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N-ethyl-*N*-nitrosourea mouse mutants in the dissection of behavioural and psychiatric disorders

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Abstract

Twin and adoption studies have consistently implicated genetics in the aetiology of psychiatric and behavioural disorders. The identification of the genes and molecular pathways that are associated with these traits using linkage studies has been difficult because psychiatric disorders are almost always non-mendelian, heterogeneous, involve multiple genetic loci and are influenced significantly by environmental factors. Mouse models that are based on intermediate signatures of psychiatric disease and pharmacological responsiveness hold promise as a complementary approach to dissecting the molecular basis of neurobehavioural disorders. This has been made possible by the development and refinement of gene targeting technologies and the use of super-efficient chemical mutagens. *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis in the mouse, when coupled to a battery of sensitive behavioural screens, is an effective way of creating and identifying novel mouse behavioural mutants. Here, the concept of screening for ENU mutants is introduced while progress with two behavioural screens, an ''anxiety'' screen and a circadian screen, are presented. It is hoped that the study of mouse mutants that have arisen from these screens will provide new insights into the genetic basis of abnormal behaviour and that they might lead to the development of novel therapeutic compounds for human psychiatric disease.

Keywords: ENU (ethyl-nitrosurea); Animal model; Mouse mutant; Phenotype-driven screen; Disease gene

1. The mouse as a model of psychiatric disease

Twin and adoption studies have implicated genetics in the aetiology of attention-deficit hyperactivity disorder, autism, bipolar disorder, depression, anxiety disorders, and schizophrenia (Folstein and Rutter, 1977; Heston, 1966; McGuffin et al., 1996, 2003; Rietveld et al., 2003). Yet, despite the great advances that have been made in genetics in the last 50 years, the underlying causative factors remain largely unknown. Many groups have undertaken linkage studies to identify genes associated with psychiatric disorders and enormous resources have been invested pursuant to this objective, but the search has not been marked by overwhelming success. With the exception of a few rare mendelian disorders (Brunner et al., 1993; Lai et al., 2001), no genes have been conclusively identified that are involved in the pathogenesis of common psychiatric disorders (Inoue and Lupski, 2003). While it is likely that future studies will be more rewarding, many linkage studies to date have been of insufficient size, produced inconsistent results, and are rarely replicated. This is not surprising given that psychiatric disorders are almost always non-mendelian, heterogeneous, involve multiple genetic loci and more often than not are influenced significantly by environment. The task of identifying genes associated with neurobehavioural traits is made simpler, though still challenging, by turning to the mouse as a model organism.

The mouse is an appropriate model organism to investigate the genetic basis of psychiatric disorders. Its genome has now been sequenced, 99% of its genes have homologues in humans, mice and humans have common neuroanatomical features (e.g., hippocampus, cerebellum, cortex, medulla, amygdala), mice have a relatively short generation time, there are a wide range of inbred strains available and transgenic technologies are available to manipulate individual genes (Tecott, 2003). Yet the key advantage over other model organisms lies with the expanded

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behavioural repertoire of the mouse. Mice exhibit a much wider range of social and emotional behaviours in comparison to invertebrate model organisms.

One might, however, take the view that psychiatric disorders cannot be modelled in other organisms because they are by their very nature uniquely human. Is it really possible to replicate the delusions associated with schizophrenia? The self loathing of depression? or the paralysing fear of an anxiety disorder? These arguments are persuasive. It is clear that the differences between the two species prevents the replication of the complex multigenic phenotypes that define human psychiatric disorders. One can, however, gain an insight into the genetic basis of behaviour and therefore behavioural disorders by focusing on: (1) shared neurobiological characteristics (so-called endophenotypes) and (2) behavioural paradigms that have been supported by rodent and human pharmacological studies.

Endophenotypes are best thought of as intermediate signatures that are linked to a neuropsychiatric condition (Gottesman and Gould, 2003). It has, for instance, been shown that impaired sensorimotor gating and working memory (Braff et al., 2001; Cannon et al., 2001) are associated with schizophrenia, that reduced P300 event-related potential correlates with a high risk of substance abuse (Almasy et al., 1999) and locomotor hyperactivity is associated with attention-deficit hyperactivity disorder (ADHD, Castellanos and Tannock, 2002). By focusing on these quantitative parameters, which can be observed and assessed in both in humans and rodents, geneticists hope to gain an insight into the genetic basis of various behaviours. Mouse models based on responses to human pharmacological compounds are also useful. An example is the Porsolt swim test which is used to study depressive behaviour in rodents. This test measures the time mice spend swimming versus the time spent floating in a cylinder of water from which they cannot escape (Porsolt et al., 1977). When administered serotonergic antidepressants mice spend more time swimming, seeking an escape route, in comparison to the time spent floating (Redrobe and Bourin, 1998; Sanchez and Meier, 1997). The role of genetics in this behaviour is supported by studies that selectively breed rodents that exhibit high and low ‘‘depression’’ (El Yacoubi et al., 2003; Overstreet et al., 1992). While the Porsolt swim test is clearly not an objective measure of mouse contentment, (if indeed mice experience contentment), it is a useful tool to help us to understand the molecular events associated with an abnormal rodent behavioural phenotype, and this in turn may have some applicability to humans.

2. Spontaneous mouse mutants

The study of spontaneous mouse mutants has led to important insights into gene function and disease phenotypes. An example of a spontaneous mutant relevant to psychiatric disorders is the mouse mutant coloboma. Coloboma was first identified because it exhibited head bobbing, hyperactivity and ocular dysmorphology (Hess et al., 1992), and was later shown to exhibit altered hippocampal plasticity (Steffensen et al., 1996) and neurodevelopmental abnormalities (Heyser et al., 1995). Genetic mapping revealed that this phenotype was due to a deletion on chromosome 2 which contained the neuron specific protein, synaptosomal-associated protein 25 (SNAP-25, Hess et al., 1996). Reduced levels of this pre-synaptic protein result in abnormal release of neurotransmitters, particularly dopamine in the dorsal striatum (Raber et al., 1997). The link between SNAP-25 and hyperactivity prompted a number of groups to undertake association studies with ADHD (Barr et al., 2000; Brophy et al., 2002; Hess et al., 1995; Kustanovich et al., 2003; Mill et al., 2002). Several of these studies have found positive associations between various polymorphisms in SNAP-25 and ADHD. While these results remain to be replicated and the biological effect of the polymorphisms needs to be explored, the colomoba mouse has initiated an interesting avenue of research.

The value of spontaneous mouse mutants is further illustrated by the mutants ducky (Barclay et al., 2001), tottering (Fletcher et al., 1996), lethargic (Burgess et al., 1997), and stargazer (Letts et al., 1998). These mutants were initially identified as a result of their ataxic phenotypes, but more importantly, they were also shown to exhibit spike wave discharges, signatures of epilepsy (Fletcher and Frankel, 1999). Positional cloning revealed that all four had mutations in Ca^{2+} channel subunits, thereby implicating Ca^{2+} channels in the pathogenesis of the disease (Chioza et al., 2002).

An important lesson to be learned from the study of spontaneous mutants, one that is also apparent from human studies, is that single-gene mutations can affect multiple biological systems. Thus, careful and complete phenotypic analysis of mouse mutants (and potential mouse mutants) should provide a more complete picture of the disparate biological functions of a particular gene. However, a major drawback of studying spontaneous mouse mutants is that the rate of induction of mutations is extremely low making it difficult to study gene function on a systematic basis. To enable this, two complementary approaches have been used for some time to increase the rate at which mouse mutations can be generated and studied: (1) gene targeting and (2) chemical mutagenesis.

3. Gene targeting and reverse genetics

The use of homologous recombination in embryonic stems cells has permitted scientists to generate targeted mutations in specific genes. This approach is referred to as reverse genetics, as one begins with a particular hypothesis about the function of a gene and proceeds to study the phenotypic consequences of a targeted mutation in that gene. Gene targeting has revolutionised mouse behavioural genet-

ics, playing a vital role in the understanding of the molecular basis of memory (Reisel et al., 2002; Silva et al., 1992; Silva, 2003), aggression (Saudou et al., 1994), cocaine addiction (Kelz et al., 1999), Huntington's disease (Mangiarini et al., 1996), familial motor neuron disease (Gurney et al., 1994) and Alzheimer's disease (Shen et al., 1997), to name but a few. Transgenic technology is becoming increasingly flexible with the use of the Cre/LoxP recombinase systems which allow one to produce tissue specific conditional knockouts (Orban et al., 1992) and the tetracycline inducible system which allows one to control the temporal specific expression of a gene of interest (Lewandoski, 2001). At the time of writing, a search of Mouse Genome Informatics (http://www.informatics.jax.org/), a site co-ordinated by the Jackson Laboratory, indicates that some 4246 mice have been created using transgenic technologies. Interestingly, however, only 181 are reported to exhibit abnormal behavioural phenotypes. This highlights one of the disadvantages of reverse genetics. Because a prior assumption is made about the function of the gene, phenotypic assessment is routinely biased by these preconceptions, and as a result interesting phenotypes may be overlooked.

4. Chemical mutagenesis and forward genetics

Forward genetics differs from reverse genetics, as it begins not with an interest in a particular gene, but in a phenotype (e.g., an abnormal behaviour). Relying on chemical mutagenesis to produce random mutations, forward genetics has been used extensively in several species to study gene function by establishing screens for various phenotypes (Driever et al., 1996; Nusslein-Volhard and Wieschaus, 1980). Pioneering studies in screening specifically for behavioural mutants were introduced by Semour Benzer in *Drosophila* (Benzer, 1971), and since these seminal experiments several groups have developed behavioural screens in a variety of species and using different chemical mutagens. The choice of mutagen for screening programmes has been given careful consideration. For example, chlorambucil has been used in the past (Rinchik et al., 1990) but this chemical can induce chromosomal rearrangements such as deletions and translocations. Phenotypes associated with mutations induced by chlorambucil could be due to contiguous gene syndromes or to positional effects and could therefore complicate gene function studies.

5. The case for ENU

The chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) has been established as the most appropriate mutagen for gene function studies in the mouse. This preference is based on several properties specific to ENU. Firstly, ENU induces mutations in rodents at a rate that is far greater than that of other mutagens. Several groups have estimated that phenotypic effects associated with every mammalian gene could be detected by screening only ~1000 animals carrying ENU-induced mutations (Coghill et al., 2002; Hitotsumachi et al., 1985). In reality, this is rather optimistic, as it would appear that ENU induces mutations at different rates in different genes. The estimate also assumes that suitable assays could be implemented that could assess the phenotypic effects of any mammalian gene. This is unlikely to be the case as disruption of any number of genes could result in very subtle, or as yet undetectable, phenotypic effects.

A second important property of ENU is that it primarily induces point mutations in genes. Mutant phenotypes will therefore be consequences of mutations in single genes, simplifying genetic and biological experiments. Mutant phenotypes associated with point mutations will potentially be more appropriate as models of human inherited disorders as this type of mutation is often associated with human genetic disease. Of further significance is that ENU can induce not only null mutations, as in conventional gene knockouts, but can also induce mutations that result in partial loss of gene function, mutations that result in an upregulation in gene expression, and mutations where the mutant form of a protein can interfere with the function of the native protein (a dominant negative mutation). By comparison of multiple mutant alleles, it should be possible to map functional domains of proteins in a systematic fashion. For example, three new peripheral myelin protein 22 (*Pmp22*) mouse mutant lines, named tr-m1H, tr-m2H and tr-m3H, provide an allelic series of mutants on a common genetic background for gene function analysis (Isaacs et al., 2000, 2002). Mutations in PMP22 in humans are associated with many peripheral neuropathies. Comparison of the phenotype of each mutant line using behavioural and pathological assessment allowed the mutants to be clearly distinguished in terms of severity of disease, which in turn could be correlated with a unique pattern of PMP22 intracellular localisation.

As ENU mutagenesis is primarily based on a phenotype-driven approach, it should be possible to associate abnormal phenotypes with previously uncharacterised genes or in genes where this phenotype/genotype correlation had not previously been identified. This has already been shown to be the case for several ENU mutants and is equally relevant for mutants with behavioural and/or neurological phenotypes. The circadian rhythms gene clock (Antoch et al., 1997; Vitaterna et al., 1994) was identified in an ENU mutagenesis screen and has provided an important entry point into the molecular basis of biological rhythms. More recently, through positional cloning projects, mutations in dynein have been associated with motor neuron degeneration (Hafezparast et al., 2003) and a mutation in *Af4*, a gene previously implicated in leukaemogenesis, results in a cerebellar ataxia with an associated cerebellar Purkinje cell degeneration (Isaacs et al., 2003).

6. Implementing an ENU mutagenesis screen

A number of institutes worldwide have established mouse ENU mutagenesis screens with the goal of modelling human disease and prescribing a function to each mammalian gene. Many groups have incorporated screens for behavioural phenotypes (See Table 1). The simplest form of screen that can be carried out is one for dominant ENU-induced mutations. With this approach, males are treated with ENU and subsequently mated with wild-type females (Fig. 1). Progeny from these crosses, which can each potentially carry mutations at up to 100 different loci, are then screened for abnormal phenotype. After confirming inheritance of a phenotype, mutant genes can be identified using a conventional positional cloning approach. Details of ENU treatment and screening protocols have been considered elsewhere (Nolan et al., 2000a,b). Screens for recessive phenotype, a far more laborious prospect involving three generations of breeding prior to phenotypic screening, are also being carried out.

In designing and implementing behavioural screens to identify mutants, a number of important factors should be kept in mind:

1. The screen should be quick and easy to conduct. It has been estimated that the specific locus mutation rate of ENU is approximately 0.00108 (Coghill et al., 2002). A rapid screen allows more mice to be screened, which in turn increases the chance that heritable phenotypic traits will be identified.
2. It is advantageous if the screening is hierarchical, consisting of primary and secondary screens. The secondary screen, which is often more time consuming than the primary one, permits enhanced phenotypic assessment, confirming or dismissing the presence of an abnormal phenotype. This limits unnecessary breeding of mice.
3. Behavioural phenotypes by their very nature are intrinsically variable. A successful screen, however, should make all attempts to limit variability so as to allow easier identification of outliers. This can be achieved by

Table 1
ENU Screens for abnormal behavioural phenotypes

Location of screen	Mutagenesis approach	Region	Website	Behavioural screens	Background strains
Mammalian Genetics Unit, Harwell, UK	Dominant	Genome wide	http://www.mgu.har.mrc.ac.uk	Locomotor activity, startle response, prepulse inhibition, morris water maze, open field, elevated plus maze, light–dark box, ethanol preference, circadian rhythms and entrainment	BALB/c, C3H
Jackson Laboratory, Bar Harbor, ME	Dominant Recessive	Genome wide	http://nmf.jax.org/index	Comprehensive cage monitoring system, prepulse inhibition, fear potentiated startle, seizure threshold	C57BL/6J
Oak Ridge National Laboratory, Tennessee, USA	Recessive	Chr 7, 10, 15, X	http://www.tnmouse.org	Novelty seeking, food neophagia, cocaine induced locomotor activity/place preference, ethanol preference, open field, light–dark box, elevated plus maze, conditioned freezing, Y-maze, startle response, prepulse inhibition, tail suspension, sexual preference	Mixed
RIKEN, Genomic Sciences Centre, Japan	Dominant Recessive	Genome wide	http://www.gsc.riken.go.jp/Mouse	Open field, passive avoidance, home cage activity, seizure induction	C57BL/6J
Samuel Lunenfeld Research Institute, Toronto, Canada	Dominant Sensitized	Genome wide	http://www.cmhd.ca	Fear conditioning, morris water maze, prepulse inhibition, social behaviour	C57BL/6J, C3H
Northwestern University Chicago, USA	Recessive Dominant	Genome wide	http://genome.northwestern.edu/	Fear conditioning, open field, elevated plus maze, circadian rhythms, psychostimulant response	C57BL/6J
Novartis Research, San Diego USA	Recessive Dominant	Genome wide	http://www.gnf.org/	Anxiety, sensorimotor gating, locomotor activity, learning and memory, circadian rhythms and pain sensitivity	C57BL/6J
UCLA School of Medicine, USA	Dominant	Genome wide	See Sayah et al., 2000	Prepulse inhibition, pain sensitivity, open field, fear conditioning, locomotor activity	C57BL/6J

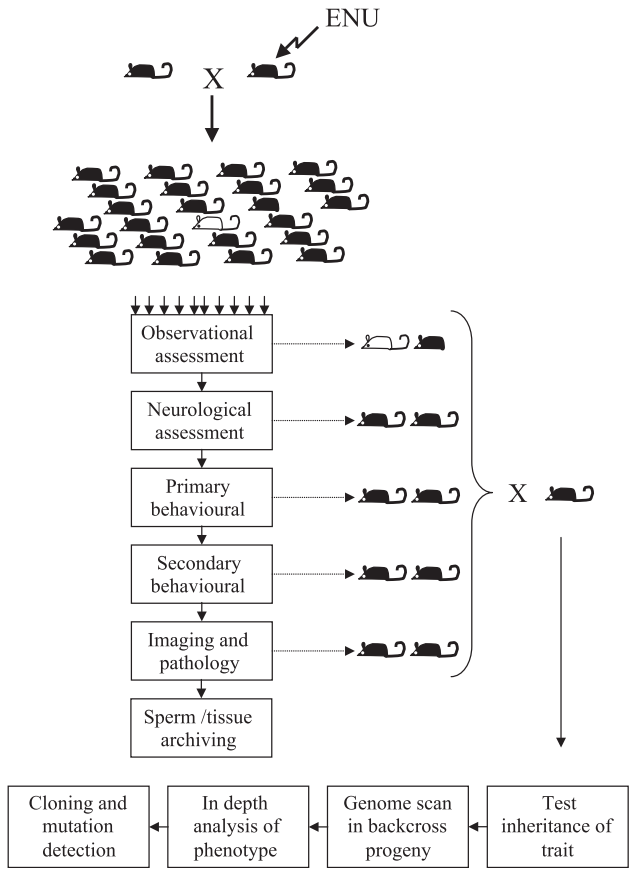

Fig. 1. Schematic representation of a screening programme for dominant neurological and behavioural mutants. Male mice are injected with ENU and are mated with wild-type females. Progeny of these crosses carry ENU-induced mutations at multiple loci. Progeny are subjected to a hierarchical battery of screens, beginning with simpler screens in all animals and progressing to more laborious screens in subpopulations. Post mortem screens can also be carried out in male progeny if sperm samples are archived, lines with an abnormal pathology can subsequently be recovered via in vitro fertilisation of archived sperm samples. Mice (founders) with abnormal phenotypes are mated with wild-type animals to test for inheritance of the trait. If the phenotype is a result of a dominant mutation, we expect this to be inherited in 50% of the founder's progeny. Once inheritance of the trait is confirmed, the mutant locus is identified using a standard genetic backcross. Generation of large backcross stocks will allow us to identify the mutated gene using positional candidate analysis/positional cloning and to carry out in depth analysis of the mutant phenotype.

implementing simple strategies such ensuring that the same person conducts the phenotypic screen, that phenotyping is conducted at the same time of day, and limiting external environmental influences.

4. Close attention should be paid to variation between inbred mouse strains. Current approaches require the introduction of a second inbred strain to permit positional cloning of any mutation. In conducting mapping crosses, care should be taken to select an inbred strain that behaves in a similar manner to that which carries the mutation. This requires the acquisition of control (or baseline) data for various inbred strains using the same screening conditions (Tarantino et al., 2000).
5. If one chooses to use a battery of tests (e.g., open field, PPI, and Morris water maze) to assess multiple phenotypes, careful consideration should be given to the order in which they are conducted to ensure that the behaviour of a mouse is not influenced by its exposure to previous testing procedures.

Below, we have selected examples of two behaviours that have been employed in both forward and reverse genetic screens. ENU-based screens for anomalies in both behaviours are currently being carried out by our group. It is our belief that the molecular analysis of both of these behaviours will benefit from the identification and characterisation of additional mutants affecting these behaviours. In identifying mutant genes, we also hope to provide additional insight into the molecular basis of behavioural and psychiatric disorders. We discuss preliminary progress with the analysis of mouse mutants in these screens and consider implications for future gene function studies.

7. Mouse mutants and anxiety

A number of mouse paradigms have been developed to explore the biological basis of anxiety. The most widely used are the open field, elevated plus maze and dark–light box (Rodgers, 2001). These devices all rely on the innate aversion of mice to open spaces and bright areas, which conflicts with their natural tendency to explore novel environments. An open field is a large square or round arena that is brightly lit and is normally white in colour. When placed in this environment, mice preferentially spend time in the perimeter rather than the centre, and those mice exhibiting high anxiety freeze and defecate. A dark–light box consists of a two-compartment box, one compartment is white and brightly lit, while the other is darkened. Mice are free to move between compartments. Those with a high anxiety tend to show an increased latency to emerge, and a preference for, the dark compartment. The elevated-plus apparatus consists of an elevated platform in the shape of a 'plus'. Two arms of the platform are open and two are enclosed. Mice with a low anxiety phenotype show a reduced latency to enter, and tend to spend more time, in the open arms of the apparatus. These experimental anxiety paradigms have been supported with the use of pharmacological compounds. Rodents when administered anxiolytic drugs spend more time in the centre of the open field, on the open arms of the elevated plus maze and on the light side of the dark–light box (Crawley, 1981; Pellow, 1986).

To date the genetic dissection of anxious behaviour in mice has relied largely on quantitative trait locus (QTL) mapping and the creation of targeted mutant mice. The significant differences in "anxiety" levels that are exhibited by a variety of inbred strains and selected lines of mice have been employed by geneticists to map QTLs that account for natural variation in behaviour. The elevated plus maze, light–

dark box and open field have been used to map QTLs on chromosomes 1, 3, 4, 5, 6, 7, 10, 11, 12, 14, 15, 18, 19 and on the X chromosome (Flint et al., 1995; Flint, 2003; Gershenfeld and Paul, 1997; Turri et al., 2001). These studies clearly indicate that anxiety-like behaviour in mice is a multigenic trait. Regrettably, cloning QTLs is an arduous task and as of yet, no genes have been identified using this approach. The use of transgenic technology has been more revealing. In excess of 60 different transgenic mice have been created that exhibit abnormal anxiety phenotypes (Clement et al., 2002). Those of most interest are summarised in Table 2. These studies have implicated the serotonergic system, the γ-amino butyric acid (GABA)ergic system, cannaboid receptors, substance P, corticotrophin releasing factor and oestrogen signalling in mouse ''anxiety''.

Despite the significant number of genes that have been associated with anxiety phenotypes using transgenic technology, the genetic factors that underlie anxiety disorders still remain largely unknown (Wood and Toth, 2001). To date only polymorphisms in the regulatory region of the serotonin transporter have been positively associated with anxiety-related traits in humans, and this accounts for only a small percentage (7–9%) of the genetic variance (Greenberg et al., 2000; Lesch et al., 1996; Melke et al., 2001). It remains likely that a significant number of other genes will be associated with anxiety-related traits. To identify these, we have embarked on an ENU screen for mice that exhibit abnormal ''anxiety''.

We have adopted a hierarchical screen. The generation of F1 subjects for screening has been described elsewhere (Nolan et al., 2000a,b). All F1 mice (BALB/c × C3H) are screened in the open field at 6 weeks of age. Those mice that exhibit high and low anxiety are then selected for secondary phenotyping. Mice that exhibit low anxiety (high centre time, high activity) are tested on the elevated plus maze which is designed to be more stressful than the open field, with very few mice emerging onto the open arms. Mice that exhibit high anxiety in the open field (low distance travelled, low rearing) are subject to assessment on the dark–light box which is designed to be less stressful, so that all but the most ''anxious'' mice emerge from the dark compartment. In this way, we aim to select only the most extreme mice for breeding. We are currently in the early stages of this project having screened approximately 600 mice on the open field arena. Fig. 2(A) shows the population distribution for distance travelled in the open field for a 5-min trial period. Generally, mice whose behaviour deviates from the population mean by more than 3 standard deviations are selected for further assessment. Of the 600 mice screened, approximately 40 mice have been selected for secondary assessment and 6 of these have been backcrossed to C3H/He to determine if the abnormal behaviour is heritable.

We have identified one line, BHV8, which appears to exhibit a heritable low anxiety phenotype. This is illustrated in Fig. 2(B) that compares the open arm time of the BHV8 line with a number of control lines. Although BHV8 shows an inherited low anxiety phenotype, certain features of this inheritance implicate the complex multigenic nature of this behaviour. Firstly, the penetrance of the low anxiety phenotype is incomplete in the population of backcross mice being studied. Although we expect 50% of animals to inherit the dominant low anxiety trait, the percentage of animals that express this phenotype is somewhat lower indicating that background modifiers may suppress the low anxiety phenotype. Secondly, the variance in behaviour between the two inbred strains, BALB/c and C3H, may be a confounding

Table 2
Targeted mutants with abnormal anxiety phenotypes

Gene knockout	Behaviour observed in knockout	Reference	Conclusion
5-HT_{1A}	↓ locomotor activity in open field ↓ open arm time in elevated zero maze ↑ latency to approach novel object ↓ open arm entries on elevated plus maze	Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998	↑ anxiety
Tac1 (encodes for substance P and neurokinin A)	↑ centre distance travelled in open field ↑ activity in open areas of elevated zero maze	Bilkei-Gorzo et al., 2002	↓ anxiety
Cannabinoid receptors (CB_1)	↓ open arm time and entries on elevated plus maze	Haller et al., 2002	↑ anxiety
Corticotropin-releasing hormone receptor 1 (Crhr1)	↓ latency to enter, and ↑ time spent in, lit side of light/dark box ↑ activity in open field	Timpl et al., 1998	↓ anxiety
Corticotropin-releasing hormone receptor 2 (Crhr2)	↓ open arm time and entries on elevated plus maze (males) ↓ time on light side of light/dark box ↑ centre entries in open field	Kishimoto et al., 2000	↑ anxiety in males
Regulator of G signalling protein 2 (RGS2)	↑ latency to enter lit side of light/dark box ↑ defecation	Oliveira-Dos-Santos et al., 2000	↑ anxiety
Neural cell adhesion molecule (NCAM)	↑ latency to enter, and ↓ time spent in, lit side of light/dark box ↑ activity on open arms of elevated plus maze	Stork et al., 1999	↑ anxiety
Estrogen receptor beta	↑ thigmotaxis in open field (females) ↓ rearing in open field (females) ↓ time in open arms elevated plus maze (females)	Krezel et al., 2001	↑ anxiety in females
Glutamic acid decarboxylase	↓ time and entries into open area of elevated zero maze ↓ time in centre of open field	Kash et al., 1999	↑ anxiety

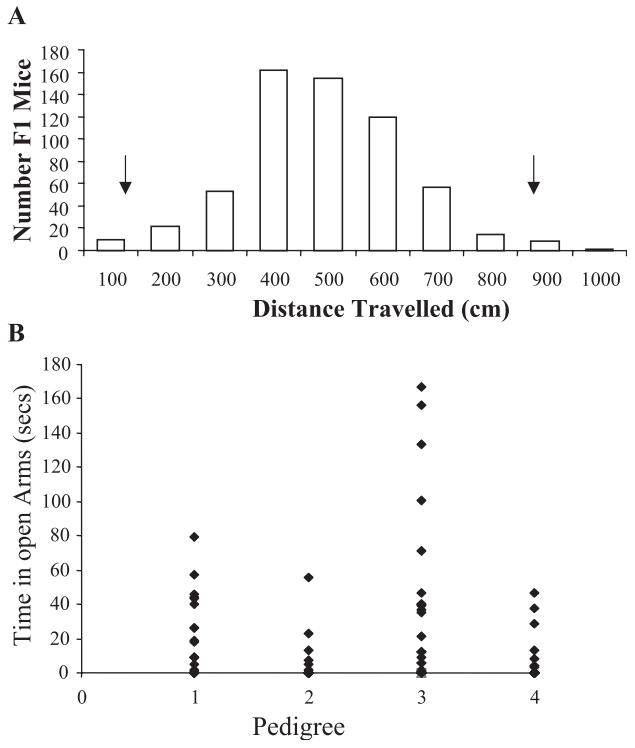

Fig. 2. Screening for anxiety phenotypes. (A) Population distribution of F1 progeny of mutagenised males for one of the parameters measured in the primary anxiety screen ($n=602$), showing distance travelled in an open field arena in a 5-min trial. Mice are selected for further analysis if their measured value lies greater than 3 standard deviations (arrows) from that of the population mean. (B) Scatterplot illustrating time spent on open arms in the elevated plus maze in C3H controls (pedigree 1, $n=23$), BALB/c controls (pedigree 2, $n=19$), BHV8 backcross animals (pedigree 3, $n=39$), and control backcross animals (pedigree 4, $n=27$). Note that a subgroup of BHV8 backcross animals shows a low anxiety phenotype (increased time spent in open arms) that is not seen in any of the control populations.

factor when backcrosses are made to determine whether an abnormal anxiety phenotype is inherited. In such cases, an alternative approach to mapping may be to treat the mutation as a quantitative trait, genotyping all mice, before analysing the data using an appropriate software package.

8. Mouse mutants and circadian behaviour

Circadian behaviour incorporates the regulation of diverse processes such as the sleep–wake cycle, locomotor activity, temperature regulation, metabolism, water/food intake and levels of circulating hormones. This behaviour is normally synchronised to external environmental cues such as sunrise/sunset, a process known as entrainment. However, in the absence of such cues, these inherent rhythms persist. Disturbances in circadian parameters have been associated with a number of psychiatric and neurological disorders in humans including seasonal affective disorder, depression, bipolar disorder and neurodegenerative disorders (Giubilei et al., 2001; Harper et al., 2001; Johansson et al., 2003; Lenox et al., 2002; von Zerssen et al., 1985). Given these associations, identification of mutations in genes with associated circadian phenotypes will provide a new source of material for the development of therapeutic targets for these and other human disorders. Indeed, polymorphisms in the pacemaker genes clock and period have already been associated with disorders such as familial advanced sleep phase syndrome (Toh et al., 2001) and seasonal affective disorder (Johansson et al., 2003) and particular allelic forms could be associated with diurnal preference in humans (Katzenberg et al., 1998). Recent studies in *Drosophila melanogaster* and in mouse have also implicated certain pacemaker genes in drug sensitisation and reward (Abarca et al., 2002; Andretic et al., 1999). The possibilities for the investigation of their role in the molecular basis of dependence and addiction pose an exciting opportunity.

The molecular dissection of circadian biology has been possible primarily through the analysis of mutant phenotypes in model organisms such as *Drosophila* (Konopka and Benzer, 1971) and *Arabidopsis* (Ahmad and Cashmore, 1993) and mammalian circadian function has depended on the analysis of targeted mutations in orthologous genes, the period and cryptochrome genes (Albrecht et al., 2001; Bae et al., 2001; Kume et al., 1999; Okamura et al., 1999; Shearman et al., 2000; Thresher et al., 1998). Nevertheless, several important insights into circadian function have been established from the analysis of mutant phenotypes in mice and other rodents. This approach has been used successfully as a complementary approach to the targeted mutagenesis approach. The cloning of the mouse clock gene (King et al., 1997; Vitaterna et al., 1994) is a milestone in the development of this approach in the mouse. The clock mutant was identified in a behavioural screen of progeny of ENU-mutagenised animals. The identification of the mutant gene has allowed us to construct a molecular feedback loop that is essentially responsible for the control of all clock output rhythms. Cloning of the spontaneous hamster mutant, *tau*, has implicated posttranslational mechanisms in clock function. The mutation, found in casein kinase I epsilon, results in a less efficient phosphorylation of the period protein and a dramatic shortening of the biological clock (Lowrey et al., 2000).

Identification of the molecular components of the biological clock is far from complete and much work has concentrated on establishing how the pacemaker interacts with environmental cues (entrainment), how the molecular clock is translated into a synchronised neural signal and how output rhythms are generated and maintained. In the first category, a subset of retinal ganglion cells responsible for non-image forming functions of the eye has been identified (Panda et al., 2002). These cells specifically express the melanopsin gene, (Provencio et al., 1998; Provencio et al., 2000), a novel opsin identified initially in frog melanophores. Mice with targeted mutations in this gene do not have any rhythm anomalies but their sensitivity

to environmental cues is reduced as are pupillary responses to light (Lucas et al., 2003; Panda et al., 2002; Ruby et al., 2002). Mouse mutants, both targeted and from phenotypic screens, have also helped in the study of synchronisation and maintenance of output rhythms (Cheng et al., 2002; Honma et al., 2002; Kapfhamer et al., 2002; Shen et al., 2001).

To further our understanding of circadian behaviour, it will be necessary to identify and characterise more mouse mutants acting through input pathways, contributing to pacemaker function and to maintenance of all output rhythms. Many groups including ours are screening progeny of ENU-mutagenised animals to increase the number of mutants (Fig. 3A and see http://www.neuromice.org). We have screened over 1300 progeny of ENU-treated animals for circadian rhythm anomalies. The screen has been very successful in detecting abnormal phenotypes. In all, we have detected 68 abnormal phenotypes (approximately 5% of the total screened). Of these, 20% show a robust inheritance and are being characterised further. Using low-resolution mapping techniques, we have found that the majority of these mutants map to novel circadian rhythm loci. Phenotypes detected fall into four categories—abnormal circadian period, abnormal phase shift, abnormal activity and multiparametric. Examples of some of the phenotypes identified are shown in Fig. 3B. As we have found with all phenotypic screens (Hough et al., 2002; Nolan et al., 2000a,b; Thaung et al., 2002), this approach is entirely effective in identifying novel molecular components of mammalian behaviours.

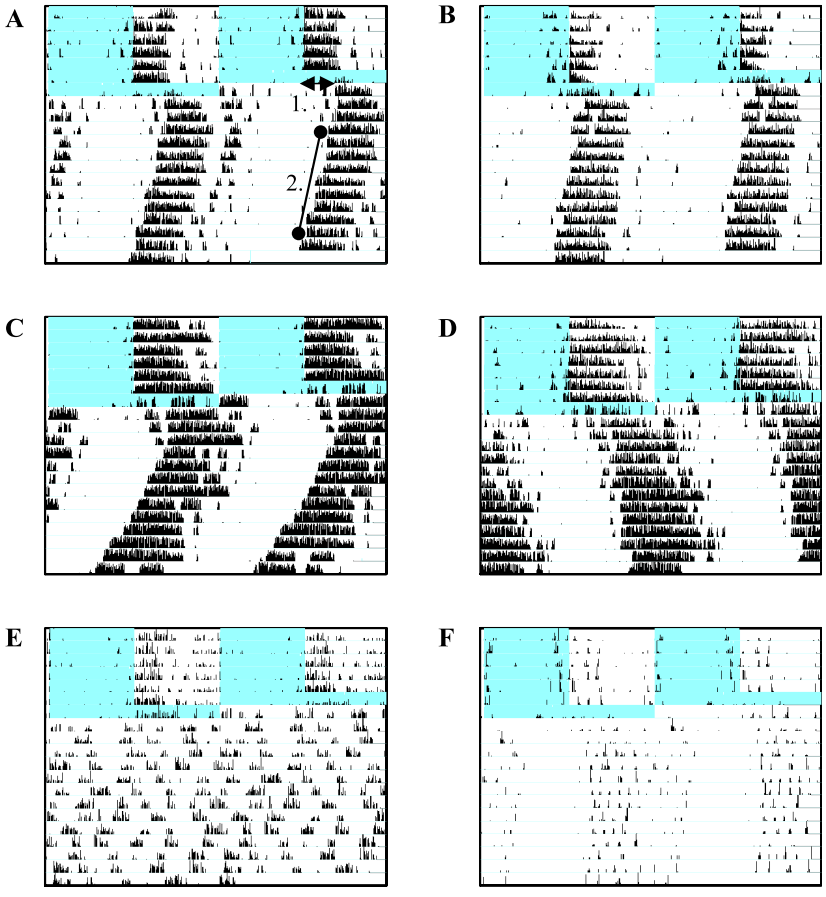

Fig. 3. Screening for activity rhythm phenotypes. (A) Typical wheel-running activity record for F1 progeny of ENU-mutagenised animals. Each image is a double-plotted actogram of wheel-running (indicated by deflections from baseline) over 21 consecutive days under light/dark (LD, days 0–7) and constant dark (DD, days 8–21) conditions. Actograms are shaded where lights are on. In LD conditions, the onset of wheel-running activity is typically coincident with lights being switched off. Upon transition from LD to DD conditions, animals receive an additional 12 h of light. Typically, this delays the onset of wheel-running activity by several hours (arrowheads, 1) and is an indication of the entrainment of biological rhythms to environmental stimuli such as light. Under DD conditions and in the absence of external stimuli the animal's endogenous clock drives biological rhythms such as wheel-running activity. Note that, for mice, the period between bouts of wheel-running activity is less than 24 h. This can be calculated by determining the slope of a line (2) drawn through activity onsets on consecutive days. (B) Double-plotted actogram of an F1 animal showing a reduced entrainment upon transition from LD to DD conditions. (C) Double-plotted actogram of an F1 animal with a shortened circadian period of wheel-running activity under DD conditions. (D) Double-plotted actogram of an F1 animal with a lengthened circadian period of wheel-running activity under DD conditions. (E) Double-plotted actogram of an F1 animal showing a reduced or absent periodicity under DD conditions. (F) Double-plotted actogram of an F1 animal showing dramatically reduced bouts of wheel-running activity under all conditions tested.

9. Implications for future studies

9.1. Histology and pathology

Human studies have implicated abnormal brain pathology in psychiatric illness. For instance, reduced hippocampal volumes have been observed in schizophrenic patients (Pegues et al., 2003; Velakoulis et al., 1999), glial cell reduction in the prefrontal cortex has been associated with mood disorders (Ongur et al., 1998) and asymmetry of the caudate nucleus has been linked to ADHD (Filipek et al., 1997; Hynd et al., 1993). Given these observations, histological and pathological studies of mouse mutants will be useful in the assessment of whether a particular mutant is relevant to a human psychiatric disorder. Methods by which we can analyse mouse mutants are becoming increasingly sophisticated with the adaption of magnetic resonance imaging (MRI) and other forms of imaging in the mouse. This technology also allows primary pathological screens to be conducted on mice carrying ENU-mutations, which can then be recovered from frozen sperm using in vitro fertilisation (Glenister and Thornton, 2000). Large stocks of mice can be created in this way permitting rapid pathological, behavioural and genotypic analysis.

9.2. Drug preference and drug challenges

Experience with ENU screens to date has shown that, by screening a relatively small population of animals, we can identify abnormal phenotypes in any assay that we carry out. In our high-throughput multi-parametric screens, we have found that approximately 2% of all animals screened exhibit stably inherited phenotypes (Nolan et al., 2000a). In screens for specific behaviours, such as the screen for circadian rhythm mutants, we have found that approximately 1% of all animals screened show a robust inheritance of the abnormal phenotype. By the same measure, it should be possible to identify single-gene mutations affecting any trait in mice depending on how sensitive the assay is. Several groups, including ours, are now trying to adapt ENU screens to study behaviours such as drug preference and drug sensitisation in mice. For example, ENU mutagenesis is being used to identify single-gene mutations affecting ethanol preference in mice (S. Knapp, E. Fisher and H. Thomas, personal communication). The suggestion from these studies is that ENU screens may provide simple and rapid entry points into the molecular basis of any behavioural trait in mice.

9.3. Environmental and genetic influences on mutant behaviour

As with human disease traits, the expression of mouse behavioural phenotypes is notoriously susceptible to environmental and genetic influences. Extensive reports and reviews on the effects of the environment on behavioural performance and implications for the development of mouse behavioural models have been dealt with in detail elsewhere (Francis et al., 2003; Wahlsten et al., 2003; Wurbel, 2001). Arguments both for the standardisation and for the diversification of behavioural testing environments are equally valid although standardisation may allow for a more systematic assessment of mutant behavioural phenotypes. Standardisation of the testing environment may also be critical, for example, in forward genetics screens where the abnormal behaviour of an individual within a population of animals is being sought after. Of equal importance, however, is the effect of the genetic background on behaviour in mouse mutants. Mutants that can express a particular behaviour on one genetic background, such as increased aggression or a deficit in learning (Gerlai, 1996; Graves et al., 2002; Young et al., 2002), may not express this behaviour when the genetic background is mixed. Considering the number of behavioural studies conducted on a mixed genetic background, it is probable that researchers may be overlooking significant behavioural phenotypes in our existing catalogue of mouse mutants.

A gene-driven adaptation of ENU-mutagenesis screening (Coghill et al., 2002) allows us to generate and study single-gene mutations initially on a uniform genetic background. This in turn could be followed by mixed genetic background studies. Using this approach, parallel archives of DNA and frozen sperm are generated from male progeny of ENU-mutagenised animals maintained on an inbred background. With sophisticated mutation-detection technologies, these DNA ‘libraries’ can be screened for mutations in any gene and mouse lines carrying this mutation can be recovered using in vitro fertilisation of the matching frozen sperm sample. Several mutant alleles could thus be generated and compared on the same genetic background, Coghill et al. predict that three or more mutant alleles at any gene locus could be identified by screening 5000 such DNA samples.

10. Conclusion

The use of forward genetics approaches, such as ENU-mutagenesis screening, to identify mouse behavioural mutants is still in its infancy although results from systematic screens would suggest that this approach is valid in identifying and elaborating upon mammalian gene function. To date, no phenotypic screen has been unsuccessful in identifying novel molecular components and mechanisms. As more sophisticated phenotypic screening tools are developed, it should be possible to identify and study mouse mutants that will aid in the molecular dissection of human inherited disorders. Using this as a complementary approach to gene targeting studies should prove to be most fruitful in establishing how aberrant gene function may contribute to human disease aetiologies.

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