A kinder, gentler genetic analysis of behavior: dissection gives way to modulation
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Many of the mutations and genetic variants that affect behavior in Drosophila have proved to be mild lesions of genes that are capable of more severe phenotypes. Examples of such variants affecting ion channels, transcription factors and protein kinases in studies of courtship and learning have anticipated recent findings on the naturally occurring variants in circadian rhythms and foraging.

Introduction
This year marks the thirtieth anniversary of the publication of Seymour Benzer’s first paper on the genetic dissection of behavior in Drosophila [1]. His approach represented a distinct departure from traditional ‘behavior genetics’, which had focused either on the behavioral influences of natural genetic variation, most of which seemed to result from relatively mild differences in multiple genes, or on spontaneous mutations that occasionally appeared in laboratory strains of flies or mice (e.g. [2,3]). Benzer adapted a paradigm from prokaryotic molecular biology—the aggressive search for single genes—by inducing mutations and testing for altered performance in behavioral tests (see [4,5]). The single-gene, induced-mutation school took no account of whether a gene would exhibit natural variation. Instead, the interest lay in which genes were essential for the behavior and which neural or biochemical components were selectively altered.

The majority of mutations uncovered in these searches caused major disruptions of their respective genes, more severe than have ever been found in the wild. The severity of a mutant phenotype is a complex function of many factors. It depends on the extent of inactivation or alteration of the gene in question, on the gene’s role in development and behavior, and on the genetic background in which it is expressed [6,7,8]. Many of the recent ‘knock-out’ mutations in the mouse have been notable for their lack of an obvious abnormal phenotype—sometimes referred to inappropriately as having ‘no phenotype’ (cf. [6] for discussion). (Knock-out refers to the engineering of a mutation that completely eliminates a gene’s product, thus producing a complete loss of function. These are known traditionally in the genetics literature as null mutations. Mutations producing partial loss of function are called hypomorphs, and can result either from lower or altered levels of expression or from a change in amino acid sequence that reduces the protein’s activity or increases its degradation.)

The probability that a mutation will have no discernible abnormal phenotype is largely a function of two factors: the means by which the mutation was obtained and the presence and arrangement of multiple forms of the gene. In flies and nematodes, most of the mutations were obtained from screening procedures deliberately designed to reveal distinctive changes in phenotype, as opposed to the mouse, in which knock-outs are generated from cloned genes in an effort to ask if mutations will produce a distinctive phenotype. Moreover, mice tend to have more versions (isoforms) of some genes than do flies and nematodes, and these isoforms are not always present in the same transcription unit such that a knock-out mutation would not eliminate the entire activity of a set of isoforms.

Despite the slash-and-burn nature of Benzer’s mutant hunting strategy in flies, some of the resulting mutations were considerably milder in phenotype, and often more informative, than the null mutations of the same gene. The milder phenotypes associated with these mutations are still more drastic than those found in nature, but they begin to approach the subtler influence of those occurring naturally. At the same time, recent molecular studies of natural variation in single genes influencing behavior have begun to close the gap between the two schools.

The importance of not being knocked out
In any kind of genetic analysis, the most unusual mutational changes often provide the most insight, and these are usually not mutations that completely inactivate a gene [9]. Mutations that have a mild or restricted effect on a gene, such as those reducing its activity or altering its transcriptional or splicing pattern, often preserve enough function to allow you to see which aspects are altered. For some genes, null mutations are lethal, whereas less drastic mutations produce informative behavioral defects.

The cacophony gene was originally isolated for its very selective effect on the male’s courtship song, producing polycyclic pulses that contrast with the 2–3 cycles per pulse found normally [10,11]. Subsequent genetic analysis of cacophony [12] showed that the mutation resided in the...
same gene as a mutation that had been independently isolated and identified as nightblind-A for its effects on optomotor behavior and visual physiology [13], and as a lethal mutation [14]. Thus, the same gene could mutate in a restricted fashion to affect the courtship song or vision, and it could also mutate to a more severe phenotype affecting viability. The gene encodes an α1 calcium channel [15*], and the optomotor mutation turns out to be a nonsense mutation in an alternatively spliced exon specific for photoreceptors, whereas the song mutation is an amino acid substitution in an evolutionarily conserved exon common to all spliced forms ([16]; LA Smith, JC Hall, personal communication).

The finding that a calcium channel could mutate to affect song production led to the examination of other ion channel mutants for similar effects. Significant effects were found in mutants of the slowpoke gene encoding a calcium-activated potassium channel [17]. Hypomorphic mutations of this gene affected song pulses even in heterozygous animals carrying one normal copy of the gene [18*]. Subtler effects were also found in hypomorphic mutations of two sodium channel genes [18*]. For each of these genes, there were examples in which parameters of song production could be altered by mutations that produced otherwise minimal effects on animal behavior.

The fruitless gene has a history somewhat similar to that of cacophony. Originally isolated as a male-sterile mutation, it was shown to cause mutant males to court other males while leaving courtship of females intact [19,20]. fruitless, which encodes a transcription factor involved in sex determination, can also mutate to produce lethality late in development [21,22*], and the viable mutations of fruitless exhibit unique but overlapping sets of defects affecting every aspect of courtship [23*]. Because the viable mutations of fruitless were produced by insertion of a transposable element and do not fall in coding regions [22*], they probably affect spatial patterns or levels of transcription or of splicing.

Variants with partial loss of function of the major serine/threonine protein kinases—cAMP-dependent protein kinase (PKA), calcium/calmodulin-dependent protein kinase type II (CaMKII) or protein kinase C (PKC)—produce similarly restricted phenotypes in tests of learning and memory. Whether produced by hypomorphic mutations or by genetically engineered transgenic flies to express specific peptide inhibitors, they share a common pattern in which null mutations are lethal and milder reductions in activity have selective effects on behavioral plasticity. Hypomorphic mutations of the catalytic subunit of PKA produce mild defects in initial learning of an olfactory avoidance paradigm when PKA activity is reduced to 40% of normal [24], and more pronounced defects in medium-term memory when activity is reduced to 16% of normal [25*]. Null mutations of the locus exhibit a lethal phenotype [26]. Transgenic flies partially inhibited for PKA also show deficits of initial learning [27]. A hypomorphic mutation of the RI regulatory subunit of PKA has little effect on the enzyme’s activity except in the absence of exogenous cAMP, when it modestly increases activity by 24%. This is enough to disrupt initial learning but has no effect on memory decay [28].

Flies engineered for partial inhibition of CaMKII or PKC also exhibit selective deficits in an associative learning paradigm based on olfactory and visual inputs associated with courtship behavior [29–31]. A modest (approximately 10%) reduction of CaMKII activity eliminates memory acquisition and retention [31,32], whereas inhibition by approximately 20% additionally eliminates the expression of conditioning during the training period [31,33]. A similar level of inhibition of PKC has no effect on acquisition and retention of courtship conditioning, but selectively eliminates the expression of conditioning during the training period [34*]. Null mutations of the structural gene for CaMKII, camk, are lethal (LC Griffith, personal communication); null mutations have yet to be obtained for PKC.

The behavioral phenotypes of the mild mutations described above are far less severe than those seen in null mutations of the same gene. The mildness of
Latitudinal cline in frequency of the (Thr-Gly)$_{17}$ (dark gray) and (Thr-Gly)$_{20}$ (light gray) alleles of the period gene. The (Thr-Gly)$_{20}$ allele, which is more prevalent in the north, is more robust in its temperature compensation. Numbers indicate chromosomes tested. Adapted from [43].

phenotype can have many origins—reduced activity of the protein, reduced levels of the protein, reduced levels of transcription or splicing, or altered patterns of expression—but their value for behavioral analysis lies in having some of the gene’s function preserved.

**Natural variation and the period locus**

Circadian rhythm mutants were among the first to be isolated under Benzer’s paradigm and have proved to be among the most informative [35,36]. For example, mutations of the period (per) gene affect all of the animal’s circadian rhythms, including the motor output patterns of eclosion, daily locomotor activity [35], and wing vibration during the male’s courtship song [37,38] (Figure 1). The range of severity in the mutant alleles also provided a clue to the gene’s function. The null per$^0$ mutation has turned out to be, not surprisingly, arrhythmic, but two other mutant alleles preserve the animal’s essential rhythmicity while modulating it slightly: a ‘short-day’ mutant per$^s$ with a circadian periodicity of 19 h, and a ‘long-day’ mutant per$^l$ with a circadian periodicity of 29 h. The respective rhythms of these mutant alleles in the courtship song are approximately 55 s for wild-type, 40 s for per$^s$ and 80 s for per$^l$. Subsequent studies have shown that per encodes a transcriptional regulator [36] and that the long-day phenotype is attributable to a low level of per expression and the short-day phenotype is attributable to amino acid substitutions in a domain of the protein that is conserved within flies [39–41].
With the per locus identified and cloned, the next step was to sample flies in the wild, assess natural variation in the gene, and ask whether there was any functional relevance of the variation to the mutant phenotypes found in the laboratory. DNA comparisons of coding regions among various isolates caught in the wild showed sequence variation [42] and, most interestingly, the predominance of one naturally occurring allele versus another correlated strongly with the temperature range of the locale in which the flies were caught [43] (Figure 2). In other words, the distribution of alleles followed a cline from north to south.

The particular portion of the sequence that correlated most strongly with the latitudinal cline was a series of Thr-Gly repeats that ranged from 14 to 23 [44]. What functional significance is there for such a correlation? Recent studies provide evidence that variations among these alleles affect the clock’s temperature compensation, one of the distinctive features of circadian oscillations, which allow even poikilothermic organisms to maintain their 24 h cycle at different temperatures [45]. Flies carrying the (Thr-Gly)20 allele, which is more prevalent in the north—where the temperature range is greater—exhibit more robust temperature compensation than flies carrying the (Thr-Gly)17 allele, which is more prevalent in the south [46]. The Thr-Gly repeat region correlates most strongly. The small differences in temperature would have been difficult to pick up and virtually impossible to map if one had started out screening natural populations for temperature compensation of rhythms. But knowing some of the functional defects of the per mutations in the laboratory and its DNA sequence, it became a simpler matter to observe in nature.

An additional issue is whether per’s role in courtship allows the differences in temperature compensation to be advertised to female flies. That is to say, do the males in different locales vary their song rhythm and do female flies pick up on these subtleties when choosing their mate? This is not yet clear, but there are suggestions of subtle song differences between males carrying different naturally occurring Thr-Gly repeats [47]. If females are able to discriminate among these song differences, as they are for per* versus normal [48], female choice may also be a factor that influences the geographical distribution of per alleles.

A single-gene basis for a natural variant: the foraging/dg2 locus

Behaviorally relevant genes are hard to find initially as natural variants because most natural variation in behavior is not primarily attributable to any one gene. In addition, if multiple genes contribute, the behavioral phenotype often disappears when they are separated from each other in the crosses required for genetic mapping. A striking exception to this trend is the foraging gene, which affects food search behavior.

Most populations of Drosophila harbor two behavioral types: rovers, who search widely for food, and sitters, who do not [49,50] (Figures 3 and 4). One behavioral type is advantageous under crowded conditions, the other under conditions of low density [51]. The difference is not a mere locomotor phenotype because it is expressed only in the presence of food. Genetic mapping revealed it to be attributable primarily to a single gene [52], and molecular analysis showed the gene to be dg2 [53], one of two loci for cGMP-dependent protein kinase (PKG) [54].

How could alterations in an enzyme as wide-ranging in its roles as a protein kinase be viable and healthy enough to survive in the wild? The explanation for the viability and specificity in the case of foraging appears to be that the magnitude of the natural enzyme variation
is relatively small: rovers have 12% more PKG activity than sitters, and the levels of \(d_g2\) protein and mRNA are correspondingly higher in rovers versus sitters \([53,55]\). This small increase may thus be sufficient to make a very distinctive difference in behavioral phenotype, but this conclusion must await determination of whether the distribution of \(d_g2\) in the nervous system is altered in the natural variants. If so, there could be a much greater difference in a small number of cells.

Protein kinases such as PKG generally have many substrates and participate in a wide range of biological processes \([55,56]\), so one would expect to find multiple phenotypes for any alteration. This is actually the case for a mouse knock-out mutant of PKG \([57]\). The distinctness of the foraging phenotype resembles that of the transgenic flies partially inhibited for CaMKII and PKC \([32,34]\) referred to above. In all of these cases, a subtle shift in kinase activity exerts a powerful effect on phenotype.

**Conclusions: from genetic dissection to genetic modulation**

Mild mutations have turned out to confer an unexpected advantage in the study of behavior, and it is perhaps because of this that they mirror the behavioral genetic variation found in nature. More importantly, they reflect the fundamental pleiotropy of most genes, especially those that have been found to affect behavior \([6,58,59]\). A mild alteration—affecting a subset of the gene’s normal functions, targets, or sites and times of expression—is often the only way a pleiotropic gene can produce a behavioral alteration without causing the animal major harm. Because they resemble natural variants more closely, they give us greater insight into the strategies by which genes modulate behavior in the real world.

Genetic dissection—with its strategy of making sharp, clean, hits—was appropriate when it was less clear that individual genes interdigitated into so many different processes. Now that we are confronting the essential network interactions of genes with nervous systems, a more sophisticated approach is required.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


5. Tully T: Discovery of genes involved with learning and memory:


A comparison of the two dominant strands of research in behavioral genetics, with particular reference to the author’s past work on sensitization in the blowfly and current work on memory phases in the fruit fly.


A thoughtful warning on the potential pitfalls attributable the effects of genetic background on behavioral phenotypes in the mouse. The principles, which apply equally to any organism, are that effects assumed to be attributable to the single mutated gene may in fact be attributable to genetic variation already present in the strain. This ‘genetic background’ effect can be particularly problematic when strains have been mixed, as in mouse knock-out breeding.


One of the few intentional studies of the effect of genetic background on a neural and behavioral mutant. The severity of phenotype in mutations affecting various brain structures in the fly can be drastically affected over a wide range by the genetic background in which they are expressed. Specific attention is focused on the mushroom-body-miniature mutation and the separability of its anatomical and behavioral phenotypes depending on genetic background.


Demonstration that the cacophony gene, identified for its effect on courtship song, encodes a calcium channel and is the same gene independently isolated as nightblind-A affecting visual physiology and behavior. A classic case of genetic pleiotropy in behavioral mutants.


Comprehensive molecular characterization of the fruitless locus, its sequence, transcription, splicing and spatial expression, its interactions with other genes involved in sex determination, and some of its behavioral phenotypes. The gene encodes a transcription factor, and the various alleles affect all steps in courtship, including song production.


Comprehensive characterization of the behavioral complexity of the various alleles of the fruitless locus: the specific courtship phenotypes of each allele individually and their interactions with each other. Illustrates all of the different ways one gene may affect courtship behavior, in some cases exerting selective effects on some steps as opposed to others.


Behavioral analysis of a cold-sensitive mutation in the catalytic subunit of protein kinase of Drosophila. Demonstrates a role for PKA in medium-term memory as well as in initial learning based on analysis of the retention curve.


Independent confirmation of the song phenotypes in long-day, short-day and arrhythmic alleles of the period gene, employing analytical techniques completely different from those used in [37], thus demonstrating the robustness and reproducibility (which had been questioned) of the original findings.


Mathematical treatment of sequence variation in the Thr-Gly region of the period gene, demonstrating that the Thr-Gly repeats themselves, rather than some other nearby sequences, correlate most strongly with the latitudinalcline previously demonstrated for natural variants of this gene. Finding this significant because these repeats have a subtle, functional impact on the courtship song and on temperature-compensated circadian oscillations.


A very significant report showing that the naturally occurring variants in the period gene produce subtle alterations in the flies' temperature compensation: circadian rhythms. The predominant allele in cold climates is better at temperature compensation over a wide range of temperatures than is the predominant allele in warm climates. Construction of transgenic strains expressing the different alleles confirms that the difference in temperature compensation is attributable to the polymorphic Thr-Gly repeat region of the gene.


Molecular identification of the foraging locus as the previously identified \textit{dg2} locus for PKG, demonstrating that \textit{sitters} have lower levels of enzyme activity, PKG protein and mRNA than \textit{rovers}, and that \textit{sitters} can be converted to \textit{rovers} by increased expression of a \textit{dg2} cDNA. Shows that natural behavioral variation can be attributable to mild differences in a widely acting gene.


Discussion of the preponderance of pleotropic mutants among those isolated for their effects on behavior, and its implication for gene identification strategies.