

Complex genetic architecture of *Drosophila* aggressive behavior

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Epistasis and pleiotropy feature prominently in the genetic architecture of quantitative traits but are difficult to assess in outbred populations. We performed a diallel cross among coisogenic *Drosophila* P-element mutations associated with hyperaggressive behavior and showed extensive epistatic and pleiotropic effects on aggression, brain morphology, and genome-wide transcript abundance in head tissues. Epistatic interactions were often of greater magnitude than homozygous effects, and the topology of epistatic networks varied among these phenotypes. The transcriptional signatures of homozygous and double heterozygous genotypes derived from the six mutations imply a large mutational target for aggressive behavior and point to evolutionarily conserved genetic mechanisms and neural signaling pathways affecting this universal fitness trait.

Epistasis (the dependence of the allelic effects on a trait at one locus on the genotype at another locus) and pleiotropy (the distribution of effects of the same allele on multiple traits) are important features of the genetic architecture of quantitative traits (1). We can use epistatic interactions to infer genetic networks affecting complex traits, but epistasis biases estimates of allelic effects from gene mapping studies when it is present but not accounted and affects predictions of long-term response to artificial and natural selection (2, 3). Pleiotropy can impose constraints to response to artificial and natural selection, affect correlated responses to selection, is the basis of genetic variation in phenotypic plasticity and sex dimorphism of quantitative traits, and is a critical feature of models for maintaining quantitative genetic variation through mutation selection balance (4, 5).

Epistasis and pleiotropy are difficult to assess in outbred populations. When allele frequencies at interacting loci are not common, epistasis makes little contribution to segregating genetic variance (6); furthermore, only large interaction effects can be detected in genome scans for pairwise epistasis in mapping studies because of the severe multiple testing penalty (1). Pleiotropy is difficult to distinguish from close linkage in populations where multiple loci affecting the traits are segregating (5). These problems can be circumvented by assessing epistatic and pleiotropic effects of single mutations in a common homozygous background. In *Drosophila melanogaster*, diallel crosses among all mutant alleles affecting the same trait have revealed extensive epistasis (7–10), whereas studies assessing the same mutations for multiple quantitative traits, including genome-wide variation in gene expression, have shown that pleiotropy is pervasive (10–13). However, pleiotropic effects of epistatic interactions have not been well-explored. Here, we assess the contribution of epistasis and pleiotropy to the genetic architecture of aggression in *Drosophila* in a diallel cross among P-element mutations associated with hyperaggressive behavior (13–15).

Aggression is important in securing food and mates, defending against predators, and establishing social hierarchies, whereas violent behaviors incur social and economic costs to human society. Variation in aggression has a significant genetic component (15–17). Evolutionary conserved genes affecting neurotransmitter signaling and metabolism affect aggressive behavior, including serotonin (18–24), monoamine oxidase A (25, 26),

dopamine (20), octopamine (20, 27), nitric oxide (28), and GABA (29). Increased aggression is associated with mutations in androgen and estrogen signaling in vertebrates (30) and sex determination in *Drosophila* (31, 32). Genes involved in brain development or synaptogenesis have been associated with aggression in mice (33) and *Drosophila* (13, 15, 20), whereas in humans, quantitative differences in size or structure of the amygdala and prefrontal cortex have been associated with aggression (34). Studies in *Drosophila* reveal the complex genetic architecture of aggressive behavior, with a large mutational target size, pleiotropic mutational effects, and evidence of epistatic interactions (13–16, 23, 35, 36). The genetic complexity of aggression, thus, shifts the focus from understanding individual loci to understanding how they interact in genetic networks and the effects of variants on networks of interacting transcripts. Here, we combine diallel cross analysis of P-element mutations associated with increased aggression with whole-genome transcriptional profiling to define the range of genome-wide epistatic effects at the transcriptome level and illustrate the relationship between transcriptional epistasis and pleiotropic effects on brain structure and aggression.

Results

Epistatic Networks for Aggressive Behavior. Previously, we identified 38 P-element mutations associated with increased aggression (13–15). We selected for analysis of epistatic and pleiotropic effects 10 autosomal mutations that had been generated in the same coisogenic *Canton S B (CSB)* background and in which the P-element insertions were within or close to an unambiguously identified gene. The genes encompassed many biological processes and molecular functions: a transcription factor, *muscle-blind (mbl)*; protein kinases *Darkener of apricot (Doa)* and *Btk29A*; a guanine exchange factor, *schizo (siz)*; an NMDA receptor subunit (*Nmdar1*); a UDP-glucose transferase, *sugarless (sgl)*; an extracellular matrix protein, *Laminin A (LanA)*; a cell adhesion molecule, *echinoid (ed)*; and two genes involved in regulating Notch signaling, the E3 ubiquitin ligase *neuralized (neur)* and the protein tyrosine phosphatase *Gp150*. We confirmed that all of the homozygous mutant alleles were more aggressive than the *CSB* control (Fig. 1A).

We constructed all 45 possible double heterozygous F1 genotypes among the 10 mutant lines and evaluated their aggressive behavior. We performed a diallel cross analysis (37) to test for nonadditive effects of the mutations on aggression. Because they were generated in a common isogenic background, the general

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combining ability (*GCA*) of each mutation represents its average degree of dominance combined with all other mutations, whereas the specific combining ability (*SCA*) of each genotype indicates whether there is epistasis (i.e., the aggressive phenotype of the double heterozygous genotype is enhanced or suppressed relative to the expected phenotype based on the additive combination of *GCA*s of the parental mutant alleles) (7–10). We found significant ($P < 0.0001$) variation in aggression among the double heterozygotes, which is attributable to variation in both *GCA* ($P < 0.0001$) and *SCA* ($P < 0.0001$) effects (Table S1). Four mutations were partially dominant (*sgl*, *mbl*, *Gp150*, and *Nmdar1*), three mutations were partially recessive (*Doa*, *ed*, and *siz*), and six mutations interacted epistatically (Fig. 1 *B* and *C* and Tables S2 and S3). We found enhancing epistasis (the double heterozygote is more aggressive than expected) between *neur* and *sgl*, *LanA* and *Nmdar1*, and *Gp150* and *Btk29A*, and we found suppressing epistasis (the double heterozygote is less aggressive than expected) between *Btk29A* and *Nmdar1*, *sgl* and *LanA*, and *LanA* and *Gp150* (Fig. 1 *B* and *C* and Tables S2 and S3). The *SCA* values for interactions between *Doa* and *ec* ($P = 0.08$) and *Doa* and *Nmdar1* ($P = 0.06$) approached formal statistical significance.

We focused on the six mutations that are hyperaggressive as homozygotes and have epistatic effects on aggression as double heterozygotes. We confirmed that the *P{GT1}* insertions cause the observed abnormalities in aggressive behavior by creating revertant alleles using crosses that preserved the coisogenic background of each line. Previously, we reported that aggressive behavior of an excision allele of *neur*^{*BG02391*} was not different from the behavior of the *CSB* control (13). Similarly, the aggressive behavior of homozygous excision alleles of *sgl*, *LanA*, *Nmdar1*, and *Gp150* reverted to the control level, whereas *Btk29A* revertants showed a slight decrease in aggression compared with *CSB* (Fig. S1).

Pleiotropic Effects on Brain Morphology. Mutants with aberrant aggressive behavior can have subtle pleiotropic effects on the morphology of the mushroom bodies and ellipsoid body (13, 15). We quantified the length and width of the α - and β -lobes of the mushroom bodies and the area of the ellipsoid body in the 6 hyperaggressive and epistatically interacting homozygous mutant lines, all 15 double heterozygotes from a diallel cross among these mutations, and the *CSB* control. The length of the α -lobes was shorter in *Gp150*, *Btk29A*, *LanA*, and *sgl* homozygous mutants and the width of the β -lobes was smaller in *Gp150* and *Btk29A* homozygous mutants compared with the *CSB* control (Fig. 2 *A–D*).

We found significant variation in α -lobe length and width, β -lobe width, and ellipsoid body area among the double heterozygotes, which is attributable to variation in both *GCA* and *SCA* effects (Fig. 2 *A–D* and Table S4). We observed significant *GCA* effects for *LanA* and *Btk29A* on α -lobe length, *neur* and *Nmdar1* on α -lobe width, *LanA* and *sgl* on β -lobe width, and *sgl* on ellipsoid body area (Fig. 2 *A–D* and Table S4). We also observed significant *SCA* effects on brain morphology not only between pairs of mutations, which both had significant homozygous effects, but also between pairs in which only one or neither had a significant homozygous effect. Thus, the variation among the double heterozygotes greatly exceeded the variation among the homozygous genotypes—a hallmark of epistasis. Although we observed epistatic interactions among the six mutations for the four aspects of brain morphology, the epistatic networks were largely distinct for each of the four traits (Fig. 2 and Tables S5 and S6) and from the network observed for aggression (Fig. 1 *B* and *C* and Tables S2 and S3).

We assessed whether variation in aggression among the 22 genotypes (*CSB*, 6 homozygous mutants, and 15 double heterozygotes) was associated with variation in brain morphology. We found a significant negative correlation ($r = -0.54$, $P = 0.008$) between the length of the mushroom body α -lobes and aggression (Fig. S2). The relationship between neuropil structure and behavior is not evident from observations on the homozygous mutations alone but is evident when epistatic interactions are taken into account. This finding suggests a role for the mushroom bodies in aggression and shows that minor alterations in brain morphology may contribute to abnormal behavior.

Pleiotropic Effects on Gene Expression. We used whole-genome expression profiling in heads of males from the 6 hyperaggressive mutant lines, 15 double heterozygotes, and *CSB* control to identify transcriptional correlates with aggression and brain morphology. We found 1,396 probe sets with differences in expression among the six homozygous mutations and *CSB* at a false discovery rate (FDR) (38) < 0.001 (including four genes tagged by the *P* elements: *neur*, *sgl*, *Nmdar1*, and *Gp150*) (Dataset S1). A total of 2,590 probe sets were significant at FDR < 0.01 , and 4,038 probe sets were significant at FDR < 0.05 . Expression levels of 1,169 probe sets were significantly (FDR < 0.001) different in all homozygous mutant lines relative to *CSB*, with 613 down- and 556 up-regulated transcripts (Dataset S2). Gene ontology (GO) analyses (39) revealed that the 613 down-regulated transcripts were enriched for genes involved in neural development and nervous system function (e.g., genes encoding the serotonin transporter, dopamine, muscarinic, GABA, and nicotinic acetyl-

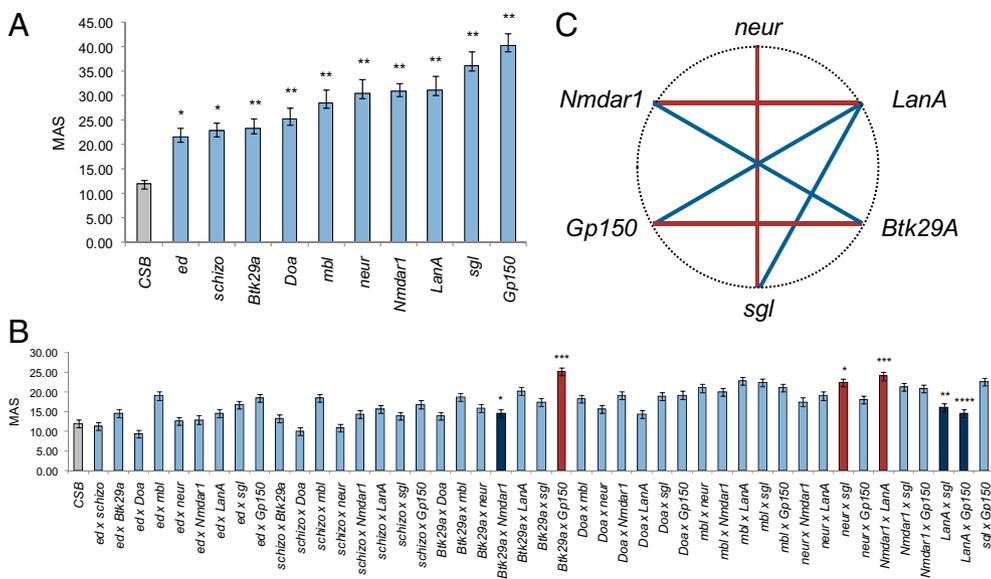


Fig. 1. Aggressive behavior of *P*-element insertions. (A) Mean aggression scores (MAS) for the control strain (*CSB*; gray bar) and 10 hyperaggressive homozygous mutant lines (light blue bars). (B) MAS for *CSB* (gray bar) and 45 double heterozygotes constructed by a half diallel cross of the 10 homozygous mutants. Light blue bars indicate double heterozygotes for which the estimate of *SCA* was not significant. Dark red and dark blue bars indicate, respectively, significant positive and negative *SCA* values. Error bars in A and B are SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (C) Pairwise epistatic interactions among six *P*-element mutations affecting aggression. The lines indicate the interacting mutations; dark red denotes positive *SCA* values, and dark blue denotes negative *SCA* values.

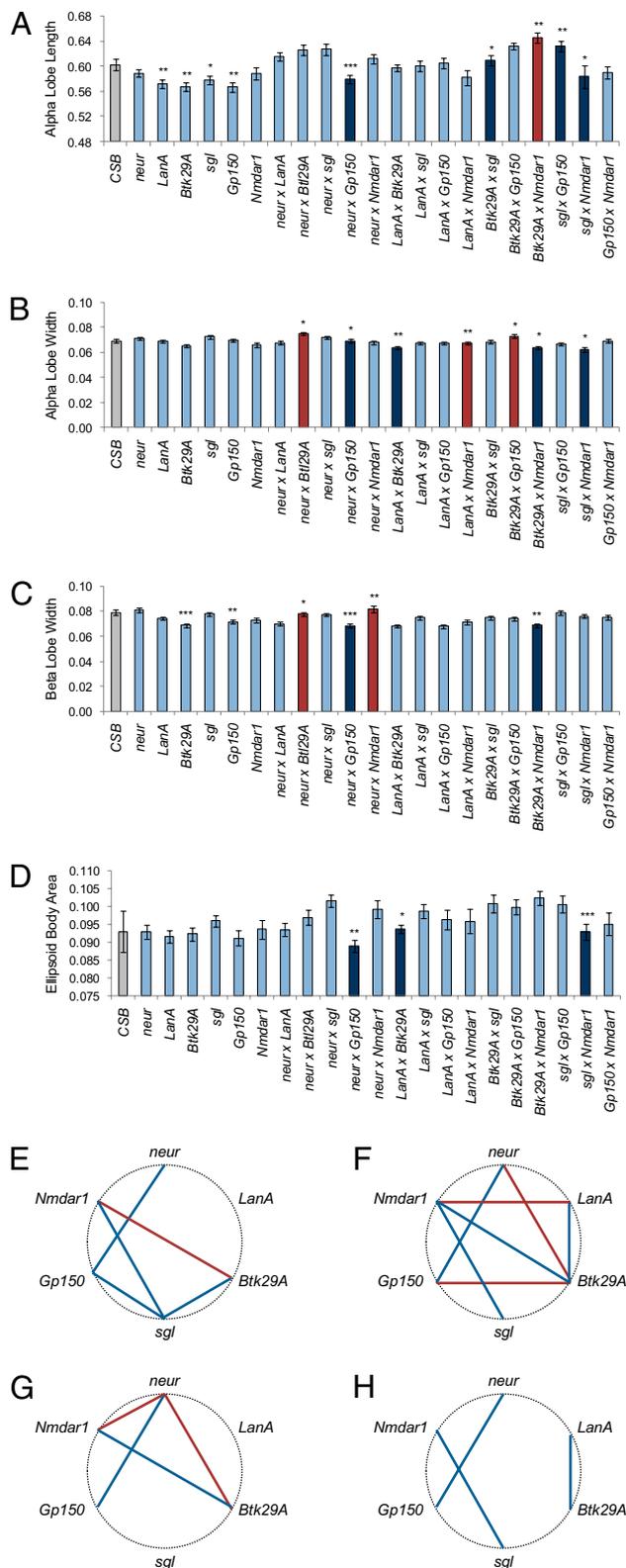


Fig. 2. Pleiotropic effects on brain morphology of *P*-element mutations affecting aggression. (A–D) Measurements of brain morphology for the control (CSB; gray bar), 6 hyperaggressive homozygous mutants (light blue bars), and 15 double heterozygotes constructed by a half diallel cross of the six mutant lines. Dark red and dark blue bars indicate, respectively, significant positive and negative *SCA* values. Error bars are SEM. Asterisks above the homozygotes indicate significant differences from the control. Asterisks above the double heterozygotes indicate significant *SCA* values. * $P < 0.05$;

choline receptors, and nitric oxide synthase). The 556 up-regulated transcripts were enriched for genes involved in transcription, translation, and metabolism. Both groups contained genes involved in known developmental pathways [e.g., the *wingless* (*wg*), *Notch*, and *decapentaplegic* signaling cascades] as well as other behaviors such as learning and memory, circadian rhythms, courtship, and processing of visual or olfactory input (Dataset S2).

Analysis of variation in gene expression among the 15 double heterozygotes revealed 1,443 significant transcripts at FDR < 0.001 (2,350 at FDR < 0.01 and 3,639 at FDR < 0.05). A total of 2,272 (42%) of the probe sets significant at FDR < 0.05 for the analyses of homozygotes and double heterozygotes overlapped; 1,766 were unique to the homozygous genotype analysis (as expected for recessive effects on transcription), whereas 1,367 were unique to the double heterozygote genotype analysis (as expected from transcriptional epistasis) (Datasets S1 and S3). We estimated *GCA* and *SCA* effects for the 3,639 transcripts for which the double heterozygotes were significant at FDR < 0.05. The *GCA* estimate was significant for 3,566 and the *SCA* estimate was significant for 830 ($P < 0.05$) of these transcripts, indicating substantial dominance and epistasis for gene expression (Dataset S3). The number of nonadditive interactions as well as the proportion of dominant and recessive effects and enhancing and suppressing epistatic interactions varied among genotypes (Fig. 3 A and B). The epistatic networks for gene expression traits were variable (Fig. 3 C–H). Genes participating in epistatic transcriptional networks were highly enriched for gene expression, mitosis, metabolism, and translation GO categories.

Single coisogenic *P*-element insertions have pleiotropic effects on expression of multiple transcripts (10–13). Our results show that genome-wide pleiotropic effects on the transcriptome are greatly amplified by double heterozygous combinations of *P*-element insertions. The extent to which these coregulated transcripts are functionally related to the manifestation of aggression and brain morphology requires an assessment of the correlation between gene expression and organismal phenotype and functional studies.

Correlation Between Gene Expression and Organismal Phenotypes.

The analyses of pleiotropic effects of homozygous and double heterozygous mutations on variation in genome-wide expression are complementary; epistasis will not be revealed by the former analyses, and recessive effects will not be revealed by the latter analyses. We assessed variation in gene expression for all genotypes and found 5,584 significant (FDR < 0.05) transcripts. To identify candidate genes for aggression and brain morphology, we assessed the correlations between variation in gene expression and phenotypic variation. We found 3,176 and 3,001 transcripts significantly ($P < 0.05$) associated with aggression and mushroom body α -lobe length, respectively, with 2,241 transcripts affecting both traits (Dataset S4). In contrast, only 112 and 86 transcripts were significantly correlated with α - and β -lobe width, respectively, and 330 transcripts were significantly correlated with ellipsoid body area (Dataset S4). Transcripts with significant associations with both aggression and α -lobe length were enriched for GO categories (39) related to nervous system development and function, protein synthesis, mitosis, and cellular signaling.

Functional Tests: Mutations. To evaluate to what extent analyses of transcriptional epistasis identify genes functionally associated with aggression, we sampled eight genes with significant differences in gene expression in analyses comprising all homozygous and double heterozygous mutations and for which variation in

** $P < 0.01$; *** $P < 0.001$. (A) α -Lobe length, (B) α -lobe width, (C) β -lobe width, and (D) ellipsoid body area. (E–H) Pairwise epistatic interactions for brain morphology. The lines indicate the interacting mutations; dark red denotes positive *SCA* values, and dark blue denotes negative *SCA* values. (E) α -Lobe length, (F) α -lobe width, (G) β -lobe width, and (H) ellipsoid body area.

tions affecting this trait. Mushroom bodies are required for aggression (20), and subtle alterations in mushroom body morphology have been observed in mutations affecting aggression (13, 15). Four of six mutations with epistatic effects on aggression had pleiotropic homozygous effects on α -lobe length, whereas two mutations had homozygous effects on β -lobe width. We observed substantial epistasis among all six mutations, however, for the length and width of the mushroom body α -lobes, β -lobe width, and ellipsoid body area. These epistatic networks differed from each other and from the network associated with aggression, which is consistent with previous observations of epistasis among mutations affecting startle response and brain neuroanatomy (9). Aggressive behavior for all 22 homozygous and double heterozygous genotypes was negatively correlated with the length of the mushroom body α -lobes, implicating these structures as important determinants for aggression.

Significant epistasis affecting brain morphology occurred not only between mutations with significant homozygous effects but also between mutations for which only one or neither had a significant effect as a homozygote. Epistatic interactions were, therefore, often of greater magnitude than homozygous effects. There are four important implications of this phenomenon. First, previous studies focusing only on mutations with homozygous effects to elucidate the biological underpinnings of quantitative traits revealed only a fraction of the underlying genetic architecture (i.e., the mutational target size is much larger than implicated in the studies that only examine homozygous mutations). Second, epistasis must be even more common than inferred from diallel cross analysis of mutations that all have homozygous effects on a trait (7–10). There are many more possible genotypes from n mutations than those mutations represented by the $n(n - 1)/2$ double heterozygotes, and the many

mutations without significant homozygous effects could still participate in epistatic interactions. Third, estimates of the effect of a single mutation are likely to vary depending on the genetic background (10, 12). Finally, we find substantial enhancing and suppressing epistasis with just six segregating loci and only pairwise epistasis among them; therefore, epistasis is also likely to occur for segregating variants affecting complex traits in natural populations, including humans. The many large genome-wide association studies in humans for disease susceptibility and quantitative traits have identified many alleles with individually small effects on the traits that together account for only a small fraction of the total phenotypic variance (43). Perhaps averaging the effects of individual SNPs across the plethora of pairwise and higher-order enhancing and suppressing epistatic interactions in which they participate causes the small effect sizes.

Several mutations with homozygous and epistatic effects on aggression had pleiotropic effects on other traits (8–10). Pleiotropy at the level of organismal phenotype was recapitulated at the level of gene expression: 4,038 transcripts were differentially expressed in heads of the homozygous mutant lines and the control. Of these transcripts, 1,169 transcripts were coordinately up- or down-regulated in all hyperaggressive homozygous mutations. Epistasis for organismal phenotypes was also mirrored by epistasis for gene expression traits. We observed epistatic interactions for over 800 transcripts, many between transcripts without significant homozygous effects and with great variation in topology among the transcriptional genetic networks.

Remarkably, homozygotes and double heterozygotes composed of only six mutations had a profound effect on the transcriptome, with significant changes in expression of 5,584 transcripts—nearly 30% of the transcripts on the array. These transcripts included 36 of 71 genes in which *P*-element mutations identified in the *CSB* background affect aggression (13–15), including 7 of 10 genes tested in this study (*mbl*, *Doa*, *neur*, *Nmdar1*, *Gp150*, *sgl*, and *ec*). The ~50% concordance between the genes implicated to affect aggression on the microarray and those genes previously identified genetically is high, considering that some of the *P*-element mutations may not exert their effect on aggression through transcription but through translation or posttranslational modification. Additionally, transcriptional changes at an earlier development time may alter structures that affect adult aggression, and transcriptional changes associated with these genes may be below our detection threshold (e.g., if they were restricted to a few cells). In addition, we observed variation in expression for all four genes implicated in variation in aggression between two WT strains (*CG11006*, *CG10754*, *mus312*, and *Rgl*) (35) as well as *Cyp6a20* (16), *npf* (23), *fru* (31, 32), *Or67d* (36), *e*, and *b* (20); mutations in all of which have been associated with aggressive behavior. Serotonin (23) and serotonin receptors (24) have also been implicated in *Drosophila* aggression; we find transcriptional variation for the serotonin receptors *5-HT1A*, *5-HT1B*, and *5-HT7* among the 22 genotypes.

Individual variation in response to pharmacological therapy is common in psychiatric patients (44). However, with a few notable exceptions (45), the underlying genetic variants affecting variation in response among individuals remain elusive (46). Our data indicate that *Drosophila* mutations with similar effects on hyperaggressive behavior indeed respond differentially to a GABA reuptake inhibitor, lithium, and valproic acid, which is used in the pharmacological treatment of anxiety disorders and bipolar disorder. Mutations in *sgl* and *Nmdar1* are less aggressive in response to all three treatments; the *Btk29A* mutation does not respond to any of the treatments, whereas the other mutations respond specifically to one (*LanA* or *Gp150*) or two (*neur*) compounds. Common evolutionarily conserved genetic mechanisms and neural signaling pathways may contribute to the manifestation of aggressive behavior across phyla ranging from flies to humans.

Methods

***Drosophila* Stocks.** We used lines with *P[GT1]*-element insertions in the *CSB* (8–10) isogenic genetic background and the *CSB* coisogenic control. We

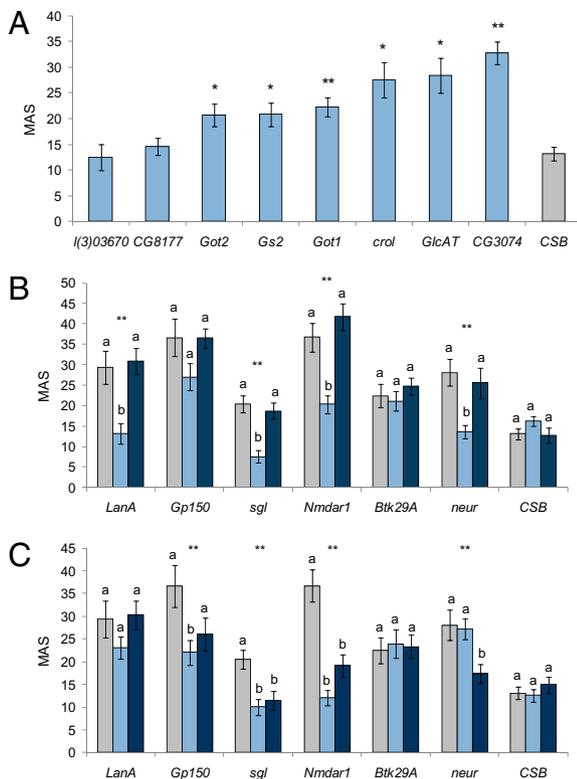


Fig. 4. Functional tests. Error bars are SEM. * $P < 0.05$; ** $P < 0.01$. (A) MAS for homozygous mutant alleles of candidate genes (light blue bars) and the control (*CSB*; gray bar). (B) MAS after treatment with DABA (light blue bars), DAPA (dark blue bars), and H₂O (gray bars). (C) MAS after treatment with lithium (light blue bars), valproic acid (dark blue bars), and H₂O (gray bars).

obtained additional *P*-element and *piggy-bac* insertion lines from the Bloomington *Drosophila* Stock Center and isogenized them by crossing to CSB males for 10 generations. We generated phenotypic revertants by mobilizing the *P* elements under conditions that preserve the same genetic background (10). We reared the flies on cornmeal/molasses/agar medium under standard culture conditions.

For pharmacological studies, *Drosophila* growth medium (Carolina Biological Supply) was supplemented with 2.5 mg/mL (13 mM) DABA or DAPA (Sigma Aldrich), 10 mM lithium chloride, or 1 mM valproic acid (Sigma Aldrich).

Phenotypic Analyses. We quantified aggression as previously described (13–15). We assessed one replicate for each tested genotype per day, with $n = 20$ replicates per genotype. All tests were performed between 8:00 and 11:00 AM.

We dissected brains from male flies, processed them for immunohistochemistry with a mouse monoclonal *antifasciclin 2* antibody, and measured the length and width of the α - and β -lobes of the mushroom bodies and height and width of the ellipsoid body of the central complex. We expressed the measurements relative to the distance between α -lobe heels (9, 13) ($n = 20$ brains per genotype).

We measured whole-genome expression using Affymetrix *Drosophila* GeneChip 2.0 arrays with three biological replicates per genotype. For each replicate, we food-deprived 3- to 7-d-old males from 8:00 to 9:30 AM, flash froze them at -80° , dissected heads, and extracted RNA from 50 heads. We prepared biotinylated cRNA probes, hybridized the RNA to microarrays, and visualized the hybridization intensities with a streptavidin–phycoerythrin conjugate.

Statistical and Bioinformatic Analyses. We used *t* tests to assess deviations of homozygous mutants from their controls for aggression and brain morphology and assess response to pharmacological treatments.

We used a half diallel crossing design (37) to measure aggression, brain morphology, and whole-genome expression among all possible non-reciprocal double heterozygotes. We computed the GCA and SCA for all genotypes (37). The GCA is the average dominance of each mutation combined with all other mutations, and significant SCA estimates indicate epistasis (7–10).

We used the weighted log(perfect match – mismatch) intensity of each probe set to quantify gene expression and scaled the expression scores to a median intensity of 500; 11,756 probe sets were expressed in adult male heads. We used one-way ANOVA models to evaluate significant variation among genotypes, FDR *q* statistics (38) to account for multiple tests, and posthoc Tukey tests to assess the contribution of each genotype. We tested for overrepresented GO categories among significant probe sets using DAVID (39). We used linear regression models to compute correlations between aggression and brain morphology and organismal phenotypes with gene expression.

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