Altered Levels of Gq Activity Modulate Axonal Pathfinding in Drosophila

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A majority of neurons that form the ventral nerve cord send out long axons that cross the midline through anterior or posterior commissures. A smaller fraction extend longitudinally and never cross the midline. The decision to cross the midline is governed by a balance of attractive and repulsive signals. We have explored the role of a G-protein, Gαq, in altering this balance in Drosophila. A splice variant of Gαq, dgαq3, is expressed in early axonal growth cones, which go to form the commissures in the Drosophila embryonic CNS. Misexpression of a gain-of-function transgene of dgαq3 (AcGq3) leads to ectopic midline crossing. Analysis of the AcGq3 phenotype in roundabout and frazzled mutants shows that AcGq3 function is antagonistic to Robo signaling and requires Frazzled to promote ectopic midline crossing. Our results show for the first time that a heterotrimeric G-protein can affect the balance of attractive versus repulsive cues in the growth cone and that it can function as a component of signaling pathways that regulate axonal pathfinding.

Key words: dgαq; Robo; Frazzled; Netrins; G-protein; midline; axon guidance

Axons use cues present at different choice points in the cellular environment to reach their targets. These cues are attractive and repulsive in nature and function at short range by contact or through long range by diffusion (Tessier-Lavigne and Goodman, 1996). The midline of the CNS in vertebrates and invertebrates serves as one such choice point for axons that need to project to their targets on the opposite side in the CNS. Cells at the midline provide cues that are both attractive and repulsive and thus enable axons to make appropriate decisions at the midline. Attractive cues are encoded by molecules called Netrins (Ishii et al., 1992; Kennedy et al., 1994; Harris et al., 1996; Mitchell et al., 1999; Hong et al., 1999). Studies in vertebrate systems have shown that altering cyclic nucleotide levels and calcium in the growth cone can convert attraction into repulsion (Song et al., 1997; Hong et al., 2000; Zheng, 2000), suggesting that G-protein-coupled signaling pathways are involved in this process. In this study we have examined the role of the heterotrimeric G-protein Gq in growth cone guidance in Drosophila. The gene dgαq encodes the α subunit of the Gq class of heterotrimeric G-proteins in Drosophila. This family of G-proteins is known to activate the phosphoinositide cascade within cells, which involves generation of inositol 1,4,5-trisphosphate (IP₃) followed by release of intracellular calcium through the IP₃ receptor (Exton, 1994). In Drosophila, the role of this gene in mediating phototransduction in the adult eye has been well established (Lee et al., 1994; Scott et al., 1995). We find that a splice variant of the Gq gene (dgαq3; Talluri et al., 1995; Alvarez et al., 1996) is expressed in the embryonic CNS during development. In this study we show for the first time that a dominant active form of dgαq3 modulates repulsive signaling in the growth cone, possibly in response to attractive cues. Our results suggest that Gq signaling could function as a part of the regulatory network that functions to tilt the balance from repulsion to attraction during midline crossing of axons.

MATERIALS AND METHODS

cDNA isolation and sequencing. Embryonic and appendage cDNA libraries were screened using a probe generated by a PCR using degenerate primers on an appendage library (Wang et al., 1999). The primer sequences were as follows: (1) 5′AC(T/C/A/G)TT(T/C)AT(T/C/A)AA(G/A)CA(A/G)ATG 3′; (2) 5′(A/G)AA(A/G)CA(A/G/T/C)TG(A/G)TA(T/C)CA(C/T)TT 3′.

They correspond to the conserved amino acid sequences “TFIKQMO” and “KWIOHCF” in the helical domain of Gq proteins. Standard PCR conditions for degenerate primers were used (Hasan and Rosbash, 1992). The PCR product was reamplified using an internal primer: 5′(T/C/A/G)TC(A/G)AA(A/T/C/G)GG(A/G)TA(T/C)TC3′, which corresponds to the conserved amino acid sequence “EYPFDL”. A 400 bp product was obtained using the internal primer. This was subcloned into the plasmid

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The samples were run on a 10% SDS-polyacrylamide gel and visualized with the antibody (Baines et al., 1999). The antibody was used at a dilution of 1:200. Anti-Gq antiserum from Santa Cruz Biotechnology (Santa Cruz, CA) was used at a dilution of 1:1000.

In situ hybridization to whole mount embryos. In situ hybridization to embryos was according to the procedure described by Tautz and Pfeifle (1989). A 210 bp dgqa3-specific probe was generated and labeled by PCR using primers to exons 11 and 14. DGQ-γ-dUTP from Boehringer Mannheim (Mannheim, Germany) was included in the PCR mix.

Western blot analysis. Protein extracts from adult tissues and 0–8, 8–16, and 16–24 hr embryos were made by homogenization in polyacrylamide gel sample buffer at twice its normal concentration (Sambrook et al., 1989). The samples were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Detection of the protein blot was achieved by procedures (Edery et al., 1994) with minor modifications. Anti-Gq antiserum from Santa Cruz Biotechnology (Santa Cruz, CA) was used at a dilution of 1:1000.

Immunohistochemical methods. Immunohistochemical staining of whole-mount embryos was according to published protocols (Gould et al., 1990). Developmental stages were identified following the description by Wieschaus and Nasenfeld-Volhard (1986). The anti-Gq antibody used from Santa Cruz Biotechnology has been directed against the C-terminal peptide (FQKKGQVDRQDPLSSLK) of mammalian Goq. This differs from the corresponding Drosophila Dgqa3 sequence by a single residue (FQVKDGQVDRQDPLSSLK). For immunohistochemistry the antibody was used at a dilution of 1:200. Anti-flg monochonal supernatant, monochonal antibody (mAb) 40–1a (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and mAb 1D4 (anti-Fasciclin II; courtesy C. Goodman laboratory, University of California, Berkeley, CA) were used at a dilution of 1:25 each. Anti-Robo (courtesy C. Goodman) and anti-Connectin antisera (courtesy of Rob White, Department of Anatomy, University of Cambridge, Cambridge, UK) were used at a dilution of 1:10. Vectastain A+B kit (Vector Laboratories, Burlingame, CA) was used for nonfluorescent immunohistochemical visualizations. The stained embryos were filleted and mounted in 90% glycerol, Fluoro-isothiocyanate (FITC) and rhodamine or Alexa Red conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA; Molecular Probes, Eugene, OR) were used at a dilution of 1:200. Specimens stained with fluorescent secondary antibodies were mounted in 70% glycerol containing 1 mg/ml of p-phenylene diamine (Sigma, St. Louis, MO) to prevent quenching.

Confocal imaging. Confocal images of antibody staining done with fluorescent secondary antibodies were viewed on Bio-Rad (poole, UK) MRC 1024. For double-labeled images, data from the two channels (605 DF32 for rhodamine and Alexa Red and 522 DF32 for FITC) were superimposed using Metamorph software version 4.0. Confocal sections of 2 μm thickness were obtained, and composite images were created by merging relevant numbers of sections. Confocal sections of 0.3 μm thickness were obtained for the images shown in Figure 2, E and F. Site-directed mutagenesis and germline transformation. The Q203L mutation requiring an A→T change was introduced in the dgqa3 cDNA by site-directed mutagenesis using the Quik-change kit by Stratagene. The primer used to introduce the mutation was 5′-CGGCGGCGTCGCTGACGATCCCG3′. The mutant cDNA was sequenced fully to ensure that no other mutations had been incorporated into the modified sequence. Both mutant and wild-type cDNAs were independently subcloned into the transformation vector pUAST (Brand and Perrimon, 1993) to obtain germline transformants. Two independent transformant lines for each construct were obtained. These were UAS-Dgqa3 (F17F2-2 on chromosome 3, UAS-Dgqa3(MM17-2 on chromosome 2, UAS-Dgqa3 on chromosome 1, and UAS-Dgqa3 on chromosome 2. Equivalent phenotypes were observed with both sets of transformant lines.

Fly stocks. All stocks were grown at 25°C. The following GAL4 stocks were used: C155-GAL4 (Lin and Goodman, 1994), f27z-GAL4 and eveng-GAL4 (courtesy of Jim Jaynes, Thomas Jefferson University, Philadelphia, PA) (Baines et al., 1999). The Ap-tau+gal stock was obtained from the laboratory of Dr. John Thomas (Salk Institute, San Diego, CA) (Lundgren et al., 1995), whereas the Ap-GAL4 stock was obtained from the Drosophila stock center (Bloomington, IN), UAS-roboF-YF (Bashaw et al., 2000) was obtained from C. Goodman’s laboratory. The Df(2R)y2w-GC stock, which carries the deficiency for dgq, was obtained from the Drosophila stock center and placed against a CyoriDgal balancer to identify homozygous deficiency embryos. For expression of AcGq3, males of the genotypic UAS-AcGq3F7-MT-GFP were crossed to homozygous females of one of the following genotypes: (1) C155-GAL4, (2) C155-GAL4; G-– Ap-tauDgal, (3) eveng-GAL4; UAS-tauDgal, or (4) eveng-GAL4. To study the behavior of Apertour neurons, an Apterous-GAL4/TopoWgal balancer was generated and subsequently crossed to UAS-AcGq3F7/Mt/CyoriDgal. To examine the genetic interactions between AcGq3 and robe1 and frazzled mutants, the following strains were generated: C155-GAL4/frz/TopoWgal/+; UAS-AcGq3/Mt/CyoriDgal. Homozygous and heterozygous mutant embryos were distinguished based on the presence or absence of marked labels in each case. For studying the interaction between robeo-Y-F and AcGq3, UAS-AcGq3F7/Mt/CyoriDgal, UAS-robo-Y-F strain was generated and crossed to C155-GAL4 females. Expression of AcGq3 was confirmed by immunohistochemical staining with anti-Gq antibody. With both C155-GAL4 and frz/+Dgal drivers, the pattern of Gq expression observed is different from the wild-type pattern.

RESULTS

Identification and expression of dgqa3 in Drosophila embryos

CDNA clones corresponding to the dgq gene were isolated in library screens using a fragment from the eye-specific splice variant dgqa1 (Lee et al., 1990). We screened libraries derived from either embryo or appendage RNAs and analyzed dgq-negative cDNA clones by restriction digests and PCR. The three classes of cDNA clones obtained are shown in Figure 1A. Of these, one class corresponds, in the region of the open-reading frame, to the previously identified splice variant transcript of the dgq gene, called dgqa3, known to be expressed in several adult tissues (Talluri et al., 1995; Alvarez et al., 1996). This class was isolated repeatedly from the embryo cDNA library, as judged by extensive PCR analysis. Specifically, primers to exons 4 and 9, 6 and 9, and 6, 11, and 11 and 14 (Figure 1A), amplified the expected fragments of 673, 321, 445, 210 bp. No amplification was observed using primers to exons 6 and 12 and exons 12 and 13, indicating that none of the embryonic cDNAs belong to the next class of cDNA isolated from the appendage library (dgqa4) (Fig. 1A). Because this was the first characterization of a dgq transcript in Drosophila embryos, we performed RT-PCRs using dgqa3-specific primers from exons 11 and 14 (Fig. 1A). As shown in Figure 1B, dgqa3-specific transcripts are present in poly(A+) RNA extracted from heads, appendages, male and female bodies, and embryos. These results were corroborated by a Northern blot analysis using the unique 3′ region of dgqa3 as a probe (data not shown). A third class of cDNA clones was found only in the appendage library and appeared identical to the adult visual Gq splice form (dgqa1) (Fig. 1A), as determined by the presence of dgqa1-specific exon 7 (by PCR), and exons 10 and 13 (by sequencing) (Fig. 1A). We have not analyzed this cDNA any further.

Next we ascertained the presence of the Dgq3 protein in Drosophila embryos by Western blot analysis of embryo extracts (Fig. 1C). The antisera used recognizes the C-terminal end of the mammalian Gq protein. In Drosophila Gq this C-terminal region is conserved only in the Dggq3 form (Fig. 1A, asterisk) (see Materials and Methods). The results obtained indicate that a 39 kDa band, corresponding to the predicted size of the Dgqa3 protein, is present in embryos throughout development from as early as 0–8 hr.
Therefore studied the expression pattern of \( \text{dgq} \) in axonal tracts of the embryonic CNS variant-specific \( \text{dgq} \) (Schmucker et al., 1992). In similar experiments with a corresponding in position to the Bolwig presence of \( \text{dgq} \) persistence till late in development, where in addition, strong expression is seen in an anterior sense organ (Fig. 2A and ventral nerve cord at stage 13 (Fig. 2B). This organ corresponds in position to the Bolwig’s organ or the larval eye (Schmucker et al., 1992). In similar experiments with a \( \text{dgq} \)-specific probe, no hybridization was observed to any region of the developing embryo (data not shown).

Expression of Dgq3 during development of the embryonic nervous system was further confirmed by immunohistochemical staining of wild-type embryos with the Gq antiserum. The CNS in \textit{Drosophila} embryos develops from a delaminated set of neuroblasts that derive from the ventral neuroepithelium after gastrulation (Goodman and Doe, 1993). These neuroblasts undergo a series of highly stereotyped cell divisions during embryonic stages 8–11, which lead to a well-defined spatial pattern (Goodman and Doe, 1993). The expression of Dgq3 at these and earlier stages appeared diffuse and non-neuronal (data not shown). The first indication of Dgq3 expression in the CNS is at early stage 12 (Fig. 2C). This is also the stage at which the pioneer neurons begin formation of axon pathways that give rise to the typical ladder-like appearance of the embryonic CNS, consisting of longitudinal tracts and anterior and posterior commissures that can be visualized with the axonal marker mAb BP102 (Fig. 2F). A similar pattern of expression of anti-Gq and the axonal marker mAb BP102 at early stage 12 suggests that Dgq3 is expressed in the pioneer growth cones that give rise to the commissures (Fig. 2E,F) (Klambt et al., 1991). At later stages of development Dgq3 protein expression increases in the axonal tracts of the CNS (Fig. 2D,G). In addition, Dgq3 expression was visible in the midgut epithelium at stages 12 (Fig. 2C) and 13 (data not shown). Specificity of the anti-Gq antibody was determined by immunohistochemical staining of embryos that were either deficient for \( \text{dgq} \) in one copy (Fig. 2G,I) or both copies (Fig. 2H,J). The likely presence of the Dgq3 protein in growth cones of early commissural axons leads us to examine the role for this gene in axonal growth and guidance.

**Neuronal expression of the activated form of Dgq3 causes abnormal midline crossing**

Axonal guidance in the \textit{Drosophila} CNS requires the interpretation of both attractive and repulsive cues, generated by cells that lie in the midline (Harris et al., 1996; Kołodziej et al., 1996; Mitchell et al., 1996; Culotti and Merz, 1998; Kidd et al., 1999). The expression pattern of Dgq3 protein suggested that it might
Figure 2.  *In situ* localization of *dgq* RNA and protein in *Drosophila* embryos.  *A, B,*  *In situ* hybridization of a *dgq*-specific probe showing RNA expression at late stage 13 (*A*) and stage 16 (*B*).  The *arrows* indicate localization of *dgq* RNA in the ventral nerve cord (*A*) and the Bolwig’s organ (*B*).  *C, D,*  *dgq* expression in the embryonic CNS observed by immunofluorescent staining using anti-Gq antibodies at stage 12 (*C, arrows*) and stage 17 (*D*).  *E, F,*  Confocal images of the developing CNS in an early stage 12 embryo after double-staining with anti-Gq (*E*) and the axonal marker mAb BP102 (*F*).  *G, H,*  Confocal images of the CNS from stage 15 embryos stained with Anti-Gq.  Embryos were either heterozygous (*G*) or homozygous (*H*) for the *Df(2R)vg-C*.  *I, J,*  Embryos shown in *G* and *H* were double-stained with mAb BP102.  Heterozygous and homozygous deficiency embryos were distinguished by the presence of actin-lacZ on the balancer chromosome, which shows up as green spots (*I, arrow*).  The commissures are poorly formed and appear thin in *J.*  Anti-Gq was visualized using rhodamine-labeled secondary antibodies, whereas an FITC-linked secondary was used for BP102.  Magnification: *A–D,* 200×; *E–J,* 600×.  *A–C* are lateral views with dorsal side up.  *D–J* are ventral views.  In all cases anterior is to the left.

be required in early growth cones for the interpretation of these cues. To address this possibility, it was essential to alter Gaq signaling in a tissue and cell-specific manner.  We therefore created transgenic strains with a dominant active form of Dgq3, in which a glutamine residue at position 203 was mutated to a leucine.  The mutation was made based on previous studies on dominant active forms of Gaq from mammalian cells and *Drosophila* (DeVivo et al., 1992; Lee et al., 1994).  As controls we also generated transgenic lines carrying the wild-type form of Dgq3.  Both activated *dgq*3 (*UAS-AcGq3*) and *dgq*3 (*UAS-Gq3*) cDNAs were placed under the control of the GAL4-inducible *UAS* promoter that would allow tissue and cell-specific expression.  To study the effect of *UAS-AcGq3* expression on axonal development, we used the *C155-GAL4* line initially, which expresses in all postmitotic neurons (Lin and Goodman, 1994).  When stained with mAb BP102, the CNS of *C155-GAL4; UAS-Gq3* embryos looked normal (Fig. 3A).  In embryos expressing AcGq3, the pattern of the CNS appeared mildly deranged in that the commissures were thicker, and the neuropil region was broader than usual (Fig. 3B).  More significant differences between the two genotypes were obvious when a monoclonal antibody against Fasciclin II (mAb 1D4) was used (Fig. 3C–H).  At stage 13, anti-Fasciclin II (anti-Fas II) marks the pioneer axons that go to form the first longitudinal axon pathway (Fig. 3C), which by stage 16, defasciculates to form three distinct fascicles (Fig. 3G).  These axons project ipsilaterally and do not cross the midline.  In embryos of the genotype *C155-GAL4; UAS-Gq3* this projection pattern was identical to wild-type embryos, indicating that overexpression of Dgq3 has no effect on Fas II-expressing axons (Fig. 3C,E,G).  However, in embryos expressing AcGq3, Fas II-positive axons appeared abnormal in all the embryos examined (Fig. 3D,F,H) with variations in the extent of abnormality.  One obvious phenotype observed was that of “stalling” of Fas II-positive axons, which could be seen clearly at late stage 13 (Fig. 3D, arrowheads).  At this stage, minute outgrowths from the cell bodies and axonal tracts were also visible (Fig. 3F, arrowheads).  Stage 15 onward, Fasciclin II-expressing axons could be seen crossing the midline (Fig. 3H, arrow).  Occasionally a whirling phenotype similar to that observed in *robo* mutant alleles was seen (Fig. 3H, asterisk) (Kidd et al., 1998a).  A quantification of these phenotypes is given in Table 1.

From these experiments the fate of the axons that cross the midline was unclear.  For this purpose we generated a strain with the *Apterous tau-βgalactosidase* (*Ap-tau-βgal*) construct in which
single axons could be observed. Ap-tauβgal marks specific Apterous-expressing neurons in each hemisegment of the embryo. Normally these axons project anteriorly on the ipsilateral side to form a distinct Apterous fascicle (Fig. 3F, arrow) (Lundgren et al. 1995). In embryos of the genotype C155; UAS-AcGq3, axons from Apterous-expressing neurons no longer remain on the ipsilateral side but are now able to cross the midline (Fig. 3I, arrows). However, unlike axons that crossover in robo mutant embryos (Wolf and Chiba, 2000), these appear to stall after reaching and crossing the midline.

Expression of AcGq3 in specific neurons leads to aberrant midline crossing

The phenotypes observed in embryos expressing AcGq3 suggest that Gq signaling can drive formation of the commissures and longitudinal tracts. This idea is supported by the phenotype observed in embryos homozygous for Df(2R)vg-C (which uncovers dgq) (Fig. 2F). In these embryos the commissures appear thinner, and there are extensive breaks in the longitudinal tracts. These phenotypes are considerably stronger than those observed for frazzled mutants, which is also uncovered by the same deficiency, indicating that the effect of removing both Dgq and Frazzled is additive. However, these defects could be either caused by erroneous signaling within neurons so that their trajectory is misinterpreted, or by a non-autonomous mechanism that affects midline guidance cues. The latter would result in misplaced neurons or glia or neurons with changed identity. In Df(2R)vg-C embryos, the pattern of neurons expressing the Even-skipped (Eve) protein appear normal (data not shown), indicating that the defects seen occur after neuronal patterning is complete.

To confirm that the phenotype seen by expression of AcGq3 in the CNS is caused by altered signaling within neurons expressing AcGq3, we used more restrictive GAL4 drivers to express UAS-AcGq3 in specific subsets of neurons of the embryonic CNS. ftzneu-GAL4 expresses in a small subset of neurons that include mostly motor neurons and some interneurons like vMP2, pCC, dMP2, and MP1 (Doe et al., 1988; Landgraf et al., 1999). These interneurons pioneer the longitudinal axon tracts, which stain positive for Fasciclin II. In addition, these axons never cross the midline. On expressing UAS-AcGq3 with ftzneu-GAL4, midline crossing by Fasciclin II-positive axons could be observed. At stage 13, the pCC axon which, normally projects anteriorly on the ipsilateral side, could be seen turning toward the midline (Fig. 4B). At stage 16, aberrant midline crossing by the medial fascicle could be observed (data not shown). The number of midline crossovers at this stage is less compared with C155-GAL4, presumably because of the restricted and comparatively weak expression of the ftzneu-GAL4 line (Table 1). Similar results were obtained with eve-neu-GAL4, which expresses in aCC, pCC, and RP2 neurons (Fig. 4C,D) (Baines et al., 1999; Featherstone et al., 2000). The pCC axon can be seen crossing the midline, whereas the aCC and RP2 projections look normal on expression of AcGq3 (Fig. 4D). Axons from Apterous-expressing dorsal cells (dc) can also change their trajectory on expression of AcGq3 (Fig. 4E).
4E,F). Instead of projecting toward the anterior and in an ipsilateral direction as is normal (Fig. 4E,F, asterisks), a fraction of the axons can be seen drifting across the midline (Fig. 4F, arrowhead). The autonomy of AcGq3 function is further supported by the observation that neurons and glia are patterned normally in C155-GAL4/UAS-AcGq3 embryos, as judged by staining with anti-Eve and anti-Repo antibodies (data not shown). Taken together these data demonstrate that specific activation of Dgq3 in ipsilaterally projecting neurons causes changes in their axonal trajectories so that they are now able to project across the midline.

### Table 1. Quantitation of the CNS phenotypes induced by AcGq3

| Genotype                                      | Stalling phenotype | Midline crossover | % Midline crossover |
|-----------------------------------------------|--------------------|-------------------|---------------------|
| C155-GAL4/UAS-AcGq3                           | 106/308* (34.41%)  | 64/133**          | 48.10               |
| UAS-AcGq3: +/+; ftz
\_ng-GAL4, UAS-tau\_Bgal | 171/854* (20.02%)   | 86/427**          | 20.14               |
| UAS-AcGq3: robo\_1/+; ftz
\_ng-GAL4, UAS-tau\_Bgal | 316/625** (5.35)^+ | 12/245** (2.10)^+| 4.8                 |
| robo\_1/+                                        | 0/161**            | 0.0               |
| C155-GAL4/+; UAS-AcGq3\_1/+; UAS-robo\_Y\_F/+ | 37/259**           | 13/245**          | 5.3                 |
| C155-GAL4/UAS-AcGq3\_fra\_1/fra\_1/+            | 37/259**           | 13/245**          | 5.3                 |

^* indicates total number of hemisegments used to quantitate the stalling phenotype observed in embryos of stage 14–16. ** indicates the total number of abdominal segments used to quantitate midline crossing. ^+ denotes expressivity, which is calculated as the ratio of the total number of midline crossovers observed and the total number of embryos showing at least one crossover.

Midline crossing by ectopic expression of AcGq3 is independent of Robo downregulation

To understand how Dgq3 acts to change axonal paths, we looked for possible interactions with genes known to affect midline guidance. Axons that cross the midline and project along the contralateral longitudinal tract normally need to downregulate expression of Robo, which acts as a receptor for the midline repellent Slt (Kidd et al., 1999). It is known that Robo downregulation requires Commissureless, but the precise mechanism is not understood (Tear et al., 1996; Kidd et al., 1998b). A possible mechanism by which AcGq3 could promote midline crossing was by downregulating Robo. To test this hypothesis we looked at Robo expression in ftz
\_ng-GAL4, UAS-AcGq3 embryos. Interestingly, we find that Robo is not downregulated visibly in axons that ectopically cross the midline under the influence of AcGq3 (Fig. 5B). The extent of Robo staining seen on these axons that aberrantly cross the midline is comparable with that seen on the longitudinal tracts. Thus, constitutive activation of Dgq3 results in aberrant midline crossing of axons by a mechanism that is independent of Robo downregulation.

Reducing robo function enhances midline crossing by AcGq3

Another mechanism by which AcGq3 could induce midline crossing is through inhibition of the repulsive signal mediated by Robo. If this were so, then reducing levels of Robo by genetic means should enhance the phenotype of AcGq3. To test this, AcGq3 was expressed using ftz
\_ng-GAL4 in embryos carrying a single copy of the robo\_1 mutant allele. robo\_1 is a recessive mutation. However, embryos with one copy of this mutation show defects similar to those in embryos of stage 14–16 (Fig. 1A). When UAS-AcGq3;robo\_1/+; ftz
\_ng-GAL4 embryos were stained with mAb 1D4, a significant increase in the number of midline crossovers was observed as compared with embryos of the genotype UAS-AcGq3;+; ftz
\_ng-GAL4 (Fig. 5D, Table 1) This suggests that activation of Dgq3 antagonizes the repulsive output through Robo resulting in excessive midline crossing. The antagonism could be mediated either through phosphorylation of Robo or signaling components that function downstream and/or in parallel with Robo.

Phosphorylation of a single tyrosine residue on Robo by Abl-son (Abl) tyrosine kinase inhibits Robo repulsive signaling and is needed for normal midline crossing to take place. Expression of a mutant form of Robo in which this tyrosine residue (Y1040) has been replaced with a phenylalanine (in a transgenic strain referred to as UAS-robo\_Y\_F), lead to constitutive Robo signaling such that no axons cross the midline, resulting in a complete absence of commissure formation (Bashaw et al., 2000). If AcGq3 acts upstream of Robo, we predicted that ectopic midline-crossing, induced by expression of AcGq3, would be reduced in presence of Robo Y\_F. In fact, in embryos expressing both AcGq3 and Robo Y\_F, no ectopic crossovers are seen (Fig. 5F, Table 1), indicating that AcGq3 could inhibit Robo signaling by promoting Robo phosphorylation. This finding is also supportive of the fact that AcGq3 exerts its effect independent of Commissureless-mediated Robo downregulation. It is possible however, that AcGq3 acts through a parallel pathway that is no longer effective in the presence of Robo Y\_F (see Discussion).

Ectopic midline crossing requires Frazzled function

Both the spatiotemporal pattern of expression and functional analysis of dgg indicate that Gq activation in vivo promotes midline crossing. Axons that cross the midline need to turn down their repulsive signaling pathway(s) as well as respond positively to attractive cues. We therefore looked to see if changes in the levels of “attractive” signaling such as the Netrin-Frazzled pathway affect the phenotype of AcGq3. Interestingly, AcGq3 phenotype shows a dosage-dependent interaction with Fra. Removal of a single copy of the Fra gene led to a threefold reduction in the number of midline crossovers induced by AcGq3 (Table 1). A further reduction was observed on removal of both copies of the Fra gene as seen in embryos of the genotype C155-GAL4/UAS-AcGq3; fra\_1/fra\_4 (Table 1, Fig. 6C). Signaling through AcGq3 is thus sensitive to levels of Frazzled in the CNS.

To examine the effect, if any, of AcGq3 on the frazzled mutant phenotype, embryos of the genotype C155-GAL4/UAS-AcGq3; fra\_1/fra\_4 were examined with anti-connectin antibody (Fig. 6) and BP102 (data not shown). Anti-connectin labels a distinct axon fascicle in the longitudinal connectives, axon projections of SP1 and RP1 neurons that project through the anterior commissure.
and a subset of axons that project through the posterior commissure to their contralateral targets (Fig. 6D, arrowhead) (Meadows et al., 1994). In embryos of the genotype C155-GAL4/+; fra3/fra3, breaks were observed in connectin-positive commissural axons and longitudinal tracts (Fig. 6E, arrowhead). Embryos of the genotype C155-GAL4/UAS-AcGq3;fra3/fra3 also show similar breaks (Fig. 6F, arrowhead), indicating that AcGq3 does not have an effect on the frazzled mutant phenotype. Similar results were obtained by staining with BP102.

DISCUSSION
Embryonic expression of Dgq
Dgq was originally identified from a head cDNA library as a homolog of mammalian Gq (Strathmann and Simon, 1990). Initial functional characterization suggested that it was a visual-specific G-protein essential for Drosophila visual transduction (Lee et al., 1990, 1994; Scott et al., 1995). However, from subsequent studies it was apparent that splice variants of dgg existed in other adult tissues (Talluri et al., 1995; Alvarez et al., 1996). In this study we have analyzed dgg expression and function during
development of the Drosophila embryonic CNS. From analysis of dgg transcripts and protein we have shown that the dggα3 splice variant is the primary embryonic form, suggesting multifunctional roles for this protein. Considering the broad expression pattern of dgg, a traditional mutagenesis approach might be unable to address late developmental phenotypes caused by dggα3 loss-of-function. The UAS-GAL4 system offered an alternate strategy that allowed us to dissect dggα3 function during axon guidance. UAS-AcGq3 essentially functions as a dominant gain-of-function allele in a tissue- and cell-specific manner.

Function of Dgqa3 in the embryonic CNS

The induction of ectopic midline crossing by AcGq3 suggests that Dgqa3 function might be required during commissural growth. What activates Dgqa3 in vivo? In Drosophila, the only pathway so far known to mediate attraction toward the midline, is the Netrin-Frazzled signaling pathway. However, null mutants for netrins and frazzled continue to show formation of commissures, albeit thin and poorly organized. The failure to show a complete absence of commissures suggests that an alternate signaling pathway or pathways exists at the midline that promotes commissural growth. The presence of a second attractive signaling pathway operating at the midline has also been suggested based on analysis of mutants

Interaction of AcGq3 with robo

The antagonism between AcGq3 and Robo suggests that AcGq3 operates by modulating repulsion from the midline during commissural growth. It has been demonstrated that Robo signaling is negatively modulated by tyrosine phosphorylation by Abelson kinase (Bashaw et al., 2000). Our results in Figure 5 suggest that AcGq3 could inhibit Robo signaling by a similar mechanism of phosphorylating Robo. It could perhaps do this by activating a kinase cascade involving a nonreceptor tyrosine kinase such as Bruton’s tyrosine kinase (BTK or Tec kinase) which, in mammalian cells, has been shown to be a direct effector of Gq signaling (Bence et al., 1997; Ma and Huang, 1998). Our results are equally consistent with the possibility that AcGq3 and Robo act through parallel pathways, such that AcGq3 induced midline crossing requires downregulation of Robo signaling.

Based on the results obtained from genetic analysis of AcGq3 with frazzled and robo, the following models can be proposed to explain the function of Dgqa3. In the first, Dgqa3 can be thought of as being a component of the attractive signaling pathway alone. Expression of the activated form of the protein functions to override the repulsive cues at the midline and promote ectopic midline crossing. In such a scenario, one would argue that the
synergism observed between AgGq3 and robo1 is a consequence of the combined effect of reduced Robo signaling and excess attractive signaling induced by AcGq3 leading to an increase in the number of midline crossovers. In the presence of UAS-RoboY-F, repulsive signaling increases to a level that cannot be overridden by AcGq3-attractive signaling. A second possibility is that Dgqa3 is a component of an attractive signaling pathway, which functions to potentiate Frazzled signaling by negatively modulating the repulsion mediated by Robo signaling. This could be through phosphorylation of Robo. A recent study using spinal axons from stage 22 Xenopus embryos has shown that the repulsive ligand Slit can “silence” the Netrin-mediated attraction through a direct physical interaction between the cytoplasmic domains of Robo and Frazzled (Stein and Tessier-Lavigne, 2001). This ligand-dependent silencing effect serves to promote repulsion of growth cones from the midline during the development of commissures. Dgqa3 might function conversely at the level of downstream effector molecules to inhibit repulsion in response to attractive cues to promote midline crossing.

In summary, our results predict the involvement of a Gq-mediated signaling pathway in regulating midline crossing in Drosophila. In addition, they also support the notion that balance between attraction and repulsion is a crucial factor that determines the final response of a growth cone to different cues. Inhibition of dgg function specifically in the growth cones should prove useful in dissecting out other components of this pathway that regulates midline crossing.

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