Molecular Genetic Advances in Sleep Research and Their Relevance to Sleep Medicine

Jonathan P. Wisor, PhD; Thomas S. Kilduff, PhD

Molecular Neurobiology Laboratory, Biosciences Division, SRI International, Menlo Park, CA

Abstract: Neurobiology has been profoundly impacted by the availability of molecular genetic techniques within the past decade, and the sleep research field is no exception to this trend. Genetic influences on normal and pathologic sleep have long been recognized, but only recently have the contributions of discrete genetic loci to the regulation of sleep timing and sleep-related physiologic phenomena begun to be demonstrated, particularly through the use of genetically engineered strains of rodents. The ability to monitor the expression of a large number of genes has also accelerated in recent years, leading to a better understanding of the molecular and neurochemical correlates of sleep and of sleep loss. This article reviews the contributions of molecular genetic studies to our understanding of the processes that underlie sleep and its pathologies, discusses their relevance to the treatment of sleep disorders, and offers some speculation on how the sleep field might further be advanced through molecular genetic analysis.

Abbreviations: 5-HT, 5-hydroxytryptamine; CREB, cyclic AMP response element binding protein; EDS, excessive daytime sleepiness; EEG, electroencephalographic; GFP, green fluorescent protein; GHB, gamma-hydroxybutyrate; Hcrt, hypocretin; HcrtR, Hcrt receptor; IL-1, interleukin-1; LT-α, lymphotoxin-α; mRNA, messenger ribonucleic acid; NREM, non-rapid eye movement; REM, rapid eye movement; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor

Key Words: Gamma-hydroxybutyrate, hypocretin, modafinil, sleep deprivation, circadian genes, molecular genetics, mutant mice, hypnons, gene expression

Citation: Wisor JP; Kilduff TS. Molecular genetic advances in sleep research and their relevance to sleep medicine. SLEEP 2005;28(3):357-367.

GLOSSARY

Forward genetics: the mapping of spontaneous or mutagen-induced genetic variability to a genetic locus.

G-protein coupled receptor: a cell surface receptor that activates an intracellular signaling protein (G-protein), which in turn may modulate a broad spectrum of intracellular biochemical reactions.

Green fluorescent protein (GFP): a bioluminescent protein originally derived from a microorganism. This protein can be targeted to a neurochemically defined cell type using a transgene in which the GFP coding sequence is driven by a promoter that confers expression exclusively to that cell type. The resulting cell type-specific bioluminescence allows the researcher to visually identify and experimentally manipulate or observe the cell type of interest.

Ion-channel: a protein or aggregation of proteins embedded in a cell membrane through which electrically charged ions pass, thereby modulating the level of excitability of the cell and its release of signaling molecules.

Knockin: a genetic manipulation that replaces an endogenous gene with a genetically engineered construct. This technique can be used to assess the effect of polymorphic variation, as opposed to that of complete loss of function (knockout), on the function of the organism. This technique is often used to introduce extra copies of a gene with the intent of increasing expression at the protein level (see overexpression).

Knockout: a genetic manipulation that abolishes gene expression, typically involving insertion of exogenous genetic material into the genome of interest.

Microarray: a microscopic array of tens to thousands of DNA probes affixed at defined locations on a silicon chip or other substrate. Each DNA probe emits light when bound by its cognate gene transcript within a biologic sample. The light signal from each location on the chip is thus a quantitative measure of the expression level of a single gene.

Overexpression: a genetic manipulation that causes expression of a pre-existing gene to be elevated above wild-type levels

Polymorphism: naturally occurring variability in the nucleotide sequence encoded by a genetic locus, which may or may not have functional consequences that are manifested at the organismic level.

Promoter: that portion of a genetic locus that is not expressed (ie, transcribed into RNA) but, rather, influences the spatial and temporal expression pattern of the locus.

Reverse genetics: genetic manipulation performed in embryonic stem cells that results in expression of a novel protein (transgenic or knockin), a change in the expression level of a preexisting gene (overexpression or underexpression) or complete failure to express a preexisting gene (knockout).

Transgenic: in general terms, an organism in which genetically engineered material has been inserted into the genome. In neurobiology, transgenic typically refers to an animal in which the inserted material produces a protein that is novel to the organism (such as ataxin or green fluorescent protein).

Underexpression: a genetic manipulation that causes expression of a preexisting gene to be suppressed below wild-type levels

Wild type: the preexisting, nonengineered genetic variant to which a novel genetic variant is compared. Depending on context, it may refer to either the DNA sequence of a gene or to animals that harbor that sequence.

INTRODUCTION

TECHNOLOGIC ADVANCES HAVE STREAMLINED GENETIC ANALYSIS IN HUMANS AND MODEL SPECIES USED FOR BIOMEDICAL RESEARCH. OVER THE PAST DECADE, THE PRODUCTION OF TRANSGENIC, "KNOCKOUT" AND "KNOCKIN" STRAINS OF RODENTS, PARTICULARLY MICE, HAS BECOME WIDESPREAD. THESE ‘REVERSE’ GENETIC METHODS HAVE PROVIDED SCIENTISTS INTERESTED IN PHYSIOLOGY AND BEHAVIOR WITH NEW TOOLS WITH WHICH TO EXAMINE COMPLEX BEHAVIORS AND ADAPTATIONS SUCH AS SLEEP. GIVEN THAT GENETIC INFLUENCES ON SLEEP AND ITS DISORDERS HAVE BEEN KNOWN FOR SOME TIME,1 2 IT SHOULD SURPRISE NO ONE THAT GENETIC MANIPULATION OF RODENTS CAN AFFECT SLEEP. INDEED, SINCE THE FIRST REPORTS OF A SLEEP PHENOTYPE IN GENETICALLY ENGINEERED MICE IN 1996,3 SLEEP PATTERNS HAVE BEEN REPORTED IN MORE THAN 50 EXPERIMENTALLY PRODUCED MUTANT LINES OF MICE AND RATS (TABLES 1-4). THIS IN VIVO APPROACH IS COMPLEMENTED BY STUDIES IN WHICH THE SLEEP-WAKE STATE DEPENDENCE OF THE EXPRESSION OF...
large numbers of genes in the rodent brain has been monitored in silico. While it would require more space than is available to enumerate all the observations that have been made about sleep using these methods, we herein summarize some noteworthy developments in which molecular and genetic analyses of rodents have advanced our understanding of sleep. Significant contributions of classical 'forward' genetic approaches (i.e., gene mapping) to the sleep research field are noted in passing but are not considered in detail, as other recent publications provide thorough reviews on this topic.1,4,5

### Table 1—Genetically Engineered Rodent Models Affecting Synaptic Signaling Systems That Have Been Subjected to Behavioral State Assessments

<table>
<thead>
<tr>
<th>Signaling Mechanism</th>
<th>Animal Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>β2 nicotinic receptor subunit null mutant mice</td>
<td>58,125</td>
</tr>
<tr>
<td>Anandamide/oleamide</td>
<td>Fatty acid amide hydrolase null mutant mice</td>
<td>126</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Dopamine transporter null mutant mice</td>
<td>45</td>
</tr>
<tr>
<td>GABA</td>
<td>α1, GABA-A receptor subunit null mutant mice</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>α2, GABA-A receptor subunit null mutant mice</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>α3, GABA-A receptor subunit null mutant mice</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>β3, GABA-A receptor subunit null mutant mice</td>
<td>64</td>
</tr>
<tr>
<td>Histamine</td>
<td>Histidine decarboxylase null mutant mice</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Histamine H1 receptor null mutant mice</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Histamine H3 receptor null mutant mice</td>
<td>127</td>
</tr>
<tr>
<td>Hypocretin (orexin)</td>
<td>Preprohycretin null mutant mice</td>
<td>9,21</td>
</tr>
<tr>
<td></td>
<td>Hypocretin (Hcrt) cell knockout mice</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Hypocretin cell knockout rat Hcrt receptor 1 (HcrtR1) null mutant mice</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Hcrt receptor 2 (HcrtR2) null mutant mice</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>HcrtR1/HcrtR2 double null mutant mice</td>
<td>16</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>Dopamine beta-hydroxylase (DBH) null mutant mice</td>
<td>128-130</td>
</tr>
<tr>
<td>Noradrenaline/Hypocretin</td>
<td>DBH/Hcrt double null mutant mice</td>
<td>131</td>
</tr>
<tr>
<td>Serotonin</td>
<td>5-HT1A receptor null mutant mice</td>
<td>132,133</td>
</tr>
<tr>
<td></td>
<td>5-HT1B receptor null mutant mice</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>5-HT2C receptor null mutant mice</td>
<td>134</td>
</tr>
<tr>
<td>Synaptic vesicle release</td>
<td>Rab3a null mutant mice</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>RIM1α null mutant mice</td>
<td>136</td>
</tr>
</tbody>
</table>

**HYPOCRETIN/OREXIN IN NARCOLEPSY AND NORMAL SLEEP**

The application of genetic techniques to the study of narcolepsy has brought to fruition the long search for the pathogenesis of this disorder. The identification of deficits in hypocretin (also known as orexin)-mediated signaling in narcolepsy and, in general, the role of hypocretin as a novel sleep-regulatory pathway, is arguably one of the most significant breakthroughs in the history of sleep research. Hypocretin was identified independently by 2 groups searching for novel brain peptides. Before it was recognized that these groups were studying the same molecules, these peptides were given the name “hypocretins” to denote the hypothalamic localization and structural resemblance to the gut peptide secretin and subsequently called “orexins” for a putative role in regulating food intake.7 One of the first studies on the hypocretin system noted that the cell bodies that synthesize hypocretin are localized to an area that past lesion studies had implicated in sleep-wake regulation and that these cell bodies project to other brain areas known to influence sleep and wake timing.8 Interest in the hypocretin system was solidified by 2 nearly simultaneous discoveries in 1999: (1) mice in which the prepro-Hcrt locus was genetically inactivated (i.e., ‘knockout’ or null mutant mice) exhibited a behavioral syndrome similar to narcolepsy9 (see below) and (2) genetically transmitted canine narcolepsy was found to result from a mutation that renders 1 of the 2 hypocretin receptors (HcrtR2) nonfunctional.10 Additional inherited mutations at the same locus have since been linked to narcolepsy in other kindreds.11 Together, these findings demonstrated a critical role for hypocretin in sleep regulation and in the pathogenesis of narcolepsy. This achievement offered hope that the pathologies underlying other sleep disorders, particularly those known to have a genetic basis such as restless legs syndrome,12 advanced13 and delayed14 sleep phase syndromes, and central sleep apnea15 might also be identified in studies of genetic animal models.

Genetic approaches in mice9,16-19 and, very recently, in rats20 have clarified a number of issues regarding the role of hypocretin in regulating sleep (Table 1). Mice that do not produce functional hypocretin peptides due to genetic inactivation of the prepro-Hcrt gene exhibit periods of behavioral arrest that bear a striking resemblance to cataplexy.9 While the circadian rhythm of sleep timing and the homeostatic response to sleep deprivation, as indicated by increased sleep time and increased slow-wave amplitude, are intact in these mice,21 sleep and wake bouts are fragmented in prepro-Hcrt knockout mice relative to wild-type mice.9,21 Prepro-Hcrt knockout mice thus exhibit a syndrome akin to narcolepsy, with symptoms analogous to both excessive daytime sleepiness (EDS) and cataplexy.9 Yet it has been argued that prepro-Hcrt knockout mice do not replicate the pathogenesis of narcolepsy because, in humans, the cells that produce the hypocretin peptides degenerate22,23 rather than simply fail to produce hypocretin. To address this concern, rodent models that mimic this deficit have been engineered. In order to model ‘human-type’ (i.e., neurodegenerative) narcolepsy, mice19 and, subsequently, rats20 were engineered so that the prepro-Hcrt promoter drives expression of the neurotoxic ataxin-3 protein. Such animals lose hypocretin-producing cells at a phase of development when endogenous hypocretin (and thus exogenously induced ataxin-3) begins to be expressed at high levels. The effects of targeted ataxin-induced cell ablation on sleep are very similar to those of the prepro-Hcrt knockout: wakefulness during the active (lights-off) period is punctuated by behavioral arrests and frequent bouts of sleep, and...
sleep during the inactive period (lights-on) is similarly fragment-
ed. The resultant phenotype is thus similar to narcolepsy, whether the endogenous prepro-Hcrt gene is inactivated or the cells that produce it are targeted for neurotoxic ablation.

With two hypocretin peptides and two hypocretin receptors, HcrtR1 and HcrtR2, known to be expressed in the brain, the question arises as to whether these receptors have distinct functional roles in the central nervous system. Although this issue has not been fully resolved to date, it has been in part addressed through in vitro studies and by transgenic approaches. In in vitro studies, it has been demonstrated that both the HcrtR1 and the HcrtR2 mediate excitatory responses, although by distinct intracellular signal transduction mechanisms. In vivo, the sleep phenotypes of HcrtR1 knockout and HcrtR2 knockout are distinct, further suggesting separate functions for the two receptors. HcrtR1 knockout apparently results in a mild phenotype consisting primarily of fragmented sleep and wake states (as reported in abstract form at the Associated Professional Sleep Societies meeting), while HcrtR2 knockout causes both sleep fragmentation and sudden behavioral arrests resembling cataplexy. HcrtR1 knockout mice apparently do not exhibit behavioral arrests. The conclusion from these reports is that HcrtR1 is critical for maintenance of sleep and wake bouts, while HcrtR2 is critical for wake-specific maintenance of skeletal muscle tone as well as state consolidation. However, this conclusion must be tempered by the fact that prepro-Hcrt knockout and double HcrtR1/R2 knockout mice both have more severe behavioral arrests than do HcrtR2 knockout mice. Thus, HcrtR1 may play some additional role in maintaining muscle tone, at least in the admittedly pathologic case of HcrtR2 knockout mice.

Another interesting transgenic tool is the hypocretin/GFP mouse, which has facilitated studies of the electrophysiologic and pharmacologic properties of hypocretin-producing cells. In these genetically engineered mice, the prepro-Hcrt promoter drives the expression of green fluorescent protein (GFP), which can then be used to identify hypocretin cells in vitro. Brain slices from these mice have been used to determine that noradrenaline and serotonin suppress the activity of hypocretin cells, and that presynaptic hypocretin receptors and metabotropic glutamate receptors on synaptic inputs to hypocretin cells modulate the activity of these neurons.

What have these findings meant for narcoleptic patients? These exciting experimental results are beginning to be translated into improvements in the diagnosis of narcolepsy and may ultimately lead to novel therapies. On the diagnosis front, genetic testing for narcolepsy in humans appears not to be an option, since genetic transmission of narcolepsy due to hypocretin or hypocretin receptor mutations has not been documented in humans. On the other hand, measurement of hypocretin peptides in the cerebrospinal fluid has been proposed as a potential diagnostic tool. The vast majority of narcoleptic patients exhibit cerebrospinal fluid hypocretin levels much lower than those of normal controls, or even below the threshold for detection. Therefore, cerebrospinal fluid hypocretin peptide levels, or perhaps ultimately plasma hypocretin, may be a useful adjunct to clinical assessment in the diagnosis of narcolepsy. As for treatment of narcolepsy, hypocretin replacement therapy (analogous to insulin therapy in diabetes) is an appealing therapeutic avenue and may at some point be feasible for those narcoleptics in whom hypocretin receptor-bearing cells are intact. The feasibility of this approach is supported by the recent finding that either intracerebroventricular infusion or overexpression of the Hcrt-1 (orexin-A) peptide ameliorates the sleep deficits in the hypocretin/ataxin-3 mouse model. One challenge to be met in this arena is the delivery of peripherally injected hypocretin or hypocretin analogues to the synaptic targets of hypocretin signaling in the brain. Although there has been one report of cataplexy suppression in narcoleptic canines by systemic hypocretin administration, a second study reported a considerably less robust effect.

Although the prospect for hypocretin replacement remains controversial, it can nonetheless be anticipated that the link between hypocretin and narcolepsy will one day be utilized to improve the lives of narcoleptic patients. Toward this end, GlaxoSmithKline has produced several small-molecule HcrtR1 antagonists and a synthetic peptide that also acts as a HcrtR1/HcrtR2 antagonist. In addition, a HcrtR2 antagonist was recently described by Banyu Pharmaceuticals. The identification of these antagonists was critically dependent on the availability of cloned hypocretin receptors and, thus, on the genetic techniques used to identify these receptors. Although the effects of these compounds on sleep are yet to be reported, the Hcrt-1 peptide has been uniformly reported to promote wakefulness and suppress sleep whether injected intracerebroventricularly or into local brain regions such as the locus coeruleus, lateral dorsal tegmental nucleus, basal forebrain, or preoptic area. Although a hypocretin agonist would be preferable for the treatment of hypocretin-deficient narcoleptic humans, the availability of antagonistic ligands is a step in the right direction.

PHARMACOGENETICS OF SLEEP-WAKE THERAPEUTICS

Considerable improvements have been made in recent decades in the treatment of insomnia and EDS as benzodiazepines have replaced barbiturates and nonamphetamine wake-promoting therapeutics, notably modafinil, have emerged. These improvements stem largely from the identification and increased exploitation of novel compounds. Yet, recognition of the clinical utility of such compounds has in some cases occurred despite any clear understanding of their neurobiologic mechanisms of action. The identification of more-effective treatments for insomnia and for EDS, as well as the identification of the etiologies of these symptoms, is thus hindered by gaps in basic sleep neurobiology. However, studies using pharmacogenetic approaches have begun to make significant inroads in this area. Pharmacogenetics can be described in general terms as the study of the effects of spontaneous or experimentally induced genetic diversity on the response to pharmacologic agents. We discuss below the use and limitations of pharmacogenetic approaches in studying the mechanisms of action of the wake-promoting agent modafinil and two classes of sleep-promoting agents: benzodiazepines and gamma-hydroxybutyrate (GHB).

Modafinil

Modafinil was first recognized as a potent suppressor of sleepiness nearly 20 years ago. Since that time, it has been widely used as a treatment for pathologic sleepiness in narcolepsy, sleep apnea, and various other conditions. The mechanism of action of modafinil remains controversial, but a number of conclusions about its mechanism of action can be drawn from pharmacogenetic studies. First, the robust wake-promoting efficacy of...
modafinil in HcrtR2-deficient canine narcoleptics demonstrates that HcrtR2 signaling is not necessary for the wake-promoting action of modafinil, despite the fact that modafinil induces Fos protein expression (a measure of cellular activity) in hypocretin-containing cells of the hypothalamus. On the other hand, genetic inactivation of the cell membrane dopamine transporter abolishes the wake-promoting effect of modafinil. Lastly, genetic inactivation of the α₁b adrenergic receptor severely blunts the behavioral response to modafinil in mice. While this last finding might be thought to demonstrate a direct role for α₁b adrenergic signaling in the response to modafinil, other studies involving α₁b adrenergic receptor knockout mice provide a cautionary note. The activity of dopaminergic projections in the brain is chronically downregulated in these mice, presumably because dopaminergic transmission is dependent upon adrenergic stimulation of dopaminergic cells. This observation raises the possibility that the blunted response to modafinil in α₁b receptor knockout mice is an indirect effect of the mutation on dopaminergic transmission and, more generally, illustrates that genetic inactivation of any one central nervous system signaling pathway may alter the activity of others in ways that are not immediately obvious. Knockout studies alone cannot ascertain a direct role for a specific pathway in the action of any pharmacologic agent. Nonetheless, the cumulative observations from knockout mice, together with a human genetic study in which polymorphisms in the catecholamine metabolizing enzyme catechol-O-methyl transferase were found to influence the efficacy of modafinil, implicate catecholaminergic transmission in the response to this drug. The recent observation that modafinil promotes wakefulness in the fruit fly Drosophila melanogaster indicates the potential of this phylogenetic model for sleep (see below) to identify additional loci that modulate the response to modafinil.

Molecular genetic approaches have identified other targets for wake-promoting therapeutics in addition to the dopamine transporter and adrenergic receptors. Table 1 summarizes some of the rodent models that have been produced to inactivate specific neurotransmitter systems that have previously been implicated in the control of behavioral states. For example, inactivation of the gene encoding histidine decarboxylase produces mice that fail to synthesize histamine. These mice fail to maintain wakefulness for an extended period of time when placed in an unfamiliar environment. Interestingly, histaminergic transmission has been identified as a downstream mediator of hypocretin signaling. These observations support the concept that histaminergic transmission is a potential target for wake-promoting, as well as hypnotic, therapeutics. The monoamine serotonin (5-hydroxytryptamine; 5-HT) has long been implicated in the development and maintenance of sleep patterns. Serotonin-transporter knockout mice exhibit more rapid eye movement (REM) sleep than do their wild-type littermates and display more frequent and longer-lasting REM bouts. 5-HT₁B receptor-deficient mice exhibit more rapid REM sleep relative to wild-type mice during the light phase and lack REM sleep rebound after REM sleep deprivation. Since mice lacking the β₂ subunit of brain nicotinic receptors fail to respond to the wake-promoting effect of nicotine, receptors containing this subunit may also be a target for interventions related to EDS. On the other hand, despite the literature implicating adenosine as a feedback molecule in homeostatic sleep regula-

<table>
<thead>
<tr>
<th>Ion Channel</th>
<th>Animal Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium channel (N-type), α₁B subunit (Cav2.2)</td>
<td>Cav2.2 null mutant mice</td>
<td>75</td>
</tr>
<tr>
<td>Calcium channel (N-type), α₁G subunit (Cav3.1)</td>
<td>Cav3.1 null mutant mice</td>
<td>137</td>
</tr>
<tr>
<td>Potassium channel subunit Kv3.1</td>
<td>Kv3.1 null mutant mice</td>
<td>76</td>
</tr>
<tr>
<td>Potassium channel subunit Kv3.2</td>
<td>Kv3.2 null mutant mice</td>
<td>77</td>
</tr>
<tr>
<td>Potassium channel subunit Kv3.3</td>
<td>Kv3.3 null mutant mice</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Kv3.1/Kv3.3 null mutant mice</td>
<td></td>
</tr>
</tbody>
</table>
In contrast to benzodiazepines, which suppress slow-wave sleep even as they decrease sleep latency and sleep fragmentation,68 GHB is a hypnotic compound that is noteworthy for its ability to induce physiologically normal slow-wave sleep.69 GHB is produced endogenously as a metabolite of the inhibitory neurotransmitter GABA.70 GHB is a very effective narcolepsy therapeutic but is atypical in that, unlike most narcolepsy therapeutics, GHB effectively treats both EDS and cataplexy when administered at bedtime rather than during the day. Furthermore, its effects emerge after several days of GHB administration, rather than as an immediate consequence of administration, and the suppression of cataplexy and EDS are sustained for several days subsequent to GHB withdrawal. The indirect and sustained efficacy of GHB in treating cataplexy and EDS in narcolepsy likely reflects profound changes in gene expression or sleep circuitry. The neurochemical and physiologic changes that underlie the sustained effects of repeated GHB administration are not known. Like its chemical relative GABA, GHB activates GABAA receptors.71 While a putative GHB receptor was recently cloned,72 most of the physiologic effects induced by GHB are abolished in mice lacking the GABAA receptor.73,74 The specificity of GHB for this receptor, and the fact that it is an agonist and not an allosteric modulator, may explain the difference between the physiologic responses to GHB and benzodiazepines.

**SLEEP WAVEFORMS ARE AFFECTED BY MANIPULATION OF SPECIFIC ION CHANNELS**

Many neurotransmitters activate specific ion channels through G protein-coupled receptors. Table 2 lists the limited number of studies that have examined sleep-wake in ion-channel knockout mice. The differential properties of voltage-dependent Ca2+ channels have been primarily ascribed to the α subunit, of which 10 different subtypes are currently known. Channels that conduct the N-type Ca2+ current possess the α1B subunit (Cav2.2). Cav2.2 deficient mice display vigilance-state differences during the light phase, including increased consolidation of REM sleep and decreased REM sleep and decreased during NREM sleep in Cav2.2 knockout mice relative to wild-type control mice.75 These results suggest that the N-type Ca2+ channel plays a role in activity and vigilance state control, most likely through effects on neurotransmitter release.

The voltage-gated potassium channels Kv3.1 and Kv3.3 are widely expressed in the brain, including areas implicated in the control of motor activity and in areas thought to regulate arousal states. Compared to wild-type mice, Kv3.1/Kv3.3-deficient mice display “restlessness” (particularly during the light period), characterized by a 40% reduction in sleep time.76 Constitutive increases of ambulatory and stereotypic activity, and accompanied by a 40% reduction in sleep time.76 Constitutive increases of ambulatory and stereotypic activity in conjunction with sleep loss has been found in Kv3.1-single mutants but not in Kv3.3-single mutants. These findings indicate that the increased motor drive and the reduction in sleep time in the double mutants is primarily due to the absence of Kv3.1-channel subunits.

Voltage-gated potassium channels containing the Kv3.2 subunit are expressed in specific neuronal populations such as thalamocortical neurons and fast-spiking GABAergic interneurons of the neocortex and hippocampus. Although Kv3.2-deficient mice have been shown to be no different than wild-type controls in response to sleep deprivation, these mice have lower EEG power density in the frequencies between 3.25 and 6 Hz NREM sleep and 3.25 and 5 Hz in REM sleep, suggesting that Kv3.2 channels may be involved in the generation of EEG oscillations in the high delta and low theta range in sleep.77

**SLEEP-WAKE REGULATORY DIFFUSIBLE FACTORS**

In addition to the fast-acting synaptically released neurotransmitters and their receptors, there is also evidence that a number of diffusible factors act as “sleep-promoting substances” and play central roles in regulating the timing of sleep and sleep-related physiologic events (see references 78-80 for review). Some diffusible factors such as cytokines may mediate the somnogenesis that occurs in association with infectious disease. By definition,78–80 an endogenous sleep substance should induce physiologic sleep. Conversely, disruption of the synthetic or signaling pathways for any putative sleep factor should either disrupt the timing of sleep or affect its expression. Mouse genetic models in which the synthesis of putative sleep factors or their signaling pathways are dis-

### Table 3—Genetically Engineered Rodent Models Affecting Endocrine and Paracrine/Autocrine Signaling Pathways That Have Been Subjected to Behavioral State Assessments

<table>
<thead>
<tr>
<th>Signaling Molecule</th>
<th>Animal Model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine A1 receptor</td>
<td>Adenosine A1 receptor null mutant mice</td>
<td>59</td>
</tr>
<tr>
<td>Adenosine A2a receptor</td>
<td>Adenosine A2a receptor null mutant mice</td>
<td>138</td>
</tr>
<tr>
<td>IL-1</td>
<td>IL-1 type I receptor null mutant (IL-1RI knockout) mice</td>
<td>139</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6 null mutant (IL-6 knockout) mice</td>
<td>140,141</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL-10 null mutant mice</td>
<td>142</td>
</tr>
<tr>
<td>IFN-RI</td>
<td>IFN type I receptor null mutant (IFN-RI knockout) mice</td>
<td>143</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Neuronal nitric oxide synthase (nNOS) null mutant mice</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Inducible (i)NOS null mutant mice</td>
<td>144</td>
</tr>
<tr>
<td>Prostaglandin D2 (PGD2)</td>
<td>Transgenic PGD synthase (PGDS) overexpressing mice</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>Lipocalin-type PGDS null mutant mice</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>DP receptor null mutant mice</td>
<td>147</td>
</tr>
<tr>
<td>TNF</td>
<td>TNF 55 kDa receptor null mutant (TNFR1-KO) mice</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>TNF2 null mutant (TNFR2KO) mice</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>TNF and lymphotoxin-alpha double null mutant mice</td>
<td>82</td>
</tr>
<tr>
<td>Growth hormone (GH) and GH-releasing hormone (GHRH)</td>
<td>Dwarf (dw/dw) rats (nonfunctional GHRH receptor)</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Dwarf (lit/lit) mice (nonfunctional GHRH receptor)</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>excess GH production mice</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>GH and insulin-like growth factor-I (IGF-I) null mutant mice</td>
<td>151</td>
</tr>
</tbody>
</table>
ruptured by induced mutations have been very useful in assessing the roles of putative sleep-promoting substances, for instance, the cytokines interleukin-1 (IL-1), tumor necrosis factor (TNF-α) and IL-10 (Table 3). The role of TNF-α has been particularly extensively studied. Mice lacking the TNF 55 kDa receptor (TNFR1) have been reported to sleep less than controls during the light period. Mice deficient for the ligands TNF-α and lymphotoxin-α (LT-α) and TNFR2 null mice had 15% less REM sleep during the baseline light period due to a reduction in REM sleep episode frequency. Whereas all three knockouts respond to sleep deprivation, EEG frequencies are differentially affected. There are likely other sleep factors that are yet to be recognized, and these novel factors may ultimately be identified by disruption of their synthesis or signaling pathways by approaches such as random mutagenesis.

CIRCADIAN GENES AND SLEEP: THE CLOCK VERSUS THE HOURGLASS

A prominent train of thought in the sleep field maintains that sleep is regulated by interacting circadian (“clock”) and homeostatic (“hourglass”) influences. In this conceptual model, the timing of sleep and sleep-related phenomena are influenced by the phase of the endogenous circadian clock and recent sleep-wake history. Interactions of these two factors can explain much of the phenomenology of sleep timing. However, recent sleep studies on mouse circadian genetic models have hinted that this interaction is perhaps more complex than originally anticipated and may even occur at the molecular level.

First, let us consider the circadian influence. Sleep, along with virtually all other organismic variables, is modulated by the master circadian clock in the suprachiasmatic nuclei (SCN) of the hypothalamus. The mammalian circadian oscillator is, in its simplest most fundamental form, an intracellular molecular feedback loop involving time of day-dependent gene transcription, protein synthesis, and the suppression of transcription by the protein products of the genes being transcribed. (A more thorough description of this process can be found elsewhere.) At the heart of this loop in mammals are the clock and bmal1 genes, two period genes (per1 and per2), and two cryptochrome genes (cry1 and cry2).

Around dawn, the transcriptional regulatory proteins BMAL1 and CLOCK activate transcription of the per and cry genes. Levels of per and cry messenger RNAs (mRNAs) surge during the day. As a result, PER and CRY protein levels increase in the cytoplasm and, ultimately, in the nucleus of the cell. Early in the night, the PER and CRY proteins suppress the transcriptional activity of BMAL1 and CLOCK. Levels of per and cry mRNAs then decline and subsequently PER and CRY proteins do the same. Freed from suppression by PER and CRY proteins late in the night, the BMAL1 and CLOCK proteins in the nucleus then ramp up transcription of the per and cry genes again at dawn, and the cycle begins anew.

Although this model has evolved from studies of activity in flies and mice, these same genes are relevant to human sleep disorders: advanced or delayed sleep phase syndrome in humans is, at least in some cases, genetically determined. In at least 1 kindred, advanced sleep phase syndrome has been traced to a mutation in the per2 locus that alters the timing of the molecular clock and, consequently, that of its circadian outputs, including sleep. In several other advanced and delayed sleep phase syndrome kindreds, circadian mutations have yet to be identified (see for review).

The cloning of the various circadian genes has been one of the most followed developments in neuroscience and has led to some unexpected observations. For instance, given that intact SCN are necessary for maintenance of rhythmicity, it was somewhat unexpected to find that circadian genes are expressed throughout, and even outside of, the brain. Also somewhat unexpected was the observation that circadian rhythms can be observed in non-SCN brain tissues maintained in isolation in vitro, albeit with much lower amplitude than in SCN cultures. These observations hinted at a role for these genes in producing oscillations outside the SCN clock.

Equally relevant to the genetic basis of sleep is its homeostatic, or sleep history-dependent, regulation. Studies on the sleep phenotypes of some circadian mutants suggest that these genes may play a role in the homeostatic, sleep history-dependent regulation of sleep. Table 4 lists some of the circadian clock-related mutants in which sleep-wake behavioral assessments have been conducted to date. The first clue that clock genes directly influence the sleep-history dependence of sleep phenomena came from a study of sleep in clock mutant mice. The clock mutation results in arrhythmicity in constant conditions. EEG studies on clock mutant mice indicate that these mice are insomniacs; they sleep approximately 2 hours less than wild-type litter mates on a daily basis. This phenotype does not represent an abnormality in circadian entrainment, as it is maintained in constant dark conditions. On the other hand, mice lacking functional cry1 and cry2 genes, which are also arrhythmic in constant conditions, exhibit the cortical correlates of high sleep drive (typically seen after sleep deprivation) under baseline conditions: they sleep approximately 1.5 hours more than wild-type litter mates in both light/dark cycles and constant-dark conditions and exhibit more consolidated sleep and higher NREM sleep EEG delta power than wild-type litter mates. Together, these observations implicate the circadian transcriptional regulatory network in what is viewed as a noncircadian function. Yet, the per genes might not influence homeostatic sleep regulation, as inactivation of either per1, per2, or both genes simultaneously has been reported to leave homeostatic sleep regulation intact. Nor does bmal1 appear to be necessary for the homeostatic regulation of sleep. Homeostatic sleep regulation is also intact in mice lacking the dbp gene, which plays a critical modulatory role in the circadian clock. Based on these observations, it can be said that some but not all of the genes that are cen-

| Table 4 | Genetically Engineered Rodent Models Affecting Transcription Factor and Circadian Clock-Related Genes That Have Been Subjected to Behavioral State Assessments |
|---------------------------------------------------------------|
| Transcription Factor Gene | Animal Model | Reference |
| c-fos | c-fos-lacZ transgenic mice | 152 |
| c-fos | null and heterozygote c-fos mice | 153 |
| Cyclic AMP response element binding protein (CREB) fos-B | CREB α/β deficient mice | 154 |
| Albumin D-binding protein (DBP) BMAL1 | dbp null mutant mice | 97 |
| clock | BMAL1 null mutant mice | 96 |
| cryptochromes mPer1 | clock non-functional mutant mice | 92 |
| mPer2 | cry1,2/- double null mutant mice | 93 |
| mPer2 | mPer1 null mutant mice | 94,95 |
| mPer2 | mPer2 nonfunctional mutant mice | 94 |
| mPer2 | mPer2 null mutant mice | 95 |
| mPer3 | mPer3 null mutant mice | 95 |
| mPer1/mPer2 | mPer1,2/- double null mutant mice | 95 |
| NPAS2 | NPAS2 null mutant mice | 102 |
tral to the circadian clock also have a role in sleep homeostasis. It is likely, then, that there are pleiotropic roles for a subset of the circadian clock genes in regulating the noncircadian aspects of sleep timing and sleep EEG phenomena.

Relevant to this story are the results from studies of sleep in the fruit fly *Drosophila melanogaster* (inasmuch as sleep is a behavioral state defined by quiescence, an elevated arousal threshold, a stereotypical posture, and a rebound after deprivation98,99). Mutations in the circadian genes *cycle*100 and *timeless*99 and the clock input gene encoding the cyclic AMP response element-binding protein (CREB) are associated with sleep phenotypes in *Drosophila*.101 Understanding the molecular features of *Drosophila* sleep should shed new light on the mechanisms and functions of clock genes in regulating sleep timing.50 A possible role for circadian genes in sleep regulation is appealing in the sense that distinct but overlapping components of this transcriptional autoregulatory network may measure temporally dependent processes: one of them being self-driven, and the other measuring sleep-dependent changes within the cell. Studies of mammalian and fruit fly circadian mutants have challenged the notion that circadian and homeostatic regulation of sleep are discrete processes and have at least raised the possibility that responding to cellular metabolic demand at the molecular level is one functional correlate of sleep.102

There are a number of caveats to bear in mind when considering this scenario. The interpretation of the sleep data as noncircadian rests on the assumption that the so-called homeostatic measures of sleep that are altered by sleep deprivation (sleep as a percentage of time, delta power, and sleep consolidation) are not dependent on the SCN (though they are modulated by the SCN103). While historically this assumption has been supported by data in rodents,104,105 three recent studies challenge this notion.106-108 At the very least, whether the sleep phenotype of any circadian gene mutant is in fact a consequence of an SCN-independent influence should be ascertained by measuring the phenotype in SCN-lesioned mutant animals.108

**GENE EXPRESSION IN BRAIN DURING SLEEP VERSUS WAKEFULNESS**

Additional evidence for a functional metabolic role of sleep comes from studies on gene expression in sleep and wake states. These studies have utilized recently developed methodologies that enable monitoring the expression of a vast array of gene transcripts simultaneously. To date, no technology is available to monitor the expression of all known transcripts expressed in the brain. However, the availability of experimental systems to monitor the expression levels of several thousand transcripts in a single tissue sample by microarray analysis has given sleep researchers plenty of information to digest. Studies in this area were motivated in part by the observation that there are changes in amino acid utilization as a function of sleep state: the rate of incorporation of amino acids into proteins in the brain is positively correlated with the occurrence of slow-wave sleep.109,110 Why study mRNA levels rather than the arguably more functionally relevant protein levels? The simple if unsatisfying answer is that we can study the levels of thousands of transcripts at once, whereas proteomics technologies (those that can measure a broad range of proteins of known amino acid sequence simultaneously) are in their infancy.111-113

Undoubtedly, there will be a review in *Sleep* on the sleep proteome in a few years, but, for now, let us consider what has been observed on a broad scale at the mRNA level.

The general strategy that has been employed in microarray studies is conceptually very familiar to sleep researchers. One simply asks the question, “What changes in gene expression occur during sleep deprivation-induced sleep loss and what changes occur during the subsequent sleep recovery?” Readers familiar with the history of the sleep field will immediately recall arguments against such an approach: sleep deprivation stresses the animal, sleep deprived rodents exhibit more locomotion than controls, sleep deprivation increases sensory stimulation above baseline levels, etc. If the reader suspends judgment on these issues, a number of intriguing observations can be made using such unbiased approaches. For example, it is apparent from these studies that sleep deprivation is a cellular-level stressor. A number of gene transcripts that are induced in other contexts by challenges to cellular survival, such as axotomy, viral infection, and abrupt changes in pH and temperature, are elevated in the cerebral cortex of animals that have been subjected to sleep deprivation relative to time-of-day controls.314,115 Also of note is that a number of genes related to synaptic plasticity are influenced by sleep.116,117

This observation is intriguing, particularly in view of the role of sleep in facilitating the performance of learned behaviors.118-121

In addition to the general question of sleep-dependent changes in gene expression, researchers are now beginning to explore variations on this theme. Knowing that noradrenergic locus coeruleus cell activity is sleep-state dependent,122 Cirelli and Tononi hypothesized that sleep-state dependent changes in gene expression in the cerebral cortex might depend on fluctuations of noradrenaline levels across sleep states.123 Using microarrays, they determined that during enforced wakefulness in the absence of noradrenergic innervation (ie, after chemical ablation of the locus coeruleus), several genes that are otherwise increased during wakefulness are no longer upregulated in the cerebral cortex. These genes are related to plasticity, signal transduction, cellular stress response, energy metabolism, and lipid metabolism. One gene that is downregulated during wakefulness (translational elongation factor 2) is disinhibited in the absence of noradrenergic innervation (ie, is upregulated in lesioned animals relative to control rats during enforced wake). The authors concluded that noradrenergic transmission in general suppresses the translation of mRNAs into proteins but activates the transcription of mRNAs and, thus, is responsible in part for the biochemical consequences of extended waking.

Given their increasing availability, gene expression microarrays (and perhaps similar techniques for protein analysis) will likely become commonplace in sleep research. Issues that could be addressed with this broad, open-ended approach include possible changes in gene expression underlying the development of tolerance to sedatives, individual differences in gene expression related to hypnotic or stimulant efficacy, and consequences of sleep apnea/hypoxia.111 Until it becomes possible to monitor gene expression in the human brain noninvasively, the clinical significance of these gene-expression assays will remain unknown. Even so, the knowledge gained with the gene expression approach, along with that from other genetic techniques, will no doubt continue to influence our thinking about the function of sleep and the nature of its disorders.

**FUTURE DIRECTIONS**

Since the mouse genome is estimated to contain approximately 30,000 genes, it can be expected that additional insights into...
the regulation of sleep and wakefulness will come from EEG studies of other knockout strains. In addition, newer techniques have been developed that allow gene deletion in particular cell types (cell-specific knockouts) or focal gene deletion in restricted brain areas in adult animals using the Cre recombinase/loxP system. This latter method avoids the problem of compensatory responses that may occur when the organism develops in the absence of a gene from conception. A particularly interesting twist of these technologies would be focal “knock in” of a gene in a localized brain region in an attempt to rescue the phenotype of a conventional “knockout.” Such techniques would go a long way to establish the circuitry that underlies the timing and functional correlates of sleep and wakefulness.

ACKNOWLEDGEMENTS

This work was supported by NIH R01AG02584, R01 MH61755 and R01HL59658. We thank Drs. Teresa Steininger and Paul Franken for their helpful comments on this manuscript.

REFERENCES

35. Hirose M, Egashira S, Goto Y, et al. N-acyl 6,7-dimethoxy-1,2,3,4-


37. Xi M, Morales FR, Chase MH. Effects on sleep and wakefulness of the injection of hypocretin-1 (orexin-A) into the laterodorsal tegmental nucleus of the cat. Brain Res 2001;901:259-64.


75. Beuckmann CT, Sinton CM, Miyamoto N, Ito M, Yanagisawa M. N-type calcium channel alpha1B subunit (Cav2.2) knock-out mice display hyperactivity and vigilance state differences. J Neurosci 2003;23:6793-7.


77. Vyazovskiy VV, Deboer T, Rudy B, Lau D, Borbely AA, Tobler I.
Sleep EEG in mice that are deficient in the potassium channel subunit K v.3.2. Brain Res 2002;947:204-11.


124. Scammell TE, Arrigoni E, Thompson MA, Ronan PJ, Saper CB,


149. Hunsley MS, Palmer RD. Norepinephrine-deficient mice exhibit normal sleep-wake states but have shorter sleep latency after mild stress and low doses of amphetamine. Sleep 2003;26:521-6.


156. Bonney LG, Simeck-Duran F, Sanford LD. Exploration and sleep in RIM1a knockout (KO) mice following exposure to novel environments. Sleep 2004;27:A384.


The weight is over.

The ResMed **Mirage Swift™ Nasal Pillows System** is here... but patients will barely know it.

Mirage Swift is so light, so flexible, and it works!

- Feather-light at approximately 2.5 oz. (70 g)
- Four tube positions for more sleeping positions
- Soft and secure headgear design
- Superior seal

The weight is over. Contact your ResMed rep now and see the Mirage Swift Nasal Pillows System.

www.resmed.com  Waking people up to sleep