Genet. Res., Camb. (2002), **80**, *pp.* 205–213. *With 4 figures.* © 2002 Cambridge University Press
DOI: 10.1017/S0016672302005888 Printed in the United Kingdom

Unusual genetic architecture of natural variation affecting drug resistance in *Drosophila melanogaster*

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(*Received 26 March 2002 and in revised form 26 June 2002*)

Summary

Naturally occurring genetic variation was quantified for survival time of adult *Drosophila melanogaster* exposed to chronic ingestion of the drugs nicotine, caffeine, dopamine, tyramine and octopamine. Responses to nicotine, tyramine and octopamine were genetically correlated in both sexes, whereas caffeine response correlated with starvation resistance. However, there is also genetic variation that is specific for each of the drugs. Females tended to be more resistant than males to nicotine and caffeine but sex-by-genotype interactions were also seen for these drugs and for the response to dopamine. An unusual and complex genetic architecture was observed in crosses between lines with different responses to caffeine ingestion. Additive and dominance components were clearly seen from the analysis of F1 individuals, but increased female resistance to caffeine in backcross generations and increased male sensitivity in F2 generations confused the interpretation of possible epistatic contributions.

1. Introduction

Dissection of the genetic architecture of complex multifactorial traits, including drug response and behavior, is a complicated task. Studies associating genes with psychological conditions such as depression and alcoholism have been undertaken in mice and humans (McLeod & Evans, 2001) but have met with mixed success, and no clear picture of the relationship between genetic and pharmacological variation has emerged. The fruit fly *Drosophila melanogaster* offers many advantages as a model system for pharmacogenetic analysis because of resources such as the genome sequence, single-nucleotide polymorphism (SNP) databases and the availability of mutant lines. Flies can also be grown in controlled environments and their genetic background can be manipulated. Here, we present an initial characterization of the architecture of survival time of *Drosophila* adults upon chronic drug exposure.

Genetic approaches have already been used to study several behaviors in flies (Sokolowski, 2001), including learning (Dubnau & Tully, 1998), reflex behaviors in decapitated flies (Hirsh, 1998; Ashton *et al.*, 2001), heart rate (Johnson *et al.*, 1998; Robbins *et al.*, 1999), alcohol-induced behavior (Heberlein, 2000) and drug response (Zimmering *et al.*, 1977). Most of these studies have adopted mendelian genetic strategies but, given anecdotal reports of the effect of genetic background, it is also important to characterize the genetic architecture of naturally occurring variation for behaviors such as drug susceptibility. The drugs that we have studied include the biogenic monoamines dopamine, octopamine and tyramine, as well as caffeine and nicotine. Biogenic monoamines are neurotransmitters involved in synaptic transmission that are highly conserved in most animals (Walker *et al.*, 1996) and are believed to modify and regulate moods, personality traits and environmental responses, as well as having several physiological effects.

Previous studies have shown that monoamines affect locomotor activity (Hirsh, 1998) and heart rate (Ashton *et al.*, 2001), and are lethal when added to the diet. Complete loss of monoamine production is also lethal (Bainton *et al.*, 2000). The addition of caffeine to the diet of *Drosophila* causes an increase in the frequency of chromosome loss in larvae and has a mutagenic effect (Clark & Clark, 1968). Caffeine is

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also lethal to adult *D. melanogaster* (Zimmering *et al*., 1977), and, at lower concentrations, decreases longevity and fecundity in *Drosophila prosaltans* (Itoyama *et al*., 1998). Furthermore, caffeine sensitivity has been shown to vary across populations and between males and females (Zimmering *et al*., 1977), but the sources of these differences are not known. As with other organisms, it presumably has a polygenic basis, reflecting variations in factors such as the rates of drug absorption, metabolism and secretion (Evans & Relling, 1999).

The effects of nicotine in *Drosophila* have not been studied in depth but, in mice, nicotine has been shown to affect the release of dopamine and serotonin when added to drinking water (Pietila & Ahtee, 2000). Given that the receptors for these neurotransmitters are highly conserved across animal taxa (Hen, 1993; Fryxell, 1995), it is reasonable to suppose that there will be some similarities in drug response between flies and mammals. In the absence of receptor mutants in flies, we have initiated a quantitative genetic analysis of pharmacological variation and show here that sex, genotype and interaction effects are prevalent for survival time on several drugs, and that the genetic effects are largely independent for each drug.

2. Materials and methods

(i) *Lines and assays*

Parental lines used in this study consisted of 16 isofemale lines of *D. melanogaster*. These flies were collected from the Kerrytown Fruit Market (Ann Arbor, MI, USA) in 1996. The stocks were maintained in vials at a density of ~ 50 flies per generation on standard cornmeal medium with yeast at 25 °C on a 12-h light–dark cycle. For the crosses, adult flies between 1 and 3 days old were taken from vials with no more than 40 flies per vial to ensure optimal growth, and were kept on standard cornmeal media for a further 3 days before scoring. These mostly non-virgin flies were then separated by sex, and ten flies of each sex were placed separately in vials with drugged food. The number of live flies was counted every 12 h until all of the flies were dead. Ten replicate vials of each line and sex were scored.

Five drug treatments were tested: octopamine, Sigma O-0250 (20 mg ml^{-1}), tyramine T-7255 (20 mg ml^{-1}), dopamine H-8502 (40 mg ml^{-1}), nicotine N-3876 (3 μl ml^{-1}) and caffeine C-0750 (10 mg ml^{-1}). Each drug was dissolved directly in molten fly food just prior to addition to empty vials. Drugged food was used between 1 and 4 days after preparation. Starvation resistance on agar medium was also measured as a control for variation in overall fitness among the lines and sexes.

Crosses of the extreme lines for caffeine resistance were produced to study the genetic architecture of drug resistance. F1 and F2 generations of the extreme parental lines (high × low) were assayed, as were the reciprocal crosses. Crosses were also made among high × high (A3 and A6), low × low (A2 and A19), no-sex-effect × no-sex-effect (A7 and A17), and sex-effect (A2 or A3) × no-sex-effect combinations of lines. In each case, one male and one virgin female were used to found five independent replicates, from which two sets of ten males and ten females were assayed for time to mortality. The replicates were established over several months, involving independently prepared food and drug batches.

(ii) *Statistical analysis*

ANOVA was performed using SAS Proc GLM on the survival time for each individual fly, computed as the midpoint of the 12-h interval in which the fly died. Vial effects were included in the statistical model to ensure that among fly variation was the major source of error. Age at death was modeled with a parametric mean, μ, sex as a fixed effect, and vial and line as random effects:

$$\text{Age at death} = \mu + \text{Line} + \text{Sex} + \text{Sex} \times \text{Line} + \text{Vial(Sex} \times \text{Line)} + \text{Error}.$$

Genetic correlations (r) between the drug treatments for each sex separately (Table 2) were calculated according to Robertson (1959) as:

$$r_{\text{drug1, drug2}} = (\text{MS}_{\text{L}} - \text{MS}_{\text{D}\times\text{L}}) \div (\text{MS}_{\text{L}} + \text{MS}_{\text{D}\times\text{L}} - 2.\text{MS}_{\text{error}})$$

where MS represents the mean square in a two-factor ANOVA for the residual error (error), line (L) or drug × line (D × L) interaction.

3. Results

(i) *Genetic variation for drug resistance*

Sixteen isofemale lines of *D. melanogaster* were assayed to gauge the amount of genetic variation present for survival time on five drug treatments. Flies between 3 and 6 days old were separated by sex and placed in vials containing standard cornmeal media mixed with one of the drugs. For some of the drugs, behavioral changes such as grogginess (nicotine and dopamine) or hyperactivity (caffeine) were observed within 8 h of transfer to the drug food. The number of flies that were alive in each vial was counted every 12 h until all of the ten flies in each vial were deceased. Concentrations of the drugs were chosen on the basis of preliminary titration experiments (data not shown) such that the mean survival time for most lines ranged

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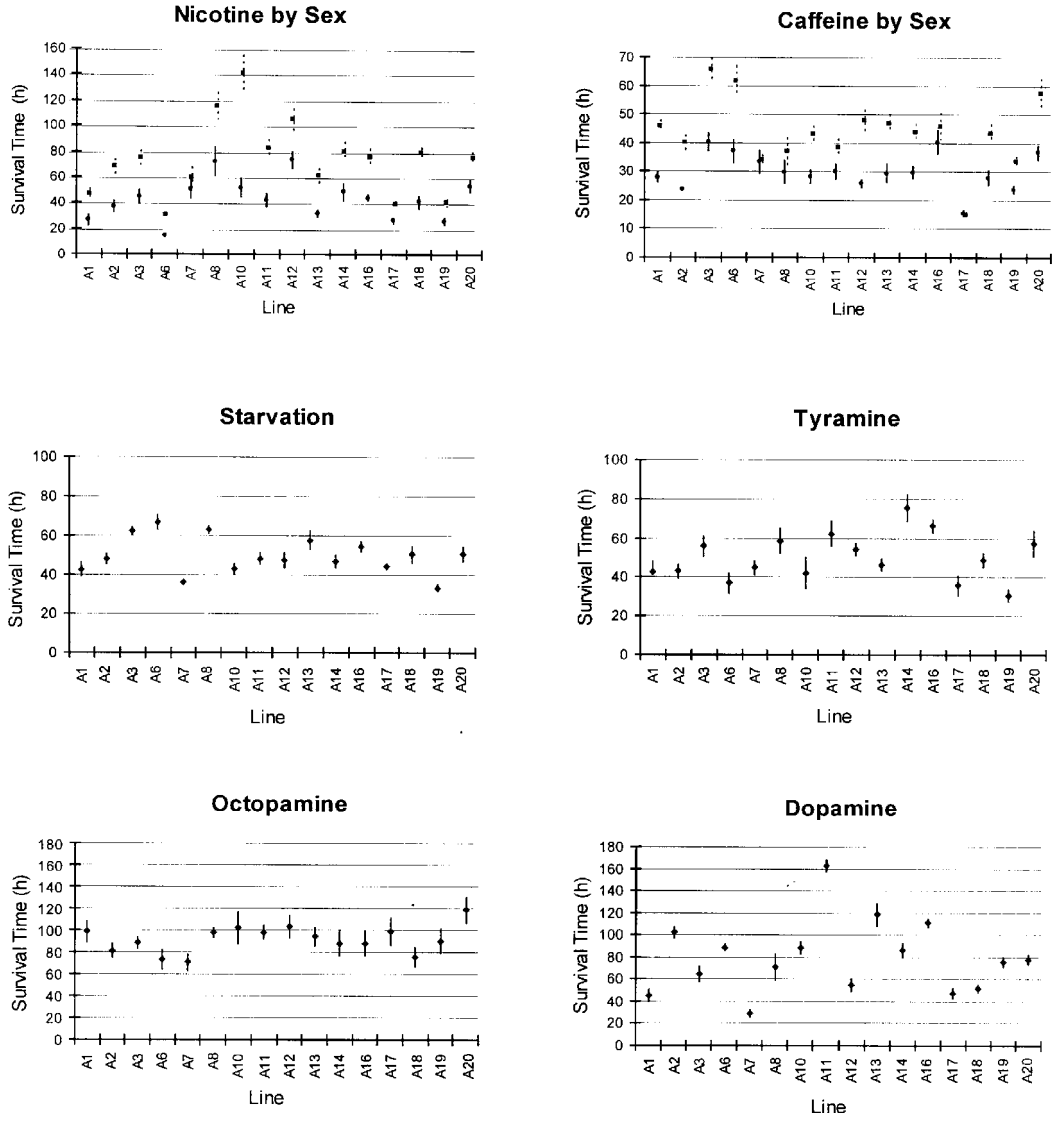

Fig. 1. Differences among lines for time to mortality upon chronic drug exposure. All plots show the mean time of death for 16 isofemale lines. Error bars indicate two standard deviations for vial effects on either side of the mean survival time for the line. The top two panels show the nicotine and caffeine responses by sex (female, squares and dashed error bars; male, circles and solid error bars), in numerical order of line identifiers to facilitate direct comparison with the other treatments. The bottom four panels show the mean survival time for both sexes together (because no overall effect was observed) for response to starvation, tyramine, octopamine and dopamine.

between 24 and 96 h. Line means are shown for starvation media, tyramine, octopamine and dopamine, and by sex for nicotine and caffeine, in Fig. 1. The range of variation was clearly greater for the latter three drugs. Very similar mean survival times for each line and sex were inferred from the point of inflexion of Kaplan–Meier survival plots. Age at death was approximately normally distributed within lines for all drugs.

ANOVA was used to assess the significance of the contributions to the variation of genotype, sex, genotype-by-sex interaction, and within and among vial effects. The F ratios associated with each effect and their significance levels are indicated along with the estimated variance component for the random effects in Table 1. For nicotine and caffeine, genotype (line), sex and the interactions between these factors were all highly significant. In general, females are twice as resistant to nicotine as males and 50 % more resistant to caffeine, so that the absolute differences between the sexes tends to increase with overall levels of resistance. There was no overall effect of sex on the response to the other drugs, although a small interaction effect (largely attributable to a few lines) was observed for dopamine and tyramine. Genotype differences were only marginally significant for the monoamines tyramine and octopamine, partly as a consequence of relatively large between-vial differences for these drugs. The heritability of survival time on each drug was estimated in Table 1 as half the

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Table 1. *Significance of variance components for drug effects.* F *ratios and significance are followed (in brackets) by the proportion of the variance that is explained by each random effect*

	Nicotine	Caffeine	Dopamine	Tyramine	Octopamine	Starvation
Line (L)	$5{\cdot}46^{b}$ (0·10)	$4{\cdot}47^{b}$ (0·04)	$15{\cdot}10^{c}$ (0·41)	$5{\cdot}76^{b}$ (0·29)	$3{\cdot}23^{a}$ (0·05)	$11{\cdot}87^{c}$ (0·21)
Sex (S)	$40{\cdot}86^{c}$	$39{\cdot}45^{c}$	$0{\cdot}47^{NS}$	$0{\cdot}12^{NS}$	$0{\cdot}01^{NS}$	$44{\cdot}80^{c}$
L × S	$12{\cdot}76^{c}$ (0·36)	$13{\cdot}44^{c}$ (0·25)	$11{\cdot}34^{c}$ (0·05)	$6{\cdot}88^{c}$ (0·10)	$1{\cdot}88^{a}$ (0·02)	$10{\cdot}32^{c}$ (0·18)
Vial (L × S)	$1{\cdot}66^{c}$ (0·03)	$0{\cdot}81^{NS}$ (0)	$0{\cdot}96^{NS}$ (0)	$3{\cdot}70^{c}$ (0·13)	$2{\cdot}94^{c}$ (0·15)	$0{\cdot}79^{NS}$ (0)
Error	(0·51)	(0·71)	(0·54)	(0·48)	(0·78)	(0·61)
Heritability	0·23	0·15	0·23	0·26	0·11	0·20

^{NS} not significant.
^{a} $0{\cdot}01 < P < 0{\cdot}05$.
^{b} $0{\cdot}001 < P < 0{\cdot}01$.
^{c} $P < 0{\cdot}001$.

proportional contribution of the genotype and genotype-by-sex variance components, and ranges up to 0·23. As reported by others (Hoffmann *et al*., 2001; Kennington *et al*., 2001; Harshmann *et al*., 1999), starvation resistance also shows considerable genetic variation, the heritability of which is higher than that of drug resistance in our experiments because of the low among-individual variance, despite the similar distributions of line means.

A major source of potential experimental error in these studies is the consistency of drug delivery to flies. For dopamine, nicotine and caffeine, vial effects were either insignificant or contributed just a few percent of the total variance, suggesting that these drugs were reproducibly dissolved in the cornmeal medium, which was prepared in multiple batches at different times. Vial effects were higher for tyramine, consistent with the low solubility of this compound, and octopamine, reflecting the general absence of sex and genotype effects for ingestion of this drug. The residual error term in each treatment indicates differences among flies within vials and presumably includes effects of ingestion as well as physiological responses to the drugs. There is no way to tease these apart, but these error terms are in the same range as those observed for many morphological traits. Even for drugs such as octopamine, which have marginally significant line effects and high error rates, clear differences in survival can still be observed between extreme lines.

(ii) *Correlation among drug responses*

To determine whether genotype-specific drug responses merely reflected generalized differences in fitness among lines, owing to, for example, fixation of deleterious alleles, the phenotypic and genetic correlations among lines were examined. Line means that have been normalized to a standard deviation of one and a mean of zero are plotted in Fig. 2, which is characterized by the prevalence with which line means cross. Associated genetic correlation coefficients are given in Table 2 for each sex, with females above the diagonal and males below it. In general, correlations between treatments are low, further implying that the genetic differences among lines that contribute to extreme drug resistance or sensitivity are different for each drug. A remarkable example of this is line A11, which is hyper-resistant to dopamine alone of the five drugs studied here.

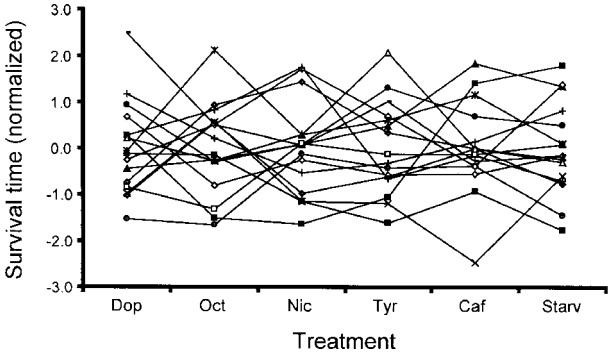

Fig. 2. Normalized phenotypic correlation among lines. The mean time of death for both sexes is pooled for each isofemale line after normalization by subtracting the overall mean and dividing by the standard deviation for each drug. Lines join normalized phenotypic means for each line. Correlated responses produce parallel lines, whereas crossing of lines indicates low or absent correlation. The order of treatments was chosen to ensure that the treatments with the most similar responses are adjacent to one another. The associated genetic correlations computed from the variance components are indicated in Table 2.

However, there are also trends that suggest some common susceptibility factors. First, caffeine and tyramine sensitivity are correlated with starvation resistance, possibly indicating that some of the response is due to the avoidance of food laced with these drugs. Starvation is not the sole cause of caffeine-induced mortality, because several of the lines survive for longer on the drugged food than on agar. Furthermore, these drugs have direct effects on viability because other lines have reduced mortality upon

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Table 2. *Genetic correlations among drug treatments by sex. Females are shown above the diagonal and males below*

	Nicotine	Caffeine	Dopamine	Tyramine	Octopamine	Starvation
Nicotine		0·062	0·078	0·282	0·330	0·052
Caffeine	0·111		0·159	0·492	−0·046	0·659
Dopamine	−0·026	0·041		0·225	0·058	0·169
Tyramine	0·488	0·265	0·268		0·149	0·453
Octopamine	0·442	−0·036	0·159	0·089		−0·007
Starvation	0·057	0·473	0·117	0·039	−0·238	

chronic drug exposure. Second, two of the lines (A17 and A19) are among the most sensitive to nicotine, caffeine, tyramine and dopamine as well as to starvation, suggesting poor general metabolic performance. In fact, one of these lines has since been lost owing to low fecundity. At the other end of the spectrum, it is noteworthy that the two lines that are most resistant to caffeine and starvation (A3 and A6) are relatively sensitive to nicotine, whereas those most resistant to nicotine have intermediate sensitivity to the other drugs. Third, a relatively high correlation was observed between nicotine and tyramine or octopamine, suggesting that these drugs might operate through related physiological systems.

(iii) *Additivity and dominance of the caffeine response*

To begin to dissect the genetic architecture of drug sensitivity, we performed a series of crosses between lines with extreme responses to caffeine. Caffeine was chosen for further study owing to the highly significant genotype, sex and interaction effects, and the absence of vial effects for response to this drug. Crosses were designed to assess the degree of dominance for both the overall resistance or sensitivity to caffeine, and for the sex specificity of the response.

As expected, the survival time of F1 progeny of crosses between resistant (A3 or A6) and sensitive (A19 or A2) isofemale lines was intermediate between that of the two parents (Fig. 3). After addition of reciprocal backcross and F2 data, generation-means analysis (Kearsey & Pooni, 1996) of these resistant-by-sensitive crosses was performed for males and females separately but, because no consistent explanatory model was observed, the results were generally uninformative and are not shown. Among-individual variance increased in the F2 generation relative to the F1 for some crosses, but the effect was too inconsistent to provide a reliable estimate of the number of genes that contribute to the variation. This might reflect insufficient power of the analysis given the high individual variability, or might reflect an effect of residual genetic variation in the inbred isofemale lines, and is also consistent with the possibility that drug sensitivity is influenced by many loci with small effects.

Crosses between the two resistant and between the two sensitive lines also indicate that different loci contribute to survival time on caffeine even in isofemale lines with similar phenotypes. In both cases, F1 progeny failed to reproduce the extreme phenotype of the two genetically distinct parents, such that a cross between resistant lines (A3 and A6) gave rise to relatively sensitive F1 means, whereas a cross between two sensitive lines (A2 and A19) gave rise to resistant F1 flies. The possibility of epistatic interactions contributing to drug sensitivity is suggested by the extraordinary observation that F1 female progeny of the two sensitive lines actually have resistance levels similar to those of the most resistant inbred lines. This was confirmed by highly significant dominance by dominance parameters in the multiple regression generation means models for these crosses.

(iv) *Sex specificity of the caffeine response*

Characterization of the genetic interactions affecting caffeine-induced mortality is further complicated by highly unusual sex-specific effects in the F2 and backcross generations of all crosses involving at least one sensitive (low survival time) parent. Backcross females in both directions are uniformly more resistant than even the resistant parent (Figs 3, 4). Just as strangely, F2 males are uniformly more sensitive than even the most sensitive parent. These results were repeatedly observed in replicates set up at different times and cannot be attributed to a batch effect of the food because, in each case, the opposite-sex progeny derived from the same parents behaved as predicted. In separate analyses, food batch was also found not to affect survival times significantly (not shown).

The unusual sex-specific nature of the response in F2 and backcross individuals was also observed in crosses designed to explore the nature of the genotype-by-sex interaction (Fig. 4). Three lines of evidence imply that the degree of sex specificity is superimposed on the overall drug response. First,

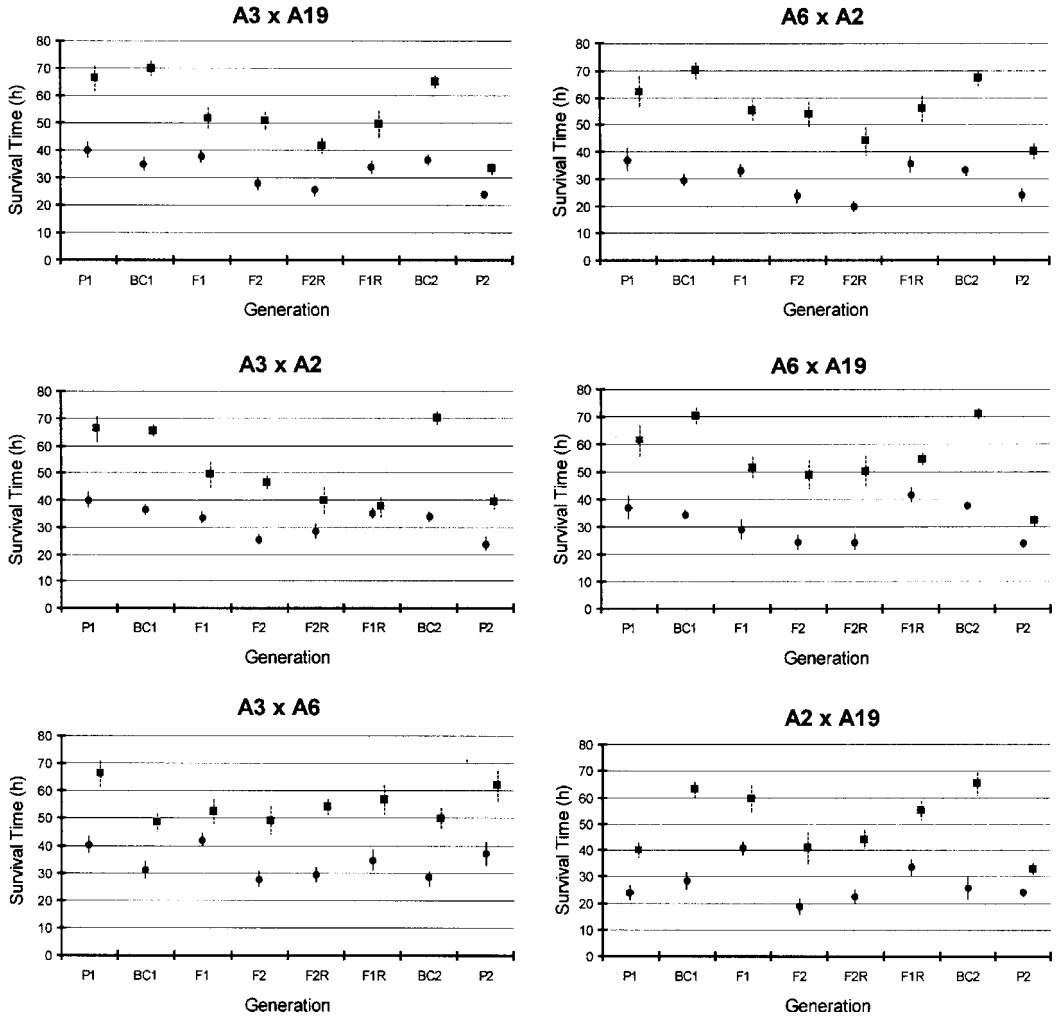

Fig. 3. Generation means of caffeine sensitivity in crosses between lines showing a difference between the sexes. Female survival times in hours are plotted as squares (error bars indicate two standard deviation units from a total of ten replicate vials for each generation), males as circles. The two parental lines for each cross are plotted at either end, with the F1 (left-hand female by right-hand male), F1R (the reciprocal cross, right-hand female by left-hand male) and F2 derived from each F1 in the middle. The reciprocal backcrosses (BC1 and BC2) are plotted adjacent to the respective parents (each backcross is pooled from all four possible crosses of parental male or female to F1 or F1R female or male; no cross effects were seen (data not shown)). F1 and F2 progeny are expected to show similar mean phenotypes in the absence of maternal, X-linked or epistatic effects. Notice the increase in survival time of backcross females, irrespective of the backcross parent (except in the cross involving two resistant lines, A3 and A6), and the general decrease in survival time of F2 males.

the two sexes are genetically correlated for all drug responses (Fig. 1). Second, a few of the lines have almost no sex effect on caffeine response, whereas the remainder have a large difference (a similar claim could be made for the nicotine response). This might imply that one or a few loci independently regulate the degree of sex specificity. Third, the sex difference is lost in the F1 generation of three of the four crosses between sex-specific (A2 or A3) and phenotypically similar non-sex-specific (A7 or A16) lines. The retention of sex specificity in the cross of A3 females with A16 males might imply a maternal effect, because it is the females that show resistance to caffeine in the F1, rather than the males (which receive different sex-chromosome genotypes). However, the unusual female backcross and male F2 effects documented above also appear in these crosses, although these results are, for simplicity, not included in Fig. 4. We do not have a good explanation for these effects, which defy standard quantitative genetic models.

4. Discussion

(i) *Genetic architecture of drug sensitivity and resistance in* Drosophila

Given the recent upsurge in interest in human pharmacogenetics, there is a pressing need to develop model systems for the study of the genetic basis of pharmacological variation. Although the biochemical

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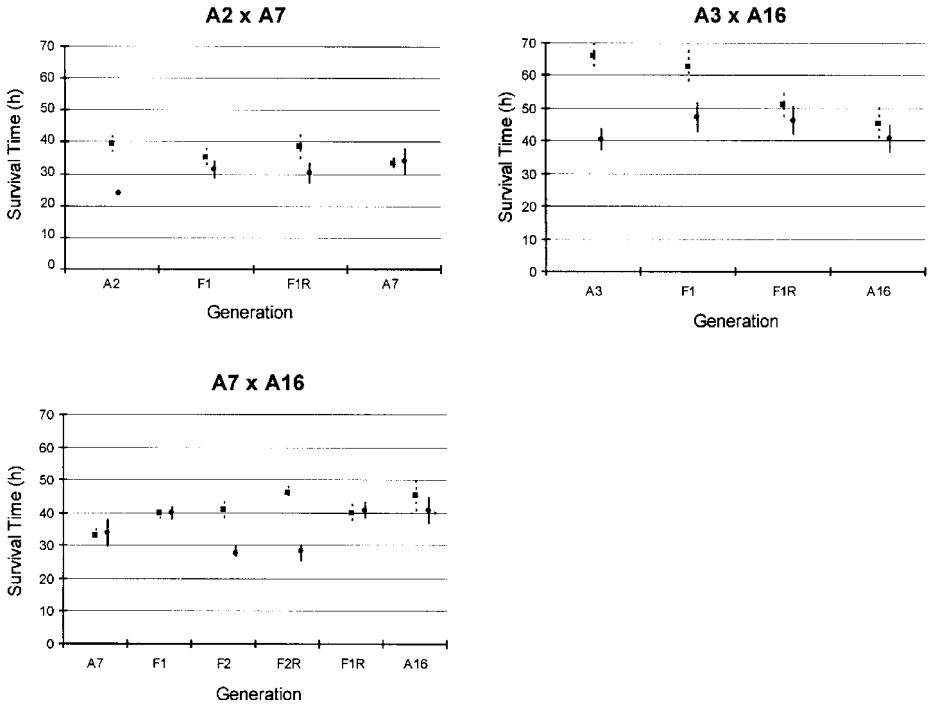

Fig. 4. Generation means of caffeine sensitivity in crosses between lines in the absence of a sex difference for at least one parent. The plots are the same as in Fig. 3, except that the backcross generations are removed in order to highlight the features discussed in the text. The top two panels show crosses between parents with similar overall caffeine sensitivity, one with and one without a sex-effect. The bottom panel shows the restoration of a sex effect in the F2 of crosses between two similar lines with no sex effect.

pathways through which monoamine neurotransmitters are metabolized are fairly well characterized in *D. melanogaster*, remarkably little is known in flies about the genetics of neurotransmitter-receptor function or, more generally, of drug activity. This is perhaps because much of the behavioral research on this organism has been driven by forward-genetic screens for perturbation of specific behaviors such as learning and vision. Nevertheless, the demonstration that there is genetic variation in flies for behaviors such as foraging and ethanol tolerance (Sokolowski, 2001; Bainton *et al.*, 2000), and for pharmacological traits such as heart rate and autonomic 'headless' behaviors (Ashton *et al.*, 2001), has encouraged us to initiate a genetic dissection of response to chronic drug exposure. In the absence of mendelian mutants, we have started by characterizing the levels of naturally occurring variation, because this will form a baseline for interpretation of the effects of gene knockouts.

A basic question in behavioral genetics is whether specific phenotypes can be disrupted by single mutations of large effect, or whether many mutations of small effect have diverse and pleiotropic effects on a variety of traits. Several mutations have been uncovered in key genes involved in signal transduction that have remarkably specific consequences, such as disruption of a specific step in learning or switching larval feeding behavior (Goodwin *et al.*, 1997; Osborne *et al.*, 1997). By contrast, mutations in enzymes that are involved in the biosynthesis and degradation of monoamines are known primarily from their effect on pigmentation (for example *ebony*, which encodes β-alanyl dopamine synthase, and *pale*, which encodes tyrosine hydroxylase) and have not been shown to disrupt pharmacology. Similarly, only a couple of the neurotransmitter-receptor genes that have been identified have been associated with lesions, and these were isolated by molecular rather than phenotypic screens, suggesting that receptor mutant phenotypes are likely to be subtle. One report has implicated the biological-clock pathway in modification of cocaine sensitivity (Andretic *et al.*, 1999), but these results might be confounded with the effects of genetic background in the experiment. One of the aims of this study has been to define a trait that might be suitable for genetic screens for aberrant response to drug exposure.

Our key findings can be summarized as follows. (i) There is ample naturally occurring genetic variation for survival time upon chronic ingestion of several drugs including nicotine, caffeine, dopamine and tyramine, although the evidence in relation to octopamine was equivocal. (ii) Survival time might not be the most biologically meaningful trait but it is easy to score and has moderate heritability and good repeatability, all of which make it simpler for genetic analysis than assays that involve measuring behavioral responses.

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In our experience, responses to volatilized drugs are highly variable from day to day and so are hard to quantify precisely. (iii) The correlations among drug responses are moderate to nonexistent, indicating that much of the genetic variation is specific for one or a few of the drugs. (iv) Females tend to be more resistant than males to nicotine and caffeine, and sex-by-genotype interactions are also seen for these drugs and for the response to dopamine. (v) Preliminary dissection of differences in caffeine sensitivity suggests a complex genetic architecture with many genes of small effect and some dominance for resistance.

(ii) *Unusual features of the caffeine response*

Dissection of the genetic architecture of behavioral responses in line crosses is complicated by relatively large vial and among-individual variance. Our results for caffeine resistance, similar to those of Kennington *et al*. (2001) in their analysis of the correlated trait of starvation resistance, failed to reveal a consistent picture of the extent or nature of epistatic effects, although these certainly seem to be present. Further dissection of this phenomenon is probably best approached by fine structure quantitative-trait-locus mapping of the loci that are responsible for modulating the survival times on each drug. Unfortunately, however, the most direct interpretation of our data is that drug sensitivity is affected by many genes of small effect. This conclusion is supported by the preliminary results of a screen for P-element insertions in an isogenic background, which suggests that mutations in at least 5% of the genome might affect drug sensitivity, often in a sex- and drug-specific manner (A. P. Wagoner & G. G., unpublished). Cloning of the genes associated with these insertions and analysis of interactions among the loci will complement classical quantitative genetic dissection of drug resistance.

The sex specificity of the response to caffeine is particularly intriguing: F2 progeny of crosses between any pair of lines always resulted in much reduced male survival times, whereas backcross females showed elevated resistance. This was true even in the case of a cross between two isofemale lines that did not show any difference between the sexes. Sex effects for caffeine resistance have been reported previously, with females of some mutant strains also living longer than males (Zimmering *et al*., 1977; Itoyama, 1998). Various explanations have been proposed for this, including differences in body size and repair efficiency between males and females. Caffeine is known to increase the frequency of chromosomal loss in both males and females, but no evidence has been found that caffeine can induce any sex-linked lethal mutations (Clark & Clark, 1968). Our interpretation is that the difference in response between the sexes might be superimposed on the general ability of the flies to resist the drugs. Nevertheless, for all of the drugs, there is a high correlation between the sexes, indicating that common factors influence the response within a line. Investigation of how sex-specific factors interact with these loci will be an essential element of dissection of drug responses, and has implications for understanding the evolutionary dynamics of variation affecting neurotransmitter activity.

We thank Elizabeth Sall and Andrea DeWees for assistance in the early stages of this project, which was funded by an NIH grant to G. G. (P01 GM45344). R. C. was supported by a fellowship from the W. M. Keck Program in Behavioral Biology at NCSU.

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