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President's Column

As my SRS presidency is ending in June, I wish to publicly thank the members of the Executive Committee for the time and energy they have devoted to our society. Through the monthly telephone conference calls the committee has been actively involved in deliberating and guiding the business of the SRS. Beyond the elected leaders are the members who volunteered to serve on the various task forces of the society. I greatly appreciate the help and support that I received from everyone.

As to the business of the society, the new Executive Committee members will assume office in June at the annual meeting. Congratulations to our new leaders: President-Elect, Ralph Lydic, Ph.D., Secretary/Treasurer, Merrill Mitler, Ph.D., and the Section Heads Robert Sack, M.D., Joyce Walsleben, Ph.D., Richard Bootzin, Ph.D., and Chiara Crelli, M.D., Ph.D. A total of 565 voting members received ballots and 208 (36%) of the ballots were returned. Also, a thank you is due to those who were not elected, but were willing to stand for election and serve the society had they been elected.

The SRS Executive Committee approved the formation of a joint SRS-ASDA Animal Rights Task Force. The charge to this task force is to promote the continued ethical use of animals within the sleep research community and to educate the public regarding the ethical use of animals by sleep scientists. The Task Force has been meeting and has offered a number of proposals for our society's adoption. These all are efforts directed to publicly indicate that the members of our society take their ethical responsibilities in using animals to study sleep very seriously.

The SRS Executive Committee received the final report of the Vision 2020 Task Force and is currently evaluating the many recommendations made by the Task Force. The full text of the Task Force report is being made available on our society's home page. Recommendations range from the more mundane, but necessary for the efficient operation of our society, to those that are highly creative and protractive. One Task Force recommendation that the Executive Committee has implemented on an interim basis is the creation of a new membership category, the Associate member. This person is actively engaged in sleep research, but is not eligible for another membership category by virtue of the fact that she or he does not have a doctorate and is not currently a student. The dues are the same as those of the Emeritus member ($29), but this category does not have voting rights. After a trial period the success of this category will be evaluated.

It has been my privilege to serve the society.

Tim Roehrs
Editor's Column

This issue of the SRS Bulletin marks the 1st anniversary of my editorship. As with any editor, it is my goal that each issue of the Bulletin is an improvement on the previous. This evolutionary process requires input from the readers; you will have an opportunity in this issue to provide additional input in the form of a short Survey (see pages 6–7).

Trainees are one segment of the SRS membership we have decided should clearly benefit from receiving the Bulletin. We added the “Student – BITS” feature, under the editorship of Tim Hays to provide a mechanism by which trainees may communicate their ideas and insights about training for a future in sleep research. Additionally, we have in the past and will continue publishing information that is directly related to training in sleep, such as pieces from the SRS Training and Education Program.

We have also added the “Laboratory Spotlight” feature, in an effort to further disseminate information about what we do. There are interesting historical perspectives associated with the establishment of many sleep research laboratories that are not apparent when reading a primary publication. The “Laboratory Spotlight” provides a means by which the members of the SRS may learn more about the variety of programs and laboratories that comprise our “family”.

The items listed above are examples of some of the changes already implemented in the Bulletin. Another, important change begins officially with this issue; there will now be three issues to each volume. The Bulletin will be published in April, August, and December. The deadlines for submitting material to the Bulletin will be March 15, July 15, and November 15 respectively. We plan to develop an email broadcast mechanism to provide at least one reminder of the submission deadline prior to that deadline.

The quality and usefulness of the Bulletin is dependent on the needs of the SRS membership, the enthusiasm of the membership for the Bulletin, and the degree to which members are willing to contribute to the Bulletin. To ascertain the desires of the SRS membership concerning these issues, we have developed a very short survey, included in this issue. We urge each of you to take a few minutes to complete this survey.

Mark R. Opp

Letter to the Editor

Predictions for Gene and Protein Expression During Sleep

Dear Sir:

We enjoyed reading the recent issue of the SRS Bulletin and the series of articles on the genetics of sleep (SRS Bulletin, Vol. 4, issue 3, pages 38–50). We agree with your editorial that the use of molecular and genetic approaches to study the intracellular events associated with sleep will continue to grow. In a recent article (Shiromani, 1998), we reviewed the status of sleep research and suggested that molecular studies of sleep share many similarities with other areas of neuroscience, in particular circadian rhythm and feeding behaviors. In light of what has been found in the fields of circadian rhythm (Dunlap, 1998) and feeding (Friedman and Halaas, 1998), we believe that sleep researchers are also very likely to discover the following:

1. Gene and protein expression are associated with a specific behavioral state (i.e., wakefulness, SWS or REM) independent of circadian time.
2. Gene and protein are expressed in cell type(s) implicated in behavioral state control.
3. Feedback loops involving positive and negative elements control transcription/translation of specific genes.
4. These loops are activated by action of putative sleep factors (neurotransmitters, adenosine, prostaglandin D2, etc.) onto specific receptors.
5. The loops would regulate the duration and amount of sleep.
6. Manipulation of gene (mutation / knockout / knockin / inducible) has a distinct effect on the behavioral state.
7. Evolutionary conservation of gene/protein across species that sleep.

Sincerely,

Mary Ann Greco, Ph.D.
Dean Wagner
Priyattam J. Shiromani, Ph.D.

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SRS Bulletin Vol 5, Issue 1, April 1999
The Sleep Study Unit (SSU) was established in the Department of Psychiatry in 1977 by Howard Roffwarg, M.D., to conduct basic research on brain mechanisms and the functions of sleep, to study sleep architecture across the life cycle in humans and to investigate sleep disturbances associated with psychiatric disorders. Although the techniques and the personnel have changed over the past 20 years, the spirit of the work remains the same — to foster a multidisciplinary environment for the study of sleep.

Roseanne Armitage, Ph.D. is the current Director of the SSU. The Unit now includes the Animal Neurophysiology Lab (Gerald Marks, Ph.D.), the Animal Models Lab (Christopher Sinton, Ph.D.) and the Human Sleep Lab (Robert Hoffmann, Ph.D., Associate Director). John Herman, Ph.D. is the Director of the Sleep Disorders Center at Children's Hospital and is also part of our research group. Our work includes collaborations with more than 20 other faculty members and labs at UT SW, including Neurology, Biochemistry, Cell Biology, Epidemiology, Psychology, Brain Imaging, The Psychopharmacology Studies Group, Outpatient Clinics in Mood Disorders, Inpatient Psychiatric Units and the Dallas Veteran's Administration Medical Center.

Human Sleep Lab

In the 1980s, John Rush, M.D. and Dr. Roffwarg formed a Mental Health Clinical Research Center at UT SW focusing on the psychobiology of mood disorders, primarily depression. Research in the SSU was a core part of the Center, establishing abnormalities in the timing and distribution of REM sleep as characteristics of unipolar depressed patients and as predictors of treatment response. This research also led to studies of childhood and adolescent depression with Graham Emslie, M.D.

Building on this foundation, we then began to investigate abnormalities in sleep microarchitecture, based on computer analysis of EEG frequencies during sleep, first with depressed adults, and then in children and adolescents. We conducted a number of studies on EEG frequency characteristics and hemispheric asymmetries during REM and NREM sleep, tympanic temperature regulation and temporal coherence of ultradian (90 min) rhythms in sleep EEG. To date, we have quantitative EEG data on over 300 patients and healthy controls. Most of the sleep microarchitectural measures showed significant differences between depressed patients and healthy controls, even when groups did not differ on standard sleep measures. The most robust group difference was lower temporal coherence in the depressed group was characteristic of more than 70% of depressed adults. Our general interpretation of these findings is that low temporal coherence reflects a breakdown in the organization of activation and arousal in depression.

One of the most exciting findings was that sex differences were significantly greater among depressed patients than in healthy controls. Depressed women showed increased fast-frequency beta activity during sleep, greater hemispheric asymmetry and lower temporal coherence during sleep compared to both depressed men and healthy women. Depressed men, on the other hand, were more likely to show reductions in delta amplitude and an abnormal time course during NREM sleep.

Our research has also included psychopharmacology studies (with Madhukar Trivedi, M.D.), evaluating treatment effects on sleep EEG regulation and the impact of serotonergic antidepressants in depressed men and women. These studies have shown that low temporal coherence persists into clinical remission and that there are significant sex differences in the effects of fluoxetine on sleep EEG in depressed adults. Moreover, a pilot study in 50 patients with early onset depression indicated that the sex differences in temporal coherence were evident in adolescents but not children with depression. We hope to follow up these findings in a larger sample of children and adolescents. A recent study (with John Rush, M.D. and Mark Fulton, M.D.) has also indicated that low temporal coherence is evident in about 25% of individuals for are at risk for depression, based on family history, but who have yet to express the symptoms of depression.

Collectively, this work suggests that sleep EEG dysregulation is characteristic of both symptomatic and remitted depressed patients and thus, may reflect a continuing biological vulnerability to new episodes of depression. In addition, low temporal coherence may be heritable and may identify those who are at risk for the first episode of depression. These studies also provide strong support that the pathophysiology of depression differs for men and women. Sex differences appear to begin at puberty and persist throughout adulthood, suggesting that gonadal hormone regulation strongly influences the organization of sleep EEG in those with depression. The developmental time course also coincides with the increased risk for depression among females throughout the reproductive years.
Our current studies address the specificity of low temporal coherence to depression, *i.e.*, whether sleep EEG dysregulation is present in other psychiatric disorders, the heritability of temporal coherence, and the clinical correlates of sleep microarchitectural abnormalities in depression. One of our major goals is to determine why women are at twice the risk for depression than men, and what are the mechanisms that underlie sleep EEG dysregulation. Part of the direction of our future work will be to evaluate sex differences in neurotransmitter regulation during sleep and the potential interaction with gonadal hormones in depressed patients and healthy controls, and in more basic work in rodents (See Animal Models Lab). Beyond the study of depression, we hope to more fully characterize sex differences in sleep EEG regulation, an area that has received little attention in either human or animal research.

**Animal Models Laboratory**

Based on data from the Human Sleep Laboratory showing lowered temporal coherence in depressed patients, we have been measuring EEG coherence in male rats with an algorithm very similar to that used in human subjects. This is a first step in the development of a possible animal model of depression. Starting from the abnormal stress response of depressed patients and based on the premise that stress and coherence may be linked, we have exposed rats to the mild stress of prolonged isolation while sleep EEG microarchitecture was being quantified. Plasma corticosterone was measured concurrently to confirm that social isolation activated the stress response under these conditions. We have found that coherence measures are affected by stress in the rat and that some of these parameters may show strain differences. To date we have identified a significant ultradian coherence rhythm of about 12 minutes, which could play a role in the rat similar to that of the 90 minute rhythm in humans. Studies are now continuing in female rats to examine sex differences in EEG coherence parameters. We are also planning to extend this investigation to perinatal stress exposure, which we predict will induce chronically lowered temporal coherence as seen in depressed patients.

A second research theme in this laboratory has been the study of the link between energy balance and sleep, which may also have implications for understanding the pathogenesis of depression. We have found that leptin, an adipose tissue-derived circulating hormone, decreases REM sleep time but increases the duration of slow-wave delta sleep in rats and that these effects are negated by a few hours of prior food deprivation. Based on previous published studies, we have interpreted these data as demonstrating the coupling between REM and NREM sleep to the somatotropic and HPA axes, respectively. Since depression is characterized by an abnormal link between REM sleep and the HPA axis, the interactions between leptin and somatostatin and/or CRF at a hypothalamic level may be affected in this disorder. In other words, the dysregulation of endocrine feedback mechanisms in these patients may impact energy balance and appetitive behavior as well as sleep. These studies are now continuing in rats exposed to perinatal stress to examine how this treatment will affect the relationship between energy balance and sleep.

**Animal Neurophysiology Laboratory**

The Animal Neurophysiology Laboratory has a well-established interest in basic neural mechanisms underlying alterations in thalamic activity accompanying the sleep/wake cycle. Intrathalamic interactions, as well as the interactions between thalamus and cortex, are popular models in which to study the cellular basis of state-dependency. Our studies have predominantly been in rat utilizing extracellular single-cell recording sometimes coupled with the iontophoretic application of receptor ligands to the neurons under study. We have found that the application of receptor agonists and antagonists can have selective effects on thalamic activities revealing the synaptic interactions subserving specific state-related neuronal firing patterns. Studies in rat also have uncovered species differences in the expression of activity in certain neuronal populations that appear to relate to the absence of interneurons in some thalamic nuclei in rat. We are very interested in pursuing the comparative approach to the study of neural mechanisms. Recently, we have begun to adapt our methods to the study of the mouse. Our plan is to investigate thalamic activity in murine strains genetically engineered to not express specific ion channels. We have examined mice that are deficient in the expression of the voltage-gated potassium channel, Kv3.1. We find that gross expression of sleep and wakefulness is not affected. However, slow wave activity in the EEG is moderately reduced and fast activity in the gamma frequency band is greatly increased. We plan to study both thalamic and cortical mechanisms in these phenomena. In an independent line of work, we have been investigating brainstem mechanisms of REM sleep in rats. Our goal is to understand the role played by neurons of the brainstem reticular formation in the control of REM sleep. We build on the finding that small intracerebral injections of cholinergic agonists can result in long-lasting elevations in REM sleep. We have found that adenosine receptor agonists can induce these same effects as can an inhibitor of adenyllycylase when injected at sites supporting REM sleep increases by cholinergic and adenosinergic agonists. These findings implicate a role for the inhibition of cAMP in the control of REM sleep. Determination of the cellular properties of neurons involved in ligand-induced increases in REM sleep will aid in identification of the neural networks subserving REM sleep control.
Student BITS (Brief Insights for Training in Sleep)

The Student BITS segment is an ongoing forum of concept and insight for issues pertaining to sleep from a sleep trainee’s perspective. The articles found here will vary in subject matter and allow pertinent sleep-related questions and topics to be addressed and brought to the forefront of discussion and thought in the sleep community. Submissions are currently being sought for the all future issues of the SRS Bulletin.

If you are interested in submitting an article for Student BITS please contact Timothy Hays; Assistant Editor, SRS Bulletin: Student BITS; e-mail: hays@ucla.edu; phone: (818) 891-7711 ext. 7576; fax: (818) 895-9575; regular mail: VAMC 151A3; 16111 Plummer Street; North Hills, CA 91343

TAKE A LOOK TO THE MOON

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At the beginning of this New Year, I was contemplating the full moon during a quiet night and a thought came to my mind:

...what a beautiful moon. This moon has always inspired musicians as Mozart to create masterpieces such as “Eine Kleine Nacht Musik” and poets as Lovecraft and his everlasting story “In the mountain of madness.” Maybe this moon also inspired Freud to elaborate an interpretation of dreams. Letting my mind wonder, I also thought: Am I the only one that looks at the moon and ask for answers such as how and why we sleep?.... Or the magic formula for getting a Grant?...

The search for the function of sleep has proposed diverse answers to these questions, since ancient times. Using different approaches to solve this problem, researchers have soared to find a clue about the mechanisms involved in the generation of the sleep-wake cycle. Seventy years after Berger’s publication of “Über das Elektroencephalogramm des Menschen” (Berger, 1929) the application of the EEG remains as an essential tool for studying sleep since the EEG provides one crucial parameter for sleep analysis. With this tool now we know that sleep and wakefulness constitute one of the most important physiological phenomena in the animal kingdom, but what else?

We are aware that the development of the knowledge about the mechanisms involved in the sleep-wake cycle arose from Bremer’s animal preparations. We are also very well aware of Moruzzi and Magoun’s pioneer experiments attempting to describe a sleep center. In the same track, what can one say about the multi-cited Asersonsky and Kleitman’s paper in which they described the now most studied sleep phase: Rapid Eye Movement Sleep (REM sleep) and the well-known and now classical theory of sleep by Jouvet involving neurotransmitters as the crucial elements in the generation of sleep.

All the results of many experiments have the purpose to postulate a specific theory answering how and why we sleep. To cite a couple of examples, Dr. Smith’s hypothesis suggesting there is an increase of REM sleep after a learning session, or Dr. Siegel’s ideas on the evolution of REM sleep which indicates that the most primitive form of sleep is observed in monotremes. The controversial question, does REM sleep = dreaming was challenged by Dr. Foulkes suggestion indicating a similar rate of dreams reports from Non-REM sleep suggesting that brain activity during dreams also occurs during Non-REM sleep, and therefore it is not restricted to REM sleep. To finish my examples, the suggestions that the activities performed during wakefulness are the result of sleep’s architecture, according to Dr. Drucker-Collin’s theory. The other amazing line of research for an endogenous sleep substance was first postulated by the classical studies published by Piéron in 1913. These kinds of studies are the starting point of a different perspective of how and why we sleep. Diverse groups have pursued to explore the generation of sleep mediated by sleep-promoting
substances. There is evidence suggesting that diverse molecules, like amines, proteins, acetylcholine and very recently by lipids, are modulating sleep. In short, we can find a considerable advance toward an understanding of the physiological, behavioral and neurochemical characterization of vigilance states and their function. Actually you can choose the theme of discussion because there is a lot of literature in the area of neuroscience. However, the questions of how and why we sleep elude our approaches.

However, all this knowledge is growing with the aid of diverse methodological techniques such as High-Performance Liquid Chromatography (HPLC) to detect the presence of new molecules that express under specific behavioral conditions. In vivo microdialysis studies are used to monitor the fluctuation of particular molecules across the sleep-waking cycle. Positron Emission Tomography (PET) is used to study the brain state associated with the states of vigilance, indicating a preferential activation of the pontine and midbrain structures during REM sleep. Extracellular and intracellular recordings in specific areas of the brain in naturally sleeping animals have revealed that these cells seem to regulate the vigilance states of the animal and perhaps humans. And last but not least, the fashion technique of this decade: the genetic studies, knockout animals, mutant and recombinant methods trying to find "the gene" related to the how and why we sleep.

The new technologies are commanding the directions of the research in area of neuroscience, especially in the sleep-waking field, and all these new methodologies are giving us new ideas to explore how the brain is while we sleep and generate new goals. You can close your eyes and try to imagine if Picron could have used all these techniques for his studies at his time! Well, this is the point where we make our presentation: We are a new generation of sleep trainees, with an overflow of new ideas and fresh perspectives. Right now we have direct access to the use of fancy technology so we can dream to find out 'how' and 'why' we sleep by using all of the experience of all the people that have thought how they can solve these questions (perhaps looking at the moon!). This has given us the information about sleep contributing to build one of the most exciting areas in neuroscience.

The most striking challenge to us is to integrate all the data, all the ideas and all the theories in only one perspective. We have the knowledge provided by Bremer's classical experiments of lesion through the modern and elegant studies in the area of genetics, but the question is: Are we capable of postulating the theory of how and why we sleep with all of this background? Yes, I know you are thinking: "...Ooops!" It is a big deal, but the cards are on the table, so let's play and let's contemplate the beauty of the moon.

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**Sleep Research Society Bulletin Survey**

In our ongoing efforts to further define a role for the SRS *Bulletin*, we feel it necessary to solicit feedback from the SRS membership, who are presumably readers of the *Bulletin*. The survey was developed to gauge a) the desire/interest of the SRS members for such a publication, and b) the willingness of individuals to contribute to its success. We also would like to know the preferred mechanism for its distribution.

There are eight (8) items that require merely "checkbox" responses, and it is anticipated that these responses may be completed in less than five minutes. The last Item (Item #9) provides an opportunity for your own comments/suggestions. This space should be used to convey to the Editor and to the Executive Committee any suggestions for improving the *Bulletin* in terms of content, features, etc. In short, this is your opportunity to address anything you feel important that was not covered by the eight survey responses. With regard to this last item, brevity and clarity count; please limit your comments/suggestions to 100 words, or less.

There are two (2) means to complete the survey. The first (highly preferred) is to complete the survey posted on the web. To do this:

1) Point your browser to:

http://psychiatry.utmb.edu/sleep

2) You will be prompted for a username and password. Please use the following:

   Username: sleep
   Password: snooze

3) When you get to the bottom of the survey, you will have the option to "Submit Form", or to "Reset Form". If you have completed the form to your satisfaction, just "Submit" it. If you need to correct a mistake, you may either do so directly at the particular response, or "Reset" the entire form.

The second (less preferred) means to complete the survey is to photocopy the facing page, complete the responses, use a separate sheet of paper for any additional comments/suggestions, and FAX both pages (without a cover sheet) to:

(409) 772-3511

Please complete the Survey by May 15, 1999. Results of the survey will be published in the next issue of the *Bulletin*. Your participation in this survey is greatly appreciated.

*Mark R. Opp*

*SRS Bulletin Vol 5, Issue 1, April 1999*
Sleep Research Society Bulletin Survey

1) What is your membership status in the Sleep Research Society (check one)?
   - Full Member
   - Emeritus Member
   - Trainee (Predoctoral / Postdoctoral Fellow / Resident)
   - Corresponding

2) There have been 12 issues of the SRS Bulletin since its inception in 1995. How many issues have you received?
   - 1 – 6
   - 7 – 12
   - none
   - don’t know

3) By which medium / mechanism would you prefer to receive the SRS Bulletin (you may check more than one response)?
   - Hardcopy, distributed via regular mail
   - Electronic, distributed via email
   - Posted on a Web site

4) Please indicate by checking the appropriate boxes which of the following features / types of information you would like to see in the SRS Bulletin.
   - President’s column
   - Editor’s column
   - Laboratory Spotlight
   - Student Bits
   - Reports from the director of the Center for Sleep Disorders Research
   - Reports from the National Institutes of Health (or other federal agencies)
   - Reports from the National Sleep Foundation
   - Job listings
   - Announcements of training opportunities
   - Notices of conferences
   - Conference preview (column from Program Committee Chair)
   - SRS Section updates
   - Website updates
   - Executive committee news
   - SRS essay contest announcements / winners
   - Taskforce reports
   - Meeting calendar
   - Call for papers for related meetings
   - Member news (promotions, appointments, awards, etc.)
   - Notice of RFAs and RFPs

5) Are you willing to submit the information necessary to publish the items listed in #4, as appropriate?
   - Yes
   - No

6) In addition to the items selected in #4, should critical review articles, which may include references and figures, be published in the SRS Bulletin (Check One. If you select “No”, please skip to Question #8)?
   - Yes
   - No

7) If you responded “Yes” to item #6, would you be willing to write a review article and have it published in the SRS Bulletin, knowing that any such article would not appear in citation databases (check one)?
   - Yes
   - No

8) Please respond to the following (check one only).
   - The SRS Bulletin, in its present format, meets a unique need for the sleep research community and should be continued
   - The SRS Bulletin has the potential to meet a unique need for the sleep research community, but not in its present format
   - The SRS Bulletin serves no useful function and should be discontinued

9) Please comment on any issues concerning the SRS Bulletin that you feel will improve the publication, including suggestions for changes in content and format (100 word limit. Please use another sheet).
PRACTICAL CONSIDERATIONS IN THE USE OF POSITIONAL CLONING

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INTRODUCTION

Recent success in cloning genes encoding components of the circadian clock have greatly accelerated research in the field of sleep research. Within a span of 10 years, our understanding of the mechanism of the circadian clock has shifted from emphasis on mutant drosophila to a set of fly/mammalian orthologous (functionally equivalent) genes that function together to form a closed circadian circuit (Darlington 1998). The powerful tool of positional cloning was a key tool in this series of discoveries. Despite the progress in understanding the molecular circuitry underlying the circadian clock, the basic molecular pathways that control the regulation of sleep are still largely a mystery. Narcolepsy is a sleep disorder causing fragmentation of sleep, abnormal manifestations of REM sleep and cataplexy. The disorder is likely to result from the disruption of normal sleep/wake regulation. The genetics of narcolepsy in humans is complex, with the majority of cases showing a genetic predisposition that must be triggered by environmental factors, and a minority of cases with familial aggregation. The autosomal recessive form of narcolepsy observed in certain dog breeds offers the opportunity to clone and study a factor underlying sleep/wake regulation (review in Faraco 1999). Our experience with using positional cloning in a non-traditional animal system has given us insights on the strengths and limitations of this revolutionary technique. In this paper, we discuss the factors that led to the development of the technique, and describe the strengths and limitations of each step in the process.

POSITIONAL CLONING

Positional cloning is a process in which mutant genes are isolated based solely on position, and does not require any knowledge of the biochemical function of the responsible gene (Collins 1991). The concept was once referred to as “reverse genetics” because, until the 1980s, the principal strategy for identifying the genetic basis of disease was to study the physiological or biochemical differences between mutant and wildtype individuals. Unfortunately, this strategy did not necessarily lead to the mutant gene. The power and success of positional cloning derives from several previous developments: the creation of a dense map of genetic markers spanning the genome (Botstein 1980, Gyapay 1994), the development of statistical methods for analysis of linkage between markers and disease phenotypes (Ott, 1974), and the development of large scale cloning and sequencing of DNA (Burke, 1987). Together, these techniques may be used to uncover the underlying genetic cause of a disease by using the location of the defective gene rather than the knowledge of its biological function. To date, this revolutionary technique has enabled the identification of 108 disease genes in humans alone (NHGRI).

MENDELIAN INHERITANCE AND LINKAGE

The theory behind pedigree analysis and positional cloning is based upon Gregor Mendel’s original observations. The first is that genes occur in pairs, with one homolog being passed to each gamete (independent segregation), and the second is that different gene pairs segregate independently of each other (independent assortment). Because most phenotypes studied by Mendel were caused by single genes, we typically call single gene disorders “Mendelian disorders”. The observation of independent segregation, followed from genes being located at a sufficient physical distance that it was likely that they would be separated by meiotic recombination. When two genetic loci are physically close it becomes less likely that they will be separated by a recombinational event: the loci are genetically linked. Linkage analysis compares the likelihood that a genetic marker will co-segregate with a mutant phenotype within a pedigree by virtue of linkage as opposed to random chance. When a genetic marker and disease gene are genetically linked, they must also be relatively physically close on a chromosome. A minimum critical region is defined through identification of recombinational breakpoints on either side of the disease locus. By definition, the mutation must lie between these two points, hence, positional cloning.

BASIC CONSIDERATIONS IN A TYPICAL POSITIONAL CLONING STRATEGY

SRS Bulletin Vol 5, Issue 1, April 1999
Phenotype

From the outset it is important to clearly define the phenotype to be studied and to determine the number of genes likely to be involved as well as the relative strength of the contributions of these genes toward the phenotype. In order for linkage analysis to be successful, there must be clear phenotypic criteria for inclusion in analysis. A common problem is the presence of relatively non-specific phenotypic traits associated with a disease. For example, symptoms of narcolepsy include SOREMps, sleep paralysis, and hypnagogic hallucinations, however these are nonspecific findings present in the general population or in other sleep disorders. For this reason, we have chosen to focus on individuals demonstrating narcolepsy with cataplexy, because this cataplexy is a pathognomonic, i.e. specific to narcolepsy.

For certain disorders phenotype may be clear-cut, however some genetic disorders have variable expressivity (severity of symptoms). Variable expressivity may be both interfamilial and intrafamilial. Variable expressivity within a pedigree may have a dramatic range: in the case of familial holoprosencephaly, it may vary from a single incisor tooth to complete holoprosencephaly including cleft lip/palate, cyclopia and perinatal lethality. This somewhat striking example illustrates the importance of carefully ascertaining the phenotype in all subjects. When a diagnostic finding has variable expressivity compounded by sporadic occurrence in the general population, it may be particularly difficult to perform linkage analysis. As an example, consider investigators attempting to study restless leg syndrome/PLMS. Although this trait appears to segregate as an autosomal dominant trait, RLS occurs at a relatively high frequency in the general population, this complicates the classification of subjects as affected or unaffected. This is further complicated by the fact that the age of onset is also quite variable.

Another confounding factor to be considered is the degree of genetic penetrance. For most recognized diseases, the predisposing genotype is both necessary and sufficient to produce the phenotype, and is therefore fully penetrant. Although the causes of non-penetration are incompletely understood, certain patterns are typical. For example, certain mutations may result in a subclinical phenotype, which can only be detected by specific tests. Incomplete penetrance may also be caused by widely variable age of onset for a disorder. In other cases, genes may play an important role in predisposition to the disease, and yet be neither necessary nor sufficient. For example, the overwhelming majority of human narcoleptic patients carry two specific HLA alleles DQA1*0102, DQB1*0602, yet this haplotype is not infrequent in the general population (for review see Faraco 1999). Furthermore, studies of monozygotic twins discordant for narcolepsy indicate that there must be environmental factors required to trigger the development of the disease.

Disease heterogeneity may also influence the clinical phenotype. Allelic heterogeneity is the result of different mutations of a given gene in different pedigrees. This often results in variability of the severity of the disease in different families. This same variability of phenotype could, however, be caused by locus heterogeneity (also called genetic heterogeneity), in which mutations at two distinct loci may cause very similar phenotypes. An example of this would be similar phenotypes caused by mutations in the human growth hormone gene or in the growth hormone receptor, both leading to dwarfism.

Taking these into consideration, it is possible that in the end, positional cloning may yield relatively little toward the understanding of sleep genes, at least in humans. The phenotypic effects of mutations or variations in such genes may be relatively subtle, or easily masked by the behavioral constraints of daily life. Consequently ascertainment of affected individuals may be difficult and measurements may be relatively complex, perhaps requiring EEG or other such tests. Such relatively small genetic effects, coupled with rare familial clustering may make these particularly challenging positional cloning targets.

Genotyping strategy

Once a strongly penetrant, sufficiently frequent trait has been selected, the next step is to select a genotyping strategy to identify the desired genetic linkage. There are several options available to investigators working with a species with a well-defined genetic map such as the fly, mouse, or human. For certain disorders, a candidate gene approach may be appropriate. Candidate genes may arise either from obvious biochemical or physiological information, such as the putative effect of the circadian CLOCK gene on human diurnal preference (Katzenberg 1998). In the positional candidate approach, genes are considered by virtue of cytogenetic or linkage data indicating that they map within a candidate region. Recent advances in mapping genes using radiation hybrid panels have resulted in the production of a map of 30,000 human genes (Deloukas 1998). This increasingly dense transcript map makes the positional candidate approach more and more fruitful.

The dense human and mouse genome maps also allow the choice of a comparatively simple genome wide scan for linkage. In 1980, restriction fragment length polymorphism (RFLP) markers were proposed as the first generation of DNA-based genetic markers (Botstein 1980). Although these proved to be very useful, RFLP markers are limited by relatively low levels of polymorphic information content, as usually there are only two versions, or alleles, present. RFLP markers are typed by Southern hybridization and thus require a relatively large amount of time and sample material for analysis.

During the late 1980s and 1990s, a second generation of DNA
markers arose. These are the simple sequence length polymorphisms (SSLP) or "microsatellite" markers consisting of di, tri or tetranucleotide repeats (Weber, 1989, Edwards 1991). These markers are ubiquitously distributed through the genome, are easily identified through oligonucleotide hybridization, and are highly polymorphic. A polymorphic (CA)$_n$ repeat occurs approximately every 30-40 kb in the human genome. These markers are typed using the polymerase chain reaction (PCR) and thus require only trace amounts of DNA, and are amenable to automation and multiplexing. The current high-density genetic maps of human, mouse and other organisms have been built using these microsatellite markers. Thousands of these have been positioned on the human genetic map. For genome-wide linkage scans, there are commercially available sets of evenly distributed SSLP markers with very high heterozygosities. Typically, a set of 300 markers spaced 10-20 cM apart is used. The same reagents may be used for either a random genome-wide scan, a directed screen surrounding a candidate region, or a combination of both. Along with the many advantages of the SSLP markers, there are certain drawbacks. Microsatellite markers have relatively high spontaneous mutation rates, which may not be surprising considering their highly polymorphic nature. Therefore they have limited use in tracing ancestral recombinational events. It is also important to include sequencing ladder size standards and controls on every gel so that linkage disequilibrium and association studies may be performed later.

Unfortunately, not all organisms have a high-resolution genetic map suitable for use in positional cloning. Investigators then need to develop alternative schemes for identification of linkage. In these cases, the candidate gene approach may be a good first attempt, and there are myriad candidates to choose from. Current large scale sequence projects have filled the human genome database with 7,700 confirmed genes and 39,000 putative genes (expressed sequence tags, ESTs), many of which have been placed on the combined genetic and radiation hybrid human map (Genome database weekly totals, Deloukas 1998, Gene map 98). Nevertheless, such a strategy would be based on cross species Southern hybridization, rely on the identification of RFLP markers, and be subject to all the inherent limitations.

The RAPD (random amplification of polymorphic DNA) method is frequently used by scientists studying microorganisms and plants to identify markers and to develop species-specific PCR fingerprints. The method is simple, PCR-based, and does not require a pre-selected probe, but results in the isolation of anonymous polymorphisms in the form of present or absent bands in a gel. The bands must be isolated and sequenced in order to learn anything about their identity. This method has been successfully used to identify polymorphic markers in the dog (Olivier 1999).

Several groups have proposed methods to directly identify markers linked to a phenotype. The first is genetically directed representational difference analysis (GRDA) (Lisitsyn 1994). In principle this method could be applied to any organism, because it does not require prior knowledge of the chromosomal location of the gene controlling the trait or the availability of a pre-existing genetic map. The method is based on the representational difference analysis subtraction technique (Lisitsyn 1993). In RDA, DNA from two individuals is first digested with restriction enzymes. Digested DNA from the "tester" sample is annealed with a vast excess of "driver" DNA that has been modified so that it may be selectively removed in subsequent steps. In the next step, a PCR reaction is performed to amplify reannealed restriction fragments that contain only tester DNA. By amplification of tester DNA and removal of driver DNA (which will include homoduplex driver DNA as well as heteroduplexes between driver and tester DNA), there is a stepwise enrichment for restriction fragments found only in the tester DNA genome. The GRDA method uses the principles of transmission genetics to create appropriate tester and driver samples for subtraction resulting in enrichment of restriction fragments genetically linked to the phenotypic trait present in the pooled tester DNA. Although GRDA has been used successfully in inbred strains of mice, the RDA technique is notoriously difficult, and in the best of cases, produces two or fewer markers, even when the genetic region is large.

Another method related to GRDA is called TRS: targeted RFLP subtraction (Corette-Bennett 1998). In theory this is also a generally applicable positional cloning method that circumvents the need for a genetic map. The technique is based on Restriction Fragment Length Polymorphism (RFLP) Subtraction, a method that produces a large number of RFLP markers spanning an entire genome. The TRS method combines a phenotypic pooling strategy with RFLP subtraction to isolate markers from a specific region. As in GRDA, the phenotypic pooling in TRS masks markers from all regions of the genome except those in the region directly surrounding the mutation of interest. The pooled sample has properties resembling those of a DNA sample from an extensively backcrossed line without actually requiring the actual genetic crosses. This method has been quite successful in haploid organisms with relatively low genomic complexity. The usefulness of this method for complex mammalian genomes remains to be tested.

The critical region

Once a region of linkage has been identified, the localization must be confirmed and refined by using more densely spaced markers and more individuals. Linkage may lead to a genetic region of 1 cM, a distance that is interrupted by recombination 1% of the time. Genetic distances have only a rough correlation to physical distances. The typical correlation quoted is approximately 1 megabase (1000 kb) per centimorgan (cM), but there are many places in the genome
where 1 cM is considerably larger than this. In mammals there may be 30-50 genes in one megabase, therefore it is essential to define the critical region as narrowly as possible.

When the limit of resolution allowable by linkage analysis has been reached, (i.e., no further recombination can be observed) other methods must be used to refine the minimum critical region. This is achieved by genotyping a dense set of markers on a large set of individuals. When marker and disease loci are close together on a chromosome, genetic crossing over will occur only infrequently and a particular marker allele may be segregating with the mutant gene more frequently than expected, a situation called linkage disequilibrium. Allelic association is a manifestation of linkage disequilibrium. When a single ancestral mutation is responsible for the majority of cases in a population, (also called the founder effect) the same allele will appear in association with the disease in unrelated families. Linkage disequilibrium and allelic association can be very useful in defining the ancestral haplotype of a mutation in relation to a number of different marker loci, and can be used for fine mapping a critical region of interest to an interval of less than one centimorgan. Observed linkage disequilibrium is limited by the allelic frequencies at each locus as well as mutation rates at the marker loci. The degree of inbreeding in a population strongly affects the physical size of a region of disequilibrium which is an important confounding factor when using relatively inbred animals such as the particular breeds of dogs used in our studies.

Building the contig

The next step is to isolate the critical region in a series of overlapping contiguous DNA clones (contig) and the production of a high resolution physical map. This is often started in parallel with linkage disequilibrium studies. The process of contig building is highly dependent on the availability of large insert genomic libraries and associated genomic resources. In the early 1990s the large-insert cloning vector of choice was the yeast artificial chromosome (YAC) and these were important to the exceptional progress in genetic and physical map development (Burke 1987). Although YAC vectors are capable of carrying inserts of 1 Mb size, the inserts frequently undergo deleitional rearrangement. The clones were also frequently chimeric, including a patchwork of large DNA segments from different parts of the genome. The current cloning vectors of choice are the BAC/PAC vectors (Shizuya 1992, Pierce 1992). These vectors carry inserts of approximately 200 kb, but do not suffer from the problems of instability or chimerism.

Typically a critical region is cloned by sequentially using the ends of cloned inserts as probes to identify the next set of overlapping clones in a process called walking. For mouse or human contig building, there are commercially available large-insert libraries. Some of these libraries are available for screening as a set of filters with clones robotically spotted in a high-density array. After screening these libraries by hybridization, the investigator may order stats of the resulting positive clones. Other large insert libraries may be screened even more easily and quickly by PCR of available pooled clone DNA. The desired clones are identified by successive rounds of PCR on increasingly specific pools of target DNA. In this manner, large contigs may be developed in a relatively short period of time.

Investigators attempting positional cloning in non-standard animals again face a significant hurdle when contig-building. In our case, there was no generally available large insert genomic library for the dog. Some investigators, particularly those studying specific dog genes, had built genomic cosmid libraries. But these libraries were not available as gridded arrays, and had relatively small insert sizes (30-50). Thus for our purposes of building a multiple megabase sized contig, we decided to form a collaboration and construct a high quality gridded dog genomic BAC library (Li, et al 1999).

Regional candidate genes

When the minimum region containing the mutation has been isolated in clones, one must identify and sift through all the genes in the region, and then find the causative mutation. This is often the rate-limiting step in a positional cloning project. Typically, a series of methods are employed including cross-species conservation screening, gene identification through CpG island isolation, exon trapping, direct DNA selection and informatics-based methods based on DNA sequence. These methods differ in effectiveness.

CpG island isolation is based on the observation that the promoters of many eukaryotic genes contain multiple methylation sites. These exist as clusters of methylated cytosine residues within CpG dinucleotides. Such genes may be identified by searching for hypermethylated areas within a contig. There is bias in the type of genes identified and so this method is of limited use for cloning disease genes.

Exon trapping relies on the presence of RNA splice signals present within the majority of eukaryotic genes, and is a useful technique that has been used to identify a number of human disease genes including the Huntingdon’s disease gene (Buckler, 1991, The Huntington’s disease research collaborative 1993). The method allows direct isolation of gene sequences from cloned DNA without knowledge of gene structure or spatial/temporal patterns of expression. Fragments are cloned into various “trapping” vectors and transfected into mammalian cells. Exons present in the fragment are transcribed and spliced to vector sequences and then identified using PCR methods. This method is limited by the inherent differences between 5’, internal and 3’ exons, which require different trapping vectors. Exons from many genes that are irrelevant to the disease process are identified with this method because it is blind to expression pattern. Finally, there are associated problems with cryptic splice sites within the vector, and genomic DNA, which lead to clones...
with no corresponding transcripts.

Direct selection of cDNAs encoded by large genomic fragments is also a highly successful strategy (Lovett, 1991). In this method, an entire population of cDNAs is modified so that it may be amplified using PCR. The highly repetitive elements contained within the cDNA pool are first suppressed with blocking DNA. Next, cDNA is hybridized in solution to biotinylated contig DNA. Fragments of genomic DNA hybridized to cDNA may then be captured, producing an enrichment of greater than 100,000-fold in two successive steps. Although this strategy requires large quantities of cDNA, it is more successful with large genomic clones than other methods and resulting clones are more likely to represent biologically relevant genes.

DNA sequence analysis is also an important tool in gene discovery. When the target region is of a reasonable size, complete sequencing may be considered, although frequently a statistical sampling of sequence is obtained instead. Sequencing requires the use of expensive tools and reagents, but the process is quite straightforward, and offers complete ascertainment of the genes in the region. Genes may be identified in DNA sequence through homology search methods and through feature recognition. Search for homology with known sequences in current databases is a simple procedure using web-based interfaces tools such as BLAST (Altschul 1990), however a high percentage of newly discovered genes encode proteins that have no recognizable homologs in current databases. Therefore tools that detect features such as exons, or protein coding sequence like BLASTX and GRAIL are also used (Gish 1993, Uberbacher 1991). The feasibility of DNA sequencing is determined by the availability of funds for reagents and equipment as well as the density of genes in the region of interest. Genomic regions with high GC sequence content may have one gene per 20 kb, but some gene-poor regions may have as little as one gene per megabase (Fields 1994, Xu 1995).

Candidate gene to disease gene

The search for disease-causing mutations follows the identification of candidate genes in the region. Mutations are distinguished from DNA polymorphisms by functional consequences, cosegregation with disease in a family, and absence in unaffected individuals. Mutations that cause a significant change in gene structure such as large deletions or inversions or expansions are often easily identified through Southern analysis. Frequently, disease-causing mutations may result in slight changes in gene structure, from small insertions or deletions to single nucleotide changes that cause missense, nonsense or splice-variants in the RNA. The search for these subtle changes in sequence is complicated by the presence of the normal allele in dominant disorders.

Mutation screening methods are typically based either on cleavage of heteroduplex wildtype/mutant DNA molecules or on detecting conformational changes produced in mutant DNA molecules. Chemical cleavage offers a high detection rate in large (kilobase) lengths of DNA, but there is differential cleavage of transition vs. transversion mutations. The process is also technically challenging and makes use of highly toxic reagents (Cotton, 1988). The most popular method of mutation screening is SSCA, or single strand conformational analysis (Orita 1989) because of its simplicity. Short single stranded (denatured) DNA molecules assume different conformations based on sequence differences. When a polymorphism or mutation is present, the corresponding DNA migrates as a shifted band during electrophoresis. Although the procedure is simple, the mutation detection rate is highly dependent on the location of the mutation in the fragment being analyzed. Sensitivity is enhanced by the use of several different electrophoresis conditions and by the analysis of very small DNA fragments (150-300 bp). The heteroduplex analysis and denaturing gradient gel electrophoresis (DGGE) methods derive from the differential electrophoretic mobility of DNA fragments containing heteroduplex-bubbles (Sheffield 1989, Keen 1991). DNA from normal and affected individuals is denatured and allowed to reanneal. Polymorphisms or mutations will produce a mismatch within the hybrid molecule. The mismatched area is susceptible to further “melting” during electrophoresis and cause the fragment to move more slowly through the gel, producing a shifted band. These two methods allow analysis of slightly larger fragments than SSCA, but the fragments are still limited to less than one kb. Specialized equipment and PCR primers are also required for DGGE analysis.

FINAL CONSIDERATIONS

Even with an identified mutation and disease gene, it may be difficult to determine how the gene or mutation leads to the disease process. Frequently there are no obvious clues to the biological function of the gene, or the functional consequences of the mutation. In some cases, the identification of the disease mutation is difficult when several “candidate” gene polymorphisms are observed within a region of interest. Different mutations may be identified within the same locus in affected individuals when allelic heterogeneity is present. Functional studies may be required to demonstrate that the putative mutation is involved in the disease process. The gold standard in this case is to demonstrate that a similar mutation can produce the corresponding phenotypic effect in the entire animal, or that the wildtype version of the gene can provide phenotypic rescue. This is usually achieved by; developing genetically modified animals such as those containing a transgene encompassing the region of interest.

From the start, the positional cloning process depends upon a good disease model with good genetic transmission properties. For the unlucky researcher, this phenotype may manifest in an inconvenient animal model with scarce genomic resources.
The process is always unpredictable, and may be quick, or may take much time and until the mutant gene has been identified, there is little to show for so much work. Despite these caveats, it is clear that positional cloning is enormously powerful and has caused a revolution in science and medicine.

REFERENCES


Genome database weekly totals http://gdwww.gdb.org/gdbreports/CountGeneByChromosome.html


Keen, J Lester, D Ingleheran A, Curtis A Bhattacharya, S 1991 Rapid detection of single base mismatches as heteroduplexes on hydrolink gels. Trends Genet 7:5.

Lisitsyn, N Lisitsyn, N, Wigler, M, 1993 Cloning the difference between two complex genomes, Science 259, 946-951.


NHGRI Inherited disease genes identified by positional cloning http://genome.nhgri.nih.gov/clone/

Olivier M, Meehl MA, Lust G 1999 Random amplified polymorphic DNA (RAPD) sequences as markers


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**SPECIAL NOTICE!**

There will be a special "Meet the NIH" session at the 13th Annual APSS meeting in Orlando. Several Program Directors from NIH institutes that sponsor sleep research will be present. This will be an excellent opportunity, particularly for young investigators to learn more about the specific interests of these institutes, the appropriate funding mechanisms, and to meet personally with Program Directors. All are encouraged to take advantage of this opportunity. Look for additional details in Orlando!
Call For Participants

1999 APSS

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THE FOURTH ANNUAL TRAINEE SYMPOSIUM SERIES
Preliminary Program

8:30-9:00am Opening & Introductory Remarks
Key Note Speech: "The Future of Sleep Research"
Michael Chase, Ph.D.

10:15-12:00 Panel Discussion: "Determinants and Implications of Sleepiness"
David Dingess, Ph.D.

12:15-1:30 Meet-the-Mentors Luncheons
"Career pathways in medical school departments…"
"Chasing dreams: Research into the psychology of sleep;"
"Hypothalamic mechanisms in sleep;"
"Insomnia: Where do we go from here? Why bother?"
"Research in sleep of geriatric populations"
"Sleepiness and drug effects;"
"Transition from graduate school to a post-doc;"
"Transition from a post-doc to a junior faculty position;"
Ruth Benca, M.D., Ph.D.
Rosalind Cartwright, Ph.D.
Dennis McGinty, Ph.D.
Daniel J. Buyse, M.D.
Sonia Ancoli-Israel, Ph.D.
Timothy Roehrs, Ph.D.
Julie Carrier, Ph.D. & Jonathan Wisor, Ph.D.
Gina Poe, Ph.D. & Michael Perlis, Ph.D.

1:45-4:00 Interactive Workshops
Trainees may attend 4 workshops of their choice:

- Basic Neuroanatomy of Sleep
- Circadian and Sleep/Wake regulation of Endocrine Function
- Developing a Good Curriculum Vitae
- Ethics in Science
- Manuscript Preparation for Journal Submissions
- Melatonin: Research and Clinical Use
- Negotiating the Sleep Boards and Sleep Disorders Fellowship Training
- Optimizing Your Presentation Skills
- Scoring and Staging Polysomnography
- Spectral Analysis

Ronald Szumusia, Ph.D.
Charles Ceisler, Ph.D., M.D.
Dale Edgar, Ph.D.
Kenneth Wright Jr., Ph.D.
Thomas Roth, Ph.D.
Robert L. Sack, M.D.
Rochelle Goldberg, M.D.
Clete Kushida, M.D., Ph.D.
Sharon Keenan, RPSGT, Ph.D.
Paul Franken, Ph.D.

5:00 - 6:30 Trainee Reception

Note: Attendance to the symposia series is limited to trainees and individuals new to the field of sleep.

For program updates and more information, please visit our website at:
http://www.websciences.org/trainee/APSS.htm

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