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Quantitative Mapping of a Digenic Behavioral Trait Implicates Globin Variation in *C. elegans* Sensory Behaviors

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SUMMARY

Most heritable behavioral traits have a complex genetic basis, but few multigenic traits are understood at a molecular level. Here we show that the *C. elegans* strains N2 and CB4856 have opposite behavioral responses to simultaneous changes in environmental O_2 and CO_2. We identify two quantitative trait loci (QTL) that affect this trait and map each QTL to a single-gene polymorphism. One gene, *npr-1*, encodes a previously described neuropeptide receptor whose high activity in N2 promotes CO_2 avoidance. The second gene, *glb-5*, encodes a neuronal globin domain protein whose high activity in CB4856 modifies behavioral responses to O_2 and combined O_2/CO_2 stimuli. *glb-5* acts in O_2-sensing neurons to increase O_2-evoked calcium signals, implicating globins in sensory signaling. An analysis of wild *C. elegans* strains indicates that the N2 alleles of *npr-1* and *glb-5* arose recently in the same strain background, possibly as an adaptation to laboratory conditions.

INTRODUCTION

Genetic variation contributes to individual differences in many behaviors, including psychiatric conditions in humans and behavioral traits in animals (Kendler, 2001; Kendler and Greenspan, 2006; Flint, 2003), but only a few behavioral traits have been traced to discrete molecular changes (de Bono and Bargmann, 1998; Osborne et al., 1997; Yalcin et al., 2004). The complex genetic structure of natural variation poses challenges for gene identification: most traits are thought to be affected by a few polymorphic genes with moderate effects, and many genes with small effects (Flint, 2003; Mackay, 2004; Kendler and Greenspan, 2006). A molecular understanding of multigenic traits is essential to determine how genetic changes arise, what genes they affect, how these genes interact, and how they influence behavior.

A common approach used to dissect complex genetic traits is QTL analysis, in which two strains are intercrossed, F2 progeny or inbred lines of progeny are characterized by genotype and phenotype, and quantitative genetics is used to find linkage to traits of interest. Classical QTL analysis has associated chromosomal regions with traits like anxiety, aggression, drug preference, and learning, but since a well-defined QTL in *Drosophila* or mouse typically covers 300–500 genes, moving from a QTL to the causative mutation is very difficult (Mackay, 2004; Mott and Flint, 2008; Flint, 2003). Indeed, the association of the G protein regulator Rgs2 with anxiety may be the only established single-gene behavioral QTL in mice (Yalcin et al., 2004). Developing methods to address the QTL-to-genetic-alteration problem is a major goal of the field. For example, new methods of interest combine QTL analysis with complementary approaches such as gene expression analysis or association studies of outbred strains (Mackay, 2004; Wang et al., 2008; Toma et al., 2002).

The resolution of QTL mapping has been greatly improved by whole-genome sequences and inexpensive resequencing, which allow strains to be genotyped at thousands of polymorphic loci. To take advantage of these high-resolution DNA maps, it is optimal to have chromosomes with many recombination breakpoints for fine genetic mapping. Recombinant inbred advanced intercross lines, or RIAILs, are modified inbred lines with many crossover points per chromosome that should allow rapid mapping of QTLs to individual genes (Darvasi and Soller, 1995; Rockman and Kruglyak, 2008). A set of high-resolution *C. elegans* RIAILs has been generated by intercrossing the standard laboratory strain (N2) with a strain isolated in a Hawaiian pineapple field (CB4856, henceforth "HW") for ten generations, inbred by selfing for ten generations, and genotyped at 1455 loci (Rockman and Kruglyak, 2009). The resulting RIAILs have been used to map several single-gene QTLs for reproductive traits and pathogen resistance (Hodgkin and Doniach, 1997; Seidel et al., 2008; Palopoli et al., 2008; Reddy et al., 2009). Here we use the RIAILs to identify two QTLs that affect a behavioral trait, and map both QTLs to single genetic changes.

C. elegans has strong behavioral responses to the gases O_2 and CO_2, which are highly variable in its natural soil and compost environments due to metabolic activity (Sylvia et al., 1998; Greenway et al., 2006). Previous studies have identified the neuropeptide receptor gene *npr-1* as a regulator of O_2 and CO_2 responses that differs between N2 and HW *C. elegans* strains (de Bono and Bargmann, 1998). N2 has a high activity *npr-1* allele (215 valine) and as a result has weak responses to

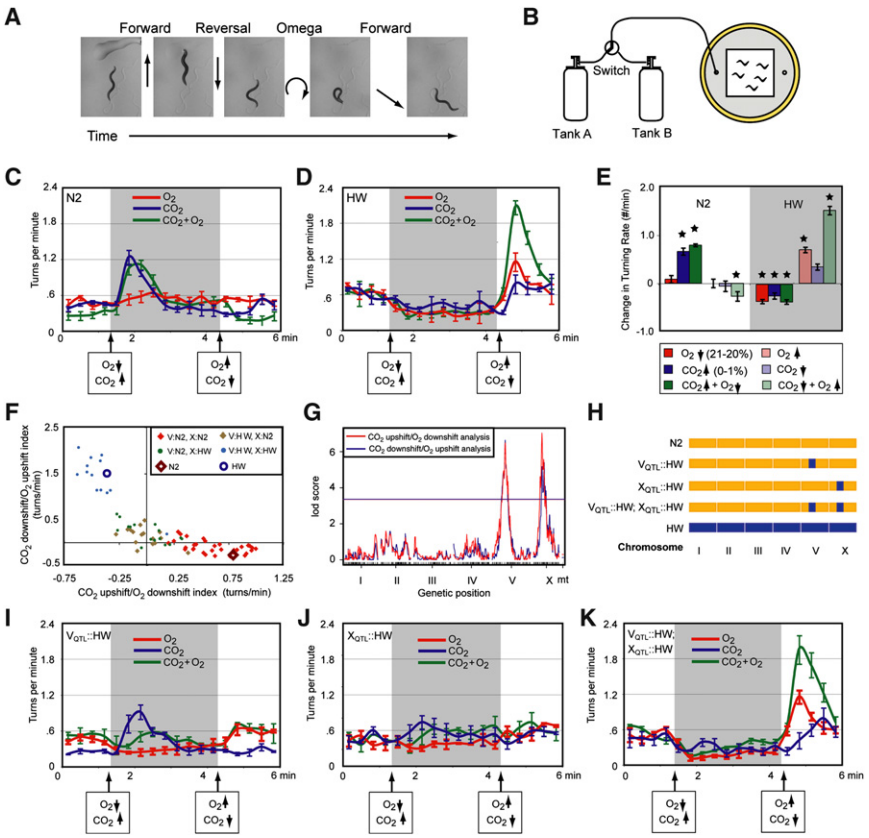

Figure 1. Quantitative Genetic Analysis of O_2 and CO_2 Responses in Two *C. elegans* Strains

(A) Forward movement interrupted by a reversal and omega turn.

(B) Behavioral arena for turning assay. Twenty to thirty animals were recorded in the presence of OP50 bacteria as O_2 and CO_2 concentrations were controlled by regulated gas flow.

(C and D) Average turn rates of N2 and CB4856 (HW) strains across nine 6 min pulse sequences (see Experimental Procedures). In all figures, O_2 changes were 21% O_2→20% O_2→21% O_2, CO_2 changes were 21% O_2→21% O_2/1% CO_2→21% O_2, and simultaneous O_2 and CO_2 changes were 21% O_2→20% O_2/1% CO_2→21% O_2. The balance in all mixtures was N_2.

(E) Average change in turning rate before and after the indicated step change (data in [C] and [D]). Stars denote significant effects of the step change ($p < 0.05$, t test).

(F) Behavioral responses of 78 RIAILs to simultaneous O_2 and CO_2 step changes. y axis, difference in turning frequency after CO_2 downshift/O_2 upshift; x axis, difference in turning frequency after CO_2 upshift/O_2 downshift. N2 and HW reference strains are included; each RIAIL is color coded by genotype at the QTLs on chromosome V and X.

(G) QTL analysis of data from (F), showing significant QTL peaks on the left arms of V and X. Horizontal line represents the genome-wide significance threshold ($p = 0.05$).

(H) Schematic of three introgression strains with small regions of HW DNA around the QTLs on V and X introduced into an N2 background.

(I–K) Turning responses of introgression strains, as in (C) and (D).

In all figures, error bars indicate standard error of mean (SEM), and Tables S1 and S2 have full datasets and statistics.

O_2 on food and strongly avoids CO_2 (Gray et al., 2004; Bretscher et al., 2008; Hallem and Sternberg, 2008). HW has a low-activity *npr-1* allele (215 phenylalanine) and as a result strongly avoids high O_2 levels on food and is not repelled by CO_2. Two other behaviors observed in HW strains, aggregation into feeding groups and accumulation at the border of a bacterial lawn, are in part caused by avoidance of high O_2 conditions (Gray et al., 2004; Cheung et al., 2005). Using QTL mapping, we find that *npr-1* cooperates with another variable gene, the globin homolog *glb-5*, to affect behavioral responses to O_2 and CO_2. Using genetic studies of 203 wild strains, we trace the history of these mutations in *C. elegans.*

RESULTS

O_2- and CO_2-Evoked Responses of Two *C. elegans* Isolates

C. elegans increases its frequency of spontaneous reversals and high-amplitude turns when exposed to repulsive stimuli and suppresses reversals and turns when exposed to attractants (Chalasani et al., 2007; Ryu and Samuel, 2002). To learn more about O_2 and CO_2 responses of *C. elegans*, we scored turning behaviors (Figure 1A) in freely moving adult animals on a thin lawn of OP50 bacteria, while switching the chamber between two different gas mixtures every 3 min for 60 min (Figure 1B). Video recordings were analyzed using automated tracking software that recorded instantaneous speed, reversals, and turns (Ramot et al., 2008). We focused on omega turns, which are high-amplitude forward turns that reorient movement by >90°.

Strikingly, N2 and HW hermaphrodites, both nominally wild-type, had opposite turning responses to simultaneous changes in CO_2 and O_2 concentrations (Figures 1C and 1D). When N2 animals were shifted from a mixture of 21%O_2/79%N_2 to 1%CO_2/20%O_2/79%N_2, they generated a burst of turns that peaked after ~30 s and fell to baseline ~60 s later (Figures 1C and 1E). The reciprocal switch back to 21%O_2/79%N_2 suppressed turning. By contrast, HW animals shifted from 21%O_2/79%N_2 to 1%CO_2/20%O_2/79%N_2 suppressed turning, and the reciprocal switch back to 21%O_2/79%N_2 caused a burst of turning that peaked after ~30 s and fell to baseline ~60 s later (Figures 1D and 1E). Since transient bursts of turning accompany the appearance of a repellent, these results suggest that HW and N2 have opposite preferences for the two gas mixtures.

To determine which gas was most important for turning behavior, CO_2 and O_2 levels were changed separately. Upon a shift from 0% to 1% CO_2, N2 responded with a burst of turning but HW did not respond; a reciprocal shift from 1% to 0% CO_2 had no effect on N2, but stimulated turning slightly in HW (Figures 1C–1E). These results suggest that N2 avoids CO_2 and that HW is weakly attracted to CO_2. A shift from 21% to

20% O_2, or from 20% to 21% O_2, had little effect on turning in N2 (Figures 1C and 1E). By contrast, HW showed a transient 2-fold increase in turning upon a 20% to 21% O_2 upshift and reduced turning upon the reciprocal O_2 downshift, suggesting a preference for 20% O_2 (Figures 1D and 1E).

Quantitative analysis of the behaviors revealed significant interactions between the gas responses (Tables S1 and S2 available online). The N2 turning response in the mixture was dominated by CO_2 and was consistent with the known avoidance of CO_2 by N2 (Bretscher et al., 2008; Hallem and Sternberg, 2008). The turning response in HW was dominated by a preference for 20% over 21% O_2 and was significantly stronger when both CO_2 and O_2 were changed simultaneously, suggesting a slight attraction to CO_2 and an interaction between the responses.

Two Genetic Loci Control the Difference in CO_2/O_2 Behavior between HW and N2

The genetic basis of the behavioral response to simultaneous changes in O_2 and CO_2 was determined by characterizing 78 RIAILs (Figure 1F). Based on their turning behaviors, the RIAILs fell into a nearly continuous distribution with three general groups: (1) HW-like lines that turned upon simultaneous O_2 increases/CO_2 decreases and suppressed turning upon simultaneous O_2 decreases/CO_2 increases; (2) N2-like lines that turned upon O_2 decreases/CO_2 increases and suppressed turning upon O_2 increases/CO_2 decreases; (3) intermediate lines that did not turn much in response to either change. The continuous distribution of phenotypes and the existence of a novel intermediate behavioral class indicate that this is a complex genetic trait, i.e., that multiple loci influence the behavior.

To identify loci that contribute to the behaviors of the RIAILs, quantitative trait locus (QTL) mapping was performed on the O_2 increase/CO_2 decrease response and on the O_2 decrease/CO_2 increase response using genotypes of the RIAILs at 1455 SNP markers. Both analyses identified two significant QTLs (Figure 1G), one QTL on chromosome V (lod scores 6.5 and 6.6 for the O_2 decrease/CO_2 increase and O_2 increase/CO_2 decrease, respectively, genome-wide corrected $p < 0.0001$ each) and one QTL on chromosome X (lod scores 7.0 and 5.2, $p < 0.0001$ and $p = 0.0003$ for the O_2 decrease/CO_2 increase and O_2 increase/CO_2 decrease, respectively). For the O_2 decrease/CO_2 increase response, the two QTLs account for 79% of the among-line variance, explaining 33% (V) and 45% (X) of the variance with no significant interaction between them ($F_1 = 3.87$, $p = 0.053$). For the O_2 increase/CO_2 decrease response, the main effects of the two loci explain 34% (V) and 35% (X) of the variance, and a significant interaction effect between the two loci accounts for 15% of the among-line variance ($F_1 = 63$, $p < 10^{-10}$; see Experimental Procedures for further discussion).

The genotypes of the two loci on V and X in the 78 RIAILs correlated with the three behavioral classes noted above (Figure 1F). Although the behavioral scores appeared to fall in a continuous distribution, most RIAILs that had N2 alleles of both QTLs behaved like N2 (n = 33), and most RIAILs with HW alleles of both QTLs behaved like HW (n = 13). RIAILs with N2 alleles at one locus and HW alleles at the other had weak responses to either O_2/CO_2 step change (n = 32) (Figure 1F).

To further analyze the mixed allele strains, HW alleles of both QTLs were individually introgressed into N2 by extensive backcrossing, yielding one strain with nearly pure N2 DNA except for the QTL region on chromosome V (referred to as V_{QTL}:HW), one strain with nearly pure N2 DNA except for the QTL region on chromosome X (referred to as X_{QTL}:HW), and one strain with nearly pure N2 DNA with both V and X QTLs from HW (referred to as V_{QTL}:HW; X_{QTL}:HW) (Figure 1H). The three strains were then tested for turning responses to O_2 step changes, CO_2 step changes, and combined O_2/CO_2 step changes (Figures 1I–1K and S1). In all three conditions, V_{QTL}:HW; X_{QTL}:HW were indistinguishable from HW, confirming that two discrete loci account for most of HW behavior (Figure 1K). The V_{QTL}:HW and X_{QTL}:HW strains with one HW locus were distinguishable from each other and from both starting strains. X_{QTL}:HW had minimal responses in all six step changes (Figure 1J). V_{QTL}:HW increased turns in response to individual CO_2 increases but did not respond when these were paired with O_2 decreases (Figure 1I).

The QTL on X Is Caused by a Point Mutation in *npr-1*

The QTL on chromosome X spans the region from 4 to 5.25 Mb, centered on the *npr-1* gene. The high-activity *npr-1(215V)* allele is required for CO_2 avoidance by N2 (Bretscher et al., 2008; Hallem and Sternberg, 2008); since HW animals have the low-activity *npr-1(215F)* allele, an *npr-1* contribution was a plausible explanation for the QTL on X. To test this possibility, a plasmid containing the high-activity N2 *npr-1(215V)* allele was injected into X_{QTL}:HW animals. The resulting transgenic animals behaved like N2 (Figures 2A and S1), indicating that expression of the N2 allele of *npr-1* is sufficient for N2-like turning behavior in X_{QTL}:HW animals. Injecting a comparable low-activity HW *npr-1(215F)* plasmid did not affect the behavior of X_{QTL}:HW animals (Figures 2A and S1). Animals bearing the EMS-induced *npr-1(ad609)* loss-of-function mutant in an N2 background behaved like X_{QTL}::HW animals (Figure 2A), confirming the importance of *npr-1* in the turning assay. These results indicate that variation in the *npr-1* coding sequence can explain the QTL for CO_2 and O_2 behavior on the X chromosome.

The QTL on Chromosome V Is Caused by a Duplication/Insertion in *glb-5*

The QTL on chromosome V was bounded by markers at 5.46 and 5.62 Mb, a region of 160 kb. Nine RIAILs had breakpoints between these markers, and finer breakpoint mapping of these strains narrowed the region to 90 kb, or 19 genes. Two interesting candidates in this interval, *glb-5* and *glb-6*, are predicted to encode proteins with globin domains that can potentially bind O_2 and CO_2 (Hoogewijs et al., 2008). The sequence of the coding region of *glb-6* was identical between N2 and HW, but the genomic sequence of *glb-5* contained a 765 bp duplication/insertion in N2 compared to HW (Figure 2B). *glb-5* cDNA analysis demonstrated that the DNA polymorphism resulted in substantially different mRNAs and predicted GLB-5 proteins in N2 and HW. The duplicated exon in N2 led to an in-frame stop codon in the *glb-5* cDNA, resulting in a truncation of the last 179 amino acids of the protein compared to HW, and the inclusion of 40 different residues (Figure 2B).

To ask whether *glb-5* regulates O_2/CO_2 responses, we first tested heterozygous offspring of V_{QTL}:HW; X_{QTL}:HW and

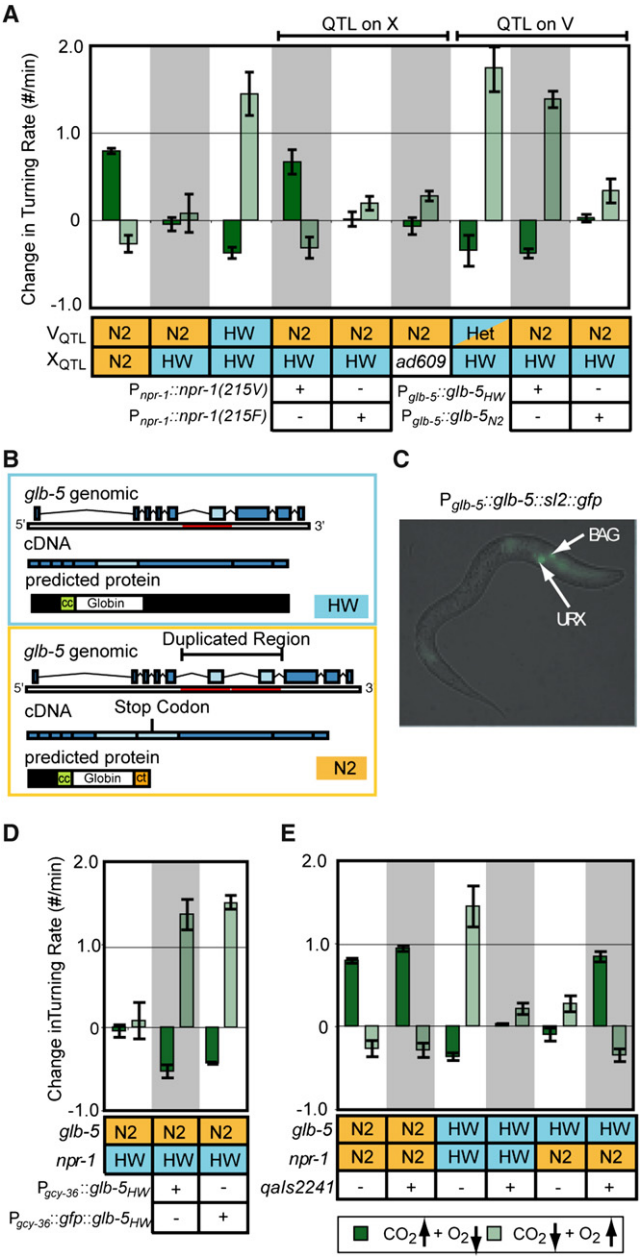

Figure 2. *npr-1* and *glb-5* Polymorphisms Account for the Two Behavioral QTLs

(A) Genetic identification of QTLs on V and X. Turning responses of strains with transgenes bearing N2 or HW alleles of *npr-1*, or N2 or HW alleles of *glb-5*; transgenes were in bicistronic transcripts followed by SL2::GFP. *ad609* is a loss-of-function allele of *npr-1* in the N2 background; "Het" indicates F1 progeny of a X_{QTL}::HW and V_{QTL}::HW; X_{QTL}::HW cross. Complete datasets are in Figure S1.

(B) Sequence polymorphisms in *glb-5* between N2 and HW. In N2, a 765 bp duplication covering the sixth exon truncates the predicted GLB-5 protein. cc, coiled-coil domain; ct, alternate C-terminal domain caused by the duplicated exon.

(C) Expression of bicistronic P_{glb-5}::*glb-5*::*SL2*::*gfp* transgene in L1 animal.

(D) Rescue of X_{QTL}:HW animals by expressing the HW allele of *glb-5* in URX, AQR, and PQR neurons (P_{gcy-36}::*glb-5*$_{HW}$ in bicistronic SL2::GFP clone; P_{gcy-36}::*gfp*::*glb-5*$_{HW}$, N-terminally GFP-tagged *glb-5*$_{HW}$).

X_{QTL}:HW animals to determine which allele of the QTL on V was dominant when *npr-1* genotype was held constant. These offspring behaved like V_{QTL}:HW; X_{QTL}:HW (Figure 2A), indicating that the HW variant on chromosome V was dominant. To ask whether the QTL corresponded to *glb-5*, a *glb-5* cDNA encoding the HW allele of *glb-5* driven by its own promoter was injected into X_{QTL}:HW animals. The resulting transgenic animals behaved like HW animals, indicating that *glb-5* transgene can mimic the presence of the HW locus on V (Figures 2A and S1). Injection of a similar transgene bearing the N2 allele of *glb-5* did not affect X_{QTL}:HW turning behavior (Figures 2A and S1). These results indicate that the 765 bp duplication/insertion in *glb-5* is the likely cause of the QTL on chromosome V.

glb-5 Acts in URX, AQR, and PQR O_2-Sensing Neurons

The expression of *glb-5* was characterized with a bicistronic transgene that encoded the active HW allele of *glb-5* followed by the coding region of GFP. In first larval stage (L1) animals, GFP was expressed prominently in the URX and BAG sensory neurons, with weaker and inconsistent expression in the ASG and ADF sensory neurons, the pharynx, and a few intestinal cells (Figure 2C). In older animals, GFP fluorescence was also observed in AQR and PQR sensory neurons. URX, AQR, and PQR are O_2-sensing neurons that are required for *npr-1* mutants to avoid high O_2 on food (Chang et al., 2006; Cheung et al., 2005; Gray et al., 2004), suggesting a possible functional link between *glb-5* and the O_2 response. Indeed, expressing the HW allele of *glb-5* in X_{QTL}:HW animals under the *gcy-36* promoter, which is selectively expressed in the URX, AQR, and PQR neurons, was sufficient for HW-like behavior (Figures 2D and S1).

The role of URX, AQR, and PQR in turning responses was confirmed using a strain in which the URX, AQR, and PQR neurons are selectively killed by the BH3 protein *egl-1* (the transgene *qaIs2241*) (Chang et al., 2006). AQR, PQR, and URX were essential for the O_2 and O_2/CO_2 responses in the V_{QTL}::HW; X_{QTL}::HW background, but they were not required for turning responses in N2 (Figures 2E and S1). An interesting result was observed in the V_{QTL}:HW animals with *glb-5*$_{HW}$ and *npr-1*$_{N2}$. Killing URX, AQR, and PQR in this strain resulted in an N2-like turning response, although the parent strain only responded to CO_2 alone (Figures 2E and S1). Thus, the cell ablations uncovered a neuronal function that depends on the specific genetic background: URX, AQR, and PQR neurons with the *glb-5*$_{HW}$ allele inhibited N2-like O_2/CO_2 responses in V_{QTL}:HW animals, but enhanced HW-like O_2 and O_2/CO_2 responses in animals with HW alleles of both *glb-5* and *npr-1.*

glb-5 Sensitizes URX Responses to Small O_2 Changes

Animals from the HW strain aggregate into feeding groups and accumulate strongly at the border of a bacterial lawn; these behaviors require the URX, AQR, and PQR neurons, low *npr-1* activity, and high O_2 levels (Coates and de Bono, 2002; Gray et al., 2004). To ask whether *glb-5* contributes to these more complex O_2-dependent behaviors, aggregation and bordering

(E) Killing URX, AQR, and PQR with *qaIs2241* eliminates the effects of *glb-5*$_{HW}$. In panels (A), (D), and (E), control strains from Figure 1 are included for reference.

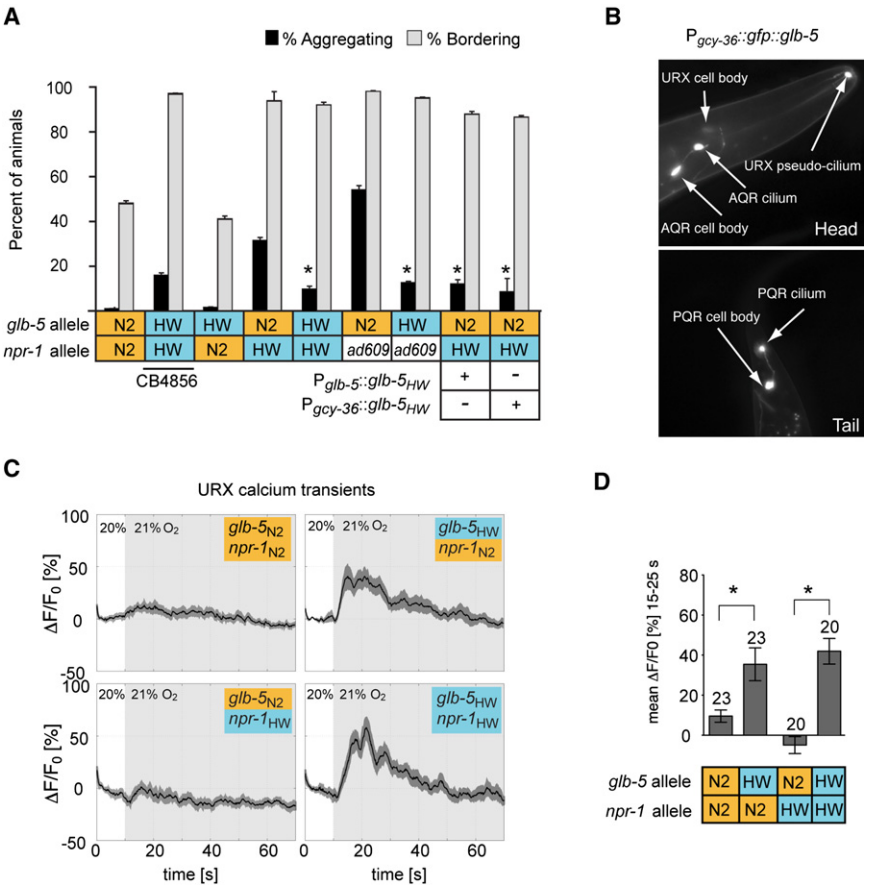

Figure 3. *glb-5* Affects Aggregation Behavior and URX Sensory Responses

(A) Aggregation and bordering behaviors in N2, HW (CB4856), introgression strains, and transgenic rescued lines. Asterisks indicate results significantly affected by $glb\text{-}5_{HW}$ genotype or transgene at $p < 0.01$ compared to the appropriate $glb\text{-}5_{N2}$ control.

(B) GFP-tagged GLB-5_{HW} protein (Figure 2D) is enriched in AQR and PQR cilia and in the anterior tip of URX.

(C) Calcium responses of URX neurons in strains with four combinations of *npr-1* and *glb-5* alleles, as indicated. Fluorescence increases in the G-CaMP indicator are caused by Ca^{2+} increases, likely associated with depolarization. Light shading indicates time at 21% O_2; dark shading indicates SEM; n = 20–23 animals for each genotype.

(D) Average calcium increase at t = 15–25 s from (C). Asterisks, results different at $p < 0.01$.

were quantified in N2, HW, and introgressed strains. Indeed, aggregation in an $npr\text{-}1_{HW}$ genetic background was significantly modulated by the *glb-5* allele: strains with $glb\text{-}5_{N2}$ aggregated more strongly than strains with $glb\text{-}5_{HW}$ (Figure 3A). $glb\text{-}5_{HW}$ also suppressed the aggregation of an *npr-1(ad609)* loss-of-function allele, but in the $npr\text{-}1_{N2}$ genetic background, there was virtually no aggregation regardless of *glb-5* genotype (Figure 3A). As expected from the dominant nature of the $glb\text{-}5_{HW}$ allele, aggregation of the $glb\text{-}5_{N2}$ strain was suppressed by expressing $glb\text{-}5_{HW}$ under its own promoter or under another promoter expressed in URX, AQR, and PQR neurons (Figure 3A). The *glb-5* allele had relatively little effect on bordering behavior, which was largely determined by *npr-1* genotype (Figure 3A).

In principle, *glb-5* could affect O_2 sensation in URX, AQR, and PQR, or synaptic release onto other neurons, among other possibilities. A functional, GFP-tagged GLB-5 protein encoded by the HW allele of *glb-5* was highly enriched in sensory endings of URX, AQR, and PQR, with less expression in cell bodies, axons, and dendrites (Figure 3B). This localization suggests a role for GLB-5 in sensory transduction.

Previous studies with genetically encoded calcium sensors have shown that the URX neurons are activated by O_2 upshifts from 10% → 21% or 15% → 21% O_2 (Zimmer et al., 2009). The *glb-5*-dependent behaviors in HW suggest that *glb-5* might affect URX responses to smaller O_2 changes. Therefore, calcium responses of URX were monitored in response to upshifts from 20% → 21% O_2, the conditions in which N2 and HW behaved differently. In the V_{QTL}:HW animals with the $glb\text{-}5_{HW}$ allele, these small O_2 20% → 21% upshifts induced rapid calcium transients in URX (Figures 3C and 3D) but in the N2 strain, similar upshifts had no apparent effect. Similar URX responses to 20% → 21% O_2 were observed in $glb\text{-}5_{HW}$ strains with either $npr\text{-}1_{HW}$ and $npr\text{-}1_{N2}$ alleles, but not in any $glb\text{-}5_{N2}$ strains (Figures 3C and 3D). These results suggest that the HW allele of *glb-5* sensitizes URX responses to small 20% → 21% O_2 upshifts.

N2 Alleles of *npr-1* and *glb-5* Are Rare in Wild Isolates of *C. elegans*

To assess the frequency and distribution of *glb-5* and *npr-1* alleles in the wild, each gene was characterized in 203 *C. elegans* isolates from Europe, North America, Africa, South America, Australia, and Japan (Table S3). The genotypes were highly skewed: 190 strains had HW alleles of both genes, and 12 strains had N2 alleles of both *npr-1* and *glb-5*, of which only one was isolated in the past 15 years (Figure 4A). One strain (TR389) had the N2 allele of *npr-1* and the HW allele of *glb-5*.

Additional genotyping at 1454 loci revealed that all N2-like strains were genetically similar (Figure 4B). Nine of the N2-like strains were identical in at least 1453 out of 1454 HW/N2 single nucleotide polymorphisms (SNPs) (group 1, Figure 4B). The remaining four N2-like strains had blocks of HW SNPs on chromosome IV and V, but were otherwise N2-like (group 2, Figures 4B and 4C). The specific blocks of SNPs in these four strains, as well as the pattern of Tc1 transposon insertions (Egilmez et al., 1995), match blocks that are present in the genome of the Bergerac strain of *C. elegans* (Figures 4C and 4D). A comparison across genotypes of these strains suggests that these four strains resulted from crosses between an N2-like strain and a Bergerac-like strain (Egilmez et al., 1995).

A

Wild strain genotyping

	npr-1 allele	
glb-5 allele	HW	N2
HW	190	1
N2	0	12

B

Strains	Group	Δ SNPs (of 1454)	*glb-5*	*npr-1*
N2, PX176, TR388	1	0	N2	N2
TR389	1	0	HW	N2
CB3191-5, CB4507	1	1	N2	N2
LSJ1	1	1	HW	HW
CB4555, DH424	2	72	N2	N2
TR403, DR1349	2	73	N2	N2

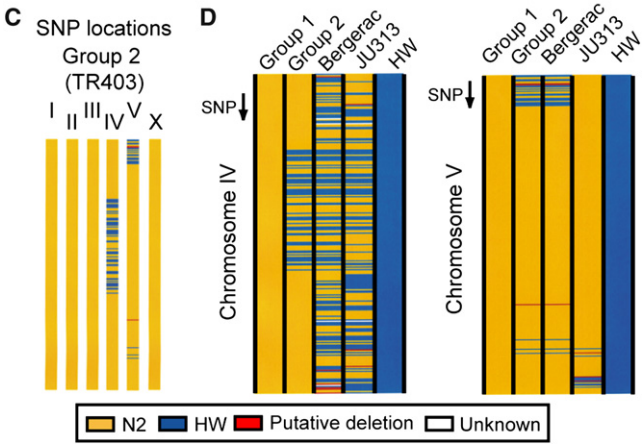

Figure 4. Analysis of Polymorphisms in Wild Strains

(A) *glb-5* and *npr-1* allele distributions in 203 wild strains.

(B) Genotype analysis of 1454 SNPs of the strains containing N2 alleles of *npr-1* or *glb-5*, and of LSJ1; data from Rockman and Kruglyak (2009). In 110 additional wild strains genotyped at these 1454 SNPs, there were on average 358 differences from N2, with a range of 112–1452 differences.

(C) SNP genotypes on all chromosomes in group 2 strains.

(D) Comparison of SNPs in group 2 strains with N2, HW, and Bergerac on chromosomes IV and V. The most similar wild strain in the region shared between group 2 animals and Bergerac on chromosome IV (JU313) is shown for reference.

Since these results suggest that N2-like strains are rare in the wild, we specifically examined additional strains from the Pasadena area where nine of the thirteen N2-like strains were isolated. Fifty-five more recently isolated strains all had HW-like alleles of *npr-1* and *glb-5* (Table S3).

DISCUSSION

N2 and HW strains of *C. elegans* have opposite behavioral responses to small changes in O_2 and CO_2. Despite the high genetic variability between these strains (Maydan et al., 2007; Wicks et al., 2001), two QTLs, one caused by a polymorphism in *npr-1* and the other caused by a polymorphism in *glb-5*, account for most of the variation in these traits.

glb-5 is a member of the globin-domain superfamily, a widespread group of heme-binding proteins that bind O_2 and typically act in O_2-affiliated roles such as transport, storage, scavenging, or sensing (Weber and Vinogradov, 2001). There are 33 distinct globin-domain-containing proteins encoded by the *C. elegans* genome (Hoogewijs et al., 2007); most are expressed exclusively in subsets of neurons, and one, GLB-10, is enriched at synapses (Hoogewijs et al., 2008; Sieburth et al., 2005). Like mammalian neuroglobins (Nienhaus and Nienhaus, 2007), the functions of neuronal *C. elegans* globins are largely unknown. Our results demonstrate a sensory role for GLB-5 that shapes behavioral responses to O_2, a new insight into a poorly understood chemosensory modality.

O_2 sensation in *glb-5*-expressing neurons requires a cGMP second messenger and specific soluble guanylate cyclase homologs (sGCs) that detect O_2 upshifts and downshifts (Zimmer et al., 2009). Members of this sGC subfamily can bind O_2 through a heme group and have been proposed to generate cGMP in an O_2-regulated manner (Gray et al., 2004; Zimmer et al., 2009). sGCs are essential for all O_2 sensitivity in URX, but the HW *glb-5* polymorphism has a more subtle effect: it increases URX sensitivity to a small 20%–21% O_2 upshift, suggesting that it alters the most-preferred O_2 level. This interpretation is consistent with *glb-5* modulation of O_2-dependent aggregation behavior, as aggregates have lower O_2 levels than lawn borders or open lawns (Gray et al., 2004; Rogers et al., 2006). One appealing reason to study quantitative genetic variation is the ability to discover biologically important molecules with subtle phenotypes, like *glb-5*, that might not emerge from classical forward genetic screens.

When and where did the polymorphisms in *npr-1* and *glb-5* arise? The genotyping of wild strains showed that N2-like alleles are evolutionarily recent. Only 13 of 203 *C. elegans* strains had an N2 allele of either *glb-5* or *npr-1*, and high-density analysis at 1454 SNP loci implies that the *npr-1* and *glb-5* polymorphisms arose recently in a common, N2-like genetic background, which in one case mated with a Bergerac-like strain. In this context, it is possible that the strains with N2-like *npr-1* and *glb-5* alleles are not actually independent wild strains, but reisolates of laboratory N2 or N2-derived strains. Many of the N2-like strains were isolated by procedures on exposed laboratory benches with a high potential for cross-contamination (P. Anderson, P. Phillips, and C. Johnson, personal communication). Molecular results also raise suspicions about the N2-like strains CB4555 and DR1349, whose genotypes are inconsistent with their recorded history but consistent with N2 cross-contamination (Hodgkin and Doniach, 1997). The fact that the four "group 2" strains can be explained as a cross between N2 and Bergerac is problematic, as Bergerac strains were maintained in the laboratory for over 30 years in association with N2; N2 and Bergerac were often intercrossed in the laboratory to generate strains with high transposon activity (Hodgkin and Doniach, 1997).

We suggest that the N2 alleles of *npr-1* and *glb-5* may have originated in the laboratory, where N2 was cultivated for nearly two decades before permanent frozen cultures were established in 1969. The N2 strain was originally isolated around 1951 by Warwick Nicholas (personal communication) from mushroom compost produced by L.N. Staniland; a sample was given to Ellsworth Dougherty at UC Berkeley, who sent a sample to Sydney Brenner, who isolated a single hermaphrodite whose progeny became N2 (Hodgkin and Doniach, 1997). From the outset, the Brenner strain had the nonaggregating behavior characteristic of the N2 *npr-1* allele (S. Brenner, personal communication). A second strain derived from the Dougherty lab, LSJ1, is identical to N2 at 1453 of 1454 SNPs, but has HW alleles of *glb-5* and *npr-1* (Figure 4B, Table S3). The existence of HW-like and N2-like alleles of *npr-1* and *glb-5* in one genetic background from the Dougherty

lab, and only HW-like alleles in most wild-caught strains, suggests that the N2 alleles arose after N2 and LSJ1 separated in the laboratory. *C. elegans* has a high mutation rate ($\sim 2 \times 10^{-8}$ mutations per site per generation) (Denver et al., 2004), so *npr-1* and *glb-5* mutations could have occurred without directed mutagenesis.

The N2 alleles of *glb-5* and *npr-1* could be random mutations fixed by genetic drift, but it would seem to be an unlikely coincidence that both mutations affect O_2 responses. Alternatively, one or both of these mutations might have conferred a selective advantage in the laboratory. N2 alleles of both *npr-1* and *glb-5* modify behaviors at 21% O_2, the normal condition in the laboratory, which is higher than *C. elegans*'s preferred level of 5%–10% O_2 (Gray et al., 2004). At 21% O_2, strains with the HW allele of *npr-1* spend less time than N2 on good food sources and are more easily killed by pathogenic bacteria (Gloria-Soria and Azevedo, 2008; Reddy et al., 2009; Styer et al., 2008); selection for successful behaviors at high O_2 could have contributed to the fixation of *npr-1* mutant alleles in N2. Whether the *glb-5* polymorphism has similar effects is unknown. In addition, strains with the HW allele of *npr-1* aggregate and burrow into the agar (de Bono and Bargmann, 1998), and experimentalists who isolate single animals from the agar surface would have favored solitary N2-like animals.

Behavioral adaptations are likely to have occurred in many experimental species during their cultivation in the laboratory. Domesticated animals show less aggressiveness, less fearful behavior, and better mating in captivity than their wild ancestors (Grandin and Deesing, 1998); similar selections are placed on laboratory strains. If recognized, laboratory adaptations can be addressed experimentally. For example, regardless of whether N2 alleles of *npr-1* and *glb-5* are laboratory adaptations, the predominance of HW alleles in wild strains suggests that the HW-like response to O_2 and CO_2 is more ecologically relevant. Therefore, in future studies of *C. elegans* behavior it should be worthwhile to characterize behaviors in strains with the HW alleles of *glb-5* and *npr-1*, particularly for behaviors that are sensitive to O_2 and CO_2.

EXPERIMENTAL PROCEDURES

Genetics and Molecular Biology

Strains were grown and maintained under standard conditions (Brenner, 1974). N2 is *C. elegans* Bristol strain N2; HW is *C. elegans* strain CB4856. Additional strains were isolated from two locations in California (see Supplementary Data) or were acquired from the CGC or from Marie-Anne Felix, Antoine Barriere, Michael Ailion, Jody Hey, and Elie Dolgin. A complete strain list and details of strain construction for introgression lines are found in Supplementary Data. Standard molecular methods were used; genotyping primers are found in Supplementary Data.

Quantitative Genetics

Seventy-eight recombinant inbred advanced intercross lines from a reciprocal cross between N2 and CB4856 were analyzed. The RIAILs were inbred from a ten generation intercross employing random pair mating with equal contributions of each pair to each generation (Rockman and Kruglyak, 2008; Rockman and Kruglyak, 2009).

Line means plotted in Figure 1F were used as phenotypes for nonparametric interval mapping in Rqtl (Broman et al., 2003). Lod scores were computed at each marker and at intervals of 1 cM; these genetic distances are estimated from the recombination fractions in the RIAILs as though they were observed in a backcross, using the Haldane map function. Technical issues are discussed in Supplemental Data.

For the O_2 increase/CO_2 decrease response, a significant interaction effect between the two QTLs accounts for 15% of the among-line variance ($F_1 = 63$, $p < 10^{-10}$). This apparent interaction may be explained by a bounded phenotype distribution. The phenotype, turns per minute, is close to zero in an $npr\text{-}1_{N2}$ background; the suppression of turning by O_2 upshift/CO_2 downshift could be masked by that low basal level, and easier to see in the more motile $npr\text{-}1_{HW}$ background.

Behavioral Assays

O_2- and CO_2-evoked turning responses were monitored on an NGM plate seeded with OP50 bacteria (grown overnight), with 20–30 adult animals confined to a 28 × 28 mm region using Whatman filter paper dipped in 20 mM $CuCl_2$. A custom-designed Plexiglass device containing an inlet, an outlet, and a 30 × 30 × 0.3 mm behavioral arena created laminar air flow over the animals. Gas tanks were ordered at primary mixture grade from Matheson TriGas. A standard tank at 21.2%O_2/78.8%N_2 was mixed 99:1 with either 100% N_2 or 100% CO_2 to create a tank at 21%O2/79%N_2 or 21%O_2/1%CO_2/78%N_2. A standard tank at 20.2%O_2/79.8%N_2 was similarly used to create tanks at 20%O_2/80%N_2 or 20%O_2/1%CO_2/79%N_2. Gas mixtures were bubbled through water at a flow rate of 50 cm_3/min, and a Hamilton MVP was used to switch between two gas tanks every 3 min for 60 min. Animals were recorded at 3 frames/s using a Zeiss microscope and Pixelink PL-A741 Monochrome Camera, and automatically tracked using Matlab software (Chalasani et al., 2007; Ramot et al., 2008).

Aggregation and bordering behaviors were measured using 60 young adult animals on a lawn of OP50 bacteria essentially as described (de Bono and Bargmann, 1998; Gray et al., 2004).

Calcium Imaging

Transgenic animals expressing the fluorescent calcium sensor G-CaMP1.0 in URX neurons were exposed to O_2 upshifts and downshifts while trapped in a custom-fabricated PDMS device (Zimmer et al., 2009). The two-layer device allowed rapid diffusion of gas mixtures from a flow chamber into a calibrated channel containing the trapped animal. Fluorescence intensities were measured with a Nikon CoolSnap camera attached to a Zeiss Axiovert microscope while switching between different gas mixtures in the flow chamber and analyzed with a script written in MetaMorph programming language. $\Delta F/F_0$ was calculated as the percent change in fluorescence relative to the mean basal fluorescence (F_0) from 1 to 4 s of each recording.

SUPPLEMENTAL DATA

The Supplemental Data can be found with this article online at http://www.neuron.org/supplemental/S0896-6273(09)00157-3.

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