Neurogenetic networks for startle-induced locomotion in *Drosophila melanogaster*

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Understanding how the genome empowers the nervous system to express behaviors remains a critical challenge in behavioral genetics. The startle response is an attractive behavioral model for studies on the relationship between genes, brain, and behavior, as the ability to respond rapidly to harmful changes in the environment is a universal survival trait. *Drosophila melanogaster* provides a powerful system in which genetic studies on individuals with controlled genetic backgrounds and reared under controlled environmental conditions can be combined with neuroanatomical studies to analyze behaviors. In a screen of 720 lines of *D. melanogaster*, carrying single *P[GT1]* transposon insertions, we found 267 lines that showed significant changes in startle-induced locomotor behavior. Excision of the transposon reversed this effect in five lines out of six tested. We infer that most of the 267 lines show mutant effects on startle-induced locomotion that are caused by the transposon insertions. We selected a subset of 15 insertions in the same genetic background in autosomal genes with strong mutant effects and crossed them to generate all 105 possible nonreciprocal double heterozygotes. These hybrids revealed an extensive network of epistatic interactions on the behavioral trait. In addition, we observed changes in neuroanatomy that were caused by these 15 mutations, individually and in their double heterozygotes. We find that behavioral and neuroanatomical phenotypes are determined by a common set of genes that are organized as partially overlapping genetic networks.

behavioral genetics | epistasis | sensorimotor integration | startle behavior | *P*-element insertional mutagenesis

A major goal of behavioral genetics is to understand the relationship between the genome and the nervous system. From a neuroscience perspective, behaviors represent the ultimate expression of the nervous system. From a genetics perspective, behaviors are complex traits for which natural variation is caused by many interacting genetic variants, with allelic effects that depend on social and external environments, sex, and genetic background (1, 2). To date, most studies that have attempted to relate genetic variation to the neural regulation of behavior have adopted a "one gene at a time" approach. Such studies have made important contributions and generated significant insights. However, recent genomic approaches, in which candidate genes affecting behaviors are identified by comparing whole-genome expression profiles of genetically divergent strains, have implicated large numbers of coregulated genes affecting behaviors that have pleiotropic effects on other traits, and that would not have been *a priori* predicted to affect behavior (3–10). In the current study, we used quantitative genetic approaches to analyze the genes–brain–behavior relationship at the level of genetic networks rather than at the level of single genes.

We used startle-induced locomotion to a mechanical disturbance in *Drosophila melanogaster* as a model behavior. Previously, we developed a simple, high-throughput and highly repeatable assay to quantify startle-induced locomotion in *Drosophila*, and used it to map 13 positional candidate genes corresponding to quantitative trait loci for this trait in a population of recombinant inbred lines; these include genes associated with bioamine synthesis as well as genes that encode transcription factors implicated in development of the nervous system (11). Transcriptional analysis of lines selected for low or high startle-induced locomotion showed 1,790 probe sets with different expression levels between the selection lines at a false discovery rate of 0.001, suggesting that a large fraction of the genome affects this behavior (5). In addition, association studies found polymorphisms associated with variation in startle-induced locomotion in *Catsup*, which encodes a negative regulator of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of dopamine (12), and in *Ddc*, which encodes dopamine decarboxylase (11). Additional studies in *Drosophila* have implicated serotonin, octopamine, and γ-aminobutyric acid as critical mediators of locomotion (13–16).

To further characterize the genetic architecture of startle-induced locomotion, we used *P*-transposable element mutagenesis in an isogenic background as an unbiased screen for loci affecting complex behaviors. *P*-element insertions often give rise to subtle effects and are thus advantageous for studies on behavior, because in contrast to null mutations they allow the development of a viable and fertile adult. We assessed startle-induced locomotion of 720 *Drosophila* lines with single *P*-element insertions and identified 267 lines with reduced startle-induced locomotion. Out of these, we selected 15 lines with autosomal *P*-element insertions to assess the extent of epistatic interactions among these mutations. Because many of the genes that affect startle-induced locomotion appear to be associated with development of the nervous system, we asked to what extent these mutations impact the neuroanatomical organization of central brain structures that mediate sensorimotor integration and locomotion: the mushroom bodies and the ellipsoid body. We found that the genetic architectures of both startle-induced locomotion and brain morphology are characterized by highly interconnected epistatic genetic networks. Among these 15 candidate genes, *robo*, an essential gene for the establishment of neural connectivity (17, 18), features as a prominent hub.

Results

Identification of *P[GT1]* Insertion Lines with Reduced Startle-Induced Locomotion. We identified a set of viable and fertile mutants with reduced startle-induced locomotion by screening 720 lines with single *P[GT1]* transposon insertional mutations in common genetic

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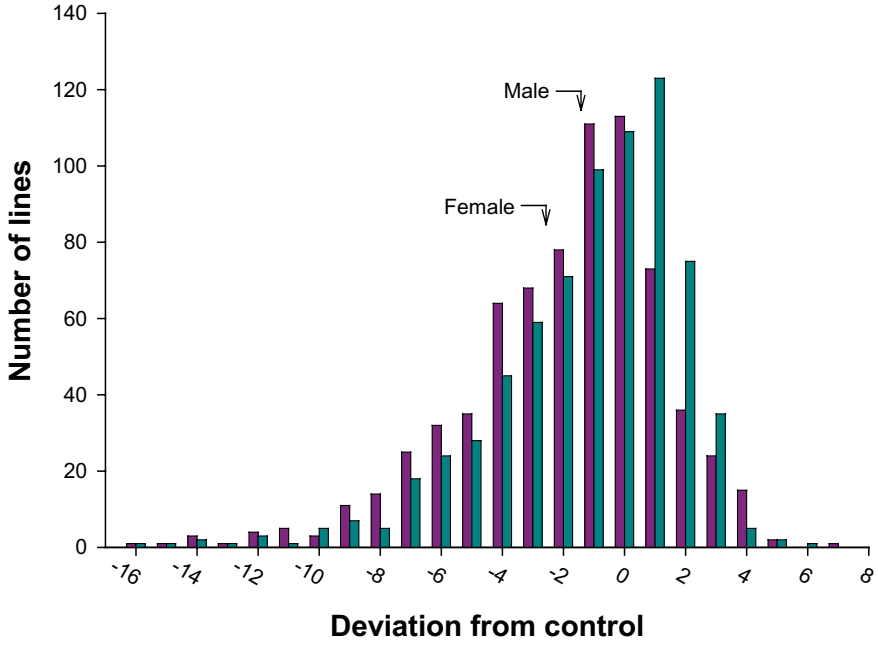

Fig. 1. Distribution of locomotion scores of 720 *P[GT1]* insertion lines. Maroon and teal bars indicate the distributions of female and male scores, respectively. Data are standardized by calculating the deviation of the score for each line from its control. Arrows indicate the mean deviation of males and females from the *Canton-S* control.

backgrounds. We found significant variation in startle-induced locomotion between the sexes and among *P*-element insertion lines, as well as significant line by sex interaction variance [supporting information (SI) Table S1]. Because control strains show 25.1 s to 28.8 s of locomotor activity in this 30-s assay, the assay is strongly biased toward detecting reduced rather than enhanced startle-induced locomotion. This is reflected in the skewed distribution of locomotion score differences between mutant lines and controls toward negative values (Fig. 1). The average deviation from the control for all 720 lines was -2.44 ± 0.12 s for females and -1.68 ± 0.12 s for males. The broad sense mutational heritability for startle-induced locomotion was $H_M^2 = 0.145$, similar to the estimate of narrow sense heritability for this trait in a segregating natural population (5). The estimate of the cross-sex genetic correlation was $r_{MF} = 0.830$, indicating a strong, but not perfect, correlation of mutational effects between the sexes. Again, this estimate is similar to that obtained from naturally segregating variation (5).

We identified 333 lines with different startle-induced locomotion scores from the *P*-element-free controls in the initial screen of 10 replicate assays per sex. We retested these candidate mutations with an additional 10 replicate assays per sex. This secondary screen confirmed that 267 mutant lines (80% of the mutations detected in the initial screen and 37.1% of all 720 lines tested) were significantly less responsive than their controls (Table S2). These 267 mutant lines included 217 unique insertion sites with 190 insertions in or near predicted genes. The insertion sites, locomotion scores, and annotated candidate genes for 151 mutations with effects that are significantly different from the control after Bonferroni correction for multiple tests ($P < 0.00015$) are shown in Table S2. Few hyperactive lines were recovered, quite possibly because the controls themselves were highly active. The insertion site of the *P[GT1]* element could be unambiguously determined in all but 5 of the 267 lines. Many genes and predicted genes were tagged by multiple insertions (e.g., *longitudinals lacking*, *Protein kinase 61C*, *Laminin A*, *Sema-5c*, *neuralized*, *CG10990*, *CG11226*). A total of 35 insertions were in regions with no annotated gene within 2 kb of the insertion site, and 63 insertions tagged computationally predicted genes. The average mutational effect was -4.8 s, that is, on average the mutations decreased startle-induced locomotion to 82% of the control value. However, some mutations of large effect decreased startle-induced locomotion to less than 50% of the control value.

Gene ontologies of candidate genes tagged by *P*-element insertions within 250 bp of the transcriptional unit but further than 2 kb from any other gene, and with mutational effects that exceed the Bonferroni-corrected significance level, are given in Table S3. Only three of these genes (*Beadex*, *Sema-5c*, and *neur*) had previously been documented to affect locomotion (19–21). Many of these candidate genes affect nervous system development and function, as well as other aspects of development, the cell cycle, and oogenesis and spermatogenesis, consistent with previous observations that genes affecting startle-induced locomotion are highly pleiotropic (5, 11, 12).

P-Element Excision and Phenotypic Reversion. To demonstrate that the observed behavioral phenotypes were due to the *P[GT1]* insertion rather than an unrelated mutation in the genome, we excised the *P[GT1]*-elements from a sample of the mutants by mobilizing the *P*-element via standard procedures, under conditions that preserve the genetic background (22). We created 25 independent revertants of six *P[GT1]* insertion lines (Fig. S1) and tested the homozygous revertants for startle-induced locomotion. For five of the six *P[GT1]* insertion lines, at least one *P*-element excision line was obtained that showed complete phenotypic reversion. For one line with a *P*-element insert in *neuralized*, we were not able to obtain excision lines that showed complete phenotypic reversion, but we did obtain partial revertants. These results allow us to infer that it is likely that impairment of startle-induced locomotion is due to the *P*-element insertion for most of the mutant lines.

Analysis of Epistasis Among Startle-Induced Locomotion-Impaired *P[GT1]* Insertion Lines. Because the *P[GT1]* insertion lines are in isogenic backgrounds, we can examine enhancer and suppressor effects among a subset of these genes, separating dominance effects from epistasis by generating all possible nonreciprocal double heterozygotes (23–25). We selected 15 representative *P[GT1]* insertion lines for this analysis based on the following criteria: (*i*) the *P[GT1]* insertions were in the *Canton-S* (*B*) background; (*ii*) the *P[GT1]* insertions were autosomal (to enable complete analysis for both sexes); (*iii*) the *P*-elements were inserted within or in proximity to an unambiguously identified gene; and (*iv*) the *P[GT1]* insertion had a large effect on the phenotype. Based on these criteria, we

selected genes coding for the transcriptional regulators *tramtrack* (*ttk*), *extramacrochaetae* (*emc*), *HLHm7*, and *longitudinals lacking* (*lola*); the calcium binding chaperone *Calreticulin* (*Crc*); the ubiquitin protein ligase *neuralized* (*neur*); the extracellular matrix protein *Laminin A* (*LanA*); and axon guidance molecules *roundabout* (*robo*) and *Sema-1a*. We also included the early developmental genes *Sema-5c* and *Darkener of apricot* (*Doa*), a protein threonine/tyrosine kinase. *Cysteine string protein* (*Csp*), which encodes a gene product essential for neurotransmitter release, and *CG8963*, which encodes a predicted transcript of unknown function with a strong effect on startle-induced locomotion, were also included. Finally, we included the two insert lines for which the closest genes encode microRNAs (*mir-279* and *mir-317*).

We crossed the 15 *P[GT1]* insertion lines to create all possible 105 nonreciprocal double heterozygotes, and quantified startle-induced locomotion. For this experiment, we extended the observation period to 45 s to increase the power to detect subtle effects. Since all insertions are in the same isogenic genetic background, the average heterozygous effect contributed by each parental line in combination with all others can be estimated as the general combining ability (*GCA*), and a phenotypic value for each double heterozygous combination can be predicted from the *GCA*s of both parents. The specific combining ability (*SCA*) of a double heterozygote is the deviation of its observed phenotype from that predicted from the *GCA*s of the parents (26). Significant *SCA* is due to epistasis, and the direction of the deviation indicates either an enhancer or suppressor effect between the two mutations (23–25).

The variation in startle-induced locomotion among the double heterozygote genotypes was highly significant ($P < 0.00001$, Table S4). The overall effect of sex was significant at $P < 0.001$, although we found significant sexual dimorphism only in 10 of the 105 double heterozygote genotypes. *GCA* and *SCA* effects were also highly significant for both sexes. Because the *GCA* × Sex and *SCA* × Sex interactions were only marginally significant, we discuss the sex-averaged data in further analyses. The analyses for each sex separately yielded similar results.

The average scores of the 15 single heterozygous genotypes and the *Canton-S (B)* control line were 39.82 s and 42.15 s, respectively. Thus, the average effect of the single heterozygotes expressed as a deviation from the control is −2.33s. With additive interactions between loci, we predict the average of the double heterozygotes to be 4.66 s less than the control, or 37.49 s. The average startle-induced locomotion score of all double heterozygotes was 35.18 s. The observed mean startle-induced locomotion of the double heterozygotes is significantly less than 37.49 s, indicating the prevalence of enhancer epistatic effects (i.e., more mutant than expected) among the double heterozygotes.

Because the *GCA* and *SCA* terms contributed significantly to the variance of startle-induced locomotion among the double heterozygous genotypes, we estimated individual *GCA* and *SCA* values for each single heterozygote and double heterozygote genotype, respectively (Table S5). We observed significant *GCA* values in 10 of the 15 lines. Positive *GCA* values indicate higher (less mutant) reactivity scores, and partly recessive effects of the mutation in combination with all other mutations. *LanA* and *Sema-1a* were partly recessive (Table S5). Negative *GCA* values indicate lower (more mutant) reactivity scores, and partly dominant effects of the mutation in combination with all other mutations. The *Sema-5c*, *mir-317*, and *robo* mutations showed partial dominance (Table S5). We found extensive epistasis among all 15 mutants, with significant *SCA* effects for 36% of the double heterozygotes (38 genotypes of 105, Table S5). Positive *SCA* effects indicate higher reactivity scores than expected, and a suppression of the mutant phenotype; conversely, negative *SCA* values indicate lower reactivity scores than expected, and enhancement of the mutant phenotype. A diagrammatic representation of the network of epistatic interactions in which all 15 independently isolated genes are interconnected is illustrated in Fig. 2. Some candidate genes, notably *robo*, *mir-317*, *Csp*, and *Crc*, feature especially prominently in the pattern of epistasis.

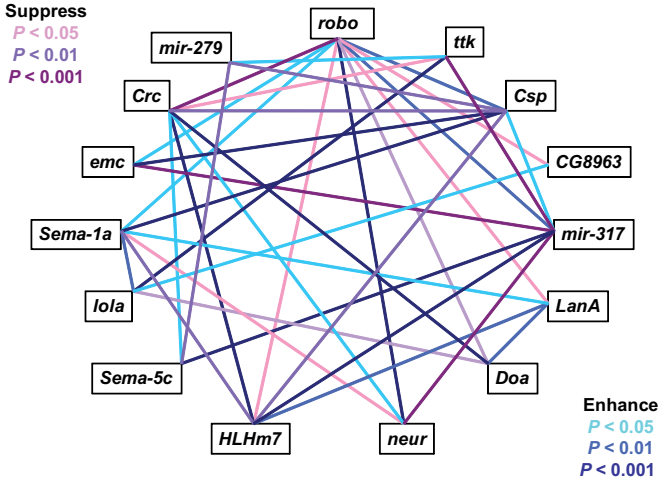

Fig. 2. Epistatic interactions for startle-induced locomotion among 15 *P[GT1]* insertion lines in double heterozygous genotypes.

Thus, our results reveal that genes that affect startle-induced locomotion form extensive epistatic networks. Several of these genes also play a role in development of the nervous system. For example, both *robo* and *lola* are essential for axon guidance during development of the nervous system (17, 18, 27, 28). Furthermore, *mir-279* has been implicated in *Drosophila* in establishing the identity of CO_2-detecting chemosensory neurons (29), and more generally, microRNAs have been implicated in neurodevelopment and morphogenesis (29–33). In addition, *mir-317* is predicted to regulate *ocelliless* (34), which has been implicated in the development of the protocerebral bridge, a component of the central complex associated with locomotion (35). These observations motivated us to ask to what extent these same mutations give rise to alterations in brain structures.

Neuroanatomy of *P*-Element Insertion Lines with Reduced Startle-Induced Locomotion. We asked to what extent neuroanatomical dimensions of central brain structures would be influenced by epistasis among the same 15 mutants that form part of the ensemble that modulates startle-induced locomotion. To determine whether these mutations give rise to alterations in brain structures, we focused on the α and β lobes of the mushroom bodies and the ellipsoid body of the central complex, because genetic and behavioral studies have shown that the mushroom bodies play a role in experience-dependent learning (36, 37) and suppress locomotor activity in *Drosophila* (38, 39), and that the central complex is a key mediator of locomotion (13, 40, 41). This analysis showed neuroanatomical defects in several of the *P*-element insertion lines, most commonly shortening of the α lobe of the mushroom body (*Sema-5c*, *ttk*, *HLHm7*, *lola*, *neur*, and *Sema-1a*; Fig. S2). We also observed missing α lobes in *neur* and missing β lobes in *LanA* (Fig. S2). These large morphological defects, however, were not fully penetrant, but were observed at frequencies of 5–10% (Fig. S2). For example, sporadic morphometric defects in *LanA* were not statistically significant due to their low penetrance. However, they were never seen in the progenitor *Canton S* (*B*) strain. Given the low penetrance, we next asked whether subtler, quantitative effects on brain structures could be seen. We used a previously established standardized method for determining morphometric parameters for these neuropils by immunohistochemistry using an anti-fasciclin 2 monoclonal antibody (21). This procedure revealed several mutations with more subtle, quantitative effects on morphology of brain structures: *lola* [length of the β lobe ($P = 0.024$) and area of the

Table 1. Standardized dimensions of mushroom body α and β lobes and ellipsoid body surface areas of homozygous mutants and control *Canton-S(B)*

Line	α lobe length	β lobe length	α lobe width	β lobe width	Ellipsoid body surface
Canton S (B)	0.550 ± 0.012	0.434 ± 0.007	0.073 ± 0.002	0.074 ± 0.004	0.096 ± 0.003
robo	0.592 ± 0.012	0.419 ± 0.005	0.073 ± 0.003	0.082 ± 0.003	0.098 ± 0.004
neur	0.514 ± 0.029	0.411 ± 0.006*	0.082 ± 0.020	0.074 ± 0.002	0.088 ± 0.006
emc	0.524 ± 0.008	0.416 ± 0.007	0.065 ± 0.002*	0.068 ± 0.003	0.094 ± 0.003
LanA	0.559 ± 0.010	0.406 ± 0.007*	0.080 ± 0.003	0.069 ± 0.004	0.096 ± 0.003
ttk	0.551 ± 0.015	0.414 ± 0.010	0.078 ± 0.002	0.075 ± 0.003	0.097 ± 0.004
Crc	0.569 ± 0.008	0.421 ± 0.005	0.074 ± 0.002	0.080 ± 0.004	0.108 ± 0.003*
Csp	0.564 ± 0.010	0.413 ± 0.006*	0.073 ± 0.002	0.075 ± 0.004	0.101 ± 0.001
mir-279	0.597 ± 0.010*	0.414 ± 0.007	0.073 ± 0.003	0.085 ± 0.003	0.100 ± 0.003
mir-317	0.514 ± 0.011	0.414 ± 0.008	0.064 ± 0.002*	0.069 ± 0.003	0.092 ± 0.002
HLHm7	0.570 ± 0.010	0.412 ± 0.006*	0.072 ± 0.002	0.078 ± 0.003	0.097 ± 0.003
CG8963	0.528 ± 0.035	0.419 ± 0.007	0.083 ± 0.006	0.095 ± 0.005*	0.094 ± 0.003
Doa	0.539 ± 0.009	0.419 ± 0.005	0.064 ± 0.002*	0.071 ± 0.002	0.085 ± 0.001**
lola	0.529 ± 0.020	0.410 ± 0.008	0.061 ± 0.002**	0.063 ± 0.002	0.082 ± 0.004
Sema-1a	0.516 ± 0.042	0.435 ± 0.008	0.067 ± 0.006	0.073 ± 0.003	0.077 ± 0.004**
Sema-5c	0.547 ± 0.009	0.438 ± 0.006	0.066 ± 0.003	0.071 ± 0.003	0.087 ± 0.003*

Dimensions (means and standard errors) are presented as ratios standardized to the distance between the peduncles. Significant differences of morphometric measurements between homozygous mutants and control are indicated at * ($P < 0.05$) and ** ($P < 0.01$) and were determined by Student's *t* test.

ellipsoid body ($P = 0.05$)]; *CG8963* [width of the β lobe; $P = 0.0019$); and *Sema-1a* and *Doa* (area of the ellipsoid body; $P = 0.0022$ and $P = 0.0014$, respectively; Table 1].

Epistatic Effects on Central Brain Structures. We observed neuroanatomical defects in 17 out of 105 double heterozygotes. These comprise defects in α lobes only (11/17), in α and β lobes (2/17), and in β lobes only (4/17). The majority of cases with defects in α lobes (10/13) involve at least one gene that displayed α lobe defects in the parental homozygous lines (*Sema-1a* combined with *Crc*, *Doa*, and *neur*; *Sema-5c* with *Crc*; *CG8963* with *lola*; *robo* with *HLHm7*; *neur* with *ttk*, *Csp*, and *Doa*). Contrary to α lobe defects, none of the β lobe defects involve *LanA*, the only gene for which a β lobe defect was recorded in the parental homozygous line. We observed instances of missing β lobes in double heterozygous combinations *CG8963*/*Csp*, *robo*/*emc*, *robo*/*Csp*, and *neur*/*mir-317*, and of β lobe fusion in *mir-317*/*ttk* and *HLHm7*/*Csp*. Five of the six combinations with β lobe defects also displayed epistatic interactions for startle-induced locomotion.

We quantified the lengths and widths of the mushroom body lobes and the vertical and horizontal diameters and surface area of the ellipsoid body (Table S6). We found significant effects for genotype, *GCA* and *SCA* values for all five morphometric measures of brain structures (Table S7). Significant *SCA* values showing both enhancer and suppressor effects were evident for many double heterozygous genotypes for all morphometric parameters (Fig. 3, Table S8). We observed epistatic interactions between *P[GT1]* insertions that both had significant effects on morphometric mea-

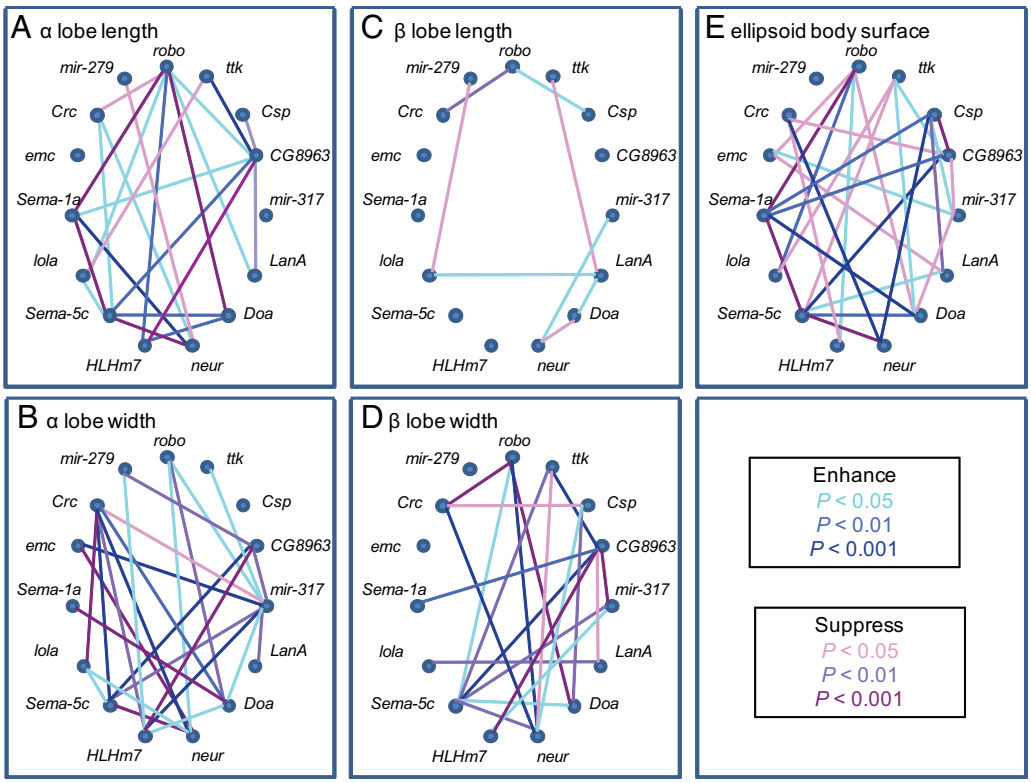

Fig. 3. Epistatic interactions for morphological measurements of the mushroom body lobes and the ellipsoid body among 15 *P[GT1]* insertion lines in double heterozygous genotypes. (*A*) α lobe length; (*B*) α lobe width; (*C*) β lobe length; (*D*) β lobe width; (*E*) surface area of the ellipsoid body.

surements in the parental homozygous lines (Table 1), between insertions in which only one mutation had significant homozygous effect, and even between pairs of insertions in which neither had significant homozygous effects on brain structure (Fig. 3, Table S6). The largest number of epistatic interactions for the length of the α lobes of the mushroom bodies involved double heterozygotes with *robo*, *Sema-5c*, or *CG8963*, whereas *mir317* featured prominently in epistatic interactions that modulate the width of the α lobes (Fig. 3, Table S6). Double heterozygotes with *neur* showed pervasive epistasis with respect to the width of the β lobes, whereas *Sema-5c* was the most frequent partner in double heterozygotes that showed enhancer or suppressor effects affecting the surface area of the ellipsoid body (Fig. 3, Table S6).

Discussion

We have identified candidate genes and characterized epistatic networks among a subset of genes that determine the magnitude of startle-induced locomotion, an important survival and fitness trait, in *Drosophila melanogaster*.

A Large Fraction of the Genome Is Associated with Startle-Induced Locomotion. About 37% of *P*-element insertion lines tested for startle-induced locomotion differ significantly from the *P*-element free, isogenic control. Previous studies also suggested that a high fraction of the genome is associated with startle-induced locomotion (5). Only three of the candidate genes affecting startle-induced locomotion had been previously linked to effects on locomotor behavior (19–21). Although many of the candidate genes affect nervous system development and function, others are in known genes that have not been found to affect locomotion, or indeed any behavior, in computationally predicted genes or in regions of the genome with no nearby annotated gene. Screening for quantitative effects of mutations in a common isogenic background is thus an effective method for functional genome annotation (24, 42).

The fact that a large fraction of the genome can affect startle-induced locomotion indicates that genes affecting startle behavior must have pleiotropic effects. Indeed, many of these mutations also affect sensory bristle number (42), olfactory avoidance behavior (24), aggression (3, 24), and ethanol sensitivity (4). Because mutations cause genome-wide disruptions in the transcriptome (43), it is clear that we need to think of all complex traits in terms of large networks of interacting genes affecting a focal trait, and joined via pleiotropy to overlapping networks of interacting genes affecting traits ranging from coregulated transcripts to whole organism phenotypes.

Startle-Induced Locomotion Is Determined by an Epistatic Network of Genes. Previous studies revealed surprisingly large networks of epistatic interactions among independently isolated mutations affecting olfactory avoidance behavior (23, 24) and a climbing assay (25). We observed even more extensive epistasis among only 15 mutations affecting startle behavior, possibly due to bias in our selection of known neurodevelopmental genes for the analyses of epistasis. Thus, epistasis seems to be a major factor shaping the genetic architecture of complex behaviors. *robo*, which is essential for axon guidance during neurogenesis (17, 18), emerges as a major hub in contributing to startle-induced locomotion, both in the epistatic network based on behavioral measurements and in epistatic networks based on corresponding morphometric measurements.

Epistatic Networks Associated with Development of the Central Nervous System. Many mutations associated with reduced startle-induced locomotion affect neurodevelopment. The mushroom bodies have a key role in experience-dependent learning (36, 37) and locomotion (38, 39). The ellipsoid body and other parts of the central complex are instrumental in directing locomotion (40, 41). We quantified the lengths and widths of mushroom body lobes and the surface area of the ellipsoid body for all 105 double heterozygotes. We found that epistatic effects that dictate neuroanatomical organization of some brain structures are even more extensive than those for startle-induced locomotion. These interactions occurred not only between mutations with significant homozygous effects on neuroanatomy, but also when only one mutation or neither mutation had significant effects as homozygotes. The effects of the mutations as double heterozygotes on startle-induced locomotion were not significantly correlated with their effects on morphometric measurements. One reason for this lack of correlation could be because the direction and magnitude of pleiotropic effects on locomotor behavior and the development of central brain structures that mediate this behavior are not consistent across the different genotypes, yielding a nonsignificant correlation between variation in brain structures and variation in locomotor behavior across all genotypes. Other possible reasons for the lack of correlation between the mutational effects on brain morphology and startle-induced locomotion are that the organization of the brain is established during development, whereas behavioral responses to changes in the environment are dynamic throughout adult life. Second, the precise contributions of the various mushroom body lobes to sensorimotor integration in response to a mechanical startle and their functional connections to the ellipsoid body are unknown. Third, we analyzed only two principal neuropils involved in startle-induced locomotion, and did not evaluate other components of the nervous system that potentially contribute to this behavior. For example, many genes that comprise the epistatic networks described here also regulate mechanosensory bristle number (42). Furthermore, *robo* (17, 18), *LanA* (44), *lola* (27, 28, 45), and *Sema-1a* (46) all play a role in embryonic ventral nerve cord development and/or motor axon guidance. Thus, many of the genes analyzed in our study have important roles in the development of mechanoreceptors, which mediate sensory input from the environment, and the thoracic ganglion, which controls motor output. These components of the nervous system are also essential for startle behavior. Documentation of both the entire genetic network and the complete neural circuitry that mediates startle-induced locomotion will be necessary to precisely determine the relationship between the genetic network that enables the expression of startle-induced locomotion and the neural circuitry responsible for its manifestation. Despite these limitations, our study shows that genes that form epistatic networks that contribute to variation in brain organization also form epistatic networks that contribute to the manifestation of behavior. These networks are not linearly correlated, but rather form overlapping ensembles that intersect at central hubs, such as *robo*.

Materials and Methods

***Drosophila* Stocks.** Single *P[GT1]* insertion lines (47) were generated in isogenic derivatives (designated as A to F) of w^{1118}; *Canton-S* as part of the Berkeley *Drosophila* Gene Disruption Project (48). We established 720 homozygous viable insertion lines with documented insertion sites. The set represents ≈490 tagged genes. To verify that observed phenotypic effects were indeed due to the *P*-element insertion, we generated phenotypic revertants by mobilizing the *P*-element under conditions that preserve the same genetic background, as described previously (22).

Behavioral Assay and *P*-Element Mutagenesis Screen. Fly culture and behavioral assays were conducted at 25°C. Flies were reared under controlled density and aged to 3–7 days post eclosion. Single flies were put in a plastic culture vial with cornmeal–agar–molasses medium for 1–2 days before measurement. The flies were subjected to a gentle mechanical disturbance by tapping the vial lightly, then placing it horizontally under a fluorescent lamp. Locomotor behavior was quantified as the number of seconds each fly was active during an observation period immediately following the disturbance (5, 11). The observation period lasted 30 s for the homozygous mutation screen and 45 s for the epistasis analysis. All tests were done between 8 a.m. and 12 p.m. We assessed startle-induced locomotion for 10 flies per sex of each line in the initial screen, and retested an additional 10 flies per sex for mutations from the initial screen with significantly different startle-induced locomotion from the control (i.e., a total of 20 flies/sex

per line). A total of 40 flies/sex were tested for the control strain each time, contemporaneously with the *P*-element insertion lines. These measurements yielded stable and highly reproducible estimates of startle-induced locomotion.

We assessed mutational variation in startle-induced locomotion by two-way mixed model ANOVA according to the model $Y = \mu + L + S + L\times S + \varepsilon$, where μ is the overall mean, *L* is the random effect of the *P*-element insertion line, *S* is the fixed effect of sex, $L\times S$ is the line by sex interaction term, and ε is the environmental variance. We also ran reduced analyses for each sex separately, and computed the variance components (σ^2) for the random effects. The mutational broad sense heritability was computed as described previously (24).

Estimation of Epistasis. We crossed 15 *P[GT1]* lines with large effects on locomotor behavior that were generated in the same genetic background, constructing all 105 possible nonreciprocal double heterozygotes. The assay included 105 double heterozygous genotypes, 15 homozygous lines, the progenitor *Canton-S (B)* strain, and 15 single heterozygous genotypes from crosses of each of 15 parental lines and *Canton-S (B).* To account for environmental variation, we tested a single set of two sexes of the 136 genotypes contemporaneously each day for 20 days, for a total of 20 replicate measurements per genotype.

We used ANOVA to assess differences in locomotor behavior of each *P[GT1]* insertion line from the appropriate control, and to assess differences in locomotion between the double heterozygous genotypes according to the model $Y = \mu + G + S + G\times S + \varepsilon$, where *G* denotes double heterozygote genotype and $G\times S$ is the genotype by sex interaction term. We further computed the general combining ability (*GCA*) and specific combining ability (*SCA*) for all genotypes (26). The *GCA* is the average dominance of each *P[GT1]* insertion in combination with all other mutations. The expected phenotypic value for each double heterozygote can be predicted from the *GCA* values of each parent. The expected phenotypic value for each double heterozygote can be predicted from the *GCA* values of each parent by adding the *GCA* values to the mean of all double heterozygotes (35.18 s). The *SCA* measures the difference of the phenotypic value of each double heterozygote from the predicted value; significant *SCA* estimates indicate epistasis (23, 24).

Whole-Mount Immunohistochemistry. Adult brains from female flies were dissected and processed for immunohistochemistry with a mouse monoclonal anti-fasciclin 2 antibody to visualize mushroom body α and β lobes and the ellipsoid body, as described previously (21). The immunostaining was documented by using an Olympus BX61 epifluorescence microscope equipped with a DP70 digital camera controlled with analySIS FIVE software.

Morphometric Analysis. Length and width of the α and β lobes of the mushroom bodies and diameters D1 and D2 of the ellipsoid body of the central complex were measured by using analySIS FIVE software and expressed as values relative to the distance between the α lobe heels (21). This internal calibration controls for differences in brain sizes when assessing variation in morphometric parameters among genotypes. Values were obtained for 10 adult brains each for all 105 double heterozygotes that were studied for locomotor behavior, as well as 15 homozygous lines and the progenitor *Canton-S (B)* strain. The analysis of a large number of brains for all genotypes averages out technique-induced variation that can result from small differences in exact mounting angle.

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