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Review

A NEUROGENETICIST'S MANIFESTO

JEFFREY C. HALL

Department of Biology, Brandeis University, Waltham Massachusetts 02454

That's my opinion anyway.

Clouseau (1964)

INTRODUCTION

This discussion of approaches and accomplishments in the neurogenetics business will focus largely on certain invertebrate nervous systems and how they function: physiology, neurochemistry, and behavior, as opposed to neural development. In turn, most of the treatment will deal with genetics as such, as opposed to genic studies that are solely molecular because they do not involve genetic variants.

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Address correspondence to Jeffrey C. Hall, Department of Biology, MS-008, Brandeis University, Waltham, MA 02454-9110. E-mail: hall@brandeis.edu

Many of the issues to be treated revolve around defining genes involved in the material control of what animals do. The concept (if any) of "material control" will come to connect with genes that in some way instruct neural substrates of behavior, such that the special features of a given kind of animal actions are regulated. In contrast (it seems), other genes specify factors that act merely to facilitate fundamental and often global features of nervous-system functioning. The basic process of identifying behaviorally interesting genes will speak to several substantive matters, in addition to the fact that defining such factors naturally creates starting points toward elucidating their underlying actions.

Some of these strictures will be honored somewhat in the breach, because asking questions about how nervous systems function such that an invertebrate can behave demands the exploitation of anatomical genetic variants (with developmental defects) and of gene manipulations (more than just chromosomal mutants). However, many of the salient achievements in this area of neurobiology, including establishment of certain principles, have involved formal genetic approaches toward understanding behavior in these "lower" organisms. I wrote some 20 years ago that "neurogenetics is just as much genetics as neurobiology" (words to this effect in Hall, 1982). Maybe that's still true; but in any case certain features of the behavioral and neuro-genetic accomplishments made in the interim speak to issues that are genetic as such—including genomic—and thus have implications that go beyond the confines of neurobiology.

This collection of potentially annoying proclamations will concentrate mostly on the behavior of *Drosophila melanogaster*. That reflects a bias, relative ignorance of other systems, or both. But perhaps the behavioral repertoire of the fruit fly is broader than that of the other prominent invertebrate member of the Genetics Security Council (Hall, 1995)—*Caenorhabditis elegans*. Another limitation of this discussion is that the genes to be featured will mostly involve so-called "forward-genetic" approaches to factors that specify and control behavior. It is still the case that most would-be behavioral genes have been identified in that manner—usually starting with mutagenesis—and that most of the discoveries made in this area have stemmed from finding genetic variants. Reverse-genetic approaches are increasingly being strategized and achieved as well. While such damages to genes are more forceful in mouse, the potential for things like gene-targeting in *Drosophila* is on the upswing. But these tactics have been minimally applied so far for anything interesting, let alone neurogenetically so. A more general component of looking askance at reverse genetics is that the results tend to be anticlimactic or frustrating. The latter problem looms especially large when a "gene knockout" leads to "no phenotype"; although that result might not even get reported, compared with those involving (for example) a putative "learning factor" being shown indeed to affect experience-dependent behavior after its gene gets knocked out. In contrast, several of the stories

whose early chapters are about behaviorists embarking on hunts for mutants ended up telling more meaningful tales.

Some readers will not find this rollickingly dense treatment of the subject to be their cup o' tea. Such persons may also roll their eyes heavenward at what they'll apprehend to be archly stated elements of the essay. That's their prerogative; but it's not necessarily warranted to recoil all the way back on one's heels at each and every piece of apparent sourness. Better to sense bitter sweetness, in that there is a putative purpose to the apparently low-pH passages, which attempt to provide some thoughts about certain perils that are associated with neurogenetic approaches. Whether or not this essay could claim to be a handbook for avoiding certain genetic and molecular biological pitfalls that can accompany studies of neural function, I gingerly avow that discussion of certain specific oversights and undersights that have crept into this area of inquiry create at least a small handful of instructive object lessons. I also sense that most of these maxims have not been even alluded to in the relevant primary literature or discussed in much detail within the information-rich reviews on these subjects, which are legion. As to the particulars of these deliberations, and of others salted throughout the piece, some of them speak to the conceptual ambiguities about which fans of neurogenetics might wish to be aware. At a minimum, readers may be brought up to date about certain accomplishments in this field, which continue to involve both innovative genetic tactics as well as substantive findings. Having said that, the essay in no way pretends to be comprehensive in terms of whatever information content it may possess.

BEHAVIOR-GENETIC INQUIRIES THAT START WITH MUTATIONS

Isolation of Mutants and Genes

The forward genetics of nervous-system function in *Drosophila* and *C. elegans* has uncovered novel behavioral-control factors that are unlikely to have been discovered otherwise. "Has uncovered" refers to connecting a neural-functional abnormality with molecules pointed to by the mutated gene. Thus, the mutation defines the stretch of DNA that one wishes to characterize, especially for sharply defined abnormalities of intriguing behaviors, including those that are arguably a material part of the animal's lifestyle (horrible word that that is). In these cases—whereby the behaviors involve more than the basest of motor activities—one is driven to clone the gene that got hit and work toward understanding how the normal form of its product functions. Such neurogenetic analyses thus proceed well beyond learning how a given piece DNA got altered when the behavioral mutant was induced by a mutagen or otherwise arose.

Oft-times such gene products are featureless and ostentatiously termed "pioneer proteins." That's good—not the phrase just quoted, but the fact that forward genetics should at least sometimes send the investigator out into terra incognita, against a backdrop of the philosophy of this approach. The maxim in question has probably been stated one-too-many times, but here goes again: When obtaining mutants that exhibit abnormalities of some process, aiming toward uncovering the mechanisms underlying it, you "start with no pre-conceived notions" about what molecular functions materially contribute to controlling the phenomenon in question. Notions will start to emerge once the mutant-defined genes are cloned; and the mechanistic directions in which the encoding products should lead could well be non-pre-conceived and perhaps involve molecules that are enticingly enigmatic (for a while). Thus you hope, in a way, that some of the genes defined initially by their mutant phenotypes will encode hitherto unknown "controlling factors." Alternative outcomes, in the behavioral subset of this forward-genetic business, would be that most of the mutated genes will, on the one hand, turn out to be readily rationalizable neurochemical entities; or, on the other hand (and worse), dreary catalytic functions that carry out housekeeping chores. The latter, when mutated, *can cause* behavioral deficits; but such genes seem unlikely to be involved in the aforementioned material regulation of the relevant neural functions. This issue will be taken up in more detail below.

Meanwhile, one needs to dig into a given gene whose mutant form causes a salient behavioral defect—if not necessarily a "specific" one (which speaks to another shibboleth that also will be coped with later). In such cloning operations, it used to be fairly tough to isolate a gene mutated solely by a series of nucleotide substitutions. One might have to "walk" by a long series of agonizing molecular steps to the part of the chromosome suspected to contain the mutated gene; then rescue the mutant with a DNA fragment (possibly a large and unwieldy such piece), sequence DNA taken from the mutated version of chromosome region (perhaps many kilobases worth of sequencing), or both. As of the mid-to-late 1990s, the wherewithal to clone a gene altered by a molecularly subtle change has improved to the extent that this point becomes almost a substantive one: Even intragenic point mutants can be connected with the pertinent piece DNA in such straightforward manners that taking this important step is no longer all that laborious, confounding, or impressive when achieved.

An alternative forward-genetic tactic, which hurdles the molecular barriers associated with inducing nucleotide substitutions, is to try and find a "physically" tagged mutant allele by transposon mobilization (e.g., Dura et al., 1993; Moore et al., 1998; Spradling et al., 1999; Bessereau et al., 2001; Dubnau et al., 2003). However, the locus may be refractory to intrusion of a molecular "insert," so this tactic is not a panacea. The genetic problem alluded to is more substantive in cases where the initial screen involves mobilizing transposons. The reason is that, even when

insertions of exogenous DNA occur, they tend to create only limited numbers and types of alleles—mutants with subnormal levels of the normal gene product. But if your genetic arsenal for the locus in question includes only such "hypomorphic" mutants, you could underinterpret the meaning of that gene. This refers to the hope that one might be able to get a handle on what the gene is doing on behalf of the relevant behavioral process, pre-cloning.

The following example may be illustrative. What if the initial mutation induced in a rhythm-related gene leads only to aperiodic behavior? This is just what happened for the first *period* (*per*) mutant in *Drosophila*, which was induced by chemical mutagenesis in 1968 then reported in 1971 by Konopka and Benzer (see Weiner, 1999, for the history). The biological, including behavioral, arrhythmicity caused by this "*period*-zero" (no periodicity) mutation could mean that the per^0 mutant is merely brain-damaged. [Lesion-effected destruction of neuronal clocks can cause behavioral arrhythmia (e.g., Weaver, 1998).] But if further *per* mutations were to be induced, some of which cause the durations of daily behavioral cycles to be altered, then one could surmise that the gene is involved in setting the pace of the biological clock that underlies the sleep-wake cycles of the organism (cf. Greenspan et al., 2001). Rhythm mutants of *this* sort—which would seemingly be defective in clock function, as opposed to missing the neural substrates of the rhythm—*were* obtained by subsequent chemical mutagenesis (Konopka & Benzer, 1971).

Such mutagens can hit any part of the gene, including of course coding regions (which sum up to the open reading frame, or ORF, contained within the locus). In contrast, most transposon mutants result from the mobile element landing in regions flanking the core of a gene's transcription unit and its ORF, or within non-coding introns; such inserts cause decrements in gene-product levels but not qualitative protein alterations (e.g., Freeman, 1991; Plasterk & van Luenen, 1997). To ameliorate this situation "gene trapping" has been developed for *Drosophila*. In this tactic, the transposon is designed to be activated only when it interrupts the gene within which it lands and thus tends to produce null mutations (Lucacsovitch & Yamamoto, 2002). However, no gene-trapped strains have been generated for specific neurogenetic purposes in invertebrates; and if they were, you couldn't really generate something like the beautiful allelic series that exists for the fly's *period* gene (mutants with fast clocks, slow clocks, or no clocks).

This state of affairs for finding transposon-tagged mutants—whereby a handful of hypomorphic neural mutants has been recovered and amorphic variants could be—has been amended by development of a tactic based on screening for over- and mis-expression variants (Rørth, 1996). This kind of mutant-hunting can lead to what are in effect altered alleles of genes (instead of molecularly ruined forms), and they are biased in a direction away from the leakies and nulls that rather commonly stem

from chemical mutagenesis (even though the latter can create things like ''neomorphic'' alleles, under whose heading might come ''over-expressors''). The relevant transgene set-up is a two-component one (Rørth, 1996), derived from the GAL4/UAS system that was appropriated from yeast (Brand & Dorman, 1995) and has been applied an endless number of times to dissect biological processes in *Drosophila*. To use this system for making mutants other than transposon-tagged hypomorphs, you obtain flies carrying a GAL4-encoding driver (best if it's well engineered and characterized in terms of when and where the *Drosophila* regulatory sequences promote expression of this yeast transcription factor). You cross them in series to a huge number of other transgenics, in each of which line a UAS-containing transgene sits at some genomic location (such elements, with their GAL4 regulatees, have been pre-mobilized to be scattered hither and yon at thousands of chromosomal sites). The latter transgenics are called EP lines (Rørth, 1996); the similar Gene Search system uses analogous GS lines (Aigaki et al., 2002). The idea is that, in certain of these doubly-transgenic flies, GAL4 will activate UAS such that a (normal) gene, if near enough to the latter's chromosomal insertion site, will be expressed in some deranged manner that's biologically detectable.

''Deranged'' is apprehended mainly to mean overexpression, although the prefix ''mis-'' would be more comprehensive. For instance, the driver-containing tissues might not encompass the usual domain of the EP-tagged gene, so GAL4-mediated activation of the latter would cause ectopic expression. In any case, the first fruit of a GAL4/EP screen for behavioral variants led to re-identification of *Drosophila's shaggy* (*sgg*) gene as flies in which—as it turned out—the *sgg*-encoded kinase is over-expressed (Martinek et al., 2001). The isolation phenotype was a shorter-than-normal cycle duration for fly's rhythm of locomotor activity. [See Dockendorff et al. (2002) for an analogous example involving identification of another rhythm-related gene, which, when overexpressed leads to longer-than-normal behavioral cycles.]

The *shaggy* investigators went onto to generate live flies in which lethal effects of the usual kind of *sgg* mutation were bypassed by transgene-induced expression of sgg^{+} during development; the expected under-expressors of this enzyme were indeed long-period for their rest-activity cycles (Martinek et al., 2001). Incidentally, SGG's substrate rhythm-wise—it must and does have several others, given the vital nature of the gene—is the TIMELESS clock protein. (This is encoded by the *tim* gene, which comes into play later to make various other points.) Therefore, SGG is in a way the companion kinase to that encoded by *Drosophila's double-time* (*dbt*) gene (a.k.a. *discs overgrown*, independently identified by the lethal mutations of Zilian et al., 1999). One of DBT's substrates is the PER protein. EP screens for variants of fly function are just getting started; the only other cases in hailing distance of functional neuroge-

netics (as opposed to the vast sea of genetic inquiry that involves neurodevelopment alone) were reported by Abdelilah-Seyfried et al. (2000) and Umemiya et al. (2002).

So, there's now more than one way to skin the cat of opportunity presented by transgenes that can create behaviorally altered phenotypes whose molecular etiology is ripe for the picking. But even when one's novel mutant was not designed to be tagged by a transposon—either an ordinary type or an EP one—contemporary mutageneses and the gene clonings that have ensued have been facilitated by genome data bases. Therefore, and as introduced above, one could stick to chemical mutagenesis alone, bearing in mind the interpretive power that's likely to be provided by a multiple allelic series. (Note that the different viable *sgg* "alleles" mentioned above had to be in part engineered after the original hypermorph was recognized by way of the initial screening.) Nowadays one can go from tiny intragenic changes to the pertinent DNA quickly. For example, high resolution genetic mapping can tell you the region of the genome in which to look for a molecular candidate in the data base (no walking necessary). Then you obtain that DNA in the mail, permitting a rapid determination of whether it is altered in material taken from your mutant (e.g., Sengupta et al., 1996; Allada et al., 1998; Rutila et al., 1998; Stanewsky et al., 1998; Dal Santo et al., 1999; Jansen et al., 1999; Dong et al., 2000; L'Étoile & Bargmann, 2000).

Thus, the conceptually feeble features of transposon mutants (too many hypomorphs!) are no longer balanced by the opportunism they present in terms of facilitating cloning. But there's more to chemical mutagenesis than the wide array of recessive mutant types that typically results from that kind of screening. If a chemical mutagen can hit the gene anywhere, among these mutations could be *dominant* ones that derange the function of the gene product. These mutations can in turn have effects that are "worse than nothing." This phrase is potentially understandable against a backdrop of the fact that a hypo- or amorphic variant, when heterozygous with the normal allele, usually leads to a minimally appreciable phenotypic problem. In contrast, for example, several *period* or *timeless* rhythm mutations in *Drosophila* cause circadian-period changes of two or more hours when a given rhythm-enabling variant is heterozygous with the normal allele. However, an arrhythmia inducing null mutation, over tim^{+} or per^{+}, leads to no change or a barely noticeable half-hour one for the flies' locomotor-cycle durations (reviewed by Hall, 2003). So, a "neo-" or "anti-morphic" mutation almost always require an amino-acid substitution. As exemplified by the *per* and *tim* variants just alluded to these kinds of intragenic changes lead to products that can interfere with the factor encoded by the normal allele, present on the other chromosome in a screen for dominant mutants. (In contrast, obviously there is no possibility for such interference when a null-mutation is heterozygous with the normal allele.)

Alternatively or in addition, neomorphic and antimorphic mutations can result in mutated proteins that can befoul the overall neurobiological process by interfering with the function of *other* gene products; this implies that the latter normally interact in heteromultimers with the protein encoded by the gene that was hit. Indeed, PER and TIM do so associate—not only with one another, but also with additional rhythm-related proteins (as is discussed in other contexts below).

These mutational sidepoints seem to be straying into authentic genetic gibberish (if only because the word "neomorph" has been used twice). But if so, necessarily so. One reason relates to the power associated with identifying and applying dominant mutations. Searches for them are extremely efficient in terms of labor and generation times. That seems like mere opportunism, but there's more. If you isolate a dominant mutant, there is a distinct chance that you found something which might not have been obtainable by recessive mutants–if the gene is a *vital* one, and homozygosity for many or most of the mutations induced would cause enough of a functional decrement to be lethal. Therefore, screens designed on behalf of novel dominant mutations can identify factors involved in the process of interest that might be missed altogether in recessively based mutant hunting (e.g., Jackson & Newby, 1993; and see Jackson et al., 2001, for where the originally dominant-abnormal, but recessive-lethal, rhythm mutation took these investigators).

One further genetic consideration harks back to the possibility that a loss of function for certain factors could lead to a minimal disruption of the process in question: "no phenotype" at the organismic level, which in the context of this subject would be normal behavior. Thus, one might conclude: "the gene is redundant!" Actually, this conceptual problem comes into play mainly in the context of reverse genetics (see below), which not uncommonly involves gene knockouts with no phenotype. If you happened to hit that same gene in this manner in a forward-genetic mutant hunt, you would not recognize the mutated strain. But if your screen judiciously includes testing mutated chromosomes heterozygous with normal ones, along the way to making such chromosomes homozygous, you might instead or in addition isolate a mutation at this locus that actively disrupts the overall process by the aforementioned interference with related functions encoded elsewhere in the genome.

This provides an example of how the investigator can nicely sidestep the *redundancy canard* (Brookfield, 1992). In this case, such a person would have shown that *certain forms of this gene can cause discernible phenotype problems*—even if losing the function entirely, with respect to a null mutation that would likely be induced in parallel, causes nothing *noticeable*. (The word just emphasized is one reason why the author just cited asked a question—"Can genes be truly redundant?"—and im-

plicitly answered it with "no.") Moreover, you wouldn't appreciate a putative loss-of-function behavioral phenotype (whether subtle or easily noticed) until you create a known genotypic null (such as by making a knockout) then test its homozygous effects, provided that such an amorphic mutation is not a recessive lethal. If that null-mutant type is not dead, and the animal seems for all the world to be behaviorally normal, the *wild-type allele of this gene would still not necessarily be redundant*—even though its *importance for phenotypic normality might have to be revealed by encountering or creating antimorphic alleles* (a term semi-synonymous with the phrase "actively disrupts... interference with..."). In other words, in the case of a gene whose *presence and robust function is not necessary for ostensible phenotypic normality*, full support of the normal state *cannot tolerate certain mutated forms* of this factor–those in which amino-acid substitutions lead, not to some sort of subnormality, but to active interference with aspects of the overall neurobiological process.

Thus, the *genetic principles* that have emerged in recent years, which can be as important to bear in mind as all the neurobiology, are propelling the forward side of behavior- and neurogenetics. The necessary arrays of mutant types *are* being found. One tends *not* to miss the identification of important factors; is inclined to *interpret better* the meaning of the gene by virtue of mutants that present altered functions, not just their absence; and has a fair chance of *avoiding* a situation in which one's mind goes into vapor-lock by invoking "redundancy!" instead of acknowledging "investigator inadequacy."

Nevertheless, these formal genetic concerns are not enough, as has been revealed by the manner in which *molecular* neurogenetic side of these behavioral investigations have come to the fore. Nowadays, there are few genetic limitations that might impede the path toward understanding these processes at the concrete level. One is indeed anxious to get to the molecular level of analysis: By definition, forward-genetic approaches—as long as they sit at the formal or abstract level—frequently do not permit even the whisper of a guess about the cellular and biochemical process in question. Two opposing object lessons come to mind in this regard: The many different kinds of mutations within the *lactose* operon of *E. coli* permitted so many firm predictions as to how the mutationally defined factors function concretely (Jacob, 1997) that the ensuing molecular genetics of *lac* were largely anticlimactic. A distinctly contrasting case is provided by the *per* variants in *Drosophila*. Although they were undeniably necessary to sustain the chronobiological investigations in question (the three kinds of mutant phenotypes would not quit), not the slightest clue about *per*-product function suggested itself until some years after the gene was cloned (see Hall, 1995, for one of the early reviews of what those strong suggestions were).

Mutants, which Are not Necessarily Behavioral Variants, with Defects in Basic Neural Functions

Actually, a few areas of neuro inquiry that started with forward neurogenetics *do* involve a priori hints and hopes as to what might be molecularly wrong with the anticipated variants. This category of phenotypes involves aspects of abnormal neural function that can be regarded as *themselves* not very interesting behaviorally. This remark (which is not meant to be pejorative) refers to neuro-functional defects that are not really behavioral abnormalities. For example, if a genetic variation causes generalized hyper- or hypo-activity one can guess that the underlying defect involves an aspect or neuronal-membrane function, or some other feature of already-known neurochemistry. Thus, the nature of the cloned gene might be (another) anticlimax. This is probably an overstatement, because at least one such neurogenetic story opened up what's by now large field: the molecular neurobiology of potassium channels. Before the pieces DNA corresponding to *Shaker* (*Sh*) mutants in *Drosophila* were cloned, one didn't really know what potassium channels were—although no one was thunderstruck when it was determined that *Sh* mutations changed their structure and function and thus began to define many features of K^+ channels in their normal forms. In any case, this story is so generically classical (starting as it did in the late 1980s) that it's not necessary to document here (just as one doesn't cite the origins, or the originators, of aspirin).

Nevertheless the case of *Sh*, and other mutants, which seem as if they would involve the nuts and bolts of nervous-system function, allow us to sharpen our focus on real behavioral mutants (in the passages below). Thus, *Sh* and companion mutants that are hyperactive—along with counterpart variants that cause hypoactivity—are rarely appreciable as behavioral mutants. This is because the mutant abnormalities involve foul-ups in neuronal or neuromuscular functions that do not speak to a normal phenotype. What is "*Shaker*-plus" behavior? It isn't anything–except the animal's generalized well being. Therefore, we can cavalierly leave aside mutants of this sort, simply in order to make the subject manageable by limiting it to cases of bona-fide behavior. This is not to denigrate the *Shaker* story or analogous ones told subsequently by forward- and molecular-genetic analysis of other anomalously excitable mutants. The case of the *Shaker* mutants provides one of the triumphs of neurogenetics, because potassium channels might not have been gotten to molecularly otherwise (or at least not as early as approximately 15 years ago). Ironically, however, certain *Sh* mutant phenotypes do speak to real behaviors, such as defective chemosensory responses that can be pitted against nicely specifiable behaviors for wild-type flies (Balakrishnan & Rodrigues, 1991). Later, we'll encounter some additional types of ion-channel mutants that initially were not "real behavioral" variants either,

but later took on that quality by virtue of phenotypic analyses beyond those involving the doleful defects by which these mutants were originally identified.

Meanwhile, the *Sh*-induced phenotype that was uncovered in the study just cited brings us to sensory neurogenetics. For this, some of the basic components of PNS excitability were anticipated to have the potential to be neurogenetically triumphant. For example, certain of the *olfactory* (*olf*) mutants in *Drosophila*, which began to be isolated in the 1970s and were reviewed later by Siddiqi (1987), could have been the stalking horses for identification of the mysterious odor receptors. However, many of these mutants fell by the wayside, even into disrepute. It turned out to be necessary and sufficient to identify the relevant invertebrate genes mainly by molecular procedures augmented by genomics (reviewed by Lessing & Carlson, 1999; Vosshall, 2000, 2001)—following the lead of mammalian folk.

It was in *C. elegans* that an olfactory-receptor gene got found by a mutational approach (Sengupta et al., 1996), coming on the heels of chemotaxis screens that had been initiated several years before. One of the genes so defined, *odr-10*, was found to make a member of the magnificent-7 family of membrane proteins, naturally (Sengupta et al., 1996). Worm genomics (Bargmann, 1998) showed this polypeptide to be a member of a large subfamily of analogous transmembrane proteins; other such subfamilies bring the number of genes up to 5% of the *C. elegans* genome—which, by the way, has defeated that of *Drosophila* by a score of 19 K to 14 K (although see Gopal et al., 2001).

The case of *odr-10* is a cause for genetic celebration. Moreover, the receptor specified by this phenogenetically defined factor is firmly tied to a known ligand; and *odr-10* presaged studies of the cellular neurobiology of "odor coding" in this organism, which soon after became quite advanced (e.g., Mori, 1999; Troemel, 1999). The results of such studies include localization of molecules such as ODR-10, and other factors identified by mutations (G-proteins, ion channels), to particular chemosensory neurons. However, this large subject demands its own review, which would cut across the molecular biology of olfaction in both nematodes, *Drosophila*, and mammals. To flog the subject ever-too-briefly, it's worth registering that the molecular genetics of a chemo system *is* most advanced, comparing the two invertebrates in question, for *C. elegans*. Among the more interesting examples are those supplied by studies which delved into differences involving olf-receptor presence in bilaterally symmetrical sensory neurons and how such highly resolved spatial-expression patterns can account for the ability of worms to discriminate between odors (Pierce-Shimomura et al., 2001; Sagasti et al., 2001; Wes and Bargmann, 2001).

One feature of the mutational side of olfactory genetics in nematode and *Drosophila* that has some special potential involves regulatory

functions that were identified by olfactorily defective variants possessing mutated transcription factors. For example, the fly's *acj6* mutant has altered odor specificity, and the transcription factor encoded by this gene influences expression of a subset of the odor-receptor genes (Clyne et al., 1999; also see Sagasti et al., 1999). Given the obviously "not-olfactory-specific" nature of *acj6*'s product, it is unsurprising that mutations in this gene also cause generic locomotor defects and neuroanatomical abnormalities that have nothing to do with the fly's sense of smell (Certel et al., 2000).

Additional chemosensory genes in *Drosophila* (reviewed by Smith, 2001) are the sequences identified by genomics that are "candidates" to produce taste receptors (Clyne et al., 2000; Dunipace et al., 2001). This purely molecular approach was augmented by digging into the one putative taste-gene that was originally known by virtue of a mutant with altered sugar responses (Ishimoto et al., 2000). Other kinds of sensory mutants, which also are real behavioral variants (see below), continue to expand our understanding of visual transduction processes (Montell, 1999; Hardie & Raghu, 2001), as well as open up analyses of mechanosensitive (Walker et al., 2000) and nociceptive phenomena (Tracey et al., 2003). With 20-20 hindsight, identification of most molecules encoded by such sensory genes are anticlimactic, in that they've involved identifications of additional ion-channel types, G-protein relatives, or previously known enzymes—factors that one would expect to be involved in how the animal receives and transduces basic information coming in from the world in order to make very simple behavioral responses to environmental stimuli. These outcomes are examples of (brace yourself) "academic" behavioral genetics and molecular neurobiology, as opposed to that which can be apprehended as more "romantic" (cf. Stent, 1969).

Mutants with Defects in Actual Behaviors

To consider the actions of animals that one might anoint as romantically intriguing and mysterious, or at least more complex, let's reconsider certain of the "merely hyperactive" mutants. As previewed above, I lied a little about some of them. So they'll now be discussed further from the perspective of "what is a behavior?" It can be thought of as something that is not only observable but also *measurable* for the wild-type animal—tough to do in a case where "this genetically normal fly is simply not shaking." Examples of these "somethings" involve sets of quantifiable responses that are normally elicited by elementary sensory stimuli (e.g., Balakrishnan & Rodrigues, 1991); more complex ones, such as those that trigger the animal's "motion detection" (e.g., Heisenberg and Wolf, 1984); or learning and memory scores that result from the organism's processing of multiple stimuli (as has been endlessly reviewed

in general terms, e.g., Dubnau & Tully, 1998; Roman & Davis, 2001). In the latter area of inquiry—mnemogenetics (Heisenberg, 1989)—the animal's handling of the sensory inputs is followed by behavioral actions above the level of quick and simple responses; thus, one measures learning and memory "performance indices" that can be nicely established as normal benchmarks against which to pit the experience-dependent behaviors of putative mutants.

Therefore, it should be noted that certain altogether too basic and obvious neuro-functional mutants *became* more than phenotypic variants harboring defects suggestive of physiological pathologies. For example, both *Sh* and *ether-à-go-go* potassium-channel mutants were found to exhibit subnormalities of experience-dependent behaviors, in the contexts of an habituatable escape response (Engel & Wu, 1998) and of *Drosophila*'s reproductive behavior (Cowan & Siegel, 1984, 1986; Griffith et al., 1994)—underpinned by the fact that "conditioned courtship" for the wild-type is firmly connected with appropriate learning or memory scores. [The bona-fide behavioral testing of *eag* was accompanied by certain reverse-genetic experiments, to be mentioned far below. Additional phenotypic assessments of *eag* mutants themselves (Dubin et al., 1998) indicated "real phenotypes" (subnormal odor responsiveness in physiological tests), as opposed to merely being induced "to go (go!)" upon exposure to ether.]

Moreover, *slowpoke* potassium-channel mutants (identified initially by generic sluggishness) turned out also to exhibit abnormalities of the *Drosophila* male's courtship song (Peixoto & Hall, 1988); whereas almost all other excitability mutants are normal (or marginally defective) for this heavily quantified behavioral character (Kulkarni & Hall, 1987; Peixoto & Hall, 1998). As will be discussed later in a reverse-genetic context, *slo* mutants are behavioral ones from an additional perspective, being abnormal for daily rhythms of locomotion (Ceriani et al., 2002).

Here's the point (although my physio-genetic friends, of whom I have none, tell me there isn't any): *Sh*, *eag*, *slo*, and the like were identified originally as patho-physiological mutants (not that there's anything wrong with that). The category circumscribing them got expanded into the behavioral realm when learning, courtship-song, and behavioral-rhythm connections were surmised, examined, and recorded—against backdrops of these phenotypes being admirably quantifiable features of the normal fly's activities.

A conceptual problem suggests itself from the would-be points just made: What if something like a *slowpoke* mutant is merely so sick in general such that "any" behavior would be subnormal if tested? (*slo* flies do exhibit generic sluggishness.) The supposition here is that you can turn pretty much *any* genetic variant that involves the general and basic functioning of excitable cells into a "behavioral mutant" if you perform tests of all actions of which the animal is known to be capable (provided, once

again, that you actually measure something vis-à-vis scores determinable for wild-type). Thus, mutants such as *slo* would be one of many that are all over the genetic world–well beyond the variants under discussion, hence well beyond neurobiology–that are *pleiotropic*. How do we separate the wheat from the chaff? In the context of this review, how might we set aside mutants defective in functions that really control a given behavior, compared with other mutations that can impair it? "Can" would mean, for example, a disruption of the final process via uninformatively indirect routes.

Well, you can't afford to set any mutant aside, necessarily, based on some early knowledge about its multiple abnormalities or guesses about a circuitous path that the mutational effect may take to get to the behavioral defect. What has emerged over two or three decades of study is that one must move *the entire broad front of mutants* forward—referring to almost all variants that are isolated in given forward-genetic screens, even though some of the functions mutated (e.g., Pickard et al., 1995) are likely to wind up having effects that are too pleiotropic and indirect (Alavizadeh et al., 2001). One nevertheless tries, by increasingly high-resolution genetic mapping, to home in on all these loci and use the information for positional cloning. As the ensuing molecular data emerge, they might encourage the belief that you're onto something (whether or not the mutants' phenotypes are wonderfully "specific"). However, one bites the bullet to imagine the following (sticking with mnemogenetic and courtship examples for the moment): Some of the conditioning or song mutants might define substances like LEARNIN or SINGIN (beguilingly brand-new entities in the subfields of behavioral inquiry); but others could prove to be defective in something altogether different—a dreary enzyme like "aldolase."

In fact, the opinion was proffered publicly that most behavioral and neurobiological mutants—which seem so interesting because of their phenotypes—will deteriorate in the end to defects in miserably ordinary functions such as the metabolic factor just named (S. Brenner said as much in an address he delivered to the Genetics Society of Canada, circa 1970). This old prediction kept making the rounds contemporarily with the following negativity: Genetic approaches to complex biological problems might have a certain "instrumental" value, by the mundane virtue of creating "tools" that could be applied to manipulate some piece of biology (Stent, 1981). But there would be no meaningful "ideological" feature to these approaches, inasmuch as the genes identified by the mutations would not connect with functions that might promise to reveal any in-depth appreciation of how an organism builds its complex structures, then operates them to mediate complex behavioral patterns.

Who knows, however, if a hapless enzyme isn't a real regulator of something like conditioned behavior? One of the old warhorses of behavior genetics is instructive in this regard—the *dunce* (*dnc*) mutants in

Drosophila. Long ago (pre-cloning) they were found to be defective in a cAMP phosphodiesterase (Byers et al., 1981), an enzyme (cA-PDE) that one didn't have to discover genetically. [There were earlier suggestions that this locus encodes a cA-PDE (Kiger & Golanty, 1977, 1979), stemming from a "segmental aneuploidy" sweep through the genome (don't ask); this is a cytogenetic approach to gene identification like those that homed in on factors mediating neurotransmitter metabolism, as discussed in the reverse-genetic section of this piece.]

The biochemical correlate of *dnc* caused a developmental geneticist (Garen, 1980) to ask: "What is the basis for your classification of *dunce* as a behavioral mutant" (as if this person was archly offended by fact that the gene encodes a mere enzyme, one previously known to influence many biological processes). The answer (from Byers, 1980) was that "a behavioral mutant is one that affects behavior"–which speaks once more to the fact that many things (anything?) *can* cause behavioral abnormalities when mutated. However, few would now argue that the manner by which cAMP levels are regulated (in part by *dunce* and its encoded product) are uninteresting in terms of how the fly and other organisms are able to learn and remember (Dubnau & Tully, 1998; Roman & Davis, 2001).

Mutant-based studies of experience-dependent provide examples of how one keeps pushing the entire front farther along—its phenogenetics then molecular and biochemical components, even if the latter involves only an enzyme. In particular, the case of *dunce* proceeded rather quickly from behavioral-genetics to neurochemical genetics. During that seminal five-year period and shortly afterward, one could not assert that an influencer of a processs, such as the mere enzyme encoded by *dunce*, is uninteresting or destined to be uninformative. As findings of this sort proceeded from the initial connections between formally defined mutations (thus the *dnc* genetic locus) to something more concrete (cAMP biochemistry), what eventually emerged was this: One *kept learning more and more about the normal neurobiological and behavioral processes*. And this kind of conceptual progress occurs in continuing studies of *some proportion of* the mutants and gene products. In contrast, at certain investigatory moments involving *other* of the mutants and molecules, *no further clues or insights* seem to be welling up from the findings. So this proportion of the particular forward behavior-genetic enterprise gets quietly put to sleep.

An example of such euthanasia seemed to be provided by 20 years of studying, off and on, the *Andante* rhythm mutants in *Drosophila* (Orr, 1982; Konopka et al., 1991; the senior author of the latter work had isolated the first such mutant in the early 1980s). Some of these genetic variants are defective in wing morphology as well as circadian behavior (Newby et al., 1991). Thus this gene would in part be concerned with "uninteresting" abnormalities of the fly's external abnormality (via a

direct effect on the developing wing: Newby & Jackson, 1995). As studies of *And* proceeded—barely, such that incipient cloning of the gene seemed to die on the vine (Jackson and Newby, 1993)—chronobiologists seemed to be learning nothing from it about normal behavioral-rhythm regulation. So *Andante* and its "moderately slow" rhythm mutants faded from the scene. However, this case is now deteriorating as a negatively construed object lesson: *And* has at last emerged as encoding a potentially interesting rhythm-modulating enzyme (Atken et al., 2003; cf. Jauch et al., 2002; Lin et al., 2002a). Meanwhile, the wing defect in the original *Andante* mutant turned out to be due to a second-site mutation at the *dusky* locus, very near to the nucleotide substitution within adjacent the *And* gene that causes moderately long-period rhythms (DiBartolomeis et al., 2002). [This ostensibly miraculous double mutant is not strange, in historical context: As described in the fine-print of Hall (1994), at least seven other neurogenetic examples exist for which a mutant's overall phenotype was caused by two apparently co-induced mutations, some of which were eventually found to be quite closely linked; moreover, the separate effects of each mutation almost mystically involved similar areas of *Drosophila* neurobiology or behavior. Thus, beware! of the distinct possibility that there is a complex genetic etiology for your newly induced neuro mutant.]

In any event, many other rhythm mutants and the genes they defined have bobbed to the top like a cork in recent years. In fact, what's been gleaned from analysis of chronobiological factors gives a lie to the notion that none of these functions would prove to be "ideological." Arguably, isolation of the *period* mutants in *Drosophila* led to a behavioral-, neuro-, and now heavily molecular-genetic story, the reading of which allows one to infer that the case is dangerously close to being sol-ved (Hall, 1998; Young, 1998, 2000; Williams & Sehgal, 2001; Young & Kay, 2001; Albrecht, 2002; Stanewsky, 2003). Much of that case has involved behavioral-genetic screening of animals and neurogenetic analysis of chronobiologically relevant neural substrates underlying rest-activity cycles; although the clock-gene story has been expanded well beyond the scope of this review and into rhythmic phenomena that go way outside the nervous system and into organisms that don't behave (as reviewed by Dunlap, 1999; Johnson and Golden, 1999; Kondo & Ishiura, 1999; Somers, 1999; Barak et al., 2000; Staiger, 2002).

One instructive feature of this story is that it showed how a pioneer protein—indeed, the one encoded by *per* itself—could begin to be understood in terms of how it functions at the level of cellular biochemistry. This occurred in conjunction with histological studies that initially involved mere characterizations: Application of antibodies against this protein (PER) showed that it defines a daily molecular rhythm (Siwicki et al., 1988). Among the cells within the fly head where PER immunoreactivity cycled in this manner are certain brain neurons (Zerr et al.,

1990). These were subsequently shown to control behavioral rhythmicity (e.g., Ewer, 1992; Frisch, 1994); and mutations which alter these locomotor cycles change the cycling "parameters" for PER within pacemaker neurons (Zerr et al., 1990) as well for *per* mRNA in fly-head homogenates (Hardin et al., 1990).

Identifications of circadian-clock factors—*per* along with a tractable handful of additional genes—were accomplished in the main by screening for behavioral-rhythm mutants followed by positional cloning (see the reviews cited in the paragraph atop the previous one). Notable among these is the aforementioned *timeless*—clock-gene number 2 in *Drosophila*'s chronogenetic history. Its mRNA as well as TIM–you guessed it, another pioneer protein–also exhibit daily oscillations of their abundances (Sehgal et al., 1995). Indeed the Zeitgeist of chrono-molecular-genetics, no matter in what organism (Dunlap, 1999), is that "clock-gene products cycle!" (not all of them, however). And the manner by which environmental stimuli (notably light) interface with the *Drosophila* clock to reset it each day, as well as how that pacemaker feeds its output into cyclical behavior, have been brought within hailing distance of elucidation by looking for additional rhythm mutants (Stanewsky et al., 1998) or stumbling into others in a reverse-genetic context (Renn et al., 1999; Sarov-Blat et al., 2000). Moreover, biological outputs from *Drosophila*'s central clock functions, beyond the classically periodic behavioral and adult-emergence phenotypes, are being defined at newly appreciated levels of neural functioning. Some of them are squarely in the circadian arena (albeit its physiological side: Krishnan et al., 1999, 2001); others not at all (Andreti & Hirsh, 2000; Megighian et al., 2001), exemplifying the tip of *per*'s pleiotropic iceberg (Hall, 2003).

One hopes that these kinds of findings provide object lessons for other forward-genetic cases well beyond behavioral rhythmicity. Thus, mutational identification of the *period* and *timeless* genes, followed by their positional cloning and recognition of the featureless PER and TIM proteins, advanced toward understanding of how these initially mysterious factors mediate a "central control" function. The rhythmic process in question was also inexplicable more broadly (a side from the mysteries of *Drosophila*'s *per* and *tim* genes in their early days). But cracking this chronogenetic case has encompassed more than the root cellular biochemistry of PER, TIM, and other clock-gene products. In addition, this case of forward genetics has expanded toward understanding how intracellular functions within certain "escape" outward into regulation of the fly's neurobiology and behavior (Jackson et al., 2001; Taghert, 2001; Park, 2002).

Perhaps this apparent success story has not been recapitulated all that many times. But an additional example in this broad area of forward behavior- and neuro-genetics is instructive from another angle. Thus, we revisit mnemogenetics. Screens for such mutants, occurring sporadically

over the last quarter of the previous century, turned up only a small handful of interesting learning and memory variants (but see below and Dubnau et al., 2003). One of the classic such mutants is *amnesiac*, which by definition learns OK but forgets too fast (Quinn et al., 1979). The *amn* locus was eventually cloned in the mid-1990s and turned out to encode a pair of gedanken peptides (Feany & Quinn, 1995). One of these substances, which we'll call AMN, is a Pituitary-Adenylate-Cyclase-Activating-Polypeptide (PACAP)-like molecule. AMN could be quickly rationalized to tap into cAMP signaling. So it fit. That is, this *amnesiac*-encoded peptide was instantly and literally fit into "the model" for cellular learning (Kandel & Abel, 1995).

Fair enough—although there are several narrow-sense problems with this scenario: Synthetic AMN (PACAP-oid) oligopeptides do not lead to mimics of electrophysiologically monitored responsiveness to (mammalian) PACAP per se at neuromuscular junctions (Zhong, 1996; Y. Zhong, personal communication). These observations correlate with the facts that AMN's similarity to bona-fide PACAP is not that strong (discussed by Taghert & Veenstra, 2002), and that PACAP-like immunoreactivity in *Drosophila* is not congruent with that of AMN in situ (Y. Zhong, personal communication). The gene's expression pattern also speaks to the matter of *amn*-associated *gal4* drivers causing lethality when they are combined with UAS-cell-killers (Waddell et al., 2000). As one's perspectives about what *amnesiac* does for the life of the fly therefore broadens, consider the following intriguing finding about this neuropeptide gene: *Developmental* expression of *amn* is sufficient to rescue learning defects caused a mutation in the gene (DeZazzo et al., 1999). The tissue expression of amn indeed occurs early and often during the fly's formative stages (DeZazzo et al., 1999), which presumably jibes with the killing observed in the doubly-transgenic flies just referred to. (In other words, if *amnesiac* were solely a "neuro-functional" factor whose product is not found during development, one might expect that killing a few AMN-containing neurons in the mature animal would result in an ostensibly healthy fly from which one would have to measure subtle behavioral impairments.)

The apparently *non-physiological etiology* of *amnesiac*'s memory impairment—despite certain intriguing neuronal-functional correlates that are exhibited by *amn* mutants (Rosay et al., 2001)—creates the same kind of interpretive annoyance as that which was provoked by extended studies of a so-called "sensory gene." The experiments involved *transient photoreceptor potential* (*trp*) mutants, whose olfactory-response anomalies are uncorrelated with expression of the encoded calcium-channel in the adult form of the chemosensory organ in question (Störtkuhl et al., 1999, discussed in more detail below). In contrast, a *cheapdate* mutation, which re-identified the pleiotropic *amn* gene when it got hit in a screen for mutants behaviorally hypersensitive to alcohol, can have its effects res-

cued solely by induced expression of amn^{+} sequences in the adult fly (Moore et al., 1998). If these findings don't already imply "enough with *amnesiac* pleiotropy!," please register as well that *amn* mutants exhibit a slower-than-normal heartbeat in *Drosophila* pupae (Johnson et al., 1997; cf. Johnson et al., 2000).

At all events, the *amn*-encoded PACAP seem not to play an acute functional role in The Learning Mechanism (T.L.M.). In this regard, the applause that greeted *amnesiac*'s cloning and product-suggesting sequencing brings to mind the story of a concert pianist who finished a recital to a modest level of hand-clapping. He exited but then rapidly returned to the stage to play an encore. Someone in the audience remarked: "Too quick!" The same could be said for *amnesiac* case and the quick inclusion of PACAP in the T.L.M.—within the piece by Kandel & Abel (1995) that was commissioned because of Feany & Quinn (1995)—before the developmental etiology of this mutant's memory deficit was demonstrated (DeZazzo et al., 1999).

The main point of the *amnesiac* story is not that one can jump the gun in terms of inferring an adult-functional role for the gene's product. Instead, the case of *amn* and its putative neuropeptide leads to consideration of another learning mutant, in particular, and what it says about forward-genetic approaches more generally. Thus consider the *linotte* (*lio*) mutant, which was identified by transposon mutagenesis and learning tests (Dura et al., 1993). The gene so tagged was reported to be identified at the same time the molecular genetics of *amn* came forth. But, unlike what was deduced from sequencing the latter's open reading frame, *lio*'s molecular correlate was inferred to be another one of these utterly unknown proteins (Bolwig et al., 1995). This will cause us to ponder how we must struggle to fit *linotte*'s product into models of learning.

Identification of the learning-relevant stretch of *lio*-locus DNA came from a transgene mutant-rescue operation in which *lio*'s coding sequences were activated in the adult flies (cf. Ewer et al., 1988, 1990, for *per*; and Moore et al., 1998, for *amn*). This mirrors a test previously performed with a *dunce* mutant (although not involved in its initial gene identification): A *heat-shock promoter* (like that used in the *per*, *amn*, and *lio* experiments) was fused to *dunce* coding sequences; late activation of the transgene largely rescued the learning defects of adult flies (Dauwalder & Davis, 1995), implying that a (straight) *dunce* mutant is not behaviorally abnormal because of developmental brain damage.

In *hsp*-based experiments exemplified by the *lio* and *dnc* ones, the inducible regulatory sequences led to *ubiquitous* expression of the sequences they are designed to control, which might be unfortunate. Therefore, temporal control of gene-rescue should in the future draw on transgenic technology that permits control of the gene's *spatial* expression as well (Bello et al., 1998; Bieschke et al., 1998; Osterwalder et al., 2001; Roman

et al., 2001; Stebbins et al., 2001). This approach, originated in mammals, has been nicely applied in rodent behavioral-genetic experiments (e.g., as reviewed by Mayford et al., 1997; Mansuy et al., 1998; more recent examples: Kida et al., 2002; Pittinger et al., 2002). These mouse manipulations are much farther along compared with flies or worms in terms of attempts to unravel developmental versus brain-functional "conundrums" about behavioral mutants (Tully, 1994).

Nevertheless, in the cases of both *dnc* and *lio* as temporally manipulated by *hsp*, the animals developed in the face of potential mutant developmental defects. For *dnc* they are a serious possibility, because 2nd-messenger signaling wouldn't be used solely for ongoing functions of the mature organism. In fact, *dnc* mutants (by themselves or in combination with certain other neural variants) exhibit other behavioral anomalies, mating-induced early death, female sterility (whose causation is not behavioral), and subtle abnormalities of neuronal morphology (e.g., Bellen & Kiger, 1987; Zhong et al., 1992; Renger et al., 2000; reviewed by Davis & Dauwalder, 1987; Wu et al., 1998). Indeed, it is now well known that factors which might seem to be neuro-functional only (involving physiology or metabolic catalysis) can influence neuroanatomy (for a tiny fraction of the relevant examples, see Budnik et al., 1989, 1990, Patil et al., 1995; Tam et al., 2000). Thus, it is reasonable that pieces of neurochemistry such as those encoded by *amn* and *trp* could be connected with developmental etiologies for the respective mutant defects (DeZazzo et al., 1999; Störtkuhl et al., 1999).

It is somewhat up in the air as to whether there are any such anatomical defects in *lio* mutants per se (see below). The key feature of transgene-rescuing *linotte*'s learning impairment is that it was sufficient for the mature fly to have normal lio^{+} function in order that it learn normally (Bolwig et al., 1995). This indicates that the LIO protein is involved in the *ongoing operation of the learning machinery*. So the question is immediately raised as to where a protein of this kind might be placed within T.L.M.?–such as the *amn*-accompanying version of it that appeared during the same year when the cloning of *lio* was reported. Needless to say, this colored diagram of how neurons learn was able quickly to insert the AMN-cum-PACAP peptide within the pertinent part of the diagrammed neuron (Kandel & Abel, 1995). But where would LIO be situated within the scheme, or even on its fringes? Nowhere, for now. Yet, if it is a neuro-functional entity, one wishes to figure out what LIO's cellular and biochemical functions entail. We would not be so concerned if the *linotte* mutant's learning defect had been shown to have a solely developmental etiology (from gene-induction experiments involving formative stages of the life cycle). In that case, the molecule could truly be "anything;" and one would not worry about LIO's cellular biochemistry in terms of how T.L.M. operates. That we should care about LIO in this regard speaks volumes to the power of forward neuro-genetics, and that

we really want some of these "value-free" mutants to shove us beyond the fringes of terra firma—in this case, outside the putative comfort zone of cAMP signaling, closely associated functions, and their known knock-on consequences for intracellular chemistry and intercellular communication.

Having said all this, it must be mentioned that whether the *lio* gene was identified as encoding a brand new protein is in dispute, because a conflicting set of studies claims that this gene corresponds to a separate but adjacent transcription unit (Dura et al., 1995; Moreau-Fauvarque et al., 2002). The latter encodes a receptor tyrosine kinase, independently identified by a neural-development mutant called *derailed*, a.k.a. *drl* (Callahan et al., 1995). It is believed by the competing investigators that the key to *lio*'s learning defect is lower-than-normal expression of *drl*; its transcription unit is located just to the other side of the P-element's insertion site (Moreau-Fauvarque et al., 2002). (When transcription units are closely juxtaposed—or overlapping or contained one within the other, as can be the case—transposon-tagging is not such a great opportunity.) It is easy to imagine that yet another kinase (LIO as DRL) would be involved in neural development, quite possibly including the neural substrates of learning. Indeed, certain *drl*-cum-*lio* mutants are reported to exhibit brain-damage within the fly's famed mushroom bodies (Moreau-Fauvarque et al., 1998). And so who cares what the gene encodes?—seriously, because essentially any way that they get disrupted anatomically causes learning impairments, as has long been known from the effects of several different kinds of mutations and of chemically induced mushroom-body elimination (e.g., Heisenberg et al., 1985; de Belle & Heisenberg, 1994; McBride et al., 1999).

The *lio* story—its own conundrum within the conundrum notwithstanding—reveals that it is not enough to contemplate "candidate genes" (which all seem to be kinases), knock them out, and find learning defects. This defines a fair fraction of the mouse story insofar as mnemogenetics is concerned (Kandel, 2001). But that approach alone is too limiting, comforting as it may be to stick with genes already known as to the molecular properties of their products. If forward genetics turns out to be "implosive" in this manner, so be it (as was mentioned above and is also discussed below, with regard to mutant screens that merely re-identify stuff that's already known). But any organism studied behavior-genetically needs to be grappled with by genetic *analysis*—mutant screens, complementation tests, and mapping—more than by application of existing clones and manufactured mutants. Perhaps mice are too refractory to large-scale mutant screenings. In fact, when such a screen for dominant mutations (see above) got underway—in a search for rhythm variants—"mouse number 25" was found to be behaviorally defective (reviewed by Kyriacou, 1994). As far as one can tell from anything in the literature, this outcome—which may have been "too quick"—led to

abandoning the screening in favor of positionally cloning the first gene that got mutated (Antoch et al., 1997; King et al., 1997).

Saturating Screens for Behavioral Mutants

Far more extensive such forward genetics is necessary, for mouse rhythms and for whatever else. This obligation is not just to provide additional genes on which to concentrate molecularly. One should keep mutagenizing in order to accumulate very large numbers of mutants. The question being asked is how many different genes are mutatable to affect a given process? What if hundreds of mutants are isolated?—as occurred starting about 30 years ago for visually defective variants in *Drosophila* and mechanosensory ones in nematode (Pak, 1991; Driscoll and Kaplan, 1997). It was extremely instructive in both cases that all these mutations, by complementation and locus mapping, defined only about a score up to a few dozen genes. It was as if the organism was approaching saturation in terms of hitting most of the factors that can disrupt the process in question. (Proviso for the worm mechano system: isolation of relatively mild "body-touch" response-defective mutants saturated that feature of the system; mutants abnormal for "nose-touch" or "harsh-touch" responsiveness: not saturated.)

Another caveat is that not necessarily everything hit during these two kinds of mutant screens in the two organisms necessarily exerts "core control" of the intracellular structures and functions within the respective sensory cells. This warning pertains especially to the approximately three-score-and-ten of would-be "visual genes" in *Drosophila* (over a denominator of more the 13-score mutants that came out of the screening referred to above). But these kinds of broad genetic results–which are really *genomic* ones—reveal that *less than everything* can "get to" these sensory phenomena and disrupt them. If this grimly intractable scenario had played out—with "everything" damaging the processes in a destined-to-be uninformative manner—then nearly all new sensory mutants of the sorts just referred to would have identified previously un-mutated loci.

To take another example from forward chronogenetics in *Drosophila*, what has been bubbling to the surface recently again seems to imply an asymptotic approach toward saturating the "clock genome" of this insect: Almost every new mutation that causes more than a marginal or iffy defect in behavioral rhythmicity turns out to be within a gene that was identified by one or more previous hits. Yet all the new mutations, which have occurred at a total of only about a half-dozen loci, were found independently of one another (Allada et al., 2001, Williams & Sehgal, 2001; Young & Kay, 2001; Hall, 1998b, 2003).

The *salutary* implosiveness of this situation is suggested further by an interspecfic anecdote: It involves, first of all, the double-time (dbt) rhythm

mutants in *Drosophila*. The first such mutation found causes, by definition, very short-period behavioral cycles (Price et al., 1998; also see Rothenfluh et al., 2000; Suri et al., 2000). The *dbt* mutations identified yet-another factor that helps operate the fly's circadian clock (Kloss et al., 1998). Now let's introduce the only other mammalian rhythm variant, aside from *Clk*, that started as a mutant: *tau*, which also happens to exhibit short-period behavioral cycles (Ralph & Menaker, 1988). Like *Clk*, *tau* was tied to its molecular correlate by positional cloning, albeit with some interesting twists (Lowrey et al., 2000) because this spontaneously occurring "fast-clock" mutant was found in a hamster. Along the way to cloning it, the *tau* gene could have been thought to encode the-usual anything: I-say-again, some brand-new clock component, hitherto unanticipated. Instead, the outcome was another case of conservatism, loosely analogous to the *intra*-organismic situation for invertebrates (after isolating a goodly number of behavioral mutations affecting a given process, most new ones simply re-define already known genes). Thus, *tau*'s product turned out to be a strong relative of *Drosophila's dbt*-encoded kinase (Lowrey et al., 2000).

As another instance of moving back and forth between discussions of rhythms variants and those connected with another "important" kind of adult behavior: Again we consider learning and memory abnormalities in *Drosophila*, now from the perspective of mutant screens and overall genetic patterns of recovered variants. Systematic searching for them was not carried out with relentless enthusiasm after the first such effort occurred in mid-1970s. A corollary was that only the *dunce* locus was hit more than once among the approximately half-dozen loci that got independently mutated (reviewed by Dubnau & Tully, 1998). Also, certain of learning variants never "became loci," in the sense that single-gene etiologies (let alone genetic map positions) for the behavioral subnormalities were not determined. Very recently, however, a large-scale screen for transposon-tagged mutants resulted in a dramatic increase in the number of variants in this area of *Drosophila* behavior: 60 new memory mutants, from testing flies in some 6,700 lines derived from transposon mobilization (Dubnau et al., 2003). Yet the saturation issue arising from these impressive results is disquieting: only three of the relevant genes were hit twice (Dubnau et al., 2003). This outcome may be connected with the relative inefficiency of transposon mutagenesis.

Let us hope, however, that this re-invigoration of forward mnemogenetics in *Drosophila* will continue and include the employment of chemical mutagens, such that saturation will eventually appear on the horizon. Another possibility is that hunting for associative-conditioning mutants will spread to other species, in conjunction with the hope that many additional rhythm mutants will be induced in animals other than *Drosophila* (cf. Vitaterna et al., 1994, Pickard et al., 1995). What is most

unlikely to occur as a result of these screens is the following: A new learning or rhythm mutant be found at rates of well less than something like one per month. Finding too many mutants is potentially disquieting (recall "mouse number 25"). But this rarely happens, and that that's so is instructive: One usually isolates only a handful of mutants per year that are defective in a given behavior (exception: nematode, with its very high rate of genetic throughput, which may be on the cusp of being rivaled by zebrafish, vertebrate-neurogenetics-wise, e.g., Baier, 2000; Li, 2001). This indicates further that not any sort of gene change can disrupt a given process.

Retrospective screening through *extant* mutants confirmed this hoped-for conclusion. Most recently this was done for circadian rhythms in *Drosophila*: a variety of "neuro" mutants, and others that the investigators imaged might present abnormalities in rest-activity cycles of behavior, were plowed through; almost all were rhythm-normal (Vosshall & Young, 1995). This harks back to similar inventoring of, on the one hand, various metabolic and physiological mutants in *Neurospora*, very few of which were abnormal in the daily growth- and differentiation-associated rhythm of this fungus (Lakin-Thomas & Brody, 1995); and, on the other, *Drosophila* mutants previously shown to suffer from known or putative neurobiological defects; the result once more: next to no newly identified learning variants (Dudai, 1977). Therefore, the notion that everything affects everything is belied—even though, speaking of the fly genes themselves, everything seems to be *expressed* everywhere and at all life-cycle stages (not that hyperbolic a claim, actually).

More Pleiotropies, Mainly Modal Ones

The foregoing discussion should not be taken to imply that a significant proportion of a given category of behavioral mutants is without pleiotropic effects, and of course we've already run across many of these in passing. It's just that extensively interlocking and potentially intractable pleiotropies are not ubiquitous. Instead, at least to a first approximation, the following state of pleiotropic affairs has emerged in recent years. These outcomes are more satisfyingly comprehensible, intriguing, and stimulating of further research than they are distressing–even though the findings may perturb those who yell at their gene: "Be specific!" The gene answers: "Sorry, but you should have expected that I'm a 'Jack of a few trades.'"

First, let's recall the *double-time* gene and its relatives from the previous section. *dbt* is a vital factor in *Drosophila*; in that certain alleles contemporaneously identified with the rhythm mutations at this locus are developmentally lethal (Zilian et al., 1999). This seems correlated with *dbt* encoding an "ordinary" kinase (Kloss et al., 1998), which could

suggest that enzyme is involved merely in "metabolically facilitating" its rhythm-related function. But this is belied by the fact that *dbt* can be mutated to cause more than an absence of rhythmicity (Price et al., 1998; Rothenfluh et al., 2000; Suri et al., 2000): other mutant alleles *alter the pace at which the clock runs*, just as does the *tau* mutation does, harking back to effects of the key *period* alleles (a fast clock or a slow one). In this regard, periodicities of circadian rhythms are distinctly species-specific, suggesting that they are tied to an organism's lifestyle (dare I say again), thus to its real behavior. I'll bet that cloning *double-time* from another *Drosophila* species, and transforming it into a *dbt*-null mutant of *D. melanogaster* (cf. Wheeler et al., 1991; Campensan et al., 2001), would cause the latter flies to exhibit circadian cycle durations like that of the donor species. The same scenario could also play out for gene manipulations of *shaggy* (Martinek et al., 2001), *Timekeeper* (Lin et al., 2002a), and *Andante* (Atken et al., 2003), each of which also encodes an intrinsically lackluster kinase with rhythm relevance—and more: Like *dbt*, this additional trio of genes defines a set of developmentally vital factors. Similarly, *vrille*, whose chronobiological and pacemaking role within the fly was tapped into initially by virtue of molecular screening (Blau & Young, 1999), turned out to have been previously known via lethal mutations (George and Terracol, 1997). But these kinds of pleiotropies are not in the least disquieting.

However, pleiotropy of this sort is difficult to appreciate as "bimodal" or the like, in that the genes discussed play fairly broad developmental roles and do one kind of job on behalf of behavior—in terms of what is known so far about their rhythm roles. Other cases involve more limited examples of discrete modalities influenced by certain neurofunctional genes that apparently have relatively limited job descriptions, pending potential tests of the following mutations on wider ranges of behavior. Thus, we have an olfactory mutation called *ota1* that turned out to disrupt the *retinal-degeneration-B* gene in *Drosophila*, long known to affect visual transduction by way of its encoded calcium-transporter (the "degeneration" just indicated is not developmental but must be light-induced). Other types of visual mutant, caused by *no-retinal-potential-A* and (as already noted) *transient-receptor-potential* mutations, were tested for olfaction and found to be sub-normal for such responsiveness (Riesgo-Escovar et al., 1995; Störtkuhl et al., 1999)—indicating roles for *norpA*'s phospholipase-C (PLC) and *trp*'s calcium channel in both processes. In this regard the expression of neither gene is limited to the visual system. In fact *norpA*'s product is so broadly produced (Zhu et al., 1993) that one might have imagined the original blind mutants to be necessarily hypmormophic alleles of a vital gene (not so: Pearn et al., 1996). With respect to *norpA* producing its PLC in the CNS (as well as the PNS, naturally)–perhaps this connects with a modest but consistent acceleration of the circadian clock's pace that is caused by mutations at this locus

(Dushay et al., 1989; Hamblen-Coyle et al., 1992); whereas anatomical blindness causes no alterations of locomotor cycle durations (Dushay et al., 1989; Frisch et al., 1994). Recall that for *trp*'s part the product's pleiotropic presence, including within the antenna, is observed during development only, not in the mature structure (Störtkuhl et al., 1999).

With regard to gene versatility in *C. elegans*, about half of the 10 genes' worth of chemotaxis mutants (Mori, 1999) are also abnormal for thermotaxis (owing to a variety of mutated molecules, some of which were mentioned above; also see Kuhara et al., 2002). In *Drosophila*, several touch-sensitive mutants were found to be hearing-defective in terms of a malfunctioning antenna (Eberl et al., 2000), which is the fly's "ear" for reception of courtship-song sounds (Ewing, 1978). Links between learning and rhythms were uncovered (Levine et al., 1994; Majercak et al., 1997) by locomotor-behavioral testing of the *dunce* and *Dco* mutants (the latter are mildly mutated in a vital gene encoding a cAMP-dependent kinase; such mutants, like the *dunce* ones, are defective in learned behavior). Similar studies of a CREB transcription factor (discussed in more detail in the reverse-genetic section) established further chrono/mnemo connections (Belvin & Yin, 1999).

The *period* clock gene is involved in circadian rhythms by its original mutant-based definition. But yet-another component of *per* pleiotropy is that these variants are also defective in a short-term behavioral rhythm (reviewed by Hall & Kyriacou, 1990). The "one-minute" rhythmicity involves systematic fluctuations in the "interpulse intervals" (IPIs) of courtship song sounds (a.k.a. pulses, the intra-song sounds that are aberrant in the *slo* mutants mentioned above). This IPI rhythm is temperature-compensated (Kyriacou & Hall, 1980). Because that's a hallmark of circadian clock function (e.g., Hall, 1997), the notion was strengthened that what underlies these two very different kinds of behavioral cycles is mechanistically related. A defecation rhythm in nematode—dare one say "interpoop interval"?—is another case of short-term rhythmicity (with ca. 50 sec cycle durations) that's temperature-compensated (Iwasaki et al., 1995). The IPI mutants and corresponding genes isolated in this roundworm may lead to an understanding of how this cyclical behavior is regulated (Dal Santo et al., 1999; Reiner et al., 1999). At the time of these worm-mutant isolations (reviewed by Iwasaki & Thomas, 1995), there were no circadian rhythms known in nematodes to test for defects in the defecation-cycle mutants, although a relative of the *per* gene was discovered—*lin-42* in *C. elegans* (Jeon et al., 1999). And now it's at last been claimed that the worm exhibits circadian rhythmicity (Kippert et al., 2002; Saigusa et al., 2002). *lin-42* is the same gene previously mutated to cause anomalies of timed developmental events in *C. elegans* (Ambros, 2000), and one wonders if the fact that *per* mutants exhibit analogous defects over the course of *Drosophila*'s 10-day development is a coincidence (Kyriacou et al., 1990).

See the pattern here? Most of these mutational effects are "modal"—bi- up to tri-modal or so—as opposed to involving any-and-all features of neural function and behavioral actions. [Indeed, poor *per* was claimed from time to time to affect a variety of additional phenomena; but almost all of this extended pleiotropy mercifully fell apart (Hall, 2003).] Moreover, the pleiotropies of mutant effects and implied gene actions are in the same ballpark in these cases (on the same pitch for any of you UK readers who are still reading).

Therefore, what emerges is more a matter of gene *versatility* than "defective functions of so-many-things disrupt everything," as if most of them play housekeeping roles. Furthermore, the implied overlaps between regulations of visual and olfactory transduction, different kinds of mechano-reception, and timed phenomena are not mind-boggling at first blush; or they can be assimilated mentally in retrospect. These cases of judicious pleiotropy are also of heuristic value, for that they take investigators into the teeth of *how* these mechanisms are shared may be instructive for understanding the various components that comprise a given pair or trio of *genetically related* mechanisms.

However, certain behavior-genetic cases call the question: "how really related?" Take *fruitless* (please), the early mutants of which exhibit striking abnormalities of male courtship in *Drosophila* (Hall, 1978; Gailey & Hall, 1989; Villella et al., 1997). Yet certain of the more recently isolated genetic variants involving the *fru* locus cause near lethality—of both developing males and females—associated with crummy- looking external morphology (Anand et al., 2001). These distressingly unrelated phenotypes can be rationalized in terms of *fru*'s multiple promoters, most of which lead to mRNAs used in both sexes and at multiple stages of development (Ryner et al., 1996; Goodwin et al., 2000; Anand et al., 2001). The remaining promoter controls production of a male-specific, and almost entirely CNS-specific protein that first appears during metamorphosis (Lee et al., 2000).

FRU proteins are not COURTIN but mere transcription-factor isoforms (Ito et al., 1996; Ryner et al., 1996). The male-specific forms function within a well-known developmental hierarchy (Ryner et al., 1996), which is devoted to sex-determination and sexual differentiation (e.g., Baker et al., 2001). But regulation of and by *fruitless* is not limited to this developmental-genetic hierarchy; and it isn't crazy that the wide array of *fru*-encoded factors would be also used for features of the fly's biology that are not related to sex and in fact are related *only genetically*, not biologically. These *fru* phenotypes and gene products form a looser connection compared with those presented by *rdgB*, *norpA*, and the like, which each involve genetic connections between certain pieces of neurobiology. Cases of developmental—then behavioral—pleiotropy for which the biological phenomena stray farther afield (maybe out of the ballpark or off the pitch) are provided by *dbt* and *vri* (see above), which are analogous to *fru* in terms of life-cycle-stage versatility.

Incidentally, the male-specific FRU^M isoform persists into adulthood as a (continuing) CNS protein (Lee et al., 2000; Lee & Hall, 2001). So this gene, too, is ripe for temporal manipulation in terms of late-developmental versus adult-function etiologies for the courtship sub-normalities and anomalies. In fact, certain pieces of *fru* phenogenetics either demand or strongly imply, respectively, that the gene plays sex-specific developmental roles and an adult neurochemical one as well. First, let's register that *fru* mutants exhibit either *defective formation* (or none) of a male-specific abdominal structure called the Muscle of Lawrence (Gailey et al., 1991; Villella et al., 1997; Usui-Aoki et al., 2000; Anand et al., 2001), which fails to make its usual appearance during metamorphosis in these mutants (Gailey et al., 1991), as opposed to developing then degenerating late. Second, certain combinations of *fru* mutations allow for mating but cause frequent sterility (post-copulation), and this is correlated with defective production of serotonin (5HT) by certain male-specific neurons in the posterior-most ganglion of the ventral nerve cord (Lee et al., 2001). The latter cells co-express FRU^M protein in wild-type males (Lee & Hall, 2001) and project 5HT-containing axons that ramify over certain internal male reproductive organs (Lee et al., 2001). But the cell-bodies and neurites in question are devoid or depleted of 5HT in the semi-sterile mutants—against a backdrop of *normal formation* of the FRU^M/5HT VNC cells and processes in *fru* mutants (Lee & Hall, 2001; Lee et al., 2001), notably the most severely gene-damaged ones that cause other developmental defects (cf. Anand et al., 2001).

This discussion leads to a short discourse on temporal expression of genes in general. A lot of them that were discovered via effects of the "most interesting" mutants (involving development, behavior–you name it), make transcription factors. Here's another iceberg-tip: *fruitless, period, timeless*, and *vrille* are among the "any number" of behaviorally relevant genes dealt with in this essay that specify one kind of transcription factor or another. The importance of such proteins for understanding life on the Third Rock has seemingly spread out into all areas of biology. Another widespreading feature of the *Drosophila* genes just named is that they are expressed at essentially *all stages* of the life cycle. This "temporal pleiotropy" will be encountered in several other fruit-fly examples, although the *C. elegans* cases are perhaps not quite so biased in the direction of this kind of promiscuity (see below). But for *Drosophila* biology in general, well beyond neurogenetics, a huge proportion of developmental mutations causing embryonic lethality define genes expressed at that stage, of course, but also later, including all the way into adulthood. Arbeitman et al. (2002) recently provided a recent a systematic documenting of this state of affairs from the perspective of genes being expressed (over and over, stage-wise) in their *normal* forms.

Why all this temporal versatility? No one knows, and the developmentalists don't seem to care. This is not an accusational snarl;

instead, an inference that can be gleaned from elements of the developmental- and neuro-genetic literature. But before we grapple with such an example (the *sevenless* gene story, recounted a short distance below) let's pause for a moment to ponder the *period* gene, from the perspectives of both its product's quality and breadth of expression patterns. Also, the redundancy issue will rear its pointy little head once again.

So, what if *per* had not been originally identified by mutations but instead in its normal form as a sequence that encodes a transcription factor in an interesting category (true: Gu et al., 2000)? Or, the gene could have been identified via an enhancer trap for which the locus-landing transposon mediates, in part, a seemingly interesting pattern of CNS expression (also true: Kaneko, 1998; Helfich-Förster, 2003). If you bothered to look at neural tissues during development you might have been taken aback by the fact that the marker reports *per* expression at all stages (see below and Liu et al., 1988, 1991; Kaneko et al., 1997; Stanewsky et al., 1997). Nevertheless, you'd be stimulated to subject this gene to reverse genetics. Perhaps an imprecise excision of the enhancer-trapping transposon would generate a knockout (or something also could be done to change the wild-type form of the gene, as we'll get to in the last major section). The result would have been a fly that's quite happy looking, thank you: no drop in viability; no overt abnormalities of appearance or outward behavior. The thing of it is is that the same kind of ostensible phenotypic normality could occur for a mutant that you'd derive from any number of presumably interesting genes—ones that got identified initially in their normal forms. "Aha!" you might say, "the product of this thing [a "PAS-domain" transcription factor in the case of PER] is redundant!" But it might never have occurred to you to test for daily rhythms of behavior (should you by some chance possess the heavy hardware and software). The only supposedly salutary feature of this scenariod is that the gene's promiscuous temporal expression might have encouraged a comfortable relegation of this gene to the "no phenotype" dumpster.

Another *Drosophila* gene supplies further object lessons about temporally indiscriminate production of its product that are much less hypothetical, however poorly they seem to be appreciated. Thus, the case of *sevenless*: The original mutation at this locus (abbreviated *sev*) caused non-formation of one particular photoreceptor type—cell 7 (R7)—from the central region of each adult ommatidium. The first report of *sev* happened to make it a bona-fide genetic locus as well as a mutant with an intriguing phenotype. In this seminal study, the investigators were using the mutation as a tool, simply to get rid of R7 and in order to ask which elements of visually mediated behavior and photochemistry were removed, and which others remained intact, in such "finely surgered" flies (Harris et al., 1976). Several years later, *sev* became one of the all-time famous genes in the area of excitable cell-fate determination, cell

interaction determination (of such fate), and... you name it (early reviews: Banerjee and Zipursky, 1990; Basler and Hafen, 1991; Greenwald & Rubin, 1992). Incredibly, *sevenless* does not make a transcription factor; instead, a receptor tyrosine kinase (RTK).

Suffice it to say here that certain *sev* mutants are genotypically null. [In fact one is a ca. 50-kb deletion which utterly removes sev^{+} and perhaps other closely linked inessential genes (Banerjee et al., 1987).] Such a *sev*-minus mutant should not have been considered an "automatic viable," because the gene product is in no way eye-specific (in this instance, meaning expressed solely within the larval eye-disc): The mRNA was reported to reappear in adults after the one stage when the protein is supposedly needed, after which it indeed ostensibly disappears from the differentiating eye. Thus, the initial papers about on *sevenless* molecular biology noted all-to-briefly that its transcript was a "head-specific" adult factor (Hafen et al., 1987; Banerjee et al., 1987); actually it turns out to be in the imaginal body as well, that is, in the ventral nerve cord as well as the brain (J. A. Pollock, personal communication). And if this weren't bad enough (for fearers and loathers of pleiotropy), the *bride-of-sevenless* gene, which provides the developmental signal received by the *sev*-encoded RTK, gets re-expressed in the adult head (Hart et al., 1990).

The following substory within the *sevenless* one is *not* provided solely to embellish an additional case of temporal and spatial pleiotropy, but rather to exemplify how a rather complicated *genetic* analysis of phenotypes associated with a neuro gene can permit an appreciation of its versatility. For the first part of this substory, it is useful to interrogate an oddity pertaining to the R7-less mutant phenotype: *How in the world was the first* sev *mutant isolated as a phototaxis variant*? This question suggests itself, because of one conclusion that stemmed from the dissection study referred to above–that *sev* is behaviorally a "UV-blind" adult (Harris & Stark, 1976). That it was nevertheless isolated in a crude white-light screening operation (Benzer, 1967) could be related to the following: There is a mutant, *Photophobe*, that "interacts" with *sev* mutants; *Ppb* more or less had to do, because it came from mutating a *sev* mutant and isolating variants that were not UV-blind (Ballinger & Benzer, 1988). Subsequently, it was shown that *Ppb* in fact causes photophobic behavior when it is in a double mutant with *certain sev* alleles; the latter ($n = 2$) are non-null. This anti-phototactic behavioral anomaly is not found in other doubly mutant combinations, even though all these *sev* variants are equivalently devoid of their formation of photoreceptor #7 (Ballinger & Benzer, 1988). The implication, then, is that the *Ppb-sev* interactions have nothing to do with the abnormality of eye development caused by the latter mutations (as simple single-mutants). Instead, the interaction may concern an *adult-brain* function of *sevenless*. This "late duty" for the gene can be inferred from the fact that *sev* indeed continues to be expressed in that tissue, post-developmentally.

The eye-development story—which began to break wide open when *sevenless* was claimed to be a "cell-specific homeotic" mutant (Tomlinson & Ready, 1986)—was followed soon after by the clone and sequence "breakthrough" alluded to above (Hafen et al., 1987). These studies were contemporary with the report of the gene-interaction sub-story, whose implications were ignored, perhaps because the case of *Photophobe* never went anywhere. At least a nod might have been given to the notion that the intrinsically ordinary RTK encoded by *sevenless* would in no way necessarily be devoted exclusively to eye development, and therefore that *sevenless* might well not be a "cell-specific" anything. Perhaps its action would have tied to such a limited features of the animal's biology, if the product of this gene had turned out to be a magical-mystery substance such as SEVENIN.

So, even if side-stepping the pleiotropy of *sev* was a sad light in its firmament of stars, one of the best features of the story involved the several instances of mutating mutants to identify factors with which the RTK in question interacts. Most such studies in *Drosophila* focused on eye development (see below), and the Ballinger & Benzer screen represents one of the relatively few cases in *Drosophila behavior*-genetics of searching for gene interactions by mutating a mutant and looking for a suppressor or enhancer. The hope would be that in some cases a novel factor gets mutationally identified, which might not have been findable by screening solely for single mutants from scratch. However, a fair fraction of mutant hunting for neurofunctional variants—based on the possibilities of two-wrongs-can-make-a-right, on the one hand, or of synergistic defects on the other—succeeded in re-identifying factors already suspected to interact with the function defined by the mutant being mutated, or at least to act in concert with it. Thus, a photoreceptor-degenerative *rdgB* mutant was chemically treated, with the investigators bearing in mind that such cellular decay is light-induced; as could be expected, some plain-only blind *norpA* mutations were induced that suppressed the degeneration. More intriguing was a *norpA*/*rdgB*-suppressor mutation ($norpA^{su}$) which by itself caused no decrement in photoreceptor responsiveness (Harris & Stark, 1977), so the double mutant was in a way fully "cured" (also see Paetkau et al., 1999). In the learning biz, screening for suppressors of *dunce* (its female sterility) led to new *rutabaga* (*rut*) mutations. Thus, the double mutants were simultaneously defective for cA-PDE and *rut*'s adenylyl cyclase (Feany, 1990). This investigator re-mutated *dnc* and generated another second-site suppressor type (Feany & Quinn, 1995), this time at the *amnesiac* locus (which facilitated the latter's cloning). Mutating the long-period per^{L} mutant led to a partial suppressor that turned out to be a *timeless* mutation (Rutila et al., 1996). Hammering the cac^{TS2} calcium-channel mutant led to a variety of (unspecified) extragenic enhancers of the base mutant's temperature-sensitive paralysis; equally appealing or moreso: four intragenic

modifying mutations were also induced, divided equally between enhancers and suppressors (Brooks et al., 2003).

Before waning eloquent with some general remarks about these successful cases of mutating *Drosophila* mutants, let's pause to give a tip of our hats to the re-identification of *amnesiac* (Feany, 1990). First, consider that any description of the newly induced *amn* variant's suppression of a *dnc* mutant's effect listens: If the latter caused a constitutive elevation of cAMP (true), then down-regulation of factors involved in short-term boostings of that small molecule's levels can rationalize the suppressive effect in the double mutant. Second, AMN as a PACAP-oid molecular is crypto-interpretable in terms of this substance stimulating a receptor that gets to adenylate cyclase (cf. Kandel & Abel, 1995). Come to think of it, this piece of *phenogenetics*—the *amn* suppression of a *dnc* effect—may be the best evidence *amnesiac*'s involvement in cAMP-related signaling. Therefore, the double-mutant phenotype won't quit, even against a backdrop of the sharp questioning that asked whether AMN = PACAP (Taghert & Veenstra, 2002).

The upshot of the mutating-of-*dunce* screens, and of others to induce genetic modifiers in *Drosophila*, was that they in the main provided proof-of-principle outcomes: gene interactions that could be readily rationalized, as opposed to sending the investigators out into unknown conceptual territory. That said, some of the interactions implied by the screening results and subsequent analyses that briefly ensued were worth following up further, puzzling, or both. For example, just how are the allele-specific gene interactions—a common result in most of the cases just cited—operating in terms of putative physical associations between the relevant proteins? And are such polypeptide interactions pertinent at all? In this regard, the PER protein was shown [around the time that Rutila et al. (1996) recovered the per^{L} with tim^{SL} (*suppressor-of-Long*) double mutant] indeed to interact with TIM (e.g., Gekakis et al., 1995; Zeng et al., 1996). But what about *norpA*'s PLC enzyme and the calcium-binding phosphatidylinositol transporter encoded by *rdgB*? These two functions are within hailing distance of each other. But no specific concept ever emerged as to how these photoreceptor factors may interact "so specifically" that the $norpA^{su}$ mutation suppresses only the photoreceptor degeneration caused by $rdgB^{KS222}$, the mutant that was mutated to recover the suppressor factor (Harris & Stark, 1977). Moreover, recall that both *norpA* and *rdgB* specify chemosensory functions as well, making one wonder whether the PLC and the transporter interact somehow in those processes. [Incidentally, *rdgB* (a.k.a. *ota1*) mutations do not lead to any apparent degeneration of chemosensory structures (Woodard et al., 1991).]

Finally, consider one further feature of the *sevenless* story, which speaks well to the gene-interaction approach toward expanding one's understanding of the manner by which a given gene product in *Drosophila* functions. In this respect, the early fruits of this part of *sev*'s tree

("let's mutate it!") are nicely summarized by Banjeree and colleagues (Rogge & Banerjee,1990; Daga & Banerjee, 1994)—as a harbinger of much-more-to-come about photoreceptor determination and differentiation. This stuff certainly came out better than the case of *Photophobe* interacting with *sevenless* (which was quickly abandoned) or the other instances (listed above) of gene interactions related to the fly's neural function; most of those small pieces fruit also died on the vine.

That state of affairs is also portrayed by the neurogenetics of *C. elegans*, but in reverse. Indeed, when contemplating inductions of modifier mutations in nematode–where to start (or end)? A significant gene-interaction cottage industry has been developed for the little worms, which seem to be high-throughput cannon fodder for mutating mutants. This has led to an endless number of enhancer and suppressor mutations. They have involved every imaginable biological process. Genotypically, the modifiers encompass extra- and intragenic modifiers, recessive mutations, dominant ones, those with or without allele-specific effects, and even "a behavioral mutant . . . that defines a gene with a wild-type null phenotype" (Greenwald & Horvitz, 1980; the gene-interaction logic presented in this paper, whose title was just quoted, is worth checking out).

Impossible as it is (for me) to give a full-justice listing of the nematologists' classy accomplishments in this gene-interaction business within the business, other examples in or close to the realm of nematode functioning can be found in the literature that goes back close to the antiquity of *C. elegans* genetics and continues apace (e.g., Riddle & Brenner, 1978; Lewis et al., 1980; Greenwald & Horvitz, 1982; Moerman et al., 1982; Waterston et al., 1982; Park and Horvitz, 1986; Sedensky & Meneely, 1987); and they continue apace (e.g., Miller et al., 1993; Lundquist & Herman, 1994; Run et al., 1996; De Stasio et al., 1997; Shreffler et al., 1995; Gu et al., 1996; Shreffler and Wolinsky, 1997; Tax et al., 1997; Hoppe & Waterston, 2000; Robatzek & Thomas, 2000; Wen et al., 2000; Branicky et al., 2001; Zwaal et al., 2001).

This inchoate (non)discussion at least had the potential virtue of concentrating on gene interactions subserving neural functioning in *C. elegans* as opposed to nematode development. This brings us to another concern, which hovers over many behavioral mutants with regard to pleiotropies of the molecules so defined: Many genes originally identified by mutations causing defects in adult functions are activated not at that stage but often as far back as embryogenesis. Who cares?—or at least: why? To try and answer the latter question, the case of *Drosophila*'s *per* gene may once-again be instructive. In this regard, the PER transcription factor makes its first appearance halfway through the embryonic period (in the CNS, e.g., James et al., 1986). And *per* mutations, despite their "early-adult-isolation" phenotypes, *could* have caused behavioral problems in mature flies because of defects in or the absence of the protein's function during embryonic neural development. Not so

(Ewer et al., 1988, 1990), but the possibility that *per* mutants are (merely?) damaged developmentally could not be dismissed out of hand (cf. Kyriacou et al., 1990). In this respect, take *optomotor-blind*, a *Drosophila* mutant whose visual-response defects are almost certainly due to abnormal anatomy within one pair of the adult fly's optic ganglia. Intriguingly, the developmental etiology of the missing "giant neurons" in this viable omb^{H31} mutant goes all the way back to aberrant expression of the *omb*-encoded transcription factor during embryogenesis of the CNS (Pflugfelder & Heisenberg, 1995). Other genetic variations at this locus cause lethality or abnormalities of external adult morphology (Pflugfelder & Heisenberg, 1995).

By the way, the research group implied by the investigators just cited has performed systematic searches for fly-neuro mutants via anatomy alone (Heisenberg & Böhl, 1979) and *then* assessed several of them for behavioral abnormalities. These are sometimes and surprisingly lacking, or at least undetectable in terms of the testing regimes applied (Heisenberg, 1980). This is another example of a value-free genetic approach, because Heisenberg and colleagues made no a-priori assumptions about which kinds of neuroanatomical mutants would even be recoverable, let alone what might be their correlated behavioral abnormalities—if any. In this respect, two correlations did become conspicuous as studies of these "brain-damaged/behaviorally defective?" mutants played out: Learning defects were found to be exhibited by certain of the anatomical brain mutants (Heisenberg et al., 1985), notably those with anatomical problems of their mushroom bodies (see above). Also, locomotor arrhythmicity was discovered for the *disconnected* (*disco*) mutants (Dushay et al., 1989; Dowse et al., 1989; Hardin et al., 1992; Renn et al., 1999). The latter's isolation phenotype was eye-brain disconnection, but a more hidden anatomical defect—the absence of certain laterally located neurons within *disco* brains—was of considerable heuristic value for delving into the neural substrates of the fly's rest-activity cycles (e.g., Kaneko, 1998; Helfrich-Förster, 1998, 2003; Blanchardon et al., 2001). Therefore, developmental mutants are of course useful for behavioral genetics. In particular for this case, it's also worth recalling that plowing through a good deal of these brain-damaged mutants (as they were originally identified) uncovered *disco* only as a severe rhythm variant (Dushay et al., 1989; Vosshall & Young, 1995). It could have turned out that "everything" affects this behavior (brain-wise). But the special nature of *disconnected* speaks to the (failed) possibility that the genetic pleiotropies discussed in this piece typically entail promiscuity: Scratch a brain via any such mutation, uncover defects in whichever behavior is being interrogated (not so).

At some time in the future, neuroanatomical variants of this kind may unravel, potentially in exquisite brain-behavioral detail, how a wide

variety of specific neural circuits underlie a given set of organismal actions (cf. Comer & Robertson, 2001). However, this facet of the behavioral *genetic* cube is *relatively* lacking of empiricism and insight compared with genetic and molecular accomplishments that have been filling in neuro-*functional* sides of the overall story. Come to think of it, specifications of circuits that subserve certain behaviors, as specifically dissected by applications of mutations and by other kinds of gene manipulations, may be catching up (exemplified by Joiner and Griffith, 1997, 1999; Renn et al., 1999; Zheng et al., 1999; Daniels et al., 2000; Waddell et al., 2000; Heimbeck et al., 2001; Pascul & Préat, 2001; de Bono et al., 2002; Marin et al., 2002; Wong et al., 2002).

One kind of ganglionic wiring diagram that has long been supposed to exist and operate on behalf of a particular feature of fly behavior is that which supposedly subserves the male's courtship song. Certain actions of the *fruitless* gene may be relevant to this gedanken circuitry, which presumably is located somewhere in the ventral nerve cord. Recall that *fru* mutants are in part developmental variants, and the male-specific FRU protein is expressed in the developing VNC, most conspicuously during metamorphosis (Lee et al., 2000). The bimodal pleiotropy for this gene—development plus behavior—includes in the latter category courtship-song defects (Wheeler et al., 1989; Villella et al., 1997; Goodwin et al., 2000). So perhaps all of this will come together someday: Is there a male-specific VNC circuit? Is its formation (then function) tweaked or disrupted in the *fru* mutants that sing abnormally or not at all (Villella et al., 1997)? How does FRU participate in specifying the cellular qualities and connectivity within the circuit?

Speaking of singing, additional *Drosophila* mutants that cut across this component of reproductive behavior create still further examples of modal pleiotropies. These cases will not merely be added to the list, because they also lead a consideration of further behavioral-genetic issues. Thus, there is the *cacophony* song mutant (cac^{S}) in *Drosophila* (Schilcher, 1977), which was mutated about the same time the locus was also hit by *nightblind-A* (cac^{nbA}) mutations that cause visual defects (Kulkarni & Hall, 1987; Homyk & Pye, 1989, and references therein). More recent studies of this gene and its mutations revealed that the original cac^{S} or newer cac^{TS2} mutant (*S* for song, *TS* for the temperature-sensitive locomotor defect by which the latter mutant allele was identified) are barely abnormal for vision; and cac^{nbA} males are minimally, if at all, defective in their singing (Kulkarni and Hall, 1987; Smith et al., 1998b; Chan et al., 2002). These distinctly different categories of phenotypes for the two kinds of mutants can be partly rationalized by certain alternative-splice forms of *cac* mRNAs (Smith et al., 1998b; cf. Kawasaki et al., 2002), all of which encode calcium-channel α-1 subunits (Smith et al., 1996). By the way, it seems non-coincidental that the aforementioned *slo* gene, which is in part a song factor (Peixoto & Hall, 1998), encodes an intragenic family

of calcium-activated potassium channel polypeptides (e.g., Atkinson et al., 1991; Brenner et al., 1996). One might pause at this point to exclaim: "Aha! why be surprised to learn that mutant forms of such channels cause anomalies in the repetitive firing of excitable cells..." (and so forth, referring to the abnormal wing vibrations that underlie the pulse-song defects exhibited by *cac* or *slo* males).

So, how are these channel genes mutated in the song-defective variants, and might such results point to "something new" about how these excitability factors function on behalf of repetitively patterned phenomena? For *cacophony*'s part, one of the song-abnormal mutants (cac^S) harbors an amino-acid (aa) substitution in a certain transmembrane region of the polypeptide (Smith et al., 1998b), but not within one of the 4 voltage-sensing segments; the other one (cac^{TS2}) is aa-substituted in a region downstream of all four clusters' worth of membrane-spanning segments (Chan et al., 2002; Kawasaki et al., 2002). Therefore, *cacophony* provides an example of how *intra*-genic forward genetics can identify perhaps unanticipated features of the system's function. Knowing only about *cac*'s product in its *normal* form *might* have prompted in-vitro mutageneses of regions within the gene corresponding to where the cac^S- and cac^{TS2}-defined sites turned out to be (discussed by Chan et al., 2002). Nevertheless, special attention seems drawn to these two residues and to nearby regions of the channel polypeptide, which might have gone unnoticed in favor of things like the voltage-sensor regions of this polypeptide (snore), alluded to above. Thus, if one is to "start somewhere" in terms of manipulating *cac* sequences, followed by bioassaying the physiological consequences of the site changes in frog eggs, in no way would such a person effect a series of "random" in-vitro mutageneses or even deal with intragenic sites already hammered on behalf of establishing structure-functional connections Instead, wouldn't generating novel kinds of molecular alterations of CAC, squarely in conjunction with the possibility of teasing out physiological correlates to the intriguing cac^S- and cac^{TS2}-induced *behavioral* phenotypes, be most compelling?

I guess the point here is that one should not rely solely on sharply targeted, go-for-the-jugular changes within neuro genes. For in-vitro mutageneses and physiological assays in heterologous cells, that tactic might well be augmented by "shotgunned" DNA alterations (the starting point of the study reported by Baylies et al., 1992, gives an example of these molecular methods). For in vivo assaying alterations of things like channel genes, it's valuable to rely neither on mutations via sharp-targeting nor on the luck of isolating phenotypically defective mutants involving a given locus at the rate of one every five years or so—as has been the case for *cacophony*. Instead, it's better once again to effect shotgun mutagenesis of *cac* in live flies and recover random mutations involving only that locus (see Bentley et al., 2000, discussed in the section about reverse genetics, for how). Then, in terms of potentially interesting pheno-

types, take what comes—behaviorally, or physiologically, or both, or neither.

The *dissonance* (*diss*) song mutant in *Drosophila* involves a story within the song story that tells a bimodal-pleiotropy tale analogous to the case of *cacophony*. Males carrying *diss* are defective in their singing by definition, but flies expressing that mutation (males or females) turned out to exhibit subnormal optomotor responses as well (Kulkarni et al., 1988). This became quasi-comprehensible when elementary genetic characterization of *diss* showed that the gene which was hit is at the long-known *no-on-transient-A* locus. Mutations there cause visual-response defects, including the electroretinogram deficits that gave *nonA* its name (see citations to the ancient literature in Kulkarni et al., 1988). However, males expressing classical *nonA* mutations were found to produce normal songs (Kulkarni et al., 1988; Rendahl et al., 1992). Whereas it is not known just how the older mutants are song-enabled but visually impaired—and whether *cac* and *nonA* are telling us something about a crypto-special relationship between the control of vision and singing behavior—the nature of this gene's product makes it understandable how the mutants would exhibit their "sights and sounds" pleiotropy: NONA, perhaps horrifyingly, is a nucleic-acid-binding protein that seems to be expressed everywhere and during all life-cycle stages (Besser et al., 1990; Rendahl et al., 1992). Nonetheless, this inferred function for the NONA protein suggests that it could participate in the post-transcriptional processing of *cacophony*'s primary transcript, which leads to a wide range of CAC isoforms (Smith et al., 1996, 1998a,b; Palladino et al., 2000; Kawasaki et al., 2002). In this respect, the "visual plus song" features of *cac* pleiotropy are very likely explainable in part by the alternative RNA splicing side of this processing; because one of the *nightblind-A* mutations within this gene knocks out only a certain splice-type isoform of CAC, which is encoded by a class of *cac* mRNA whose expression is highly enriched in the fly's visual system (Smith et al., 1998b).

Naturally Occurring Variations in Behavioral Genes

More about the *non-on-transient-A* gene: Wouldn't its ubiquitously expressed, RNA-binding product be in the animal for nothing more than post-transcriptional gene-expression housekeeping? Perhaps the protein encoded by *nonA* is helping out with such dingy chores, but the following results and arguments indicate that this gene is about more than pathetic pleiotropy. The experiments involved interspecific gene transfers, the *nonA* version of which harks back to what was done with *period* genes near the beginning of the 1990s: Given that song rhythmicity is species-specific (most recently: Demetriades et al., 1999), and that hybrids showed the genetic etiology of the behavioral difference between two *Drosophila* species to map to the chromosome where *per* sits (Kyriacou &

Hall, 1986), one wondered whether entire etiology might reside within this locus. That turned out to be the case (Wheeler et al., 1991): *per* cloned from *D. simulans*, then transformed into *melanoagster* males lacking the gene's function, generated rhythms that were completely *simulans*-like. These results may have created some degree of evolutionary intrigue; although its disappointing that this part of the *per* story has gone nowhere since. What mainly happened during the 1990s is that attacks on the very phenomena—are there really song rhythms and do *per* mutations in *D. melanogaster* affect that behavior?—were beaten back, primarily by virtue of independent confirmation of the original results (Alt et al., 1998).

The interspecific transfers of various *per* forms also established yet another object lesson. It was applied to the case of *nonA*, by investigators who noticed that song traces recorded from $nonA^{diss}$ mutant males look suspiciously like those of *D. virilis* ones (in their genetically normal form). This prompted cloning of $nonA^{+}$ from *D. virilis*, which diverged from *melanogaster* eleventy-zillion years ago, and *transfer of it into* non-A *null hosts* of the latter species. The "barely-partial-hybrid" males, in that their genome was overwhelmingly *melanogaster*, had their visual defects rescued *and* sang in a *virilis*-like fashion (Campesan et al., 2001). This outcome re-focuses interest on the original *dissonance* mutation. It can now be argued that it occurred in a gene which is connected with courtship-song in a manner that means more than "can go awry if mutated." Restoring the damaged or missing function in a merely *can*-affect situation would only be *facilitative* for the normal behavior. The result would have been restoration of behavioral normality to the partially hybrid flies and that they would merely have sung like wild-type *melanogaster* males. The actual interspecific gene-transfer results suggest that the NONA proteins are instead *instructive* with respect to building or operating the cellular substrates of courtship song. As a final fillip, one might go onto say "building *and/or* operating," because of this further piece intrigue: *hsp* activation of $nonA^{+}$ sequences during development *or* in adulthood were each sufficient for males to generate normal song sounds (Rendahl & Hall, 1996).

As I continue this natural-variant discourse, I remember a human geneticist pronouncing publicly a while back that it's wrongheaded to study "severe" mutants with behavioral effects: They are "unnatural," could not survive in the wild, and do not not involve real behavioral genetics (blustered by L.J. Eaves at a conference in 1986; see Hall et al., 1990). The cases of interspecific gene transfers belie this notion. Chemically induced, severely mutated alleles of genes like *per* and *nonA* greatly facilitated their molecular identification; this was accomplished by introducing pieces of DNA into these mutants, which clearly restored normal behavior or not, as the case may be (e.g., Zehring et al., 1984; Rendahl et al., 1992). Armed with DNA encompassing the normal alleles,

the behavioral geneticist could use that material to tap into natural variants of these genes. These altered "alleles," cloned from different species, were then bioassayed behaviorally by generation of further transgenics. Such results have been significant from a general evolutionary perspective (Costa & Kyriacou, 1998; Rosato & Kyriacou, 2001) and in particular because they revealed one of the genes arguably to be a real behavioral one, despite its ordinary function and pleiotropic expression.

In this spirit, certain *intra*-specific natural variations proved equally interesting and informative. The behavioral phenomena involve polymorphisms of foraging for food: A given *Drosophila* larva taken from the wild exhibits either "roving" behavior, or it "sits" locally and eats there. The latter phenotype is unrelated to general sluggishness, and the locomotor difference—which is mirrored by the actions of rover versus. sitter adults—is observed only in the presence of food (reviewed by Sokolowski, 1998, 2001). Similarly, a given nematode exhibits either solitary or social feeding behavior (de Bono et al., 1998). Both species' worth of behavioral differences, if heritable (they are), could readily be imagined to be due to "small" allelic differences at *several* genetic loci. This would have been the same wrong guess made about the etiology of the interspecific song-rhythm difference described above. Both cases of naturally occurring intraspecific variations came down to one "major gene" in *D. melanogaster* and *C. elegans*. Respectively, the genes encode a cGMP-dependent kinase (*foraging* in flies: Osborne et al., 1997) and a neuropeptide receptor (*npr*-1 in worms: de Bono & Bargmann, 1998). Both gene products may play on the same field (cGMP signaling), given that *npr-1* encodes the nematode relative of a G-protein-coupled neuropeptide receptor in mammals (reviewed by Sokolowski, 2002). Only in the *C. elegans* case has the polymorphism been pinned down intragenically (as a single amino-acid substitution). But in both cases the surprisingly simple and tractable reason for the behavioral variations were proved transgenically: Introducing a *rover* allele of the *Drosophila foraging* gene into a for^{sit} strain turned the host larvae into rovers (Osborne et al., 1997); and the *solitary* allele of the nematode *npr-1* gene transformed a wild-derived social strain into one for which the foraging worms do not aggregate to feed but disperse across the food surface (de Bono & Bargmann, 1998).

The main thing that happened molecularly after *Drosophila*'s *foraging* gene was identified at that level was that the story slipped sideways into bees; this was a correlative study of *for* product levels monitored in hive-workers versus. foraging adults (Ben-Shahar et al., 2002). However, social versus. solitary feeding in *C. elegans* has recently resurfaced in the context of *npr-1* variants. They have been heavily analyzed in combination with a variety of sensory mutants (and others), leading to the suppositions that aversive sensory inputs to certain nociceptive neurons promote aggregation of the worms plus the group feeding that ensues (de Bono et al., 2002).

In addition, a genetically manipulable cGMP-gated ion channel, which functions in neurons that receive signals via the worm's bodily fluids, was found to be involved in activating social feeding; and NPR-1 activity suppresses this behavior by antagonizing the signaling that works through this channel (Coates and de Bono, 2002). A bonus accompanying the latter study was the demonstration of an acute influence of inducible *npr-1*$^{+}$ on solitary feeding in the context of worms lacking NPR-1 activity being social feeders (Coates & de Bono, 2002). Thus the etiology of such variants would not be developmental, and in this regard we should recall the effects of adult activation of the *period*, *dunce*, and *amnesiac* genes (with the proviso that, for *amn*, we are talking about behavioral responses of *Drosophila* to alcohol and not about experience-dependent behavior).

Prior to the *npr-1* follow-up studies just described, Zheng et al. (1999) happened to come at foraging-related locomotor control from a different angle: manipulating a glutamate receptor (GluR) in *C. elegans* that can influence the long forward movements, interrupted by short reversals, that characterize this behavior. This study also exemplifies nicely conceived dominant mutations (as introduced above more generally), which negatively act upon the phenotype in terms of inappropriately activated forms of the GluR.

Sprining forward in time slightly from the most recent iterations of the *npr-1* story, there are now cGMP-dependent-kinase variants in nematode that have been shown to have firm connections with food-related locomotion (reviewed by Shafer, 2002). These "PKG" mutants, involving a gene called *egl-4,* were not recognized by natural variants; nor was the locus initially known from feeding perspectives (e.g., Daniels et al., 2000). Nonetheless, *egl-4* loss-of-function mutations cause increased "roaming" in the presence of food, compared with the "dweller" behavior that worms of various genotypes can switch into (this include wild-type, and other mutant types with aberrant chemosensory structures are biased toward locomotor dwelling). This emerging story is rich with details, including those dealing with PKG's involvement in the mechanism of sensory adaptation and pinning down the sensory neurons within which this enzyme functions to regulated roaming versus. dwelling (l'Étoile et al., 2002; Fujiwara et al., 2002). Suffice it to say here that the effect of nematode PKG on food-related locomotion is opposite to that in insects, for which relatively high levels of this kinase activity are correlated with roving (Osborne et al., 1997) or with shifting from (hive) "working" to foraging behavior (Ben-Shahar et al., 2002).

But I digress—having probably strayed too far from spontaneously occurring behavior-genetic variants. Therefore, I'll wind up this section by mentioning another feature of the fly's behavioral repertoire: geotaxis. The history of its genetic study seemed to imply implacably complicated multi-genic control, but harder genotype/phenotype connections have begun to be unraveled by teasing out the influences of a tractable handful

of putatatively "major" genes (Toma et al., 2002). These were not tapped into by inducing mutations. However, the starting strains for this case of combining the behavioral genetics of geotaxis with molecular genetics (Toma et al., 2002) did not involve variants taken straight from natural populations, but *Drosophila* lines selected in the lab for relatively robust versus modest geotactic responses (Ricker & Hirsch, 1988).

Developmental versus Functional Behavior Genes: Transcripton Factors versus Neurochemistry?

Let's reflect for a moment on the kinds of gene functions to which one was guided by these behavioral polymorphisms. In the context of such variations that involve bona-fide behaviors (see below), the post-formative operation of the insect and roundworm nervous systems would seem to fall under the influence of the enzyme and the neuropeptide receptor, respectively. This makes one wonder whether *neuro-functional* factors—revealed by temporal gene manipulations carried out in the appropriate genetic background (here, for^{sit} or $npr\text{-}1^{soc}$)—will frequently connect with neurochemical phenomena like those defined by signal-transduction events or neurotransmitter actions (see Renn et al., 1999; and Waggoner et al., 2000, for further genetic examples of the latter). In contrast, behavioral mutants defining genes that specify the behaviorally relevant portions of the nervous system during development may mostly revolved round the action of transcription factors. Some of these will be encoded by "master genes" (vomit) that function rather high up in genetic hierarchies, such as the aforementioned case of *fruitless* (Baker et al., 2001). Whereas *fru*'s male-specific product is still found in the *adult* nervous system ("early" transcription factors almost always are), it's not that easy to imagine how this putative gene-regulator would function to control courtship behavior in an acute manner, as the male follows the female around, sings to her, and so forth. One would similarly infer a developmental etiology for male mating inability that is caused by a transcription-factor mutation in *C. elegans*, among whose effects are abnormalities of "sex muscle" differentiation and gonad morphology (Lints & Emmons, 2002). Genetic control of mating behavior *as it occurs* is more easily contemplated for a nematode mutant in which males exhibit subnormal sensory perception of the hermaphrodite, exemplified by a membrane function encoded by the *location-of-vulva-1* gene (Barr & Sternberg, 1999). Actually, the *mab-23* mutant alluded to immediately above may exhibit mating problems for acute neurochemical reasons as well as anatomical ones: dopaminergic "ray neurons," which are involved in a nematode male's sensing contact with an hermaprodite, exhibit abnormal patterning of this neurotransmitter's distribution (Lints & Emmons, 2002).

There are other exceptions to a scenario in which genetic defects in transcription factors would necessarily cause behavioral—once again meaning reproductive—anomalies because of problems involving the formation of sexually related cells and structures. Thus, mating defects exhibited by certain *fruitless* mutants in *Drosophila* strongly imply that FRU^M protein regulates neurotransmitter synthesis in a well-defined subset of the male's posterior CNS (Lee & Hall, 2001; Lee et al., 2001). The substance in question (5HT) seems to be sitting there waiting to be used as mating comes into action and is sustained—in that FRU^M seems to control the ongoing production, if not moment-to-moment amount, of a hypothetical mating-regulator: 5HT in the mature male (see above).

Changing gene regulation (a key feature developmental specification over the course of a normal animal's ontogeny) is also pertinent to the shorter-term actions of certain behavioral genes: PER protein undergoes abundance oscillations over the course of a day (as reviewed early-on by Hall, 1995). One category of what that molecular rhythmicity regulates affects involves product oscillations of downstream factors, a.k.a. clock-regulated genes (e.g., Claridge-Chang et al., 2001; McDonald & Rosbash, 2001; Ceriani et al., 2002; Stempfl et al., 2002; Lin et al., 2002b; Ueda et al., 2002). Then there are the CREB transcription factors, which seem to participate heavily in the fly's ability to remember and which arguably alter expression of target genes in the temporal context of long-term memory (see below). But the *per* and *dCreb2* genes—as transcription-controlling entities that regulate the actions of behaving animals, as opposed to influencing behavior via neural development—may be exceptional. This is because the behavioral changes in both cases are not all *that* acute, since they occur over hours or days of time.

Other exceptional cases are of the opposite variety, in that they involve factors one would predict to function in the mature animal: the aforementioned TRP in *Drosophila*, whose aforementioned involvement in olfactory adaptation depends on a role played by this calcium-channel type during development (Störtkuhl et al., 1999); and the *amnesiac* neuropeptide, which is associated with adult memory defects that have a developmental etiology in *amn* mutants (DeZazzo et al., 1999).

Now we return briefly to a consideration of factors that are more easily apprehended as functioning during the animal's performance—the G-kinase and neuropeptide receptor involved in foraging for food. These forward-genetic stories suggest new ways to identify factors that participate in the control of behavior and also how to move the subject into an ethological area of sorts. These of the animals' activities seem to be part of their "real lifestyle." Also, such behaviors are genuinely specifiable by quantification of one allele's effect versus that of the other—although in these foraging examples one is not pitting a mutant's actions against those of *the* wild-type. But we're about to leave the realm of "kindler, gentler" behavioral genetics (Greenspan, 1997), with its alternative alleles

that each allow for solid functioning of the gene in question, for the harshness of reverse genetics.

BEHAVIORAL GENETIC INQUIRIES THAT START WITH NORMAL GENES

Clone it, then Mutate it Chromosomally

If we accept that most behavioral genes are pleiotropic in terms of adult phenotypes, and that an appreciable subset of these factors are even worse because of the completely different biological consequences of their expression during development, then the identification of such genes by mutations can be so problematical as to be woefully inadequate. In other words, a gene that's potentially significant with respect to a particular behavior could easily get lost in the noise of mutant screening: gross or widespread anatomical defects could occur for most newly induced alleles, or a broad array of adult functions could go awry. For example, a mutated "learning gene" in *Drosophila* might disrupt basic olfactory processing as well as experience-dependent behavior; and because most of the learning and memory assays for this organism depend on the fly's ability to deal with (conditioned) odor stimuli, such a mutant would fall by the wayside as mnemo-defective for a trivial reason—even though the encoded molecule might subserve functions in neural "learning centers" that function separately from and deeper than levels involved in olfactory inputs. A flip side of this coin is that one might mutate a gene during a screen in which a wide variety of behaviors is monitored (e.g., Homyk et al., 1980), but the genotypically altered animal would show no phenotypic change of any discernible kind. Therefore, it becomes important to deal with genes that could be concerned with behavioral processes by means that are not limited to induction of mutations followed by phenotypic testing.

As the molecular genetics of invertebrates surged, including establishment of nearly complete genomics of *Drosophila* and *C. elegans* (*C. elegans* Genome Consortium, 1998; Wilson, 1999; Celniker, 2000; Rubin & Lewis, 2000), among the genes identified in their normal form are large numbers of factors that suggest themselves to be neurobiologically and behaviorally significant. Well before one could look in databases for such candidates—and be confronted with hundreds of mystery ORFs in both species—investigators were routinely identifying wild-type forms of genes with neuro potential by experimental means: for example, proceeding from antigens with potentially interesting PNS or CNS expression patterns to the encoding sequences (Zipursky et al., 1984); looking for such final gene products by interactions between proteins or fragments thereof (a behavioral example from *Drosophila*: Gekakis et al., 1995; a more

recent one, which approaches "genome proteomics," from *C. elegans*: Walhout et al., 2000); recognizing the provocative expression pattern of a reporter factor in (non-homozygosed) enhancer-trap (about which Freeman, 1991, furnished an early review); performing molecular subtractions or differential displays involving nucleic acids taken from certain "excitable tissues" or behaviorally relevant structures (e.g., Palazzolo et al., 1989; Pikielny et al., 1994; Xu et al., 2002) or even time of day (Rouyer et al., 1997; Blau & Young, 1999; Shaw et al., 2000); plus the now-upon-us onslaught of "microarray" applications to ask, for example, which ESTs correspond to mRNAs whose levels are altered in a given behavioral circumstance. The first bolus of microarray results with behavioral implications dealt with screens for transcripts whose abundance is relatively high at one daily timepoint, low at another (Claridge-Chang et al., 2001; McDonald & Rosbash, 2001; Lin et al., 2002b; Ueda et al., 2002; also see Tom et al., 2002). Analogous molecular findings emerged more recently, in the context of flies trained to learn and remember versus naïve (Dubnau et al., 2003).

A few elements of both the chrono- and mnemo-chips led quickly to actual genetic tests of the behavioral meanings of certain molecules: Transcripts encoded by a *Pdp1* (McDonald and Rosbash, 2001; Ueda et al., 2002) and *slo* (Ceriani et al., 2002) genes fell out of the relevant screens by virtue of exhibiting daily abundance oscillations. These genes specify, respectively, a transcription factor isoform and the aforementioned potassium-channel subunit. [In reality, *slo*'s potential relevance was pointed to in the main by robust daily cycling of *slob* mRNA (McDonald & Rosbash, 2001, Claridge-Chang et al., 2001; Ueda et al., 2002), which encodes a "SLO binding protein."] A *Pdp1* null mutation (Cyran et al., 2003) and more than one mutant allele of *slo* (Ceriani et al., 2002) was each found to cause abnormal behavioral rhythmicity. Chipping away at *Drosophila* memory identified—among several categories of molecules—factors involved in translational regulation subcellular localization of mRNAs; several of the corresponding genes had been previously identified with regard to the control of body-plan formation in early embryos. One such genetic locus was coidentified in the companion screen for transposon-induced memory mutants (Dubnau et al., 2003). This resulted in instant validation of the behavioral significance of that molecule (encoded by the *pumilio* gene). Another factor identified only in the microarray screen (encoded by the *staufen* "embryo gene") was quickly testable for its memory role, owing to availability of a temperature-sensitive allelic combination. [$stau^{C8}/stau^{D3}$ adults are produced when cultures producing this trans-heterozygous type are grown at a low temperature; after training such flies in that condition and shifting them to a high temperature during their "retention interval," this $stau^{TS}$ mutant type exhibited markedly subnormal memory (Dudnau et al., 2003).] The current "line" for flogging these approaches—if not the "bottom" one—is that if

the genetics of an organism is rolling, molecular searching for molecules that *may* be significant for the process which prompted design of the screens *can* lead swiftly to the requisite bio-behavioral results.

What about worm microarrays and neural function? A few years after Kim (1999) briefly laid out this approach for *C. elegans*, the first behavioral exploitation of it appeared in Zhang et al. (2002). The identification by these investigators of genes whose expression is affected by a long-known mechanosensory mutation involved interesting findings as such (the uncovering of 50 factors previously unknown to be "*mec-3*-dependent"). This study also happened to hark back to one of the earliest and most successful searches for behavioral mutants in this organism, which involved a particular kind of sensory stimulus and the worm's responses thereto (reviewed by Driscoll & Kaplan, 1997).

As is painfully obvious by now, one of the best ways to assess the meaning of a normal gene identified in one or the other of these molecular screens is to mutate it or otherwise impinge upon its expression. For making mutants per se, it is possible to generate them in rather systematic fashion—even in invertebrates, for which gene replacement technology lags behind the situation in mouse (however, see below). For example, a transposon-tagged enhancer trap, which was tapped into not by a phenotypic defect but instead the inferred expression pattern of the normal gene nearby (Freeman, 1991), might lead to behavioral abnormalities by simple virtue of making the molecular "insert" homozygous. A corollary of sorts would involve first finding the piece DNA only (no potential phenotypic variant at hand), followed by mobilizing transposons to obtain a putatively damaged gene.

Such "site-selected" mutagenesis (originally Ballinger & Benzer, 1989; Kaiser & Goodwin, 1990) has worked numerous times (Lineruth et al., 1992; Pereira et al., 1992; Segalat et al., 1992; Littleton et al., 1993; Milligan & Kaiser, 1993; Rushforth et al., 1993; Zwaal et al., 1993; Clark et al., 1994; Finbow et al., 1994; Dalby et al. 1995; Guo et al., 1996; Eggert et al., 1998; reviewed by Kaiser, 1990; Sentry et al., 1994; Plasterk, 1995). Among the loci successfully mutated by selected transposon landings are genes suspected to be in part neuro factors at or before the time of identifying in their normal molecular forms (Dura et al., 1993; Zinsmaier et al., 1994; Goodwin et al., 1997; Taillebourg & Dura, 1999; Palladino et al., 2000). I plowed through this potentially irritating laundry list, because it seems a common misapprehension that site-selected mutagenesis doesn't work very well in *Drosophila* and that one must therefore rely solely on other reverse-genetic tactics.

One of them relies on the aforementioned transposon mobilization. As for enhancer-trap screening, you cause transposons to go mobile (by the usual fruit-fly genetic mumbo jumbo) and collect derivative strains in which the new inserts are heterozygous with normal, transposonless chromosomes. Thus, if such a strain harbors a tagged form of your

gene-of-interest, and if that transposon turns out to damage the function such that the insert homozygote is lethal, you nevertheless would have recovered this line. (The same is so for enhancer trapping in which mobilized transposons landing near "something" that mediates an interesting expression pattern; such inserts are typically identified in animals for which the new insert is heterozygous with a chromosome free of genetic variants.)

Two transposon-mutagenesis operations are exemplary from a behavioral perspective: An A-kinase/regulatory-subunit transposon variant, which turned out to be homozygous viable and learning-impaired (Goodwin et al., 1997); and an adenosine deaminase mutant (*dADAR*), in which the site-selected transposon was expected to cause decrements of post-transcriptional RNA editing, in part with regard to transcripts encoding ion channels (Palladino et al., 2000). Thus, perhaps the *dADAR* mutants (some of whose derivatives were null variants) could have been lethal; but instead they were only sick and said to exhibit "behavioral deficits." In reality, though, the only impairments discerned in this study were of the pathophysiological variety, à la the generically anomalous or degraded locomotor activities of *Shaker* or *slowpoke* mutants. Inasmuch as *cacophony*'s primary gene product is RNA-edited (Smith et al., 1996; 1998a; Palladino et al., 2000), it wouldn't be shocking if more mildly mutated *dADAR* alleles were found also to cause genuine behavioral anomalies (cf. Kulkarni & Hall, 1987; Smith et al., 1998b; Chan et al., 2002).

What if homozygosity for a newly obtained transposon insert leads to the opposite of lethality or ill-health, that is, to no apparent problems even when a variety of behaviors are systematically tested? Perhaps mutations at such a locus can't cause any such abnormalities, no matter what. Therefore: "redundant!" However, the burden remains on the invoker of such claptrap, because that investigator must keep plugging away to assess "all" aspects of the organism's behavior and neuromuscular physiology, to such an extent that this experimentalist will be just as physically exhausted as he or she was mentally addled at the moment of inferring redundancy. To be fairer, such exhaustive testing is a conceptual impossibility, let alone a practical one. A more sanguine state of affairs for an apparently phenotypeless mutant obtained by reverse genetics is that the variant is not genotypically severe enough. Thus, it is essentially always necessary to remobilize the transposon. For a fair fraction of genomic insert sites (but by no means all of them), some such genetic events will "rip out" at least a portion of the gene. Owing to the all-too-common hypomorphy of transposon mutants, an imprecise-excision can create a more severely mutated allele. At last, then, a phenotypic abnormality might reveal itself. Examples are provided by the case of *dADAR*'s RNA-editing regulator; by an (initially) transposon-tagged mutation at a locus encoding a

"cysteine-string" (CSP) protein (Zinsmaier et al., 1994); and by similar inserts near a couple of rhythm-related genes (Dockendorff et al., 2002; Cyran et al., 2003). The excision-produced *csp*-null was notable for its temperature-sensitive patho-physiological defect (in the context of synaptic functions mediated by the CSP polypeptide). This seems counterintuitive, if one imagines that a temperature-sensitive mutant must involve a qualitatively altered form of the protein, as was found to be so for *Acetylcolinesterase*, *Choline-acetyltransferease*, or *norpA* TS mutants (respectively, Mutero et al., 1994; Tajima & Salvaterra, 1990; Masai & Hotta, 1991; see below for more about the first two of these three genes). However, a null mutation can cause an *overall process* to exhibit temperature sensitivity, if one component of a set of interacting factors is removed, rendering the remainder of the "complex" labile to something like heat stress.

What if all remobilizations involve precise excisions of the transposon-tagged variant?—not a rare outcome. Chemical mutagenesis "over the transposon-insert" may be necessary to induce a null allele. That's laborious and requires some luck. An alternative tactic works for certain genetic loci, those that happen to be within genomic regions rich with previously isolated deletions or associated with other kinds of chromosome aberrations. For these fortunate cases, one may be able to create a synthetically deleted genotype, by crossing two kinds of deletion-bearing strains to each other, such that a predictable fraction of the offspring will be homozygously deleted of the gene of interest. In such cases, the "locus-minus" state results from the relatively short region of overlap between the two deletions, each of which by itself is totally lethal when homozygous (simply because almost all cytologically observable deletions remove one to several vital genes). Fruit-fly examples of heterozygosity for overlapping deletions that created genotypic nullness for the pertinent genes are provided by cytogenetic manipulations devoted to the *dunce* memory gene and the *period* clock one (Kiger, 1977; Smith & Konopka, 1981). In advance of showing that certain point mutations at these loci are molecularly null (per^{01}: Baylies et al., 1987; Yu et al., 1987; dnc^{M14}: R. L. Davis, personal communication), the loss-of-function phenotypes were discerned by fertility and learning tests (Kiger, 1977; Byers et al., 1981), or behavioral rhythm monitoring (Smith & Konopka, 1981) of flies suffering from the synthetic deletions. In the same spirit, combining a deletion that removes, among other loci, the *Appl* gene of *Drosophila* with a genetic duplication that covers part of the deletion-defined region (but not *Appl*) led to a null variant; it exhibited only the subtlest of behavioral defects (Luo et al., 1992). That the *Appl* minus flies were alive was perhaps surprising, for they lacked a pan-neural protein, one that happens to be similar to the well-known amyloid-protein-precursor well known to Alzheimer folk. Speaking of mammals, the overlapping-deletion ploy was miraculously effected in mouse, once; the animals

homoygously deleted for the "brain gene" in question *seemed* to exhibit no phenotypic impairments (Kingsley et al., 1990).

These examples of inessential genes—which, even when removed, cause essentially no overt biological problems (though see Bellen et al., 1987, for a complicated fly in the *dunce* ointment)—are the tip of the following iceberg: As seems nowadays a haplessly quaint notion, it was thought for some time that there is a one-to-one relationship between genes and chromosomal bands in *Drosophila*, so that this organism would become and do all that it is and does with a mere 5,200 genes. The relevant results and suppositions came from genetic analyses of how many lethal or anatomical mutants could be identified with respect to preexisting or newly induced mutations in a given genomic interval. The X-chromosomal region in which the *per* gene lies is instructive, because this became a famous such interval by virtue of "saturating it with lethals" (reviewed by Judd and Young, 1973; also see Young & Judd, 1978). But what if additional mutations were being induced all along and discarded, because behavioral phenotypes were off these investigators' radar screens? When *per* got mapped to this very region of the X chromosome (contemporary with the saturation screening), it provided one of the first object lessons pertaining to a potentially large number of genes that cannot mutate to lethality. Several years later, the floodgates opened when it began to be realized that more than 60% of *Drosophila*'s genes are inessential for viability. Even before this state of affairs began to get summarized (e.g., Miklos & Rubin, 1996), investigatory genome expansion was well underway thanks to the stealth of behavioral genetics—which dribbled along below almost all radar screens as *Drosophila*'s developmental genetics held sway (e.g., Lewis, 1995). All the while, though, more behavioral mutants than one can name here were being shown—as in the case of *per*—to define genes that are inessential for viability.

One more transgenic approach to the reverse genetics of invertebrates should be mentioned. This recently developed strategy allows for actual *gene replacement*, establishing the potential for disruption of *Drosophila* genes that may rival what's eminently do-able in mouse. The documented genes that have been replaced are factors such as *yellow* and *pugilist* (Rong & Golic, 2000, 2001), which involve rather dreary fly phenotypes. Actually, *yellow* provided one of the first intensive studies involving single-gene behavioral genetics in *Drosophila*, when mutant males were found to exhibit subnormal courtship (Bastock, 1956). Those results were barely exploited subsequently, although it was suggested 24 years later that *yellow*'s behavioral deficit might occur because of subnormal levels of norepinephrine (Burnet et al., 1980). This represents a possible connection between a neurochemical entity and a particular set of behaviors that was tenuously formed but then not strengthened subsequently. A long time later, the product of the *yellow* gene was indeed found to be expressed with in the fly's nervous system (Radovic et al., 2002; Drapeau et al.,

2003), although it is far from clear how its membership in the ''royal jelly family'' of proteins would connect with production of norepinehprine. By the way, it should be noted that norepinephrine (NE) is commonly believed not even to exist in *Drosophila*. For example, Monastirioti (1999) makes mention of the lack of detectability for a requisite NE-synthetic enzyme activity (dopamine-β-hydroxylase) in *Drosophila*. But NE's nonexistence in this species is apparently a misapprehension (Tunnicliff et al., 1969; Watson et al., 1993), and a recent search of the *D. melanogaster* genome database led to uncoverage of sequences for two genes that would be predicted to encode dopamine-β-hydroxylase (U. Heberlein, personal communication).

Plain-old chemical mutagenesis can be applied in reverse-genetic operations. The pioneer work of this kind stemmed from identifying *Drosophlia* loci in their normal forms that encode the neurotransmitter metabolizing enzymes previewed above: acetylcholinesterase (*Ace*), choline acetyltransferase (*Cha*), and DOPA decarboxylase (*Ddc*). This was accomplished by segmental aneuploidy (alluded to above), which included identification of chromosomal deletions causing reduced enzyme activity in flies heterozygous for the key deletion; it was a necessarily recessive lethal (because it was missing a fair handful of loci) but led to enzyme decrements when the deletion was over a normal chromosome (Hodgetts, 1975; Hall & Kankel, 1976; Greenspan, 1980). Mutagenesis of the latter material, and eventual crosses such chemically hit normal chromosomes to deletion-bearing flies, led to isolation of mutations of the genes in question (reviewed by Hall et al., 1979; Wright et al., 1981). The correct guess in each case was that such mutations would be lethal, which facilitated phenotypic screening. The behavioral significance of these mutants—which possessed severe decrements in acetylchloline synthesis or degradation, or synthesis of serotonin and dopamine—might seem impossible to assess. This was accomplished, however, by the isolation and application of temperature-sensitive alleles that allowed for turning off the functions after development took place at permissive temperatures (Hall et al., 1980; Greenspan, 1980; Greenspan et al., 1980; Gorczyca & Hall, 1984; Tempel et al., 1984). This kind of strategy is well known.

Another way to skin this essential-gene cat was to generate *mosaic* flies in which lethally-mutated tissues in various portions of the CNS were carried to adulthood (Greenspan, 1980; Greenspan et al., 1980; Gailey et al., 1987). I won't burden you with what was learned from these conditional mutants and mosaics about the behavioral significance of these pieces of neurochemistry. The findings are too old, and they also haven't gone much of anywhere during the last 15 years. This was especially so for acetylcholine-related variants; and manipulations of amine-containing substances in *Drosophila* went off in other directions (e.g., Monastirioti 1999; Andretic & Hirsh, 2000; Kutsukaka et al., 2000),

into *C. elegans* (e.g., Waggoner et al., 1998; Sawin et al., 2000), or both. Also, elements of these kinds of studies strayed from behavior into pathopharmacology (McClung & Hirsh, 1999; Li et al., 2000; Hirsh, 2001). Some cases of neurogenetic inquiry seem to get dropped for no good reason, as opposed to involving any specific decision about "this part of the front" being judiciously deemed unworthy of being pushed farther forward.

Other instances of reverse genetics picked up steam as the apparent fruits of certain neurochemical mutants were dying on the vine. The further studies of this general sort also involved chemical mutagenesis but a different manner of mutant identification. This strategy was prompted by asking whether an allele to be induced in an as-yet unmutated gene might not cause a blatant neurobiological defect. Stated another way, one could place mutated chromosomes over the appropriate deletion, screen for certain phenotypic defects, but have *guessed* wrong as to what would *go* wrong in terms of neural functions. This problem was circumvented by more value-free reverse-genetic screens for the necessary mutants. Investigators looked for variants that were *devoid of the antigenicity* presented by the normal gene product. This kind of biochemical-genetic screening was especially successful in terms of isolating mutants with defects in phototransduction (Montell, 1999). In fact, the seminal case started with phenotypic assumptions in this ballpark—that mutants involving a photoreceptor-specific antigen would cause visual-response defects (Van Vactor et al., 1988; also see Reinke et al., 1988). Plowing through thousands of putants probably induced such (putative) mutants, which were then discarded. This was revealed by going for something closer to the jugular: looking for mutants at a level nearer the genotypic one, that is, antigen-minus variants. They were found by Van Vactor et al. (1988) and turned out to be fairly normal behaviorally (unless one tested these reverse-genetic variants at very low light intensities). The further cases of visual-mutant abnormalites (reviewed by Montell, 1999; Hardie & Raghu, 2001) were sometimes as expected (phototransduction subnormalities caused by G-protein defects: z z z z z), but sometimes not—such as the matter of retinal mutations that could be surmised "only" to impinge on functional features of phototransduction, but also cause deteriorations of photoreceptor cellular integrity (e.g., Dolph et al., 1993; Kurado & O'Tousa, 1995; Kiselev et al., 2000; Yoon et al., 2000).

If it's the case that all-out gene replacement in *Drosophila* will remain rather demanding as such (given the tactics in question) or beyond the capabilities of most (one really has to be a transposon pro), then an alternative reverse-genetics approach is worth considering. It involves, once again, plain-old chemical mutagenesis and identification of novel mutants at the genomic level—yet without grinding through hundreds & hundreds of strains for something like gene-product lowering. Instead, one effects a relatively rapid-throughput chromatographic procedure to

identify site changes within a "given stretch" of DNA—such as some portion of the gene-of-interest's ORF (Bentley et al., 2000). The relevant DNA heteroduplex mismatches tells one that a mutation occurred within that genomic region, and thus that a fly *heterozygous* for the induced change is in hand (the adjective just emphasized implies that one proceeds a minimal number of generations postmutagenesis and also that any putatively lethal mutation will nevertheless be isolated). The tractable "demominator" for "chemical-mutagenesis/targeted-recovery" (Bentley et al., 2000) involves screening through numbers of putants, which isn't that much more work than the fly-handling necessary for the "gene-targeting/homologous-recombination" approach (Rong & Golic, 2000, 2001; Rong et al., 2002). Also, when you're aiming toward targeted recovery, whopping doses of mutagen can be applied, because you neither have to nor want to homozygose the chemically treated chromosomes (Bentley et al., 2000). Therefore, this strategy may be just as good for neurogenetics as the one that involves transposon-mediated gene replacement (Rong & Golic, 2000, etc.). One hopes that applications of this tactic for homing in on a certain gene, starting with plain-old EMS-mutagenesis, will proceed beyond the "object-lesson" detection of chemically induced mutations in the dismal *awd* gene (Bentley et al., 2000), whose one saving grace is that its original name was *Killer-of-prune*.

Clone it, then Impinge on the Gene's Expression or Function by Transgene Manipulations

Further strategies for reverse genetics in invertebrates have attempted to attenuate or otherwise alter *normal* gene functioning *without* actually mutating it, or at least not doing so to create a chromosomal mutant. For example, the available *vrille* variants were either lethal intragenic mutations or a deletion of the locus (George & Terracol, 1997). When heterozygous with vri^{+}, such recessively-lethal nulls did cause mild behavioral rhythm changes, implying marginal clock-pace quickening. It was more useful to achieve a doubly transgenic setup for cellularly limited overexpression of vri^{+} sequences, which led to more substantially *lengthened* periods of rest-activity cycles or arrhythmicity (Blau & Young, 1999). Experiments of a roughly similar sort caused another potassium-channel type—a rather occult one for which there are no *Drosophila* mutants—to become a behaviorally relevant physiological factor. Thus, dORK (an open-rectifier channel presumed to mediate functions similar to the neuronal leak conductance) was applied in transgene-manipulated forms designed to achieve "electrical silencing" of circadian-pacemaker neurons, which led to marked locomotor-activity abnormalities (Nitabach et al., 2002). This is not to say that dORK carries out any *normal* function in these cells (the protein may not even be present there

in wild-type brains). In other words, this was not a "dORK study" but rather the application of that factor as a neuronally disrupting tool. Perhaps in this regard, ectopic expression of the channel-encoding transgene scrapes up against the edges of pseudo-reverse genetics. What if such expression gums up the chronobiological works in some unknown and unwanted manner?

Therefore, it may be equally valuable to disrupt the brain's clock neurons and physiological functions that are normally possessed by them. For this, recall *Drosophila*'s pigment dispersing factor (PDF)—even though that's tough because it deliberately wasn't named in previous referrals (Renn et al., 1999). This is because the fly's PDF is a misnomer, stemming from identification of peptide hormone (PDH) that does regulate pigment dispersal in crustacea. In this regard, anti-PDH leads to staining of certain neurons in the dorsal brain of adults that do not contain bonafide PDF, as shown by Renn et al.'s demonstration that a *Pdf*-null mutation leaves that part of the expression pattern intact; and by Park et al. (2000), who were unable to detect dorsal-brain marker expression as driven by the gene's promoter. For nongeneticists performing immunohistochemistry on excitable tissues, one wonders whether *not* worrying about the demands and opportunities created by these genetic controls for antibody specificity is pleasurable. Yes, such persons can pre-absorb such reagents with antigen, but that's destined to reveal little for polyclonal sera; or effect double-labeling via IHC and in-situ hybridization (ISH) to the antigen-encoding mRNA (cf. Sauman & Reppert, 1996), but such trouble is rarely taken. (By the way, Park et al. also showed that no *Pdf* ISH signals appeared in the fly's dorsal brain.)

In any case, cloning *Pdf* in *D. melanogaster* (Park & Hall, 1998) led to the wherewithal to manipulate it as well as determine in situ expression patterns of this gene's products. For the former operations, one had in mind the potential role of PDF itself in the fly's chronobiology; because PDH-like antigenicity is co-expressed in several neurons that make the *period* gene product (Helfrich-Förster, 1995). One way to deal with *Pdf* was to mediate its ectopic expression in the CNS, including usage of a pan-neural driver (fused to, you guessed it, *gal4*). The results were an array of locomotor anomalies, monitored in the usual way one does this when studying rest-activity rhythms (Helfrich-Förster et al., 2000). Thus, *Pdf* would seem to be "involved in" the fly's behavioral rhythmicity. But the interpretation of such results is hampered by the usual problem: can disrupt, but does the normal gene really control? By analogy, ectopic *Pdf* expression in neurons way beyond the usual *per* ones can affect behavior (and, incidentally, it was weirdly anomalous in the study just referred to, as opposed to involving changes in the usual parameters by which daily locomotor cycles are characterized). But does this imply that Pdf^{+} participates in the regulation of normal chrono-behavior? Perhaps not; and

so it was arguably better to stumble into the PDF-less mutant introduced above (reverse-genetics by luck in this case), then show that mutation causes flies to degenerate from weak locomotor rhythmicity to aperiodic behavior in constant darkness (Renn et al., 1999). In other words, an elementary elimination of the function seems to promote more interpretable effects on behavior than does adding the gene product to regions of the nervous system where it is never found normally. The latter (transgenic) situation could involve the anomalous activation of motor pathways having nothing to do with PDF actions, in the sense that the *Pdf*-null mutation might have had no effect on locomotion or rhythmic features thereof. Having said this, it is worth reiterating that loss-of-function genotypes and phenotypes can be problematical, if elimination of a molecule fouls up a behavior by some indirect (gratuitous?) effect that will be ultimately uninformative as to how the normal process is regulated. However, this seems more of a worry for the first-blush phenotypes of variants resulting from forward-genetic screens—absent, at that moment, any information on the gene product or where it is expressed. In contrast, the behavioral effects of the *Pdf* mutation are nicely interpretable in context of the neuropeptide's co-expression with clock-gene products in a well-defined subset of brain neurons.

These examples of reverse genetics, whether pseudo- or non-, have queued up to form a lengthening line of transgenically effected encroachments on the normal functions of mutationaless genes that were surmised to include behavioral regulation in their job descriptions. For example, *Drosophila* sequences encoding CREB transcription factors were manipulated by *hsp*-including transgenes in flies that were otherwise *dCreb2*$^{+}$. Among the key such manipulations were those that drew on background knowledge about a CREB-*negative* isoform. This provided, on paper, a transgenic natural dominant-negative, and the resulting inductions of the transgene indeed induced deficits in long-term memory (Tully et al., 1994; Yin et al., 1994; reviewed by Yin & Tully, 1996). [Later an actual mutation of the endogenous gene (called *dCREB2*) was found; it leads to near-lethality, and the rare survivors have so far been behaviorally tested only for circadian behavioral rhythmicity (Belvin & Yin, 1999).]

Equally interesting were the effects of supplying too much of a CREB-*activator* isoform, in conjunction with a learning regimen during which the flies were not provided with inter-training consolidation periods that are ordinarily necessary for robust long-term memory (Yin et al., 1995). The flies were thereby shown to exhibit impressively peculiar "photographic memory" in these experiments, exemplifying how reverse genetics can sometimes involve supra-normal gene function. An old-time geneticist would term this *dCreb 2* "allele" a *hyper*morphic one, although in this case a heavily engineered "mutant."

Sadly, this set of results has not been exploited. For instance, these forms of CREB would seem to be high-level gene-regulatory factors. But

what are the *effectors*—the presumed targets of these transcription factors, involved in more proximate mediation of information storage? Actually, the same kind of inadequacy characterizes other states of current behavior-genetic understanding. That the *fruitless* male courtship gene encodes yet another transcription factor just pushes the question about what *fru* is doing one step further. There are no incipient answers yet, absent any knowledge about what downstream genes are (probably) regulated by the male-specific FRU proteins. One waits with trepidation for micro-arrays to identify transcripts that are differentially present in *fru* mutant versus genetically normal males (probably in the context of male- versus female-"specific" mRNAs). And given that learning-related candidates have been revealed by abundances of various molecules going up or down after training flies for long-term memory (Dubnau et al., 2003), it would seem a short step to take to apply micro-arrays to material taken from flies "plus and minus" activation of CREB activator or repressor.

Other cases of reverse genetics in the learning business seem more in the realm of actually functional effectors (entities acting downstream of the regulatory factors). In these experiments, dominant-negative forms of certain catalytic or signal-transduction factors had to be designed (absent any natural or induced variants). Thus constructs were made, according to predictions that they would impinge on functioning of the function in question, then put into *Drosophila* transgenically. Activations of such engineered material were aimed at assessing the roles played by CaM-kinase-II and protein-kinase-C in the learning and memory which is associated with male courtship behavior (Griffith et al., 1993; Kane et al., 1997); and by a Gα protein isoform in shock-odor conditioning (Connolly et al., 1996). The resulting behavioral defects were variously informative and intriguing. However, and by analogy to reverse-genetic findings involving the visual system, the fact that a "molecular-switch" defect in CaM-kinase (CaMKII)-attenuated flies would disrupt their learning ability was not surprising (as reviewed by Griffith, 1997). Moreover, subnormal function of this enzyme would seem almost certainly to cause more pleiotropic biological defects, as is the case for *unc-43* mutants of *C. elegans* (Reiner et al., 1999).

The full scope of the CaMKII transgenics' impairments is not known in *Drosophila*, although the relevant males at least were not grossly impaired in sensory responses or motor actions (so they probably perceived cues from the female and could track her movements well). Additional manipulations and learning tests involving CaM-kinase (Joiner & Griffith, 1997), and those based on the dominant-negative PKC transgene (Kane et al., 1997), were extra informative, for they showed that the fly can exhibit memory deficits even after learning in an apparently normal manner during the initial stages of the courtship testing. Joiner and Griffith (1999) went on to show that the neural substrates for these

distinguishable experience-dependent phenomena are separable within the brain and discuss how the mnemo-behavioral distinctions just noted might be artificial.

Further reverse-mnemogenetic disruptions CaM-kinase-II relied on transgenic technology that involves the aforementioned warhorses of *Drosophila* molecular genetics. Thus, certain of the learning and memory tests just described were more refined than the whole-animal expression of dominant-negative constructs. For this, the (basic) GAL4/UAS system (no EP transgenes involved or necessary) was applied. The behavioral example in question was provided by the application of GAL4-containing enhancer-trapped strains in which certain portions of the CNS produce the yeast transcription factor; these transgenics were combined with constructs in which cis-acting UAS targets of GAL4 had been fused to sequences encoding the peptide fragment designed to impinge on CaM-kinase activity (Joiner & Griffith, 1997, 1999, 2000). These transgene combinations permitted various local-brain expressions of dominant-negativity, a reverse-genetic example of "dissecting" particular neural substrates of the experience-dependent behavior. The PKC experiments also involved GAL4, but in this case ubiquitously induced adult expression of it, in doubly transgenic *hsp-gal4/UAS-PKC*^{inhib} flies (Kane et al., 1997).

As mentioned before, putting the disruptive agent all over the animal is not necessarily a sanguine state of affairs, but at least the *hsp* manipulator provides command of life-cycle stage. Presumably, the wherewithal to effect such control—not devoted to the gene of interest, but instead to the disruptor—is now upon us (cf. Osterwalder et al., 2001; Roman et al., 2001; Stebbins et al., 2001). Temporal control can also be effected when the *gal4*-including transgene is combined with a UAS-*shi*^{TS} construct in which this mutated form of the dynamin-encoding *shibire* gene causes a dominant-negative block of synaptic transmission; crucially, this neurofunctional or neuromuscular-communicational abnormality can be brought to bear after development is over, given the TS-ness of this *shi* variant (Kitamoto, 2001). In this respect, relatively refined control of *gal4* + UAS-*shi*^{TS} bearing flies was effected for learning and memory experiments in which the driver was "mushroom-body predominant" in terms of spatial patterns (Dubnau et al., 2001; McGuire et al., 2001). These studies came on the heels of the first application of manipulable *shi*^{TS}, which also occurred in a mnemobiological context (Waddell et al., 2000). This study of *amnesiac* included the oddity of *acute* neurochemical disruptions (including those that were driven by an intra-*amn*, *gal4*-containing element) causing memory loss, in the context of an apparent developmental etiology for that behavioral deficit in *amn* mutants (DeZazzo et al., 1999).

In any case, it is obviously superior to combine temporal with spatial control of the deleterious agent in question. Previous studies along these

lines that relied on GAL4-driven expression of tetanus toxin (reviewed by Martin et al., 2002) were accompanied by the aforementioned "conundrum" as to whether the behavioral defects were caused by neuroanatomical or neurofunctional abnormalities (e.g., Kaneko et al., 2000). The same kind of conceptual problem for interpreting the eventual defect in behavior (e.g., Renn et al., 1999; Waddell et al., 2000; Park et al., 2002) obtains when expression of cell-killer factors is driven by sequences within a gene that naturally begins to be expressed at an early developmental stage (respectively, in these two cases: James et al., 1996; DeZazzo et al., 1999). A nematode case of cell killing gives us a reprise of the foraging behavior story, for which cells important in the underlying circuit were uncovered by effecting directed expressions of a caspase encoded by the *ICE* gene (Zheng et al., 1999).

Enhancer trapping applied to the analysis of neuro genes could trigger consideration of where this approach comes from—such as what are the actual enhancers. These are typically unknown, because the genetic locus trapped (by an ordinary *lacZ* containing transposon or one that bears *gal4*) is rarely even identified, let alone delved into. In contrast one does try to identify the gene, then burrow into analysis of it, in studies stemming from behavioral mutant hunts that are based on transposon mobilizations; even though nailing the gene and regulatory sequences responsible for the phenotypic change can be tough (e.g., Bolwig et al., 1995, versus Dura et al., 1995).

However, some *gal4*-including analyses of neural functions rely on dedicated fusions of regulatory material from a "real gene" (e.g., Renn et al., 1999; Emery et al., 2000) and it's useful at least to compare these so-called "dedicated" gene-regulating tactics to similar experiments that relied instead on an enhancer trap at an anonymous genetic locus (e.g., compare Blanchardon et al., 2001 with Kaneko et al., 2000). Here's a further wrinkle in this regard: fusion of dedicated regulatory sequences (taken from a known gene) to *gal4*, followed by generation of transformed strains, can be confounded by enhancer trapping. In other words, the expression pattern driven by the "5′-flanking-*gal4*" construct may not faithfully reflect the gene's normal expression pattern for some of the transgenic lines. Typically, this problem is revealed by the doubly-transgenic tissues mimicking the real gene's pattern plus extra location, which are of course unwanted. [See Kaneko & Hall (2000), who exemplify the inter-line variations of marker signals that can be problematical—in this case, as driven by a series of *per-gal4* transgenic lines and those in which a *tim-gal4* fusion was inserted at an array of separate chromosome locations.] This difficulty can apparently be bypassed, however: "Insulator sequences" have been discovered in *Drosophila*, which are interesting for substantive reasons (Bell & Felsenfeld, 1999). They are also useful practically for designing transgenics, in that one can flank a given *gal4*-fusion construct with insulators (e.g., Barolo et al., 2000; Stebbins et al., 2001),

probably to ameliorate "position effects" that can confound studies of transgenes containing expressible genes. (Effects of position in the current discussion refer to unwanted enhancer trapping that will accompany the *gal4* driving mediated by the chosen regulatory sequences, for an appreciable proportion of the transgenes' chromosomal landing sites.)

For those of you who may sneer at the supposed obviousness of why and how gene-regulatory sequences are manipulated, skip the following passages. They are provided because there does not seem to have been sufficient discussion of the possibility that behavioral neurogenetics in *Drosophila* has relied overly on application of enhancer-trap strains as such, compared with dedicated manipulation of specific well-characterized genes. The enhancer traps so applied tend, for example, not to be thoroughly characterized as to all the fly tissues in which they drive expression at all life-cycle stages. In contrast, exploiting regulatory sequences at a real locus usually occurs against a backdrop of the gene's spatial and temporal expression pattern being already documented. Thus, the usual starting point for "the-hell-with-enhancer-trapping" is to use enough regulatory sequences—usually meaning a more-than-tiny stretch of 5′-flanking DNA—such that the normal expression pattern of the gene is mimicked by the *gal4*-fusion construct combined with an array of UAS-marker transgenes (e.g., Kaneko & Hall, 2000). The "classical" such marker is β-galactosidase (β-GAL) activity or immunoreactivity as encoded by *E. coli*'s *lacZ* gene (again, Kaneko & Hall, 2000, provide one among a host of examples). However, GFP marking seems to be taking over—but this can lead to visualization troubles when neurons express fluorescence within neurites as well as cell bodies: determining the basic, ganglion-by-ganglion pattern of GAL4-driven expression can be difficult as one views the tangle of vines created by the GFP signals. For you confocal fans (who don't like to elicit β-GAL activity or antibody-mediated colorimetric staining), the potential problem of a GFP mess has been alleviated by creation of a *Drosophila* transgenic carrying a UAS-*gfp* construct that encodes a nuclear GFP (Barolo et al., 2000). Speaking of neurite marking—it can be maximized by application of another kind of manipulated *gfp* gene, which encodes a fluorescent protein engineered to be membrane-bound (Lee & Luo, 1999). This tactic for determining the neuronal "wiring diagrams" of cells in which a *gal4*-containing transgene of interest is—by the way—likely to be superior to that which draws on UAS constructs encoding the axon-filling tau polypeptide (once more, see Kaneko & Hall, 2000, for an example): this mammalian protein seems to disrupt normal axonal morphology, at least in certain cases of applying to fly-neuro histology (Williams et al., 2000).

Did you like that further digression? To return to the matter of GAL4-encoding driver and further experiments that can exploit them: The in-

vestigator then proceeds from the descriptive histological studies just discussed to combining the driver with a series of additional UAS-including constructs, those in which these drivee sequences are fused to those from the gene's ORF (will that combination fully rescue the effects of a mutation at the locus?); or to a disrupter factor (e.g., a synaptic disruptor or cell-killer). This sets up the wherewithal to "promoter-bash" the regulatory sequences such that perhaps only a subset of the wild-type expression pattern (temporally or spatially) can be brought to bear on things like rescuing only a subset of the (typically) pleiotropic effects of the gene's mutations. For instance, bashing the *Pdf* promoter eliminated VNC expression of the peptide (observed in wild-type *Drosophila* in the posterior-most ganglion thereof) but left its brain expression normal (Park et al., 2000). A much more extensive and behaviorally related dissection of gene-regulating enhancers is provided by Boll & Noll (2002).

However, the approach just outlined mainly involves gedanken experiments. Most of the "tissue dissection" studies at issue have applied enhancer-traps *strains* alone. No identifications of the regulatory sequences detected by the relevant transposon mobilizations were effected, abnegating the wherewithal for further manipulations of the presumed enhancer and adjacent sequences. Furthermore, many enhancer traps (let's say a *gal4*-containing ones) drive a rather broad expression pattern of the companion (UAS-containing) transgene; much of a given such pattern would be not relevant to the phenotypes in question. What one needs to do is apply a rather large collection of enhancer-trap strains. This can permit homing in on the particular subset of the nervous system that underlies whatever phenotype is at issue: rescue of a mutation's effects on certain features of a behavioral syndrome with which the gene is involved (when UAS is fused to the gene's ORF), or disruption of behavior by some kind of damage to or alteration of a certain portion of the CNS (when UAS is fused to something like shi^{TS}). One is creating Venn diagrams of the expression-overlap among the *gal4* strains, which in principle allows for localization of the relevant neural substrate (see Ferveur & Greenspan, 1998, for an example of this transgenic-mosaic tactic, which got documented in a manner more extensive and detailed than most).

It seems equally warranted to take a *known* regulatory sequence, fuse it to *gal4*, and quickly proceed to the tissue-expression plus phenotypic analyses of promoter-bashed derivatives. In the old days, this was done all the time—but not in conjunction with the versatility provided by the GAL4/UAS system. Instead, people would bash the 5′-flanking region of their gene in conjunction with *lacZ* fusions, usually to examine expression patterns alone (no behavior) and not infrequently achieving some subset of the wild-type pattern (e.g., Mismer & Rubin, 1988; Mismer et al., 1989; Fortini & Rubin, 1990; Kitamoto & Salvaterra, 1993, 1995).

Incidentally, this kind of whittling away at the regulatory material seems more necessary in *Drosophila* compared with nematode, given one's sense that there is more versatile-cum-promiscuous expression of neuro genes in the fly and heightened specificity in the worm. Genes encoding ion-channels, receptors, signal transduction factors, and catalytic ones—stuff that should pertain in part to the function of excitable cells—are inclined to present relatively limited locations of expression in *C. elegans* (e.g., Troemel et al., 1994; Yu et al., 1997; Jansen et al., 1999; Sagasti et al., 1999; Satterlee et al., 2001). Even the dreaded transcription factors—a given neuro one of which seems to be all over the fly—can show quite limited patterns of spatial expression in the worm (e.g., Sengupta et al., 1994; Hobert et al., 1997). One wonders whether the "extra 5K genes" in *C. elegans* (see above) permits many of them to do more dedicated jobs, compared with the auxiliary versatility that's putatively demanded of many genes in *Drosophila*.

Somebody must have so surmised before (that is, how this "higher invertebrate" gets by with only about 14,000 genes). No matter; or at least consider the following provisos: Not all *C. elegans* genes are "exquisitely specific." There's the pan-neural *unc-14* one (Ogura et al., 1997); the fact that the aforementioned NPY receptor is expressed in "too many" neurons for one to glean the neural substrate of foraging behavior (de Bono & Bargmann, 1998); and that factors regulating egg-laying behavior tend exhibit to rather broad expression patterns (e.g., Nelson et al., 1998 a,b; Daniels et al., 2000; Waggoner et al., 2000). Also, even the specificity-ridden cases exemplified above frequently involve several life-cycle stages worth of product production. Moreover, most of these patterns seem to be determined by fusing regulatory sequences from a given worm gene to marker-encoding material (to make GFP, naturally). What if the 5′-flanking DNA is inadequate? One could so determine by in-parallel assessments of antigenicity for the encoded product, but this seems not be done that often. An argument against the necessity for such efforts (which might have to include the extra step of double-labeling) would be that the same 5′-flanking sequences fused to the gene's ORF are able fully to rescue the neural defects caused by mutations at the locus. But what if not all such phenotypic abnormalities are known? The rescue-based argument forms a circle; because if other structures, functions, and behaviors come under the sway of the gene, it could be that they were not rescuable by the (straight genomic-DNA) transgene—squarely in the context of the promoter-*gfp* fusion not revealing the complete expression pattern.

In any case, the nematode situation allows promoter fusions to mediate specificity of expression and function in conjunction with asking elementary neurally related questions: as just noted, inferring neural substrates of the behavior in a mutant-rescue test, against a backdrop of

the expression pattern assessed in promoter/marker transgenic; or effecting experimental manipulations, such as those that would façilely fuse a not-necessary-to-bash promoter with a disruptive agent. Sometimes, however, it is warranted to make heterologous promoter-including fusions, as exemplified in the combo chemo/thermosensory study of a calcineurin-encoding gene (*tax-6*) in nematode. Coding sequences of this gene were directed to a variety of tissues and cells, by fusing this *tax-6* material to various regulatory sequences, ranging from those that are active broadly, or in all body-wall muscles, or in particular subsets of olfactory neurons. An analogous example in *Drosophila* involved fusing regulatory material from a so-called eye gene to *period* coding sequences; the former (called *glass*) "of course" is also expressed within the CNS, so the fusion construct led brain expression of *per*. This seemed to mimic an intra-CNS subset of the normal *per* pattern, which was sufficient to effect weak rescue of the behavioral arrhythmicity that's caused by a *per*-null mutation (Vosshall & Young, 1995).

Here's a final fillip to the gene-regulation substory as it applies to neuro genes: One should never forget that *Drosophila* provides a staggering number of chromosome aberrations. Many of the fly's genetic loci are rich with chromosomal breakpoints, not only within genes, but also "just this side" of their core sequences. It follows that certain locus-associated lesions should disrupt regulatory sequences, and that is the case. This has allowed for expressional and behavioral phenotypic assessments to go hand in hand in certain studies (e.g., Qiu & Davis, 1993; Lee & Hall, 2001). What subset of the normal tissue pattern might be removed or remain under the influence of a given breakpoint that falls within (for example) the 5′-flanking region? In parallel, what aspects of the phenotypic syndrome caused by mutations at the locus are abnormal or not (respectively, in the two cases just referred to: learning and other biological defects; or various courtship subnormalities and anomalies)?

This part of the gene-analysis narrative brings us back to considering "genes of interest" as such—compared with wallowing in *gal4* drivers, so many of which are inherently unappealing (most of them being woefully underanalyzed or overinterpreted as to spatial specificity). Thus, instead of just mucking around with functions related to the neural substrates of a given behavior, by flinging a bunch of enhancer traps and UAS-disruptor at the problem, another way to manipulate expression of the actual gene is to apply double-stranded RNA corresponding to sequences within its transcription unit. This RNA-inhibition (RNAi) tactic—which originally involved physical injection or even feeding of such material (e.g., Vaz Gomes and Wahlestedt, 2000; cf. Dong et al., 2000)—provides yet another way to interfere with a function, absent the availability of genetic variations at the relevant genetic locus. The RNAi ploy requires refinement for late phenotypes. For example, getting this

material into animal by injecting it into embryos might have no chance to produce behavioral defects several developmental stages later, except for certain genes with neuro-anatomical relevance. For instance, introducing just the right stuff into a *Drosophila* embryo (e.g., Yang & Erickson, 2000) could lead to a phenocopy of *optomotor-blind* (cf. Pflugfelder & Heisenberg, 1995). Also, feeding *C. elegans* the interfering nucleic acid (Timmons & Fire, 1998) doesn't always work for neural genes (Timmons et al., 2001), although this has potentially been taken care of by further refinements (Simmer et al., 2002). [Other nematode examples are provided by Hunter (1999), Fraser et al. (2000), Grishok et al., (2000), Gönczy et al. (2000).]

One can do more than introduce RNAi exogenously. Thus, another form of this strategy involves generating stably transformed lines in which a transgene is engineered to produce double-stranded "foldback" mRNA (e.g., Lam & Thummel, 2000; Fortier & Belote, 2000; Kennerdell & Carthew, 2000; Piccin et al., 2001; Kalidas & Smith, 2002; Ueda et al., 2002). A proof-of-principle result was provided by *per* RNAi transgenics, against a backdrop of hypomorphy for this gene causing slowdowns of the circadian clock's pace (e.g., Smith & Konopka, 1982). Indeed, the appropriately transformed flies, in which the inverted-repeat sequences were fused downstream of UAS ones, exhibited longer-than-normal cycle durations in their locomotor rhythms (Martinek and Young, 2000). That the driver of UAS-*per*(RNAi) in this study was a GAL4-encoding transgene, speaks to the versatility of the approach, because the expression-disruptor can be combined with any number of such drivers. A certain degree of spatial refinement can be achieved, because even if the enhacer fused to *gal4* drives expression "all over," only the cells naturally expressing the gene from which RNAi was derived will be disrupted. Temporable manipulability can be effected in the RNAi systems as well (see Allikian et al., 2002, for a non-behavioral example in *Drosophila*; cf. Tavernarakis et al., 2000, for a loosely analogous case in *C. elegans*). Therefore, developmental lethality that might obtain if the molecular inhibition were to be driven relatively early in the life cycle (by the gene's own regulatory sequences) can be bypassed as one waits for the live flies to emerge for behavioral testing. This potential for manipulabilty of neural expression and behavior is probably the best feature of RNAi, because what we have in this one case so far in terms of nervous-system function involves only a decrement of gene function (Martinek & Young, 2000). The *period* gene whose RNA was doubled-up in this case is not all that exemplary: This particular clock factor is beautifully set up in terms of a relationship between behavioral cycle durations and grossly appreciated levels of *per* product (e.g., Coté & Brody, 1986); but an RNAi-induced loss of function may be necessary for many genes, if the molecularly-induced change is to cause noticeable phenotypic defect.

A LAST GASP OF WOULD-BE SUMMARY

Did you notice that the previous section ended with a thud? Here are some general remarks of this sort, perhaps forming more of a wheeze than a plop. First, let's recycle the oft-stated claim, given the many genetic inroads which have already been made toward elucidating neurofunctional processes, that neurogeneticists are onto something.

Certainly such investigators are rarely concerned anymore with *whether* genotypic variations and the normal factors so defined can meaningfully disrupt and materially control the structure & function of the nervous system. Instead, *how* is this so? Can all the key genetic players that regulate a given process be identified? Arguably, yes, if you're willing to keep mutagenizing and screening to the extent that the process approaches genetic saturation, and that you don't deny the behavioral roles of vital genes.

In this regard, does the supposedly "value-free" nature of forward genetics make "no assumptions?" Perhaps not: screening for mutations affecting a particular behavior seems to assume that a newly induced variant will not be wildly pleiotropic and of course not lethal—in a limited screen that doesn't include testing the effects of mutated chromosomes heterozygous with normal ones. In principle, the nature of certain genes might have the property of most changes within them damaging the relevant function to the extent that nearly all processes which could go awry are befouled, thus obscuring a real "close-control" function of a particular behavior via one very local subset of the gene's spatial domain (although, again, inspection of mutant-heterozygote effects could get round this problem).

Recalling where this neurogenetic discussion started (inducing mutations and isolating behavioral variants) leads to the following reminders about additional issues that were raised as the subject played out: (1) appreciation of the quintessential power of multiple alleles for an individual behaviorally relevant gene (not all mutations in most genes will be without relatively distinctive or subtle effects); (2) the manner by which differing forms of genes come into play when factor interactions are discovered by mutating mutants (might special mutations be identified in this manner, possibly by virtue of allele-specific effects in double mutants?); (3) the examples that were presented for several behavioral genes, though possibly widespread in terms of their temporal plus spatial expressions, which are capable of causing "oligo-modal" effects on behavior when mutated in certain ways (as opposed to "all genes are expressed everywhere and affect everything"); (4) the wherewithal for many kinds of gene and mutant manipulations to dissect "when and fromwhere" the factor contributes to a given feature of the phenotypes that come under its away.

Forward genetics in the neuro business seems on the wane, at least relative to other tacks being taken toward discovering attractive genes. Nonetheless, molecularly based screens for factors putatively underlying a given behavior, searching which is supposed to lead in part to reverse genetics, embrace certain value-free viewpoints of their own. Gene products that pass muster in terms of when and where they are expressed (life-cycle stage, time of day, post-conditioning, neural tissue, sex, and so forth) could be anything and frequently include molecular surprises. Here I refer to factors that are identified in reverse-genetic enterprises that commence with screening operations, compared with going after an already-known molecule (previously recognized in another organism, or understood to be in "yours" from genomics)—with the intention of eventually mutating screen-derived candidates and testing the effects of knockouts or other variants on a behavior of interest. But even when more broadly based searches for genes connected with these actions of the animal are performed (by molecular subtraction, differential display, or micro-array application), the "some-assumptions" bugaboo rears up. For example: Will all genes involved in learning change in terms of product levels post-training? Or will everything underlying circadian behavior involve genes whose RNAs and proteins cycle in their abundance? (The answer to the latter question is already known to be "no.") Just as one must sift through a collection of newly isolated behavioral mutants—experimentally scratching one's head for a while as to which genetic variants define "really relevant" genes—any molecularly based screen runs up against the usual wheat-from-the-chaff challenge.

In this respect—what, if anything, is a "behavioral gene?" Asking such a question prompts me to reiterate that I lied in the introductory passages (probably did in many others as well). Thus, maybe the subject implied by the question just asked should involve just as much developmental neurogenetics (including the implied dissection of neural circuits subserving behavior) as gene-based studies that deal with functions occurring in conjunctions with the mature animal's actions (or, by certain implications of mnemogenetics, that are modulated after the occurrence of certain behaviors).

Baker et al. (2001) take up the behavior-gene issue in a more digestible manner than I'm doing now. One thing these authors wrote about was to suggest certain operational and boundary conditions as to how a developmental-neural gene can be viewed and even demonstrated to be a significant behavioral factor, one which acts fairly high up in the relevant regulatory processes. Or at least a gene apprehended and manipulated appropriately can potentially be demonstrated to function above the level of "can disrupt the behavior when mutated." The *fruitless* gene was gingerly offered as an exemplary higher-up candidate (Baker et al., 2001). In this regard: you, *fru*, are a behavior gene at least in part via

your CNS expression during development, even though you also continue to produce a neural-specific factor in adult flies that is already known to regulate one piece of mature neurochemistry (ignoring for the moment the sex-unrelated vital function harbored within this genetic locus). One awaits temporal and spatial dissections of *fru*, to determine experimentally when and wherefrom arises a given feature of the reproductive behavioral syndrome exhibited by the array of *fruitless* mutants. The case of *fru* also reminds us that we should graciously accept (let alone exploit) the genuine behavioral roles played by certain transcription factors, intrinsically vile as they may be. (Too many of the "really interesting genes" remind one of the American economy: our people don't actually *do* anything; we just push information around.) By analogy, we do not necessarily rend our garments if a gene defined by a behavioral mutation encodes a supposedly ordinary enzyme [although, if such a forward-genetic operation should lead you to aldolase (cf. Ahn et al. 1994), you have permission to go to a high place within your science complex and hurl yourself into the atrium or courtyard that probably exists there].

As these necessary *analyses* of *extant* behavioral mutants and genes proceed and deepen it will nevertheless remain warranted to continue mutating the animals into oblivion, with respect to whatever set of behavioral phenotypes interests a given collection of neurogeneticists. For *fru*'s part, why not also mutate those mutants for suppressors and enhancers of the courtship or mating deficits and anomalies? In the course of these indispensibly endless screening operations, more and more of the hits and repeat-hits are likely to fall within the genome's transcription units whose coding potentials are without precedent in terms of known protein functions (the so-called "Unknowns" with which any genomic database is replete). As increasing numbers of such molecules become "candidate factors" by the results of parallel searches for *fru*-connected genes in their normal forms, the RNAs and proteins will be assessed for their expression patterns, and what encodes these gene-products will succumb to reverse-genetic manipulations. Ultimately, it seems eminently possible to unravel the manner by which all *fru*-controlled behaviors are specified developmentally then regulated in the mature animals.

There are of course a hell of a lot of animal behaviors beyond the sex-specific ones controlled by the *fruitless* gene in *Drosophila*. And the stories being written about a goodly number of such factors involve tales that are nearer to their climax. All in all, then, investigatory intersections among genes, nerves, and behavior seem as if they are very likely to continue contributing to a process of demystifying the relevant neuronal functions and eventually the underlying neural circuitries. If the case of behavioral rhythmicity can be cracked in this manner—and it has been—then why not also the academics of sensory input and processing,

neurochemical control of central functions, on up to things like the figurative and literal romance of sexual behavior?

Until we meet again, and the case is sol-ved.
Clouseau (1975)

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