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Searching for Genes Underlying Behavior: Lessons from Circadian Rhythms

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Abstract

The success of forward genetic (from phenotype to gene) approaches to uncover genes that drive the molecular mechanism of circadian clocks and control circadian behavior has been unprecedented. Links among genes, cells, neural circuits and circadian behavior have been uncovered in the Drosophila and mammalian systems, demonstrating the feasibility of finding single genes that have major effects on behavior. Why was this approach so successful in the elucidation of circadian rhythms? This article explores the answers to this question and describes how the methods used successfully for identifying the molecular basis of circadian rhythms can be applied to other behaviors such as anxiety, addiction, and learning and memory.

In the 1970's Seymour Benzer and his colleagues uncovered a remarkable number of genes that underlie neural and behavioral functions. They treated the fruit fly Drosophila with mutagens and systematically screened them for behavioral abnormalities (1, 2). The discovery, in one of these screens, of flies with mutations in the *period* gene—which show longer or shorter cycles of the flies' endogenous 24-hour clock—by Konopka and Benzer (3) remains the exemplar for genetic dissection of behavior (4). Why was the search for circadian mutants so successful, and why were unbiased approaches for gene discovery so important?

Today, we understand the molecular mechanism of the circadian clock in a number of model organisms ranging from bacteria to humans (5, 6). In retrospect, it is clear that the genes regulating circadian rhythms would not have been easily uncovered without the use of forward genetic screens (2). In each model organism (Drosophila, Neurospora, cyanobacteria, Arabidopsis, and mouse), novel pathways were identified by the cloning of circadian mutants (5). Even today, with the benefit of complete genome sequences, the function of most of these "clock genes" would have been difficult to work out without those screens because our preconceived notions of the properties of a clock gene were largely incorrect. For example, a long history of anatomical and physiological experiments in mammals [beginning with the localization of the central clock, the suprachiasmatic nucleus (SCN), in 1972 (7)] .indicated that clock genes should be tissue-specific and restricted to the SCN. In addition, it was assumed that clock genes would be transcribed in a circadian pattern. Both of these assumptions were incorrect, at least in part. The *Clock* and *Period* genes are expressed ubiquitously, and *Period*, but not *Clock*, is expressed in a circadian pattern (8-10). We now realize that clock genes are really housekeeping genes and are

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integral to the most basic functions of cells and that virtually all cells in the body contain cell-autonomous circadian oscillators (11-13).

The genetic screens for circadian mutants were successful because we have a deep understanding of circadian phenotypes and robust assays. Of the measurable parameters of circadian rhythms (period, phase, and amplitude), the choice of circadian period as a primary phenotype has proven to be key. Circadian period length is a fundamental aspect of the clock that can be easily and accurately measured by 24/7 automated monitoring (14). Parameters such as amplitude are inherently ambiguous because they can be influenced by processes downstream of the circadian oscillatory system (that is, by output pathways). Phase measures can also be ambiguous because phase can be influenced by changes in input pathways that entrain the oscillator. Under steady-state conditions, period length (even when measured at the behavioral level) is directly correlated with the period of the underlying circadian pacemaker system (15) and thus is a very sensitive measure of the rate-limiting molecular steps in the circadian pathway.

Another key to success has been the accuracy with which circadian period length can be measured. The onset of rhythms in activity--in particular, wheel-running by rodents--is a remarkably precise phenotype (14). The inbred mouse strain C57BL/6J, for example, shows an average period length for circadian wheel-running in constant darkness of 23.7 hours with a standard deviation (SD) of 0.17 hours, or 10 minutes (16). This is a relative standard deviation (RSD = SD/mean) of only 0.72% (Fig. 1A and table S1). [The RSD of circadian rhythms of individual mice is even lower, about 0.2%, which is second in precision only to the neural oscillator driving the electric organs in fish (17)]. Thus, in genetic screens more than 99% of C57BL/6J mice have circadian periods between 23.1 and 24.3 hours (which represents +/- 3 SD from the mean); and, any mutagenized mouse with a period outside this range is likely to be a mutant. (The precision of circadian rhythms in mice is strain-dependent, and C57BL/6J is one of the most precise for period length.) Indeed, phenotype-driven genetic screens based on period length have been the most successful for the discovery and functional assessment of circadian clock genes (2).

How can one apply what we have learned from circadian clock genetics to discover genes underlying other complex behaviors in the mouse? Over the past decade, my colleagues and I have systematically applied forward genetic screens in the mouse using the point mutagen, *N*-ethyl-*N*-nitrosourea (ENU), to find mutants that affect learning and memory, anxiety, locomotion, vision, and response to psychostimulant drugs (18). To select appropriate screens, we looked for behaviors in which the phenotype was well established and for which we had an understanding of the neural loci and circuitry underlying the behavior. We required that the behavioral assay be amenable to automated data acquisition. The phenotypic screen also had to be scalable to achieve a throughput of more than 200 mice screened per week, so that ~10,000 mice could be screened per year, which is needed to have a reasonable probability of recovering mutants. This requirement for high throughput places restrictions on the types of behavioral assays one can employ. Tests such as the Morris water maze for learning and memory are too time- and resource-intensive (19). Thus, contextual and cue-dependent fear conditioning assays (in which animals learn to associate fear with a particular environment or signal) have been the most practical to use in assessing learning and memory (18). Although rodents will self-administer drugs, mimicking human addiction (20), this method is also too cumbersome to use for screening. Instead, locomotor responses induced by psychostimulants have been used for drug addiction screens (18).

The behavioral assays chosen for mutant screening should be valid indicators for the behavioral phenotype of interest. Contextual fear conditioning requires both a hippocampal pathway for memory and an amygdala pathway for the fear/emotional component (21), but

the memory aspect of this task can be assessed separately. In our screens of mutagenized mice for genes required for long-term memory, we have tested for defects in the long-term component of fear conditioning (22). We already know that many long-term memory mutants in Drosophila are sensitive to gene dosage, indicating that these mutations may point to a rate-limiting step in the underlying memory process (23).

On the other hand, psychostimulant-induced locomotion and sensitization of this response are thought to be elements of the addictive response, but, unlike drug self-administration tests, this assay alone cannot assess addictive behavior (20). Thus, in such screens, one may or may not be able to recover mutants that affect addiction because addictive behavior is not directly measured.

The validity of the available screens may also be questionable for other behavioral phenotypes. For example, two common tests for anxiety, open-field behavior and the elevated-plus maze, use a measure of exploration or activity that can be easily influenced by many factors (such as ataxia or sensory changes) in addition to the anxiety phenotype that the tests are intended to assess. Thus, an important lesson learned from circadian mutants is that a deep understanding of the phenotype and a phenotypic measure (such as period length) that reflects rate-limiting processes are critical for the success of behavioral screens.

A second lesson, already well known from classical genetics studies in model organisms and humans and reinforced by the isolation of circadian mutants, is that point mutations can be more informative than loss-of-function mutations. Although loss-of-function mutations are necessary to prove that a specific gene is necessary for a phenotype, in practice such mutations can be limiting because the complete loss of the gene is often lethal. In addition, paralogous genes may take over the function of the deleted gene, and there may be other compensatory mechanisms. Hypomorphic and dominant negative mutations can circumvent some of these problems, and the recovery of an allelic series of mutations can also be informative for understanding the structure and function of genes. Indeed, the short-period, long-period, and loss-of-rhythm mutant alleles in the original Drosophila *period* locus indicated that *per* was not only essential for circadian behavior but also influenced the rate of the process (3). The strongest period-altering mutant alleles in mammals are caused by point mutations rather than loss-of-function alleles; for example, the *casein kinase 1e tau* ($Csnk1e^{tau}$) and the $Clock^{\Delta 19}$ mutations (8, 24) which produced the highest mutant deviations from the wild-type mean on a normalized scale (Z-scores) when compared with those seen with null mutations (Fig. 1A). Interestingly, loss-of-function mutations of *Clock* and *Csnk1e* have subtle phenotypes because paralogous genes have overlapping function (25, 26). Both $Csnk1e^{tau}$ and $Clock^{\Delta 19}$ harbor dominant negative mutations (26, 27), and the dominant negative action of these mutant alleles on paralogous genes explains their strong phenotypes. Given that there are worldwide efforts to create null mutations for every gene in the mouse genome in ES cells (28, 29), point mutagenesis with ENU provides a complementary and mutually reinforcing approach for expanding the allelic spectrum of mutations available in the mouse (29-31).

A third lesson from circadian screens is that the variance of the behavioral phenotype is critical for the discovery of strong mutants. When we compare the precision of a phenotype using the relative standard deviation, the circadian period phenotype is almost two orders of magnitude more precise than assays for fear conditioning, psychostimulant response, as well as other circadian parameters such as amplitude or activity level (Fig. 1A). The precision of the assay is key for the isolation of circadian mutants such as $Clock^{\Delta 19}$ or *Overtime* ($Fbxl3^{Ovtm}$) that caused mutant period phenotypes that were 6 to 10 SD (Z-scores of 6- to 10) away from those of wild-type mice (16, 32) (Fig. 1A), where there is virtually no overlap in the period lengths of the mutant and wild-type populations. For this to be the

case, the difference in the average values of the mutant and wild-type populations must exceed 6 SD (so that +/- 3 SD for each population will not overlap) (Fig. 1B). We have not recovered such strong mutations for other behavioral phenotypes in the mouse. The strongest effect sizes for fear conditioning mutants in mice are about 1- to 3 SD (Z-scores of 1- to 3) (33) (Fig. 1A; Supplemental Table 1). Under these conditions, mutant and wildtype mice have significant phenotypic overlap (Fig. 1, C and D), and it is not possible to score individual mice as either mutant or wildtype (unless each mouse is test crossed, which is very costly). These phenotypic effect sizes are similar to those of strong quantitative trait loci (QTL) which originate from naturally occurring allelic variants (34, 35) . In order to map such subtle mutants, quantitative genetic mapping approaches must be used because the mice cannot be scored qualitatively. The resolution of such crosses will be limited by the inherent ambiguity in quantitative versus qualitative scoring of phenotypic traits.

An additional problem is that mutations can be suppressed or modified by genetic background (36, 37), a common issue with behavioral mutants. In order to locate mutagen-induced mutations, the mice need to be crossed with other strains of mice that differ enough from the mutant strain to provide informative genetic markers. QTLs that are independent of the behavioral mutation can segregate in these mapping crosses, confounding mapping of the mutant locus (35, 38). Thus, there is a tradeoff between choosing a strain for mapping that is divergent enough to provide sufficient polymorphic markers and the increased likelihood of QTLs arising from the divergent genetic background. Typically, one chooses other standard inbred mouse strains for mapping crosses because of the availability and high resolution of genetic markers. For example, in a typical cross of C57BL/6J to other common inbred strains such as A/J, BALBc/J, or DBA/2J, half of the available microsatellite or single-nucleotide polymorphism (SNP) markers would be polymorphic. The variance of the phenotype in these hybrid crosses will increase because QTLs from the parental strains (Haldane's rule) will segregate in crosses with the induced mutation, exacerbating the phenotypic overlap and confusing identification of the mutant mice. To circumvent some of these issues, particularly the increase in phenotypic variance and the suppression of mutant phenotypes, one can use very closely related mouse strains for mapping. This approach was not feasible until recently because of the paucity of genetic markers between very closely related strains; however, with the availability of high density SNPs, even such related strains (C57BL/10J for mapping mutants isolated on a C57BL/6J isogenic background, for example) will have informative markers. The use of closely related strains can succeed in genetic mapping experiments when conventional approaches have failed.

A final consideration arises: If the phenotypic effect sizes of mutagen-induced behavioral mutants are as subtle as QTLs, then why bother with induced mutants instead of attempting to clone QTLs for the same phenotype (39)? The answer to this question is simple. QTLs or naturally occurring allelic variants are extremely difficult to clone because there are so many sequence variants between the parental strains of mice [about one polymorphism every 440 basepairs (bp) in high SNP regions] (34, 40). Thus, a typical QTL mapping experiment will narrow the region responsible for a phenotypic variation to ~40 Mb (34, 35), within which there will be literally tens of thousands of sequence variants. By contrast, with ENU-induced mutations isolated on an isogenic C57BL/6J strain background, there will be only about one sequence variant in 100,000 bp, and for overt (causative) mutations the rate is 10 times lower (one in a million bp of coding sequence) (41). The subtle phenotypic effect size (~1 SD), however, means that the mapping resolution of a mutation-induced allele and a QTL will be comparable because both must be treated as quantitative traits.

How can the mutation be found within an ~40 Mb region? Until recently, with a region this large, it would be extremely difficult to locate and clone the sequence alteration responsible for the behavioral defect unless one could find obvious candidate genes to analyze.

However, massively parallel sequencing (42) and targeted selection of genomic sequences (43, 44) now allow sequencing of *all* of the genes (exons and flanking regulatory regions) in a 50 Mb interval (400 to 450 genes on average in the mouse). The exome of 400 genes can be sequenced with current Roche 454 FLX sequencing methods at >25 times coverage in a single run; eight such samples could be sequenced in a single run by Illumina/Solexa (or Applied Biosystems SOLiD) sequencing at an even lower cost per sample. The sequence capacity of these instruments is already increasing so that the cost of brute-force sequencing of the exome of all genes in a large genomic interval will be reasonable and will permit the positional cloning of essentially any ENU-induced mutation in the future.

The prospects for forward genetic and positional cloning approaches to complex behavior in the immediate future are bright. ENU-induced mutants isolated on isogenic mouse strains with available finished genomic sequence will enable the identification of critical behavioral genes by brute-force sequencing of all candidate genes in a large genomic interval with sequence capture methods coupled with massively parallel sequencing. With these methods, even the most subtle behavioral mutants in the mouse can be successful targets for gene discovery, and the black box that underlies the genetic architecture of many complex behaviors will soon be opened.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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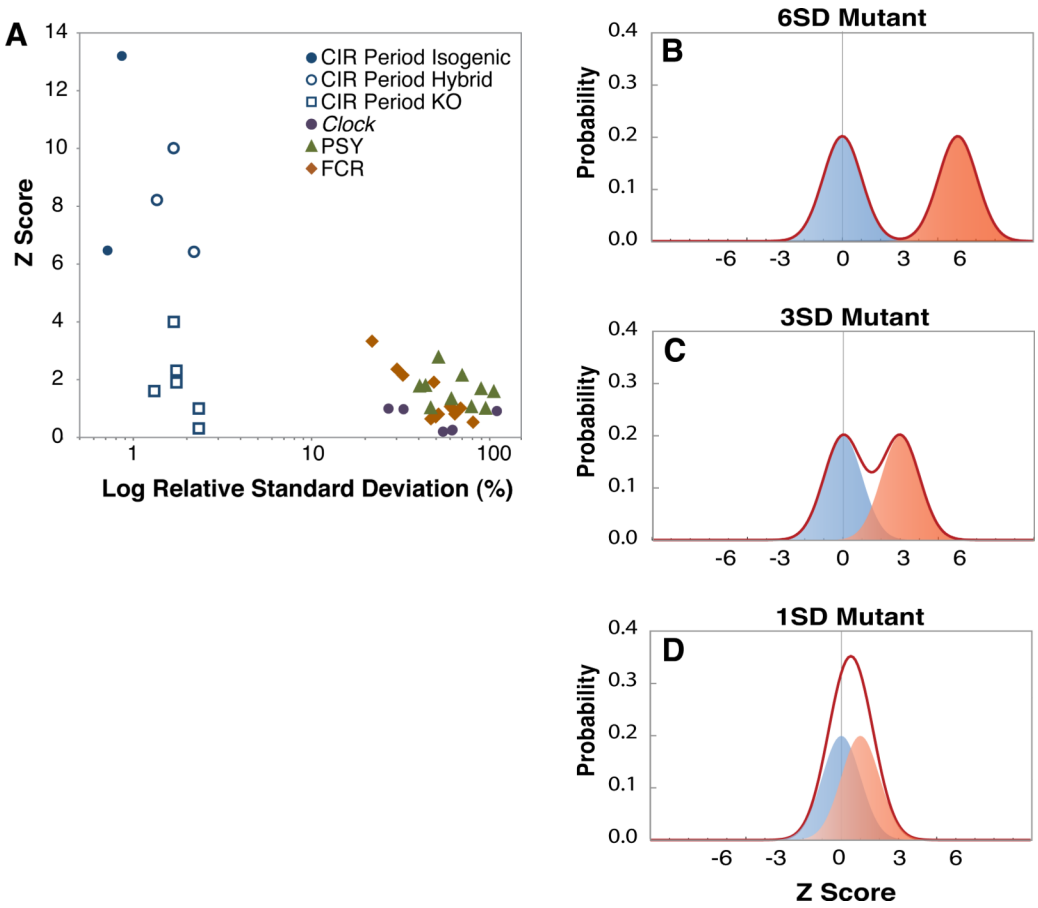

Figure 1.

(A) The relationship between the precision of the phenotypic assay (relative standard deviation, RSD, %) and the phenotypic effect size (Z score deviation from wild type) for circadian rhythm, fear conditioning and psychostimulant response mutants. The circadian period measurements (circles, squares) have the lowest RSD compared to fear conditioning (FCR-diamonds) and psychostimulant response (PSY - triangles) mutants as well as other circadian measurements such as activity level and circadian amplitude (purple circles). Circadian point mutants (closed circles) discovered in isogenic forward genetic screens have the highest mutant Z scores and the lowest RSD values. The same mutations in hybrid genetic backgrounds have slightly higher RSDs. Circadian knockout (KO) mutations (open squares, hybrid genetic background) have lower mutant Z scores compared to point mutants isolated in forward genetic screens. See Supplemental Table 1 for detailed information and references on mutants. **(B, C, D)** Phenotypic distribution of Z scores of wild type (blue shading) and mutant animals (red shading) that differ by 6, 3 or 1 standard deviations, respectively, from the mean of wild type. The graphs illustrate the expected 1:1 distribution of wild type and mutant populations for a backcross assuming a dominant mutation. Solid red line indicates the sum of the wild type and mutant populations.