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Large-Scale Mutagenesis and Phenotypic Screen for Nervous System and Behavior in Mice

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Abstract

Significant developments have occurred in our understanding of the mammalian genome, thanks to informatics, expression profiling and the sequencing of the human and rodent genomes. While these facets of genomic analysis are being addressed, analysis of *in vivo* gene function, however, remains a formidable task. Particularly relevant to the nervous system and behavior, evaluation of the phenotype of a mutant provides powerful access to gene function. Here, we discuss the complementary mouse genetic approaches of gene-driven, targeted mutagenesis and phenotype-driven, chemical mutagenesis. We highlight a NIH-supported large-scale effort to use phenotype-driven mutagenesis screens to identify mouse mutants with neural and behavioral alterations. Such single gene mutations can then be used for gene identification using positional candidate gene cloning methods.

Introduction

The Human Genome Project and related efforts have produced major advances in our understanding of mammalian genomes¹. The sequencing of the human, mouse, and rat genomes²⁻⁴ has opened the way for comparative analysis and identification of mammalian genes in ways not possible previously. Sequence search databases⁵⁻⁷ and comparison tools^{8,9} have developed rapidly to “mine” the genomes¹⁰. Tools for genetic and physical mapping of mammalian genes have also developed as a consequence of the Genome Project¹¹⁻¹⁴. Recently, new methods of examining gene expression patterns promise new insights into gene function^{15,16}. The rapid scientific progress in all these fronts holds the promise that the full complement of mammalian genes can be identified.

An inventory of genes, even genes that are expressed in the nervous system, however, does not provide for a complete understanding of the genetics underlying nervous system function or complex phenotypes such as behaviors. There is still a need for research to identify the functions of the expressed genes. Conversely, in many aspects of neuroscience there is evidence of genetic influence, yet the identity of these genes remains unknown. There are numerous and varied human conditions such as affective disorders¹⁷, autism¹⁸, addiction¹⁹, or blindness²⁰, for which the occurrences cluster in families yet the genetic alterations responsible for the familial vulnerability are unknown or poorly understood. Thus, there is an information gap between the genes expressed in the nervous system and the neuroscientific processes likely to be influenced by these genes.

Comparison and expression analysis tools primarily provide methods to infer gene function. Ultimately, however, confirmation of a gene's function results from demonstration of the phenotypic consequences of an alteration of that gene *in vivo*. Experimentally this can be accomplished readily in the mouse, in which efficient methods to induce mutations exist²¹. Mice offer an experimentally accessible genome with a high degree of homology to the human²². However, the number of genes for which the phenotypes of a mutant have been described is quite low²³. This lack of functional annotation of the mammalian genome has been termed the "phenotype gap"^{10,24,25} and is a particular problem for neuroscience²⁶.

Bridging the phenotype gap: Mutant Mice

Mutagenesis is undertaken with one of two goals in mind. First, in order to understand the mechanism of action of a gene thought to be an important component in a particular process, mutagenesis of that gene is performed and the mutant and wild-type animals are compared. This gene-driven, "reverse genetic" approach, has led to the understanding of the function of many genes and gene products²⁷⁻³⁰. Alternatively, mutagenesis is undertaken in order to identify genes that are essential for a particular process in the nervous system. This phenotype-driven, "forward genetic" approach begins with random mutagenesis, followed by screening of the mutagenized animals for a defect in the particular nervous system process under study. After mutants are identified, mapping of the mutant genes and finally cloning of the genes is performed. This approach was first demonstrated in *Drosophila* by Benzer and co-workers³¹ and has been useful to identify genes essential for processes as diverse as circadian rhythms³², development³³, and vision^{34,35}.

In the past, identification of genes causing mutant phenotypes by positional cloning has been an arduous process³⁶. However, with the availability of high quality genetic and physical maps of the mouse genome, as well as the near completion of "finished sequence" of the mouse genome (90% finished as of December 2005), positional cloning has been rapidly accelerated. The workman-like efforts of physical mapping and transcription unit analysis no longer are required. The remaining rate-limiting steps are the ability to generate mapping crosses with sufficient genetic resolution for positional candidate gene cloning. Overall, this has reduced the time (and cost) by about two years for each mutant.

Mutagenesis and screening offers several advantages. Usually, subtle point mutations rather than large deletions or rearrangements are generated by the mutagens in current use. These mutations are capable of causing a variety of perturbations of the affected function. One of the most useful of these is a dominant negative effect in which the mutant gene product interferes with the function of the wild-type gene product³⁷. Point mutations can produce an effect on the nervous system where 'knockouts' are without noticeable effect. Two examples of this are the *Weaver* mutation^{38,39} and the control of cardiac contractility by *PI3Kgamma*⁴⁰. In fact, the study of a series of alleles of a particular gene is of immense use in understanding the details of the function of the gene. Mutagenesis can produce a series of alleles that produce a variety of effects. One reason why this is possible is that mutagenesis and screening do not require assumptions to be made about the function of the gene under study; the process simply identifies affected mutant animals. A favorable consequence of this fact is that novel genes, that would not be identified based on homology or known functions, can also be discovered. For example, both the *Leptin* gene⁴¹ and the *Clock* gene⁴²⁻⁴⁴ were identified on the basis of mutant phenotypes (*obese* in the case of *Leptin*) that identified important components of processes in the nervous system.

Since both the genomic sequence and methods for directed mutagenesis are available, a systematic phenotypic assessment of targeted mutants could be undertaken. Indeed, such projects are currently underway, for example "The Knockout Mouse Project"⁴⁵. The difficult question of "What mutation should be made?" remains, however. The answer to

this question is difficult because, in the absence of knowledge of the gene's function, it is not possible to predict what effect a particular mutation will have on the function of the encoded gene product. This is a particular problem for 'knockout' or null mutations produced by gene targeting. A null mutation is a fundamental starting point for genetic analysis, however, many such mutants may either have no obvious defects, or alternatively result in embryonic lethality. The availability of point mutations have been very useful in these situations.

QTLs—the *Other* Forward Approach

Quantitative Trait Locus (QTL) analysis has been particularly productive for the mouse because there exist many inbred strains and thousands of DNA polymorphisms have been discovered among over 50 inbred mouse strains⁴⁶⁻⁴⁹. QTL studies focus on identification of the numerous loci that quantitatively (rather than qualitatively) impact phenotypes of interest. QTL approaches have been refined in recent years with methods allowing for identification of complex interactions^{50,51} and improvements in mapping^{46,52}. Like chemical mutagenesis, QTL analysis represents a forward, or phenotype-driven, genetic approach, but these two approaches are complementary rather than redundant.

QTL studies have some unique limitations. Mapping QTLs can be difficult, since between any two inbred strains there are usually many regions of the genome that differ, and even after one particular genomic region is isolated as having the responsible gene (i.e., after mapping the QTL) it is often difficult to find that gene. Other limitations of the QTL approach involve their quantitative nature: phenotypic effects are small and thus very large test population sizes are required to achieve statistical power. This statistical power limitation also impacts genetic mapping and ultimately gene identification. The greatest successes in identification of QTLs involve loci with substantial phenotypic impact.

Unlike single-gene mutagenesis approaches, QTL analyses focus on multigenic interactions and on existing (rather than inducing) genetic variants. Consequently, these two types of approaches can be complementary in elucidating the genetics underlying a neural process. In some instances, the presence of QTLs has become evident in the process of analyzing a mutation^{53,54}. Important modifying loci have been identified as a result, such as the *Mom-1* modifier of *Apc*^{Min55}. Thus, QTL analyses will ultimately be important to a full understanding of the genetic regulation of neurobehavioral phenotypes.

The NIH Neurogenomics Project at Northwestern University

Internationally, there have been multiple large-scale mutagenesis efforts to identify genes by phenotype, and thus by function. Other mutagenesis initiatives^{56,57} have necessarily been broad in scope, with only attempts to detect severe neurological impairments (ataxia, spontaneous seizures) or briefly monitor spontaneous behaviors⁵⁸. By contrast, The Neurogenomics Project is one of three mutagenesis centers specifically focused on neuroscience (Table 1). All three of these centers are supported by a trans-NIH initiative⁵⁹.

The overall objective of the Neurogenomics Project has been to produce, identify, and distribute mice with new neurobehavioral mutations in five phenotypic domains: Learning and Memory, Circadian Rhythmicity, Vision, Responses to Stress, and Response to Psychostimulants. New mutations are produced by chemical mutagenesis with N-ethyl-N-nitrosourea (ENU). Mice are bred to produce stable mutant lines, and these progeny are phenotypically screened for alterations in these domains to identify putative mutants ("putants"). The heritability and mode of inheritance of the traits are determined by breeding "putants". High-priority mutations are genetically mapped to low resolution (50-100 meioses). Data and information regarding the mice, as well as the mutants themselves, are

made freely available to the greater scientific community at our website (<http://genome.northwestern.edu/neuro>).

The Neurogenomics project's organization (Figure 1) and goals (Box 1) reflects our overall strategy-- to focus on five phenotypic domains within neuroscience for conducting a 3-generation recessive screen. These five screens are each "vertically" organized (Figure 1) and move from the primary screen to test-cross validation to follow-up phenotypic assays. The order of testing is designed so that the behavioral tests that are most easily compromised by the environmental and handling conditions will be tested first, in order to minimize interactions among tests. For example, the vision screen (electroretinogram) is performed after all of the other tests have been completed since it involves anesthetizing the animals.

To date, the Project has focused on a recessive screen involving three generations of breeding to produce homozygotes for the ENU-induced mutations. Third generation (G3) mice are produced using a backcross breeding scheme (Figure 2), wherein the Generation 2 (G2) females (daughters) are crossed back to the Generation 1 (G1) pedigree-founding males (sires). Each G1 pedigree represents the progeny of a single mutagenized gamete or "genome" from the original G0 mouse. The efficiency of scanning of the G1 pedigree can be calculated⁶⁰. We try to screen five G3 progeny for each of four G2 females for a total of 20 G3 progeny per pedigree, yielding an 85% probability of recovering a homozygous mutation at any locus in that pedigree.

Using the ENU mutagenesis protocol that we employ with C57BL/6J inbred mice, the forward mutation rate is estimated to be 0.015 mutations per locus per gamete (or 1 mutant in 650 gametes screened). To scan the genome, we have set a goal of screening 2000 mutagenized gametes, which corresponds to ~2.5X coverage at 85% efficiency. To screen 2000 gametes requires a total of 40,000 mice screened (2,000 pedigrees × 20 G3 mice per pedigree). To achieve this goal, we must have the capability of mutagenizing and screening 10,000 mice per year over four years. This rate is equivalent to screening 200 mice per week, hence all of our phenotypic screens require a throughput of greater than 200 mice per week. We have accomplished this goal by developing a number of new phenotypic assay systems using video-based data acquisition and analysis methods. Many of these phenotyping platforms are now available commercially. The systems used are summarized in Table 2. Further information regarding phenotyping protocols is also available at <http://www.genome.northwestern.edu/neuro/protocols.cfm>.

To maximize the genomic coverage (by increasing the number of mutagenized gametes contributing to our population screened) a dominant G1 screen is also being conducted (Figure 2). Dominant mutations are also of particular interest since these are unlikely to arise with a simple loss-of-function, thus these types of mutant alleles are functionally informative in a different way from a null. Heritability testing and mapping of dominant mutations is also more straightforward than with a recessive mutation.

As of June 2005, we have tested nearly 30,000 progeny of ENU mutagen-treated mice (Table 3), for a total of more than 130,000 phenotypic assays completed. Putative mutants have been identified in all phenotypic domains, and bred to test for heritability of the trait by "vertical" transmission (from a putative mutant line founder to its progeny). Breeding of "putants" also generates more carriers of new mutations, allows determination of the mode of inheritance, and can create a segregating generation to genetically map the mutations. Difficulty breeding "putant" lines has resulted in loss of some lines, although breeding of presumptive carrier family members or assisted reproductive technologies such as transplantation of ovaries from infertile mutant females have been useful.

Table 3 includes examples of mutants that have been identified from our screens. Until the mutations have been mapped and cloned, and the altered gene identified, it is not clear whether the mutant defines a new gene or represents a mutation in a previously identified one. Hence, all mutant lines are provisionally given a name descriptive of the mutant phenotype (see <http://www.informatics.jax.org/mgihome/nomen/> for mouse gene nomenclature information). Should the mutation prove to define a novel gene, this name could become the gene name (as was the case for *Clock*⁴⁴), else this name may become the allele designation. For updated details concerning the phenotypes or the progress of the screens, see <http://www.genome.northwestern.edu/neuro> or <http://www.neuromice.org>.

Phenotype, Phenotype, Phenotype

For such a phenotype-driven approach to be successful, the quality of the phenotypes studied is of central importance. A true understanding of the underlying physiology and significance of the phenotype is necessary. In developing our screening assay for circadian rhythms, for example, we benefited from decades of research which provided us with knowledge of the functional properties of the circadian pacemaker⁶¹ and thus enabled us to focus our phenotypic screen on a fundamental property, the free-running period in constant darkness. Had we limited our screen to examining the entrained behavior of the mice while housed in a light-dark cycle, the *Clock* mutant would not have been discovered⁴⁴.

In selecting our phenotypic assays for the NIH Neurogenomics Project, then, we applied strict criteria. The assay should directly measure a central aspect of the system targeted (as discussed above). Neurobehavioral domains were chosen for which we had access to expertise (Figure 1) to help insure this was true. The domains were also chosen to be mutually reinforcing—access to related phenotypes aid in data interpretation. The assays must be amenable to high through-put, with computerized data acquisition preferable. The assays should be rapid, and non-invasive so that mice can be bred after testing. The measures should be quantitative, with low variance, so that both high- and low-scoring outliers can be detected readily. And finally, the results from individual mice must be meaningful and informative, as a single individual in a screen may have a mutation of importance.

While our primary screen methods have been adjusted to satisfy the particular needs for high-throughput screening, other more conventional methods and involved assays are used to characterize putative mutants and mutants in detail. Thus, for example, progeny of animals with altered fundus or electroretinogram responses to light may be tested for visual responses to light or detailed histological analysis of the retina^{20,62}, or progeny of mice with altered psychostimulant response may be tested in a sensitization or conditioned place preference assay.

A Neuroscience Community Resource

Because this project was established as a resource-generating undertaking by a cooperative agreement with NIH, we expected that we would make all mutants available as widely and as readily as possible. To minimize complication that might produce delays, excessive intellectual property restrictions or restricted availability of unique resources, a determination of “exceptional circumstances” has been made relative to the Bayh-Dole Act⁵⁹. Hence, NIH retains IP rights to these mutants to insure that the mutants can remain publicly available. In this way, licensing or patent claims cannot become an impediment to their distribution to the scientific communities.

To facilitate the distribution of mice and the dissemination of information regarding the ENU-induced mutants, a web resource has been created at www.neuromice.org.

Neuromice.org is a consortium of the three NIH-funded neuroscience-focused mutagenesis centers, and provides a searchable, web-accessible database with information on mutants available at all three sites (see Box 2). As of December 2005, over 195 different mutant lines are available through Neuromice.org. These mutants represent valuable research resources in a number of ways.

Each mutant, because it was identified on the basis of an altered neuroscience-related phenotype, represents a unique functional “tag” to a mutated gene. In some cases this may represent a new allele of a known gene. Analysis of allelic series may shed light into the functions of different domains of a gene. In other cases, the mutations may link known genes to new functions, or may provide evidence of function for transcripts for which no function was known. In yet other cases, mutations may link to completely unknown genes, as was the case for *Clock*^{42,43}. Each mutant represents a neuroscience relevant gene-to-function link. Thus, identification of each of the genes mutated fits another piece in the neurogenomics puzzle. These mutant mice also have value as research model organisms that can duplicate human disease. ENU-induced mutations are generally point mutations. Thus, changes in function in only a single gene, or even in only a single domain of a gene, are possible. Further, a variety of changes in function are possible; these include partial loss of function, gain of function, even dominant negative (reverse) function⁶³. Targeted mutations most typically are complete loss of function, which account for only about 50% of cases in human genetic disease. Radiation-induced mutagenesis typically results in chromosomal rearrangements (deletions, translocations, inversions), affecting multiple genes in a region, which also represent a minority of human genetic disorders. Indeed, it has been estimated that as much as 70% of mutations with discernable phenotypes identified in humans are the result of single base-pair changes⁶⁴. ENU-induced mutations, thus, can be highly similar to those underlying human genetic diseases. For example, the ENU-induced *Noerg-1* mutation of *Rhodopsin*, which causes severe attenuation of the electroretinogram and retinal degeneration (Figure 3), results in the identical amino acid change documented in a case of human familial blindness⁶². Thus, ENU-induced mutations can model human disease with high fidelity.

Mutants with known functional alterations can be exploited to address biological questions. For example, analysis of the *Clock* mutant mouse, which was originally identified on the basis of its altered circadian rhythm phenotype⁴⁴, has allowed researchers to test hypotheses regarding the relationships between the circadian and other physiological systems, uncovering some surprising links. *Clock* mutant mice have been shown to sleep less⁶⁵, demonstrating a greater link between the circadian clock and sleep homeostasis. *Clock* mice have been found to have altered sensitivity to the chemotherapeutic agent cyclophosphamide⁶⁶, indicating a role for the circadian system, specifically the CLOCK/BMAL1 complex, in chemotherapy toxicity and target cell survival. *Clock* mutants have been found to be more active and exploratory⁶⁷. *Clock* mutants have greater sensitivity and sensitization to cocaine as well as alterations in dopaminergic neuron activity and dopamine expression and synthesis in the ventral tegmental area⁶⁸. *Clock* mutants also develop overeating, obesity, and aspects of metabolic syndrome⁶⁹. As the *Clock* mutant has been characterized from a variety of perspectives, it has become clear that this mutant may be a useful experimental organism for research in a number of aspects of behavior and physiology, beyond the circadian system. The same is likely to be true for other neurobehavioral mutants. For this reason, more than any other, distribution of the ENU-induced mutants so that they are studied by a number of researchers, is vital. It is in this manner that these mutants may achieve their heuristic potential as neurogenetic tools.

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Box 1. Goals of the NIH Neurogenomics Project**1. Conduct a three-generation recessive screen**

- Mutagenize C57BL/6J mice, using $3 \times 100 \text{ mg kg}^{-1}$ ENU
- Screen using a battery of neural and behavioral assays
- Organize the screen into five domains with 'deep' phenotypes
- Screen 10 000 mice per year (200 per week)
- Screen 40 000 mice in total (to cover the total mouse genome ~ 2.5 times)

2. Confirm mutants by vertical transmission of the phenotype**3. Map mutations at low resolution****4. Distribute mutant lines via www.neuromice.org**

- Ensure there are no intellectual-property restrictions on the mice

Box 2. [Neuromice.org](http://www.neuromice.org): a mouse resource center for neuroscientists

[Neuromice.org](http://www.neuromice.org) (Figure I) is an online ‘virtual store’ for mice with mutations affecting the nervous system and behavior. These mice are important model organisms for neuroscience and genetic research. Established by NIH, it is a consortium of three neuroscience-focused mutagenesis centers, formed to facilitate the distribution of the new mutant lines to the wider scientific community. Mouse lines available from all three centers are included in a single web-accessible database. Visitors to the site (<http://www.neuromice.org>) can browse through information such as detailed phenotypic descriptions, phenotypic testing protocols, chromosomal location or gene identity. After registering with the site (providing contact information such as their e-mail address), users can set up automatic searches and thus receive e-mail notification of new mouse lines matching their search criteria. Mouse orders also can be placed online. To date >160 different mutant lines with a wide range of neurobehavioral phenotypes are listed at the site.

[Neuromice.org](http://www.neuromice.org) represents a logical extension of the mission of the three consortium member centers through enhancing availability of research resources. These centers (the NIH Neurogenomics Project at Northwestern University, the Neuromutagenesis Program of the Tennessee Mouse Genome Consortium, and the Neuroscience Mutagenesis Facility of the Jackson Laboratory) were established as part of a trans-NIH initiative to generate tools and resources for mouse genetics, genomics and their application to other areas of biology (<http://www.nih.gov/science/models/mouse/>).

Figure 1. Organization of the Neurogenomics Project

Components comprising the major operational elements of the Neurogenomics Project are shown. Four cores at Northwestern provide support to multiple phenotypic screens: The Administrative Core, the Mutagenesis and Production Core, the Bioinformatics Core, and the Genetics and Distribution Core. The screens in five phenotypic domains occur in parallel: Circadian Rhythms, Learning and Memory, Vision, Neuroendocrine Responses to Stress, and Psychostimulant Response. All primary screens are conducted at Northwestern University. In addition, laboratories of different Project investigators were designated to follow up on putative mutants and confirmed mutants for each domain, with more extensive phenotypic characterization of the mutants. These investigators are: Joseph S. Takahashi, Northwestern University, Eric Kandel, Columbia University, Val Sheffield, University of Iowa, Lawrence H. Pinto, Northwestern University, Eva Redei, Northwestern University, and Marc Caron, Duke University.

Figure 2. Breeding Scheme for ENU mutagenesis and production of mice to screen for mutations
Adapted from ^{21,71}. At Generation 0 (G₀), ENU (3×100 mg/kg) is given to C57BL/6J (B6) male mice so that they will genetically transmit mutations (red M). These G₀ males are bred with wild-type (+) B6 females to produce G₁ mice. For a recessive screen, G₁ males each found a pedigree. They are first bred with B6 wild-type females to produce G₂ mice. G₂ daughters, 50% of whom will be carriers of a given ENU-induced mutation (M), are backcrossed with their G₁ father to produce G₃s. These G₃ mice can be screened for dominant and recessive mutations. For a dominant screen, G₁ mice that would be heterozygous for any ENU-induced mutations can be screened.

Figure 3. The *Noerg-1* mutation of *Rhodopsin*

Fundus photographs (upper) and electroretinograms (lower) of adult (75-96 day old) wild-type and mutant mice. Note the thinned retinal vessels and absence of electroretinogram in the mutant. Scale bars on each fundus photograph indicate 1 mm. The electroretinogram responses evoked by stimuli of different illuminations are indicated by different color traces as follows: red, 0.00066 cd·sec/m²; brown, 0.0083 cd·sec/m²; yellow, 0.158 cd·sec/m²; green, 0.331 cd·sec/m²; grey, 0.637 cd·sec/m²; pink, 1.19 cd·sec/m²; blue, 27.88 cd·sec/m². Details of the *Noerg-1* mutation are described in ⁶².

Figure I.
The logo of Neuromice.org

Table 1
Neuroscience-focused mouse ENU mutagenesis and screening centers

Center name, location and URL	Genomic approach	Phenotypic screens	Center director
Neurogenomics Project Northwestern University Center for Functional Genomics Evanston, IL, USA http://www.genome.northwestern.edu/neuro	Genome-wide recessive Using C57BL/6J mice Genome-wide dominant Using C57BL/6J mice	Overt Hearing Elevated plus maze Open-field behavior Fear conditioning Cocaine response Fundus Electroretinogram Circadian rhythms	Joseph S. Takahashi
Neuroscience Mutagenesis Facility Jackson Laboratory Bar Harbor, ME, USA http://nmf.jax.org/index.html	Genome-wide recessive Using C57BL/6J mice	Seizure threshold Auditory Vision and eye Overt Gait Home-cage activity	Wayne N. Frankel
Neuromutagenesis Project Tennessee Mouse Genome Consortium (TMGC) Memphis, TN, USA http://tnmouse.org/neuromutagenesis	Regional recessive Inversion or deletion stocks used to screen regions of chromosomes 10,15, 19 and X	Aging Hearing Cocaine response Ethanol response Seizure threshold Eye General behavior Neurohistology Social behavior	Daniel Goldowitz

Table 2
Phenotypic assay systems used in high-throughput neuroscience screens

Phenotypic domain	Assay	Notes	Software package
Circadian rhythm	Wheel-running behavior	Described in [70]	ClockLab ^a
Learning and memory	Fear conditioning	Video-image-based scoring of freezing	FreezeFrame ^a
Preliminary assessment	Open-field activity	Video-image-based scoring of exploration	LimeLight ^a
Preliminary assessment	Elevated plus maze	Video-image-based scoring of exploration	LimeLight ^a
Psychostimulant response	Hyperlocomotion behavior	Video-image-based tracking of locomotion	BigBrother ^a
Vision	Electroretinogram	Described in [20]	Developed by Lawrence Pinto and colleagues
Vision	Fundus photography	Described in [20]	Not applicable

^aSoftware from Actimetrics Inc. (<http://www.actimetrics.com/>).

Table 3
Progress of the Neurogenomics Project^a

Phenotypic domain	ENU progeny screened	Putative mutants	Putative mutant lines with progeny	Confirmed mutants	Representative mutants identified
General assessment	29 860	80	38	14	<i>baldy, compass, corkscrew, cue-ball, dali, dill, gandalf, pisa, tiresias</i>
Preliminary assessment	29 860	153	83	12	<i>Big Boy, butterball, lardy, Mighty Mouse</i>
Learning and memory	23 123	165	106	19	<i>fearful, Metus 1, No Fear 1</i>
Psychostimulant response	20 997	168	86	9	<i>High Response 1 High Response 2</i>
Neuroendocrine response to stress	13 118	126	54	2	This screen has been discontinued
Vision	15 582	108	60	6	<i>Noerg-1, biga-1, nob-4, smaller-1, smaller-2, smaller-3</i>
Circadian rhythms	12 157	363	225	46	<i>Overtime, part-time, Half-time, Past time, Time course, Time machine, Time share, Time Trial, Time Traveler</i>

^aNumbers are as in December 2005.