

Mutant Mice and Neuroscience: Recommendations Concerning Genetic Background

Viewpoint

Banbury Conference on Genetic Background in Mice*

Mouse mutants derived by targeted mutagenesis in embryonic stem (ES) cells offer many advantages to the study of the molecular and cellular mechanisms underlying behaviors such as learning and memory, circadian rhythms, motor coordination, and aggression, as well as other neuroscience research areas such as brain development. The beginning of any new field, however, is often marked by a period in which key issues are debated, and as a consequence the approach is sharpened and focused. Theoretical and practical issues related to the impact of genetic background on the analysis of mutant mice have been a central topic of discussion in this new field (Crawley, 1996, 1997; Crusio, 1996; Gerlai, 1996; Lathe, 1996; Wehner and Silva, 1996). Analysis of the literature reveals that there is no consensus on the nature of appropriate controls for genetic background. This report summarizes a recent Banbury workshop held in Cold Spring Harbor, New York, on December 8–11, 1996, to discuss these issues in an effort to come to a consensus within the field. The recommendations that follow reflect the need for rigorously controlling the genetic background of experimental animals, and the practical issues surrounding the implementation of the appropriate controls.

Three principles emerged from our meeting. First, all reports of genetic experiments must include a detailed description of the genetic background of the animals studied. This description should be exact and include enough detail to allow rederivation of the mice used. Second, the genetic background chosen should not be so complex as to preclude others from reproducing and expanding the experiments reported. Third, use of a common genetic background would facilitate the comparison of results across experiments and among laboratories. For a variety of reasons described below, we recommend that mutations be maintained in congenic lines, and that mutants be analyzed in a defined hybrid (and preferably F1) genetic background.

Controlling for Genetic Background Is Essential

The complexity of biological interactions among genes and proteins is at the heart of issues concerning the importance of genetic background. For example, in defining the impact of the mutation of a protein kinase, it is important to consider the levels of second messengers that activate it, the activities of opposing phosphatases, the availability of substrates, and the general state of the cellular processes that it regulates, which could also be controlled in parallel by many other kinases (Pawson, 1995). These and other factors are part of the genetic background in which the mutation is studied. Mutations can have very different phenotypes in different backgrounds (e.g., Abeliovich et al., 1993; Smithies and Maeda, 1995; Threadgill et al., 1995). Because the

identity of the genetic elements governing these other factors (modifiers) is usually unknown, it is important to keep them constant when evaluating the impact of a mutation. Only if the same genetic background is used across experiments can differences between the phenotypes obtained be ascribed to the mutations rather than to different genetic backgrounds. Adoption of a common genetic background does not preclude comparison of the effects of a given mutation in different backgrounds.

Genetic background can be used as a tool in the analysis of a mutation (e.g., quantitative trait loci analysis and enhancer/suppressor screens; Takahashi et al., 1994). By placing the same mutation in different genetic backgrounds, it is possible to study facets of gene function that would elude studies in any single background. Additionally, powerful new mapping and cloning strategies may allow the identification of modifiers from different backgrounds (Dietrich et al., 1993; Gould et al., 1996). Genetic interactions between a mutation and the genetic background may account for the variable penetrance of human genetic diseases, and it is important to study and understand the nature of these interactions.

Most targeting experiments to date have relied on the use of ES cells derived from substrain 129 mice. However, the 129 substrains are a complex collection of various backgrounds, and so ES cells derived from them are likewise genetically complex (Simpson et al., 1997). In addition, recent analysis revealed that some commonly used ES cell lines are polymorphic at a number of loci, showing that they were not derived from inbred strains (Simpson et al., 1997). This raises the possibility that random segregation of these polymorphic loci to either mutants or controls could affect the phenotypes of the resulting animals and complicate the interpretation of experiments. Nevertheless, it is still possible to use the 129 ES cell lines currently available without compromising experiments, because congenic mutant lines can be generated by backcrossing to standard inbred mice. The extent of backcrossing required will depend on the degree of polymorphism. Genetic markers can be used to accelerate this process (see below). It is important to note that there are ES cell lines derived from inbred 129 substrains (Simpson et al., 1997).

Although there are ES cell lines from inbred 129 substrains, the mouse community in general, and neuroscientists specifically, would benefit greatly from the availability of a selection of ES cells from other inbred mouse strains, which would simplify the design of experiments and facilitate the reproduction, continuation, and cross-referencing of genetic studies. However, developing robust ES cells that are pluripotent and do well under extended culture conditions will require the focused attention of expert laboratories. The development of this resource is so important that national and international funding organizations must be encouraged to direct research support to this area. Newly derived ES cell lines could then be made generally available to the community.

*Editor's note: These recommendations are those of the conference and the contributors and do not necessarily reflect the policy of *Neuron* or Cell Press.

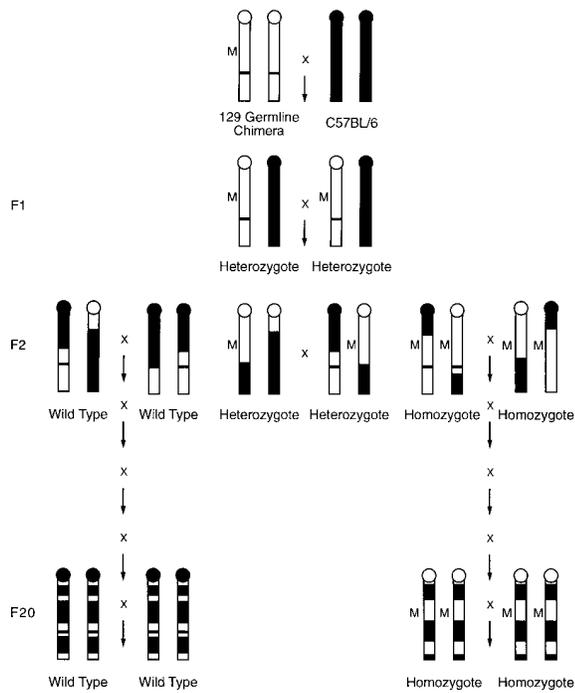


Figure 1. F2 Mice Are Not Well Suited for Strain Derivation

One example of a common strategy to maintain mutant strains is shown. Although cost effective, this strategy is beset with several fundamental problems. Starting from F2 mice, averaging 50:50 129 (white chromosome regions) and C57BL/6 (black chromosome regions), generations of inbreeding will inevitably result in homozygous and WT control lines of very different genetic backgrounds.

Mutations Should Be Maintained as Standard Inbred Congenic Lines

There are many different ways to make errors in the maintenance of mutant lines, most of which stem from violations of two principles mentioned in the introduction: the exact genetic background of a mutation should always be known, and it should be easily reproducible. Maintaining a mutant line by inbreeding homozygous mice (Figure 1) should be avoided as it violates both principles. Over consecutive generations, random segregation events lead to progressive changes in the genotype of these hybrid lines. During this time, any deleterious aspect of the homozygous targeted mutation may result in selection for background genes that change the mutant phenotype. After 20 generations of brother-sister matings, a new inbred line is generated. Such new inbred strains, even in the absence of a targeted mutation, often contain deleterious allele combinations, resulting in deficits such as reproductive suppression. Additionally, there is no appropriate control for the mutant mice because the exact genotypes are not known at all of the polymorphic alleles randomly segregating during the propagation of such a line (Figure 1). Simply generating a similar line with wild-type (WT) littermates of the mutants is not an adequate solution because of random segregation and fixation of alleles, and because the starting mice differ at the genes linked to the targeted locus. Mutations currently maintained in this manner

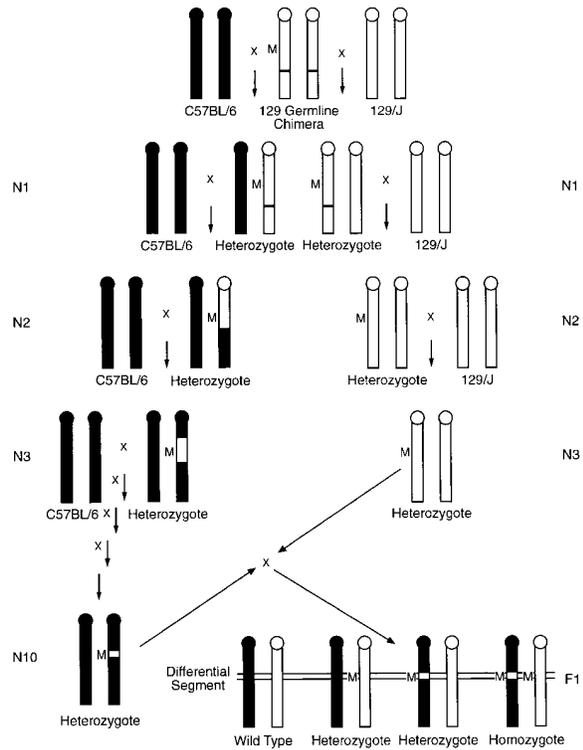


Figure 2. Simultaneous Derivation of Two Congenics Permits the Ongoing Generation of F1 Hybrids

Chimeras are mated and repeatedly backcrossed with the inbred C57BL/6 strain (black chromosomes), and the targeted mutation (M) is maintained heterozygous and then studied homozygous in that background. Similarly, the targeted mutation is backcrossed in the 129/J genetic background (white chromosomes). ES cell chromosomes are pictured white to indicate their 129 origin, but with a polymorphic region distinguishing them from 129/J mice. The polymorphic region is eliminated during backcrossing. From heterozygotes of these two different backcross strains, it is possible to derive defined 50:50 F1 homozygous and WT mice for study (e.g., N10N3F1). However, it is important to note that even after 10 backcrosses (N10), there will be a small region around the targeted locus that differs between F1 homozygotes and control WT littermates (differential segment). A solution to this potential problem is suggested in the text and Figure 3.

could be readily transferred to standard inbred backgrounds, with the aid of speed congenics (Lander and Schork, 1994).

Figure 2 depicts a breeding strategy designed to address the problems mentioned above. In brief, we recommend that targeted mutations be maintained as congenic lines. This is accomplished by consistently backcrossing onto defined inbred backgrounds. Inbred strains are homozygous at the vast majority of loci, eliminating variability that may confound the mutant phenotype. Continuous backcrossing reduces the chance of genetic drift and the size of the "differential segment" (see below).

However, because of random allele fixation during derivation, these lines can also be homozygous for certain alleles that cause phenotypic abnormalities, such as loss of spatial learning, resistance to kainic acid injury, high seizure susceptibility, etc. (Müller et al., 1994;

Crawley, 1996; Wehner and Silva, 1996; Crawley et al., 1997). Thus, it is often impossible, however, to study certain phenotypes in inbred genetic backgrounds because the parental strain is already affected. For example, most 129 and DBA strains show poor hippocampal-dependent learning (Upchurch and Wehner, 1988; Wolfer et al., 1997); BALB/c and C3H have visual problems (Upchurch and Wehner, 1988); and C57BL/6 mice become deaf to certain frequencies at an early age (Willott, 1986) and are poor avoidance learners (Schwegler and Lipp, 1983). Inbred lines also tend to be very sensitive to environmental stressors, which can often result in considerable within-subjects variability (Falconer and MacKay, 1996). In addition, any single genetic background can either overshadow or exacerbate a specific mutant phenotype, due to complex epistatic genetic interactions between alleles in that background and the targeted locus.

Analyzing Mutations in a Hybrid Background

Hybrid crosses tend to eliminate homozygosity of alleles responsible for the abnormalities described above. In addition, the phenotypes of different hybrids (i.e., C57BL/6 129/J versus 129/J BALB/c) should be more alike than the phenotype of different inbred lines. For example, even though C57BL/6 is the only inbred line known to perform well in the Morris water maze, all F1 hybrid lines tested so far perform better than the C57BL/6 mice (Upchurch and Wehner, 1988). Therefore, even if genetic background is different between mice, the use of hybrids will facilitate the comparison and integration of results across experiments and among laboratories.

Having already discussed the advantages of a common genetic background, what genetic background should be used? We suggest that a 50% C57BL/6 and 50% 129/J hybrid background may be a reasonable choice. One reason for choosing these strains is that laboratories that may ultimately use inbred C57BL/6 ES cell lines, as well as laboratories currently using established 129 ES lines, can both easily derive these mice. Also, hybrid mice similar to those depicted in Figure 2 can be produced easily and quickly soon after the derivation of a new targeted mutation (see below and Figure 3). As already noted, there is considerable variability among 129 substrains (Simpson et al., 1997). Thus, to have a truly common background, a specific 129 substrain needs to be chosen. The 129/J substrain is fully inbred (Simpson et al., 1997) and could be used for maintenance of mutant strains. Another closely related substrain that is also an excellent choice is 129/JEm. This strain was recently derived from 129/J such that it no longer segregates at the tyrosinase (Tyr) locus.

F1 mice may not always be ideal. For example, the study of olfaction-dependent pregnancy block requires certain inbred strains (Brennan et al., 1990). Additionally, it would be costly to study double mutants in an F1 hybrid background because the double mutants would be only 1/16 of the F1 progeny of double heterozygous parents. Nevertheless, whenever possible, it would be best to use F1 mice of the C57BL/6 129/J background.

The derivation of F1 hybrid mice requires that the mutation is present in both C57BL/6 and 129/J inbred lines (Figure 2). Even with speed congenics, this transfer process may take as long as a year. It is possible to

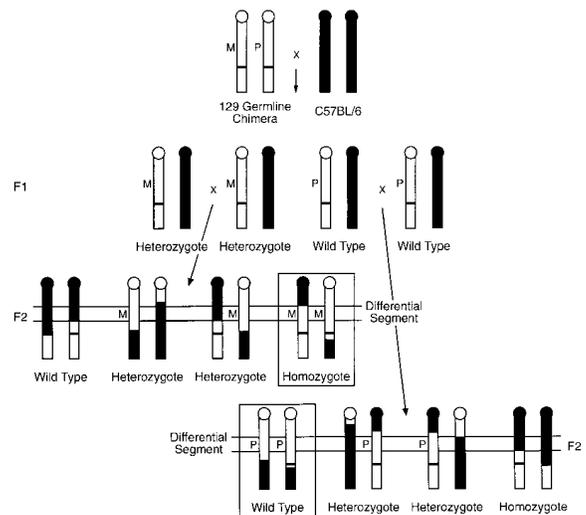


Figure 3. F2 Hybrids Have Value for Initial Studies and Differential Segment Analysis

F2 animals offer the earliest opportunity to examine a new mutant allele homozygous on a 50:50 hybrid background. By mating germ line chimeras derived from a 129 ES cell line (substantively white chromosomes) to C57BL/6 animals (black chromosomes), an F1 generation of genetically identical hybrid heterozygotes is produced. Depending on the nature of the targeted allele (M), these animals may be suitable for study. Intercrossing the F1 heterozygotes will generate the first homozygotes for the targeted allele (upper box). Such F2 animals have a variable, but on average, 50:50 hybrid background. However, the WT littermates of the homozygous mutants may not be ideal controls because the region around the targeted locus differs between WT and homozygous mutant littermates (differential segment). In homozygotes, the differential region is derived from the ES cell genetic background, while in WT mice, it is not. More appropriate WT controls may be prepared by mating the F1 mice not carrying the targeted mutation. To identify the appropriate control animals (lower box), a polymorphic probe (P) must be used to tag the 129 WT genomic region corresponding to the targeted allele. This strategy for differential segment analysis of F2 mice is not restricted to initial analysis but can be employed at any time a targeted mutation is maintained on its original genetic background.

transfer a mutation to another background by backcrossing, but how complete should the transfer be? A congenic line made with unrelated strains is statistically expected to be 99.9% from the host after 10 generations of backcrossing (Mouse Nomenclature Guidelines, 1997). At the beginning of the backcrossing procedure, each additional backcross makes a significant contribution. However, after the fifth backcross generation, the returns of additional backcrossing decrease precipitously. We propose that incipient congenic colonies could be used even after five backcrosses, although clearly the backcrossing procedure should continue indefinitely.

Because of the time required to derive the mutant mice, many gene-targeting studies have used an alternative strategy: chimeras with 129-derived ES cells are mated with C57BL/6 mice, and the resulting heterozygotes are intercrossed to produce F2 homozygous mutants (Figure 3). These mice are on average 50% 129 (from the ES cells) and 50% C57BL/6. Despite its intrinsic problems (see below), this breeding scheme may

be a reasonable compromise between the conflicting demands of time and rigorous definition and control of genetic background. Note that we are not recommending the establishment and study of hybrid lines.

In contrast to F1 mice that have one whole chromosome from each parent (Figure 2), F2 animals have a scrambling of parental genes that is on average 50% from each parent (Figure 3). The intrinsic variability of F2 animals could mask a weak phenotype. Therefore, whenever possible, it is preferable to use F1 homozygotes.

For both F1 and F2 hybrid mice, WT littermates of the homozygous mutants may not be ideal controls. The region immediately surrounding the targeted locus is necessarily derived from the genetic background of the ES cells (e.g., 129) in homozygous mutants, while in their F2 WT littermates, the same region is always derived from the other parental strain (e.g., C57BL/6; Figure 3). Although all other genomic regions are randomly assorted between mutants and WT mice, genes linked to the targeted locus could have an impact on the analysis of the mutant phenotype, because there are differences between C57BL/6 and 129 inbred strains (Collinge et al., 1994; Wehner and Silva, 1996; Logue et al., 1997; Owen et al., 1997; Wolfer et al., 1997). Genes within the genomic region linked to a given targeted locus could be responsible for some of these differences between strains, and thus in some cases confound the interpretation of the phenotype of the mutants.

The best controls for the F2 homozygotes are WT mice that also have the genomic region linked to the targeted locus derived from the 129 ES cell strains. Such WT animals can be produced from crosses of F1 WT mice in which the locus of interest derives from the genetic background of the ES cells (Figure 3). The identification of these mice requires the isolation of polymorphisms within or near the targeted locus. If the phenotype of the two types of WT mice does not differ for the phenotypes studied, future experiments could simply use WT littermates as controls, thus avoiding the costly and laborious use of independently derived WT animals.

Other Types of Transgenic Experiments Benefit from Defined Backgrounds

Many of the problems and possible solutions discussed above for mice derived by targeted mutagenesis also apply to other kinds of transgenic mice. Mutant mice (and rats) can also be generated by random insertion of genes microinjected into the pronucleus of single zygotes. The situation is further complicated by new experimental strategies involving the derivation of compound mutant mice that result from crossing random-insertion transgenics with targeted mutants (e.g., Tsien et al., 1996). Controlling for genetic background may be difficult in these experiments if the random-insertion and targeted-mutagenesis mice involved are maintained in non-inbred backgrounds. For example, without a common genetic background, it will be difficult to compare the overexpression of a gene (in random-insertion mice) with its deletion (in targeted mice). Similarly, experiments using random-insertion animals to rescue genes deleted in targeted mice would be hard to interpret if non-inbred genetic backgrounds are used. Without rigorous control for genetic background, the rescue could

be due to the genetic background of the compound transgenic and not to the rescue transgene.

New genetic strategies allow restriction of mutations to particular regions of the brain (Tsien et al., 1996) or the localized induction of genes (Mayford et al., 1996). Even in these experiments, genetic background remains an issue of central importance. As discussed above, if the genetic background of the mice is neither exactly defined nor easily recreated, it will be difficult to repeat and expand on these experiments, no matter how exciting the results.

Considering all of the reasons discussed above, as well as our recommendations concerning targeted transgenic mice, we suggest that random-insertion transgenic mice should be derived in the C57BL/6 (or 129/J) inbred strain. In the future, methods other than the traditional pronuclear injection may become available for the generation of these mice (e.g., loxP-directed insertion of transgenic constructs into predetermined genomic sites in ES cell lines). Alternatively, congenic lines carrying the various insertions could be derived in the C57BL/6 background after the initial generation of the mice. The congenic mice could be used readily for crosses with targeted animals, and could also be used to generate F1 hybrids with mice of the 129/J genetic background. With a common genetic background, results with targeted animals could easily be integrated with findings from random-insertion studies. Maintenance of all of these transgenic lines should also follow the same general guidelines discussed above for gene-targeted mice.

The Importance of Nomenclature

Mutants tend to be identified by the name of the manipulated gene, regardless of genetic background. This is a problem when seemingly identical mutations result in distinct phenotypes in different laboratories. Unfortunately, genes and the proteins that they encode are frequently thought of as autonomous functional entities with a defined role in complex biological phenomena. The implication of this simplistic view is that a genetic mutation should have similar impact regardless of the genetic background used. To emphasize the role of genetic background and to avoid ambiguity, authors should use appropriate abbreviations that denote both the gene manipulated and the genetic background of the mutants.

Recommendations versus Rules

It is important to note that many published studies have not followed the recommendations discussed above. This, however, does not mean that these studies should be discounted or mistrusted. In many cases, the conclusions were based on evidence from multiple studies involving a variety of approaches. Therefore, it is unlikely that genetic background was a confound in most of those experiments. Although the issues discussed above are not to be taken as ironclad requirements, they should be considered in the future design and description of neurogenetic studies. In evaluating these experiments, it may not be wise to use rigid prescriptions. Instead, each study should be evaluated for its own merits and in the context of other available information. For example, the nature of the experimental question, the known variability of the phenotype tested, and the natural range of phenotypes found among related non-mutant lines

all can affect the impact that genetic background may have on the interpretation of the results. Clearly, a subtle behavioral phenotype resulting from a mutation of a poorly characterized gene should be interpreted with great caution.

Summary

Controlling genetic background during the construction and testing of mutants is complex. Here, we propose that the genetic background of the mutants should always be described in detail, and that any background used should be easily recreated from available stocks. We also propose that both transgenic and gene-targeted mice be generated and maintained in inbred genetic backgrounds (i.e., either 129/J and/or C57BL/6). We propose the study of F1 hybrid mice whenever possible (50% C57BL/6 and 50% 129/J). It is important to standardize the genetic background of the mutants studied to facilitate the comparison of results between experiments and among laboratories.

Contributors

The following scientists made significant contributions to the recommendations in this article: Alcino J. Silva, Elizabeth M. Simpson, Joseph S. Takahashi, Hans-Peter Lipp, Shigetada Nakanishi, Jeanne M. Wehner, Karl P. Giese, Tim Tully, Ted Abel, Paul F. Chapman, Kevin Fox, Seth Grant, Shigeyoshi Itoharu, Richard Lathe, Mark Mayford, James O. McNamara, Roger J. Morris, Marina Picciotto, John Roder, Hee-Sup Shin, Paul A. Slesinger, Daniel R. Storm, Michael P. Stryker, Susumu Tonegawa, Yanyan Wang, and David P. Wolfer.

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