

NEUROSCIENCE PERSPECTIVES

Flanking Gene and Genetic Background Problems in Genetically Manipulated Mice

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Mice carrying engineered genetic modifications have become an indispensable tool in the study of gene functioning. The interpretation of results obtained with targeted mutants is not completely straightforward, however, because of genetic complications due to linkage and epistasis. Effects of closely linked genes flanking the targeted locus might sometimes be responsible for phenotypic changes ascribed to the null mutation. The effects of the latter might also be modified by the general genetic background. This review presents some examples and discusses some simple strategies to deal with these complications.

Key Words: Mutants, linkage, dominance, genetics

In the last decade, research that uses mutant mice has boomed to the extent that research in many areas has become almost unthinkable without genetic mouse models. In a sense, we are living in a golden era for genetics, which seems to be pervading almost any field of scientific endeavor. In another sense, however, this image might be rather misleading. More often than not, when researchers nowadays talk of “genetics,” what they actually mean is physiology, biochemistry, or, in the fields of behavioral neuroscience or biological psychiatry, neurochemistry. Actual knowledge of genetics and genetic mechanisms is often surprisingly limited; this is exemplified by the often rather sloppy use of genetic terminology (Crusio 2002; Wotjak 2003). The first time I was confronted with this phenomenon was a number of years ago, when as a young postdoctoral fellow I was asked to give a presentation on the molecular bases of dominance. Not having received much training in molecular biology myself, I asked a colleague who was working in molecular genetics for a reference that would allow me to find some literature about this subject. To my surprise, my colleague started talking about mutant and wild-type bacteria in a nutrient solution, one or the other subsequently becoming “dominant.” When I replied that this was ecology, not genetics, and that I was interested in dominance in the Mendelian sense (not to be found in haploid bacteria anyway), my colleague’s answer was that high school was quite a long time ago, so could I please explain what Mendelian dominance was again? Similarly, another colleague once started to talk to me about a certain gene, stating that some individuals had seven alleles, whereas others had nine. Only after a lot of confusion did it turn out that what this colleague called “alleles” were the different bands visible on a gel after digestion of the DNA with restriction enzymes.

This is not to say that these colleagues were bad scientists. These examples merely serve to illustrate the fact that even basic knowledge of classical genetics has come to be seen as old-fashioned, outdated, and unnecessary. As I hope to make clear in the following review, this has been to the obvious detriment of the use of genetic animal models, sometimes leading researchers on the wrong track and providing misleading or wrong interpretations of results.

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Received October 17, 2003; revised December 19, 2003; accepted December 30, 2003.

0006-3223/04/\$30.00
doi:10.1016/j.biopsych.2003.12.026

Complications in Induced-Mutation Experiments

An apt illustration of how the neglect of classic genetics can lead to problems has been the development and use of so-called gene knockout techniques, whereby homologous recombination is used to block the function of a known gene (Wynshaw-Boris et al 1999). Two common but fundamentally different genetic processes might complicate the interpretation of the results of such an induced-mutation experiment: linkage and epistatic interaction. The first might lead to false results, when an effect of a closely linked gene is attributed to the induced mutation. This will be referred to hereafter as the flanking gene problem. The second process, epistasis, can change the effects of an induced mutation, depending on the genotype at other, often unlinked loci. This complication is referred to as the genetic background problem, although the word “problem” is perhaps not quite appropriate here. In contrast to the flanking gene problem, complications due to the genetic background do not lead to false results, although they might lead to misinterpretations. Instead, they only illustrate the well-known fact that genes should not be studied in isolation but have to be seen in the context of the other genes that an organism carries, as well as the environment in which it lives. In addition, if a mutation has widely divergent effects on a phenotype, depending on the general genetic background, then this might actually help us in the genetic dissection of the physiologic pathway leading from the mutated gene to the ultimate phenotype. In fact, the genetic background problem has an environmental equivalent, which has not often been recognized as a complication yet, because the effects of environmental variations are often under-appreciated (van der Staay and Steckler 2002; Würbel 2002). Nevertheless, the phenotypic expression of a genetic mutation frequently depends not only on the general genetic background but also on the general environment in which the mutated animal lives. Rampon and colleagues, for instance, reported that a region-specific null mutation in the gene for the *N*-methyl-D-aspartate receptor 1 subunit in area CA1 of the hippocampus had deleterious effects on learning but no effects when animals were raised in enriched environments (Rampon et al 2000). Similarly, Tremml et al (2002) could correct some of the learning deficits observed in β -amyloid precursor protein knockout mice by environmental manipulation.

In contrast to the above-mentioned experimental complications, the two other common problems are not the consequence of experimental bias but rather of basic errors of experimental design. One common mistake is to compare newly generated mutants with animals from some conveniently available 129 strain; however, given the large genetic, behavioral, and neuroanatomic variations between the different inbred strains belonging to the 129 family (Festing et al 1999; Montkowski et al 1997; Paylor and Crawley 1997; Simpson et al 1997; Wolfer et al 1997), it should be realized

that this comparison is only valid if the particular 129 strain used is identical to the one from which the embryonic stem (ES) cells were derived. Finally, another sign of the ignorance of classic genetics is that many reports of knockout or transgenic mouse studies describe the genetic constitution of the animals so poorly that it is impossible for any reader to understand what really has been going on. Such reports can be found in the highest-impact journals in the field, and there is a clear need for journal referees and editors to apply more stringent criteria here.

The rest of this review will concentrate on complications due to flanking genes and the genetic background.

Linkage and Flanking Genes

Typically, a null mutation is induced in an ES cell, which then is injected into a blastocyst to generate a chimeric animal. Almost invariably, the ES cells are derived from a different inbred strain than the blastocyst. The chimera is then mated and, if the mutated ES cells have passed into the chimera's germline, a mutant line is established. In subsequent experiments, homozygous null mutants (if they are viable) are compared with homozygous wild-type animals to establish the function of the silenced gene. After this technique was first introduced, it took several years for anyone to notice that there was a systematic bias in this type of experiment: genes flanking the induced null mutation would also be homozygous for alleles derived from the ES cell donor in homozygous null mutants (Gerlai 1996). It took several more years for it to be realized that relatively simple crosses, based on Mendelian principles, could check for this bias (Bolivar et al 2001; Wolfer et al 2002). Because the problem has been discussed at length elsewhere (Banbury Conference 1997; Gerlai 1996; Wolfer et al 2002), I will describe it only briefly here.

For practical reasons, most null mutations have been generated with ES cells derived from one of the inbred strains of the 129 family (Gerlai 1996; for a review of the genetics of these strains, see Simpson et al 1997). If the chimera is backcrossed to the very same 129 strain that provided the ES cells, there is no further problem, genetically speaking, because this would lead to the establishment of a co-isogenic strain (i.e., a strain that is identical to the parental strain, with the exception of a genetic change at only a single locus). Unfortunately, most 129 strains are generally poor breeders and possess several undesirable phenotypic characteristics, such as absent corpus callosum and, frequently, poor learning performance (Balogh et al 1999; Lipp and Wahlsten 1992; Livy and Wahlsten 1991; Montkowski et al 1997; Wolfer et al 1997). Tellingly, hardly any behavioral data were available on these strains before their sudden popularity as ES cell donors. In any case, the chimeras produced are usually mated to wild-type (i.e., nonmutated) mice of another inbred strain, frequently C57BL/6J. If the chimera transmits the null mutation through its germline, its offspring will be heterozygous for the mutation, and the subsequent F₂ generation will consist of mutants, wild types, and heterozygotes in Mendelian ratios. When this technique first came into use, any phenotypic difference between mutation carriers and noncarriers was ascribed to the genetic lesion. Of course it was realized that the F₂ would also segregate for all other genes for which C57BL/6 and 129 differ, but because segregation is a stochastic process, this would not be expected to lead to any systematic differences between carriers and noncarriers. Unfortunately, this is only true for genes that are located on chromosomes other than the one carrying the mutation. Gerlai, in a debate in the journal *Trends in Neurosciences* (Crawley 1996; Crusio 1996; Gerlai 1996; Lathe 1996),

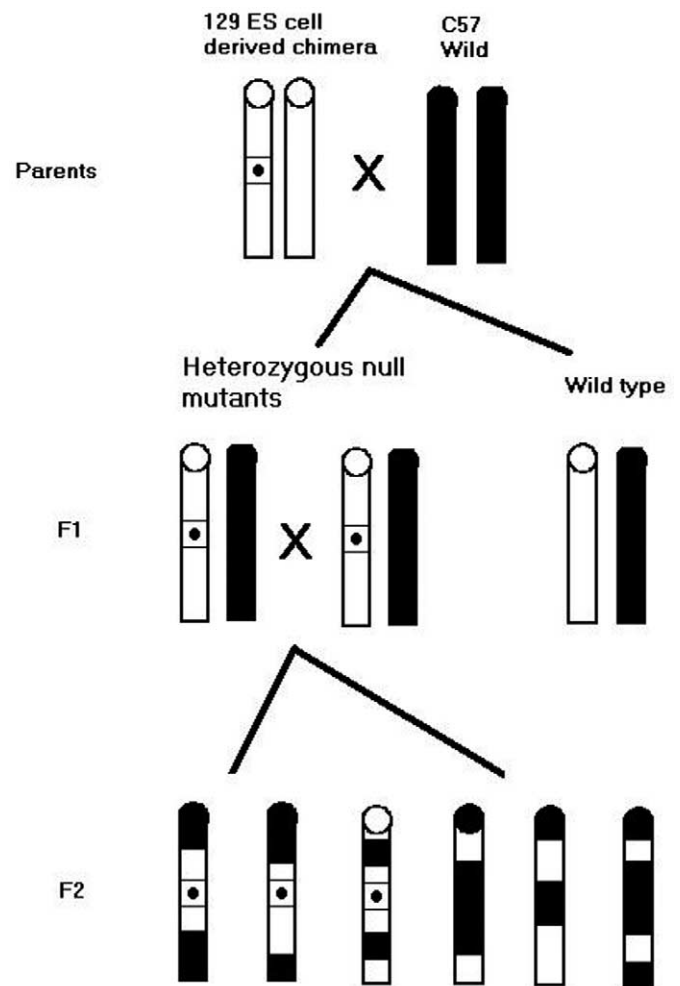


Figure 1. Chromosomal constitution of F₂ mice from a cross between a C57BL/6 mouse and a 129-derived germline chimera generated by gene targeting. 129-derived chromosomal segments are represented as white, C57BL/6-derived segments as black; the circle on top of the chromosomes represents the centromere (all mouse chromosomes are acrocentric). The induced null mutation is represented by a black dot. Because of crossing-over and recombination, F₂ chromosomes will be a mixture of the parental chromosomes. It becomes immediately obvious that F₂ chromosomes carrying the null mutation will also differ from chromosomes carrying the wild-type allele by many 129-derived alleles for genes localized in the chromosomal region flanking the targeted gene. For ease of representation, only the one chromosome carrying the targeted gene is shown; in the mean, mutants and nonmutants are not expected to differ systematically for any genes located on the other chromosomes. Modified after Gerlai 1996.

was the first to clearly point out the bias in these experiments: mutant carriers will also carry 129-derived alleles for any genes that are linked to the locus of the null mutation (see Figure 1).

An illustrative example is provided by the *Kcc2* knockout mouse that was generated by Eric Delpire and collaborators (Woo et al 2002). This gene codes for a K-Cl cotransporter and is expressed exclusively in neurons. When the null mutation was transferred to an inbred background (C57BL/6), it turned out that even after 11 generations of backcrossing the null mutation was still co-segregating with fur color (agouti; Eric Delpire, personal communication, August 22, 2003). Had this been a behavioral or neural characteristic, any researcher would naturally have assumed a functional implication of *Kcc2*. It is unlikely that this gene influences coat color, however. Indeed, it happens that the

agouti locus maps very closely to the *Kcc2* locus on mouse chromosome 2 (at a distance of approximately 5 cM), so that the co-segregation of *Kcc2* with agouti would only have been disrupted in the unlikely event of a recombination between these loci. It is perhaps worth noting that the region between the agouti and *Kcc2* loci (and the 129-derived region flanking *Kcc2* might well be considerably larger) contains literally dozens of other genes, too (cf. http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?chr=mouse_chr.inf).

The above example is not unique. Elegant experiments performed by Lorraine Flaherty and collaborators (Bolivar et al 2001) have shown several cases in which flanking alleles, but not the targeted mutations, influence a behavioral phenotype. In fact, knockout congenic strains are becoming a powerful tool to find quantitative trait loci (Bolivar et al 2001; Spyropoulos et al 2003). (Congenic strains are obtained by repeatedly backcrossing mice to an inbred strain. After 10 generations, the contribution of genes from the donor strain that are unlinked to the selected locus or loci will, in the mean, be less than .1%.) The initial response of many researchers working with targeted mutations that the probability of the occurrence of flanking gene effects would be low is clearly unwarranted.

It is often thought that the solution to the flanking gene problem is to transfer the mutation to an inbred background by repeated backcrosses (Banbury Conference 1997). In fact, because of crossing-over, the flanking region will become smaller with successive generations as long as the breeding population contains heterogenic genetic material for this region, regardless of whether a backcross procedure is being applied. Generally, this process will be slightly more rapid when backcrosses are used, but not much so. In fact, even after 12 generations of backcrossing, the average expected size of the flanking region will be approximately 16 cM, or more than 1% of the genome. It is important to note that this is an average value, which might vary widely in the cases of individual congenic lines. Indeed, much larger flanking gene regions than this have been reported in some congenics (Bolivar et al 2001). Application of speed-congenics techniques, whereby individuals are chosen for breeding in subsequent generations on the basis of their genotype at markers close to the targeted locus (Behringer 1998), might be an effective strategy to reduce the size of the flanking region, although its size will never become zero. Backcrossing is therefore not a solution to the flanking gene problem, although it can be a valuable technique to investigate genetic background effects in a systematic way.

Unfortunately, whatever breeding strategy is used, in practice it will be impossible to get rid of all 129-derived alleles flanking a targeted locus. The only way to obtain a co-isogenic strain (i.e., a strain differing from the original strain on only one locus) would be to backcross the original chimera to the inbred strain from which the ES cells were derived. As noted above, this solution is impractical for most of the popular 129-derived ES cells, given the many peculiarities of 129 inbred strains. Nevertheless, this might still be a viable strategy for some phenotypes that fall within the normal range in some 129 strains or if ES cells derived from other strains (such as C57BL/6J; see Seong et al 2004) were to be used.

Experimental Solutions to the Flanking Allele Problem

Recently, we proposed several relatively simple experimental solutions to the flanking gene problem (Wolfer et al 2002). Some of the proposed solutions were approximate, representing the

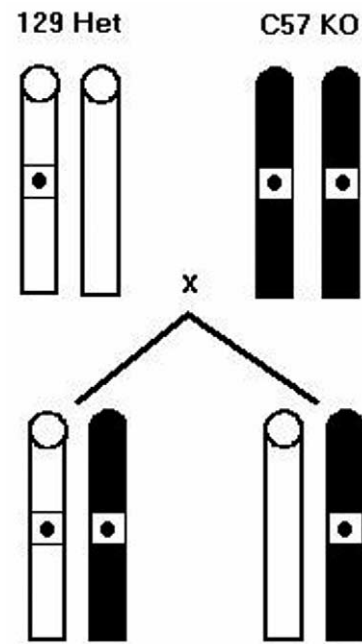


Figure 2. Checking for flanking allele effects if dominance is absent or incomplete. Homozygous mutants on a congenic C57BL/6J background are crossed with heterozygous (Het) mutants on a co-isogenic 129 background. The former are obtained by backcrossing mutants to C57BL/6J animals for several generations, ideally 10 or more. The latter are obtained by crossing the original germ-line transmitting chimera to 129 animals of the same strain as the embryonic stem cell donor. The offspring of the cross between these two lines is genetically identical for all loci, including those flanking the mutation. They will only differ at the mutant locus, where half of the offspring will be homozygous and the other half heterozygous for the mutation. Any difference between the two groups (which can be distinguished by polymerase chain reaction) will only be attributable to this difference and cannot be due to flanking allele effects. 129-derived chromosomal segments are represented as white, C57BL/6-derived segments as black; the circle on top of the chromosomes represents the centromere (all mouse chromosomes are acrocentric). The induced null mutation is represented by a black dot. As in Figure 1, only the one chromosome carrying the targeted gene is shown. KO, knockout.

best attainable in most situations (i.e., those situations in which the original chimera does not exist any more and has not been backcrossed to the ES cell donor). For an unbiased test of flanking gene effects, one needs a 129 co-isogenic strain as well as a C57BL/6J congenic strain for the null mutation under investigation, except in the case of a completely dominant mutation, in which only a 129 co-isogenic strain is needed. Unfortunately, the 129 background sometimes does not support low-viability mutations, and in those cases only the approximate solutions presented by Wolfer et al (2002) can be used. If, however, a 129 co-isogenic strain can be produced, crosses can then be carried out, depending on whether the phenotype associated with the null mutation is inherited in a completely dominant fashion or otherwise (additive, recessive, or incompletely dominant; Figures 2 and 3). As crucial comparisons are carried out on a 129 × C57BL/6 F₁ background, the unfavorable characteristics of the 129 strain are avoided, because they generally are recessive. Whether a phenotype is completely dominant can already be determined at the earliest stages of the production of a new targeted mutant, when the original chimera is crossed to a C57BL/6J animal. It should be pointed out that the mode of inheritance of the phenotype might differ, depending on the particular background strains used.

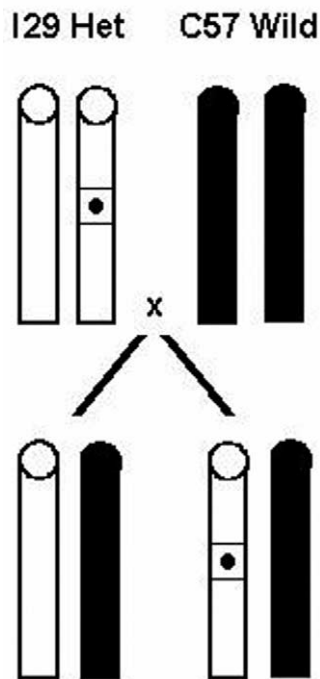


Figure 3. Checking for flanking allele effects if dominance is complete. Heterozygous (Het) mutants on a co-isogenic 129 background (see legend of Figure 2) are crossed with wild-type (Wild) C57BL/6 animals (or any other inbred strain that one wishes to use). The resulting offspring will be either homozygous wild-type or heterozygous for the null mutation and can be distinguished by polymerase chain reaction. Any difference between homozygotes and heterozygotes will be due to the null mutation, because they are otherwise genetically identical. 129-derived chromosomal segments are represented as white, C57BL/6-derived segments as black; the circle on top of the chromosomes represents the centromere (all mouse chromosomes are acrocentric). The induced null mutation is represented by a black dot. As in Figure 1, only the one chromosome carrying the targeted gene is shown.

The methods described by Wolfer et al (2002) and illustrated in Figures 2 and 3 were designed in such a way that comparisons between different genetic groups can always be done on littermates. This follows the recommendations of the Banbury Conference (1997). This practice is crucial in situations in which a mutation is maintained on a heterogeneous background. In this case, there is a risk that groups of mutant and nonmutant animals differ for other genes, too. This need not be due to any systematic bias but might simply come about by sampling. Of course, the larger sample sizes are, the smaller this risk will be. If mutant and control animals would come from different parents, this risk would become much higher. Breeding mutant and control animals as separate lines is, of course, completely unacceptable in this situation because it will effectively lead to the establishment of two different (recombinant) inbred strains that will differ for large chunks of chromosomes carrying numerous genes. Furthermore, because parents will differ genetically, their parental care might be quite variable, too, with possible effects on the phenotype of the offspring. It should perhaps be noted here that the use of littermates implies adding "litter" as a factor in the statistical analysis of the data.

In case a mutation is maintained in a highly congenic strain, or even an isogenic strain, the reasons given above for using littermates no longer apply. To simplify breeding, researchers might in this case opt to maintain separate mutant and wild-type lines. Comparisons of mutant and control animals will now be

unbiased by any genetic confounds or by variations between litters; however, one important qualifier here is that the mutation can now have not only direct but also indirect influences on the phenotype of an animal, for instance if the mutation influences maternal behavior or milk composition.

The General Genetic Background

As explained above, genetic background effects concern the phenomenon that the expression of a mutation depends on the genotype at other loci, regardless of whether they are linked or localized on completely different chromosomes. In other words, these effects are due to epistatic interactions. Several examples are known from the classic behavior genetics literature (Ehrman and Parsons 1981; Fuller and Thompson 1978). An early example from the literature on targeted mutations was the observation that the phenotypic effects (namely, the developmental time point at which mutated animals died) of an epidermal growth factor null mutation depended strongly on the strain background on which the mutation had been transferred (Sibilia and Wagner 1995; Threadgill et al 1995). The implication is that there are genes, called modifier genes, which interact epistatically with the targeted locus. The first efforts to map, identify, and characterize such modifier genes have recently met with success (Nadeau 2003).

An example from the behavioral domain was provided by LeRoy et al (2000), who studied neuronal nitric oxide synthase (*nNOS*) knockout mutants. Initial reports had shown a strong increase in aggressive behavior in these mutants on a mixed 129S4/SvJae-C57BL/6J background (Nelson et al 1995). After only five generations of backcrossing to the nonaggressive C57BL/6J strain, LeRoy et al obtained mutants that had aggression levels indistinguishable from C57BL/6J. Although no markers from the flanking region were reported, a crossing-over event diminishing the size of the probably already small flanking region in these mutants even more is rather improbable. It therefore appears that the *nNOS* null mutation is not sufficient to increase aggression in the pacific C57BL/6J animals but can do so on a background containing a large contribution from the more aggressive 129S4/SvJae strain.

Given these findings, the question poses itself: what is the ideal genetic background for targeted mutation experiments? Unfortunately, the answer must be that there is none, for several reasons. First, there is no a priori reason to designate any particular genetic background, be it inbred or hybrid, as "standard." Second, all inbred strains have one drawback or another. Some have visual problems, others become deaf after puberty, and still others have learning deficits or brain abnormalities (Staats 1985). The Banbury Conference (1997) recommended the use of hybrid backgrounds, specifically between the 129 and C57BL/6 inbred strains. Hybrid backgrounds are indeed a great improvement over inbred ones, because they will not suffer from the generally recessive problems signaled above, but even they are not necessarily ideal. Hybrids between 129 and C57BL/6, for instance, spontaneously develop humoral autoimmunity (Spyropoulos et al 2003). This increases in animals carrying a null mutation for the complement system protein C1q, but not if this null mutation is backcrossed onto either a pure C57BL/6 or a pure 129 background (Spyropoulos et al 2003), which provides yet another example of an interaction with the genetic background.

The only solution, therefore, appears to be to test the effects of a mutation on several different backgrounds. For the sake of reproducibility, these should be clearly defined backgrounds, according to the recommendations of the Banbury Conference (1997). Careful analysis of differential phenotypic expressions of

the mutation depending on the particular background might actually help in the genetic dissection of the phenotype. Currently, no examples of such analyses are available, but an analogous situation was described by van Abeelen (1989), who used the differential behavioral effects of identical pharmacologic treatments in C57BL/6 and DBA/2 mice to uncover hippocampal mechanisms that were difficult to study otherwise. For example, van Abeelen showed that intrahippocampal injections with an anticholinesterase (physostigmine) decreased exploratory behavior in an open field in both strains, whereas adequate doses of an anticholinergic compound (scopolamine) increased scores in the normally low-scoring DBA/2J but decreased them in the normally high-scoring C57BL/6J. In the latter case, equal treatment resulted in a reversal of the original strain difference. From these experiments, van Abeelen concluded that the hippocampal cholinergic system was well balanced in C57BL/6J animals but imbalanced in DBA/2J, which had an excess of acetylcholine that could be corrected by appropriate drug treatments; a well-balanced cholinergic system led to higher levels of exploratory activity.

Conclusions

Often, new techniques are greeted with considerable enthusiasm—and even hype—in the research community, only to become part of the standard toolbox of the researcher once not only their advantages but also their drawbacks become more firmly established. The induction of targeted mutations has not been an exception (Gerlai 2001). Initially, it was greeted as a technique that would allow researchers to establish rapidly the functions of any gene of interest and advance our understanding of complex processes, such as spatial cognition and emotionality. Enthusiasm was dampened somewhat when the first limitations of the method received more attention and when the realization struck that trying to understand higher cognitive processes by single-gene analysis is somewhat akin to deducing the orbit of the earth from information gleaned from its constituent subatomic particles (Crusio 1999). The time has now come to abandon the hype and to integrate this valuable technique with all the others that are available to the behavioral neurogeneticist.

Many of the ideas expressed above were developed over the years in stimulating discussions with my colleagues and friends Robert Gerlai (Indianapolis, Indiana), Hans-Peter Lipp, and David Wolfer (Zurich, Switzerland). I also thank the anonymous referees for their numerous valuable suggestions regarding the manuscript; any errors or omissions, however, are completely my responsibility.

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