UNDER CONSTRUCTION

Circadian Rhythms Although nine genes have been proposed to be components of the mouse circadian clock, definitive genetic evidence for their participation is not available for the majority of these genes. For example, the null mutants of *mPer1* and *mPer3* produced by gene targeting do not revealed an abnormal circadian phenotype. To understand the function of the known genes implicated in circadian regulation and to discover new circadian genes, we will screen 10,000 progeny of mutagenized mice per year for circadian rhythms by measuring locomotor activity rhythms.

CIRCADIAN ASSAY

Specific Aims:

In the past few years, tremendous progress has been made to identify genes which appear to be key components of the mammalian circadian clock. Although nine such genes have been identified, definitive genetic evidence for their participation in circadian clock function is not available for many. In addition, the search for circadian clock genes has not been exhaustive and it's likely there are more than just nine genes governing circadian behavior. We have previously used a mutagenesis and screening approach to identify mice with abnormal circadian phenotypes due to dominant or semi-dominant mutations. Here in the Center for Functional Genomics we use a mouse production strategy which will allow us to identify both dominant and recessive mutants.

We will assay 10,000 mutant mice per year for abnormal circadian phenotypes as determined by wheel running behavior. Data collected will measure four major circadian phenotypes: 1) the free running period of locomotor activity, 2) the phase angle of entrainment to the light-dark cycle, 3) the robustness or period amplitude of the circadian rhythm of activity and 4) the average daily level of activity. With this screen we will identify novel circadian rhythm mutants which will identify both new circadian rhythm genes and new mutant alleles of previously cloned circadian rhythm genes. Both types of mutants will help to elucidate the molecular mechanisms underlying mammalian circadian behavior.

Background and Significance

Many biochemical, physiological and behavioral processes oscillate with a daily rhythm. Under normal environmental conditions, these rhythms are entrained to environmental cues, such as the light-dark cycle. In constant darkness, however, these rhythms free run with a period close to, but not exactly, 24 hours. Organisms as diverse as cyanobacteria, plants, insects and mammals all share similar clock mechanisms and several share similar clock components.

Genetic control of circadian rhythms The first mutation known to affect the circadian clock in mammals, *tau*, occurred spontaneously in the golden hamster [74]. Twelve years later *tau* was cloned through considerable effort [75]. In contrast, a study using ENU mutagenesis in the mouse identified [9] and cloned [76,77]. The first mammalian circadian rhythm gene, *Clock* within a considerably

shorter period of time. The other seven mammalian circadian rhythms genes (*Per1, Per2, Per3, Timeless, Bmal1, Cry1* and *Cry2*) have been identified by various biochemical and bioinformatics methods using *Drosophila* as a guide. The circadian function of only three (*mPer2*, *Cry1* and *Cry2*) has been genetically verified by the creation of targeted mouse mutants.

Assays for Circadian Behavior. In selecting a phenotypic assay for the circadian clock that could be used in a high throughput screen for abnormal circadian rhythm phenotype, there are two primary considerations. First, the assay of choice should be rapid, non-invasive, inexpensive and capable of automation. Second, the assay should be a true reflection of the function of the circadian clock underlying the measurable rhythm. In mice, wheel-running behavior satisfies both sets of considerations. The discovery of *tau* and *Clock* [9,74] were identified by altered wheel running activity. Wheel-running meets the criteria of a good circadian measure because it allows simple, continuous, automated data collection via computer over many cycles under both entrained and free-running conditions, and it allows for easy quantification of various rhythm parameters. Alterations in the basic characteristics of the circadian rhythm of activity are indicative that the central clock and the rhythms under the clock's control have been altered. Thus, wheel-running rhythms are excellent markers of a functioning mammalian circadian clock.

Experimental Plan

The screening protocol we will use on G3 mice will be one we have successfully used to identify the *Clock* mutant. The screen is extremely efficient since it is fully automated and enables us to collect data on circadian phenotypes from hundreds of animals at a time. The basic experimental procedure involves the continuous monitoring of the rhythm as measured by wheel running behavior in two different phases. During the first phase of the assay, the mice will be placed in a cage with a running wheel and maintained under the same LD 12:12 cycle they have entrained to since birth. After collecting data for 7 days under LD conditions, the animals will be released into constant darkness (DD) for a period of 20 days. The animals will then be returned to the LD cycle for other phenotypic screens.

We will screen for animals showing one or more abnormal circadian phenotypes in wheel running behavior, as measured by free-running period, phase angle of entrainment to the LD cycle, period amplitude and average daily activity levels. Each of these measurements is extracted from wheel activity records using ClockLab software (Actimetrics, Evanston, IL) which allows for the automated collection and analysis of the data. Animals showing circadian phenotypes that are more than 3 standard deviations from the mean quantitative measure for that phenotype will be further analyzed to determine if the abnormal phenotype is heritable. Each of the specific phenotypic assays is described below in more detail.

Free Running Period Once normal mice are transferred from a LD cycle to DD the rhythm of wheel running behavior will continue to free run with a period of about 24 hrs. Since there may be some after-effects of the LD cycle on the endogenous nature of the free-running period, it's necessary to monitor rhythmicity for an extended period of time. We have chosen a period of 20 days

in DD as a compromise for accurately determining circadian period and in the necessity to move the animals through all the screening procedures in a timely manner.

Circadian period can be extracted from the constant darkness portion of wheel activity records by either Chi^2 periodogram analysis or linear regression analysis of the onsets of activity. Both of these analytical methods are optimized in ClockLab.

To date, the single most important measurement used to detect circadian rhythms mutants has been free running period. Both the hamster *tau* and the mouse *Clock* homozygotes' periods differ from those of wild-type animals by almost four hours. However, important mutations may also be identified with less extreme period phenotypes. For example, period measurements from *Cry1* and *Cry2* knockout mice only varied from wild-type mean period measurements by one hour**.** We will select animals that show a free-running period that is more than 1 hour from the mean (6 standard deviation units).

Phase Angle of Entrainment The phase angle of entrainment reflects the difference between the onset of activity and the onset of the dark period in nocturnal rodents. In addition to determining this value while the animals are exposed to the LD cycle, the value is also determined by examining the onsets of daily of activity after the transfer to DD to verify that the exposure to the light was not masking the true onset of activity. This is accomplished by determining the linear regression of activity onsets for the first 7 days in constant darkness and extrapolating this line to the last day of the LD cycle.

Animals with an abnormal phase angle of entrainment could be carrying a mutation affecting a photoreceptor or some component of the input pathway to the circadian pacemaker. A mutation in the period of the circadian clock itself could also lead to a change in the phase angle of entrainment, and this would be directly assessed when the animals are transferred to DD.

Circadian Amplitude In order to assess the amplitude of the dominant circadian component for the activity rhythm, power spectral density of the circadian peak by fast Fourier transformation (FFT) will be measured. Period amplitude is a measurement of the robustness of a rhythm. Low period amplitude could reflect an unstable circadian clock or a mouse with low activity levels. An extremely low amplitude indicates that the animal is arrhythmic. Animals with a period amplitude greater than 3 standard deviations from the mean of the wild-type mice will be considered an interesting potential mutant for further studies.

Average Daily Activity Levels The fourth phenotype of the circadian rhythm of wheel running behavior measured is the average daily activity level during constant darkness. This is calculated by summing the total number of wheel revolutions during DD and dividing by the number of days in DD. Low activity levels could indicate other physical abnormalities with the mutant mouse and

serves as a assay for general physical robustness. Additionally, low activity levels may also correspond with lower circadian amplitudes, demonstrating the inter-relatedness of the circadian measurements.

Preliminary studies

As previously noted, we have successfully used a mutagenesis and phenotype screening approach to first identify a circadian mutant mouse and then used progeny from this mutant to clone the first mammalian circadian clock gene. In this proposed mutagenesis screen we will also use C57BL/6J mice; a mouse strain which shows robust circadian rhythmicity under both LD and DD conditions.

As a first step in our mutagenesis screen we have begun to more thoroughly characterize the circadian behavior of wild type C57BL/6J mice. Twenty-four wild type male and twenty-four wild type female C57 BL/6J mice where placed on running wheels as described above and the four major circadian phenotype measurements were collected and plotted as frequency histograms (Appendix II, figure F). Circadian measurements from wild type male and female mice were not significantly different, so the data from the two sexes was pooled. These wild type mice ran with a free running period of 23.70 $\pm$ 0.117 hrs. Note that the standard deviation for this measurement is only about 7 minutes. These mice also entrained to the LD cycle at 17.14 $\pm$ 1.009 hrs. These mice are fairly robust runners as indicated by their amplitude (16% + 5.3) and total activity (32,686 $\pm$ 8746 counts/day) measurements. The robust nature and small variability in both the free running period and phase angle of entrainment indicates that this assay and this strain are ideally suited for a mutagenesis/screening program aimed at screening for abnormal circadian clock phenotypes. We plan to assay at least 100 wild type males and 100 wild type each year to obtain wild type baseline data throughout the 5-year screen..

We have also analyzed wheel records of G1 mutant mice. Circadian phenotypic measurements were collected from 175 G1 female and 180 G1 male mice. The results are plotted as frequency histograms (Appendix II:E). We noted a significant difference in wheel running activity levels between the sexes, so we analyzed the male and female data separately. Both G1 males and G1 females ran with a free running period of about 23.6 + 0.17 hrs and a phase of entrainment of about 17.7 hrs. Again, as with the wild type mice, amplitude (~18-19%) and wheel running activity measurements were fairly robust.