tissue specific expression of the yeast transcription factor GAL4. GAL4 binds to its target sequence (GAL4 sites or upstream activating sequence/UAS) and activates the transcription of downstream elements. (C) *timeless*-GAL4 is active in *timeless*-expressing cells in the larval brain. Larval brains expressing *tau-lacZ* under the control of *timeless*-GAL4 were stained with antibodies against β-Gal (blue) and antibodies against TIMELESS (red) at ZT23. Note the nuclear localization of TIMELESS and the cytoplasmic localization of β-Gal in the same neurons. (D) *gmr*-GAL4 is not active in *timeless*-expressing cells. Larval brains expressing *tau-lacZ* under the control of *gmr*-GAL4 were stained with antibodies against against β-Gal (blue) and antibodies against TIMELESS (red; arrow) at ZT23. The TIMELESS positive neurons are in the same position as those in (C). The blue signal shows the photoreceptor axons which grow from the developing retina into the brain.

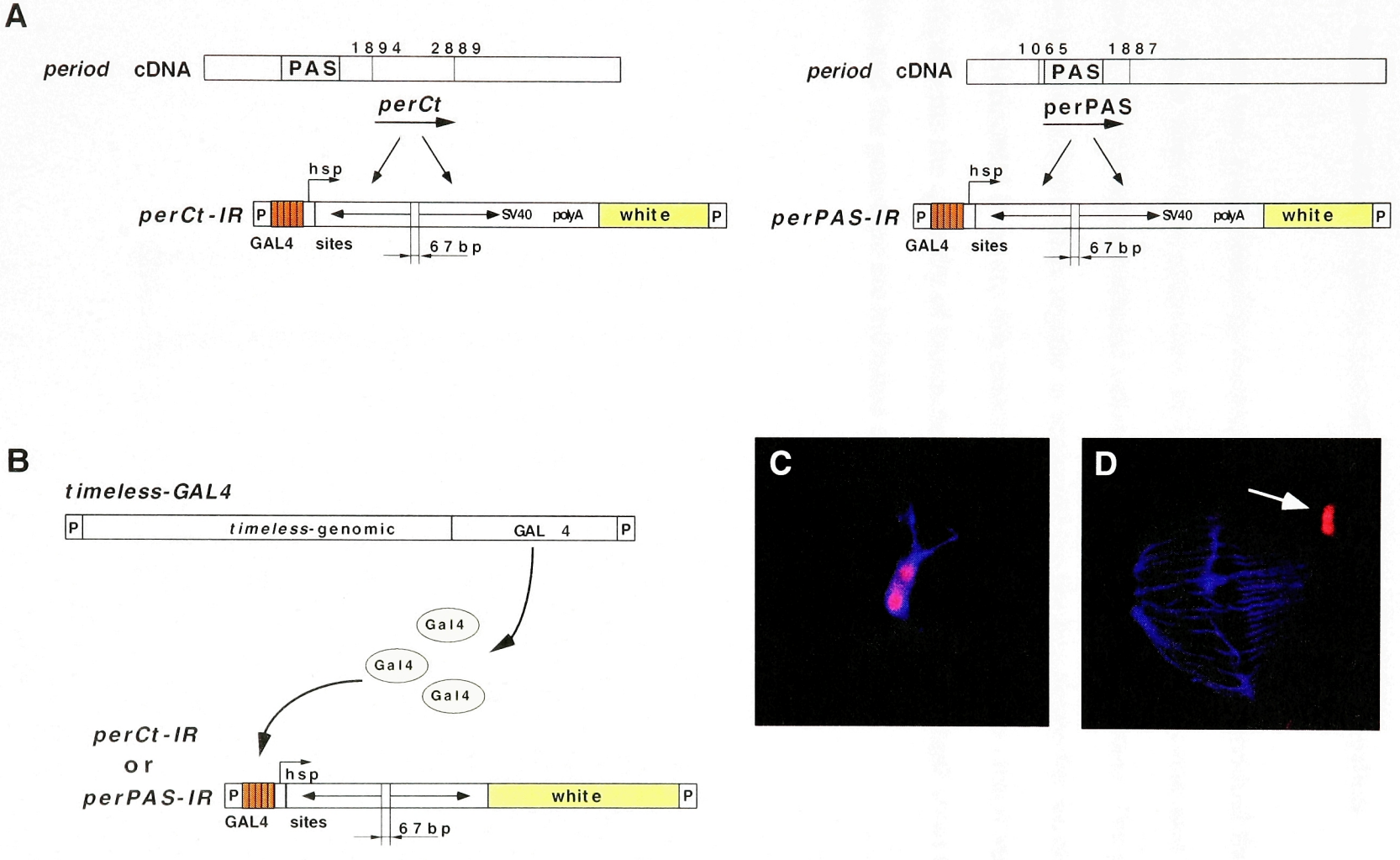

A
period cDNA
1894 2889
PAS
perCt
hsp
perCt-IR
P
SV40 polyA
white
P
GAL4 sites
67 bp
period cDNA
1065 1887
PAS
perPAS
hsp
perPAS-IR
P
SV40 polyA
white
P
GAL4 sites
67 bp
B
timeless-GAL4
P
timeless-genomic
GAL 4
P
Gal4
Gal4
Gal4
perCt-IR
or
perPAS-IR
hsp
P
white
P
GAL4 sites
67 bp
C
D

Figure 26: Expression of *perCt-IR* and *perPAS-IR* under the control of *timeless*-GAL4 lengthens periods of locomotor activity rhythms.

Each histogram describes the locomotor activity of an individual fly over time. Adult flies were entrained in a 12 h light: 12 h dark cycle and their locomotor activity was monitored subsequently in constant darkness. The phase of the previous light: dark regime is indicated by the bar above the locomotor record. Horizontal lines are 48h intervals, and the height of the closed vertical bars represents the quantity of locomotor activity. The period length (tau) of the rhythm and the genotype are indicated on top of each record.

timeless-GAL4/+ tau=24 h

perCt-IR I1/ timeless-GAL4 tau=26 h

perPAS-IR G2/ timeless-GAL4 tau=26 h

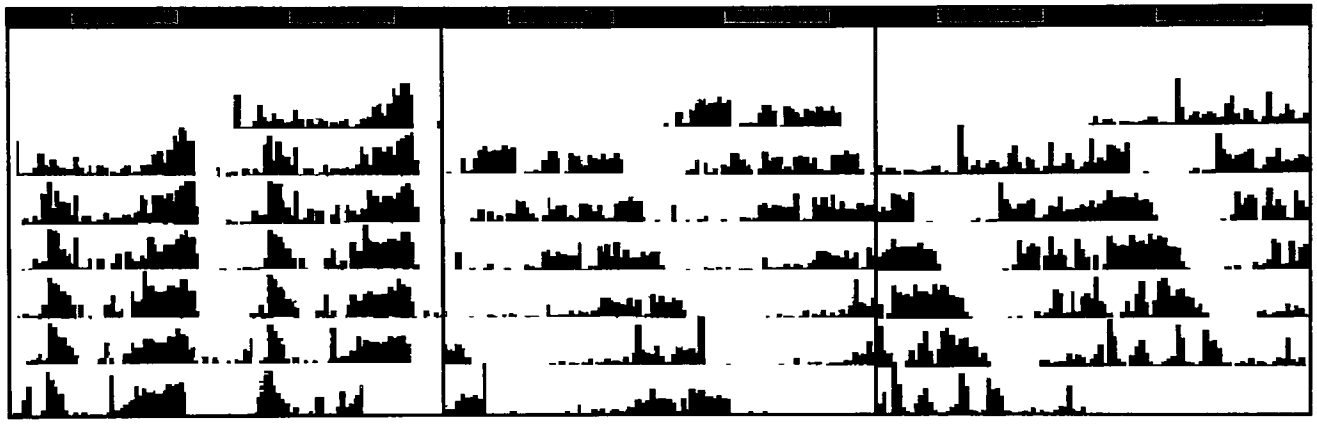


Figure 27: RNA levels from the *period* gene are reduced in flies expressing *perCt-IR*.

(A) RNAse protection assay on total head RNA from flies expressing *perCt-IR* under the control of *gmr*-GAL4 (lanes 3 and 4). As wild type control, RNA from flies carrying *gmr*-GAL4 alone was used (lanes 1 and 2). Flies of the respective genotypes were entrained in a 12 h light: 12 h dark cycle and collected on the 3rd day at time points ZT2 and ZT14. (B) The histogram illustrates relative *period* RNA levels for *perCt-IR*/*gmr*-GAL4 at ZT2 and ZT14 compared to wild type (*gmr*-GAL4/+). For the quantification, the samples of the RNAse protection assay were analyzed by a phosphorimager (Molecular Dynamics). RNA levels were assessed in reference to tubulin RNA and subsequently normalized to *period* RNA levels in *gmr*-GAL4 at ZT14. Each bar represents the average of two independent experiments. (C) RNAse protection assay as in (A). Genotypes are indicated on top of the gel. The bar on top of the gel indicates the light (gray) and the dark portion (black) of the LD cycle. (D) The histogram illustrates the quantified and normalized data from the gel shown in (C) for *perCt-IR B1* (closed boxes). *gmr*-GAL4/+ served as wild type control (closed circles). Numbers below the graph represent time points in LD. (E) Quantified and normalized data from the RNAse protection assay in (C) are shown for *perPAS-IR G2* (closed boxes). Closed circles represent the wild type control.

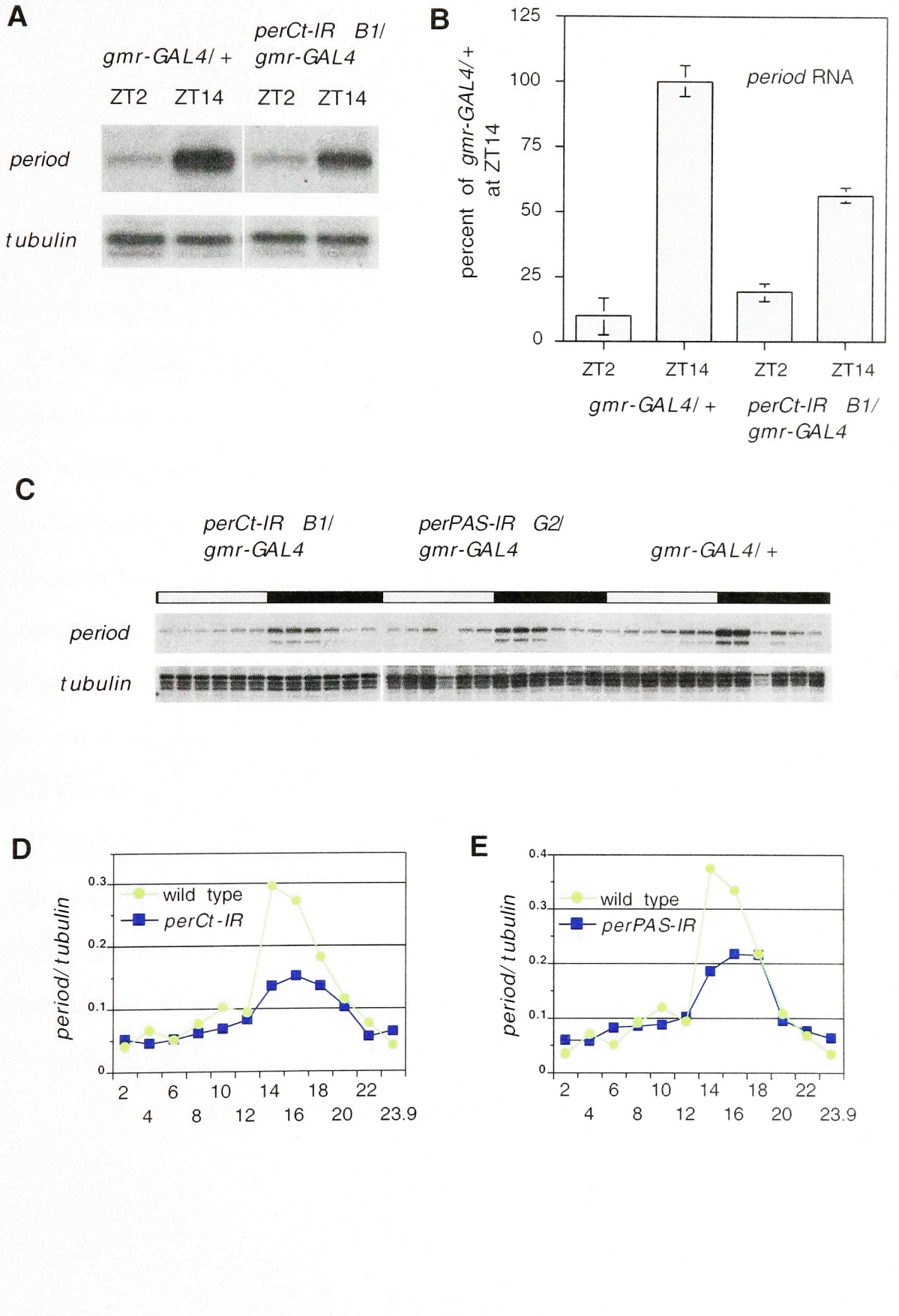
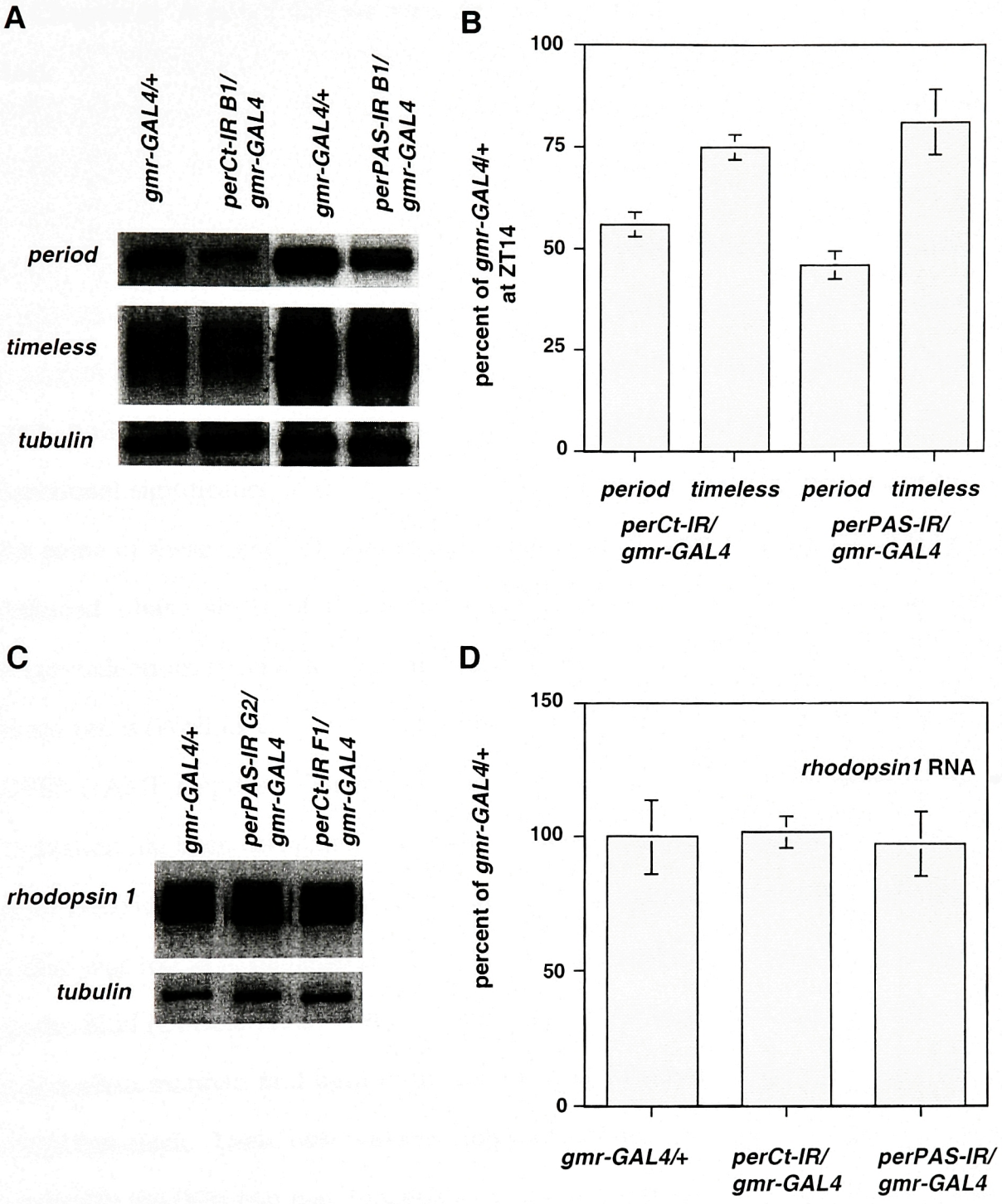

A
gmr-GAL4/+
perCt-IR B1/ gmr-GAL4
ZT2 ZT14 ZT2 ZT14
period
tubulin
B
percent of gmr-GAL4/+ at ZT14
125
100
75
50
25
0
period RNA
ZT2 ZT14 ZT2 ZT14
gmr-GAL4/+
perCt-IR B1/ gmr-GAL4
C
perCt-IR B1/ gmr-GAL4
perPAS-IR G2/ gmr-GAL4
gmr-GAL4/+
period
tubulin
D
period/tubulin
wild type
perCt-IR
0.3
0.2
0.1
0
2 4 6 8 10 12 14 16 18 20 22 23.9
E
period/tubulin
wild type
perPAS-IR
0.4
0.3
0.2
0.1
0
2 4 6 8 10 12 14 16 18 20 22 23.9

Figure 28: Specific inhibition of *period* RNA by *perCt-IR* and *perPAS-IR*.

RNA abundance from the *timeless* gene is closer to its wild type peak at ZT14 than *period* RNA abundance, and *rhodopsin1* RNA levels are not affected in flies expressing *perCt-IR* or *perPAS-IR* under control of *gmr*-GAL4. (A) RNAse protection assay on total head RNA of flies expressing *perCt-IR* and *perPAS-IR* respectively under the control of *gmr*-GAL4. *gmr*-GAL4/+ flies served as wild type control. Flies were collected at ZT14 after 3 days of entrainment. For each construct the result from one individual transgenic line is shown as an example. (B) Relative amounts of *period* and *timeless* RNA in flies expressing *perCt-IR* and *perPAS-IR* respectively at ZT14. RNAse protection analysis was done as in (A). Subsequently, samples were quantified by a phosphorimager and *period* and *timeless* RNA levels were assessed in reference to tubulin and subsequently normalized to the respective RNA level in wild type (*gmr*-GAL4/+) at ZT14. Each bar represents the average of three different transgenic lines for each construct. (C) RNAse protection assay on total head RNA using *rhodopsin1* RNA as a probe. *gmr*-GAL4/+ serves as wild type control. Flies were entrained for 3 days and subsequently collected at ZT14. (D) Relative levels of *rhodopsin1* RNA in flies expressing *perCt-IR* or *perPAS-IR*. The samples of the RNAse protection assay were quantified relative to tubulin RNA levels, and subsequently normalized to *rhodopsin1* RNA levels in wild type (*gmr*-GAL4/+). Each bar represents the average from three independent transgenic lines.


A
gmr-GAL4/+
perCt-IR B1/ gmr-GAL4
gmr-GAL4/+
perPAS-IR B1/ gmr-GAL4
period
timeless
tubulin
B
percent of gmr-GAL4/+ at ZT14
100
75
50
25
0
period
timeless
period
timeless
perCt-IR/ gmr-GAL4
perPAS-IR/ gmr-GAL4
C
gmr-GAL4/+
perPAS-IR G2/ gmr-GAL4
perCt-IR F1/ gmr-GAL4
rhodopsin 1
tubulin
D
percent of gmr-GAL4/+
150
100
50
0
rhodopsin1 RNA
gmr-GAL4/+
perCt-IR/ gmr-GAL4
perPAS-IR/ gmr-GAL4

Chapter 8: A potential role for a *Drosophila* MAP Kinase in the circadian clock

Introduction

A complex network of signal transduction cascades is involved in the light response of the Suprachiasmatic Nucleus (SCN). It has been known for some time that light induces the expression of a set of immediate-early genes, *e.g.* c-Fos and JunB, within the SCN (for a recent example Morris *et al.* 1998). The functional significance of this induction has been elucidated only more recently for some of these genes. For example, Wollnik et. al were able to inhibit light-induced phase shifts of the mammalian clock by administering antisense oligonucleotides specific to c-Fos and JunB, thereby preventing the expression of these genes (Wollnik *et al.* 1995). Phosphorylation of the transcriptional regulator CREB (cAMP response element binding protein) at serine^{133} and presumably its activation has been correlated with light-induced transcription in the SCN (Ginty *et al.* 1993) implicating CREB as a transducer of the light signal. Full activation of CREB was found to be dependent on the light-induced activity of a MAP kinase in the SCN (Obrietan *et al.* 1998). Interestingly, MAP kinase activity oscillates in a circadian manner, and light induction of MAP kinase activity is gated by the circadian clock. These observations suggest that the MAP kinase is under clock control in the SCN and may function as an input component.

The finding that serum shocks induce the circadian expression of various genes in rat fibroblasts (Balsalobre *et al.* 1998) opened a new way of studying the mechanism by which signaling events affect the biological clock. Akashi and Nishida (2000) investigated the molecular basis underlying this serum shock and

were able to demonstrate that MAP kinase activity is involved in the induction of circadian gene expression (Akashi and Nishida 2000). Although this study was performed on mouse fibroblast NIH-3T3 cells, the circadian expression of the mPER1 and mPER2 genes in these cells suggests that this assay reconstitutes events normally taking place in SCN neurons. This observation also supports the notion that MAP kinase activity may be involved in resetting the circadian clock in mammals through its impact on transcriptional activity of circadian gene expression.

Results

Light stimulates MAP kinase activity in *Drosophila*

We were interested in the possibility that the MAP kinase pathway in *Drosophila* has a function similar to circadian rhythmicity in the SCN. As the activation of MAP kinase is mediated by phosphorylation, a phospho-MAP kinase specific antibody, anti-dpERK (Sigma; Gabay *et al.* 1997), can be used to monitor MAP kinase activity *in situ* or in extracts. In order to test whether light stimulates MAP kinase activity, flies were subjected to a 2 min light pulse at ZT17. Protein was extracted from heads of these flies at different times following the light pulse and subjected to Western blot analysis using the anti-dpERK antibody. 15 minutes after onset of the light pulse, dpERK specific immuno-reactivity is elevated demonstrating an increase of activated MAP kinase (Figure 29A). 25 min after the onset of the light pulse, however, MAP kinase activity has began to decline. In order to test whether this response is specific to brief stimuli of light or can be maintained for longer times, MAP kinase activity was investigated in an LD cycle. As shown in Figure 29B, MAP kinase activity is

higher during the light part demonstrating that elevated MAP kinase activity can be maintained during longer exposures to light. To test whether cycling MAP kinase activity is under circadian control, MAP kinase activity was assayed under constant conditions. The oscillation of MAP kinase activity was abolished in DD (data not shown) suggesting that MAP kinase cycling in LD is solely a response to environmental conditions.

The blue light receptor CRYPTOCHROME is an important component of the entrainment pathway in flies (Stanewsky *et al.* 1998; Emery *et al.* 1998; Emery *et al.* 2000a; Emery *et al.* 2000b). To investigate whether the light response of MAP kinase activity is contingent upon proper functioning of the light receptor CRYPTOCHROME, MAP kinase activation in the *cryptochrome*^{b} mutant was investigated. The lack of functional CRYPTOCHROME failed to abolish activation of MAP kinase in response to light (data not shown). Presumably, visual light reception or another unknown light receptor is sufficient to trigger MAP kinase activation.

The finding that MAP kinase activity is increased in response to light is reminiscent of the scenario in the SCN. However, MAP kinase activity in flies did not appear to oscillate under constant environmental conditions nor was the response to light pulses gated by the circadian clock (data not shown). This finding is another example of the differences between the two systems.

A mutation in the gene encoding the *Drosophila* MAP kinase alters phase responses to photic stimuli

To test the functional significance of the induction of MAP kinase activity upon light pulses, effects of reducing MAP kinase activity on circadian rhythms

were examined. Due to its essential function in development, strong mutations in the *rolled* gene, which encodes a *Drosophila* MAP kinase are lethal (Biggs III *et al.* 1994). A mild, homozygous viable mutation, *rolled*^{1}, was therefore employed in this study. The light induced increase in MAP kinase activity suggests a function for *rolled* in the input pathway. To test a possible defect in the input pathway in *rolled*^{1} mutant flies, the phase response of these flies to photic stimuli (PRC) was measured. The time points for the PRC were chosen such that the maximum responses would be observed. As shown in Figure 30A, the maximal phase delay and the maximal phase advance are increased in *rolled*^{1}. As *rolled*^{1} has been shown in other systems to be a mild hypomorphic mutation the increased phase shift suggests a negative regulatory role for the MAP kinase in the resetting of the circadian clock in *Drosophila*. This result deviates from what would have been expected from the putative role of the MAP kinase in the SCN where it appears to function as a positive regulator of circadian gene expression.

Light induced MAP kinase activation is dependent upon proper cAMP metabolism

If MAP kinase activity is a regulator of an entrainment pathway, then it must be part of a signal transduction pathway that connects photoreception with TIMELESS degradation. Because pleiotropic functions of MAP kinase activity impede a behavioral analysis, other components in this entrainment pathway may provide more detailed insight into its mechanism. Mutations in genes of the same signaling pathway often cause related phenotypes, thus, all mutants whose PRC is similar to that of *rolled*^{1} are candidates for components that act in concert with MAP kinase activity in an entrainment pathway. Lethality associated with

components of the canonical MAP kinase pathway prevented testing of their effects on entrainment. Interestingly, the *dunce*^{1} mutation caused a similar increase in the phase delay upon light pulses (Figure 30B; Levine *et al.* 1994). The *dunce* gene, which is probably best known for its role in learning and behavior, encodes a cAMP specific phosphodiesterase (Nighorn *et al.* 1991). Consistently, previous studies have implicated cAMP metabolism in the entrainment mechanism of the SCN (Prosser and Gillette 1989; Prosser and Gillette 1991). To investigate a possible functional connection between cAMP metabolism and MAP kinase activity in the entrainment pathway, light induced MAP kinase activation was tested in a *dunce*^{1} mutant background. As shown in Figure 31, light stimulation of MAP kinase activity is reduced in *dunce*^{1}. Additionally, differential MAP kinase activity during an LD cycle is absent in a *dunce*^{1} mutant background (data not shown). These observations indicate that *dunce* plays a positive regulatory role in the light induced activation of the MAP kinase. However, it is unclear whether *dunce*'s role in the entrainment pathway acts through *rolled* activation or whether these are two separate aspects of *dunce* function.

Discussion

An inherent difficulty in establishing a role for the MAP kinase pathway in a behavioral system is the broad spectrum of essential functions of this pathway. The problem is twofold. (A) Various genetic techniques have to be employed in order to circumvent problems with viability. Otherwise only weak alleles that may not uncover all aspects of MAP kinase function can be used for a behavioral assay. Additionally, a behavioral phenotype may also be caused by

differentiation defects of the neurons involved in this particular behavior. However, *in vitro* systems offer the opportunity to administer temporarily specific inhibitors and activators of the MAP kinase as illustrated by the advances made in the understanding of the MAP kinase in the induction of circadian gene expression (see introduction of this chapter, Akashi and Nishida 2000). (B) The global involvement of the MAP kinase pathway in cellular signaling events may prevent the investigation of one specific aspect of MAP kinase function in the system of interest, since a network of signaling events may depend on MAP kinase function at several positions.

These difficulties notwithstanding, application of the anti-dpERK antibody can demonstrate a correlation between MAP kinase activation and the process of interest. Under well controlled conditions this can be strong evidence for a role of the MAP kinase pathway in a given process. The correlation between MAP kinase activation and phase shifts as a response to photic stimuli is suggestive but not conclusive since MAP kinase activity may as well be involved in other light related processes. For example, light may trigger clock-unrelated physiological changes in the eye necessary for vision. Alternatively, MAP kinase activation may be involved in the fly's startle response to light. However, the abnormalities observed in the PRC of the *rolled*^{1} mutant flies indicate that the MAP kinase can influence circadian behavior. It should be pointed out that the interpretation of the data is complicated since the parental strain of the *rolled*^{1} mutant was not available to us as a wild type control. It is therefore possible that the phenotype is not caused by *rolled*^{1} but by other mutation(s) in the genome of the strain.

Another way of revealing functions of individual protein components in a genetic system are genetic interactions. The gene activity of one component in a given pathway can be altered, *e.g.* reduced, such that any further alteration of the activity of this pathway would result in an enhancement or suppression of the associated phenotype. In such a sensitized genetic background, a change in gene activity of other components of this pathway by 50% or even less may cause a visible modification of the phenotype. Such a genetic interaction had been observed between the MAP kinase pathway and the *Neurofibromin-1* gene in *Drosophila* (Williams *et al.* 1999). NEUROFIBROMIN-1 is a guanosine triphosphatase-activating protein that has been implicated in the regulation of cAMP metabolism in *Drosophila* (Guo *et al.* 1997; The *et al.* 1997). Flies homozygous mutant for *Neurofibromin-1*^{P1} are viable but lack circadian rhythmicity of locomotor activity, which provides clear evidence for an essential role of *Neurofibromin-1* in the regulation of circadian behavior. The *Neurofibromin-1*^{P1} conferred arrhythmia can be suppressed by loss of function mutations in the Ras/MAP kinase pathway and by expression of a constitutively active catalytic subunit of the cAMP-dependent protein kinase (PKA). These observations place all three signaling molecules (NEUROFIBROMIN-1, MAP kinase, and PKA) in a genetic pathway that presumably partakes in the self-sustained circadian oscillator. Although the results described here also implicate MAP kinase activity in the regulation of circadian behavior, they rather suggest a role of the MAP kinase in the input pathway than a role in the oscillator itself. The period length of *rolled*^{1} mutant flies was indistinguishable from wild type, and MAP kinase activity was induced by light but failed to cycle in DD. However, *rolled*^{1} is a weak allele and probably fails to uncover all clock-related

functions of this MAP kinase. Thus, it is likely that MAP kinase is involved in the input as well as in the self-sustained oscillator.

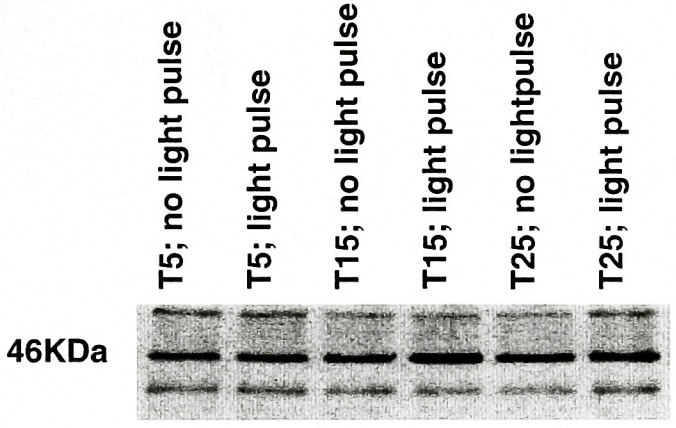
Since *Neurofibromin-1*, which regulates cAMP metabolism, and components of the MAP kinase pathway interact genetically and MAP kinase activation can depend on proper function of the phosphodiesterase, DUNCE, clock-related aspects of MAP kinase function may depend on cAMP levels. A similar molecular scenario has been suggested for long-term facilitation in *Aplysia* (Martin *et al.* 1997). However, the available data are not sufficient to decide whether cAMP levels regulate MAP kinase activity directly. Alternatively, cAMP signaling and MAP kinase signaling could converge at a component further downstream. A candidate is CREB, which may be regulated by both PKA as well as MAP kinase. Consistently, the *dCREB2* gene has been shown to partake in circadian gene expression (Belvin *et al.* 1999).

The data suggest that MAP kinase activity is part of a complex circadian signaling network. The investigation of this network is hindered by pleiotropic functions of some of its constituent protein components. However, genes like *Neurofibromin-1* may be specific to the regulation of the circadian aspect of MAP kinase function thereby providing an entry point in the molecular characterization of this network.

Figure 29: Light stimulates *Drosophila* MAP kinase (*rolled*) activation.

(A) Wild type flies were entrained for 3 consecutive days in an LD cycle and subjected to a 2 min light pulse at ZT17 of the last day. Flies were collected at the indicated times, "T" is the time after the onset of the light pulse in minutes. Protein extracts from heads were subsequently subjected to Western blot analysis using the anti-dpERK (Sigma) antibody. Unpulsed flies were used as negative control. (B) Wild type flies were entrained as in A and collected at the indicated times of an LD cycle (ZT0 is lights on). Proteins were extracted from heads and subjected to Western blot analysis as in A. Note the higher intensity of the signal at times when the light is on (ZT4, 8, and 12).

A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T5; light pulse
T15; no light pulse
T15; light pulse
T25; no lightpulse
T25; light pulse
46KDa

B

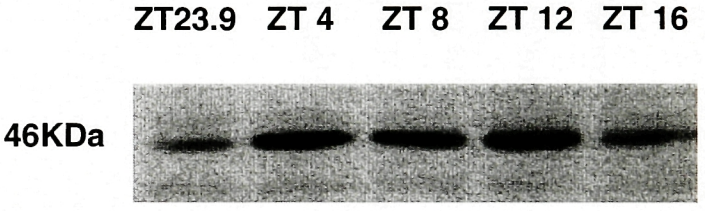
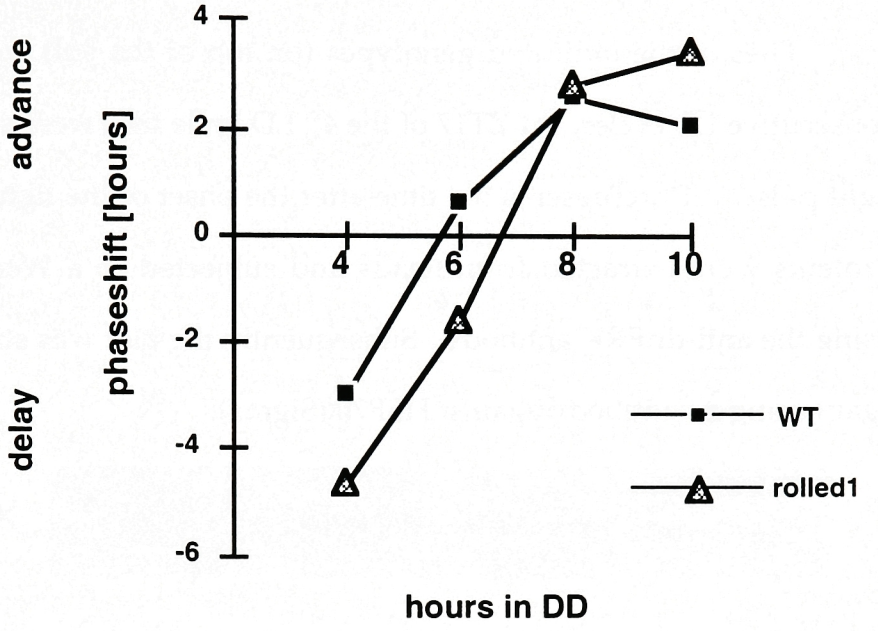

ZT23.9
ZT 4
ZT 8
ZT 12
ZT 16
46KDa

Figure 30: Altered phase response to photic stimuli in the MAP kinase mutant *rolled*^{1}.

Flies were entrained for 3 consecutive LD cycles and subjected to 10 minute light pulses in the night of the 4th day. The phase response to these light pulses was plotted against the time at which they were administered. Hours in DD indicate the time after lights off. Data for wild type are represented by solid squares and data for *rolled*^{1} by triangles. (B) Phase response curve for the mutant *rolled*^{1} from Levine *et al.* 1994.

A


advance
delay
phaseshift [hours]
4
2
0
-2
-4
-6
4
6
8
10
WT
rolled1
hours in DD

B

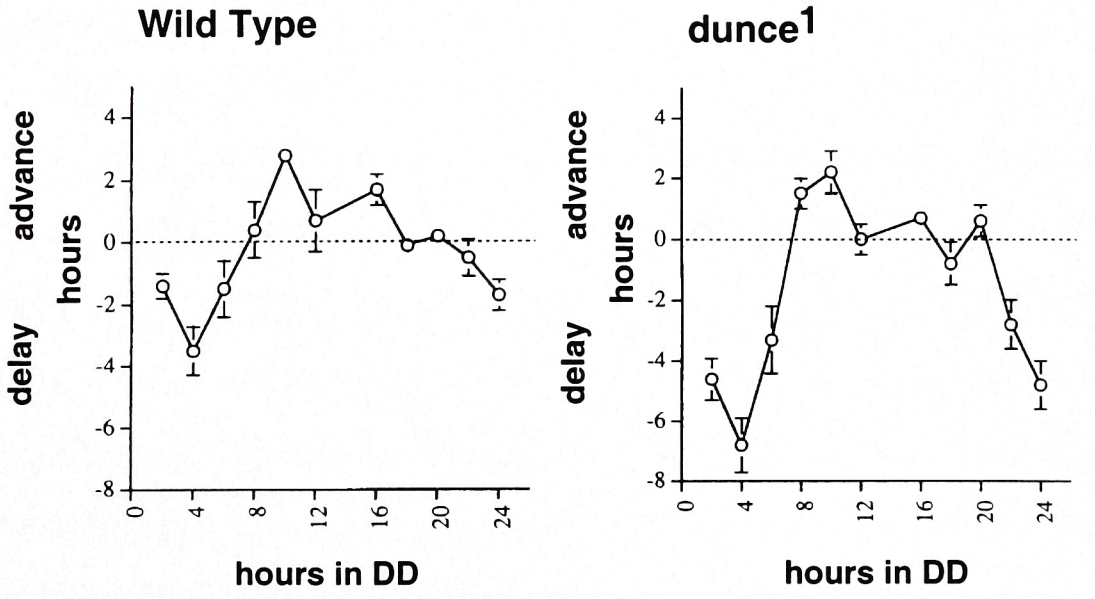
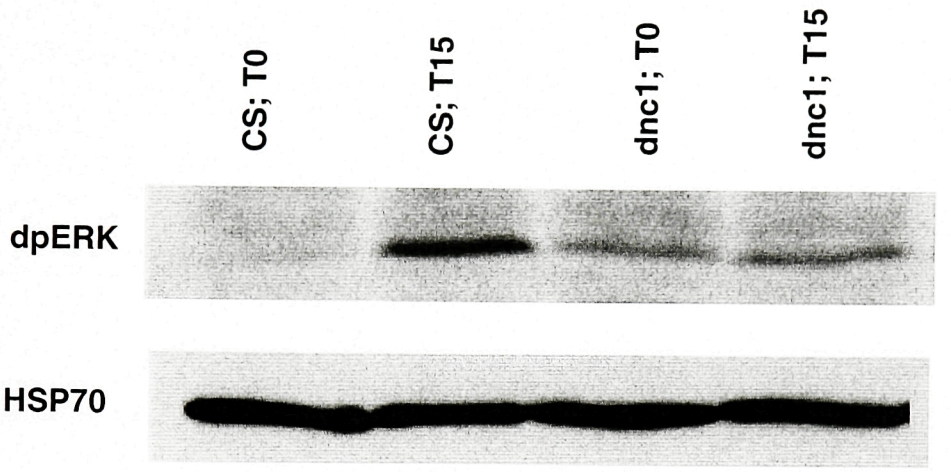

Wild Type
dunce1
advance
delay
hours
4
2
0
-2
-4
-6
-8
0
4
8
12
16
20
24
hours in DD

Figure 31: Light induced MAP kinase activation is reduced in *dunce*^{1}.

Flies of the indicated genotypes (on top of the gel) were entrained for 3 consecutive LD cycles. At ZT17 of the 4th LD cycle flies were subjected to a 2 min light pulse. "T" represents the time after the onset of the light pulse in minutes. Proteins were extracted from heads and subjected to a Western blot analysis using the anti-dpERK antibody. Subsequently the blot was stripped and probed again using an antibody against HSP70 (Sigma).


CS; T0
CS; T15
dnc1; T0
dnc1; T15
dpERK
HSP70

Chapter 9: Discussion and Perspectives

As described in the introduction, unanswered questions regarding circadian behavior suggest the existence of additional clock-related genes. The goal of this study was therefore to identify new components regulating circadian behavior in *Drosophila.* However, it is very likely that most if not all regulators of circadian behavior amenable to classical genetic analysis have already been identified. This is suggested by the fact that screening in different organisms yielded orthologs of the same set of genes. If there were many more genes involved in clock function and accessible to classical mutagenesis, such mutagenesis screens should have identified mutations of different genes. Thus, the function of additional clock-related genes may be masked by their earlier role in development or by redundancy. Alternative approaches are therefore necessary to identify such genes.

To this end, an over-expression screen for abnormal circadian locomotor activity was conducted. As discussed in chapter 4, this screen yielded several new loci. While a *bona fide* clock function has yet to be established for some of the identified genes, the data strongly suggest that *shaggy* is a crucial regulator of the circadian oscillator in flies, and this final chapter is devoted to a further consideration of the function of this gene in circadian rhythmicity. SHAGGY may regulate the nuclear translocation of the PERIOD/TIMELESS heterodimer through its effect on TIMELESS phosphorylation (Figure 32). Although it has been known for some time that TIMELESS and PERIOD heterodimerization is essential for nuclear translocation (Saez and Young 1996; Vosshall *et al.* 1994), the precise regulation of this step is still unclear. As discussed below, *shaggy* may shed light on the regulation of nuclear translocation of TIMELESS and PERIOD.

It has been hypothesized that TIMELESS and PERIOD accumulate in the cytoplasm until a threshold concentration is exceeded, which would allow TIMELESS and PERIOD to enter the nucleus. Interestingly, deletion of the cytoplasmic localization domain (CLD) of PERIOD or TIMELESS obviates the requirement for dimerization and allows nuclear translocation of TIMELESS and PERIOD individually (Saez and Young 1996). This observation prompted Saez and colleagues to propose a model, in which heterodimerization masks the CLD, thus allowing nuclear entry of the heterodimer. Implicit in this model is that protein accumulation in the cytoplasm precedes heterodimerization, and only once the critical threshold is reached will heterodimerization occur. This could be tested by determining the precise timing of complex formation *in vivo* using fluorescence resonance energy transfer (reviewed in Da 1999).

Immunohistochemical studies on the timing of the nuclear translocation showed that all of the accumulated cytoplasmic protein enters the nucleus within a relatively short period of time (Curtin *et al.* 1995). Remarkably, protein that is translated after the initial nuclear transfer enters the nucleus without prior cytoplasmic accumulation. TIMELESS and PERIOD levels peak several hours after the initial nuclear entry, a substantial portion of protein heterodimerizes and enters the nucleus immediately after translation without prior cytoplasmic accumulation. This suggests that after the initial nuclear translocation something must change, *i.e.* the system must have a memory. This is further supported by the following thought. If protein concentrations were the sole explanation for the delayed nuclear entry, it would be expected that only the amount of protein that is in excess to the threshold concentration enters the nucleus and not the majority of the available protein. Thus, a mechanism must exist that differentiates

between the initial and the subsequent nuclear translocation of PERIOD/TIMELESS. One could envision a model in which the gating of nuclear entry itself is under circadian control.

Although circadian control of SHAGGY has not been demonstrated yet, *shaggy* function could be involved in the circadian gating of nuclear entry. Once the critical protein concentration is reached, SHAGGY mediated phosphorylation of TIMELESS could become temporarily up-regulated, and alleviation of cytoplasmic retention could ensue. This may occur either by increasing the affinity of phosphorylated TIMELESS for PERIOD or by a direct effect of TIMELESS' phosphorylation state on the nuclear transfer. SHAGGY activity may remain elevated until all of the current cycle's protein has entered the nucleus. Subsequently, SHAGGY activity may decline, thus preventing immediate nuclear entry in the next cycle. The nature of a potential circadian regulation of SHAGGY is highly speculative. One could envision a mechanism that depends on the formation of complexes including TIMELESS and PERIOD. For the initial formation of this complex, TIMELESS and PERIOD levels need to reach a threshold concentration. The complex may recruit and activate an additional component that up-regulates SHAGGY. Once activated, SHAGGY may remain up-regulated for several hours and its activity may decline only after the completion of one cycle. Implicit in this idea is a physical contact between the TIMELESS/PERIOD complex and SHAGGY. However, immunoprecipation experiments failed to detect a physical contact between SHAGGY and any of the other known clock components. Although this may be due to insufficient sensitivity of the experimental approach, it suggests that SHAGGY is not part of a permanent complex with PERIOD and TIMELESS.

Naturally, examining the daily pattern of SHAGGY activity would be the first step to test this model. A possible biochemical test of oscillating SHAGGY activity may be confounded by the ubiquitous expression of *shaggy* and the plurality of SHAGGY isoforms. SHAGGY in tissues other than the ones with a functional clock as well as non-oscillating isoforms of SHAGGY will probably mask any possible oscillating activity. However, SHAGGY is a highly regulated enzyme in other systems (reviewed in Harwood 2000) and may therefore also be regulated in its role as a clock component. The kinase activity of GSK-3 is known to be regulated by phosphorylation at different residues. Inhibition of GSK-3 by protein kinase B mediated phosphorylation, for example, is an important step in the down-regulation of glycogen synthase in response to insulin (reviewed in Harwood 2000). Thus, Phospho-SHAGGY specific antibodies may furnish a tool to monitor SHAGGY activity *in situ*.

Another approach to investigate the control of SHAGGY activity in the circadian oscillator would be to ask whether protein components that are known to regulate SHAGGY in other systems also impinge on circadian behavior. In preliminary experiments it was found that over-expression of *dishevelled* in LNs lengthens the circadian period of locomotor activity (L. Saez, unpublished observation). Consistently, *dishevelled* has been known to down-regulate SHAGGY activity upon activation by *wingless*. However, attempts to establish that loss of *dishevelled* function affects circadian behavior have not been successful. Moreover, testing of a temperature sensitive allele of *wingless* also failed to reveal abnormal circadian behavior. Thus, circadian regulation of SHAGGY may involve components different from the *wingless* signal transducers.

Traditionally, GSK-3 has been viewed as a negative regulator whose basal activity ceases upon activation of a given signaling event. However, accumulating evidence shows that GSK-3 can also act as positive regulatory element. In *Dictostelium*, for example, activation of the dual specificity kinase ZAK1 stimulates GSK-3 activity, possibly through direct phosphorylation, which in turn promotes morphogenesis of the unicellular amoeba to a multicellular structure (Leung *et al.* 1999). If GSK-3 does indeed regulate circadian behavior in a way similar to the one we envision, then this would be yet another example for the complexity of GSK-3's regulation and action.

Our study falls short of determining the phenotype of complete loss of *shaggy* function. Although *shaggy*'s potential role in cell viability may prevent the behavioral analysis of a complete loss function, further reduction of *shaggy* function could be achieved by tissue-specific gene interference. For example, the dominant negative construct or *shaggy* specific inverted repeats could be expressed under the control of *timeless*-GAL4 in the "heat-shock-rescued" *shaggy* mutant. Alternatively, the potential GSK-3 sites in TIMELESS could be removed, and the ability of this GSK-3 site deficient *timeless* transgene to rescue *timeless*^{0} could be tested. If the entire effect of *shaggy* on the circadian oscillator is based on direct phosphorylation of TIMELESS through SHAGGY, substituting the respective sites should eliminate *shaggy*'s effect on the molecular oscillator. The depicted model would predict that the nuclear localization of such a recombinant TIMELESS molecule would be substantially delayed or even fail to occur entirely.

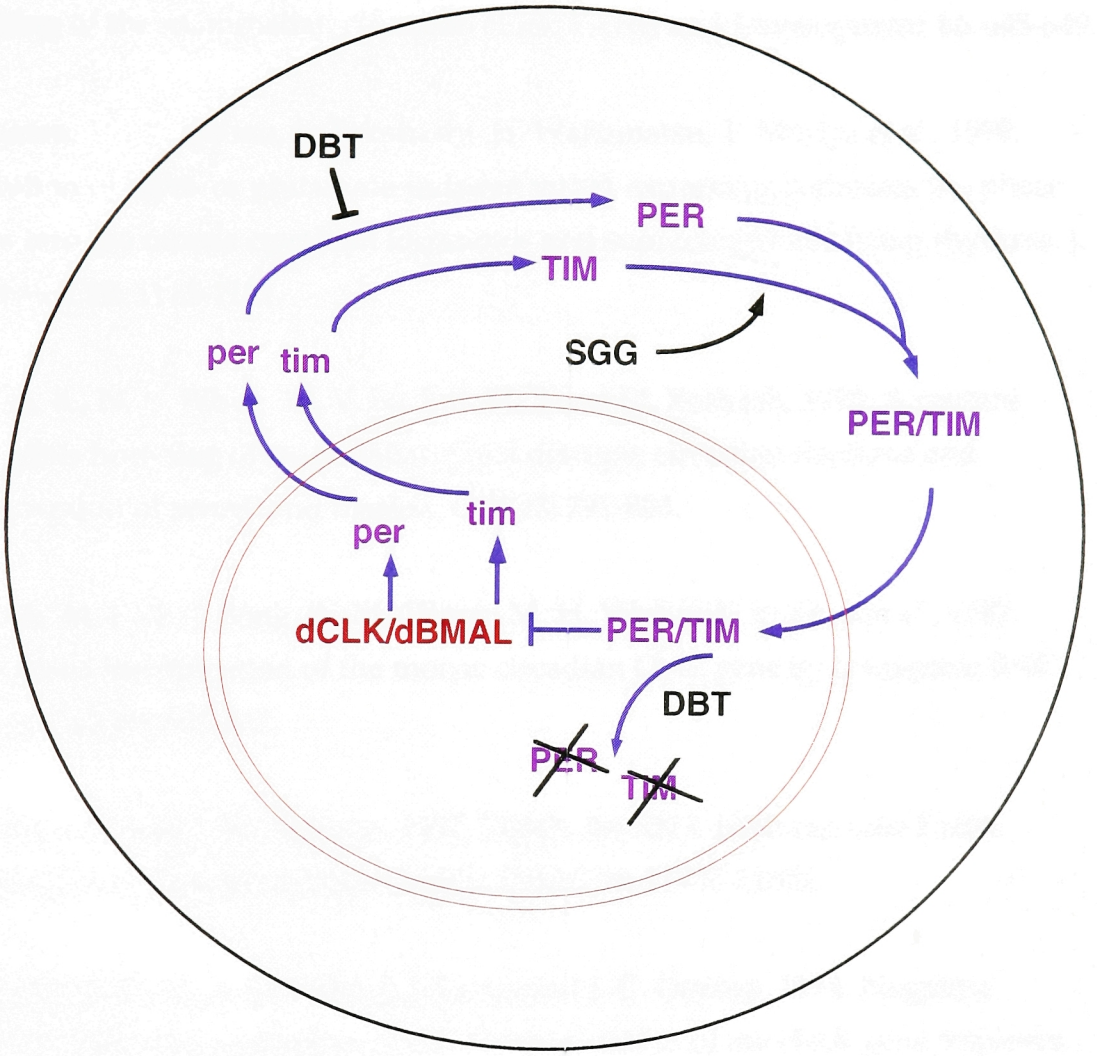
It would be very interesting to see whether the circadian function of GSK-3 is conserved among species. Generating a mouse model with elevated GSK-3

levels in the SCN should be a first step towards the answer to this question. An additional interesting aspect of investigating GSK-3's role in the mammalian oscillator is that it may shed light on the function of the mammalian *timeless* homolog. As described in the introduction, a circadian function of a mammalian *timeless* homolog still remains to be established. However, if GSK-3 impinges on the circadian oscillator in mammals through its effect on TIMELESS phosphorylation, interfering with wild type GSK-3 function should reveal the role of mammalian TIMELESS.

The investigation of *shaggy*'s role in the circadian oscillator is another example for pleiotropic gene functions in animal behavior. As was pointed out in the introduction, several aspects of circadian regulation of behavior still await elucidation and probably depend on the function of components that have yet to be implicated in circadian rhythmicity. It is likely that the function of other regulators of circadian rhythmicity has been masked either by pleiotropic functions in development/viability or by redundancy. Future molecular approaches, such as micro-arrays (Spellman *et al.* 1998; White *et al.* 1999), and additional over-expression screens may obviate these problems and lead to the isolation of protein components that further our understanding of circadian behavior.

Figure 32: SGG's role in the circadian oscillator.

Schematic model for the circadian clock mechanism in *Drosophila*. Names of proteins are in uppercase, names of genes and RNAs are in lowercase. Arrows represent a positive regulation, bars at the end of lines represent a negative regulation, and the two concentric circles in the middle of the diagram represent the nuclear envelop. For a description of the model see Figure 1 and text. Based on the data presented in this thesis, SHAGGY has been added to this model. SHAGGY impinges on the molecular oscillator through its effect on the phosphorylation pattern of TIM. Abbreviations are: per - *period*; tim *timeless*; dbt *double-time*; dclk - *dclock*; dbmal *dBmal*; SGG – SHAGGY.


DBT
PER
TIM
per
tim
SGG
PER/TIM
tim
per
dCLK/dBMAL
PER/TIM
DBT
PER
TIM

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