

Quantitative Trait Loci for Locomotor Behavior in *Drosophila melanogaster*

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ABSTRACT

Locomotion is an integral component of most animal behaviors and many human diseases and disorders are associated with locomotor deficits, but little is known about the genetic basis of natural variation in locomotor behavior. Locomotion is a complex trait, with variation attributable to the joint segregation of multiple interacting quantitative trait loci (QTL), with effects that are sensitive to the environment. We assessed variation in a component of locomotor behavior (locomotor reactivity) in a population of 98 recombinant inbred lines of *Drosophila melanogaster* and mapped four QTL affecting locomotor reactivity by linkage to polymorphic *roo* transposable element insertion sites. We used complementation tests of deficiencies to fine map these QTL to 12 chromosomal regions and complementation tests of mutations to identify 13 positional candidate genes affecting locomotor reactivity, including *Dopa decarboxylase (Ddc)*, which catalyzes the final step in the synthesis of serotonin and dopamine. Linkage disequilibrium mapping in a population of 164 second chromosome substitution lines derived from a single natural population showed that polymorphisms at *Ddc* were associated with naturally occurring genetic variation in locomotor behavior. These data implicate variation in the synthesis of bioamines as a factor contributing to natural variation in locomotor reactivity.

LOCOMOTION is an integral component of most animal behaviors: movement is required for localization of food and mates, escape from predators, defense of territory, and response to stress. Locomotor behavior can also be regarded as a major component of fitness, given its central role in survival and reproduction (GILCHRIST *et al.* 1997). Many human neurological diseases (*e.g.*, Parkinson's disease and Huntington's disease) are associated with locomotor deficits, and hyperactivity and hypoactivity are, respectively, associated with activity disorders and depression (AMERICAN PSYCHIATRIC ASSOCIATION 1994). Locomotion is a complex behavior, and variation in nature is likely attributable to the joint segregation of multiple interacting quantitative trait loci (QTL), with effects that are sensitive to the environment. Thus, understanding the genetic architecture of locomotor behavior is important from the dual perspectives of evolutionary biology and human health. However, our current knowledge falls short of the level of detail with which we ultimately seek to describe variation in this trait: Which genes contribute to natural variation in locomotion? What are their homozygous, heterozygous, epistatic, and pleiotropic effects? How do these effects vary in a natural range of environments? And what are the molecular

polymorphisms responsible for natural allelic variation? Although to date no complex trait in any organism has been dissected at this level of detail, the greatest opportunity for success will come from genetic analysis of model organisms with excellent genetic and genomic resources, such as *Drosophila melanogaster*. Further, basic biological processes, including development of the nervous system, are evolutionarily conserved between flies and mammals (ADAMS *et al.* 2000). Thus, the same genes affecting *Drosophila* locomotion may well be relevant in humans.

Indeed, *Drosophila* provides a model for several human neurodegenerative diseases with marked locomotor impairments. Parkinson's disease is the most common neurodegenerative movement disorder and is associated with progressive degeneration of nigrostriatal dopaminergic neurons and accumulation of Lewy bodies, neuronal cytoplasmic inclusions containing ubiquitinated proteins (OLANOW and TATTON 1999). Consistent with a causal role of dopamine in the pathogenesis of Parkinson's disease, treatment with the precursor L-dopa ameliorates the symptoms in early stages of the disease. Dopamine has also been implicated in locomotion of mice (ZHOU and PALMITER 1995) and *Drosophila*.

In *Drosophila*, tyrosine is converted to L-dopa by tyrosine hydroxylase (encoded by *pale*), which is then decarboxylated by *Dopa decarboxylase* (encoded by *Dopa decarboxylase, Ddc*) to yield dopamine (MONASTIROT

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1999). Mutations with reduced levels of dopamine, as well as pharmacological interventions altering dopamine levels, are consistently associated with alterations in locomotor activity, although the direction of the effect is variable. Analysis of lines artificially selected for increased and decreased locomotor activity in an open field environment (CONNOLLY 1966, 1967) indicated an inverse relationship between level of activity and dopamine levels (TUNNICLIFF *et al.* 1969). Pharmacologically increasing the level of dopamine in the highly active selected line was associated with a reduction in locomotor activity, but not in the control line, indicating an genotype-by-environment interaction (CONNOLLY *et al.* 1971). *tyrosinase 1* (*tyr1*) mutants have reduced levels of dopamine and increased levels of spontaneous locomotor activity (BURNELL and DALY 1982; MEEHAN and WILSON 1987). Conversely, mutations in *pale* are associated with reduced locomotion, and the effect is rescued by administering L-dopa (PENDLETON *et al.* 2002).

The neurotransmitters serotonin (5-hydroxytryptamine, 5-HT), octopamine (the invertebrate homolog of noradrenaline), and γ -aminobutyric acid (GABA) also affect *Drosophila* locomotion. *eagle* (*eg*) mutants have reduced numbers of 5-HT cells in the ventral nerve cord and are inactive (LUNDELL and HIRSCH 1998). *inactive* (*iav*) mutants have reduced octopamine levels and are hypoactive (O'DELL and BURNET 1988). Pharmacological treatment of flies with GABA transport inhibitors decreases locomotor activity in a dose-dependent fashion, while cotreatment with a GABA antagonist restores locomotor activity, consistent with the inverse relationship between GABA levels and locomotion observed in vertebrates (LEAL and NECKAMEYER 2002).

The mushroom bodies and components of the central complex (which consists of a network of four neuropilar regions: the protocerebral bridge, fan-shaped body, ellipsoid body, and the paired noduli) control *Drosophila* locomotor behavior. *mushroom body miniature* (*mbm*) mutations lack Kenyon cells and exhibit increased locomotor activity, as do animals in which the mushroom bodies have been chemically or genetically ablated (MARTIN *et al.* 1998). Mutations in seven genes with neuroanatomical alterations in the structure of the central complex walk more slowly than wild-type flies. They are less active or quickly lose activity or fail to start walking or flying under circumstances in which wild-type flies would readily do so (reviewed by STRAUSS 2002). These genes include *no bridge* (*nob*, STRAUSS *et al.* 1992; MARTIN *et al.* 1999), *ocelliless* (*oc*, MARTIN *et al.* 1999), *central complex* (*cex*, MARTIN *et al.* 1999), *highwire* (*hiw*, WAN *et al.* 2000), *eyeless* (*ey*, CALLAERTS *et al.* 2001), *single minded* (*sim*, PIELAGE *et al.* 2002), *C31* (STRAUSS 2002), and *C141* (STRAUSS 2002). These observations are consistent with the hypothesis that the central complex is required for maintenance of walking activity, while the mushroom bodies act as inhibitors of locomotion (MARTIN *et al.* 1999; STRAUSS 2002).

Locomotor activity is not a single trait, but can (and has) been examined according to the time course of behavior (from minutes to days), differentiating among speed, amount, and directionality of activity and taking external stimuli such as the presence of food or stress of handling into account. Flies tend to react vigorously to a handling disturbance; the activity immediately following such a disturbance (MEEHAN and WILSON 1987), the time to settle to the endogenous level of activity, the level of expression of locomotor activity at steady state (CONNOLLY 1967), and the amount, speed, and direction of activity at any of these time points (BURNET *et al.* 1988; GOTZ 1989; STRAUSS *et al.* 1992; STRAUSS and HEISENBERG 1993) are all measures of activity. However, the various aspects of locomotor behavior are not perfectly correlated, implicating different genetic mechanisms underlying particular measures of activity. For example, the lines selected for activity in an open field environment (CONNOLLY 1966) differed in baseline activity but not in the change in activity over time following introduction to a novel environment (CONNOLLY 1967). Amount and speed of activity are under independent genetic control (BURNET *et al.* 1988). Further, some mutations affect activity only in a context-dependent manner. Mutations at the *period* (*per*) locus affect the circadian rhythm, but not the total amount of activity (KONOPKA and BENZER 1971). Variants of the *foraging* (*for*) gene affect the distance larvae and adults travel away from a food source, but these differences are not expressed in the absence of food (SOKOLOWSKI 1980; DEBELLE and SOKOLOWSKI 1987; PEREIRA and SOKOLOWSKI 1993).

There is abundant naturally segregating variation for locomotor activity in *Drosophila* (CONNOLLY 1966, 1967; VAN DIJKEN and SCHARLOO 1979; BURNET *et al.* 1988), yet we know virtually nothing of the genes and pathways responsible for this variation. There is a trade-off between quantifying all the aspects of locomotor behavior outlined above and assessing behavior from a large number of strains. Quantitative genetic analysis requires the latter; therefore, we developed a rapid and high-throughput assay to quantify the level of activity immediately following a disturbance (*sensu* MEEHAN and WILSON 1987), which we call locomotor reactivity. This is a general behavioral response that includes many constituent and more specific behaviors. Thus, genetic variation in locomotor reactivity could arise from variation in genes affecting the many biological processes affecting the development of the structures required for the execution, perception, and response to the stimulus. Our approach is to use quantitative genetic analysis of natural variants as an unbiased screen for genes and pathways affecting variation in locomotor behavior. Having identified these genes, one can subsequently examine the allelic effects in detail by determining which particular aspects of locomotor behavior are affected. We recognize that this approach is at variance

with (and anathema to) that of behavioral geneticists whose goal is to identify genes specifying the neural circuits that provide the potential for specific behaviors, where it is necessary to define and measure highly specific components of behavior (BAKER *et al.* 2001).

We assessed variation in locomotor reactivity in a population of recombinant inbred (RI) lines derived from the wild-type strain Oregon-R (Ore-R) and a strain selected for low male-mating propensity, 2b (KAIDANOV 1990), and mapped four QTL affecting this trait by linkage to *roo* transposable element markers (NUZHIDIN *et al.* 1997). We refined the QTL map positions using quantitative complementation tests of deficiencies, followed by complementation tests of mutations of positional candidate genes (LONG *et al.* 1996; MACKAY and FRY 1996; PASYUKOVA *et al.* 2000; FANARA *et al.* 2002; DE LUCA *et al.* 2003; HARBISON *et al.* 2004; MOEHRING and MACKAY 2004) to identify 13 candidate genes contributing to the observed variation between the Ore and 2b parental strains. One of the implicated candidate genes is *Ddc*, which catalyzes both the decarboxylation of L-dopa to dopamine and 5-hydroxytryptophan to serotonin in *Drosophila* (MONASTIRIOTI 1999; STEWARD 2000). We used linkage disequilibrium mapping (LD) on a sample of 164 alleles from a single population to show that molecular polymorphisms in *Ddc* are associated with natural variation in locomotor behavior.

MATERIALS AND METHODS

Genome scan for QTL affecting locomotor behavior: *Drosophila stocks:* We mapped QTL affecting variation in locomotor behavior between two isogenic strains: 2b, a derivative of a line originally selected for low male-mating propensity (KAIDANOV 1990), and Ore-R, a standard wild-type and unrelated stock (LINDSLEY and ZIMM 1992). We used a mapping population of 98 RI lines previously derived from these strains (NUZHIDIN *et al.* 1997). The genotype of each RI line was determined using 80 *roo* transposable element markers with polymorphic insertion sites that differ between the parental lines and the fourth chromosome visible mutation *shaven* (also known as *sparkling*), giving 81 informative markers with an average spacing of ~3 cM (NUZHIDIN *et al.* 1997). The *roo* transposable element markers are located at cytological positions 1B, 3E, 4F, 5D, 6E, 7D, 7E, 9A, 10D, 11C, 11D, 12E, 14C, 15A, 16D, 17C, 19A, 21E, 22F, 27B, 29F, 30AB, 30D, 33E, 34EF, 35B, 38A, 38E, 43A, 43E, 46A, 46C, 48D, 49D, 50B, 50D, 50F, 57C, 57F, 60E, 61A, 63A, 65A, 65D, 67D, 68B, 68C, 69D, 70C, 71E, 72A, 73D, 76A, 76B, 77A, 77E, 78D, 82D, 85A, 85F, 87B, 87E, 87F, 88E, 89B, 91A, 91D, 92A, 93A, 93B, 94D, 96A, 96F, 97D, 97E, 98A, 99A, 99B, 99E, and 100A. We maintained all stocks in vials containing 10 ml cornmeal–agar–molasses medium at 25° under 12-hr light–dark cycles.

Assay to quantify locomotor behavior: We placed single 3- to 7-day-old adult flies, collected under CO₂ exposure, into vials containing 5 ml of standard cornmeal–agar–molasses media and left them overnight to acclimate to their new environment. To quantify locomotor reactivity, we subjected each fly to a mechanical disturbance by tapping the vial twice against a table and recorded the amount of time the fly was active in the 30 sec immediately following the disturbance. This was accomplished by starting a timer and a stopwatch at the

beginning of the assay. The timer indicated the duration of the assay. The stopwatch was started and stopped when the fly was active or immobile, respectively, for the duration of the assay. The measure of locomotor reactivity was thus a score ranging from 0 to 30, representing the time in seconds that the fly moved within the assay period, as recorded by the stopwatch. All measurements were taken in a behavioral chamber at the same time of day (8 AM–12 PM, 2–6 hr after lights on) under constant temperature (25°) and humidity (75%). Activity scores were obtained for 20 males and 20 females for each genotype tested in a randomized design.

Quantitative genetic analysis: We evaluated variation in locomotor reactivity among the RI lines using two-way analysis of variance (ANOVA) according to the mixed model $Y = \mu + L + S + L \times S + E$, where μ is the overall mean, L and S are the main effects of line (random) and sex (fixed), $L \times S$ is the genotype-by-sex interaction term, and E is the within-genotype environmental variance. Broad-sense heritability (H^2) for locomotor reactivity in this population of RI lines was computed as $(\sigma_L^2 + \sigma_{L \times S}^2) / (\sigma_L^2 + \sigma_{L \times S}^2 + \sigma_E^2)$, where σ_L^2 is the among-line variance component, $\sigma_{L \times S}^2$ is the variance attributable to the line \times sex interaction, and σ_E^2 is the within-line environmental variance component. The genetic correlation (r_{GS}) across sexes was calculated as $r_{GS} = \sigma_L^2 / (\sigma_{LM} \sigma_{LF})$, where σ_L^2 is the among-line variance component from the analysis pooled across both sexes, and σ_{LM} and σ_{LF} are the square roots of the among-line variance components from the separate analyses of each sex. These ANOVAs and those described below were computed using SAS software (SAS INSTITUTE 1988) using the GLM and VARCOMP procedures.

QTL mapping: We mapped QTL affecting locomotor reactivity by linkage to *roo* polymorphic markers, using composite interval mapping (CIM) (ZENG 1994) of RI line means as implemented by QTL Cartographer software (BASTEN *et al.* 1994, 1999). QTL intervals were detected testing the hypothesis that there is no QTL for activity within the interval between two markers, using multiple regression to control for effects of other QTL. The likelihood-ratio (LR) test statistic is $-2 \ln(L_0/L_1)$, where (L_0/L_1) is the ratio of the null hypothesis (there is no QTL within the test interval) to the alternate hypothesis (there is a QTL within the test interval). Marker cofactors were chosen for the trait using forward selection–backward elimination stepwise regression. LR test statistics were computed every 0.1 cM using a conditioning window of 10 cM, such that only markers 10 cM away from the markers flanking the test interval were included in the model. To control for multiple testing, we randomly permuted the trait and marker data 1000 times. We computed the maximum LR across all intervals for each permuted data set to generate an empirical distribution of LR statistics under the null hypothesis, where there is no association between the genotypic and phenotypic data. LR statistics computed from the original data that exceeded the 950th LR statistic determined for the permuted data were considered to be significant at an experiment-wise 5% type I significance level (CHURCHILL and DOERGE 1994; DOERGE and CHURCHILL 1996).

Tests for epistasis: All possible pairwise epistatic interactions between markers were evaluated using ANOVAs on RI line means according to the model $Y = \mu + M_i + M_j + S + M_i \times S + M_j \times S + M_i \times M_j + M_i \times M_j \times S + E$, where M_i and M_j are the two focal markers evaluated for epistasis, S is the main effect of sex, and E is the error variance. We evaluated the significance of the $M_i \times M_j$ and $M_i \times M_j \times S$ interactions, using the conservative Bonferroni correction for multiple tests and a $Q = 0.05$ false-positive discovery rate criterion (STOREY and TIBSHIRANI 2003).

High-resolution QTL mapping: *Drosophila stocks:* Deficiency and mutant stocks used in complementation tests were

obtained from the Bloomington *Drosophila* Stock Center and are listed in supplemental Tables 1 and 2 at <http://www.genetics.org/supplemental/>.

Deficiency complementation mapping: We used deficiency complementation mapping (PASYUKOVA *et al.* 2000) to map the four QTL affecting locomotor reactivity with higher resolution. We chose 61 deficiency stocks (*Df/Bal*, where *Bal* indicates a dominantly marked *Balancer* chromosome), uncovering QTL regions affecting locomotor reactivity. We extended the deficiency complementation analysis to the markers immediately adjacent to those defining the 2-LOD support intervals, because QTL boundaries as determined by CIM can fluctuate slightly, depending on the model parameters (PASYUKOVA *et al.* 2000). We crossed males of each of the deficiency stocks to both Ore-R and 2b virgin females and measured locomotor activity, as described above, for 20 males and 20 females from each of the four F₁ genotypes of each cross (*Df/Ore*, *Df/2b*, *Bal/Ore*, and *Bal/2b*). The design was completely randomized for each QTL region. Only females ($n = 20/\text{F}_1$ genotype) were tested for the X chromosome deficiencies. All deficiencies exhibiting quantitative failure to complement Ore-R and 2b QTL (see below) were then retested in exactly the same manner, bringing the total sample size to 320 individuals tested for each significant deficiency (40 individuals/sex \times two sexes \times four F₁ genotypes).

Statistical analysis: The test for quantitative failure to complement compares the difference in mean activity levels of the *Df/Ore* and *Df/2b* flies to the difference between the activity of *Bal/Ore* and *Bal/2b* individuals. We infer quantitative complementation if the difference in mean activity between *Df/Ore* and *Df/2b* genotypes is equal to the difference in mean activity between the *Bal/Ore* and *Bal/2b* genotypes. We infer quantitative failure to complement when the difference in activity of the *Df* genotypes (*Df/Ore*–*Df/2b*) is greater than the difference in activity of the *Bal* genotypes (*Bal/Ore*–*Bal/2b*). The statistical analysis of the complementation data is a three-way, fixed-effects ANOVA model: $Y = \mu + S + L + G + S \times L + S \times G + L \times G + S \times L \times G + E$, where μ is the overall mean, S is the main effect of sex, L is the main effect of the parental line (Ore-R or 2b), G is the main effect of the genotype (*Df* or *Bal*), and E is the error variance. Significance of the $L \times G$ (and/or $S \times L \times G$) interaction is indicative of quantitative failure to complement (and/or sex-specific failure to complement), provided that subsequent tests reveal that (*Df/Ore*–*Df/2b*) > (*Bal/Ore*–*Bal/2b*). We repeated the complementation tests for deficiencies exhibiting significant failure to complement in the initial experiment. These ANOVAs included a term for the random effect of replicates nested within $S \times L \times G$. The QTL locations were initially defined by the breakpoints of the deficiencies exhibiting significant failure to complement, pooled over both replicates. These locations were further delimited by the right and left breakpoints of overlapping non-significant (complementing) deficiencies to the left and right, respectively, of the significant deficiency (PASYUKOVA *et al.* 2000).

Complementation tests to candidate genes: Quantitative complementation tests of 27 mutations at 26 candidate genes in the QTL regions defined by deficiency mapping were used to identify putative candidate genes within each QTL. All mutant stocks were heterozygous over balancers, with the exception of *nob^{KS49}*. The experimental design, sample sizes, and analyses were the same as described above for the deficiency complementation tests, except for *nob^{KS49}*, where the control cross utilized the Berlin strain in which the *nob* mutation was induced. All candidate genes that exhibited significant failure to complement on the initial test were retested, as described above for the deficiency complementation tests.

Natural variation in locomotor reactivity: *Drosophila* stocks: One of the positional candidate genes corresponding to a

QTL affecting locomotor reactivity was *Ddc* (2-53.9, 37C1). Previously, we established isofemale lines from gravid females collected from the Raleigh, North Carolina, farmer's market in 1999. We extracted 164 isogenic second chromosomes from these lines and substituted them into the genetic background of the highly inbred Samarkand (Sam) strain by standard techniques using balancer chromosomes (LYMAN *et al.* 1999; DELUCA *et al.* 2003). In addition, we substituted the chromosome containing a γ -ray-induced, recessive lethal null *Ddc* allele (*Ddc²⁷*) into the Sam genetic background.

Complementation tests: We used complementation tests to assess whether naturally occurring *Ddc* alleles potentially contributed to quantitative variation in locomotor reactivity. We crossed each of the 164 second chromosome substitution lines ($C2_i$, $i = 1$ –164) to both Sam; *Ddc²⁷* and Sam; *Ddc⁺* stocks (where *Ddc⁺* denotes the Sam *Ddc* allele) and collected Sam; *Ddc²⁷/C2_i*; and Sam; *Ddc⁺/C2_i*; F₁ individuals, respectively, from each cross. We measured locomotor reactivity, as described above, for 20 males and 20 females of each F₁ genotype. The experimental design was nearly balanced, with a total sample size of 13,068 rather than 13,120 individuals. The experimental design was completely randomized. We analyzed these data using the three-way factorial, mixed-model ANOVA: $Y = \mu + L + S + C + L \times S + L \times C + S \times C + L \times S \times C + E$, where μ is the overall mean, L is the random main effect of the C2 line, S is the fixed main effect of sex, C is the fixed main effect of the cross (*Ddc²⁷* or *Ddc⁺*), $L \times S$, $L \times C$, $S \times C$, and $L \times S \times C$ are the interaction terms, and E is the error variance. Significance of the $L \times C$ interaction indicates that the difference in the phenotype between second chromosome alleles varies between the mutant and control backgrounds and that wild-derived alleles fail to complement the *Ddc* mutant allele for locomotor reactivity. Similarly, significance of the $L \times S \times C$ interaction indicates sex-specific failure of the wild-derived alleles to complement the *Ddc* mutant allele for locomotor reactivity. In either case, *Ddc* is a potential candidate gene affecting natural variation in locomotor reactivity.

The total genetic variance of locomotor activity (σ_G^2) in this population was estimated as $\sigma_G^2 = \sigma_L^2 + \sigma_{L \times S}^2 + \sigma_{L \times C}^2 + \sigma_{L \times S \times C}^2$, where σ_L^2 is the among-line variance component, $\sigma_{L \times S}^2$ is the variance attributable to the line–sex interaction, $\sigma_{L \times C}^2$ is the line–cross interaction variance, and $\sigma_{L \times S \times C}^2$ is the three-way line–sex–cross interaction variance. The total phenotypic variance (σ_P^2), was estimated as $\sigma_P^2 = \sigma_G^2 + \sigma_E^2$, where σ_E^2 is the environmental variance component.

Genotype–phenotype associations: Previously, we genotyped the 164 wild-derived *Ddc* alleles from the Raleigh population for 36 molecular markers [31 single nucleotide polymorphisms (SNPs) and 5 insertion/deletion polymorphisms] (DELUCA *et al.* 2003). Two of the marker pairs were in perfect LD, giving 34 different association tests (DELUCA *et al.* 2003). We assessed whether molecular polymorphisms at *Ddc* were associated with variation in locomotor reactivity by three-way factorial ANOVA of line means, according to the fixed effects model $Y = \mu + S + C + M + S \times C + S \times M + C \times M + S \times C \times M + E$, where μ is the overall mean, S is the main effect of sex, C is the main effect of the cross, M is the main effect of the marker (or haplotype), $S \times C$, $S \times M$, $C \times M$, and $S \times C \times M$ are the interaction terms, and E is the error variance. Significance of the M term indicates that the difference in phenotype between marker alleles varies, suggesting an allelic association in locomotor reactivity with a particular marker. In addition to the single-marker analysis, we also performed a three-way ANOVA to test the effect of global (across all 36 polymorphisms) haplotype variation in *Ddc* on locomotor reactivity using the following model: $Y = \mu + S + C + H + S \times C + S \times H + C \times H + S \times C \times H + E$, where the main effects of sex and cross are as described above, and H represents the fixed effect of haplotype class

(1–103). Significance of the H term indicates differences in phenotype between the global *Ddc* haplotype classes, suggesting an effect of molecular variation in *Ddc* on variation of locomotor reactivity.

We used permutation tests to determine empirical distributions under the null hypotheses of no association between *Ddc* genotypes and locomotor reactivity. To assess whether any of the individual markers were significantly associated with variation in locomotor reactivity, we permuted the trait phenotypes among the 36 markers 1000 times and performed single-marker association analyses on the permuted data. We recorded the lowest P -value for the effect of marker from each permutation run. The 50th lowest P -value corresponds to $\alpha = 0.05$ under the null hypothesis; any marker with a P -value less than this is individually significant (DE LUCA *et al.* 2003). To assess whether we observed more nominally significant marker–phenotype associations than expected by chance, we recorded the number of significant associations at $P < 0.05$, $P < 0.025$, and $P < 0.01$ for each permuted data set and determined whether the observed number of significant markers at each P -value (0.05, 0.025, and 0.01) exceeded the expected number determined by permutation. Finally, to assess whether the observed association of locomotor reactivity with *Ddc* population haplotypes was less than expected by chance, we permuted the trait phenotypes among the global haplotypes 1000 times and recorded the lowest P -value for the effect of haplotype for each permuted data set.

RESULTS

Genome scan for QTL affecting locomotor behavior: *Quantitative genetic variation in locomotor behavior:* We observed a highly significant difference in locomotor reactivity behavior between the Ore-R and 2b isogenic lines ($F_{1,96} = 156.68$, $P < 0.0001$; Figure 1). The mean (\pm SE) level of locomotor reactivity of Ore-R was 16.6 ± 0.79 sec (15.3 ± 0.75 sec for males and 18.0 ± 0.83 sec for females), more than twice the mean level of reactivity of the 2b strain, 6.8 ± 0.77 sec (7.9 ± 0.70 sec for males and 6.0 ± 0.84 sec for females). Not surprisingly, there was highly significant genetic variation in locomotor reactivity among the RI lines derived from Ore-R and 2b ($F_{97,3724} = 9.50$, $P < 0.0001$; Figure 1). In addition, the line–sex interaction term was significant ($F_{97,3724} = 1.53$, $P = 0.0007$), indicating variation in sex dimorphism of reactivity in this population of RI lines. The estimate of broad-sense heritability of locomotor reactivity in the RI lines was $H^2 = 0.26$. The correlation in locomotor reactivity between the sexes ($r_{GS} = 0.82 \pm 0.058$) was high and positive, but significantly different from unity. Thus, the effects of genes affecting this trait are not identical in males and females.

QTL mapping: Given the significant genetic variation among the RI lines in locomotor reactivity, we used QTL mapping to identify genomic regions responsible for the observed differences in activity between the parental lines. We used CIM to map four QTL affecting reactivity levels based on permutation-derived significance thresholds at cytological locations 1B–3E, 27B–29E, 30D–38A, and 99A–100A (Figure 2 and Table 1). Consistent with the inference of sex-specific effects on locomotor

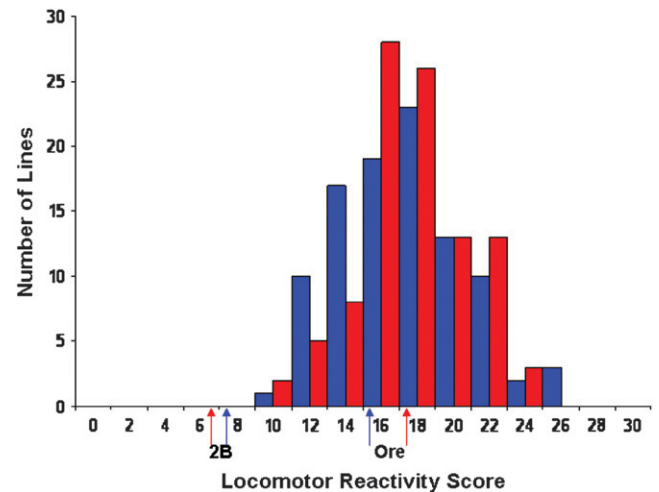


FIGURE 1.—Variation in locomotor reactivity for males (blue) and females (red). Mean reactivity scores are depicted for the parental strains, Ore-R and 2b, and for the mapping population of 98 RI lines.

reactivity from the quantitative genetic analyses, the 1B–3E QTL was formally significant only in males; the 27B–29E QTL was significant only in females; and the remaining two QTL had similar effects in both sexes. The physical regions encompassed by the QTL range from 2600 to 9200 kb and include between 574 and 1553 positional candidate genes (Table 1). It is possible that additional QTL affect variation in locomotor reactivity in this mapping population, but their effects are too small to be detected, given the sample size used in this experiment.

Epistasis: We tested for pairwise epistatic interactions between all possible marker combinations. We observed four blocks of significant epistatic interactions between markers 21E and 65D–67D; 50D–50F and 65A; 50D–50F and 91A–92A; and 61A–65A and 72A–88E. Six pairs of markers in these blocks were significant using a conservative Bonferroni correction for multiple tests, and the rest were significant using a false discovery rate (STOREY and TIBSHIRANI 2003) criterion of $Q \leq 0.05$, corresponding to a P -value of ≤ 0.0011 . None of the blocks of markers contributing to epistatic interactions had significant additive QTL effects in the CIM analysis.

High-resolution QTL mapping: *Deficiency complementation mapping:* As noted above, our initial genome scan localized QTL affecting locomotor reactivity to relatively broad genomic regions. Therefore, we implemented deficiency complementation mapping using 61 deficiency stocks spanning the QTL intervals (supplemental Table 1 at <http://www.genetics.org/supplemental/>) to map the QTL with higher resolution (Table 2, Figure 3). We extended the deficiency complementation analysis to the markers immediately adjacent to those defining the 2-LOD support intervals, because QTL boundaries as determined by CIM can fluctuate slightly, depending on the model parameters (PASYUKOVA *et al.* 2000). The QTL

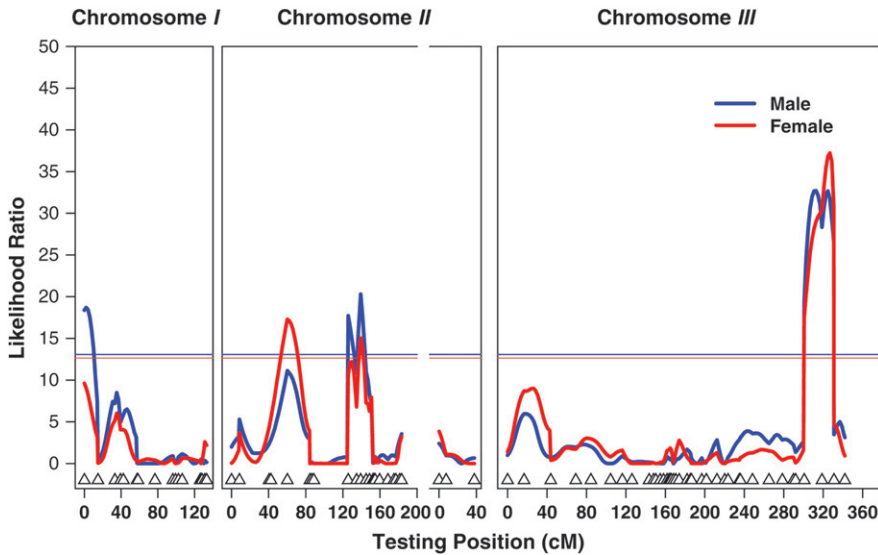


FIGURE 2.—QTL for locomotor reactivity. Triangles on the x -axis depict the locations of 700 transposable element markers in centimorgans. The y -axis is the LR test statistic. The horizontal lines denote the LR statistics corresponding to permutation-derived experiment-wise 5% significance thresholds, shown separately for males and females.

from 1B–3E was refined to a region just adjacent to original QTL, from 4F11–5A2. The peak on the second chromosome spanning 27B to 29E fractionated into three smaller QTL, including 23E1–23E6, 24C3–24E4, and 28C1–28E7. The QTL peak spanning 30D to 38A reduced into six smaller regions, including 32F1–33B3, 35B10–35D1, 36D1–36E2, 37B2–37F1, 38B1–40B1, and 42B3–42E1. The QTL with a large effect on the third chromosome spanning 98A to 100A split into two regions, 99A6–99C1 and 100D2–100F5. Overall, the four main QTL that spanned over 18.2 Mb fractionated into 12 much smaller regions that harbor between 29 and 407 positional candidate genes (Table 2). This range is skewed because of the 407-gene region from 38B1 to 40B1, which spans the centromere and for which additional deficiencies were not available at the time of testing. Including this region, the average number of positional candidate genes identified using deficiency complementation mapping is 101.8 loci/QTL. This reduced number greatly facilitates the task of choosing candidate genes for further study. While each refined QTL region could represent the effect of a single gene, it is also possible that there are closely linked genes within the QTL regions that contribute to variation in locomotor reactivity.

Mutant complementation mapping: We used complementation tests to 27 mutations at 26 positional candidate genes within QTL defined by deficiency complementation mapping to identify mutations that fail to complement the Ore-R and 2b QTL alleles for locomotor reactivity (supplemental Table 2 at <http://www.genetics.org/supplemental/>). We preferentially chose candidate genes for complementation tests that were known to function in the nervous system, were required for normal development (including leg development genes), were involved in response to stress, and/or were previously implicated in behavior. Mutations in the following 13 genes failed to complement Ore-R and 2b QTL alleles for locomotor reactivity: *nob*, *turtle* (*tull*), *TBP-related factor* (*Trf*), *crooked legs* (*crol*), *shuttlecraft* (*stc*), *Catecholamines up* (*Catsup*), *Lim3*, *Ddc*, *derailed* (*drl*), *tyr1*, *Aconitase* (*Acon*), *teashirt* (*tsh*), and *Drop* (*Dr*) (Figure 4, supplemental Table 3 at <http://www.genetics.org/supplemental/>). Among the 13 mutations implicated in this study only *nob*, *tyr1*, and *Dr* have been previously found to affect locomotor behavior. The other 10 loci are novel candidate genes affecting locomotor reactivity. We did not test candidate genes in the 23E1–23E6 QTL, as no genes in this region fulfilled our criteria (all are predicted genes of unknown function). Further, none of the mutations at positional

TABLE 1

Summary of QTL mapping results

| QTL | Marker | 2-LOD interval | Male LR ^a | Male <i>a</i> | Male ^b <i>a</i> /σ _P | Female LR ^a | Female <i>a</i> | Female ^b <i>a</i> /σ _P | kb ^c | No. of loci ^d |
|-----|--------|----------------|----------------------|---------------|--|------------------------|-----------------|--|-----------------|--------------------------|
| 1 | 1B | 1B–3E | 18.69* | −1.70 | −0.284 | 9.62 | −0.83 | −0.138 | 3200 | 712 |
| 2 | 27B | 27B–29E | 10.42 | 1.001 | 0.167 | 17.30* | 1.13 | 0.188 | 3200 | 368 |
| 3 | 35BC | 30D–38A | 19.38* | −1.54 | −0.257 | 15.04* | −1.13 | −0.189 | 9200 | 1553 |
| 4 | 99A | 98A–100A | 32.70* | 2.16 | 0.361 | 37.23* | 1.66 | 0.277 | 2600 | 574 |

^a Peak LR (* denotes significant LR).

^b Additive effect (*a*) of QTL (Ore-R – 2b) in phenotypic standard deviation units (σ_P).

^c Size of QTL region given by the 2-LOD support intervals in kilobase pairs.

^d Number of genes within 2-LOD support intervals of each QTL based on DRYSDALE *et al.* (2005).

TABLE 2
High-resolution QTL mapping from complementation tests of deficiencies

| Chromosome | Deficiency name | <i>P</i> -value $L \times G$ | QTL | No. of loci ^b |
|------------|--|------------------------------|-------------|--------------------------|
| X | <i>Df(1)JC70/FM7c</i> | 0.0004 | 4F11–5A2 | 43 |
| 2 | <i>Df(2L)S2590/CyO</i> | 0.0039 | 23D2–23E3 | 53 |
| 2 | <i>Df(2L)ed-dp/SM1</i> | 0.0024 | 24C3–24E4 | 73 |
| 2 | <i>Df(2L) Trf-C6R31/CyO</i> | 0.0078 | 28C1–28E7 | 106 |
| | <i>Df(2L)XE-2750/CyO</i> | 0.0004 | | |
| 2 | <i>Df(2L)PrI/CyO</i> | 0.0001 | 32F1–33B3 | 58 |
| 2 | <i>Df(2L)TE35BC-24/CyO</i> | 0.0001 ^a | 35B10–35D1 | 37 |
| 2 | <i>Df(2L)H20/CyO</i> | 0.0064 ^a | 36D1–36E2 | 29 |
| 2 | <i>Df(2L)prA16/CyO</i> | 0.0001 ^a | 37B2–37F1 | 175 |
| | <i>DF(2L)TW158/CyO</i> | 0.0022 | | |
| 2 | <i>Df(2L)TW161/CyO</i> | 0.0001 ^a | 38B1–40B1 | 407 |
| 2 | <i>Df(2R)ST1/CyO</i> | 0.0008 | 42B3–42E1 | 87 |
| 3 | <i>Df(3R)Dr-τ1/TM3</i> | 0.0001 | 99A6–99C1 | 87 |
| | <i>Df(3R)01215/TM3</i> | 0.0083 | | |
| 3 | <i>Df(3R)faf-BP/TM6B</i> | 0.0048 | 100D2–100F5 | 66 |
| | <i>Df(3R)04661/TM3</i> | 0.0003 ^a | | |

^a Significant $L \times G \times S$ interaction in addition to significant $L \times G$ term.

^b Number of genes in QTL intervals based on DRYSDALE *et al.* (2005).

candidate genes corresponding to the QTL at 36D1–36E2 and 42B3–42E1 exhibited failure to complement.

Linkage disequilibrium mapping: Complementation tests: *Ddc* was one of the candidate genes affecting variation in locomotor reactivity between Ore-R and 2b. We conducted complementation tests to determine whether *Ddc* could potentially be associated with naturally occurring variation in locomotor reactivity. We crossed a *Ddc* null allele (*Ddc*²⁷) and a wild-type *Ddc* allele, both of which had been substituted into the inbred Sam genetic background, to a panel of 164 wild-derived isogenic second chromosomes extracted from the Raleigh population, which had also been substituted into the Sam background. The analysis of these data is given in Table 3. There is considerable naturally segregating genetic variation in locomotor reactivity attributable to genes on the second chromosome, as shown by the highly significant effect of the second chromosome substitution line. In addition, the significant main effect of cross indicates that the mean locomotor reactivity of lines differs according to whether they are heterozygous for the null or for the wild type *Ddc* allele: in fact, mean locomotor reactivity is reduced in the *Ddc*²⁷ background. The major terms of interest from this analysis are those involving the interaction between line and cross ($L \times C$ and $L \times S \times C$), both of which are highly significant. These are the complementation terms, which indicate that the difference in means between the *Ddc*²⁷ and *Ddc*⁺ alleles varies among the chromosome substitution lines and that the sex dimorphism of the complementation

effects also varies among the wild-derived chromosomes. That is, there is genetic interaction between the *Ddc*²⁷ and *Ddc*⁺ chromosomes and the QTL affecting locomotor reactivity on the wild-derived chromosomes.

These data do not prove that *Ddc* is associated with natural variation in locomotor reactivity, but rather that *Ddc* cannot be excluded as a potential candidate gene, as would have been the case if the interactions between line and cross were not significant. This is because the *Ddc*²⁷ and *Ddc*⁺ alleles are not on co-isogenic chromosomes. Therefore, there are several possible and not mutually exclusive interpretations of the observed quantitative failure to complement: (1) allelic interactions between naturally occurring *Ddc* alleles and the mutant and wild-type *Ddc* alleles; (2) epistatic interactions between other QTL on the wild second chromosomes affecting locomotor reactivity and the mutant and wild-type *Ddc* alleles; and (3) interactions between any of the QTL affecting locomotor reactivity that differ between the chromosomes harboring the *Ddc*²⁷ and *Ddc*⁺ alleles and the QTL affecting variation in locomotor reactivity on the wild second chromosomes. If the former interpretation is true, then we expect that molecular polymorphism at *Ddc* will be associated with naturally occurring variation in locomotor reactivity.

Genotype–phenotype associations: We tested this hypothesis directly by evaluating associations between polymorphic markers at *Ddc* in this population of 164 second chromosome lines and variation in locomotor reactivity. Five markers exhibited an association with locomotor

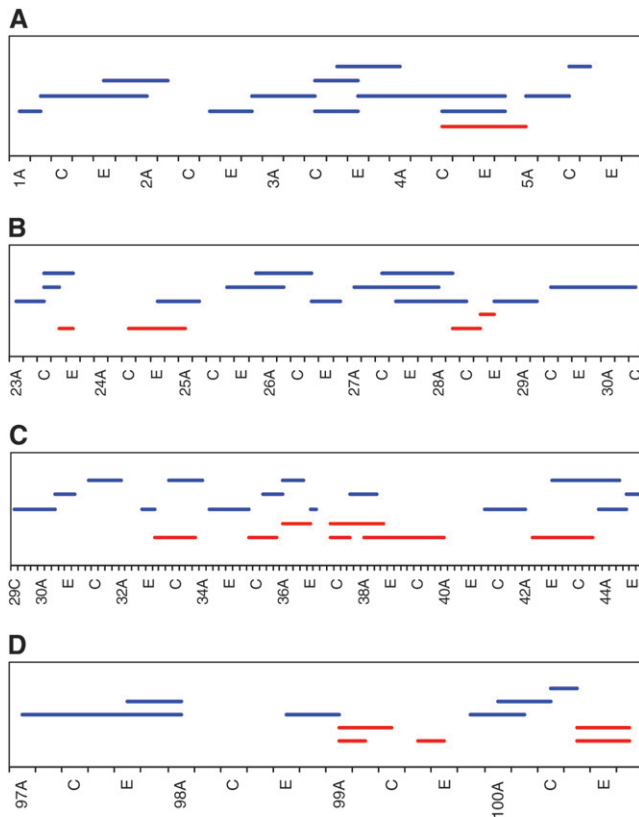


FIGURE 3.—High-resolution QTL mapping using complementation tests of deficiencies. Each horizontal bar shows the cytological breakpoints of the tested deficiencies (supplemental Table 1 at <http://www.genetics.org/supplemental/>). Red bars indicate deficiencies exhibiting quantitative failure to complement QTL affecting locomotor behavior, and blue bars indicate quantitative complementation. (A–D) The four genomic regions to which QTL affecting locomotor reactivity were mapped. The scale denotes sections and subsections of polytene chromosomes. (A) 1B–5F. (B) 23A–30C. (C) 29C–44F. (D) 97A–100F.

reactivity at the nominal 5% significance level: G236C ($P = 0.0458$) and A507T ($P = 0.0201$) in the promoter region, T1042G in intron 1 ($P = 0.0015$), In2622Del in intron 3 ($P = 0.0064$), and A3554G, a synonymous SNP in exon 4 ($P = 0.0209$) (Figure 5). The T1042G polymorphism was formally significant at an experiment-wise $P = 0.05$ both using a conservative Bonferroni-corrected significance threshold ($P = 0.0015$, based on 34 different association tests) and using a significance threshold derived by permutation. We also used permutation to test whether there were more significant marker-trait associations than expected by chance. The permutation-derived probabilities of observing five marker-trait associations at $P < 0.05$, four marker-trait associations at $P < 0.025$, and two marker-trait associations at $P < 0.01$ were, respectively, $P = 0.071$, $P = 0.039$, and $P = 0.040$. Thus, we observed more marker-trait associations at $P < 0.025$ than expected by chance, also implicating *Ddc* as a candidate gene affecting natural variation in locomotor reactivity. The four markers significantly associated with variation in locomotor

behavior at $P < 0.025$ are in strong LD with each other. Estimates of LD were significant at $P < 0.0001$ (Fisher's exact test) between A507T and T1042G, A507T and In2622Del, T1042G and A3554G, and In2664Del and A3554G (DELUCA *et al.* 2003). Indeed, LD extends throughout the 5.5-kb region including the *Ddc* transcription unit (DELUCA *et al.* 2003). There are 103 population haplotypes at *Ddc* formed by considering all 36 polymorphic sites in the sample of 164 alleles, and these haplotypes are strongly associated with variation in locomotor reactivity ($P = 9.7 \times 10^{-14}$). This association is highly significant ($P < 0.001$) on the basis of a permutation test.

DISCUSSION

Genetic architecture of locomotor behavior: We mapped four additively acting QTL affecting variation in locomotor reactivity between Ore-R and 2b. However, the genetic architecture of locomotor behavior in these strains is much more complex than suggested by the initial QTL mapping, since the four QTL detected by recombination mapping fractionated into a minimum of 12 QTL on the basis of quantitative complementation tests of deficiencies (PASYUKOVA *et al.* 2000), and there was evidence for epistasis between gene regions without main QTL effects. Further, several of the QTL defined by deficiency mapping harbored multiple candidate genes as indicated by complementation tests to mutants. It should be noted that there were a few small gaps in the deficiency coverage and that only a small fraction of positional candidate genes were tested for complementation to mutations, either because mutations were unavailable or because they did not have high priority according to our criteria for inclusion in this study. Similar complexity has been observed in previous analyses of variation in longevity (PASYUKOVA *et al.* 2000; MACKAY *et al.* 2005), starvation resistance (HARBISON *et al.* 2004), and mating behavior (MOEHRING and MACKAY 2004) in these lines. In addition, the number of QTL detected in any line-cross analysis is always a lower bound to the number of QTL affecting naturally occurring variation in the trait (MACKAY 2001). More QTL, with smaller effects, contributing to variation in locomotor behavior between Ore-R and 2b could be detected by increasing the number of recombinants as well as the number of individuals tested per recombinant genotype. Also, these strains represent only a subset of naturally occurring variation, and expanding the study to other strains is likely to uncover additional QTL that do not segregate in these strains. When one adds to these considerations the fact that locomotor reactivity is only one of many measures of locomotor behavior, the inescapable conclusion is that a large number of loci must affect natural variation in this important suite of traits. This, in turn, implies substantial

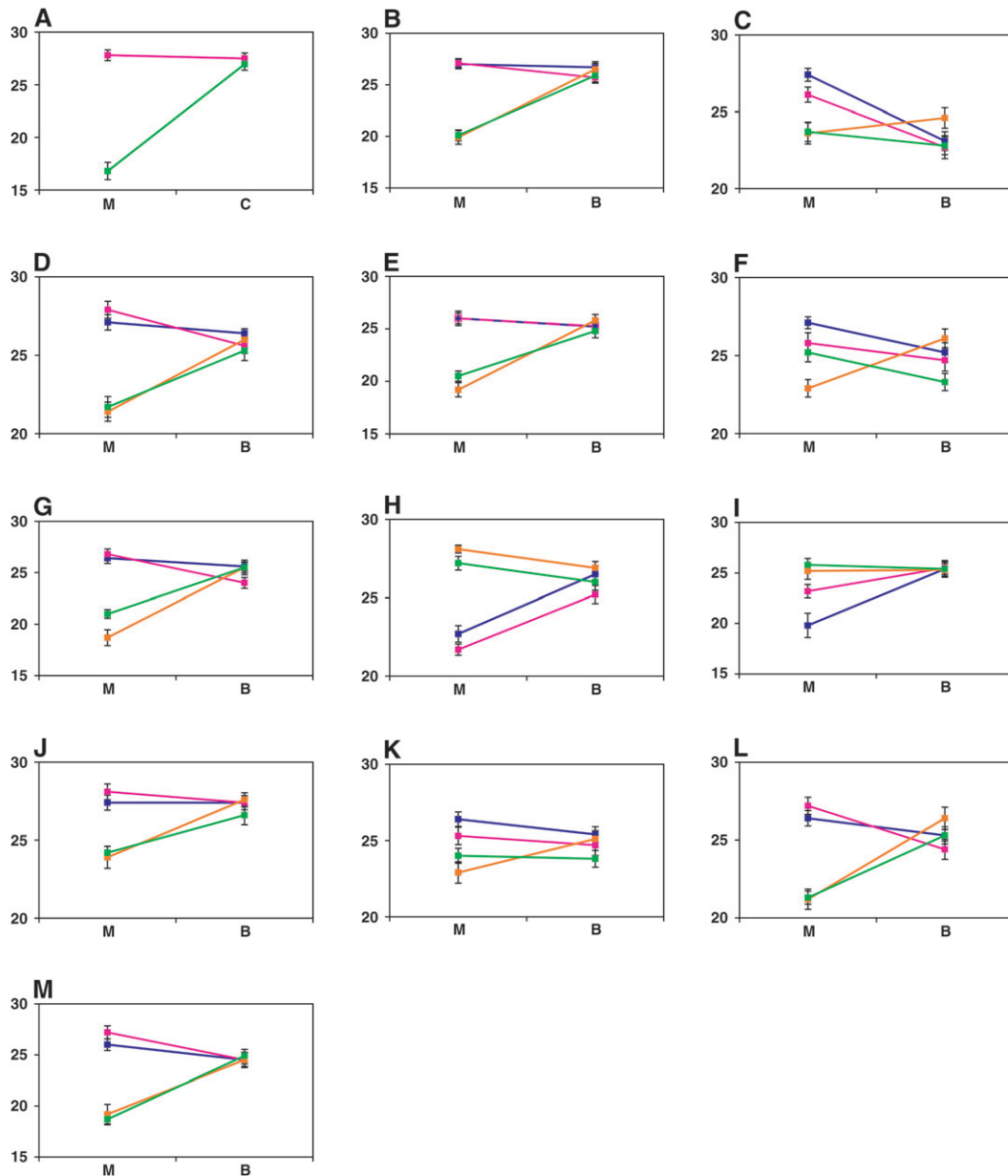


FIGURE 4.—Mutant complementation tests. Means and standard errors of locomotor reactivity (in seconds, y-axis) are given for the genes that exhibited failure to complement Ore-R and 2b alleles, separately for crosses to mutants (M) and balancers (B) (x-axis). Males (blue) from the crosses to Ore-R. Females (pink) from the crosses to Ore-R. Males (gold) from the crosses to 2b. Females (green) from the crosses to 2b. (A) *nob^{KS49}*. (B) *tul⁰¹⁰⁸⁵*. (C) *Trf¹*. (D) *cro⁰⁴⁴¹⁸*. (E) *stc⁰⁵⁴⁴¹*. (F) *Catsup¹*. (G) *Lim³*. (H) *Ddc²⁷*. (I) *dr²*. (J) *tyr¹*. (K) *Acon⁰⁷⁰⁵⁴*. (L) *tsh⁰⁴³¹⁹*. (M) *Dr¹*.

pleiotropy between the genetic architectures of multiple complex traits, as previously inferred from the analysis of effects of *P*-element-induced mutations affecting olfactory behavior (ANHOLT *et al.* 1996), sensory bristle number (NORGA *et al.* 2003), and resistance to starvation stress (HARBISON *et al.* 2004).

Quantitative complementation tests: The major challenge to understanding the genetic basis of complex traits in most species has been the difficulty in mapping individual genes corresponding to QTL by recombination alone (MACKAY 2001). In *Drosophila*, we can circumvent this difficulty by utilizing complementation

tests of deficiencies to rapidly map QTL to small genomic regions and complementation tests of mutations to identify positional candidate genes corresponding to QTL (LONG *et al.* 1996; MACKAY and FRY 1996; PASYUKOVA *et al.* 2000; FANARA *et al.* 2002; DELUCA *et al.* 2003; HARBISON *et al.* 2004; MOEHRING and MACKAY 2004). These approaches are not without caveats. Until recently, deficiency mapping has been limited because there are gaps in deficiency coverage, and the breakpoints have been ascertained only cytologically. More importantly, deficiencies have been induced in a heterogeneous collection of genetic backgrounds and are

TABLE 3

ANOVA of complementation tests of isogenic second chromosomes to *Ddc*²⁷ and *Ddc*⁺ alleles

| Source | d.f. | MS ^a | F | P | σ^2 ^b |
|--------------------------------|--------|-----------------|-------|---------|-------------------------|
| Line (<i>L</i>) | 163 | 183.007 | 3.95 | <0.0001 | 1.7150 |
| Sex (<i>S</i>) | 1 | 4.292 | 0.27 | 0.6072 | Fixed |
| Cross (<i>C</i>) | 1 | 821.598 | 18.82 | <0.0001 | Fixed |
| <i>L</i> × <i>S</i> | 163 | 16.175 | 1.20 | 0.1267 | 0.0627 |
| <i>L</i> × <i>C</i> | 163 | 43.661 | 3.23 | <0.0001 | 0.7544 |
| <i>S</i> × <i>C</i> | 1 | 37.528 | 2.78 | 0.0976 | Fixed |
| <i>L</i> × <i>S</i> × <i>C</i> | 163 | 13.520 | 1.57 | <0.0001 | 0.2500 |
| Error | 12,412 | 8.612 | | | 8.6142 |

^a From type III sums of squares.^b Variance component.

often marked with recessive and dominant mutations, and the Balancer chromosome is from an unrelated genetic background with additional mutations. The heterogeneous genetic backgrounds pose a problem for comparing effects across overlapping deficiencies, since variable effects could be due to multiple linked QTL or differences in genetic background. Recently, DrosDel and Exelixis deficiencies have been generated in co-isogenic backgrounds without additional mutations, have molecularly defined breakpoints (PARKS *et al.* 2004), and will prove a major boon for future deficiency complementation mapping efforts. Similarly, the drawback for using quantitative complementation tests of mutations at positional candidate genes has been the limited number of mutations at positional candidate genes embraced by a QTL (ideally, one would like to perform complementation tests to all positional candidate genes), because those that exist are not typically derived in an isogenic background. The Exelixis collection of co-isogenic *P* and *piggyBac* insertions (THIBAUT *et al.* 2004) and the ongoing effort to mutate all *Drosophila* genes (SPRADLING *et al.* 1999; BELLEN

et al. 2004) will greatly enhance future efforts to identify all positional candidate genes that fail to complement QTL alleles.

Candidate genes affecting variation in locomotor behavior between Ore-R and 2b: We have identified 13 candidate genes affecting variation in locomotor reactivity between Ore-R and 2b using complementation tests of mutations at positional candidate genes (supplemental Table 3 at <http://www.genetics.org/supplemental/>). Of these, three have been previously implicated to affect adult locomotion (*nob*, *tyr1*, and *Dr*). *nob* mutants are inactive and have neuroanatomical defects in the protocerebral bridge and fan-shaped body of the central complex, the brain region required for maintenance of locomotor activity (STRAUSS *et al.* 1992; MARTIN *et al.* 1999; HITIER *et al.* 2000; STRAUSS 2002). *tyr1* affects catecholamine biosynthesis. *tyr1* mutations have reduced levels of phenol oxidase (PENTZ *et al.* 1990) and dopamine (BURNELL and DALY 1982) and are hyperactive (BURNELL and DALY 1982; MEEHAN and WILSON 1987). Mutations at *Dr* are “uncoordinated.” *Dr* is a highly pleiotropic homeobox gene that encodes a RNA polymerase II transcription factor involved in brain and muscle development and in establishing the dorsal identity of neuroblasts (ISSHIKI *et al.* 1997; JURATA *et al.* 2000).

Six candidate genes have mutant phenotypes consistent with an involvement in regulating locomotion, although effects on locomotor behavior have not been quantified previously (*tutl*, *drl*, *Catsup*, *Lim3*, *Ddc*, and *Acon*). *tutl* plays an essential role in establishing neural circuits for coordinated motor output: *tutl* mutations exhibit an abnormal response to tactile stimulation, an inability to regain an upright position from an inverted position, and an inability to fly (BODILY *et al.* 2001). *drl* encodes a protein tyrosine kinase, which acts in embryos to guide axons across the midline in the anterior commissure and represses crossing the midline in the posterior commissure. Derailed may have a similar

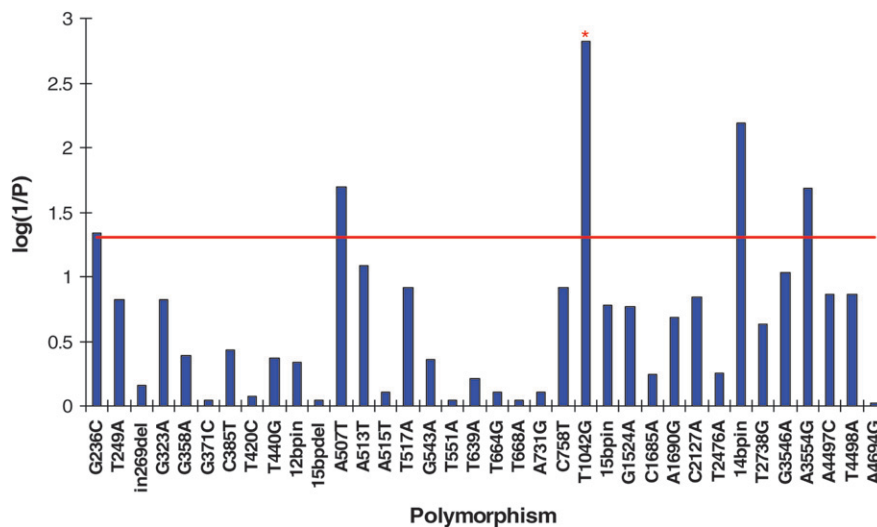


FIGURE 5.—Association of polymorphic markers at *Ddc* with variation in locomotor reactivity among 164 second chromosome substitution lines. The 36 polymorphic markers at *Ddc* are given on the x-axis, and *P*-values, transformed to $\log(1/P)$, from the ANOVA tests of association of the markers with locomotor reactivity are given on the y-axis. The red horizontal line denotes the nominal 5% significance level. Marker T1042G, denoted by *, is formally significant on the basis of a permutation test for individual markers, as well as the Bonferroni-corrected experiment-wise 5% significance level.

function in commissure choice in adults, where it is required for appropriate projections between mushroom bodies (BONKOWSKY *et al.* 1999). *drl* mutants show structural brain defects in the adult central complex and mushroom bodies and have defects in olfactory learning and memory (BOLWIG *et al.* 1995).

Variation in levels of dopamine (TUNNICLIFF *et al.* 1969; CONNOLLY *et al.* 1971; BURNELL and DALY 1982; MEEHAN and WILSON 1987; PENDLETON *et al.* 2002) and other biogenic amine neurotransmitters (O'DELL and BURNET 1988; LUNDELL and HIRSCH 1998) has been associated with variation in *Drosophila* locomotor activity. Four of the candidate genes affecting variation in locomotor reactivity between Ore-R and 2b affect dopamine and biogenic amine synthesis. *Catsup*, *Ddc*, and *Lim3* are members of the *Ddc* cluster (STATHAKIS *et al.* 1995). Mutations in many of the genes in this cluster show defects in cuticle formation, sclerotinization (hardening) or melanization of the cuticle, and formation of melanotic pseudotumors, all of which are hallmarks of abnormal catecholamine metabolism (WRIGHT 1996). *Catsup* encodes a metal ion transporter and is a negative regulator of tyrosine hydroxylase, which catalyzes the rate-limiting step of dopamine biosynthesis (STATHAKIS *et al.* 1999). Mutations in *Catsup* have significantly elevated tyrosine hydroxylase activity, resulting in unusually high levels of dopamine. *Ddc* is required in catecholamine and serotonin biosynthesis (MONASTIRIOTI 1999). *Lim3* is a homeobox gene that encodes an RNA polymerase II transcription factor required for motor neuron development (THOR *et al.* 1999; HOBERT and WESTPHAL 2000). *Acon* encodes a product involved in amino acid biosynthesis and the tricarboxylic acid cycle and is putatively required for biogenic amine synthesis (DRYSDALE *et al.* 2005).

The remaining four genes are novel candidate genes affecting locomotor behavior (*Trf*, *croI*, *stc*, and *tsh*). These genes all encode RNA polymerase II transcription factors that have been implicated in nervous system development. *Trf* is expressed in the embryonic central nervous system and in adult male germ cells (DAVIDSON 2003). *Trf* mutants are male sterile and exhibit an ether-induced leg-shaking behavioral phenotype (CROWLEY *et al.* 1993). *croI* is required for proper leg development and ecdysone-regulated gene expression during metamorphosis (D'AVINO and THUMMEL 1998). Mutants of *croI* specifically affect the number of ecdysone-induced genes, including genes controlling motor axon guidance and synaptogenesis (KRAUT *et al.* 2001). *stc* is the only known homolog of human transcription factor NF-X1 (STROUMBAKIS *et al.* 1996). *stc* is expressed in the embryonic central nervous system, where it is required for motor neuron development. *tsh* is a homeobox gene required for trunk segment identity and interacts with the Wnt-signaling pathway to modulate the response of Wnt signaling (GALLET *et al.* 1998; SHARPE *et al.* 2001).

These results show that “wild-type” allelic variation at genes previously identified through mutational analysis contributes to quantitative genetic variation, and conversely, quantitative genetic analysis is an effective method for functional genome annotation, identifying novel loci affecting behavior (SOKOLOWSKI 2001). Mutations at many essential genes are homozygous lethal; quantitative complementation tests are particularly useful in determining effects of these genes on complex traits.

All of the candidate genes affecting differences in locomotor reactivity between Ore-R and 2b have pleiotropic effects during development. Thus, these genes may act throughout development to lay the blueprint of the nervous system that is responsible for manifesting adult behavior. Further, consistent with our inference of substantial pleiotropy among the genetic architectures of multiple complex traits, many of the candidate genes affecting variation in locomotor reactivity in these lines have also been implicated as candidate genes affecting other complex traits. *Ddc* (DELUCA *et al.* 2003), *stc* (PASYUKOVA *et al.* 2004), *Catsup* (MACKAY *et al.* 2005), and *Lim3* (MACKAY *et al.* 2005) are all candidate genes affecting variation in longevity between Ore-R and 2b, and *croI* is associated with variation in starvation stress resistance between these strains (HARBISON *et al.* 2004).

There is some overlap of genes found in this QTL screen and those that have been implicated from mutational screens, but there are many genes with mutational effects on locomotor behavior (*e.g.*, *per*, *ey*, *oc*, and *hiw*) not detected in the QTL screen for variation in locomotor reactivity between Ore and 2b. This highlights the relationship between mutational and quantitative genetic approaches, where the former approach identifies genes involved in the manifestation of the normal behavior and the latter identifies genes affecting natural variation in the behavior (MACKAY 2001). These approaches are complementary because (1) genes required to produce the behavior may be so critical they do not vary in nature due to the selective constraint against changes that would greatly decrease overall reproductive fitness; (2) natural variants may specifically affect behavior, but the behavioral phenotypes may not be detectable in a mutagenesis study because null mutations are lethal or have a pronounced effect on a different phenotype; and (3) quantitative methods can detect genes only with naturally occurring allelic variation in the founder population.

Mutagenesis studies have identified single genes affecting locomotion, providing insight into the genetic framework in which the locomotor circuit is built. The definition of quantitative trait genes affecting locomotion will further characterize the components responsible for variation in nature, enhancing the picture of how genes and environment interact to produce complex behavior. It should be emphasized that, to understand behavior, one may have to go beyond understanding the function of individual components (genes and cells)

and venture into the territory of neural function and emergent properties (HOBERT 2003). More specifically, we ultimately need to determine how the temporal and spatial expression of neural networks produces movements characterizing adult behavior (SOKOLOWSKI 2001).

Linkage disequilibrium mapping: Identifying candidate genes contributing to variation in behavior between two strains conveys no information about the contribution of the candidate genes to naturally occurring variation in the trait. LD mapping is necessary to assess the contribution of variation at candidate genes to natural variation in locomotor behavior. We observed more significant associations of polymorphic markers at *Ddc* with variation in locomotor reactivity than expected by chance and also observed that population haplotypes derived from all genotyped markers were significantly associated with variation in locomotor behavior. Although LD in *Drosophila* generally decays rapidly with physical distance in normal regions of recombination (LONG *et al.* 1998; ROBIN *et al.* 2002), there was considerable LD throughout the 5.5-kb region, including the *Ddc* transcription unit (DELUCA *et al.* 2003). Since genotypes of all polymorphic sites were not determined, and LD did not decay markedly within the surveyed region, all we can deduce is that one or more polymorphisms at *Ddc*, or polymorphisms in LD with polymorphisms at *Ddc*, affect naturally occurring variation in locomotor behavior. Future efforts to identify causal polymorphisms must obtain complete sequences of all *Ddc* alleles, extending sufficiently 5' and 3' of the coding region for LD to decay across the sequenced region. In addition, larger samples will be required to detect rare and informative recombinants. Nevertheless, these data implicate variation in the synthesis of bioamines as a factor contributing to natural variation in locomotor reactivity.

A previous study identified three molecular markers in *Ddc* that were associated with variation in life span among the same set of chromosomes (DELUCA *et al.* 2003). None of the markers associated with variation in locomotor behavior were associated with variation in life span. *Catsup* is another positional candidate gene affecting dopamine synthesis, locomotor reactivity, and longevity. Similarly, different molecular polymorphisms in *Catsup* have been shown to be independently associated with natural variation in longevity and locomotor behavior (CARBONE *et al.* 2006). Thus, analysis of natural alleles of highly pleiotropic genes will prove invaluable in parsing the mechanisms underpinning the effects on constituent traits (SOKOLOWSKI 2001).

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