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Epistatic partners of neurogenic genes modulate Drosophila olfactory behavior

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The extent to which epistasis affects the genetic architecture of complex traits is difficult to quantify, and identifying variants in natural populations with epistatic interactions is challenging. Previous studies in Drosophila implicated extensive epistasis between variants in genes that affect neural connectivity and contribute to natural variation in olfactory response to benzaldehyde. In this study, we implemented a powerful screen to quantify the extent of epistasis as well as identify candidate interacting variants using 203 inbred wild-derived lines with sequenced genomes of the *Drosophila melanogaster* Genetic Reference Panel (DGRP). We crossed the DGRP lines to *P[GT1]*-element insertion mutants in *Sema-5c* and *neuralized* (*neur*), two neurodevelopmental loci which affect olfactory behavior, and to their coisogenic wild-type control. We observed significant variation in olfactory responses to benzaldehyde among F_1 genotypes and for the DGRP line by mutant genotype interactions for both loci, showing extensive nonadditive genetic variation. We performed genome-wide association analyses to identify the candidate modifier loci. None of these polymorphisms were in or near the focal genes; therefore, epistasis is the cause of the nonadditive genetic variance. Candidate genes could be placed in interaction networks. Several candidate modifiers are associated with neural development. Analyses of mutants of candidate epistatic partners with *neur* (*merry-go-round* (*mgr*), *prospero* (*pros*), *CG10098*, *Alhambra* (*Alh*) and *CG12535*) and *Sema-5c* (*CG42540* and *bruchpilot* (*brp*)) showed aberrant olfactory responses compared with coisogenic controls. Thus, integrating genome-wide analyses of natural variants with mutations at defined genomic locations in a common coisogenic background can unmask specific epistatic modifiers of behavioral phenotypes.

Keywords: Chemosensation, *Drosophila melanogaster* Genetic Reference Panel, genetic architecture, genome-wide association study, quantitative traits

The potential for adaptive evolution depends on genetic variation within a population. How such variation is maintained remains an enigma (Charlesworth 2015; Félix & Barkoulas 2015; Mackay 2010). Waddington (1942) proposed that organisms harbor a reservoir of hidden (cryptic) genetic variation, which can be mobilized as a consequence of environmental changes or genetic perturbations (Gibson & Dworkin 2004; Waddington 1942). Furthermore, it has been proposed that such modifiers can buffer the genome against newly arising mutations through suppressing epistasis (Mackay 2014; Swarup *et al.* 2012; Yamamoto *et al.* 2009). Identifying epistasis is challenging when neither the genetic background nor environmental conditions can be controlled. In addition, the magnitude of the effects of epistasis on quantitative trait phenotypes depends on the allele frequencies of the interacting genes (Mackay 2014). Thus, changes in allele frequencies between interacting partners that give rise to nonlinear or even antagonistic phenotypic effects can account for irreproducible results in genome-wide association (GWA) studies in human populations, and suppressing epistasis can contribute to the 'missing heritability' of quantitative traits (Manolio *et al.* 2009).

Epistasis can be more readily detected in genetic model organisms in which crosses between mutations can be made and for which allele frequencies are balanced in quantitative trait locus (QTL) mapping populations (Mackay 2014). Indeed, significant epistatic interactions have been found in QTL-mapping studies on sporulation efficiency (Deutschbauer & Davis 2005; Gerke *et al.* 2009) and gene expression (Brem *et al.* 2005) in yeast; thermal preference in *Caenorhabditis elegans* (Gaertner *et al.* 2012); bristle number (Dilda & Mackay 2002), wing shape (Chandler *et al.* 2014; Gilchrist & Partridge 2001), longevity (Magwire *et al.* 2010), enzyme activity (Clark & Wang 1997), metabolic rate and flight velocity (Montooth *et al.* 2003) and locomotor behaviors (van Swinderen & Greenspan 2005; Yamamoto *et al.* 2008) in *Drosophila melanogaster*; body weight and adiposity traits (Cheverud *et al.* 2001; Jarvis & Cheverud 2011; Leamy *et al.* 2011; Stylianou *et al.* 2006), litter size (Peripato *et al.* 2004) and production of serum-like growth factor-1 (Hanlon *et al.* 2006) in mice; growth rate in chickens (Carlborg *et al.* 2006; Pettersson *et al.* 2011); growth rate (Kroymann & Mitchell-Olds 2005; Wentzel *et al.* 2007) and metabolites (Rowe *et al.* 2008) in *Arabidopsis thaliana*; and, differences in whole plant and inflorescence architecture between maize and teosinte (Doebley *et al.* 1995).

Furthermore, studies on phenotypic variation in *D. melanogaster* have inferred pervasive epistasis for recovery time from a chill-induced coma, and starvation stress resistance, as well as startle, olfactory and aggressive behavior (Huang *et al.* 2012; Shorter *et al.* 2015; Swarup *et al.* 2013). Despite evidence that epistasis is an important feature of the genetic architecture of complex traits, genome-wide identification of specific genes that engage in epistatic interactions with defined target genes remains a challenge.

Here, we describe an experimental design that enables us to identify epistatic modifiers of defined target alleles using the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.* 2012; Huang *et al.* 2014). The DGRP consists of 205 inbred wild-derived *D. melanogaster* lines with fully sequenced genomes, in which all 4853802 single nucleotide polymorphisms (SNPs) and 1296080 non-SNP variants (insertions, deletions and copy number variants) as well as 16 polymorphic inversions have been identified (Huang *et al.* 2014). We previously inferred that epistasis is an important component of the genetic architecture of olfactory behavior, both for natural variation in the DGRP (Swarup *et al.* 2013) as well as for new mutations (Fedorowicz *et al.* 1998; Sambandan *et al.* 2006). Two of these mutants with large effects on olfactory behavior had *P[GT1]*-insertions at the neurogenic *Sema-5c* and *neuralized* (*neur*) loci and were characterized in greater detail (Rollmann *et al.* 2007,2008).

Here, we asked whether and to what extent the insertional mutations at *neur* and *Sema-5c* genetically interact with variants affecting olfactory behavior in the DGRP by performing quantitative complementation tests (Long *et al.* 1996; Mackay 2014). We crossed 203 DGRP lines to the *neur* and *Sema-5c* mutant alleles as well as to Canton S(B), the coisogenic line in which they were induced (Norga *et al.* 2003). We evaluated the extent to which the mutant alleles had the same effect in the DGRP lines, and found that nonadditive genetic variance accounted for over 70% of the total genetic variance for each mutation. We then performed GWA analysis and showed that none of the top interacting variants were in or near the focal genes; therefore, the nonadditive genetic variance is attributable to epistasis and not dominance. We identified polymorphisms in 24 and 31 candidate genes that contribute to variation in epistatic interactions with *Sema-5*c and *neur*, respectively. We show that these genes can be used to construct interaction networks in which additional putative epistatic partners can be computationally recruited. We also show that several candidate epistatic partners of the target genes also affect the behavioral phenotype. Thus, generating heterozygotes between defined transposon insertion mutants and natural variants in controlled genetic backgrounds is an effective strategy for genome-wide identification of candidate epistatic modifiers.

Material and methods

Fly stocks

All flies were reared on cornmeal–molasses–agar medium at 25°C, 60–75% relative humidity and a 12-h light/dark cycle. Males of 203 DGRP lines (Huang *et al.* 2014) were crossed to females of homozygous *Sema-5c*^{BG2386} and *neur*^{BG2391} *P[GT1]*-element insertion lines in the Canton S(B) coisogenic background (Bellen *et al.* 2004; Lukacsovich *et al.* 2001) as well as to Canton S(B) coisogenic females. To analyze effects of candidate epistatic modifier genes on olfactory behavior, we obtained homozygous mutant lines of nine genes from the Exelixis collection (Thibault *et al.* 2004) and the corresponding coisogenic control line. We obtained a *UAS-RNAi* line for *bruchpilot* (*brp*) along with the isogenic progenitor w^{1118} control from the Vienna Drosophila Resource Center and crossed these to an *ubiquitin-GAL4* driver line (w^{1118}; *Ubi-Gal4*) backcrossed in the Canton S(B) background.

Behavioral assay

Benzaldehyde was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Olfactory behavior to 1% (v/v) benzaldehyde was measured between 1300 and 1600 h for five replicate groups of fifty 4- to 7-day-old flies, males and females separately, for the homozygous *Sema-5c*^{BG2386} and *neur*^{BG2391} *P[GT1]*-element insertion lines and Canton S(B) coisogenic control, and for three replicate sets of fifty 4- to 7-day-old females per replicate of the F_1 progenies of DGRP lines crossed with *Sema-5c*^{BG2386}, *neur*^{BG2391} and Canton S(B), using a high throughput modification of the classical dipstick assay (Anholt *et al.* 1996), exactly as described previously (Swarup *et al.* 2011). In this assay, response indices are computed after flies have been allowed to distribute between two interconnected tubes, either near or away from the odorant source, where the response index (RI) = number of flies in the collection tube/total number of flies. RI = 1 indicates a maximal aversive response to the odorant, RI = 0.5 indicates indifference to the odorant, and RI = 0 indicates that all flies remain near the odor source. Replicates were run on different days to randomize environmental variation. For functional validation of candidate epistatic modifier genes, we quantified olfactory responses for at least five replicates of females from Exelixis mutants and 10 replicates of *ubiquitin-GAL4 x UAS-brpRNAi* females.

Statistical analyses and genome-wide associations

To analyze variation in olfactory behavior among the DGRP lines, we ran ANOVA models of form: $Y = \mu + L + G + L \times G + E$, where L (random) denotes DGRP lines; G (fixed) denotes either the *Sema-5c* and Canton S(B) genotypes or the *neur* and Canton S(B) genotypes; $L \times G$ (random) is the line by genotype interaction term (i.e. denotes nonadditive effects) and E is the within-line variance. To evaluate the effects of mutations or RNAi knockdown of candidate genes on olfactory behavior, we performed Dunnett's tests compared with the appropriate controls.

We performed GWA analyses using DGRP freeze 2.0 sequences using the pipeline from the DGRP website (http://dgrp2.gnets.ncsu.edu/). Briefly, this analysis evaluates the strength of association of alternative DGRP alleles with quantitative trait phenotypes for each segregating variant, after accounting for any effects of *Wolbachia* infection, karyotype of common polymorphic inversions and polygenic relatedness. We performed GWA analyses separately for DGRP/*Sema-5c*, DGRP/*neur* and the difference between DGRP/Canton S(B) and DGRP/*Sema-5c* and DGRP/Canton S(B) and DGRP/*neur* F_1 females. The latter two analyses specifically test for variants with nonadditive effects because the variation in the difference between the control and mutant phenotypes is equivalent to the genotype by line interaction term. All analyses were performed using genotype means. Gene interaction networks were constructed using GeneMANIA (Warde-Farley *et al.* 2010).

Results

Nonadditive variance for olfactory behavior

Previous studies identified *P*-element insertion mutants that affect olfactory avoidance behavior in response to benzaldehyde (Anholt *et al.* 1996; Sambandan *et al.* 2006) and showed extensive epistatic interactions among them (Fedorowicz

et al. 1998; Sambandan *et al.* 2006). Two of these *P*-element mutants at the *Sema-5c* and *neur* loci had large effects on olfactory behavior. The *Sema-5c* mutant also affected startle response (Rollmann *et al.* 2007; Yamamoto *et al.* 2008) and the *neur* mutant had pleiotropic effects on startle behavior and aggression (Rollmann *et al.* 2008; Yamamoto *et al.* 2008). Both mutations showed structural alterations in the mushroom bodies of the brain (Rollmann *et al.* 2007; Rollmann *et al.* 2008).

We first confirmed the effects of the homozygous *Sema-5c*^{BG2386} and *neur*^{BG2391} *P[GT1]*-element insertion lines on olfactory behavior compared with the coisogenic parental Canton S(B) strain. The Canton S(B) control showed strong avoidance behavior to 1% (v/v) benzaldehyde, as expected (Sambandan *et al.* 2006), while behavioral responses were greatly reduced in the *Sema-5c*^{BG2386} and *neur*^{BG2391} mutants, confirming the impairment of the behavioral phenotype described previously. We estimated the homozygous (*a*) and heterozygous (*d*) effects of the mutations in the Canton S(B) genetic background, where *a* is one half of the difference between the mean olfactory behavior of the homozygous Canton S(B) and mutant genotypes and *d* is the difference between the mean olfactory behavior of the heterozygotes and the average of the two homozygotes (Falconer & Mackay 1996). The effect of the *Sema-5c* mutation is additive in females ($a = 0.1336$; $d = 0.0208$) and nearly recessive in males ($a = 0.1108$; $d = 0.0888$) and the effect of the *neur* mutation is semi-dominant in both females ($a = 0.1176$; $d = -0.0815$) and males ($a = 0.0827$; $d = -0.0675$) (Fig. 1a).

To identify naturally occurring modifiers of the *Sema-5c*^{BG2386} and *neur*^{BG2391} alleles with nonadditive effects, we crossed each mutant and the control to each of the DGRP lines and tested the F_1 offspring for olfactory behavior (Fig. 1b). To simplify the experimental design and facilitate identification of modifiers on the *X* chromosome, we restricted our screen to F_1 females. We observed significant phenotypic variation across the DGRP lines for the Canton S(B), *Sema-5c*^{BG2386} and *neur*^{BG2391} crosses (Fig. 1c,d; Tables 1 and S1, Supporting Information) with average response index scores generally indicative of avoidance behavior (RI > 0.5).

We performed analyses of variance to partition the variation in olfactory behavior into sources attributable to the main effect of Genotype (Canton S(B) and the mutation), DGRP Line and the Genotype by Line interaction term (Table 1). We observed significant effects of Genotype in both analyses, but this term only explained 4.13% and 1.21% of the total sums of squares for *neur* and *Sema-5c*, respectively. We observed significant genetic variation in olfactory behavior in both comparisons, with the total genetic variance accounting for 20.9% and 30.4% of the phenotypic variance for *neur* and *Sema-5c*, respectively. With additive gene action, we expect that the differences in phenotypic values between the Canton S(B) × DGRP crosses and the mutant × DGRP crosses would be constant; i.e. there would be no variation in the difference between the DGRP/Canton S(B) and DGRP/mutation genotypes, as assessed by the significance of the Genotype by Line interaction terms. However, this is not what we observed. There is extensive variation in the difference between these phenotypic values, which is evidence of nonadditivity (Fig. 1c,d; Tables 1 and S1). Remarkably, 72.4% and 73.5% of the total genetic variance in olfactory behavior is attributable to naturally occurring variation with nonadditive effects on *neur* and *Sema-5c*, respectively (Table 1).

GWA analysis for nonadditive modifiers

The nonadditive genetic variance in olfactory behavior in the two mutant backgrounds could be due to alleles in *neur* or *Sema-5c* causing variation in the degree of dominance at these loci (allelic failure to complement) or by unlinked modifiers of the focal loci (epistatic failure to complement). As the DGRP lines are sequenced, we can distinguish between these two possibilities by performing GWA analyses. The limited number of DGRP lines and large number of polymorphic markers (~2.5 million) prevents GWA from reaching significance at a strict Bonferroni-corrected threshold unless effects are large. Therefore, we used a nominal significance threshold of $P < 10^{-5}$ to report associations, based on the appearance of deviations from the linear expectation of quantile–quantile plots in this *P*-value range (Fig. S1). When we performed GWA analyses across the DGRP lines crossed to *Sema-5c*^{BG2386} and *neur*^{BG2391}, we identified 53 and 80 candidate genes, respectively, that harbor polymorphisms associated with variation in olfactory response to benzaldehyde (Tables S2 and S3). These candidate genes were different for *Sema-5c*^{BG2386} and *neur*^{BG2391}, with only *dunce* (*dnc*) in common between the two analyses.

Next, we correlated phenotypic differences between the control and mutant DGRP crosses with genome-wide DNA sequence variation to identify candidate genes representing nonadditive modifiers of the *Sema-5c*^{BG2386} and *neur*^{BG2391} alleles (Fig. 2). Associations observed across mutant sensitized DGRP backgrounds are confounded by main effects and possible epistatic interactions, whereas analyses of the differences between the phenotypic effects of the DGRP × mutant hybrids and their DGRP × Canton S (B) reflect only epistasis. We identified 39 polymorphisms, including 36 biallelic SNPs and three indels, in 24 genes that modify the effects of the *Sema-5c*^{BG2386} allele at $P < 10^{-5}$ (Fig. 2a; Table S4). These genes include *brp*, involved with maintenance of the presynaptic active zone for neurotransmitter release (Wagh *et al.* 2006). We also identified SNPs in chemosensory receptors, notably an intronic SNP in *Gr39a* and a synonymous SNP in *Ir56c*; a nonsynonymous SNP in *Tetraspanin 39D* and several predicted genes of unknown function. We identified 78 polymorphisms, including 66 biallelic SNPs, 3 multiple nucleotide polymorphisms (MNPs), and 9 indels in 31 genes that modify the effects of the *neur*^{BG2391} allele (Fig. 2b; Table S5). The associated genes include *dunce*, which encodes a cyclic AMP phosphodiesterase (Byers *et al.* 1981); *Dop1R2*, which encodes a dopamine receptor; *rickets* (*rk*), which encodes a neuropeptide receptor for the molting hormone bursicon (Loveall & Deitcher 2010), two long noncoding RNAs, two microRNAs (*mir-968* and *mir-1002*), and several predicted genes of unknown function.

Seven candidate genes with polymorphisms that were associated with phenotypic variation in olfactory behavior

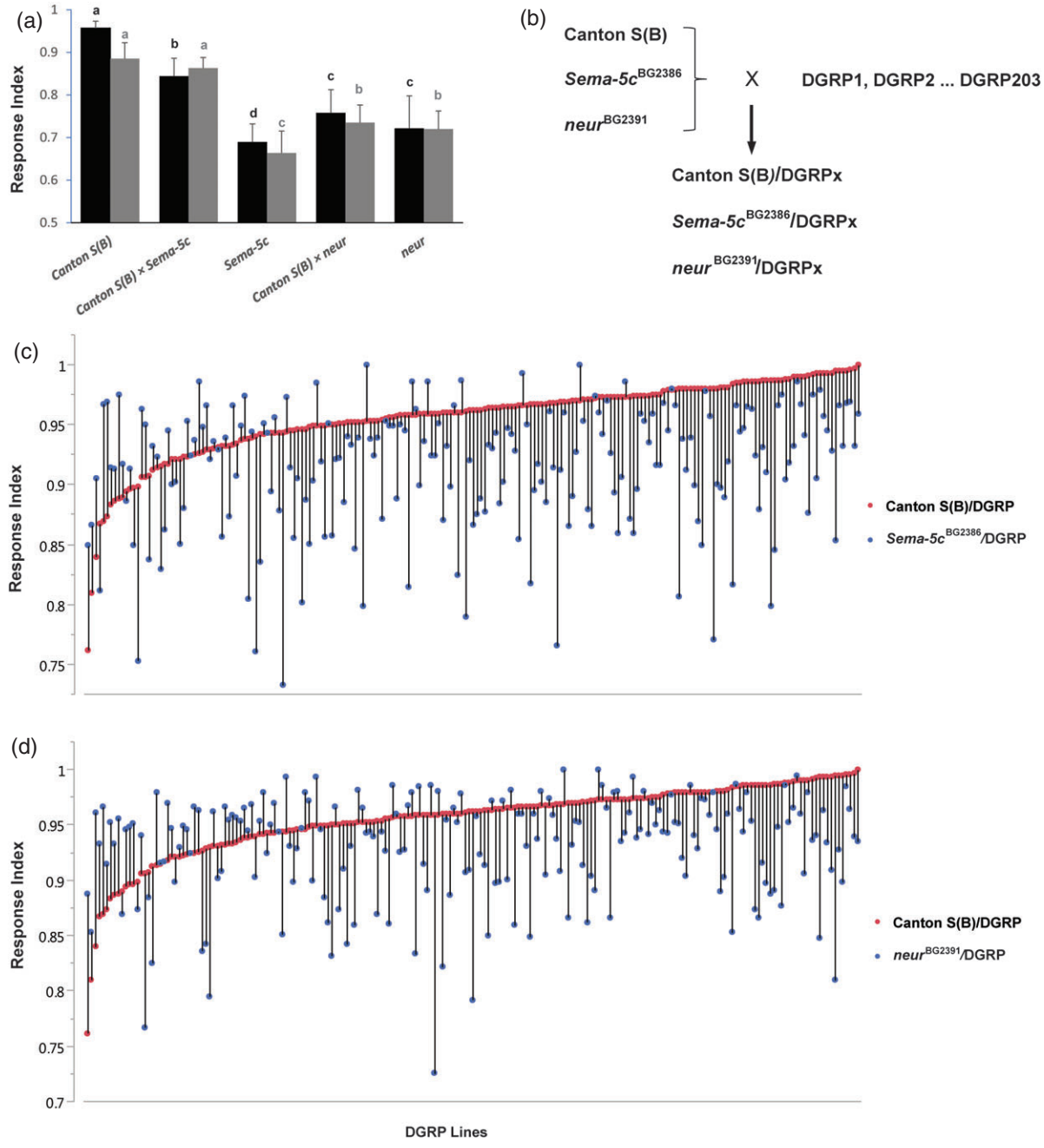

Figure 1: Genome-wide screen for nonadditive modifiers of the *Sema-5C* and *neur* loci in the DGRP. (a) Behavioral responses to benzaldehyde of female (black bars) and male (gray bars) control Canton S(B) flies and coisogenic *Sema-5c* and *neur* mutants. Error bars represent standard errors of the mean. Statistically significant differences ($P < 0.05$) for each sex are determined by Tukey's test and indicated by letters above the bars, where different letters denote significantly different line means. (b) Schematic representation of the crossing scheme for genome-wide identification of candidate nonadditive modifiers. (c) Response indices for behavioral responses to 1% (v/v) benzaldehyde of F1 hybrids from 203 DGRP lines crossed to Canton S(B), indicated by red symbols, and F_1 hybrids from 203 DGRP lines crossed to the *Sema-5c*^{BG2386} mutant, indicated by the blue symbols. Differences between the control and mutant F_1s are indicated by black lines that connect the corresponding red and blue symbols. (d) Response indices for behavioral responses to 1% (v/v) benzaldehyde of F1 hybrids from 203 DGRP lines crossed to Canton S (B), indicated by red symbols, and F_1 hybrids from 203 DGRP lines crossed to the *neur*^{BG2391} mutant, indicated by the blue symbols. Differences between the control and mutant F1s are indicated by black lines that connect the corresponding red and blue symbols.

Table 1: Analyses of variance of DGRP lines crossed to Canton S(B) and coisogenic *P*-element insertional mutations (*neur*^{BG2391} and *Sema-5C*^{BG2389})

F_1 genotype	Source	df	SS × 10^3	MS × 10^3	*F*	*P*-Value	σ^2 × 10^4	SE × 10^4
DGRP/Canton S(B)	Line	202	807.192	3.996	2.06	< 0.0001	5.827	1.149
	Residual	524	1014.988	1.937			19.234	1.179
	Total	726	1822.18				25.061	
DGRP/*neur*^{BG2391}	Line	202	1568.732	7.766	1.9	< 0.0001	10.196	2.35
	Residual	525	2140.425	4.077			41.098	2.55
	Total	727	3709.157				51.294	
DGRP/ *Sema-5C*^{BG2389}	Line	202	1954.522	9.676	2.81	< 0.0001	17.19	2.84
	Residual	512	1760.256	3.438			34.405	2.15
	Total	714	3714.778				51.595	
DGRP/Canton S(B) & DGRP/*neur*^{BG2391}	Genotype	1	237.965	237.965	47.35	< 0.0001	Fixed	Fixed
	Line	203	1344.442	6.623	1.30	0.0305	2.205	1.188
	Genotype × Line	201	1022.676	5.088	1.69	< 0.0001	5.791	1.512
	Residual	1049	3155.591	3.008			30.185	1.321
	Total	1454	5760.674				38.181	
DGRP/Canton S(B) & DGRP/ *Sema-5C*^{BG2389}	Genotype	1	91.405	91.405	12.98	0.0004	Fixed	Fixed
	Line	203	2116.264	10.425	1.46	0.0035	3.097	1.396
	Genotype × Line	201	1431.433	7.122	1.89	< 0.0001	8.614	1.681
	Residual	1037	3900.884	3.762			26.789	1.176
	Total	1442	7539.986				38.500	

df, degrees of freedom; MS, type III mean squares; *F*, *F* statistic; σ^2, variance component; SE, standard error of variance component.

among the DGRP × *Sema-5c* hybrids were also significantly associated when we performed the GWA on the difference between the DGRP × Canton S (B) control and DGRP × *Sema-5c*, namely *numb*, *Aats-val*, *Kdm4B*,*Toll-6*, *CG42540*, *CG42684 and RhoGAP19D*. In addition, 21 candidate genes with polymorphisms that were associated with phenotypic variation in olfactory behavior among the DGRP × *neur* hybrids were also significantly associated when we performed the GWA on the difference between the DGRP × Canton S (B) control and DGRP × *neur*, including *RluA-2*, *CR44216*, *CG10019*, *CG42669*, *CG13800*, *mod(mdg4)*, *CG6074*, *DNApol-alpha73*, *CG17991*, *DopR2*, *Ref1*, *Alh*, *CG10098*, *CR42738*, *CG14610*, *CG2656*, *pros*, *mgr*, *mRpL40*, *Sirt4* and *dnc*.

Notably, none of the top ($P < 10^{-5}$) variants associated with nonadditive modifiers of *Sema-5c* and *neur* were located in or near these genes. Therefore, it appears that the nonadditive variance we observe is primarily because of epistatic modifiers; variants contributing to any nonadditive variance from variation in the degree of dominance cannot be detected at the reporting significance threshold.

Genetic networks of modifier loci

We used the GeneMANIA program (www.genemania.org; Warde-Farley *et al.* 2010) to explore to what extent candidate epistatic modifier genes for the *Sema-5c*^{BG2386} and *neur*^{BG2391} alleles identified by our GWA analyses form interactive networks, using as input all candidate modifier genes with nominally significant associations at $P < 10^{-5}$ (Table S4 and S5). This program visualizes physical interactions among gene products, genetic interactions, and coexpression among candidate genes. These analyses show that candidate genes that epistatically modify the effects of *Sema-5c*^{BG2386} (Fig. 3) and *neur*^{BG2391} (Fig. 4) in our GWA analyses participate in known genetic and physical interaction networks as well as gene coexpression networks with the focal genes and each other. These networks also recruit additional genes that interact physically or genetically or are coexpressed with these candidate genes, or *Sema-5c* or *neur*. The interaction network for *Sema-5c* contains 91 coregulated pairs, 23 pairs of physically interacting gene products and 13 known genetic interactions (Fig. 3), whereas the interaction network for *neur* contains 59 coregulated pairs, 19 pairs of physically interacting gene products and 10 known genetic interactions (Fig. 4).

To further explore the functional significance of candidate epistatic partners, we asked whether mutations in candidate genes that were identified in our GWA analyses, showed coregulated expression with the target gene and were homozygous viable also altered olfactory behavior. We tested olfactory responses of females of coisogenic Exelixis mutants and their control at 0.3% (v/v) benzaldehyde, which was a maximally discriminating concentration for this genetic background. The selected genes, which were connected in the GeneMANIA networks and have coregulated expression with *Sema-5c*, included *CG42684*, *CG34408*, *CG42540* and *brp* (Fig. 5a) and selected genes for behavioral testing for *neur* included *mgr*, *pros*, *CG10098*, *Alh*, *CG12535* and *dnc* (Fig. 5b). *pros* and *mgr* are located in close proximity and opposite orientation and the associated SNP (*3R*_7233525) is located in the upstream regions of both genes. The polymorphic marker at *3R*_2931095 is a deletion in *Alh* associated with variation in olfactory behavior in DGRP × *neur*^{BG2391} F_1 females. A second associated SNP in *Alh* is upstream of this gene and of *CG10098*.

A mutant of *CG42540*, a candidate epistatic modifier of *Sema-5c*, showed aberrant olfactory behavior compared with

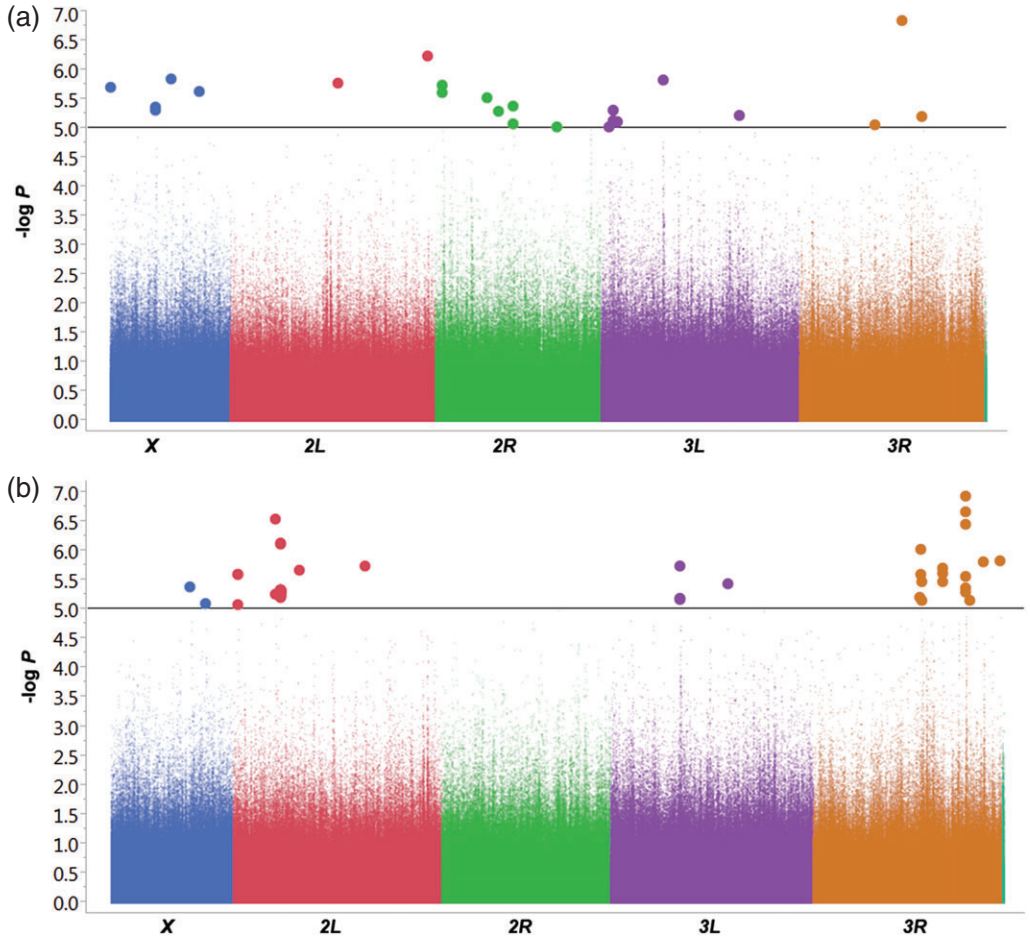

Figure 2: Manhattan plots for genome-wide associations for differences in olfactory behavior for F1 hybrids from 203 DGRP lines crossed to Canton S(B) and *Sema-5c*^{BG2386} (a) or Canton S(B) and the *neur*^{BG2391} mutant (b). The *X*-axes represent the color-coded major chromosome arms of the Drosophila genome and the *Y*-axes the probability for association. The horizontal line marks the $P < 10^{-5}$ nominal reporting threshold.

the coisogenic control (Fig. 5a; Table 2). The presynaptic gene product encoded by *brp* was of special interest, but the *brp* Exelixis mutant was not homozygous viable. However, knockdown of *brp* expression with RNAi resulted in a statistically significant effect on olfactory behavior compared with the control (Fig. 5a; Table 2). Mutants of five of the six tested genes (*mgr*, *pros*, *CG10098*, *Alh*, *dnc* and *CG12535*) implicated as modifiers of *neur* showed aberrant olfactory behavior compared with the control (Fig. 5b; Table 2). Thus, a total of eight out of the ten candidate epistatic partner genes tested were themselves functionally implicated in modulating the behavioral phenotype.

Discussion

There is growing evidence that epistatic interactions contribute to the genetic architecture of complex traits (Carlborg *et al.* 2006; Chandler *et al.* 2014; Chari & Dworkin 2013; Clark & Wang 1997; Doebley *et al.* 1995; Gaertner *et al.* 2012; Gibson & Dworkin 2004; Hanlon *et al.* 2006; Huang *et al.* 2012; Jarvis & Cheverud 2011; Kroymann & Mitchell-Olds 2005; Leamy *et al.* 2011; Mackay 2014; Ober *et al.* 2015; Pettersson *et al.* 2011; Rowe *et al.* 2008; Swarup *et al.* 2013). Pervasive epistasis adds an additional layer of complexity to the genotype-phenotype relationship. Thus, epistatic interactions can confound interpretations of causality in human GWA studies, and suppressing epistasis may be a contributing factor to the 'missing heritability' conundrum (Manolio *et al.* 2009; Zuk *et al.* 2012).

Epistatic modifiers are challenging to detect in human populations because of 'statistical noise' contributed by heterogeneous genetic backgrounds and uncontrolled environmental effects. Here, we document an experimental design in *D. melanogaster*, where statistical noise can be minimized through strict control of genetic background and growth conditions as well as replicate measurements on large numbers of individuals of the same genotype, to detect individual

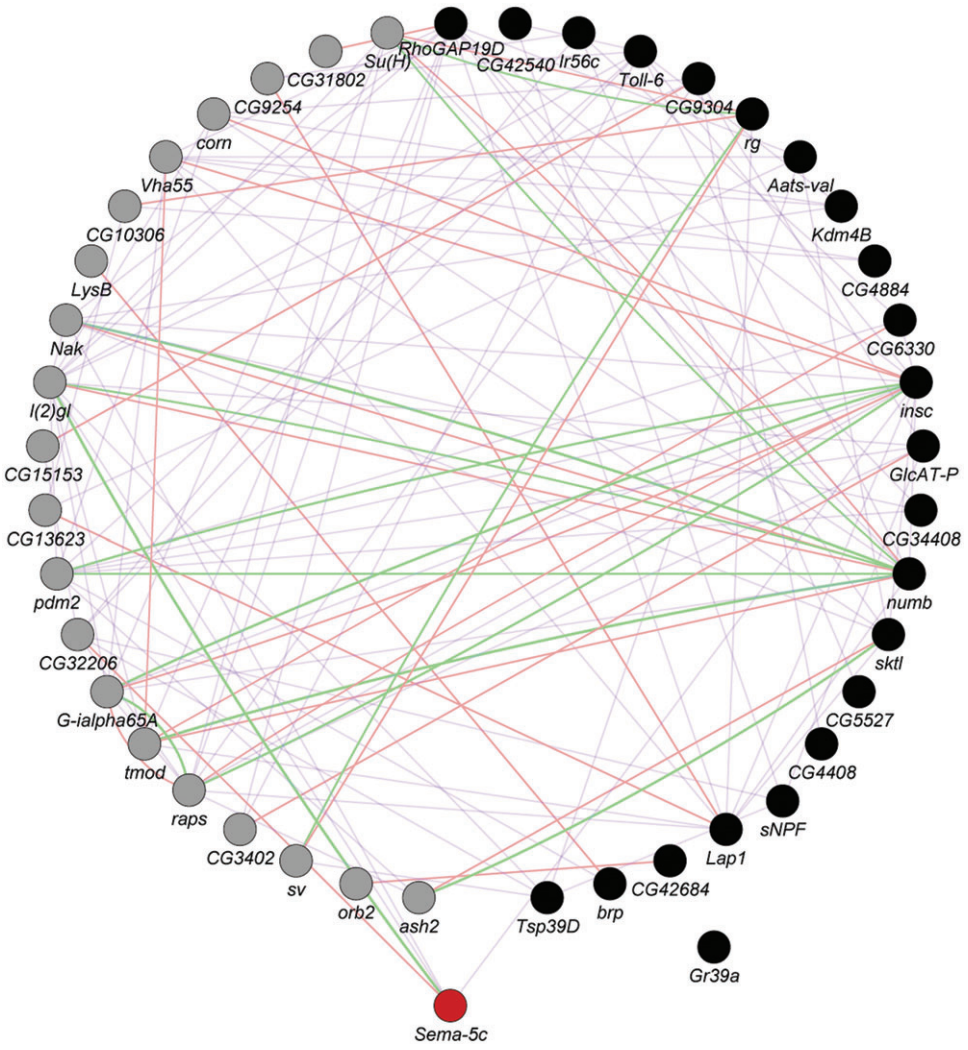

Figure 3: Genetic interaction network for *Sema-5c* derived from GWA analyses of the differences between Canton S(B) and DGRP crosses and corresponding *Sema-5c*^{BG2386} and DGRP crosses. The focal gene is indicated on a red background. Candidate epistatic partners identified by our GWA analysis are indicated on a black background. Computationally recruited interacting partners are indicated on a gray background. Purple edges represent coexpressed pairs, red edges physically interacting pairs, and green edges known genetic interactions.

modifiers of defined neurogenic loci that affect a behavioral fitness phenotype, the response to odorants. However, we are aware that epistasis between naturally segregating variants and a large effect mutant locus may present a different scenario from epistasis between naturally segregating variants.

We were able to identify specific candidate epistatic modifiers on the phenotypic effects of defined target loci by combining analyses of natural variants in fully sequenced inbred lines with coisogenic transposon mutations at defined loci. This approach can, in principle, be expanded and applied to a broad range of phenotypes to document the contributions of nonadditive effects on the genetic architecture of complex traits, not only at a statistical, but also at a molecular level.

Gene networks can be constructed based on physical interactions, genetic interactions or coexpression. Regarding the *neur* and *Sema-5c* loci we identified genetic interactions between genes that belong to coregulated expression modules and showed that, although we did not functionally confirm epistasis directly, 80% of candidate epistatic modifiers of *neur* and *Sema-5c* tested themselves affect olfactory behavior. This is similar to the validation rate of candidate genes identified by previous GWA studies on the DGRP (Arya *et al.* 2015; Dembeck *et al.* 2015; Harbison *et al.* 2013; Jordan *et al.* 2012; Swarup *et al.* 2013; Weber *et al.* 2012). Furthermore, previous transposon insertion based screens for olfactory behavior have shown that only 4–6% of transposon insertion lines affect olfactory behavior (Anholt *et al.* 1996; Sambandan

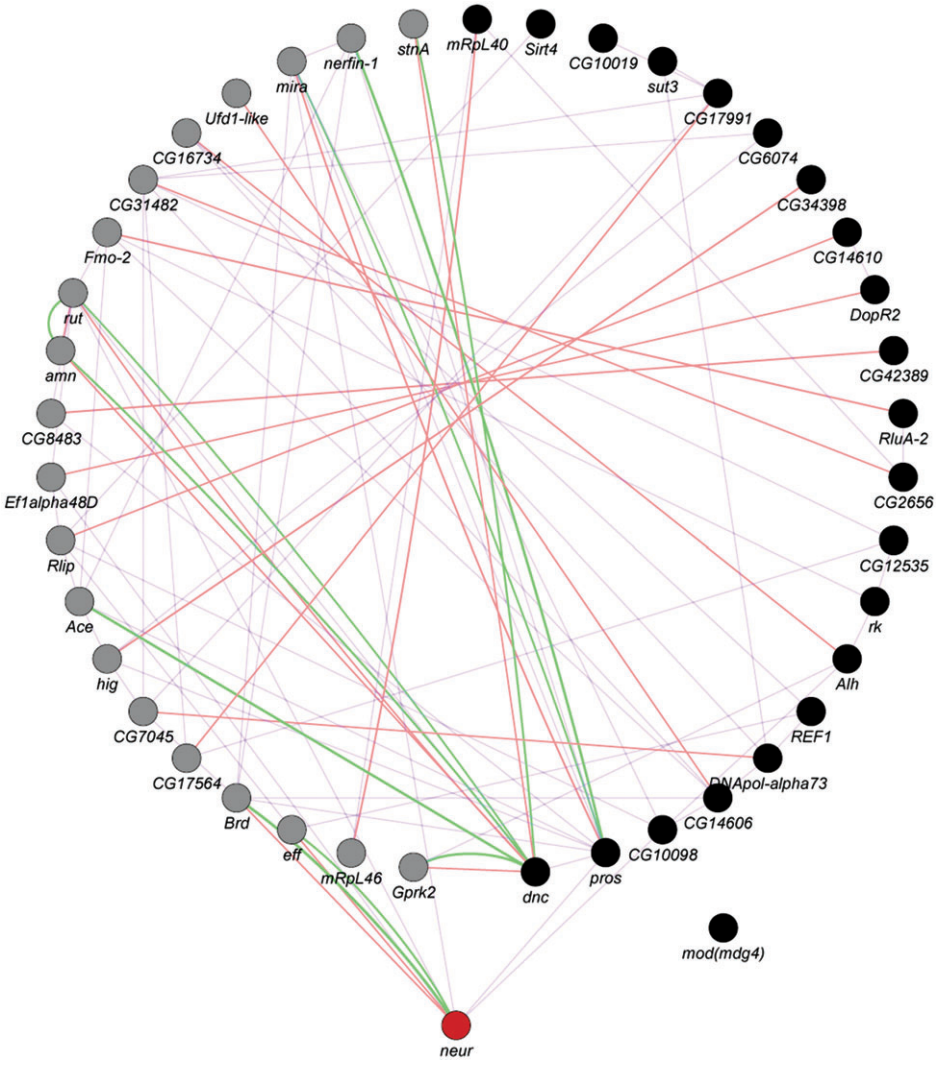

Figure 4: Genetic interaction network for *neur* derived from GWA analyses of the differences between Canton S(B) and DGRP crosses and corresponding *neur*^{BG2391} and DGRP crosses. The focal gene is indicated on a red background. Candidate epistatic partners identified by our GWA analysis are indicated on a black background. Computationally recruited interacting partners are indicated on a gray background. Purple edges represent coexpressed pairs, red edges physically interacting pairs and green edges known genetic interactions.

et al. 2006) compared with an 80% success rate in our experiment. One caveat, however, is that the Exelixis transposon mutants used to functionally test *pros* and *mgr* might affect the expression of both genes, limiting unambiguous assignment of the causal epistatic partner(s) for *neur*. This is also the case for the mutants corresponding to *Alh* and *CG10098*.

We note that the number of genes tested represents only a subset of all putative epistatic modifier loci identified in our genome-wide screens. The gene action of *neur* or *Sema-5c* on the phenotype is likely to represent the sum of multiple nonlinear gene–gene interactions, including both antagonistic suppressor and enhancer effects (Fig. S2). In a previous study (Sambandan *et al.* 2006), we did not observe direct epistatic interactions between *Sema-5c* and *neur* and there is no overlap between their interacting partners (Table S4 and S5). However, we cannot exclude higher order epistatic interactions between the two networks that involve computationally recruited genes. In addition, we note that the majority of epistatic effects are such that the minor allele in the mutant × DGRP F1 background is associated with a decrease in olfactory behavior; i.e. it is the major allele that suppresses (canalizes) the effect of the mutation. The interconnectivity of the transcriptome and the prevalence of epistasis support the hypothesis that networks of epistatic interactions may provide a protective buffering capacity that confers homeostasis to the transcriptome in the face of genetic perturbations or environmental fluctuations (Swarup *et al.* 2012;

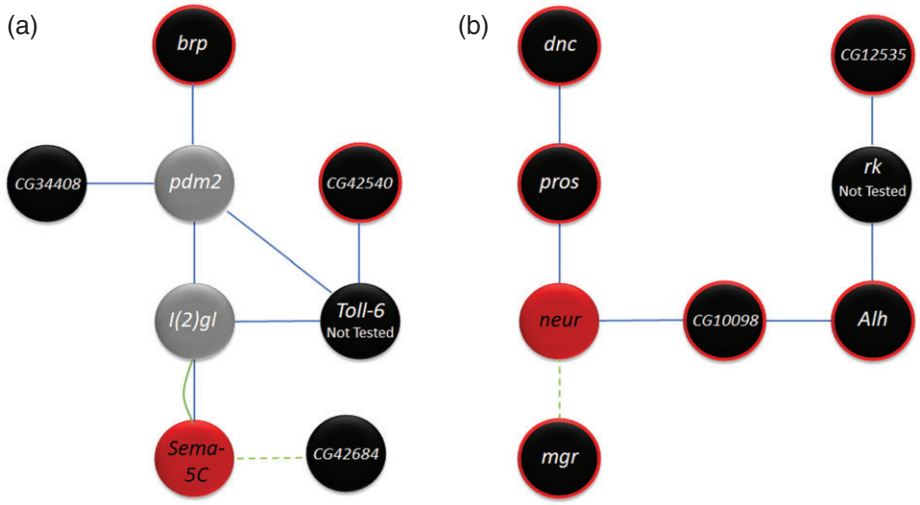

Figure 5: Reduced networks of candidate epistatic partners for *Sema-5c* (a) and *neur* (b). The focal genes are indicated on a red background. Candidate epistatic partners are indicated on a black background. Interconnecting genes in the network that were not identified by our GWA analyses are indicated on a gray background. Red borders indicate genes that were functionally validated with RNAi or mutants. Blue edges indicate coexpression and the green edge indicates a known genetic interaction. The dotted green edges indicate previously unknown epistatic interactions postulated from our GWA results.

Table 2: Functional effects of candidate epistatic partners on olfactory behavior

Focal gene	Candidate epistatic partner	*P-value*	*n*
neur	*Alh*	0.0010*	5
neur	*CG10098*	0.0065*	5
neur	*CG12535*	0.0104*	5
neur	*dnc*	0.0678	3
neur	*mgr*	<.0001*	2
neur	*pros*	<.0001*	5
Sema-5C	*brp*	0.0005*	10
Sema-5C	*CG34408*	0.9880	5
Sema-5C	*CG42540*	0.0499*	5
Sema-5C	*CG42684*	1.0000	5

**P*-values for olfactory responses of mutants vs. control by Dunnett's test.

Yamamoto *et al.* 2009). This concept is likely not specific to flies, but applicable across phyla, including humans.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Quantile–quantile (Q–Q) plots for GWA analyses. (a) Q–Q plot for GWA analysis of olfactory responses of F_1 progeny from *Sema-5c*^{BG2386} and DGRP crosses. (b) Q–Q plot for GWA analyses of the differences of olfactory responses of F_1 progeny between Canton S(B) and DGRP crosses and corresponding *Sema-5c*^{BG2386} and DGRP crosses. (c) Q–Q plot for GWA analysis of olfactory responses of F_1 progeny from *neur*^{BG2391} and DGRP crosses. (d) Q–Q plot for GWA analyses of the differences of olfactory responses of F_1 progeny between Canton S(B) and DGRP crosses and corresponding *neur*^{BG2391} and DGRP crosses.

Figure S2: Epistatic reaction norms for olfactory responses between mutants and control for major and minor alleles of candidate epistatic modifier SNPs. The response indices of the major or minor alleles for a particular SNP are calculated by averaging the response index scores from all lines with the major or minor alleles, respectively. Crosses between DGRP lines and Canton S(B) are indicated in blue, crosses between DGRP lines and *neur* mutants are indicated in red, and crosses between DGRP lines and *Sema-5c* mutants are indicated in green. *P*-values for all epistatic interactions illustrated are $< 10^{-5}$.

Table S1: Phenotypic values for olfactory behavioral responses to 1% (v/v) benzaldehyde.

Table S2: Top polymorphisms ($P < 10^{-5}$) identified by GWA for olfactory behavior of progeny from 203 DGRP lines crossed to *Sema-5c*^{BG2386}.

Table S3: Top polymorphisms ($P < 10^{-5}$) identified by GWA for olfactory behavior of progeny from 203 DGRP lines crossed to *neur*^{BG2391}.

Table S4: Top polymorphisms ($P < 10^{-5}$) identified by GWA for differences in olfactory behavior between progeny from 203 DGRP lines crossed to Canton S(B) and *Sema-5c*^{BG2386}.

Table S5: Top polymorphisms ($P < 10^{-5}$) identified by GWA for differences in olfactory behavior between progeny from 203 DGRP lines crossed to Canton S(B) and *neur*^{BG2391}.