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Non-mammalian genetic model systems in sleep research

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Introduction

Among the most notable advances to the sleep research field in the past decade has been the addition of several new model systems that are amenable to genetic analysis. These include the fruit fly *Drosophila melanogaster*, the round worm *Caenorhabditis elegans*, and the Zebrafish *Danio rerio*. Though this field is young, research in these model systems has already yielded important contributions into our understanding of the regulation and function of sleep and sleep-like states. Looking forward, these model systems hold promise to contribute for many years to the discovery of new sleep regulators, to understanding the function of genes discovered in human genetic research, and to testing hypotheses regarding the function of sleep.

There are three principal uses of genetic model systems relevant to human sleep disorders: discovery of new genes, testing the *in vivo* function of gene variants associated with human sleep traits, and testing of hypotheses regarding the function of sleep.

The goal of this chapter is to review the chief utilities of non-mammalian model system in the study of sleep. The criteria for sleep or a sleep-like state have been extensively reviewed (1, 2) as have the experiments supporting the idea that sleep is fundamentally conserved in phylogeny (1, 3–6). We will therefore start with the premise that sleep can be studied in any of the key non-mammalian model genetic systems including *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Danio rerio*.

Gene Discovery

The oldest use of genetic model systems has been to discover genes that function in processes of interest. Such functions are often then shown to be conserved in mammalian systems. The process of finding new genes is often referred to as “forward genetics”, where one screens for a phenotype of interest following random mutagenesis of the genome. Forward genetics is hypothesis-independent in that one has no a priori bias as to which genes will mutate to cause the phenotype of interest. The power of such an approach is that it can lead to novel and unexpected insights. An example of the utility of forward genetics is

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illustrated from genetic research in the circadian field. Here, nearly all of the components that function in the circadian clock have been discovered using forward genetics in model systems, primarily in *Drosophila*. Most of these genes have clear mammalian homologues that have been shown to also function in the mammalian clock. The clinical utility of this approach is demonstrated by the work of Jones, Ptacek, Fu, and colleagues who have found that human families with familial advanced sleep phase syndrome or FASPS have mutations in these same clock components (7, 8).

The use of forward genetics to identify non-circadian sleep regulators has been undertaken in *Drosophila*. Such screening has identified the genes *shaker* and *sleepless* as key genes promoting sleep. *shaker* encodes a voltage gated potassium channel (9) whereas *sleepless* encodes a GPI-linked protein that regulates expression and function of Shaker ion channels (10, 11). *Shaker* and *sleepless* mutants were the first mutants studied because of their extreme mutant phenotypes, i.e., very short sleep, which facilitated their cloning and characterization. Several other short sleeping mutants have been identified among a collection of second chromosome mutants but the molecular basis of these mutants remains unknown (12). Short sleepers are the only phenotypes that have been reported in screens to date. Future research should include analysis of mutants with long sleep and with abnormal sleep architecture.

The forward genetics approach must deal with the issue of pleiotropy and redundancy. Pleiotropy refers to the idea that more than one phenotype is caused by a particular genetic perturbation. A gene may be involved in the same molecular process in different cell types; it may be involved in multiple molecular processes; or the molecular process to which the gene contributes may be important for normal development in addition to adult behavior. Phenotypes that are pleiotropic may preclude analysis of sleep, which in non-mammalian species requires that the animal be able to move normally. There are a number of ways to address the issue of pleiotropy including conditional activation or inactivation of a gene product to circumvent developmental lethality (13) and the use of tissue-specific RNA interference (14) or somatic recombination (15, 16) to limit potentially deleterious gene expression to specific tissues. RNA interference and conditional activation can be combined to great effect to deduce the sleep function of a gene within specific tissues (see examples in Table 1).

Redundancy in genetic analysis refers to the idea that one or more genes have over-lapping functions in the process of interest such that removing only one gene has no discernible phenotype. To get around redundancy one can mutate both genes functioning in a given process. One of the strengths of using forward genetic approaches in *Drosophila* or *C. elegans* from a perspective of eluding redundancy issues is that the fly and worm genome is less duplicated than vertebrate genomes, i.e. it is more likely that a single gene will fulfill a function that is fulfilled by more than one homologous vertebrate genes (17, 18). For examples, whereas there are three genes encoding the protein PERIOD in mammals, there is only one in *Drosophila*, and whereas there are four genes encoding EGF receptors in mammals, there is only one in both *Drosophila* and *C. elegans*.

Another approach to the issue of redundancy and pleiotropy is to over-express rather than knockdown a gene of interest. An example of this approach is illustrated by studies of the EGF signaling pathway in *C. elegans*. The *C. elegans* genome encodes only one EGF ligand, called LIN-3, and only one EGF receptor, called LET-23. Animals with complete loss of function of either *lin-3* or *let-23* die due to the involvement of these gene products in signaling events controlling development. To get around this pleiotropy, Van Buskirk and Sternberg over-expressed the *lin-3* gene product to induce behavioral quiescence (19). They

then showed small but significant effects of partial loss of function mutations in LET-23/EGF on the natural sleep-like lethargus period.

A gene discovery approach that is driven by molecular rather than behavioral phenotypes entails the use of mRNA expression profiling to identify either individual genes or patterns of gene that change expression in association with behavioral state. This approach has been used to identify candidate genes that might regulate sleep. The function of these genes in regulating sleep is then tested with targeted genetic experiments. Examples of this approach include the analysis of arylalkylamine *N*-acetyltransferase (20), the analysis of the heat-shock protein 70 endoplasmic reticulum chaperone BiP (20, 21), and the analysis of the nuclear factor Kappa-B homologue relish (22).

An approach that combines unbiased phenotypic analysis with gene expression studies was recently demonstrated in *Drosophila*. The approach was one of quantitative trait loci (QTL) analysis, where one can simultaneously assess the contribution of all genes in the genome to a quantitative phenotype of interest. The combination of phenotypic assessment of 40 wild-derived *Drosophila* lines with gene expression profiles of these lines allowed the authors to identify genes whose expression correlated with sleep phenotypes (23). As in the cases above, they then turned to a targeted approach for manipulating the function of one of these genes. Using this approach, they showed that mutations of each of three genes—*bicoid-interacting protein 3*, *Tetraspanin 42Ef*, and *AKT1*—in an otherwise isogenic genetic background conferred a sleep phenotype as predicted by the QTL analysis.

Syndecan, a trans-membrane protein that regulates metabolism in the fly, was also identified in the sleep QTL analysis (23) and demonstrates a link between reduced energy metabolism and increased sleep (24). This result obtained in *Drosophila* may be relevant to human metabolism and sleep; a minor allele in a single nucleotide polymorphism (SNP) of the human homologue of the *syndecan* SDC4 is associated with higher resting energy expenditures and short sleep duration (24).

In the fields of *C. elegans* and *D. rerio*, large scale forward genetic screens for sleep phenotypes have not yet been reported. In zebrafish, an alternative to a genetic screen was recently described, where the authors screened a chemical library for compounds affecting sleep (25). This novel approach led to the appreciation of novel signaling pathways regulating sleep. We anticipate that in the future screens of chemical libraries will be applied both to *Drosophila* and *C. elegans*.

In-depth characterization of gene function

The opposite approach to forward genetics is reverse genetics. Here, the function of a specific gene is perturbed in a very pointed hypothesis-driven way. The advantage of this approach is that one can delve into greater depth in characterizing the phenotype of the animals with the mutated gene as one has a hypothesis that predicts specific behavioral outcomes. This way, more subtle phenotypes that would not be identified in a random mutagenesis, can be uncovered. There are a number of examples of using model systems to study the function of specific genes in sleep regulation. Examples include the study of dopamine transporter *Drosophila* mutants (26), the study of ecdysone signaling in fruit flies (27), the study of octopamine neurotransmission (28, 29), Epidermal growth factor signaling in both flies (30) and worms (19), and cAMP signaling in both flies (31) and worms (32). Table 1 summarizes insights gained from both forward genetics and reverse genetics approaches in non-mammalian model systems.

One of the most powerful uses of non-mammalian model organisms is in testing the in vivo significance of genes whose function in regulating sleep was first suggested based on

humans genetic studies. Again, this approach was initially illustrated in the circadian field. After identification of families with Familial Advanced Sleep Phase Syndrome bearing mutations in components of the circadian clock that were first identified in model organisms, the identified mutations were introduced into two model systems, the mouse and the fruit fly, in order to prove that the gene variants that had been identified were causal to the phenotype of interest (mutation of casein kinase 1 delta (8); and mutation of PER2 (33)). Hence, that work is a beautiful example of coming full circle (fig 1): Genes of interest are first identified in the model organism through phenotype-driven forward genetics research; variants of these genes are then identified in humans displaying particular phenotypes of interest; and finally these gene variants are placed back into the model system for in depth phenotypic characterization. As shown in fig 1, human gene variants can be identified by approaches that include genetic linkage, genome wide association studies, and whole genome sequencing.

Another recent example illustrates the power of the approach that is outlined in Figure 1. Two members of a family with a naturally short sleep phenotype were found to harbor a mutation resulting in an amino acid change in DEC2, a protein which was previously characterized as a component of the circadian clock (34) but which had never been shown to affect sleep. The authors introduced the *dec2* gene with the human variant into both mice and fruit flies to show that that variant indeed can cause a short sleep phenotype (35). In the absence of this demonstration of causality using model organisms, it would be very difficult to prove that the variant they identified is contributing to the human sleep phenotype.

Another example of the use of non-mammalian systems in understanding genes that function in human sleep is illustrated by the work on hypocretin. Hypocretin signaling was first identified by convergent lines of work in mice (36) and in dogs (37) and later shown to be central to the pathogenesis of narcolepsy (38). But the first insight into the understanding the primordial role of hypocretin in regulating vertebrate behavioral states was gained from work in zebrafish. The fish system has the advantages of a simpler yet similar neuroanatomy to that of mammals, and with respect to the hypocretin signaling system, has the added advantage of only one hypocretin receptor (mammals have 2 receptors). Prober and colleagues showed that over-expression of hypocretin leads to hyperactivity and reduced sleep at a time (lights off) when the animals are normally quiescent (39). Yokogawa and colleagues showed that deletion of the single hypocretin receptor resulted in behavioral state instability during the fish's normal sleep time (40). This work then established an ancient role for hypocretin both in promoting arousal and in stabilizing behavioral states.

While a potential disadvantage of zebrafish is the difficulty of performing traditional electrophysiological recordings from live animals, a recent technological advance has made this apparent disadvantage far less of a concern (41). These authors expressed in hypocretinergic neurons a protein genetically engineered to emit light when bound to calcium. Because zebrafish are small and relatively transparent, light emitted by deep brain tissue can be recorded outside the animal. They were thus able to follow the physiological activity of these neurons in awake, unrestrained, behaving animals. This method, which can be used to monitor the activity of any neuron cell group for which there is a specific promoter, has the advantage over traditional electrophysiology by being noninvasive and by simultaneous monitoring of all cells in a functional group. The same method has been used in *Drosophila* (42), though it has not yet been used for the study of sleep.

Testing functions of sleep

Recent years have witnessed an increasing appreciation of the effects of acute total sleep deprivation and of chronic partial sleep restriction on human disease. The question of the

function of sleep is therefore no longer purely an academic one and has tremendous implications both to public policy particularly in the occupational health arena and to possible therapeutic interventions to prevent or slow disease. Research in simple non-mammalian animal models may allow for testing specific hypotheses regarding the function of sleep.

Among the many theories regarding the function of sleep, two have stood out in recent years. First, sleep as a restorative state for brain energetics and second sleep as a state promoting nervous system plasticity. The idea of sleep is restorative to brain metabolic storage pools have been tested in *Drosophila*, where glycogen levels fluctuate in the brain but not the body in a circadian fashion (43). Glycogen stores measured from whole animals (brains and bodies) correlate with sleep amounts in male flies (44) and brain glycogen stores decrease after 3 hours of acute sleep deprivation (43). In contrast to the brain glycogen response to acute sleep deprivation, whole body glycogen is reduced by repetitive mechanical stimulation, regardless of whether the stimulation is applied during the wake or sleep period. The ability to study mutants in *Drosophila* allows one to get past correlation and to test causal relationships between energy stores and sleep or sleep homeostasis (44, 45).

With respect to synaptic plasticity, this theory has its roots in the observation of increased sleep and sleep pressure during times in which animals are developing. With rare exceptions (46) maximal rest or sleep occurs at birth, with the amount gradually decreasing as animals grow to the adult stage. This is true not only for terrestrial mammals, but also for zebrafish, who show longer quiescent bouts as larvae, (39, 40), fruit flies, whose sleep amounts decline as they age (20, 47), and round worms, which show long periods of behavioral quiescence predominantly during the larval stages (32). Alternative specific hypothesis regarding the cellular mechanisms of brain plasticity have been proposed (48, 49). Both theories propose that changes in synaptic strength occur as a function of behavioral state although in one case the change is predominantly in the positive direction (i.e. synapses are potentiated) whereas in the other changes are in the negative direction (i.e. synapses are depressed).

Work in model genetic organisms has provided some illumination onto these theories. Circadian changes in *Drosophila* neuronal terminals have been observed (50, 51). Gilestro and colleagues (52) showed that independent from circadian effects, sleep results in reduced expression of both pre-synaptic and post-synaptic proteins. Donlea and colleagues (53) showed that sleep deprivation results in an increase in synaptic terminals of *Drosophila* lateral neurons, again suggesting that wake and sleep promote synaptic growth or depression, respectively. Using zebrafish, Applebaum and colleagues (54) exploited the animal's transparency to study the dynamics of synaptic structure in specific neurons as a function of the circadian time and of behavioral state. They saw a clear reduction in synapses during the nighttime relative to the daytime. Sleep deprivation had only a modest effect on synapses suggesting that at least in the zebrafish hypocretineric synapses they studied, the predominant inputs to synaptic change are circadian and not sleep/wake cycles.

The *C. elegans* sleep-like state lethargus occurs not on a circadian time frame but rather, on a developmental time frame. However, analogous to the circadian system, cycling of expression of the worm orthologues of PERIOD is observed to occur with larval cycles (55). With respect to the total number of cells and to the size of its nervous system, *C. elegans* is the simplest animal to date for which sleep or a sleep-like state has been described. Understanding the events that occur during lethargus may therefore give clues as to the core function of sleep. Studies in this respect are on-going but a few preliminary comments can be made. First, the observation of sleep-like behavior predominantly during development and not in the adult worm suggests a role for this behavior in development as has been

proposed (56). Second, over one fourth of the 302 neurons in the *C. elegans* adult nervous system are born during larval development. These neurons must get incorporated into the existing circuitry, necessitating the formation of new synapses as well as the elimination of some old synapses. Thus, *C. elegans* is an excellent model system for the study of nervous system plasticity and its relationship to sleep-like states. Third, lethargus marks the time in which the animal completes the synthesis and assembly of a new cuticle and escapes from the old cuticle. Molting is a process that is executed primarily by epithelial cells. Hence, there is a clear relationship of *C. elegans* sleep-like state to events that occur outside the nervous system. This observation leads to reconsideration of the dogma that sleep serves a function unique to the nervous system (57). Finally, the association with the molt, which is presumably a biosynthetically active process in the worm is in keeping with theories suggesting that sleep and sleep-like state are associated with anabolic cellular processes (58).

Concluding comments

With the explosion in the number of genome wide association studies and the anticipated frequent use of whole genome sequencing to identify human gene variants associated with disease, it has become increasingly important to identify the proper approach for testing the *in vivo* significance of identified variants. Model genetic systems including mice, fruit flies, round worms, and zebrafish allow one to perform such testing. *C. elegans* and *Drosophila* offer the additional advantage of rapidity of experiments and of their use in the identification of new sleep regulating genes, variations in which may also affect human sleep.

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Figure 1.
Human variants associated with sleep phenotypes

Table 1

Genes identified as sleep regulators in non-mammalian model systems.

Organism	Gene	Molecular identity	Phenotype [#]	Insight gained
<i>Drosophila</i>	<i>Shaker</i>	<i>Pore-forming subunit of a Voltage gated potassium channel</i>	Less sleep	Membrane excitability affects sleep/wake
	<i>Sleepless</i> (10)	Novel GPI-anchored protein that regulates Shaker	Less sleep	
	<i>Hyperkinetic</i> (59)	Accessory subunit of Shaker potassium channel	Less sleep	
	<i>Fumin</i> (26)	Dopamine transporter	Less sleep and hyper-responsiveness	Dopamine promotes arousal indicating an evolutionarily conserved role
	<i>EcR</i> (27)	Ecdysone receptor	Less sleep	steroid signaling promotes sleep
	<i>Rutabaga</i> (31)	Adenylate Cyclase	More sleep	cAMP signaling promotes wakefulness indicating an evolutionarily conserved role
	<i>Dunce</i> (31)	cAMP-specific phosphodiesterase	Less sleep	
	<i>D-CREB</i> (31)	Cyclic AMP Response Element Binding Protein	More sleep	
	<i>Activating transcription factor-2</i> (60)	cAMP response element (CRE) binding protein activated by stress/locomotor activity	Less sleep [^]	Activation of ATF-2 promotes sleep
	<i>Syndecan</i> (24)	Trans-membrane protein involved in energy metabolism	More sleep	A reduction in metabolic rate promotes sleep
	<i>Spargel</i> (24)	PGC-1	More sleep	
	<i>Rhomboid</i> (30)	Membrane-bound protease that activates EGFR ligand	More sleep [^]	EGFR activation promotes sleep
	<i>Resistant to dieldrin</i> (61)	GABA _A receptor	Less sleep [^]	GABA promotes sleep indicating an evolutionarily conserved role of GABA in this process
	<i>Tyrosine decarboxylase 2</i> (28)	Enzyme which converts tyrosine to tyramine, the substrate for octopamine	More sleep	Octopamine promotes wakefulness
	<i>tyramine β hydroxylase</i> (28)	Enzyme which converts tyramine to octopamine	More sleep	
	<i>5-HT1A</i> (62)	Serotonin receptor	Less sleep	Serotonin promotes sleep indicating an evolutionarily conserved role
<i>C. elegans</i>	<i>Fmr1</i> (63)	RNA-binding protein	More sleep	Fmr1 promotes wakefulness
	<i>pde-4</i> (32)	cAMP-specific phosphodiesterase	Hyper-responsiveness	cAMP promotes wakefulness
	<i>acy-1</i> (32)	Adenylate cyclase	Hyper-responsiveness [*]	
	<i>kin-2</i> (64)	Regulatory subunit of cAMP-dependent protein kinase	Hyper-responsiveness	
	<i>egl-4</i> (32)	cGMP dependent protein kinase	Hyper-responsiveness and reduced quiescence	cGMP promotes sleep

Organism	Gene	Molecular identity	Phenotype [#]	Insight gained
	<i>let-23</i> (19)	EGF receptor	Reduced quiescence	EGF signaling required for function of sleep-inducing neuron ALA
	<i>plc-3</i> (19)	Phospholipase C-gamma	Reduced quiescence	EGF signaling in ALA is via phospholipase C gamma.
	<i>lin-3</i> (19)	EGF receptor ligand	Increased quiescence [*]	
<i>D. rerio</i>	<i>Hypocretin</i> (39)		Reduced quiescence [*]	Hypocretin promotes wake
	<i>hypocretin receptor</i> (40)		Fragmented nocturnal sleep	Hypocretin signaling is required for behavioral state stability

Unless otherwise indicated, phenotype is that of a reduction in gene function;

^ Based on RNAi knockout;

* Based on analysis of gain-of-function phenotype