Soluble Guanylate Cyclase Is Required during Development for Visual System Function in *Drosophila*

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A requirement for nitric oxide (NO) in visual system development has been demonstrated in many model systems, but the role of potential downstream effector molecules has not been established. Developing *Drosophila* photoreceptors express an NO-sensitive soluble guanylate cyclase (sGC), whereas the optic lobe targets express NO synthase. Both of these molecules are expressed after photoreceptor outgrowth to the optic lobe, when retinal growth cones are actively selecting their postsynaptic partners. We have previously shown that inhibition of the NO–cGMP pathway *in vitro* leads to overgrowth of retinal axons. Here we examined flies mutant for the α subunit gene of the *Drosophila* sGC (*Gcα1*). This mutation severely reduced but did not abolish GCα1 protein levels and NO-stimulated sGC activity in the developing photoreceptors. Although few mutant individuals possessed a disorganized retinal projection pattern, pharmacological NOS inhibition during metamorphosis increased this disorganization in mutants to a greater degree than in the wild type. Adult mutants lacked phototactic behavior, and the off-transient component of electroretinograms was frequently absent or greatly reduced in amplitude. Normal phototaxis and off-transient amplitude were restored by heat shock-mediated *Gcα1* expression applied during metamorphosis but not in the adult. We propose that diminished sGC activity in the visual system during development causes inappropriate or inadequate formation of first-order retinal synapses, leading to defects in visual system function and visually mediated behavior.

Key words: phototaxis; electroretinogram; nitric oxide; cGMP; photoreceptor; invertebrate

The formation of appropriate synaptic connections during development provides the foundation for nervous system function and behavior in the adult. Nitric oxide (NO) has been implicated as a key regulator of synaptogenic events in a number of systems, most convincingly in the developing visual system (for review, see Cramer and Sur, 1998; Mize and Lo, 2000). Pharmacological inhibition of NO synthase (NOS) prevents elimination of inappropriate retinal projections in chick (Wu et al., 1994; Ernst et al., 1999) and the proper segregation of retinal inputs to the lateral geniculate nucleus in ferret (Cramer et al., 1996; Cramer and Sur, 1999). Endothelial NOS–neuronal NOS double-knock-out mice also display increased inappropriate retinocollicular projections (Wu et al., 2000). Although these studies support a role for NO in vertebrate visual development, they do not identify potential effector molecules through which NO could be acting *in vivo*. However, very recent work strongly suggests that NO regulates the sublamination of retinal inputs to the lateral geniculate nucleus in ferrets by activating soluble guanylate cyclase (sGC;

Leamy et al., 2001). A well characterized receptor for NO (Miki et al., 1977), sGCs function as heterodimers comprising a large α subunit and a small β subunit, both containing putative catalytic domains (Harteneck et al., 1990; Buechler et al., 1991). NO binding to sGC stimulates production of the second messenger cGMP (Arnold et al., 1977), which can then interact with molecular targets such as protein kinases (Farber et al., 1979; Paupardin-Tritsch et al., 1986), phosphodiesterases (Hartzell and Fischmeister, 1986), and ion channels (Johnson et al., 1986; Nawy and Jahr, 1990).

Both NO and sGC play an important role in *Drosophila* visual system development. During metamorphosis the photoreceptors display a period of NO-sensitive sGC activity, beginning after retinal axon outgrowth and ending before synapse formation between the photoreceptors and optic lobe targets (Gibbs and Truman, 1998). A similar type of NO sensitivity is observed in other insect systems, where many developing neurons respond to NO with increases in cGMP synthesis after arrival at their postsynaptic targets (Truman et al., 1996; Ball and Truman, 1998; Schachtner et al., 1998; Wildemann and Bicker, 1999; Zayas et al., 2000). NADPH-diaphorase staining and NOS-like immunoreactivity are present in target regions of the *Drosophila* optic lobe throughout metamorphosis, providing a potential source of NO to sGC-expressing photoreceptors (Gibbs and Truman, 1998; Gibbs, 2001). We have previously shown that pharmacological inhibition of NO or sGC signaling *in vitro* during this window of sensitivity disrupts the organization of the retinal projection pattern and leads to the overgrowth of retinal axons beyond their normal targets in the medulla (Gibbs and Truman, 1998). The retinal overgrowth observed with NOS inhibition *in vitro* can be rescued with the addition of a cGMP analog, suggesting that sGC

activity in the photoreceptors is sufficient to maintain retinal axons in the appropriate target region before synaptogenesis (Gibbs and Truman, 1998).

The genetic tools available in *Drosophila* provide an opportunity to examine sGC function *in vivo*. In addition, the fruit fly is a model for understanding the physiological basis of invertebrate visual system function and visually mediated behavior (Benzer, 1967; Alawi and Pak, 1971; Heisenberg, 1972; Inoue et al., 1985; Shieh et al., 1997). Genes for the sGC α (*dgcα1*) and β (*dgcβ1*) subunits have been cloned from *Drosophila* (Yoshikawa et al., 1993; Liu et al., 1995; Shah and Hyde, 1995), and coexpression studies demonstrated that the DGCα1/DGCβ1 heterodimer can be stimulated with NO (Shah and Hyde, 1995). In the present study we examined visual system organization and function in flies carrying a mutation in the α subunit gene, *Gcα1*. The results suggest that diminished sGC activity during metamorphosis leads to inappropriate or inadequate connections between photoreceptors and optic lobe neurons, further supporting a requirement for sGC signaling in *Drosophila* visual system development.

MATERIALS AND METHODS

Fly stocks. All flies were raised at 25°C on standard medium. For metamorphic time points, white puparia were collected and staged from the time of collection as hours after puparium formation (APF). The *Gcα1* mutants and transgenic flies were obtained from the laboratory of Dr. Charles Zuker (University of California at San Diego). The *claret1* and *brevis1* progenitor strains came from the Bloomington Stock Center. Canton-S and w1118 strains were also used as controls. The levels of GCα1 protein were indistinguishable across these control strains, as determined by Western blot of adult heads (data not shown).

Isolation of guanylate cyclase mutants. Two genetic screens were performed to isolate mutations in the guanylate cyclase gene, *Gcα1*, located by *in situ* hybridization to 99B4-5 on the cytological map. Both used a Western blot assay to detect loss of GCα1 protein. Mutagenesis was performed by feeding *claret1* (*ca1*; F3 screen) or *brevis1* (*bv1*; F2 screen) males 25 mM ethylmethane sulfonate (EMS) in 1% sucrose (Grigliatti, 1986). See Flybase for descriptions of the mutations.

The F3 screen used the temperature-sensitive dominant lethal mutation [*l(3)DTS41*] in the *TM9* balancer chromosome. Mutagenized *ca1* males were mass-mated to *TM9/Bsb* virgin females, and the bottles were kept at 23°C. Single F1 males were then mated to two *TM9/Bsb* virgin females in a vial and kept at 29°C for 2 d. Vials were then cleared of the parents and returned to 29°C for 2 more days before being moved to 25°C. The F2 *Bsb/ca* progeny were transferred to new vials, and the resulting F3 *ca* flies were tested by Western blot for reduced or missing protein. The alleles *Gcα1^1* (GC 207), *Gcα1^2* (GC 738) and *Gcα1^3* (GC782) induced in the *ca* marked chromosome came from 11,692 F3 lines tested. Stocks were made by selecting *ca1* males and females.

For the F2 screen, mutagenized *bv1* males were mass-mated to *Gcα1^1*, *ca1* virgin females. All crosses were done at 25°C. Single F1 males were crossed to two *Gcα1^1*, *ca1* virgin females, and the F2 wild-type flies were tested for the GCα1 protein. Stocks were made by selecting *bv1* flies for any lines that were missing the protein. Two alleles, *Gcα1^4* (GC193) and *Gcα1^5* (GC253), induced in a *bv1* marked chromosome, came from 8445 F2 lines tested. Additional Western blots confirmed that the three lines from the F3 screen and the two lines from the F2 screen were indeed GC mutants. *Gcα1^4* is no longer available. *Gcα1^1* was sequenced and found to be a single-base change (AG/GT to AG/AT) in the donor sequence at the splice junction of intron 1. This leads to an insertion of intron sequences in the cDNA and a premature termination codon (L. Sun and C. Zuker, personal communication).

Transgenic flies. A PCR fragment representing the full-length guanylate cyclase gene, confirmed by sequencing, was cloned into the *Xba* site of a P{CaSpeR-hr}heat shock transformation vector (Thummel and Pirrotta, 1992). P element-mediated germ line transformation and fly manipulations were performed using standard techniques (Karess and Rubin, 1984.).

Western blot analysis. Single adult fly heads were collected from each mutant line and sonicated in SDS-Laemmeli buffer. The samples were loaded on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose using a Bio-Rad (Hercules, CA) transblot. The membrane was blocked for 1 hr at room temperature in PBS and 0.05% Tween 20 (PBST) containing 5% nonfat dry milk. The blots were incubated overnight at 4°C in PBST containing 5% nonfat dry milk and anti-Gcα1 antibody, which was made against a CT peptide. After washing in PBST (four times for 5 min each), the membrane was incubated for 1 hr with peroxidase-conjugated affinity-purified goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). After washing with PBST (four times for 5 min each), the antibody complexes were detected using chemiluminescence (SuperSignal; Pierce, Rockford, IL).

NO stimulation and cGMP immunocytochemistry. The protocol for stimulation with NO donors and cGMP immunocytochemistry has been described previously (Truman et al., 1996; Gibbs and Truman, 1998). The CNS and attached eye disks were dissected from pupae at 24 hr APF and placed in a solution of PBS containing 1 mM sodium nitroprusside (SNP; Sigma, St. Louis, MO) and 1 mM isobutylmethylxanthine (IBMX; Sigma) for 15 min. For heat shock induction experiments, dissection and stimulation with SNP and IBMX took place 2 hr after a 45 min heat shock at 37°C. Tissue was then fixed overnight at 4°C in 4% paraformaldehyde in PBS. After rinsing in PBS plus 0.3% Triton X-100 (PBS-TX), the tissue was blocked for 30 min at room temperature in 5% normal donkey serum in PBS-TX. Nervous systems were incubated overnight at 4°C with a sheep anti-cGMP antiserum (1:10,000; a gift from J. De Vente, Maastricht University, Maastricht, The Netherlands), rinsed in several changes of PBS-TX, and incubated again overnight at 4°C in a Texas Red-conjugated donkey anti-sheep secondary antibody (1:500; Jackson ImmunoResearch). The tissue was rinsed and mounted on poly-L-lysine-coated coverslips and dehydrated through an ethanol series. It was then cleared in xylene and mounted in DPX mountant (Fluka, Buchs, Switzerland). Optical sections were collected on a Bio-Rad MRC 600 confocal microscope and projected in NIH Image.

CNS culture and chaoptin immunocytochemistry. The CNS culture technique was described by Gibbs and Truman (1998). Briefly, CNS–eye disk complexes were dissected from white puparia and cultured in a droplet of D22 *Drosophila* medium (Sigma). The medium was supplemented with 7.5% fetal calf serum and a 1% final concentration of an antibiotic-antimycotic mixture (Sigma) comprising 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B. The competitive NOS inhibitor L-nitro-arginine-methylester (L-NAME) was made as a 1 M stock solution in tissue culture-grade water and diluted to working concentrations of 1 mM and 100, 10, and 1 μM in D22. Control cultures received the maximum volume of sterile water. Cultures were kept at 25°C in a humidified incubator and aerated with 95% oxygen and 5% carbon dioxide. After 24 hr, the tissue was transferred into fresh media containing 1 μg/ml 20-hydroxyecdysone and cultured for a remaining 72 hr. The tissue was then fixed overnight at 4°C in PBS containing 4% paraformaldehye.

After a series of washes in PBS with 0.3% Triton X-100, fixed tissue was incubated overnight at 4°C with a mouse monoclonal antibody to chaoptin (monoclonal antibody 24B10, 1:500; a gift from Dr. S. L. Zipursky, University of California, Los Angeles, CA). A peroxidase-conjugated goat anti-mouse secondary antibody was used at 1:500 (overnight, 4°C). After rinsing for at least 1 hr with several changes of PBS-TX, antibody complexes were visualized by incubating the tissue in PBS-TX containing 0.5 mg/ml diaminobenzedine (Sigma), 2 mg/ml β-D-glucose, 0.4 mg/ml ammonium chloride, 0.03% nickel chloride, and 0.2 U of glucose oxidase. The tissue was mounted as described above.

After culturing, nervous systems were analyzed for retinal axon overgrowth. Each experiment was repeated at least three times, and samples were scored blind as to treatment. Nervous systems were assigned a score reflecting the amount of retinal disorganization in the medulla. Parameters were as follows: 0, normal retinal pattern; 1, slight disorganization in pattern and crowding of photoreceptor terminals; 2, further disruption and gaps appearing in the pattern; 3, growth of retinal axons beyond the medulla but not the lobula; and 4, growth of retinal axons beyond the lobula. For more details, see Gibbs and Truman (1998). Each experiment was repeated at least three times, and samples were scored blind as to treatment. A mean score was then calculated for each group, and the results were analyzed using StatWorks (Cricket Software, Malvern, PA). In addition, the percentages of total nervous systems showing retinal axon growth beyond the medulla were compiled for mutant and wild-type nervous systems. These data were pooled and used to calculate the percent change in retinal axon overgrowth in the mutants in relation to the wild type (percentage of total *Gcα1^1* mutant nervous systems showing retinal overgrowth/percentage of total wild-type nervous systems

showing retinal overgrowth). The statistical significance of these differences was determined using Fisher's exact test.

Phototaxis and geotaxis assays. Behavioral assays for fast phototaxis were performed in the dark, using a countercurrent apparatus and protocol as described by Benzer (1967). Eleven 18×150 mm plastic test tubes were joined by a sliding Lucite rack (five on top and six on bottom). The apparatus was laid horizontally in front of a fluorescent light source that was horizontal and perpendicular to the tubes. Trials were performed with mixed male–female populations of ~100 flies 1 week after eclosion. After 10 min of dark adaptation, flies were given 15 sec to move from one tube into the next toward the light before being transferred horizontally to the neighboring tube. For testing negative phototaxis, the apparatus was placed in the reverse orientation, with the light source behind the flies. The distribution of flies in all six tubes was determined after five runs and calculated as a percentage of the total population per tube. Three trials were performed for each strain of flies (~300 flies total per strain). SEs were calculated from these averages. Geotaxis assays were performed in total darkness, orienting the countercurrent apparatus vertically. Populations of ~30 flies were used per trial and given 15 sec per run. As with the phototaxis assays, three trials were run with three different populations of flies per strain. The distributions were then calculated and averaged for each strain.

Heat shock experiments were performed with flies carrying the $Gc\alpha1^1$ allele and a wild-type $Gc\alpha1$ transgene under heat shock control. Two sets of flies carrying the $Gc\alpha1$ transgene and one set of *claret*^{1} controls were raised at 18°C. It was determined that a heat shock of 45 min at 37°C produced maximum levels of transgene expression at 3–4 hr after heat shock, as assayed by Western blot (Fig. 1*A*) and NO-induced cGMP immunocytochemistry, which was maintained until 6–7 hr after heat shock, when it began to decline (S. M. Gibbs, unpublished data). For the phototaxis experiments, 45 min heat shocks at 37°C were delivered every 8 hr for the first 48 hr of metamorphosis to one group of *hs-gc*$^+$; $Gc\alpha1^1$ mutants. In the acute experiments, a single 45 min heat shock was delivered to adult flies, and they were tested for phototaxis 3 hr later, when transgene expression would be peaking. In the final experiment, 45 min heat shocks were given every 12 hr over 48 hr to adult flies, and they were tested for phototaxis 24 hr after the last heat shock.

Electroretinograms. Adult males 4–7 d old were anesthetized with CO_2. The legs and wings were removed, and the fly was immobilized and dark-adapted for 10 min. Extracellular recordings were performed using a glass microelectrode filled with physiological saline (in mM: 130 NaCl, 4.7 KCl, and 1.9 CaCl$_2$). The recording electrode was always placed subcorneally in the center of the eye; a second electrode in the thorax served as the reference electrode. The light stimulus was a 565 nm green light-emitting diode (LED) positioned 2 cm from the eye. Light pulses of 1 sec were presented at varying intensities. Electroretinogram (ERG) responses were collected using a computer sampling at 500 Hz (LabView), and analysis was performed in LabView. Heat shock experiments were performed as described for the phototaxis assays. Mutant and heat-shocked flies or controls were always recorded in an alternating sequence with the same electrode.

RESULTS

A mutation in *Gcα1* decreases sGC activity in the visual system during metamorphosis

Genetic mutants in $Gc\alpha1$ were produced with EMS mutagenesis. Four alleles of $Gc\alpha1$ were recovered. On the basis of decreases in intensity of GCα1 immunoreactivity on a Western blot of adult heads, the allelic series was $Gc\alpha1^5 = Gc\alpha1^1 = Gc\alpha1^3 > Gc\alpha1^2$. The differences in the levels of GCα1 protein were virtually indistinguishable among $Gc\alpha1^5$, $Gc\alpha1^1$, and $Gc\alpha1^3$ (data not shown). Almost all of the following experiments were performed with $Gc\alpha1^1$, the first to be isolated. In a Western blot of protein extract from wild-type adult heads, the anti-GCα1 antibody recognized a band of ~76 kDa, approximately the size reported previously (Liu et al., 1995; Shah and Hyde, 1995). In contrast, GCα1 antibody staining was greatly reduced in $Gc\alpha1^1$ flies (Fig. 1*A, third lane*). A transgene containing the wild-type $GC\alpha1$ was expressed with a 1 hr heat shock in the $Gc\alpha1^1$ mutant, which increased the anti-GCα1 staining to wild-type levels (Fig. 1*A, fourth lane, arrow*). The $Gc\alpha1^1$ mutation not only reduced GCα1
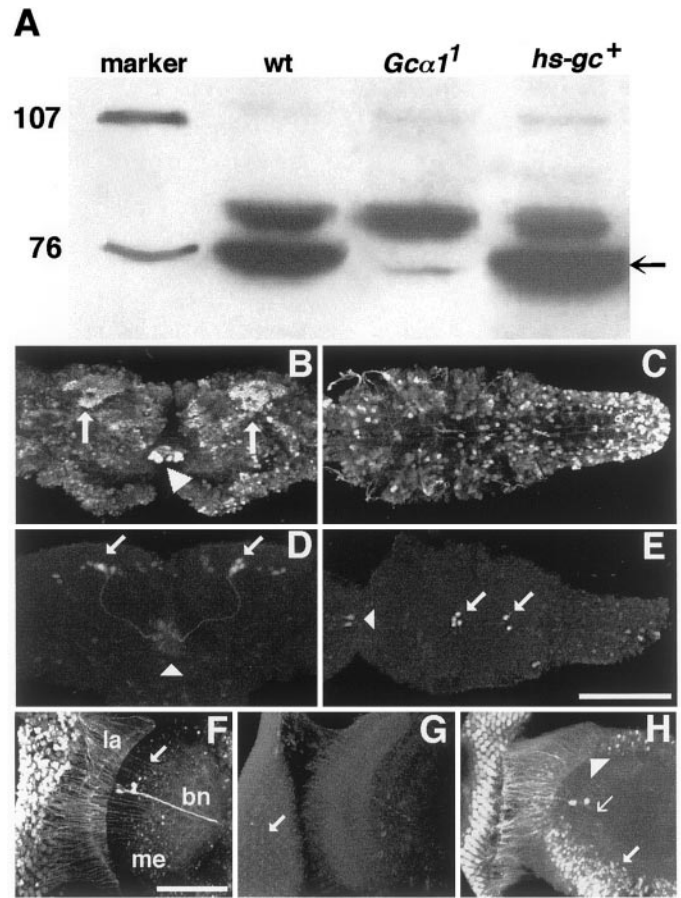

Figure 1. GCα1 expression and activity in wild-type and mutant flies. *A*, Western blot analysis of GCα1 expression. The polyclonal Gcα1 antibody recognized a band of ~76 kDa in adult wild-type heads (*wt, second lane*). Anti-Gcα1 staining was greatly reduced in heads from $Gc\alpha1^1$ mutant adults (*third lane*). Heat shock-induced expression of a Gcα1 transgene restores GCα1 staining in $Gc\alpha1^1$ (*fourth lane, arrow*). *B–H*, cGMP production in the CNS at 24 hr APF, as revealed by cGMP antibody staining after treatment with 1 mM SNP and IBMX. *B*, cGMP-IR was present in the wild-type brain. It is very prominent in the mushroom bodies (*arrows*) and in the neurons at the bast of the ring gland (*arrowhead*). *C*, cGMP-IR was also distributed throughout the wild-type ventral nervous system. *D*, cGMP-IR was dramatically reduced in the brain of $Gc\alpha1^1$ mutants at 24 hr APF. Two clusters of neurons in the medial anterior brain remained cGMP-positive (*arrows*), sending projections to the ring gland, which also showed faint cGMP-IR (*arrowhead*). *E*, A pair of cells in the subesophageal ganglion remained cGMP-positive in $Gc\alpha1^1$ (*arrowhead*). Three pairs of cells at the ventral midline also showed cGMP-IR (*arrows*). *F*, In the wild-type visual system, cGMP-IR was observed in photoreceptors R1–6 projecting to the lamina (*la*) and R7/8 terminating in the medulla (*me, arrow*). Bolwig's nerve (*bn*) also showed cGMP-IR. *G*, cGMP-IR was greatly reduced in the visual system of $Gc\alpha1^1$ at 24 hr APF after exposure to 1 mM SNP and IBMX. Faint staining was seen in photoreceptor cell bodies of the developing eye disk (*arrow*). *H*, NO-induced cGMP-IR was restored in photoreceptors R1–6 (*arrowhead*) and R7/8 (*small arrow*) of $Gc\alpha1^1$ after heat shock expression of $Gc\alpha1$. cGMP-IR also appeared in interneurons of the medulla (*large arrow*). Scale bars, 50 μm. *B* and *C* represent projected stacks of optical sections (see Materials and Methods). In *F* and *H*, the posterior- and anterior-most sections have been omitted to show the detail of projections to the lamina and medulla.

staining on a Western blot but also greatly attenuated the synthesis of cGMP in the nervous system in response to NO (Fig. 1*D,E*). The activity of sGC in the developing *Drosophila* nervous system was visualized by exposing the tissue to the NO donor

SNP (1 mM) and the phosphodiesterase inhibitor IBMX (1 mM), followed by immunocytochemistry with an anti-cGMP antibody (Truman et al., 1996; Gibbs and Truman, 1998). At 24 hr APF, the CNS of the *Gcα1¹* mutants showed cGMP immunoreactivity (cGMP-IR) in only a few neurons of the central brain, suboesphageal ganglion, and ventral nerve cord in response to SNP and IBMX (Fig. 1*D,E*). This represents a small subset of the cGMP-positive population of cells observed in the wild-type CNS at 24 hr APF after similar treatment (Fig. 1*B,C*). Most cells that showed cGMP-IR in *Gcα1¹* appear to be the same cells that remain cGMP-positive in the wild-type CNS after treatment with the sCG inhibitor 1H-[1,2,4-oxadiazolo-[4,3,a]quinoxalin-1-one and are frequently cGMP-positive in nervous systems that have not been exposed to SNP and IBMX (Gibbs, unpublished observations). A receptor-type guanylate cyclase has been cloned from *Drosophila* (Liu et al., 1995), and it is possible that the cGMP-IR in these cells reflects the activity of this GC.

The photoreceptors possess NO-sensitive sGC activity during the first half of metamorphic development, after they arrive at their optic lobe targets (Gibbs and Truman, 1998). An example of cGMP staining after SNP and IBMX exposure is shown for the visual system at 24 hr APF (Fig. 1*F*). cGMP immunoreactivity was observed in the cell bodies of photoreceptors in the developing retina as well as their axons projecting into the lamina and medulla regions of the optic lobe. These represent the axons of both classes of photoreceptors, R1–6 and R7/8. The axons of Bolwig's nerve were also cGMP-positive. In contrast, a very low level of cGMP-IR was observed in the visual system of *Gcα1¹* after SNP and IBMX treatment at 24 hr APF, primarily in the cell bodies of the eye imaginal disc (Fig. 1*G, arrow*). This faint cGMP-IR most likely reflects the activity of remaining low levels of GCα1 protein in the *Gcα1¹* mutant (Fig. 1*A*). Heat shock-induced expression of a wild-type *Gcα1* transgene restored the strong response of the photoreceptors to NO in *Gcα1¹* at 24 hr APF. Two hours after a single 45 min heat shock, cGMP production was observed in the photoreceptor cell bodies and axons and the larval pioneers after treatment with 1 mM SNP and IBMX (Fig. 1*H*). The intensity of cGMP-IR induced with heat shock was not observed, however, without SNP and IBMX exposure (data not shown). Surprisingly, after heat shock expression of *Gcα1*, strong cGMP-IR was also observed in the medulla interneurons of the optic lobe of the *Gcα1¹* mutants (Fig. 1*H, arrows*). This was in contrast to the wild-type visual system, in which NO-induced cGMP accumulation is very low in the medulla at 24 hr APF, becoming more prominent by ~48 hr APF (Fig. 1*F*). Because both α and β subunits are required for soluble guanylate cyclase activity in response to NO (Shah and Hyde, 1995), we conclude that the GCβ1 protein must already be present in the medulla interneurons at 24 hr APF. The delayed onset of NO sensitivity in these cells may then reflect the regulated expression of GCα1 rather than GCβ1.

Gcα1¹ mutants show minor defects in the retinal projection pattern and increased sensitivity to NOS inhibition *in vitro*

We have previously shown that cells of the lamina and medulla express NOS and that pharmacological inhibition of NOS or sGC *in vitro* causes disorganization and overgrowth of the retinal projections in the wild-type visual system (Gibbs and Truman, 1998). On the basis of these previous *in vitro* results, we predicted that flies mutant for *Gcα1* would display similar defects in visual system organization. We examined the retinal projection pattern
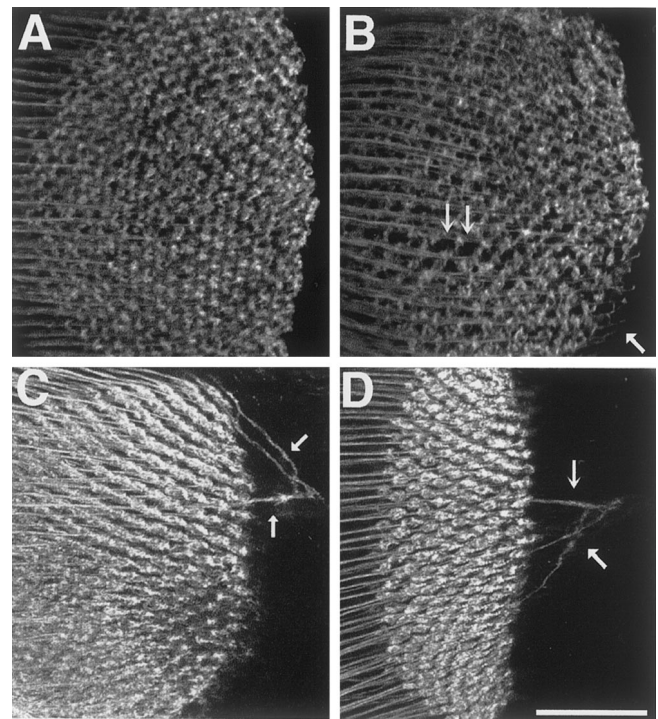

Figure 2. *Gcα1¹* mutants show slight defects in visual system organization. *A,* The projections of R7/8 form a well organized pattern in the medulla of wild-type flies at 48 hr APF, as visualized by whole-mount chaoptin immunocytochemistry. *B,* The projection pattern of R7/R8 in *Gcα1¹* at 48 hr APF was less well organized. Gaps in the pattern were observed (*double arrows*), as well as a few retinal axons projecting slightly beyond the border of the medulla (*arrow*). This phenotype was observed in a small percentage of mutants (6 of 130). *C, D,* Further examples of the *Gcα1¹* projection pattern in the medulla at 48 hr APF, as visualized with fasciclin II immunocytochemistry. Retinal axons were seen projecting well beyond the medulla in these individuals (*arrows*); however, this phenotype was not typical (*n* = 4 of 70). Scale bar, 10 μm.

of R7/8 in the medulla of all four *Gcα1* alleles throughout metamorphosis and in adults, using whole-mount immunocytochemistry with antibodies to chaoptin and fasciclin II, both of which label photoreceptor axons (Zipursky et al., 1984; Schneider et al., 1995; Hiesinger et al., 1999). In general, the mutants did not show dramatic disruption of the projection pattern of R7/8, although minor defects were noted in some cases (Fig. 2; 10 of 200 flies). At 48 hr APF, the chaoptin-stained retinal projections of a few *Gcα1¹* mutants were slightly disrupted when compared with the wild type (Fig. 2*A*), producing gaps in the pattern and extension of retinal axons slightly beyond the medullar margin and producing a "ragged" border (Fig. 2*B*). Similarly, staining with a fasciclin II antibody at the same stage revealed what appeared to be a few retinal axons projecting well beyond the medulla in some individuals (Fig. 2*C,D*). Although this phenotype was not widely observed, it was never seen in age-matched wild-type flies (*n* > 200) and may reflect the variable penetrance of the *Gcα1¹* mutation.

The *Gcα1¹* flies express low levels of GCα1 protein, which is also reflected in very low levels of NO-sensitive sGC activity in the developing visual system (Fig. 1). sGC is an enzyme; thus even a small amount is capable of producing a greatly amplified cGMP signaling cascade in the presence of sufficient ligand. We therefore predicted that although this residual sGC activity was normally preventing retinal axon overgrowth in most of the

$Gc\alpha1^1$ mutants, it would not be enough to compensate for decreases in NO production *in vitro*. CNSs and attached eye disks from wild-type (Canton-S) and mutant white puparia were placed in culture with the hormone 20-hydroxyecdysone (1 μg/ml), which promotes metamorphic development of the nervous system *in vitro* (Awad, 1995; Gibbs and Truman, 1998), or hormone plus the competitive NOS inhibitor L-NAME. After 96 hr in culture, the tissue was then processed for chaoptin immunocytochemistry. For analysis we focused on the projection pattern of R7/8 in the medulla, which is easily resolved in whole-mount preparations. Nervous systems were assigned a score of 0–4 based on the severity of disorganization in the projection pattern. The scores for all samples were averaged to obtain a mean score for a given treatment, called the disruption index (see Materials and Methods; Gibbs and Truman, 1998), and the percentages of nervous systems showing any retinal growth beyond the medulla were determined for mutant and wild-type nervous systems for each treatment.

A delay in neural development occurs with culturing, so that after 96 hr *in vitro,* the visual system has progressed to a stage comparable to 48–50 hr APF (Gibbs and Truman, 1998). In control cultures without inhibitor, a normal, well organized projection pattern was typically seen in the medulla, and no retinal fibers were seen growing past medullar targets in either wild-type or mutant visual systems (Fig. 3*A,D*). Although not significantly different, the disruption index for the mutants under control conditions was slightly higher than that for the wild type, suggesting that the $Gc\alpha1^1$ mutation caused subtle projection pattern defects in a low percentage of individuals, as was observed with whole-mount analysis of noncultured nervous systems (Fig. 2). When a low level of L-NAME was added to the cultures (1 μM), essentially no pattern disruption was observed in the medulla of wild-type nervous systems (Fig. 3*B*). However, this concentration of L-NAME caused the growth of many retinal axons beyond the posterior medulla of the $Gc\alpha1^1$ optic lobe (Fig. 3*E*). This effect was more severely pronounced in the mutants with 10 μM L-NAME. Under these conditions, the retinal fibers produced a dense, disorganized tangle in the medulla and extended many projections into the lobula (Fig. 3*F*). This was not the case for wild-type nervous systems, in which treatment with 10 μM L-NAME produced a slight disorganization of the projection pattern, but no retinal fibers were observed projecting beyond the medulla (Fig. 3*C*).

The compiled quantitative results from the *in vitro* experiments are shown in Figure 3, *G* and *H*. The disruption index for $Gc\alpha1^1$ was significantly higher than that for wild-type controls for all tested concentrations of L-NAME (Fig. 3*G*; $p < 0.001$, Student's unpaired *t* test). The percent increase in $Gc\alpha1^1$ mutant versus wild-type nervous systems possessing retinal axons growing past the medulla for each treatment is shown in Figure 3*H*. An increase in retinal overgrowth was observed for the mutants for all treatment groups. However, significant increases in the percentage of flies with retinal axon overgrowth were seen with mutants exposed to 1 μM L-NAME (150%; $p < 0.02$, Fisher's exact test) and 10 μM L-NAME (300%; $p < 0.0001$, Fisher's exact test). Thus, although overall levels of disruption in the organization of the retinal projection pattern were increased in the $Gc\alpha1^1$ mutants (Fig. 3*G*), the extended growth of photoreceptors beyond the medulla was only significantly increased when nervous systems were exposed to the two lowest levels of NOS inhibitor (Fig. 3*H*). These results suggest that although the $Gc\alpha1^1$ mutants do not normally display high levels of retinal overgrowth, the

decreased GCα1 and subsequent lower cGMP production in these flies sensitizes the developing visual system to the disruptive effects of NOS inhibition *in vitro*. However, the residual expression and activity of GCα1 in these hypomorphic mutants appear to be sufficient to normally prevent significant retinal axon overgrowth in most individuals (Figs. 1, 2).

$Gc\alpha1$ mutants are deficient in phototactic behavior

To examine visual system function in the $Gc\alpha1$ mutant adults, we first used a standard behavioral assay for fast phototaxis, using a countercurrent apparatus as described by Benzer (1967). In these experiments, a population of ~100 flies was placed in the first of six clear tubes, laid onto a horizontal surface, and transferred through the tubes at 30 sec intervals as they moved toward a light source. An average of 65% of the total population of wild-type flies consistently moved toward the light after each transfer to end up in the last tube (tube 6) by the end of the trial. All four of the $Gc\alpha1$ mutant strains showed a reduction in positive phototaxis at 1 week after eclosion when compared with the *claret*[1] progenitor strain (Gibbs, 2001), but the $Gc\alpha1^1$ mutants were the most severely compromised (Fig. 4). A combined 12–13% of $Gc\alpha1^1$ flies made it to the last two tubes (tubes 5 and 6) of the apparatus in repeated trials, compared with 65% of *claret*[1] controls. This behavior was not the result of negative phototaxis, as tested by reversing the orientation of the countercurrent apparatus relative to the light source (Fig. 4*A*). The profile of $Gc\alpha1^1$ also remained unchanged in the absence of light (Fig. 4*A*). Although visually mediated, phototaxis requires the integration of many behaviors (Rockwell and Seiger, 1973). However, $Gc\alpha1^1$ adults showed normal performance in a geotaxis assay, demonstrating that these flies do not possess gross motor impairments (Fig. 4*B*).

We next investigated whether restoring sGC activity during the period of retinal innervation would improve the phototactic performance of the $Gc\alpha1^1$ mutants, using a heat shock-inducible $Gc\alpha1$ transgene. In this experiment, a 45 min heat shock was given every 8 hr for the first 48 hr of pupal development, approximately encompassing the window of NO-sensitive cyclase activity observed in the photoreceptors. Phototaxis was tested 1 week after adult eclosion, 9 d after the last heat shock (Fig. 4*C*). At the completion of this experiment, 51% of the total population of $hs\text{-}gc^+; Gc\alpha1^1$ adults exposed to heat shock during metamorphosis converged in the last two tubes of the countercurrent apparatus. This was compared with 13% for the non-heat-shocked $hs\text{-}gc^+; Gc\alpha1^1$ adults and 65% for the *claret*[1] progenitor strain. In addition, the percentage of flies in the first two tubes was nearly identical between ca^1 controls and heat-shocked $hs\text{-}gc^+; Gc\alpha1^1$ adults. Thus positive phototaxis was restored in the $Gc\alpha1^1$ mutants to near wild-type levels when $Gc\alpha1$ was expressed during the first half of metamorphosis, and the phototactic profile was made to resemble that of the wild type. An improvement in positive phototaxis was not observed when adult $hs\text{-}gc^+; Gc\alpha1^1$ flies were tested 3 hr after a single acute heat shock (Fig. 4*D*). In addition, increased positive phototaxis was not seen in $hs\text{-}gc^+; Gc\alpha1^1$ mutants that were exposed to heat shock every 12 hr for 48 hr as adults and tested 24 hr after the last heat shock. In this case only 17% of flies accumulated in the last two tubes, comparable with results obtained with non-heat-shocked mutants (data not shown). These results strongly support the hypothesis that the function of sGC in the phototactic response is developmental, rather than a physiological requirement for sGC activity at the time the behavior is being performed.
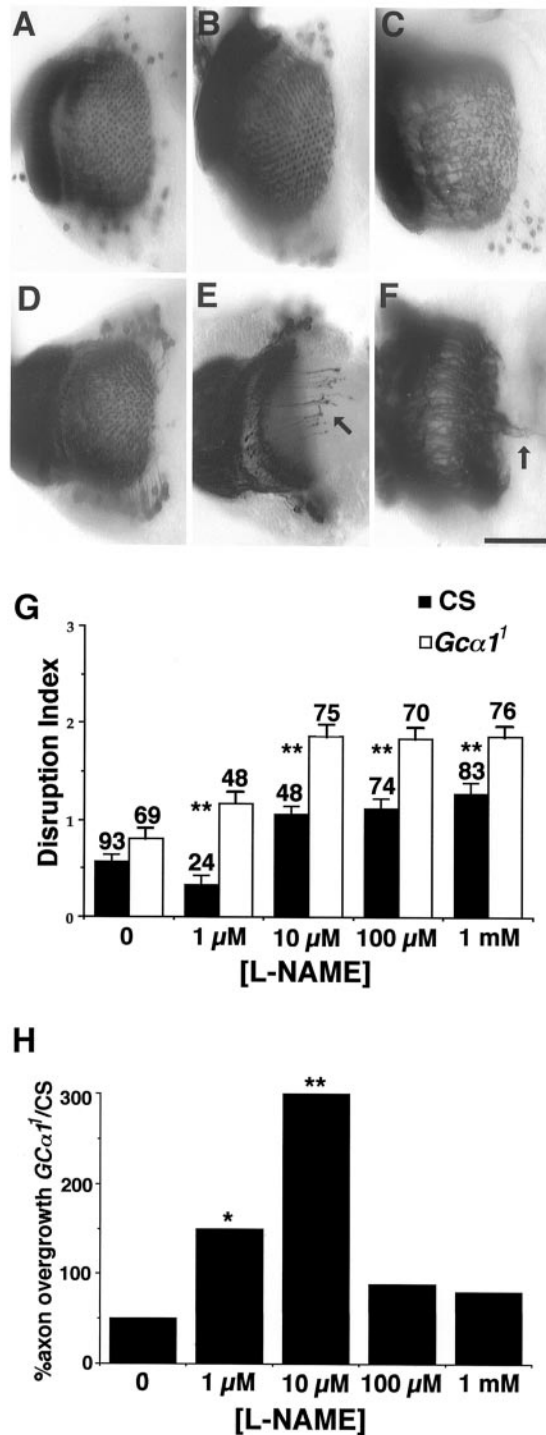
Figure 3. The visual system of *Gcα1¹* shows increased sensitivity to NOS inhibition *in vitro*. CNSs from white puparia were cultured for 96 hr with varying concentrations of L-NAME before being processed for chaoptin immunocytochemistry. *A*, Wild-type control, cultured without L-NAME. *B*, Wild type, 1 μM L-NAME. *C*, Wild type, 10 μM L-NAME. *D*, *Gcα1¹* control, cultured without L-NAME. *E*, *Gcα1¹* cultured with 1 μM L-NAME. The focal plane is positioned at the posterior portion of the medulla to show several retinal axons projecting beyond the posterior medulla (*arrow*). *F*, *Gcα1¹* cultured with 10 μM L-NAME. The projection pattern was severely disorganized, with bundled retinal axons projecting beyond the medulla and into the lobula (*arrow*). Scale bar, 50 μm. *G*, *Gcα1¹* had a significantly greater disruption index than wild type for every tested concentration of L-NAME (**p < 0.001, unpaired Student's *t* test). Numbers of optic lobes scored are given above SE bars for each group.

The ERG response in *Gcα1¹* mutants is reduced

We performed ERGs in a further attempt to examine the effects of the *Gcα1¹* mutation on visual system function. The ERG primarily characterizes the electrophysiological response to light of a subset of photoreceptors, R1–6, and their postsynaptic cells in the lamina. The ERG consists of a corneal positive "on-transient," followed by a sustained negative wave that lasts throughout the period of illumination, and then a corneal negative "off-transient." The off-transient results from the summation of postsynaptic potentials in the monopolar neurons L1 and L2 of the lamina and can thus be used as an indicator of synaptic efficacy (Coombe, 1986).

For these experiments, two sets of *hs-gc⁺*; *Gcα1¹* flies were grown at 18°C, along with a control group (*claret¹*). One set of mutants received a 45 min 37°C heat shock, repeated every 8 hr from 4 to 48 hr APF. ERGs from all three groups were recorded 5–7 d after adult eclosion. Recordings from *Gcα1¹* mutants were always preceded by *claret¹* controls. All the ERGs from *claret¹* flies contained a normal on-transient (Fig. 5*A, arrowhead*), followed by a sustained depolarization of the photoreceptors in response to a 1 sec pulse from a green LED. A large, rapid off-transient followed termination of the light stimulus in *claret¹* controls (Fig. 5*A, arrow*). In contrast, 82% of the ERGs from the *Gcα1¹* flies lacked off-transients or had off-transients that were greatly reduced in amplitude (Fig. 5*B,C, arrows*). We also observed more than one peak in the reduced off-transients of some *hs-gc⁺*; *Gcα1¹* mutants (Fig. 5*D, large arrow*). However, the amplitude and shape of the off-transient appeared normal in *hs-gc⁺*; *Gcα1¹* flies that received heat shocks during the first half of metamorphosis (Fig. 5*E, arrow*). Both heat-shocked and non-heat-shocked mutants also showed a normal sustained depolarization during the light stimulus (Fig. 5*B,C*; data not shown), consistent with previous findings that photoreceptor function is normal in *Gcα1* mutants (K. Scott, L. Sun, and C. Zuker, personal communication). The mean off-transient amplitude of the *Gcα1¹* flies (1.54 ± 0.25 mV) was less than half that of *claret¹* controls (3.34 ± 0.23 mV). In contrast, *Gcα1¹* flies that had *Gcα1* supplied with heat shock during the first half of metamorphosis expressed a mean off-transient amplitude that was comparable with that of wild-type controls (3.94 ± 0.27 mV). The effect of the *Gcα1¹* mutation appeared to be limited to the off-transient amplitude, because both the mean duration of the off-transient peak and the mean amplitude of the sustained component were not significantly different between all three populations (data not shown). Thus, both positive phototaxis and a normal ERG trace can be restored to adult flies expressing very low levels of sGC activity when *Gcα1* is supplied developmentally as the retinal growth cones are undergoing target selection in the optic lobe.

DISCUSSION

For many years the fly has been used as a model system for understanding visual function and visually mediated behavior (Benzer, 1967; Alawi and Pak, 1971; Heisenberg, 1972; Inoue et al., 1985). More recently, the highly organized architecture of the *Drosophila* visual system has provided a model for understanding

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H, The percentage of total nervous systems containing retinal axons growing past the border of the medulla was significantly increased in the *Gcα1¹* mutants by 150% when cultured with 1 μM L-NAME (**p < 0.02) and by 300% when exposed to 10 μM L-NAME (***p < 0.0001, Fisher's exact test).
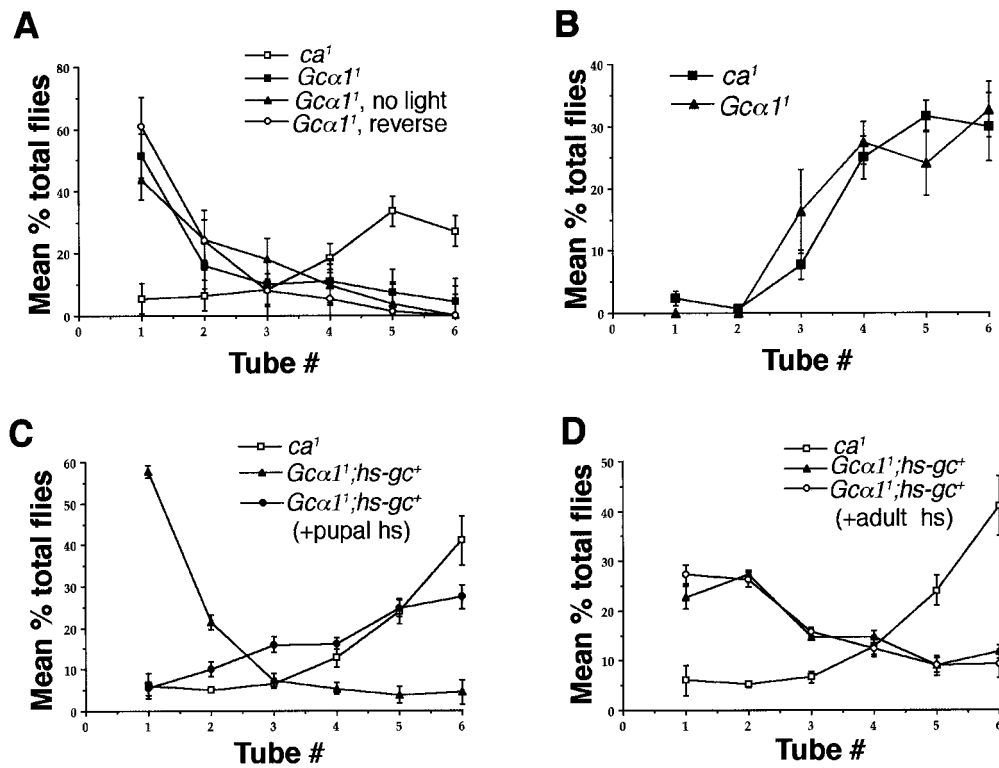
Figure 4. Heat shock expression of *Gcα1* during metamorphosis restores positive phototaxis in *Gcα1¹* adults. *A*, Phototactic profiles for *Gcα1¹* remained weak in the absence of light (*no light*) and when the light source was placed in the opposite orientation to the countercurrent apparatus (*reverse*). *B*, *Gcα1¹* showed positive geotaxis that was indistinguishable from *claret¹* controls. *C*, The phototactic profile of *Gcα1¹* more closely resembled that of the *claret¹* strain after *Gcα1* was expressed with heat shock every 8 hr during the first 48 hr of metamorphosis. *D*, The phototactic profile of *Gcα1¹* did not change when *Gcα1* was expressed with a single heat shock in adult flies. Error bars indicate SE.
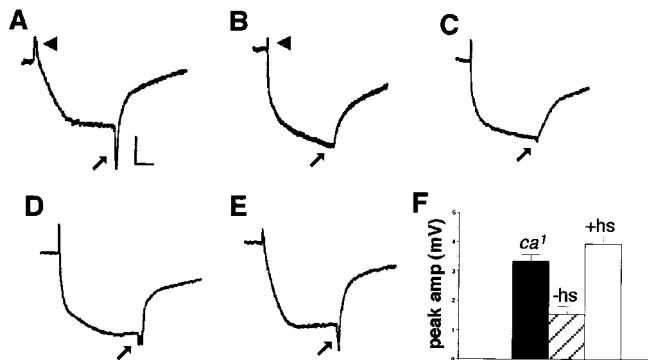

Figure 5. *Gcα1¹* flies display abnormal ERGs with reduced off-transients. *A*, ERG recording from a *claret¹* adult in response to a 1 sec light pulse, showing a normal on-transient (*arrowhead*) and off-transient (*arrow*). Calibration: *vertical*, 3 mV; *horizontal*, 200 msec. *B*, ERG from an *hs-gc⁺; Gcα1¹* mutant, showing a normal on-transient (*arrowhead*) and sustained depolarization. No off-transient could be detected (*arrow*). *C*, ERG from another *hs-gc⁺; Gcα1¹* mutant also displaying a greatly reduced off-transient (*arrow*). *D*, ERG from a third *hs-gc⁺; Gcα1¹* mutant, showing an off-transient of reduced amplitude containing two peaks (*arrow*). *E*, ERG from an *hs-gc⁺; Gcα1¹* mutant that received a heat shock every 8 hr for approximately the first 48 hr of metamorphosis, containing an off-transient of normal shape and amplitude (*arrow*). *F*, The mean amplitude of the off-transient peak in the *hs-gc⁺; Gcα1¹* mutants was significantly less ($n = 17$) than those from *hs-gc⁺; Gcα1¹* mutants that received heat shocks during the first half of metamorphosis ($n = 18$) or *claret¹* controls ($n = 9$). Error bars indicate SE.

axon targeting and synaptic patterning during development (for review, see Wolff et al., 1997). We have previously demonstrated the presence of NO-sensitive sGC activity in the photoreceptors between 12 and 48 hr of metamorphosis and showed that pharmacological inhibition of NO–sGC signaling during this temporal window disrupts the retinal projection pattern (Gibbs and Truman, 1998). In the present study, we analyzed hypomorphic

genetic mutants in the sGC α-subunit gene *Gcα1* to further explore the role of sGC signaling in the formation of synaptic connections between photoreceptors and optic lobe interneurons. Using *in vitro* culture, behavioral assays, and electrophysiology, we demonstrated that decreased expression of *Gcα1* causes minor defects in visual system organization and major defects in adult visual system function. Importantly, we show that these defects could be rescued when the wild-type *Gcα1* gene was expressed during a specific developmental period, when the location and organization of first-order retinal synapses is being established.

The α subunit of a soluble guanylate cyclase has been repeatedly cloned from *Drosophila* (Yoshikawa et al., 1993; Liu et al., 1995; Shah and Hyde, 1995; C. Zuker, A. Becker, R. W. Hardy, and L. Sun, unpublished data). However, there is controversy as to the expression pattern of *Gcα1*. Using Northern blot analysis, Yoshikawa et al. (1993) and Shah and Hyde (1995) demonstrated *Gcα1* mRNA in wild-type but not eyeless adult fly heads, suggesting that *Gcα1* is expressed primarily in the adult retina. Shah and Hyde (1995) also used an antibody to localize the GCα1 protein to the retina. Although Liu et al. (1995) showed abundant *Gcα1* mRNA in a Northern blot of adult heads, the same probe failed to hybridize to adult retinal tissue *in situ*, and no retinal staining was found with a GCα1 antibody. Here we present evidence that *Gcα1* is expressed in the photoreceptors during development. A genetic mutation that diminished GCα1 protein levels, *Gcα1¹*, reduced NO-induced production of cGMP in developing photoreceptors, which was restored with heat shock-mediated expression of wild-type *Gcα1*. Thus we conclude that *Gcα1* is normally expressed in the photoreceptors from ~12 to 48 hr APF, and we infer that the β subunit is also present at this time. We have not determined whether the observed loss of NO sensitivity in the photoreceptors at ~48 hr APF and into adulthood (Gibbs and Truman, 1998; Gibbs, unpublished observations) is attributable to changes in *Gcα1* expression. However,

spatiotemporal changes in α and β subunit expression may provide a molecular basis for regulating the timing of NO-sensitive sGC activity in the photoreceptors and other cells of the developing visual system.

Reports of *Gcα1* expression in the adult retina, (Yoshikawa et al., 1993; Shah and Hyde, 1995), and cGMP-mediated enhancement of the photoresponse in isolated *Drosophila* photoreceptors (Bacigalupo et al., 1995) implicated cGMP as a putative mediator of a phototransduction mediator in flies. Studies show that NO and cGMP can modulate the locust photoresponse (Elphick et al., 1996; Schmachtenberg and Bicker, 1999) and signaling in other insect sensory systems (Nighorn et al., 1998; Gibson and Nighorn, 2000; Ott et al., 2000). The *Gcα1* mutants were initially generated to further establish the role of cGMP in phototransduction; however, both intracellular and extracellular recordings from these flies have revealed a normal response of the photoreceptors themselves to light (Fig. 5; K. Scott, L. Sun, and C. Zuker, personal communication). In other studies, inositol trisphosphate and diacylglycerol were shown to be primarily responsible for generating the depolarizing potential in the *Drosophila* retina (Zuker, 1996; Chyb et al., 1999). Our results suggest that the requirement for cGMP in the *Drosophila* visual system is developmental, rather than physiological. First, we never observed cGMP in the photoreceptors after 48 hr APF, in the presence or absence of NO stimulation (Gibbs and Truman, 1998; Gibbs, unpublished data). Second, although mutant adults lacked positive phototaxis, a basic visually mediated behavior, positive phototaxis was only restored to *Gcα1* adults when *Gcα1* was supplied with heat shock during the first 48 hr of metamorphosis, encompassing the period of observed NO-sensitive sGC activity in the photoreceptors. In contrast, phototaxis did not improve when *Gcα1* was expressed acutely or chronically in adult mutants. Our ERG results also support the hypothesis that sGC signaling is required during the first half of metamorphosis, when retinal growth cones are selecting postsynaptic partners in the optic lobe and NO-sensitive sGC activity is observed (Meinertzhagen and Hanson, 1993; Gibbs and Truman, 1998). The sustained depolarization of the photoreceptors was normal in *Gcα1* adults, but the off-transients were frequently undetectable or greatly reduced in amplitude. In addition, some off-transients contained two peaks. The postsynaptic responses of the laminar monoplar cells are responsible for generating the off-transient in *Drosophila* (Coombe, 1986). Thus, the decreased and aberrant off-transients observed in *GCα1* implicates a defect in the postsynaptic response of laminar monopolar cells to retinal input in these mutants. This abnormal postsynaptic response could arise from disorganized retinal synapses, perhaps as a result of subtle deviations in growth cone behavior in the absence of normal cGMP levels. When *Gcα1* was expressed with heat shock in *Gcα1* mutants during the first half of metamorphosis, the off-transient shape and amplitude were indistinguishable from those of the wild type. The behavioral and electrophysiological results support the hypothesis that cGMP signaling is required in the photoreceptors to promote the appropriate wiring of first-order retinal synapses during metamorphosis. However, because the *Gcα1* mutation and the heat shock *Gcα1* expression were global and not eye-specific, we cannot exclude the possibility that formation of downstream connections in the optic lobe and brain also require GCα1. Our behavioral results in particular may reflect the effects of *Gcα1* expression on these connections. However, from 12 to 48 hr APF we observed NO-sensitive cGMP production almost exclusively in the photoreceptors and not in other visual centers

until later in development (Fig. 1*B*; Gibbs, unpublished data). This was also the developmental window during which heat shock *Gcα1* expression rescued both the phototactic and ERG phenotypes, which strongly suggests that the photoreceptors themselves require GCα1 to ensure the appropriate wiring of first-order retinal synapses.

Despite the profound defects in visual system function, we did not observe a dramatic and consistent disorganization of the retinal projections in *Gcα1*. This contrasts with our previous results, in which pharmacological inhibition of NO–sGC signaling caused severe disruption of the wild-type projection pattern *in vitro* (Gibbs and Truman, 1998). There are several possible explanations for these results. First, the *Gcα1* mutants are hypomorphs and do show low levels of GCα1 protein and NO-sensitive sGC activity in the photoreceptors during metamorphosis. Residual enzymatic activity of these low levels of functional sGC could produce adequate levels of retinal cGMP to prevent significant axon overgrowth provided that the ligand, NO, is present at wild-type levels. This is supported by our experiments in which nervous systems from *Gcα1* pupae were exposed to low levels of an NOS inhibitor. Under these conditions, the resulting disorganization and overgrowth of the retinal axons were much greater than in wild-type controls. These results suggest that residual GCα1 activity and production of cGMP by endogenous NO in *Gcα1* is sufficient to prevent overgrowth of retinal axons *in vivo* but cannot compensate for suppression of NO signaling *in vitro*. Second, we have only examined the *Gcα1* visual system at the whole-mount level. A more detailed analysis of the retinal projections and cartridge organization, perhaps using electron microscopic techniques, may reveal further architectural defects in the *Gcα1* mutants. Finally, other signaling pathways have been shown to contribute to formation of the *Drosophila* retinal projection pattern, and these may compensate for decreased sGC activity during visual system development. Molecules such as Hedgehog (Huang and Kunes, 1996), the EGF-like molecule Spitz (Huang et al., 1998), and N-cadherin (Lee et al., 2001) are all involved in regulating retinal axon targeting and connectivity. The most well characterized signaling pathway shown to regulate retinal targeting in *Drosophila* involves the recruitment of p21-activated kinase (Pak) to the growth cones of growing photoreceptors by the adaptor protein Dock (Garrity et al., 1996; Rao and Zipursky 1998; Hing et al., 1999) and subsequent Pak activation by the combined signaling of Dock and the guanine nucleotide exchange factor Trio (Hing et al., 1999; Newsome et al., 2000). Interestingly, the retinal projection phenotypes of *pak*, *dock*, and *trio* mutants resemble the disruptive effects of NO and sGC inhibition *in vitro*. However, whereas the Pak–Dock pathway seems to regulate the initial stages of retinal axon outgrowth and targeting, the NO–cGMP pathway seems to be required later for maintaining the position of retinal growth cones within the target before synaptogenesis.

We propose that *Gcα1*expression and subsequent sGC- and NO-induced cGMP activity in the photoreceptors regulate synapse formation between photoreceptors and optic lobe neurons by exerting subtle effects on retinal growth cone behavior during cartridge assembly. Expression of NO-sensitive sGC activity was never seen during retinal axon outgrowth but only in photoreceptors that had arrived at their respective optic ganglia. This makes it unlikely that NO and cGMP are acting in a chemoattractive manner to guide retinal growth cones to the optic lobe. The metamorphic period when NOS expression in the optic lobe and NO-sensitive sGC expression in the photoreceptors was ob-

served (12–48 hr APF; Gibbs and Truman, 1998; Gibbs, 2001) correlates temporally with when retinal growth cones are actively seeking out optic lobe cells with which they will form synaptic cartridges (Meinertzhagen and Hanson, 1993). Our current and previously published results support a model wherein NO from the target acts to stimulate cGMP synthesis in newly arrived retinal growth cones, stabilizing them and preventing further axonal extension but still allowing lateral movement within the target region. When NO production or sGC activity is inhibited pharmacologically, this stabilization is lost, and the photoreceptors resume longitudinal growth (Gibbs and Truman, 1998). Our current results show that although genetically reducing sGC activity leads to more subtle defects in visual system architecture, perhaps at the level of cartridge organization, the overall effects of this mutation on adult visual system function are profound. NO and cGMP have been proposed to regulate vertebrate visual system development by acting as effectors of activity-dependent refinement mechanisms (Ernst et al., 1999; Mize and Lo, 2000; Leamy et al., 2001). However, this is not likely to be the case in *Drosophila*, because the visual system can develop normally in the absence of histamine, the primary visual neurotransmitter (Melzig et al., 1998). Instead, the effects of NO-induced cGMP production may act to regulate growth cone behavior. cGMP has been shown to modulate the response of sensory growth cones and pyramidal cell dendrites to semaphorin (Song et al., 1998; Polleux et al., 2000). Additionally, sGC mediates the NO-induced behavior of filopodia in cultured *Helisoma* neurons (Van Wagenen and Rehder, 1999, 2000). In nematode worms, mutations in a cyclic nucleotide-gated channel affect sensory axon outgrowth during development and in adults (Coburn and Bargmann, 1996; Coburn et al., 1998). Calcium is a well documented mediator of growth cone activity (Davenport and Kater, 1992; Rehder and Kater, 1992; Gomez and Spitzer, 1999), and cGMP can produce growth cone calcium transients *in vitro* (Van Wagenen and Rehder, 1999; Kafitz et al., 2000). For cGMP to influence growth cone dynamics, it must ultimately affect the cytoskeleton. In cultured neuroblastoma cells, intracellular injections of cGMP cause the motile structures of growth cones to freeze and retract, whereas cAMP promotes outgrowth (Bolsover et al., 1992). More recent studies showed that cGMP-dependent protein kinase-mediated phosphorylation inactivates the small GTPase RhoA and prevents it from inhibiting myosin light chain phosphatase, thus decreasing contractility (Surks et al., 1999; Sauzeau et al., 2000; Sawada et al., 2001). Although cGMP could conceivably affect the neuronal cytoskeleton via this mechanism to modulate growth cone behavior and axon outgrowth, its role in the developing *Drosophila* visual system remains a question for further research.

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