Behavior Genetics, Vol. 35, No. 3, May 2005 (© 2005)
DOI: 10.1007/s10519-005-3216-1

Drosophila soluble guanylyl cyclase mutants exhibit increased foraging locomotion: behavioral and genomic investigations

Craig A. L. Riedl,[1] Scott J. Neal,[1,2] Alain Robichon,[3] J. Timothy Westwood,[1,2] and Marla B. Sokolowski[1,4]

Received 1 Dec. 2004—Final 1 Feb. 2005

Genetic variation in the gene *foraging* (*for*) is associated with a natural behavioral dimorphism in the fruit fly, *Drosophila melanogaster*. Some larvae, called 'rovers', have increased foraging locomotion compared to others, called 'sitters', and this difference is directly related to *for*-encoded cGMP-dependent protein kinase (PKG) activity. Here we report that larvae with mutations in the gene *dgcα1*, which encodes a soluble guanylyl cyclase (sGC) subunit, have increases in both PKG activity and foraging locomotion. This is contrary to our original prediction that, based on the role of sGC in the synthesis of cGMP, *dgcα1* mutant larvae would have deficient cGMP production leading to decreased PKG activation and thus reduced larval foraging locomotion. We performed DNA microarray analyses to compare transcriptional changes induced by a *dgcα1* mutation in both rover and sitter wildtype genetic backgrounds. In either background, we identified many genes that are differentially transcribed, and interestingly, relatively few are affected in both backgrounds. Furthermore, several of these commonly affected genes are enhanced or suppressed in a background-dependent manner. Thus, genetic background has a critical influence on the molecular effects of this mutation. These findings will support future investigations of *Drosophila* foraging behavior.

KEY WORDS: behavior; cGMP; *Drosophila*; foraging; guanylyl cyclase; genetic background; microarray.

INTRODUCTION

Wild populations of the fruit fly, *Drosophila melanogaster*, exhibit a natural dimorphism in larval foraging behavior (Sokolowski, 1980). Some larvae, called 'rovers', travel significantly farther while feeding on a nutritive yeast paste than others, called 'sitters'. When tested on a non-nutritive substrate, there are no significant differences in the distances moved, indicating similar general locomotor abilities in the two variants. Foraging behavioral variation is strongly influenced by allelism in the gene, *foraging* (*for*) (de Belle *et al.*, 1989). The *for* gene encodes a cyclic guanosine-3′,5′-monophosphate (cGMP) dependent protein kinase (PKG) (Kalderon and Rubin, 1989; Osborne *et al.*, 1997). The rover phenotype is associated with relatively higher PKG activity, conferred by the dominant allele for^R, while animals with for^s alleles have lower PKG activity and are sitters (de Belle *et al.*, 1989; Osborne *et al.*, 1997).

Our understanding of the neuromolecular mechanisms by which *for* genetic variation is translated into behavioral effects is still developing. An important step forward is the identification of other genes and proteins that may interact with *for*-PKG to

[1] Department of Biology, University of Toronto at Mississauga, 3359 Mississauga Road, Mississauga, ON, L5L 1C6, Canada; e-mail: msokolow@utm.utoronto.ca
[2] Canadian *Drosophila* Microarray Centre, Department of Biology, University of Toronto at Mississauga, 3359 Mississauga Road, Mississauga, Ont., L5L 1C6, Canada.
[3] INRA Centre de Recherche de Sophia-Antipolis, 400 route des Chappes, BP 167, 06903, Sophia-Antipolis Cedex, France.
[4] To whom correspondence should be addressed at Department of Biology, University of Toronto at Mississauga, 3359 Mississauga Road, Mississauga, Ont., Canada L5L 1C6.

produce the phenotype. Genetic screens for mutations that modify foraging behavior have had some success (Pereira *et al*., 1995; Shaver *et al*., 2000) but identifying the genes involved and their protein products can be difficult. Another approach is to test the behavior of larvae with mutations in candidate genes that encode proteins reported to affect PKG signaling in other systems. Since PKG is activated by cGMP, one potential candidate protein is guanylyl cyclase (GC), which catalyses the formation of cGMP (Hardman and Sutherland, 1969). Here we report the results of our investigations into the behavioral effects of mutations in the gene *dgcα1*, which encodes a *Drosophila* soluble guanylyl cyclase (sGC) alpha subunit (Liu *et al*., 1995; Shah and Hyde, 1995; Yoshikawa *et al*., 1993).

Guanylyl cyclases (GC) form a family of widely expressed enzymes that catalyze the formation of cGMP from GTP. GC's can be divided into two main classes: soluble (sGC) and particulate (pGC) (for recent reviews of GC see Lucas *et al*., 2000; Wedel and Garbers, 2001). pGC's are membrane-integrated receptors with many diverse isoforms and functions (Garbers, 1999). There are two classes of sGC's: the typical NO-sensitive form (reviewed in Koesling *et al*., 2004), and the newly identified atypical NO-insensitive sGC's (Morton, 2004a; Nighorn *et al*., 1999; Prabhakar *et al*., 1997). Typical sGC enzymes are cytoplasmic and can be stimulated by the gaseous neurotransmitters NO and, less effectively, CO (Stone and Marletta, 1994). They are heterodimeric proteins composed of one α and one β subunit, and activation by NO is mediated by a prosthetic heme moiety (Koesling, 1998). In contrast, atypical sGC may form homodimers and are not sensitive to, or are only poorly activated by, NO (Morton, 2004a). Recent studies in the nematode worm, *Caenorhabditis elegans*, have indicated a role for atypical sGC in foraging behaviors (Cheung *et al*., 2004; Gray *et al*., 2004) and demonstrate that the enzyme is responsive to atmospheric O_2 levels (Gray *et al*., 2004). Three genes encoding atypical, NO-insensitive sGC's have been identified in the *D. melanogaster* genome (Morton, 2004a), and recent *in vitro* work suggests that they may likewise function as molecular O_2 sensors (Morton, 2004b). The implications, *in vivo*, of this O_2 sensitivity are not yet understood.

Since PKG activity is sensitive to cellular cGMP levels, the *dgcα1* protein product, an α subunit of a NO-sensitive sGC (Liu *et al*., 1995; Shah and Hyde, 1995; Yoshikawa *et al*., 1993), may have a role in the regulation of PKG, and thus may influence foraging behavior. *dgcα1* is expressed in neurons and preliminary immunohistochemical staining has shown that it is present in the larval brain (J. Ewer, *pers. comm*.). It also has a neurodevelopmental role resulting in defective visual systems in adult flies with *dgcα1* mutations (Gibbs *et al*., 2001). We predicted that the *dgcα1* mutations could also affect larval foraging behavior, since a loss of GC activity, leading to lower cellular cGMP and a corresponding reduction in PKG activity, could result in more sitter-like larval foraging behavior. Alternatively, DGCα1 may not interact with the *for* signal transduction cascade and thus no difference in PKG activity or foraging behavior would result.

Interestingly, the *dgcα1* mutant phenotypes failed to support either of our predictions. Using three independently generated mutant *dgcα1* alleles ($dgc\alpha1^1$, $dgc\alpha1^3$, and $dgc\alpha1^5$) (Gibbs *et al*., 2001), which we crossed into different rover and sitter genetic backgrounds, we observed that the mutant larvae exhibit increased foraging locomotion and have higher PKG activities compared to wild-type controls. To further probe the mechanisms of this phenotype, we performed microarray analyses of the transcriptional changes induced by a *dgcα1* mutation in both wildtype rover and wildtype sitter genetic backgrounds. In both backgrounds the mutation induces altered transcription of several genes. Remarkably, of the total number of genes exhibiting altered transcription in either background, only a small subset were commonly affected in both genetic backgrounds, and several of these were changed in a background-dependent manner.

METHODS

Fly Stocks

Three *Drosophila* EMS-induced *dgcα1* mutations, two of which were generated in a ca^1 marked strain: $dgc\alpha1^1$and $dgc\alpha1^3$; and one, $dgc\alpha1^5$, which was induced in a bv^1 marked strain, as well as their respective controls (ca^1 or bv^1 marked strains) were used (Gibbs *et al*., 2001). For information about the marker mutations see Lindsey and Zimm (1992). The *dgcα1* gene is located at polytene position 99B7 on chromosome *3* (Liu *et al*., 1995; Shah and Hyde, 1995). Because the marker mutations are very close to *dgcα1* (ca^1 is at polytene position 99B8-10 and bv^1 is at polytene position 100B2-C3) and due to the lack of an easily selectable phenotype for the *dgcα1* mutations, we did not endeavour to remove the marker mutations by recombination. Mutant and control

chromosomes were substituted into different genetic backgrounds with known *for* genotypes (wildtype rover (for^R) and wildtype sitter (for^s), and an induced mutant sitter (for^{s2}) which was induced on a for^R background) (de Belle *et al*., 1989). Because the chromosomes containing these *for* alleles were not coisogenic, variability potentially exists at many background genetic loci. As a result, the rover and sitter strain genetic background differences are likely to arise from more than just allelic variation at *for*. Here we refer to the different genetic backgrounds by the *for* allele contained, e.g., the wildtype rover genetic background is called the for^R background. Thus, for example, the genotype of the $dgc\alpha1^1$ mutant flies after chromosome substitution into a natural rover background is for^R; $dgc\alpha1^1 ca^1$ and its control is for^R; ca^1. Similar genotypes were generated using for^s and for^{s2} genetic backgrounds and the other *dgcα1* mutant and control chromosomes. After the crosses were complete, the presence of the mutations in the relevant strains was confirmed by Western Blot analyses using standard techniques (Sambrook and Russel, 2001) using two different antibodies kindly provided by W.L. Pak (Purdue) and C.S. Zuker (UCSD) (Gibbs *et al*., 2001; Liu *et al*., 1995). All strains were reared in 170 mL plastic bottles containing 40 mL of a standard yeast–agar–sucrose medium and maintained at 25°C in 12L:12D (standard conditions).

Behavioral Analysis

Larval foraging behavior was quantified as described in Sokolowski *et al*. (1997). Briefly, mid-third instar larvae were collected, quickly washed with distilled water, and placed individually in shallow wells (0.5 mm deep × 86 mm diameter) containing a nutritive yeast paste (approximately 2 water: 1 yeast, by weight). The wells were then covered with Petri dish lids to prevent dehydration and the larvae were allowed to feed freely for 5 min. After this, the trails left by the larvae as they moved through the paste were traced onto the Petri dish lid. The lengths of paths were then recorded for analysis. General larval locomotion was assayed in a similar manner except that the larvae were placed on a non-nutritive agar medium for 5 min while their movements were traced.

PKG Activity Assay

PKG activity was assayed as described elsewhere (Osborne *et al*., 1997). Briefly, 10 adult heads were homogenized in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, PMSF (1 μg/ml), leupeptin (1 μg/ml), aprotinin (4 μg/ml), and 0.05% Triton X-100. The larval brains were homogenized by repeated pipetting in an Eppendorf tube with the above homogenization buffer. Samples were then microcentrifuged for 5 min and the supernatant was removed for analysis of cGMP-dependent protein kinase activity (Casnellie, 1991; Fiscus and Murad, 1988). To reduce cross-activation, we used a specific inhibitor of the cAMP dependent protein kinase (Drain *et al*., 1991). The PKG substrate (histone H2B) was covalently bound to *N*-hydroxysucinimidyl sepharose (H8280) allowing extensive washing while reducing loss. This mild modification permitted us to drastically increase the specific activity of ATP to 10,000 cpm/pmol final (5 μM concentration) while minimizing contamination by background activity. At final concentration, the reaction mixture contained: 25 mM Tris (pH 7.4); 1 mM EDTA, 2 nM EGTA; 7 mM magnesium acetate; 0.5 mg/ml histone H2B; 1 nM rabbit cAMP-dependent protein kinase inhibitor (PKI P-0300; Sigma) (K_i: 3 nM); 10 μM cGMP (Sigma); and 5 μM adenosine 5′-triphosphate (ATP) with γ^{32}P-ATP at 5000 cpm/pmol in a final volume of 100 μl. ^{32}P-ATP was supplied by New England Nuclear (NEG-002a, 300 Ci/mmol).

NO-Induced GC Activity

NO-stimulated GC activity was assayed as described in Zhang *et al*. (2002). Briefly, 100 adult fly heads were homogenized on ice in 1 ml 25 mM Tris–HCl (pH 7.2), 1 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, PMSF (1 μg/ml), leupeptin (1 μg/ml) aprotinin (1 μg/ml) in a glass Potter Duall. The extract was centrifuged at 4°C for 10 min at 10,000 *g*. The assay was carried out with 100 μl of the supernatant for 10 min in 1 ml final volume with buffer Tris 25 mM (pH 7.2), 1 mM $MgCl_2$ and 5,000,000 cpm α^{32}P-GTP in 50 μM cold GTP. This high specific activity was used in order to increase the sensitivity of the dosage. 1 mM 3-isobutyl-1-methylxanthine, 1 mM cGMP, 1 mM creatine phosphate, 2 units/tube of creatine phosphokinase type I (Sigma) was also added with or without 0.1 mM sodium nitroprusside (SNP). The isolation of isotopic cGMP was performed as described elsewhere (Shah and Hyde, 1995). Briefly, 500 μl of 120 mM zinc acetate was added to each tube followed by 500 μl of 144 mM sodium carbonate. The mixture was precipitated by centrifugation, and the supernatant was

further purified by anion exchange acid alumina chromatography. Isotopic cGMP was eluted with 100 mM sodium acetate and the radioactivity counted. This procedure allowed us to recover 50% of isotopic cGMP. The cGMP was also counted by radioimmunoassay to confirm the validity of the method.

RNA Isolation

This and other protocols used in this study are available at the Canadian *Drosophila* Microarray Centre (CDMC) website (http://www.flyarrays.com), and have been previously described (Neal *et al*., 2003). Briefly, larvae were homogenized in TRIzol reagent (Invitrogen Canada Inc.) with a handheld PRO200 homogenizer fitted with a Multi-Gen7 generator (Pro Scientific Inc.) for 10 s at settings 4–5. The RNA extraction was performed according to the manufacturer's guidelines with the final pellet dissolved in 18 MΩ water (Sigma). RNA concentration and quality were evaluated by using spectrophotometry.

Microarray Hybridizations and Data Analysis

The D12k version 1 cDNA-based microarray produced by the CDMC was used for the microarray experiments and information on the production and use of a similar CDMC array has been published (Neal *et al*., 2003). Microarray experiments were performed according to the methods previously described (Neal *et al*., 2003). Briefly, fluorescently labeled cDNA was generated from 80 μg total RNA using SuperScript II reverse transcriptase (Invitrogen). The cDNAs from one Cyanine-3-dCTP (Perkin Elmer) and one Cyanine-5-dCTP reaction were combined and were co-hybridized to a D12k1 microarray on which approximately 10,500 genes are represented. Images of the hybridized arrays were acquired using a ScanArray 4000 XL laser scanner (Perkin Elmer) and were quantified using QuantArray 3.0 software (Perkin Elmer).

Microarray analysis and Lowess sub-grid normalization were performed with GeneTraffic Duo (Iobion Informatics/Stratagene). The normalized data were analyzed using the Statistical Analysis of Microarrays (SAM) software from Stanford University (Tusher *et al*., 2001). The *delta* threshold was adjusted such that less than one result was expected to arise by chance. Gene lists generated in SAM were filtered in GeneTraffic to include only those genes that displayed at least a 1.3-fold difference and whose coefficient of variance was less than 125%.

All of the quantified microarray data and original TIFF images are available from the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). The D12k1 array platform (accession GPL1467) has recently been updated with current annotation. The data from each microarray hybridization may be retrieved from GEO using the series accession code GSE3126 or the individual hybridization accession codes GSM38619 to GSM38626, inclusive.

To investigate the altered transcription of genes that we predicted could directly interact with DGCα1 signaling, we specifically analysed the expression of the following genes (based on their annotated functions at http://flybase.org): *CG3618*, *CG10738*, *CG3216*, *CG31183*, *Gyc76C*, *Gyc88E*, *Gyc89Da*, *Gyc89Db*, *Gyc-α99B* (*dgcα1*), *Frq*, *Gs-α60A*, *Ac13E*, *Ac76E*, *CG32158*, *capt*, *Ac78C*, *ACXC*, *5-HT7*, *Ac3*, *5-HT1A*, *for*, *Pkg21D*, *CG4839*, *Pka-C2*, *Pka-C3*, *Pka-R1*, *Pka-R2*, *dnc*, *Pde8*, *Pde9*, *CG9296*, *Pde1c*, *CG31757*, *Pde11*, *Pde6*, *dNOS*, and *Ho*. Some potentially related genes (*i.e., CG9783*, *Gyc32E*, *Gyc-β100B* (*dgcβ1*), *GC32305*, *rut*, and *Pka-C1*) were not represented on the array.

Polymerase Chain Reaction (PCR) Primer Design

The Whitehead Institute's Primer3 software (Rozen and Skaletsky, 2000) was used to design primers based on sequence data acquired from GenBank (http://www.ncbi.nlm.nih.gov). The user-defined parameters were: (a) amplicon length = 100–250 bp; (b) oligo length = 18–22 (20 optimal); (c) melting temperature = 57–63°C (60°C optimal); (d) GC content = 35–65% (50% optimal); (e) maximum polynucleotide tract = 4. OligoAnalyzer 3.0 (http://scitools.idtdna.com/Analyzer/) was used to detect potential secondary structure and dimer formation. The sequences for the RpL32 primers have previously been published (Gim *et al*., 2001). The primer sequences used for each of the test genes were as follows: *Pepck* (CG17725) 5′-GGCATGAAGGATAAGGTGGA-3′ and 5′-TGTCTACGCGGGAAGATAGC-3′; *Lsp1γ* (CG6821) 5′-CTATGACTTCTACACCTACGGC-3′ and 5′-CAGGTAGTTGCCCACAATCT-3′; *vanin-like* (CG32754) 5′-GAAACGTGCGAAGAGGAGTC-3′ and 5′-CCACTAGTTCCCTCGTCCAA-3′; *pst* (CG8588) 5′-TGCTGCTAATGGAGTTGTGC-3′ and 5′-AGAGCTTTTCTGCCGTCTTG-3′.

Real-Time Reverse Transcriptase (RT) PCR

A two-step approach was taken in which the initial reverse transcription was followed by the quantitative PCR amplification. Total RNA (10 μg) was treated with 10 units of DNase I (Fermentas Life Sciences) as recommended by the manufacturer. DNA-free RNA (500 ng) was reverse transcribed using a $dT_{20}VN$ primer (Sigma Genosys) with SuperScript II for 2 h at 42°C. EDTA was added to a final concentration of 5 mM and the reaction was diluted 1:8 for future use.

Real-time PCR reactions (25 μL) were assembled using components of the Brilliant® SYBR® Green QPCR Core Reagent Kit (Stratagene); 1X Core PCR Buffer, 200 μM each dNTP, 2 mM $MgCl_2$, 0.75 μL 1:500 ROX, 1.25 μL 1:1000 SYBR Green I, 8% glycerol and 1.25 units SureStart Taq polymerase. Primer mix (2.5 pmol each primer) and 1 μL of diluted cDNA/RNA template were added to individual reactions.

The Mx4000® Multiplex Quantitative PCR System (Stratagene) was used for sample processing and analysis. Samples were incubated at 95°C for 10 min prior to thermal cycling (40 cycles of: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s). Triplicate endpoint observations were made at each annealing and extension step. Dissociation curves were also plotted for each product. PCR efficiency was determined from the ROX-normalized fluorescence measurements using the program LinRegPCR (Ramakers *et al*., 2003) and was incorporated into the final calculation of fold induction from the ΔC_t values. Each experiment was performed in triplicate on four independent pairs of samples from each genetic background. The expression of each test gene was normalized to the level of Act5C transcript within each sample prior to comparisons between samples.

Statistical Analysis

For behavioral and biochemical data, ANOVA was followed by Student–Newman–Keuls (SNK) tests to determine significant differences between the means. The significance level used was 0.05 for all tests. qRT-PCR data were analysed using the following non-parametric tests: Wilcoxon Sign-Rank test (for the genes *vanin-like*, *pst*, and *Pepck*) or Kruskal–Wallis (due to the unequal sample sizes in the data for gene *Lsp1γ*). All statistical analyses were performed using either SAS or SAS/JMP software (SAS institute Inc., 1999).

RESULTS

Mutations in *dgcα1* Induce Increased Larval Foraging Locomotion

The *dgcα1* mutations induced increased larval foraging locomotion on a nutritive yeast substrate in the different genetic backgrounds (Figure 1). The rover–sitter foraging behavioral differences associated with the *for* genotype were maintained even in

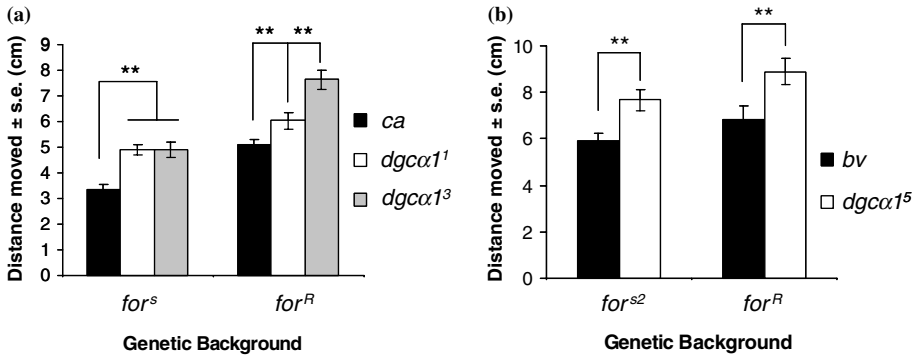

Fig. 1. Larval locomotion while foraging is increased by mutations in *dgcα1*. Larvae were placed in shallow wells containing a nutritive yeast media and were allowed to move freely for 5 min. The distances moved during this time were recorded for analysis. (a) Foraging locomotion is altered in larvae with $dgc\alpha1^1$ or $dgc\alpha1^3$ alleles, both of which were generated on chromosomes containing a ca^1 mutation. On both rover (for^R) and sitter (for^s) genetic backgrounds, the *dgcα1* mutant larvae travel significantly farther while foraging than do the *ca* control larvae. Similar results were observed on an induced mutant sitter (for^{s2}) genetic background (data not shown). (b) The foraging behavior of larvae with mutant allele $dgc\alpha1^5$, which was generated on a bv^1 mutant chromosome, is also significantly increased. The behavior of $dgc\alpha1^5$ larvae on for^R and for^{s2} sitter backgrounds is shown here. The behavior of for^s; $dgc\alpha1^5$ bv^1 larvae could not be assessed because they are developmentally delayed. (**indicates significant differences: ANOVA, SNK, $p < 0.05$, $13 < n < 49$).

the presence of a *dgcα1* mutation. No significant differences were observed when larval locomotion on a non-nutritive agar surface was tested, indicating that the observed foraging differences are not accompanied by a general change in mobility (data not shown).

PKG Activity is Increased in *dgcα1* Mutants

To test whether the previously reported relationship between higher PKG activity and increased larval locomotion while foraging is maintained in the *dgcα1* mutants, we performed PKG enzyme activity assays. The *dgcα1* mutations induce significantly increased PKG activity in head extracts from natural sitter larvae (Figure 2a). There was also a trend towards higher PKG activity in extracts from rovers with *dgcα1* mutations, although these differences were not always significant. In PKG assays performed on dissected larval CNS's the trend was similar to that observed in the adult heads, but the differences were not significant (Figure 2b), perhaps reflecting the increased difficulty associated with dissecting the CNS and the reduced sample size and volume. Since foraging locomotion in wild-type larvae is directly related to PKG activity (Osborne *et al.*, 1997), the increased PKG activity observed in the *dgcα1* mutants may mediate the mutant foraging phenotype. It is interesting to note that, in both the adult and larval samples, the *dgcα1*-induced PKG activities are similar on rover and sitter backgrounds, whereas the PKG activities of the controls are different. Thus, the magnitudes of the changes are largest, and more significant, in the sitter background.

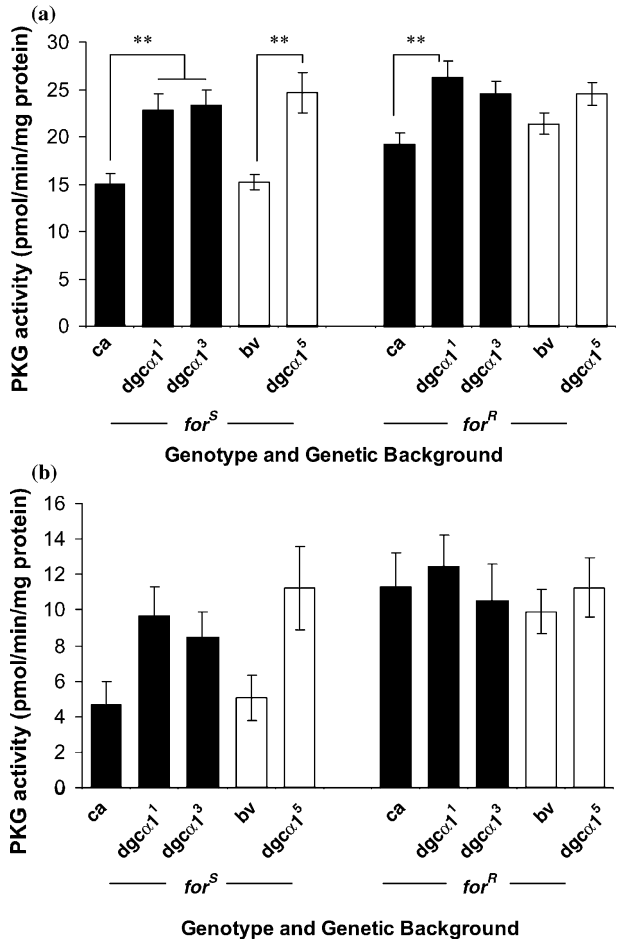

Fig. 2. Overall PKG enzyme activity is increased by mutations in *dgcα1* on both natural rover (for^R) and sitter (for^s) genetic backgrounds. Mutations $dgc\alpha1^1$ and $dgc\alpha1^3$ and their control strain, all marked with a ca^1 allele (mutants and control charted in black), and $dgc\alpha1^5$ and its control strain, marked with a bv^1 mutation (white bars), are shown. (a) PKG activity of adult head extracts. All *dgcα1* mutations elicited significant increases in PKG activity on sitter backgrounds. In rovers the trend was maintained, although the difference was not always significant (**ANOVA, SNK, $p < 0.05$, $n = 8$). (b) Results from PKG activity assays performed on dissected larval CNS. The trend of increased PKG activity was generally maintained, although the differences were not significant (ANOVA, SNK, $n = 5$). As observed with adult head homogenate, the greatest differences are observed in sitter backgrounds.

NO-Induced cGMP Production is Reduced in *dgcα1* mutants

To test whether compensatory activity by another NO-responsive GC may explain the increased PKG activity, we analysed NO-stimulated cGMP production in adult head extracts. The NO donor, SNP, stimulated less cGMP production in *dgcα1* mutants compared to controls (Figure 3). Although this trend was consistent for all alleles, the reduction was not always statistically significant. This indicates that the NO-sensitive GC activity is impaired, and is not likely the sole source of the increased PKG activation and larval foraging locomotion. However, since some NO-stimulated cGMP production was observed, sGC cannot be completely discounted. The presence of residual NO-induced cGMP production in the *dgcα1* mutants is consistent with previous observations (Gibbs, *et al.*, 2001).

Microarray Results

There is the potential for a high degree of functional degeneracy in cGMP signaling systems since

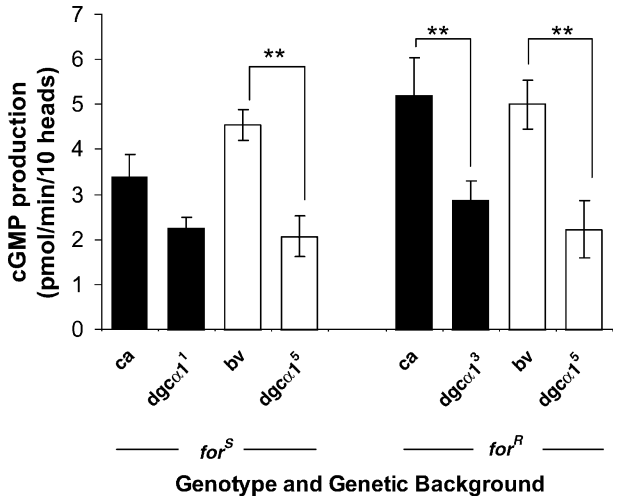

Fig. 3. NO-stimulated cGMP production is decreased in *dgcα1* mutants on both rover and sitter genetic backgrounds. Extracts from adult heads were treated with the NO donor SNP and subsequent cGMP production was quantified. Flies with *dgcα1* mutations that were generated on chromosomes marked with either ca^1 (black bars) or bv^1 (white bars) have reduced cGMP production compared to controls on both rover (for^R) and sitter (for^s) genetic backgrounds. Although this trend was consistent, the reductions were not always statistically significant (**ANOVA, SNK, $p < 0.05$, $n = 6$ for all but the *ca* controls where $n = 3$).

the fly genome encodes several other GC isoforms and phosphodiesterases (PDE), as well as three proteins with putative PKG activity (from *D. melanogaster* genome annotations at http://flybase.org/annot/). Additionally, there may be cross-talk between cAMP- and cGMP-mediated signaling systems (Pelligrino and Wang, 1998). Thus, the increased PKG activity may be elicited by many potential mechanisms, such as the compensatory activation of another GC, or the suppression of a PDE, etc. To further clarify the situation, DNA microarray studies were performed to compare transcriptional changes induced by the $dgc\alpha 1^3$ mutation in both natural rover and natural sitter genetic backgrounds. Mutations in $dgc\alpha\ 1^3$ were chosen since they elicited the strongest behavioral phenotype.

Total RNA was extracted from four independent paired samples of control and *dgcα1* mutant larvae from both rover and sitter genetic backgrounds. Subsequently, four hybridizations to *Drosophila* 12k1 microarrays were performed for each genotype. The normalized data for each genetic background were subjected to a one-class analysis using the program SAM (Tusher *et al.*, 2001). This analysis revealed 338 affected genes in rovers (261 enhanced and 77 suppressed in the $dgc\alpha 1^3$ mutants) and 601 affected genes in sitters (333 enhanced and 268 suppressed in the $dgc\alpha 1^3$ larvae). These lists were filtered in GeneTraffic to include only those genes that had a minimum 1.3-fold change and COV $< 125\%$ (Jin *et al.*, 2001; Neal *et al.*, 2003). This yielded reduced lists of 212 (164 enhanced, 48 suppressed) and 297 (155 enhanced, 142 suppressed) genes affected in rover and sitter backgrounds, respectively.

Since the *dgcα1* mutations have similar phenotypes (increased larval foraging locomotion and PKG activity) in both genetic backgrounds, the two gene lists were intersected to isolate genes whose transcription is altered in both rovers and sitters. This identified only 60 genes, whose expression data was clustered (Figure 4) using the Pearson algorithm (Eisen *et al.*, 1998). The $dgc\alpha 1^3$ mutation induced the enhanced expression of 37 genes in both backgrounds, while 14 were consistently suppressed. Surprisingly, nine genes were affected in a background-dependent manner, i.e. their transcription was consistently altered, but in opposite directions depending on the genetic background. Information from Gene Ontology (http://www.geneontology.org) annotations suggests that many of the altered genes are involved in metabolic processes (Table I).

Four genes displaying altered transcription were selected for confirmation by Sybr Green quantitative real-time reverse transcriptase PCR (qRT-PCR). They were selected based on their possible biological relevance to foraging behavior (based on genomic annotations). The qRT-PCR experiments confirmed the directional changes detected using microarrays. *Larval serum protein 1γ* (*Lsp1γ*), *vanin-like*, and *pastrel* (*pst*) were all confirmed to be up-regulated in the $dgc\alpha 1^3$ mutants (Numerical scores represent the mean normalized C_t values ± standard error, which are inversely related to RNA abundance: *Lsp1γ*: $\text{mean}_{mut} = 25.31 \pm 0.63$, $n = 8$ and $\text{mean}_{wt} = 36.64 \pm 0.5$, $n = 2$, Kruskall–Wallis $p = 0.05$; *vanin-like*: $\text{mean}_{mut} = 27.52 \pm 0.14$, $n = 8$ and $\text{mean}_{wt} = 29.79 \pm 0.65$, $n = 8$, Wilcoxon test $p = 0.008$; *pst*: $\text{mean}_{mut} = 32.99 \pm 0.30$, $n = 8$ and $\text{mean}_{wt} = 36.00 \pm 0.37$, $n = 8$, Wilcoxon test $p = 0.004$; all one-tailed tests based on *a priori* hypotheses from the microarray study). The fourth gene, *Phosphoenolpyruvate carboxykinase* (*Pepck*), was differentially affected by the GC mutation in the two backgrounds. It was up-regulated in $dgc\alpha 1^3$ mutant rovers whereas it was down-regulated in sitters with the mutation. qRT-PCR analysis revealed a similar trend, although the differences were not significant, possibly as a result of the smaller sample sizes available for the statistical test since the scores had to be analyzed separately for the rover vs. sitter

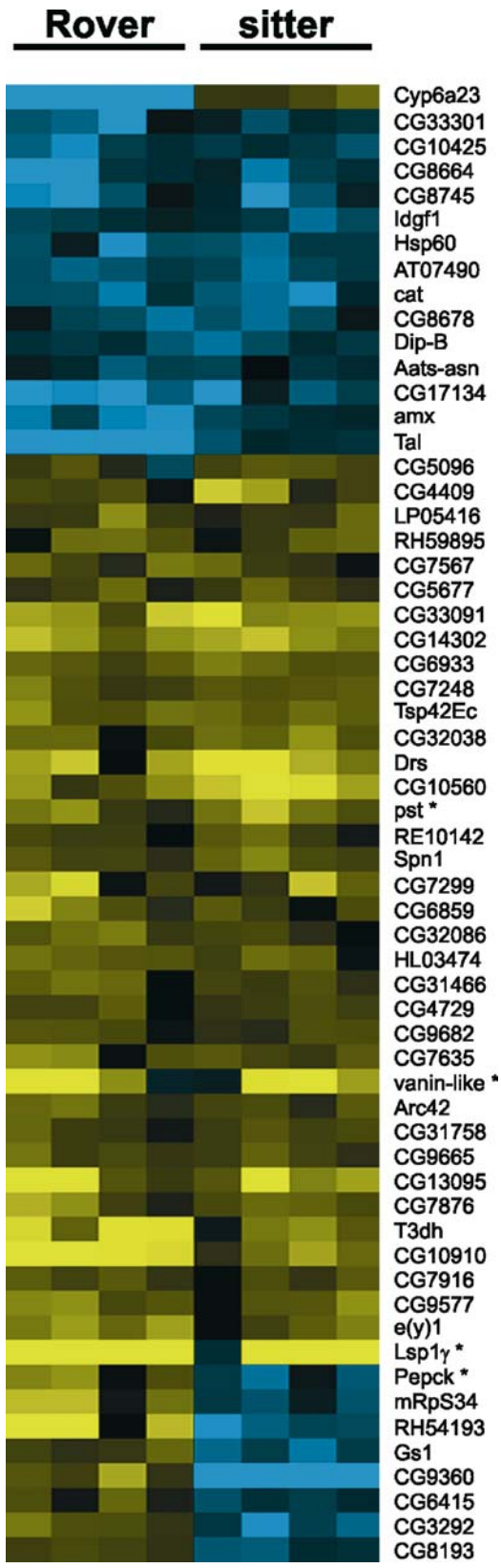

Fig. 4. A Pearson cluster was formed using data from 60 genes that were differentially expressed in $dgc\alpha1^3$ mutants from both rover (for^R) and sitter (for^s) genetic backgrounds. Columns in the figure represent individual hybridizations whereas each row corresponds to the $\log_2$-normalized ratio of a single gene's expression in mutants over control animals. The ratios are represented by the spectrum of colors from blue (down-regulated in mutant) to black (unchanged) to yellow (up-regulated in mutant). The saturation threshold was set to the equivalent of a fourfold change. Levels of 37 transcripts were increased and 14 were decreased in the mutants in both rover and sitter background comparisons. The transcription of nine genes was altered in a background-dependent manner: eight were enhanced in rovers but suppressed in sitters while one was suppressed in rovers, but enhanced in sitters. The expression changes of the four genes indicated (*) were confirmed by qRT-PCR.

backgrounds (Rovers: $\text{mean}_{\text{mut}} = 25.28 \pm 0.29$, $n = 4$, $\text{mean}_{\text{wt}} = 27.34 \pm 0.54$, $n = 4$, Wilcoxon test $p = 0.06$; sitters: $\text{mean}_{\text{mut}} = 26.59 \pm 0.34$, $n = 4$, $\text{mean}_{\text{wt}} = 26.12 \pm 0.54$, $n = 4$, Wilcoxon test $p = 0.875$).

The expression patterns of genes known or predicted to have GC, PDE, PKG, nitric oxide synthetase, heme oxygenase, adenylyl cyclase, or cAMP-dependent protein kinase activity were specifically investigated (data not shown). Putative gene functions were identified based on the *D. melanogaster* genomic annotations (release 3.1) at http://flybase.org/annot/. No consistent changes were observed in the expression of 37 genes fitting into the above categories. It is important to note, however, that six of the genes currently predicted or known to encode such enzymes were not represented on the array (see Methods).

DISCUSSION

Understanding the molecular and neurological mechanisms by which genetic information, together with environmental effects, is translated into behavioral variation represents a significant challenge for modern Biology. Complex behavioral phenotypes are thought to result from the reception, integration, and responses to information from several sources (i.e., various cues from the internal and external environments, as well as genetic effects) (Sokolowski and Riedl, 1999). Thus, they are sensitive to subtle developmental or functional differences at many levels in many systems. Through their integration of converging signals, behavior traits can exhibit a large degree of plasticity (Anholt and MacKay, 2004; Dubnau and Tully, 1998; Sokolowski, 2001; Tully, 1996). For example, an individual may have an increased likelihood of displaying a specific behavioral trait in a given external environment but changes in

Table I. Sixty Genes, Identified by Microarray Analyses, have Altered Expression Induced by the *dgcα1* Mutations in Rover and Sitter Genetic Backgrounds

Gene Symbol[1]	Gene ID	Change in mutant[2]		Polytene[1] position	Selected annotated functions[1,3]
		Rover	Sitter		
Cyp6a23	CG10242	–	+	51D1	Cytochrome P450 enzyme
	CG33301	–	–	31A1	
	CG10425	–	–	96C8	
	CG8664	–	–	15F9	Glucose/ribitol dehydrogenase, oxidoreductase activity
	CG8745	–	–	70C4–70C5	
Idgf1	CG4472	–	–	36A1	Amino acid metabolism, imaginal disc growth
Hsp60	CG12101	–	–	10A4	Response to heat and stress
	AT07490	–	–		DGC2 putative GC14703
cat	CG6871	–	–	75E1	Antioxidant activity
	CG8678	–	–	39B3	
Dip-B	CG9285	–	–	87F13	Proteolysis and peptidolysis
Aats-asn	CG10687	–	–	37C1	Asparagine-tRNA ligase activity
	CG17134	–	–	32B1	Pepsin (A1) aspartic protease, eukaryotic and viral aspartic protease active site, acid proteases
amx	CG12127	–	–	8D10	DNA replication, ectoderm and mesoderm development
Tal	CG2827	–	–	60A12	Transaldolase activity
	CG5096	+	+	31D11	Cell adhesion; development; neural transmission
	CG4409	+	+	53C4	
	LP05416	+	+		cDNA clone from larval-early pupal stage
	RH59895	+	+		Putatively Cyp4d20
	CG7567	+	+	99B4	
	CG5677	+	+	95F3	Signal peptide processing
	CG33091	+	+	96C8	
	CG14302	+	+	91C5	
	CG6933	+	+	77A2	Structural constituent of peritrophic membrane
	CG7248	+	+	68E3	Structural constituent of peritrophic membrane
Tsp42Ec	CG12847	+	+	42E5	
	CG32038	+	+	67B6	
Drs	CG10810	+	+	63D2	Defense response to fungi
	CG10560	+	+	96D1	
pst	CG8588	+	+	65F6–65F7	Learning and memory effects
	RE10142	+	+		
Spn1	CG9456	+	+	42D6	Proteolysis and peptidolysis
	CG7299	+	+	32A4	
	CG6859	+	+	71B5	Peroxisome organization and biogenesis
	CG32086	+	+	68C13	
	HL03474	+	+		cDNA clone from fly head, brain & sensory organs
	CG31446	+	+	89A2	
	CG4729	+	+	72E4	Phospholipid and glycerol acyltransferase
	CG9682	+	+	99E3	
	CG7635	+	+	18A6	Cytoskeleton organization and biogenesis
vanin-like	CG32754	+	+	5E1	Hydrolase activity, cell motility, vitamin biosynthesis
Arc42	CG4703	+	+	92B4	RNA polymerase II transcription mediator activity; acyl-CoA dehydrogenase activity
	CG31758	+	+	33A3	Serine-type endopeptidase inhibitor activity
	CG9665	+	+	73D7–73E1	
	CG13095	+	+	29D2	Aspartic-type endopeptidase activity
	CG7876	+	+	18B1	Chitin binding domain, Tachycitin
T3dh	CG3425	+	+	58D3	Type III alcohol dehydrogenase
	CG10910	+	+	55B1	
	CG7916	+	+	34D7	
	CG9577	+	+	19B3	Fatty acid beta-oxidation
E(y)1	CG6474	+	+	16E1	Regulation of transcription from Pol II promoter
Lsp1γ	CG6821	+	+	61A6	Larval serum protein 1γ

Table I *(Continued)*

Gene Symbol[1]	Gene ID	Change in mutant[2]		Polytene[1] position	Selected annotated functions[1,3]
		Rover	Sitter		
Pepck	CG17725	+	–	55D3	Gluconeogenesis
mRpS34-RA	CG13037	+	–	72E1–72E2	Mitochondrial ribosomal protein S34
	RH54193	+	–		
Gs1	CG2718	+	–	21B1	Amino acid biosynthesis
	CG9360	+	–	10E2	Oxidoreductase activity, acting on CH-OH group of donors
	CG6415	+	–	32A4	Glycine catabolism
	CG3292	+	–	58D1	Alakline phosphatase activity
	CG8193	+	–	45A1	Defense response, monophenol monooxygenase activity

[1]Where known.
[2]Direction of change in relative transcript abundance in *dcgα1* mutants compared to control, on rover or sitter background: '+' = increased in mutant, '–' = reduced in mutant.
[3]From FlyBase, A Database of the Drosophila Genome (http://flybase.org).

the internal environment (e.g., hunger) can alter the response to these same external cues (Graf and Sokolowski, 1989; LaGraize *et al*., 2004; Sokolowski and Riedl, 1999). Such informational integration may be partially mediated by molecules that respond differently to a given signal depending on the co-ordination of that signal with information from other sources (Bourne and Nicoll, 1993). Previous studies have indicated that *Drosophila* sGC is finely regulated by diverse coincidental signals (Zhang *et al*., 2002), thus promoting it as a possible signal-integrating molecule that may help generate appropriate behavioral responses.

Behavioral and Biochemical Studies

We have investigated the foraging phenotypes of larvae with one of three mutations in the gene *dgcα1*, which encodes an α subunit of *Drosophila* sGC (Gibbs *et al*., 2001; Liu *et al*., 1995; Shah and Hyde, 1995; Yoshikawa *et al*., 1993). Since PKG activity is directly related to larval locomotion while foraging (Osborne *et al*., 1997), and is stimulated by cGMP, we proposed that *dgcα1* mutations leading to decreased cGMP production would suppress PKG activity, thereby reducing larval foraging locomotion. Paradoxically, the *dgcα1* mutations stimulated foraging-specific locomotion in both rover and sitter genetic backgrounds. This behavioral effect was accompanied by increased PKG enzyme activity in the mutants, consistent with previous observations of the link between PKG activity and foraging behavior (Osborne *et al*., 1997).

Mutations eliciting large changes in protein abundance or function, such as the *dgcα1* mutations described herein, may have multiple, sometimes unexpected, pleiotropic consequences making it difficult to accurately predict a mutant behavioral phenotype (Greenspan, 1997; Tully, 1996). This difficulty is compounded when dealing with signaling systems, such as the cGMP signaling cascade, that appear to have multiple points of inbuilt degeneracy. For example, a search through the genome of *Drosophila melanogaster* (http://flybase.org/annot/) yields 12 genes with known, or predicted guanylyl cyclase activity. Of these, the majority are pGCs, while five encode sGC peptides. There are also several genes encoding PDEs (enzymes that convert cGMP to GMP), as well as three potential PKG genes. Additionally, cross-talk between cGMP and cAMP transduction cascades may further complicate the situation (Pelligrino and Wang, 1998). Work in other systems has demonstrated that various GC isoforms can have overlapping expression and this may allow one form to compensate for the loss of another (Lee *et al*., 2004; Rivero-Vilches *et al*., 2003). This compensation is not expected to be perfect, however, since the compensating GC may not be as responsive to the appropriate regulatory systems.

One possible explanation for the observed increases in larval foraging locomotion is that a second GC over-compensates for the lack of *dgcα1* expression in the mutant strains (Lee *et al*., 2004). This over-compensation may be due to less stringent or inappropriate regulatory systems in the relevant cells or tissues. Previous experiments on flies with these same *dgcα1* mutations have indicated that while

NO-stimulated cGMP production is, in general, greatly reduced in the mutants, it is not completely absent: some cells in the adult brain of *dgcα1* mutants continue to produce cGMP in response to NO donors (Gibbs, *et al*., 2001). This activity may be due to residual activity of *dgcα1*, however an alternate hypothesis is that the cGMP is synthesized by atypical sGC activity. Atypical sGC's are suppressed by O_2, which binds to an associated heme group, and this suppression may be reduced by the displacement of O_2 by NO (Langlais *et al*., 2004; Morton, 2004b). Thus, in the mutants, compensatory activation of atypical sGC's may increase cGMP levels in relevant cells thereby stimulating PKG activity leading to increased larval foraging locomotion. The observed reduction of NO-stimulated GC activity in *dgcα1* mutants in the present study corresponds well with previous reports that the activity is greatly attenuated, but not absent (Gibbs, *et al*., 2001). Though sGC activity is generally reduced, enhanced activity (either by typical or atypical sGC) in select cells may occur, and may affect the foraging phenotype.

Additionally, there are many NO-independent mechanisms by which PKG activity can be increased. For example, a pGC, or an atypical sGC, may be stimulated to produce more cGMP, or a PDE could be suppressed, furthermore, PKG transcription itself could be enhanced (see Wang and Robinson, 1997, for review). Using DNA microarray analysis, we did not observe increased transcription of any known or predicted GC or PKG gene, although not all of them were represented on the array (one pGC and the sGC β subunit gene were absent). As stated above, the compensatory activity may be limited to a small number of cells and thus transcriptional increases may be below the threshold of detection by this technique. Furthermore, increased cGMP production may be a result of post-translational GC activation and would thus not be revealed by micorarray analysis. We did, however, identify several other genes that are differentially transcribed in the mutants, as discussed below.

Finally, although we chose to investigate the foraging phenotypes of *dgcα1* mutants based on the proposed activity of DGCα1 directly upstream of *for*-encoded PKG, the possibility of a less direct relationship exists. For example, *dgcα1* mutations may be affecting sensory system function or development (Ball and Truman, 1998; Elphick and Jones, 1998; Müller and Hildebrandt, 2002; Nighorn *et al*., 2000; Redkozubov, 2000; Truman *et al*., 1996; Wildemann and Bicker, 1999a, b) to produce an animal that is more sensitive to signals inducing foraging locomotion. Consequently, the relevant PKG activation may be increased in some other effector system due to over-stimulation by aberrant neural signaling caused by the mutations. Furthermore, besides *for*, the genome of *D. melanogaster* contains two other known or predicted PKG genes (Pkg21D and CG4839). Thus, it is also possible that the *dgcα1* mutant phenotype may be mediated by a PKG other than the one encoded by *for*. While we cannot exclude this possibility, the maintenance of the rover–sitter behavioral dimorphism even in the presence of the *dgcα1* mutations indicates that the *for* genotype plays an important role. Detailed larval expression data for *dcgα1*, *for*, and other PKG genes, and especially, the identification of the specific expression patterns required to mediate foraging behavioral variation, will be useful in determining the precise nature of the interaction between *dgcα1* and *for* signaling.

Microarray Analysis

Using DNA microarray analyses, we compared the transcriptional changes induced by the $dgc\alpha1^3$ mutation in both natural rover and natural sitter genetic backgrounds. As stated above, we observed no significant or consistent differences in the transcription of genes encoding proteins that we predicted might interact with sGC. Since microarray analysis detects only transcriptional differences, compensatory alterations in protein function or other neuromolecular mechanisms may have occurred but were not detectable by this method. The microarray screen did, however, identify a number of genes whose expression was consistently altered in the presence of a $dgc\alpha1^3$ mutation and therefore may affect the foraging behavioral phenotype. The genetic background, either rover or sitter, of the $dgc\alpha1^3$ mutation had a surprising effect on the induced transcriptional changes. Only a subset of the genes altered in either rovers or sitters (60 of 212 or 297, respectively) were commonly affected in both backgrounds. Furthermore, the expression of nine of these commonly affected genes was altered in opposite directions depending on the background.

Since $dgc\alpha1^3$ has a similar phenotype in both rovers and sitters we anticipate that future investigations into the commonly altered genes will provide the clearest insight into the mechanisms of the effects. We used RT-PCR analysis to independently validate the microarray results of four of the genes. Table I presents a preliminary characterization of the genes based on their reported molecular functions. It is

interesting to note that several of these are involved in metabolic processes and thus present intriguing candidates for this food-related behavioral phenotype. Investigation of these genes will help elucidate both the mechanisms by which *dgcα1* mutations induce increased larval foraging locomotion, and also into the regulation and effectuation of natural rover and sitter differences.

The influence of genetic background on transcriptional differences between animals with or without a $dgc\alpha1^3$ mutation presents an interesting and unexpected result. This is observed most strikingly in the subset of nine genes whose expression was significantly altered in both rovers and sitters, but in opposite directions depending on the genetic context. The profile of expression differences was also dependent on background: for example, in sitters, about half of the affected genes were down regulated (142/297) whereas in rovers only about a quarter of the genes affected were suppressed (48/212), of the 60 commonly affected genes, 22 were suppressed in sitters while only 15 were reduced in rovers. Even though the significance to larval foraging behavior of these differences in the expression response profiles is not yet clear, it is apparent that critical information may be gained by studying the effects in multiple genetic backgrounds. While the importance of genetic background on mutant phenotypes has been previously demonstrated (de Belle and Heisenberg, 1996; Gerlai, 1996), this is the first case, that we are aware of, in which the presence of a mutation affects the activity of a set of genes in opposite directions depending on the genetic background. Thus, we propose that genetic background should be an important consideration in future microarray experiments.

CONCLUSION

This work outlines a behavioral phenotype associated with mutations in a *Drosophila* sGC α subunit gene, *dgcα1*. We observe that *dgcα1* mutants exhibit significantly increased larval locomotion while foraging. In addition to this mutant foraging behavior we observe increased PKG activity, but decreased NO-stimulated GC activity in the mutants. To gain insight into the mechanism by which this phenotype is elicited, we performed DNA microarray analyses of transcriptional changes induced by the mutations on both natural rover and natural sitter genetic backgrounds. The array analyses provided no evidence of compensatory changes in other enzymes we predicted to interact in the cGMP signaling cascade. We did, however, detect transcriptional changes in other genes, several of which encode metabolic proteins and this information will be used in future studies into the mechanisms effecting the natural foraging behavioral dimorphism. Finally, we observed that the genetic background of the *dgcα1* mutation has a strong influence on the transcriptional responses of some genes. This suggests that genetic background should be considered when DNA microarray analysis is used to investigate the effects of mutations.

ACKNOWLEDGMENTS

We thank Dr. Charles Zuker for generously providing the *dgcα1* mutants and antibody. We also thank Dr. William Pak for providing another antibody to DGCα1. We acknowledge and thank the members of the Sokolowski lab, past and present, for their help with behavior testing and fly stock maintenance. We are grateful to Dr. John Ewer for his information on larval *dgcα1* expression, and to Mark Fitzpatrick for assisting with the RT-PCR statistical analysis. This work was supported by funding from the CIHR and a Canada Research Chair in Genetics to MBS. The CDMC is supported by a CIHR multi-user grant to J.T.W, H. Lipshitz, H. Krause and others.

REFERENCES

Anholt, R. R., and Mackay, T. F. C. (2004). Quantitative genetic analyses of complex behaviours in Drosophila. *Nat. Rev. Genet.* **5**:838–849.

Ball, E. E., and Truman, J. W. (1998). Developing grasshopper neurons show variable levels of guanylyl cyclase activity on arrival at their targets. *J. Comp. Neurol.* **394**:1–13.

Bourne, H. R., and Nicoll, R. (1993). Molecular machines integrate coincident synaptic signals. *Cell* **72**(Suppl), 65–75.

Casnellie, J. E. (1991). Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Methods Enzymol.* **200**:115–120.

Cheung, B. H., Arellano-Carbajal, F., Rybicki, I., and De Bono, M. (2004). Soluble guanylate cyclases act in neurons exposed to the body fluid to promote *C. elegans* aggregation behavior. *Curr. Biol.* **14**:1105–1111.

de Belle, J. S., and Heisenberg, M. (1996). Expression of *Drosophila* mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc. Natl. Acad. Sci. USA* **93**:9875–9880.

de Belle, J. S., Hilliker, A. J., and Sokolowski, M. B. (1989). Genetic localization of foraging (for): a major gene for larval behavior in *Drosophila melanogaster*. *Genetics* **123**:157–163.

Drain, P., Folkers, E., and Quinn, W. G. (1991). cAMP-dependent protein kinase and the disruption of learning in transgenic flies. *Neuron* **6**:71–82.

Dubnau, J., and Tully, T. (1998). Gene discovery in *Drosophila*: new Insights for Learning and Memory. *Annu. Rev. Neurosci.* **21**:407–444.

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**:14863–14868.

Elphick, M. R., and Jones, I. W. (1998). Localization of soluble guanylyl cyclase α-subunit in identified insect neurons. *Brain Res.* **800**:174–179.

Fiscus, R. R., and Murad, F. (1988). cGMP-dependent protein kinase activation in intact tissues. *Methods Enzymol.* **159**:150–159.

Garbers, D. L. (1999). The guanylyl cyclase receptors. *Methods* **19**:477–484.

Gerlai, R. (1996). Gene-targeting studies of mammalian behavior: is it the mutations or the background genotype?. *Trends Neurosci.* **19**:177–181.

Gibbs, S. M., Becker, A., Hardy, R. W., and Truman, J. W. (2001). Soluble guanylate cyclase is required during development for visual system function in Drosophila. *J. Neurosci.* **21**:7705–7714.

Gim, B. S., Park, J. M., Yoon, J. H., Kang, C., and Kim, Y. J. (2001). *Drosophila Med6* is required for elevated expression of a large but distinct set of developmentally regulated genes. *Mol. Cell. Biol.* **21**:5242–5255.

Graf, S. A., and Sokolowski, M. B. (1989). Rover/sitter *Drosophila melanogaster* larval foraging polymorphism as a function of larval development, food-patch quality, and starvation. *J. Insect Behav.* **2**:301–313.

Gray, J. M., Karow, D. S., Lu, H., Chang, A. J., Chang, J. S., Ellis, R. E., Marletta, M. A., and Bargmann, C. I. (2004). Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature* **430**:317–322.

Greenspan, R. J. (1997). A kinder, gentler genetic analysis of behavior: dissection gives way to modulation. *Curr. Opin. Neurobiol.* **7**:805–811.

Hardman, J. G., and Sutherland, E. W. (1969). Guanyl Cyclase, an enzyme catalyzing the formation of guanosine 3′,5′-monophosphate from guanosine triphosphate. *J. Biol. Chem.* **244**:6363–6370.

Jin, W., Riley, R. M., Wolfinger, R. D., White, K. P., Passador-Gurgel, G., and Gibson, G. (2001). The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*. *Nat. Genet.* **29**:389–395.

Kalderon, D., and Rubin, G. M. (1989). cGMP-dependent protein kinase genes in Drosophila. *J. Biol. Chem.* **264**:10738–10748.

Koesling, D. (1998). Modulators of soluble guanylyl cyclase. *Naunyn Schmiedebergs Arch. Pharmacol.* **358**:123–126.

Koesling, D., Russwurm, M., Mergia, E., Mullershausen, F., and Friebe, A. (2004). Nitric oxide-sensitive guanylyl cyclase: structure and regulation. *Neurochem. Int.* **45**:813–819.

LaGraize, S. C., Borzan, J., Rinker, M. M., Kopp, J. L., and Fuchs, P. N. (2004). Behavioral evidence for competing motivational drives of nociception and hunger. *Neurosci. Lett.* **372**:30–34.

Langlais, K. K., Stewart, J. A., and Morton, D. B. (2004). Preliminary characterization of two atypical soluble guanylyl cyclases in the central and peripheral nervous system of *Drosophila melanogaster*. *J. Exp. Biol.* **207**(Pt 13), 2323–2338.

Lee, J., Kim, S. W., Jung, T. K., Oh, Y., Park, C. S., Ma, S. K., Kim, N. H., and Choi, K. C. (2004). Indomethacin decreases particulate guanylyl cyclase activity in rat kidney. *Clin. Exp. Pharmacol. Physiol.* **31**:207–211.

Lindsey, D. L., and Zimm, G. G. (1992). *The genome of Drosophila Melanogaster*. NY, USA: Academic Press.

Liu, W., Yoon, J., Burg, M., Chen, L., and Pak, W. L. (1995). Molecular characterization of two *Drosophila* guanylate cyclases expressed in the nervous system. *J. Biol. Chem.* **270**:12418–12427.

Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schultz, S., Chepenik, K. P., and Waldman, S. A. (2000). Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev.* **52**:375–413.

Morton, D. B. (2004a). Invertebrates yield a plethora of atypical guanylyl cyclases. *Mol. Neurobiol.* **29**:97–115.

Morton, D. B. (2004b). Atypical soluble guanylyl cyclases in *Drosophila* can function as molecular oxygen sensors. *J. Biol. Chem.* **279**:50651–50653.

Müller, U., and Hildebrandt, H. (2002). Nitric oxide/cGMP-mediated protein kinase A activation in the antennal lobes plays an important role in appetitive reflex habituation in the honeybee. *J. Neurosci.* **22**:8739–8747.

Neal, S. J., Gibson, M. L., So, A. K.-C., and Westwood, J. T. (2003). Construction of a cDNA-based microarray for *Drosophila melanogaster*: a comparison of gene transcription profiles from SL2 and Kc167 cells. *Genome* **46**:879–892.

Nighorn, A., Byrnes, K. A., and Morton, D. B. (1999). Identification and characterization of a novel β subunit of soluble guanylyl cyclase that is active in the absence of a second subunit and is relatively insensitive to nitric oxide. *J. Biol. Chem.* **274**:2525–2531.

Nighorn, A., Gibson, N. J., Rivers, D. M., Hildebrand, J. G., and Morton, D. B (2000). The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. *J. Neurosci.* **18**:7244–7255.

Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J., and Sokolowski, M. B. (1997). Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* **277**:834–836.

Pelligrino, D. A., and Wang, Q. (1998). Cyclic nucleotide crosstalk and the regulation of cerebral vasodilation. *Prog. Neurobiol.* **56**:1–18.

Pereira, H. S., MacDonald, D. E., Hilliker, A. J., and Sokolowski, M. B. (1995). Chaser (Csr), a new gene affecting larval foraging behavior in *Drosophila melanogaster*. *Genetics* **141**:263–270.

Prabhakar, S., Short, D. B., Scholz, N. L., and Goy, M. F. (1997). Identification of nitric oxide-sensitive and -insensitive forms of cytoplasmic guanylate cyclase. *J. Neurochem.* **69**:1650–1660.

Ramakers, C., Ruijter, J. M., Lekanne Deprez, R. H., and Moorman, A. F. M. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**:62–66.

Redkozubov, A. (2000). Guanosine 3′,5′-cyclic monophosphate reduces the response of the Moth's olfactory receptor neuron to pheromone. *Chem. Senses.* **25**:381–385.

Rivero-Vilches, F. J., de Frutos, S., Saura, M., Rodriguez-Puyol, D., and Rodriguez-Puyol, M. (2003). Differential relaxing responses to particulate or soluble guanylyl cyclase activation on endothelial cells: a mechanism dependent on PKG-I alpha activation by NO/cGMP. *Am. J. Physiol. Cell Physiol.* **285**:C891–C898.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**:365–386.

Sambrook, J., and Russell, D. (2001). *Molecular cloning. A laboratory manual* (3rd ed.). Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

SAS Institute Inc. (1999). *SAS/STAT user's guide, version 8*. Cary, NC: SAS Institute Inc.

Shah, S., and Hyde, D. R. (1995). Two *Drosophila* genes that encode the α and β subunits of the brain soluble guanylyl cyclase. *J. Biol. Chem.* **270**:15368–15376.

Shaver, S. A., Riedl, C. A. L., Parkes, T. L., Sokolowski, M. B., and Hilliker, A. J. (2000). Isolation of larval behavioral mutants in *Drosophila melanogaster*. *J. Neurogenet.* **14**:193–205.

Sokolowski, M. B. (1980). Foraging strategies of *Drosophila melanogaster*: a chromosomal analysis. *Behav. Genet*. **10**:291–302.
Sokolowski, M. B. (2001). *Drosophila*: genetics meets behaviour. *Nat. Rev. Genet*. **2**:879–890.
Sokolowski, M. B., Pereira, H. S., and Hughes, K. (1997). Evolution of foraging behavior in *Drosophila* by density-dependent selection. *Proc. Natl. Acad. Sci. USA* **94**:7373–7377.
Sokolowski, M .B., and Riedl, C. A. L. (1999). Behaviour-genetic and molecular analysis of naturally occurring variation in Drosophila larval foraging behaviour. In R. Gerlai and W. Crusio (eds.), *Molecular-genetic techniques for brain and behaviour*. London: Elsevier Scientific, pp. 517–532.
Stone, J. R., and Marletta, M. A. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* **33**:5636–5640.
Truman, J. W., De Vente, J., and Ball, E. E. (1996). Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects. *Development* **122**:3949–3958.
Tully, T. (1996). Discovery of genes involved with learning and memory: an experimental synthesis of Hirschian and Benzerian perspectives. *Proc. Natl. Acad. Sci. USA* **93**:13460–13467.
Tusher, V. G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**:5116–5121.
Wang, X., and Robinson, P. J. (1997). Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J. Neurochem*. **68**:443–456.
Wedel, B. J., and Garbers, D. L. (2001). The guanylyl cyclase family at Y2K. *Annu. Rev. Physiol*. **63**:215–233.
Wildemann, B., and Bicker, G. (1999a). Developmental expression of nitric oxide/cyclic GMP synthesizing cells in the nervous system of *Drosophila melanogaster*. *J. Neurobiol*. **38**:1–15.
Wildemann, B., and Bicker, G. (1999b). Nitric oxide and cyclic GMP induce vesicle release at *Drosophila* neuromuscular junction. *J. Neurobiol*. **39**:337–346.
Yoshikawa, S., Miyamoto, I., Aruga, J., Furuichi, T., Okano, H., and Mikoshiba, K. (1993). Isolation of a *Drosophila* gene encoding a head-specific guanylyl cyclase. *J. Neurochem*. **60**:1570–1573.
Zhang, L., Tinette, S., and Robichon, A. (2002). Drosophila NO-dependent guanylyl cyclase is finely regulated by sequential order of coincidental signaling. *J. Cell. Biochem*. **85**:392–402.

Edited by Yong-Kyu Kim