Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait

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Identifying the genes involved in polygenic traits has been difficult. In the 1950s and 1960s, laboratory selection experiments for extreme geotaxic behavior in fruit flies established for the first time that a complex behavioral trait has a genetic basis. But the specific genes responsible for the behavior have never been identified using this classical model. To identify the individual genes involved in geotaxic response, we used cDNA microarrays to identify candidate genes and assessed fly lines mutant in these genes for behavioral confirmation. We have thus determined the identities of several genes that contribute to the complex, polygenic behavior of geotaxis.

Introduction

Pioneering experiments on *Drosophila melanogaster* and *Drosophila pseudoobscura* investigated the nature of the genetic basis for extreme, selected geotaxic behavior. These experiments constituted the first attempt at the genetic analysis of a behavior. Selection and chromosomal substitution experiments successfully showed that there is a genetic basis for extreme geotaxic response in flies[1–5] and, by implication, for behavior in general. These experiments also added to our understanding of the role of variation in phenotypic evolution and selection[6–8]. Despite their seminal contributions in behavioral genetics, population genetics and the study of selection, by their nature these experiments could not identify specific genes[9].

These results highlight both the success and the limitation of behavioral selection experiments. Although selection results tend to be representative of the natural interactions of genes that produce behavior[10] and can demonstrate that a trait has a genetic basis, they do not pinpoint specific genes that influence the trait. This is partly due to the involvement of many genes and the relatively minor role of each in complex polygenic phenotypes—a problem that is especially acute for the intrinsically more variable phenotypes that are associated with behavior. The advent of cDNA microarray technology offers an easily generalized strategy for detecting gene expression differences and can complement other means of identifying the genes that underlie complex traits[11]. An expression difference may occur in a gene that is not itself polymorphic, but that gene may contribute to the realization of the phenotypic difference.

Results

Geotaxis behavior for selected lines

As a starting point for identifying genes that affect a complex trait, we analyzed the selected, established *Hi5* and *Lo* extreme geotaxic lines (refs 5,12; I. Park and J.H., unpublished data) for changes in gene expression between strains of *Drosophila melanogaster* subjected to long-term selection and isolation (Fig. 1). We could not test the unselected strain that was used as the source population for the original experiments, because it no longer exists.

We used a two-step approach. First, we determined the differential expression levels of mRNAs isolated from the heads of *Hi5* and *Lo* flies using cDNA microarrays and real-time quantitative PCR (qPCR). Second, we tested independently a subset of the differentially expressed genes for their influence on geotaxis behavior by running mutants for these genes through a geotaxis maze. We reasoned that some of the differences in gene expression between strains might be related to phenotypic differences and that it should therefore be possible, at least in part, to reconstruct the phenotype with independently derived mutations in some of the differentially expressed genes.

cDNA microarray and qPCR

Initially, we used cDNA microarrays[13] that contained about one-third of the predicted genes in the genome to identify roughly 250 genes that showed an approximately twofold or greater expression differential between the *Hi5* and *Lo* lines. We did these experiments in duplicate with different sets of flies and removed the few genes that behaved inconsistently from further analysis. The number of genes that showed consistent differential expression was about 5% of those assayed. Thus, gene expression in these strains has been modified as the result of laboratory selection. The polymorphisms responsible for this differential gene expression probably derive both from variation that was present in the initial selected populations and from spontaneous mutations that occurred during the course of the selection experiments. Not all of these differentially expressed genes would be

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Table 1 • Comparison of cDNA microarray and qPCR ratios of mRNAs

	Experimental group					Control group					
Gene	*cry*	*Pdf*	*Pen*	*pros (l)*	*pros (sl)*	*cnk*	*Csp*	*for*	*mth*	*nmo*	*per*
Array (*Lo*/*Hi5*)	3.57	1.85	0.18	3.22	3.22	0.92	1.03	1.27	1.11	1.01	–
qPCR (*Lo*/*Hi5*)	5.96	2.02	–	3.71	1.57	0.69	1.00	1.42	1.62	1.01	1.74

The average coefficient of variance for the qPCR results from each selected line was 19.33% with a range of 17.32–23.08% for *Hi5*, and 22.86% with a range of 21.96–24.17% for *Lo*. Because arrays were repeated only twice, no estimate of variance was possible. We report no *Pen* qPCR data because, of six primer pairs tested, none amplified efficiently enough to obtain consistent results, although the direction of change for those that gave some amplification was in the predicted direction. *pros* has two splice variants[17], short (s) and long (l), which the array did not resolve. We therefore designed a separate primer pair for each form, but the pair for the short form, designated (sl), amplifies both.

expected to be involved in the selected geotaxis response: some might be present in the strains as a consequence of genetic drift, whereas others could be 'hitchhiking' through genetic linkage with selected loci. However, some of the differentially expressed genes might influence the trait.

To select candidate genes for further analyses, we first identified the differentially expressed genes for which mutant *D. melanogaster* lines exist. We then chose those mutant lines that have known neurological defects, which were most of those available. We also chose a set of control genes that are not differentially expressed between the *Hi5* and *Lo* lines, and that cause neurological defects when mutated. Before the behavioral analyses, we used qPCR assays to confirm the microarray results for each gene. Given that there were only a limited number of existing mutants among the differentially expressed genes and that we could test them directly by qPCR, we thought that the combination of these two independent techniques would produce more reliable results than additional microarray replications. The qPCR results were in agreement with the array data (Table 1).

In total, we chose ten mutant fly lines for behavioral analyses. Four comprised our experimental group: *cryptochrome* (cry^b, null mutant)[14], *Pendulin* (Pen^{k14401}, extreme hypomorph)[15], *Pigment-dispersing factor* (Pdf^{01}, null mutant)[16] and *prospero* ($pros^{17}$, null mutant)[17]. Five comprised our initial control group: *connector enhancer of ksr* (cnk^{k16314}, extreme hypomorph)[18], *Cysteine string protein* (Csp^{P1}, hypomorph)[19], *foraging* (for^{189Y}, extreme hypomorph)[20], *methuselah* (mth^1, extreme hypomorph)[21,22] and *nemo* (nmo^{P1}, extreme hypomorph)[23]. We also included a *period* (per^{01}, null mutant)[24] mutant line as a 'control' for the involvement of the circadian mechanism in geotaxis behavior, because two circadian rhythm genes (*cry* and *Pdf*) had been implicated by the array (*per* was not represented on the microarray). We also tested transgenic fly lines that express wildtype alleles for two of our candidate genes: $Pdf^{+t3.530}$ (transgenic insertion of wildtype gene)[16] and $pros^{+t30.8}$ (transgenic insertion of wildtype gene; K. Hill, M. Hoffmann and C.-Q. Doe, unpublished data).

Geotaxis behavior for mutant fly lines

To examine the geotaxis influence of each gene, we transferred each mutation into a common genetic background, wildtype *Canton-S* (*CS*), that was different from either of the selected lines. We tested the resultant strains (Table 2) in a geotaxis maze. We placed the mutants on a neutral background to assay for those genes that have the most robust phenotypic effect that is independent of the combination of alleles in the selected lines. We also tested the effects of varying the gene dosage of *Pdf* and *pros*. For *Pdf*, we constructed lines with Pdf^{01} (henceforth referred to as Pdf^-) and the wildtype transgenic insertion $Pdf^{+t3.530}$ (henceforth referred to as Pdf^{+t}) to titrate its effect on the behavior. Likewise, for *pros* we used the mutant allele $pros^{17}$ and the transgenic insertion $pros^{+t30.8}$ (henceforth referred to as $pros^{+t}$).

The *Pen* and *cry* mutants deviated significantly from *CS* (Table 3 and Fig. 2*a*). Pdf^- flies also deviated significantly from *CS*. There were also effects on geotaxic behavior in Pdf^- flies owing to alterations in gene dosage and sex (genotype × sex interaction, $F = 3.85$, $P < 0.0015$; Table 4 and Fig. 2*b*). The sex-specific effect of varying *Pdf* gene dosage was graded, with the homozygous Pdf^- males showing the same response as *Hi5* males. In males, the effect was $Hi5 = Pdf^-/Pdf^- > Pdf^{+t}/+$; $Pdf^-/Pdf^- = Pdf^{+t}/Pdf^{+t}$; $Pdf^-/Pdf^- > Pdf^-/+ = CS = Pdf^{+t}/+ = Pdf^{+t}/Pdf^{+t} > Lo$, where nonsignificance is indicated by '=' and significance is indicated by '>' (Table 4 and Fig. 2*b*). Thus, although Pdf^-/Pdf^- males did not differ significantly from *Hi5* males, adding one copy of the transgene significantly lowered their score. Adding two copies continued this downward trend, but not significantly. The addition of either one or two normal alleles at the *Pdf* locus caused a further, significant decrease. The transgene alone did not significantly alter behavior in males with the *CS* background, but there was a trend downward with each copy.

In females, there were two key sets of significant changes, which we present separately for clarity. First, $Hi5 > Pdf^-/Pdf^- = Pdf^{+t}/+$; $Pdf^-/Pdf^- > Pdf^-/+ = CS$; thus, adding the transgene to Pdf^-/Pdf^- females had no effect, and the heterozygote ($Pdf^-/+$) without the transgene had a significantly lower score than that of $Pdf^{+t}/+$; Pdf^-/Pdf^- females. Second, $Pdf^{+t}/+$; $Pdf^-/Pdf^- = Pdf^{+t}/Pdf^{+t}$; $Pdf^-/Pdf^- > Pdf^{+t}/+ = Pdf^{+t}/Pdf^{+t}$ (Table 4 and Fig. 2*b*); although Pdf^{+t}/Pdf^{+t}; Pdf^-/Pdf^- did not differ significantly from $Pdf^-/+$ or *CS*, adding one copy of the transgene to *CS* caused a significant drop in score from Pdf^{+t}/Pdf^{+t}; Pdf^-/Pdf^- females. Thus, the transgene alone had no effect in the *CS* background, but a continued

Table 2 • Genotypes of mutants and variants

All genotypes except *Pdf*

♂	$cnk^{k1631}/+$	cry^b/cry^b	for^{189Y}/for^{189Y}	w/Y; mth^1/mth^1	w/Y; $nmo^{P1}/+$	w/Y; $Pen^{k14401}/+$	per^{01}/per^{01}	$w\ pros^{+t30.8}/Y$	$pros^{17}/+$
♀	$w/+$; $cnk^{k1631}/+$	cry^b/cry^b	for^{189Y}/for^{189Y}	w/w; mth^1/mth^1	w/w; $nmo^{P1}/+$	w/w; $Pen^{k14401}/+$	per^{01}/per^{01}	$w\ pros^{+t30.8}/w\ pros^{+t30.8}$	$w/+$; $pros^{17}/+$

Pdf genotypes

♂	w/Y; $Pdf^{+t3.530}/Pdf^{+t3.530}$	$Pdf^{+t3.530}/+$	$Pdf^{01}/+$	w/Y; $Pdf^{+t3.530}/Pdf^{+t3.530}$; Pdf^{01}/Pdf^{01}	$Pdf^{+t3.530}/+$; Pdf^{01}/Pdf^{01}	Pdf^{01}/Pdf^{01}	
♀	w/w; $Pdf^{+t3.530}/Pdf^{+t3.530}$	$w/+$; $Pdf^{+t3.530}/+$	$Pdf^{01}/+$	w/w; $Pdf^{+t3.530}/Pdf^{+t3.530}$; Pdf^{01}/Pdf^{01}	$w/+$; $Pdf^{+t3.530}/+$; Pdf^{01}/Pdf^{01}	Pdf^{01}/Pdf^{01}	

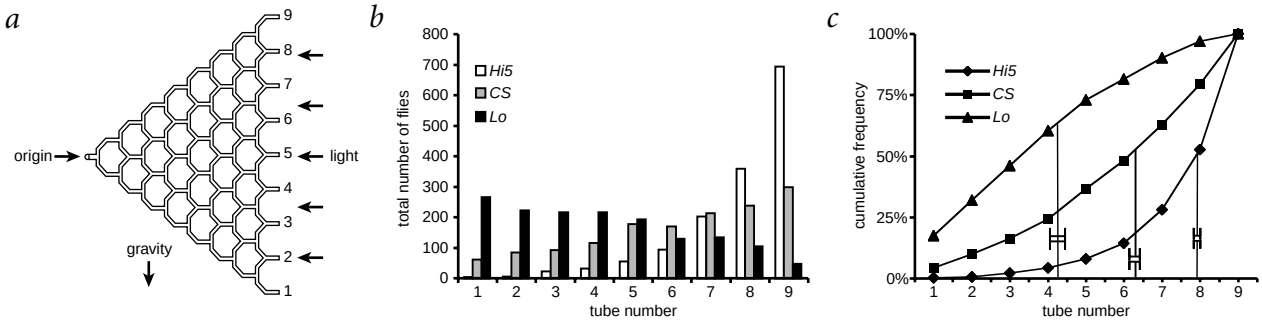

Fig. 1 Comparisons of geotaxis scores of *Hi5* and *Lo* geotaxic lines and wildtype *CS* lines in a nine-choice maze. ***a***, The nine-choice geotaxis maze[31]. ***b***, Distribution of the total number of flies across the maze. ***c***, Cumulative frequency distributions of tube scores. The mean score of flies in each line is indicated by a vertical to the *x* axis. Double-hatched lines are standard deviations of means of all runs for each genotype. The effects test for genotype was significant, $F = 486.2$, $P < 0.0001$. The effects test for sex and genotype × sex were nonsignificant. Average scores: *Lo*, 4.03 ± 0.11; *CS*, 6.19 ± 0.27; *Hi5*, 7.90 ± 0.19. All three genotype means were significantly different from one another.

increase in *Pdf* titers based on Pdf^{+t} did eventually cause a significant alteration in score. Geotaxis is therefore very sensitive to small changes in *Pdf* titers, with either increasing or decreasing amounts causing significant changes. The same was not found for *pros*, for which neither a reduction in titer in the heterozygous mutant nor an increase in titer in the transgenic insertion of a wildtype gene altered the geotaxis phenotype by itself.

Discussion

Our findings indicate that differences in gene expression can be used to identify phenotypically relevant genes, even when no large, single-gene effects are detectable by classical, quantitative genetic analysis. Three of the four genes implicated by our microarray and qPCR measurements caused differences in geotaxis, whereas none of the six control genes had an effect. Only those genes that had larger differences in expression according to the microarrays, or that were significantly different according to qPCR results (*cry*, *Pdf* and *Pen*), significantly changed geotaxis scores. The converse was not true, because altered geotaxis behavior did not always accompany larger differences in mRNA levels, as shown by *pros*, although this might reflect the sensitivity of *pros* to aspects of the genetic context that are not represented in the *CS* strain. All of the genes tested for which there was little or no difference in mRNA levels between the selected *Hi5* and *Lo* lines also showed no influence on geotaxis behavior.

The directionality of behavioral and mRNA differences proved to be consistent with predictions that were based on expression levels. Homozygous null mutants of *Pdf* and *cry* showed a significant increase in geotaxis score, which is consistent with a lower level of expression of these genes in *Hi5* relative to *Lo*. Similarly, the heterozygous *Pen* mutant showed a significant downward shift in geotaxis score, which is consistent with a lower level of *Pen* expression in *Lo* relative to *Hi5*. Thus, the change in behavior of the tested mutants corresponds to the direction predicted by differences in transcript level in the selected *Hi5* and *Lo* lines.

Whereas the *cry*, *Pen* and female *Pdf* mutants produced the anticipated effect on behavior, the magnitude of behavioral effect was smaller than in the original selected lines. This probably reflects the difference between the aggregate effect of an ensemble of genes in the selected lines as opposed to the individual effect of a single mutant gene in a neutral background. In addition, their relatively small effects are exactly the results that one would predict in a polygenic system such as geotaxis behavior, in which many genes have small contributions to the overall phenotype[2–4]. The three genes identified in this study would not have been predicted on the basis of their previously defined functions.

Pen encodes a nuclear importin, homozygous mutants of which have pleiotropic phenotypes including hypertrophied brains[25]. The other two identified genes are part of the circadian-rhythm machinery: *cry* encodes a highly conserved photopigment protein that mediates a component of entrainment in *D. melanogaster*[26], and *Pdf* encodes a neuropeptide that mediates circadian locomotor activity[16]. Although the mechanistic basis for geotaxis and for the involvement of these genes in it remains unknown and is currently under investigation, previous findings provide some clues. There may be an anatomical association between *Pdf* expression and geotaxis, because the handful of neurons containing the PDF neuropeptide are located next to the area of the brain that receives inputs from mechanosensory neurons[16,27]. A more general link between geotaxis and other nervous system functions derives from the fact that the circadian gene products regulate the expression of many genes in the nervous system[28,29].

These results show that the two separate approaches to behavioral genetics—the classical Hirschian quantitative analysis of genetic architecture and the modern Benzerian approach of single-gene mutant analysis[10]—are complemen-

Table 3 • Mean geotaxis maze scores not including *Pdf* genotypes

	Experimental group					Control group					
Mutants	*cry*	*Pen*	$pros^{17}$	$pros^{+t}$	*CS*	*cnk*	*Csp*	*for*	*mth*	*nmo*	*per*
Score[a]	6.64 ± 0.15	5.61 ± 0.06	6.28 ± 0.08	6.08 ± 0.07	6.19 ± 0.08	6.10 ± 0.08	6.06 ± 0.10	6.30 ± 0.08	6.06 ± 0.09	5.92 ± 0.08	6.31 ± 0.06
Deviation from *CS*	S	S	NS	NS	–	NS	NS	NS	NS	NS	NS

[a]The score (tube number) is based on the mean of individually combined morning and afternoon runs, $F = 7.06$, $P < 0.0001$. For these genotypes there was also a small main effect for sex ($F = 5.11$, $P < 0.025$), but it accounted for less than 3% of the model variance. $pros^{+t}$ refers to the transgenic wildtype insertion $pros^{+t30.8}$. NS, not significant; S, significant (see Methods).

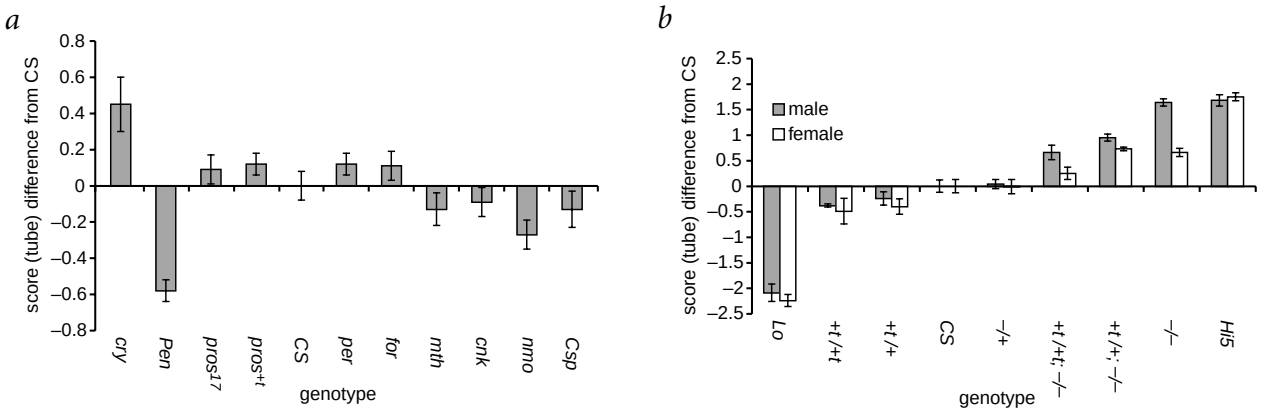

Fig. 2 Difference in average geotaxis scores between a tested genotype and *CS*. ***a***, All genotypes tested except *Pdf*. ***b***, *Pdf* gentoypes and their dosage effects. '*+t*' (transgenic insertion) refers to Pdf^{+t}; '–' refers to Pdf^{-}.

tary and can be unified. Here we used the results of a Hirschian approach of laboratory selection for natural variants to identify single gene differences, such as one would find in a Benzerian approach. Our results are consistent with the suggestion that naturally occurring variants in behavior correspond to mild lesions in pleiotropic genes[30].

Finally, our results show that differences in gene expression identified by cDNA microarray analysis can be used as a starting point for narrowing down candidate genes involved in complex genetic processes. Such an approach is analogous, as well as complementary, to the current method of mapping quantitative trait loci to large chromosomal intervals and then making educated guesses about which genes within those intervals may be involved in the trait.

The combination of selection, with its ability to exaggerate natural phenotypic variation, and global analysis of differences in gene expression by cDNA microarray analysis offers a promising approach to previously intractable molecular analyses of behavior. The geotaxis genes that we have identified might have been the direct targets of selection, or they might be downstream of the direct targets. Additional studies using the *Hi5* and *Lo* selected lines will be required to distinguish between these possibilities and to determine the causal role that these genes have in the context of the selected lines.

We have gone from the selection of a 'laboratory-evolved' behavioral phenotype, to screening for mRNA differences, to partially reconstituting the phenotype using mutants. This shows the feasibility of combining genomic and classical genetic approaches for the breakdown and partial reassembly of an artificially selected behavioral trait.

Methods

***D. melanogaster* cultures and stocks.** All assays, both molecular and behavioral, used flies cultured on yeast, dark corn syrup and agar food at 25 °C, 50–60% humidity in a 12 h/12 h light/dark cycle (lights on at 08:00).

We obtained cnk^{k16314}, Pen^{k14401} and $pros^{17}$ from Bloomington *Drosophila* Stock Center; cry^{b} and per^{01} from J.C. Hall (Brandeis Univ.); Csp^{P1} from K. Zinsmaier (Univ. of Pennsylvania); for^{189Y} from M. Sokolowski (Univ. of Toronto); mth^{1} from S. Benzer (California Inst. of Technology); nmo^{P1} from K.-W. Choi (Baylor College of Medicine); Pdf^{01} and $Pdf^{+t3.530}$ (a transgenic duplication) from P. Taghert (Washington Univ.); and $pros^{+t30.8}$ from C. Doe (Univ. of Oregon).

Geotaxis maze design. The geotaxis maze is a modification of a previous design[31]. The dimensions are the same, but instead of tygon tubing, the maze comprises two solid halves of plexiglass bolted together to form the complete maze. Flies start on one side and move through the maze toward a light source and into collection tubes on the other side (Fig. 1*a*). Runs consisted of females and males of all genotypes, aged 2–6 d, separated in groups of 75 and tested in the morning and the afternoon at 25 °C. We cleaned the maze between each trial by washing it with dilute Alconox (Alconox) and rinsing it extensively with deionized water. At the end of a given day, we discarded the flies and collected a fresh set to run the next day; we repeated this five times for each genotype.

Microarrays and qPCR. For microarrays and qPCR, we isolated the heads from flies frozen in liquid nitrogen and extracted RNA by homogenization as described[32]. The array experiments were done as described[13].

For the reverse transcriptase (RT) reactions and real-time qPCR assays, we obtained three separate sets of 20 heads (10 male, 10 female) from both the *Hi5* and *Lo* lines, and another set from the *CS* line. We isolated total RNA as described[32] and ran 6 RT reactions for each RNA pool in the selected lines and 36 for *CS* based on protocols for Superscript II (Invitrogen), using oligo d(T)s, 100 ng of total RNA and 0.02 pg of an artificially

Table 4 • Mean geotaxis maze scores of *Pdf* genotypes

	Lo	Pdf^{+t}/Pdf^{+t}	$Pdf^{+t}/+$	*CS*	$Pdf^{-}/+$	Pdf^{+t}/Pdf^{+t}; Pdf^{-}/Pdf^{-}	$Pdf^{+t}/+$; Pdf^{-}/Pdf^{-}	Pdf^{-}/Pdf^{-}	*Hi5*
♂ Score[a]	4.14 ± 0.17	5.85 ± 0.03	5.99 ± 0.13	6.23 ± 0.12	6.27 ± 0.09	6.89 ± 0.15	7.18 ± 0.08	7.87 ± 0.07	7.91 ± 0.11
♀ Score	3.91 ± 0.12	5.66 ± 0.25	5.75 ± 0.15	6.15 ± 0.13	6.14 ± 0.14	6.40 ± 0.12	6.88 ± 0.04	6.81 ± 0.08	7.90 ± 0.08
Deviation from *CS*	♂ S	NS	NS	–	NS	S	S	S	S
	♀ S	NS	NS	–	NS	S	S	S	S

[a]The score (tube number) is based on the mean of individually combined morning and afternoon runs. See text for detailed interactions. *Hi5* and *Lo* are added for comparison. NS, not significant; S, significant (see Methods).

constructed mRNA based on the gene encoding human β-actin, which was added to each reaction as an RT standard. Our rationale was that typical standards, such as *RP49*, have changed (2.7-fold) between *Hi5* and *Lo* owing to selection. We combined *CS* RT reactions for use as a qPCR standard curve, designed all primers for qPCR with Applied Biosystems Primer Express 1.5 software and carried out qPCR according to the manufacturer's protocols for the Applied Biosystem GeneAmp 5700 sequence detection system, with the following modifications: the reaction volume was 25 μl with a 3.2-fold dilution of SYBR Green PCR master mix and 5 μl of a 1/10 or 1/20 dilution of RT. We carried out qPCRs on initial RT reactions twice using primers for the gene encoding human β-actin and, on this basis, pooled RT reactions with less than 20% variance from the same starting RNA. We then did two more rounds of PCR and pooled the reactions. This resulted in two separate pools of *Lo* and three pools of *Hi5*, which we used for all subsequent PCRs with four reactions per qPCR assay for each unique pool. For *Csp* and *nmo*, which were expressed equally in the arrays, we show further confirmation of equality of pools in Table 1.

Scoring and statistics. Geotaxis maze scores are calculated as the weighted average of the number of flies in each of nine collection tubes, with tube 1 being the bottommost and tube 9 being the topmost. Thus, a score of 9 would be a perfect negative geotaxis score and a score of 1 would be a perfect positive geotaxis score. The unit of statistical analysis was the average score of each run. Each genotype result consists of 5 groups of 75 flies for each sex, run once in the morning and once in the afternoon. Because of the markedly skewed data and the importance of determining the difference between morning and afternoon runs, each run yielded two scores (morning and afternoon). The effect of time was nonsignificant across both experiments, as indicated by the results of a genotype × sex × time multiple analysis of variance, with repeated measures on time. We therefore averaged morning and afternoon results for an n number of 1 trial per day (total $n = 5$ for each genotype × sex) and conducted analyses using a full factorial least-squares regression model. We then used Tukey HSD post-hoc comparisons for interactions between groups (genotype × genotype or genotype × sex as needed), and used the Dunnett procedure in the control group comparison. The experiment-wise error rate for post-hoc comparisons was $P = 0.05$.

Selected lines. *Hi5* and *Lo* refer to the extreme, negatively geotaxic (moving up away from gravity) and the extreme, positively geotaxic (moving down towards gravity) selected lines, respectively. Both of these lines stabilized for the extreme behavior in the early 1980s (ref. 12), and the *Lo* line has remained stabilized without selection. The *Hi* line reverted partially about 5 years ago; to obtain its full, extreme geonegative phenotype, reselection experiments over five generations produced a new subline named *Hi5* (I. Park and J. Hirsch, unpublished data).

Standardizations of fly lines and genotypes. We standardized mutant and transgenic lines as follows. We obtained *per* on a *CS* background. For all other lines, the two major chromosomes, which did not contain the mutations or constructs, were substituted directly for the corresponding *CS* chromosome using balancers, thus transferring 100% of the genetic material for these chromosomes. Lines containing a P-element insert with a *mini-w* marker (*cnk*, *Csp*, *mth*, *nmo*, *Pen*, Pdf^{+t} and $pros^{+t}$) were placed on a *w CS* background and backcrossed 4–5 times with *w CS*. The third chromosomes containing Pdf^{01} and $pros^{17}$, which contain the mutations, retained their original background. The chromosome bearing the *cry* mutation initially contained an *ss* marker mutation, which we removed by crossover, thus subjecting the third chromosome containing the *cry* mutation to one backcross with *CS*. The *w CS* line was significantly different from *CS* ($F = 4.3$, $P < 0.013$) in geotaxis score. However, the model variance accounted for (24%) was low, both by itself and particularly when compared with the 40–97% in other groups tested, and none of the *w* genotypes in the tested mutants had *w* phenotypes, as the P-element inserts produced nearly wildtype eye color. We tested all mutations that are recessive lethal (*cnk*, *Csp*, *nmo*, *Pen* and *pros*) as heterozygotes.

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Competing interests statement

The authors declare that they have no competing financial interests.

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