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Soluble Guanylyl Cyclases in Invertebrates: Targets for NO and O₂

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MAMMALIAN SOLUBLE GUANYLYL CYCLASES

Soluble guanylyl cyclase (sGC) is probably the most prevalent target for NO in almost all species and tissues (Lucas et al., 2000). The native enzyme is usually a heterodimer (see below for exceptions to this) containing a single heme group (Lucas et al., 2000). In mammals, four subunits have been identified: $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$. The major functional enzyme is the $\alpha 1/\beta 1$ heterodimer (Lucas et al., 2000). The $\alpha 2$ is very similar in sequence to the $\alpha 1$ subunit and the $\alpha 2/\beta 1$ heterodimer has similar properties to the $\alpha 1/\beta 1$ enzyme (Russworm et al., 1998, Gibb et al., 2003). One significant difference is that the $\alpha 2$ subunit interacts with the scaffold protein PSD95 and hence likely has a different subcellular distribution (Russworm et al., 2001). All four mammalian subunits are homologous proteins with a similar arrangement of functional domains (Figure 1). The subunits can be divided into a C-terminal catalytic domain that catalyses the conversion of GTP to cGMP and an N-terminal regulatory domain that functions as a heme binding region and is required for NO activation of the enzyme (Figure 1). A variety of studies have identified several residues in each of these domains that are required for function and are indicated in Figure 1. The $\alpha 1/\beta 1$ heterodimer binds a single heme group per heterodimer and the $\beta 1$ subunit is primarily responsible for this interaction (Lucas et al., 2000). The Fe²⁺ ion in the center of the heme group interacts with His105 (identified as H105 in Figure 1) of the $\beta 1$ subunit (Zhao et al., 1998). The heme group interacts with three residues in the $\beta 1$ subunit via hydrogen bonds that form the YXS/TXR motif (Schmidt et al., 2004). In addition, two cysteines (at positions C78 and C214 in the rat $\beta 1$) subunit that are necessary for NO activation (Freibe et al., 1997). The relative positions of these residues are shown in Figure 1.

Modeling of the catalytic domain of sGC, based on the crystal structure of the catalytic domain of mammalian adenylyl cyclase, predicts that there are 17 residues that contact the GTP substrate (Liu et al., 1997). The active site is at the interface between the two subunits, so each subunit provides a subset of specific residues (10 for the β subunit and 7 for the α subunit, which are marked on the catalytic domain in Figure 1). This model predicts that a single GTP molecule will bind per heterodimer (Liu et al., 1997).

The $\beta 2$ subunit has quite different functional characteristics and appears to be a member of a different subfamily of sGC subunits, which we have termed the atypical sGCs (Morton, 2004a). Although the $\beta 2$ subunit will form active heterodimers with both the $\alpha 1$ and $\alpha 2$, it has the unusual property that it is also active as a homodimer (Koglin et al., 2001; Gibb et al., 2003). Each of these enzymes are sensitive to NO, but they are all less potently activated by NO compared to the $\alpha 1/\beta 1$ and $\alpha 2/\beta 1$ heterodimers (Gibb et al., 2003). The regulatory domain of the $\beta 2$ subunit contains His104, Cys78, Cys214 and the YXS/TXR motif (Figure 1), which suggests that the $\alpha 1/\beta 2$ and $\alpha 2/\beta 2$ heterodimers bind a single heme group, but it is not known

whether the $\beta 2$ homodimer binds one or two heme groups. The catalytic domain of the $\beta 2$ subunits contains all 17 of the residues that bind the GTP substrate, providing a structural basis for the formation of an active homodimer (Morton 2004a). This is similar to the situation with the receptor GCs, which also form active homodimers and contain all 17 residues that interact with GTP. Modeling of the catalytic domain of receptor GCs suggests that two GTP molecules bind per dimer (Liu et al., 1997), a prediction which is supported by the finding that the receptor GC, GC-A, shows kinetics with positive cooperativity (Wong et al., 1995). Members of both conventional and atypical sGC families have also been identified in a variety of invertebrates (Table 1; Morton, 2004a; Vermehren et al., 2006). Phylogenetic and sequence comparisons have also been made across a broad array of prokaryotes and eukaryotes (Schaap, 2005).

CONVENTIONAL INVERTEBRATE sGCS

Structures

Genes that code for sGCs have been identified in both nematodes and insects (Morton, 2004a). Insects have members of both conventional and atypical sGC families, whereas all the sGCs identified in *C. elegans* are members of the atypical sGC family (Table 1; Morton 2004a.). Insects appear to express one conventional α subunit and one conventional β subunit (Vermehren et al., 2006). Both subunits are similar in primary sequence to their mammalian orthologues, with a similar regulatory and catalytic domain (Morton and Hudson, 2002). All of the individual amino acid residues that have been identified as critical to the function of conventional sGCs are conserved between the mammalian and insect orthologues (Figure 1; Morton and Hudson, 2002). In the regulatory domain of $\beta 1$, these include His105 that forms the heme axial ligand and the YXS/TXR motif (Figure 1). In addition, Cys78 and Cys214 that are necessary for NO activation are also conserved in the insect conventional sGC β subunits that have been identified (Morton and Hudson, 2002). These sequence comparisons predict that the insect conventional heterodimers bind a single heme group per dimer. Although there is no direct evidence to support this, the sGC inhibitor 1H-[1,2,4]oxadiazolo [4,3,-a] quinoxaline-1-one (ODQ) that acts by oxidizing the heme group is an effective inhibitor of the insect conventional sGCs suggesting that they do contain heme (Morton et al., 2005a). One notable difference among the insect β subunit regulatory domains is that although the *Manduca* MsGC- $\beta 1$ regulatory domain contains the same number of residues as mammalian β subunits (Nighorn et al., 1998), the *Drosophila* Gyc β -100B subunit contains an insertion of 118 amino acids at the center of the regulatory domain (Figure 1; Shah and Hyde, 1995). This results in a β subunit of 86 kDa, which is larger than the α subunit whereas all other β subunits are smaller than the β subunits. Interestingly, the *Anopheles* β subunit also has an insertion of 91 residues in the same position although there is no sequence conservation of the inserts between the two flies (Caccone et al., 1999). The insect α subunits are very similar in primary sequence to the mammalian $\alpha 1$ subunits. None of the insect α subunit sequences reported contain C-terminal sequences that would predict that they can bind to PDZ domains in a similar fashion to the mammalian $\alpha 2$ subunits (Russworm et al., 2001).

The catalytic domains of both the α and the β subunits are highly conserved across species with all the residues that interact with GTP from each subunit conserved in the insect orthologue (Figure 1; Morton and Hudson, 2002). Several studies have described the biochemical properties of insect conventional sGCs by transiently expressing cloned subunits in heterologous cells. These studies have generally shown that the insect enzymes have very similar properties to their mammalian orthologues. Both *Drosophila* and *Manduca* conventional sGCs form obligate heterodimers, which are potently activated by a variety of NO donors and are inhibited by ODQ, which has a similar potency on insect and mammalian sGCs (Shah and Hyde, 1995; Nighorn et al., 1998; Morton et al., 2005a).

Function

Most functional studies aimed at investigating physiological functions of the conventional sGCs have focused on the use of sGC inhibitors, such as ODQ and sGC activators, such as NO donors. One general theme that has emerged from these studies has been the proposed role of the sGCs in a variety of developmental events in the nervous system. Examples include neuronal outgrowth, synapse formation and neuronal and glial migration in a variety of insect species (Truman et al., 1996; Wright et al., 1998; Gibbs and Truman, 1998; Gibson et al., 2001, Bicker 2005). Although these studies provide strong evidence to support the role of the NO/cGMP pathway in neuronal development, it should be pointed out that most of the pharmacological manipulations used are not specific for the conventional sGCs. As described below, in addition to the conventional sGCs insects also express another family of sGCs, the atypical sGCs (Morton, 2004a). In *Drosophila* these enzymes are slightly stimulated by NO donors and are also strongly inhibited by ODQ (Morton et al., 2005a). At the present time the only specific pharmacological agents available for the conventional sGCs are the new family of NO-independent sGC activators such as YC-1 and BAY 41-2272 (Morton et al., 2005a).

Genetic manipulations provide an alternative, non-pharmacological, approach for assessing sGC function. This is potentially complicated by the fact that *Drosophila* that are homozygous for a point mutation within the coding region of the NOS gene die in the early first instar (Regulski et al., 2004), raising the possibility that null mutations in the conventional sGC subunit genes would also be lethal. It is not known which tissues require the presence of NOS during early development. It is also not known whether any or all of the essential actions of NO require sGCs. However, studies in progress (Vermehren and Morton, unpublished data) show that fly lines that have a transposon within introns of the genes for either the α or the β subunit of the conventional sGC are also homozygous early larval lethal. Because the insertion is also within the intron of a gene on the opposite DNA strand it is not known whether the lethality is due to disruption of the sGC genes. By contrast, fly lines with point mutations that lead to greatly reduced levels of expression of *Gyca-99B* were viable, but failed to develop connections between their photoreceptors and the post-synaptic cells (Gibbs et al., 2001). This defect was rescued with exogenous, global expression of *Gyca-99B* (Gibbs et al., 2001). These results were consistent with the inhibitor-based studies cited above that pharmacologically demonstrated a requirement for sGC activity in photoreceptor development (Gibbs and Truman, 1998).

ATYPICAL sGCS

cDNA cloning and expression studies

The first atypical sGC was demonstrated biochemically in lobster muscle (Prabhakar et al., 1997). In this study, two distinct peaks of GC activity were separated chromatographically from soluble fractions of lobster muscle, one of which was stimulated by NO and the other was NO-insensitive (Prabhakar et al., 1997). Whether the NO-insensitive peak represented an sGC orthologue or whether it was similar to MsGC-I (Simpson et al., 1999), a *Manduca* GC more similar to receptor GCs but lacking a transmembrane domain is not known. The first cloned atypical sGC, MsGC- β 3 (Table 1), was identified in the insect, *Manduca sexta* (Nighorn et al., 1999). As described above, conventional sGCs are obligate heterodimers that are potentially activated by NO. When MsGC- β 3 was transiently expressed in heterologous cells its properties were strikingly different: in cell free homogenates, it was enzymatically active in the absence of additional subunits and it was insensitive to NO donors (Nighorn et al., 1999). Subsequent analysis showed that it formed active homodimers and could form heterodimers with either of the conventional subunits that showed neither basal nor NO-stimulated activity (Morton and Anderson, 2003).

The mammalian $\beta 2$ subunit also appears to belong to this family of atypical sGC subunits and shares a number of biochemical properties with MsGC- $\beta 3$. The sequence first reported for $\beta 2$ predicted a protein lacking the equivalent residues corresponding to the first 62 residues of the $\beta 1$ subunit (Yuen et al., 1990). When this version of $\beta 2$ was co-expressed with the $\alpha 1$ subunit the resulting enzyme was substantially less sensitive to NO than the $\alpha 1/\beta 1$ heterodimer (Gupta et al., 1997). When co-expressed with the $\alpha 1/\beta 1$ heterodimer, the $\beta 2$ subunit acted as a dominant negative subunit reducing the NO sensitivity of the combined subunits (Gupta et al., 1997). We showed that MsGC- $\beta 3$ could act in the same way when co-expressed with both the α and β subunits from *Manduca*, also reducing the NO activation of the conventional sGC (Morton and Anderson, 2003). The results described by Gupta et al. (1997) were subsequently questioned as others failed to measure enzyme activity when the $\beta 2$ subunit was co-expressed with $\alpha 1$ (Denninger and Marletta, 1999). A more recent report describes the cloning of another cDNA for the $\beta 2$ subunit that contains residues that correspond to the 62 amino acid N-terminus of the $\beta 1$ subunit (Koglin et al., 2001). This variant of the $\beta 2$ subunit is, like MsGC- $\beta 3$, active in the absence of additional subunits, but unlike MsGC- $\beta 3$ is slightly stimulated by NO donors in cell-free homogenates (Koglin et al., 2001). RT-PCR studies showed that the $\beta 2$ subunit was expressed in the brain, but in situ hybridization experiments failed to reveal specific staining, so the cellular or regional location of the subunit is unknown (Gibb and Garthwaite, 2001). More recent experiments have revealed further subtleties in the properties of the $\beta 2$ subunits (Gibb et al., 2003). The studies by Gupta et al. (1997) and Koglin et al. (2001) used cell homogenates from heterologous cells transiently expressing the subunits to measure enzyme activity. Gibb et al. (2003) by contrast used intact cells that were transiently transfected with the subunits and then the accumulated levels of cGMP were measured. This more recent study showed that the shorter version of the $\beta 2$ subunit when co-expressed with either the $\alpha 1$ or the $\alpha 2$ subunit was active and although it was stimulated by NO, it showed a much reduced level of activation compared to either the $\alpha 1/\beta 1$ or $\alpha 2/\beta 1$ combinations (Gibb et al., 2003). The $\beta 2$ variant ($v\beta 2$, with the additional 62 amino acids) was active in intact cells in the absence of additional subunits and again was stimulated to a lesser extent than the conventional subunit combinations (Gibb et al., 2003). All the combinations involving $\beta 2$ or $v\beta 2$ subunits showed a marked bell-shaped dose-response curve for NO activation, suggesting rapid desensitization (Gibb et al., 2003). These data indicate that depending on which splice variant is expressed, $\beta 2$ can function as either a homodimer or a heterodimer, but in either case it is less sensitive to NO stimulation than the conventional subunits.

Sequencing the genomes of *C. elegans* and *Drosophila melanogaster* revealed a much broader array of atypical guanylyl cyclase subunits than previously imagined (Morton, 2004a). The genome of *C. elegans* contains seven genes (Table 1) that code for guanylyl cyclase subunits (Birnbay et al., 2000) and all are predicted to be NO insensitive (Morton et al., 1999). Although their primary sequence is more similar to β subunits than α subunits, they have all been predicted to form various heterodimers in vivo (Morton, 2004a). Although no biochemical evidence has been obtained to support these predictions, genetic studies confirmed heterodimer formation between two subunits GCY-35 and GCY-36 (Cheung et al., 2004). The *Drosophila* genome predicts three genes that code for atypical subunits (Table 1), Gyc-88E, an orthologue of MsGC- $\beta 3$, and two additional genes: Gyc-89Da and Gyc-89Db (Morton and Hudson, 2002; Morton, 2004a; Langlais et al., 2004). Additional genomic sequences have suggested that all insects have an orthologue of MsGC- $\beta 3$ /Gyc-88E but other insects appear to have a single gene coding for an orthologue of Gyc-89Da/89Db (Vermehren et al., 2006). *Drosophila pseudoobscura* has both Gyc-89Da and Gyc-89Db orthologues whereas the mosquito (also a member of the order Diptera), *Anopheles gambiae*, has a single orthologue suggesting that there was a relatively recent gene duplication of Gyc-89Da/89Db (Vermehren et al., 2006). A recent phylogenetic analysis of sGCs has grouped two of the *C. elegans* sGCs, GYC-31 and GYC-33, close to the insect atypical sGCs, while the remaining 5 *C. elegans* sGCs were grouped closer to the mammalian $\beta 2$ subunits (Fitzpatrick et al., 2006). The domain

structure of the atypical sGCs is similar to that of the conventional sGCs with both an N-terminal regulatory domain and a more C-terminal catalytic domain (Figure 1). One notable difference is the Gyc-88E has a large C-terminal domain (Figure 1). This has no known function and does not contain any known functional protein domains. The *Manduca* and *Anopheles* orthologues to Gyc-88E also have a C-terminal domain that is a similar size (about 300 residues). There is little sequence similarity between the C-terminal domains between these species although there is a region of about 20 residues that is highly conserved (Langlais, et al., 2004). Interestingly, the $\beta 2$ subunit and a *C. elegans* orthologue, GCY-31, also contain a C-terminal domain although there is no sequence conservation in this domain between insects, mammals and *C. elegans*. Although no functional role for this domain has been identified, in MsGC- $\beta 3$ there is evidence that it may play an auto-inhibitory role (Morton and Anderson, 2003).

All the *Drosophila* subunits have been transiently expressed in heterologous cells and their biochemical properties described (Langlais et al., 2004; Morton et al., 2005a). Gyc-88E, like MsGC- $\beta 3$ and the mammalian $\nu\beta 2$ subunit, was active in the absence of additional subunits (Langlais et al., 2004). In cell-free homogenates, Gyc-88E was similar to $\nu\beta 2$ and was slightly stimulated by some, but not all, NO donors (Langlais et al., 2004; Morton et al., 2005a). Gyc-89Da and Gyc-89Db were inactive on their own, but enhanced the activity of Gyc-88E when they were co-expressed (Langlais et al., 2004; Morton et al., 2005a). These heterodimers were also slightly stimulated by NO and were more sensitive to NO donors than the homodimeric Gyc-88E (Morton et al., 2005a). The NO activation was inhibited by ODQ, a property shared by the conventional sGCs, suggesting that they contain a heme group (Morton et al., 2005a). The heterodimers showed a pronounced bell-shaped dose response curve to the NO donor DEA-NONOate, which was interpreted as indicating that rapid desensitization occurred, similar to the mammalian $\beta 2$ subunits (Morton et al., 2005a). In vivo, we found that Gyc-88E was frequently co-expressed with either Gyc-89Da or Gyc-89Db suggesting that the native enzymes were likely to be heterodimers (Langlais et al., 2004; Morton et al., 2005a). Although the maximum stimulation of the atypical heterodimers to NO donors was only 2–4 fold compared to at least 50 fold stimulation for the conventional heterodimers, the only qualitative difference in biochemical properties revealed by these studies was the response to the NO-independent activator BAY 41-2272. This compound was a potent activator of the conventional sGCs, but had no effect on the atypical subunits (Morton et al., 2005a).

Analysis of the critical residues shown in Figure 1 is consistent with most of the biochemical properties of the atypical sGCs. They all contain the heme interacting residues, His104 and the YXS/TXR motif supporting the prediction that they contain a heme group. As both Gyc-88E and Gyc-89Da/89Db contain these residues it is not clear whether the heterodimers would contain one or two heme groups per heterodimer. All three atypical sGC subunits lack Cys78 and Cys214, required for NO activation in $\beta 1$. Whereas *Manduca* MsGC- $\beta 3$ lacks these cysteines and is NO-insensitive and the mammalian $\beta 2$ subunit contains this cysteines and is slightly NO-sensitive the *Drosophila* Gyc-88E, Gyc-89Da and Gyc-89Db are slightly NO-sensitive, suggesting that these residues are not absolutely required for NO activation. In the catalytic domain, Gyc-88E contains all the residues that interact with GTP consistent with its ability to form homodimers and Gyc-89Da and Gyc-89Db only contain the residues found in α subunits consistent with their formation of obligate heterodimers.

Oxygen sensing by the atypical sGCs

The poor responsiveness of the atypical sGCs to NO raises the possibility that this is not the natural ligand. This idea was supported by the finding that in *C. elegans*, a related atypical sGC subunit, GCY-35 (Table 1), was required for oxygen sensitivity (Gray et al., 2004).

Conventional sGCs are unusual for heme proteins in that they do not bind oxygen (Lawson et

al., 2003). By contrast, the regulatory domain of GCY-35 is capable of binding oxygen via a heme group, suggesting that it could act as a molecular oxygen detector and its activity would be regulated by oxygen concentration (Gray et al., 2004). It has not been possible to test this directly as none of the *C. elegans* sGC subunits exhibit any enzymatic activity when expressed in heterologous cells (Morton, 2004a).

The activity of the *Drosophila* atypical sGCs expressed in heterologous cells, by contrast was regulated by oxygen. When COS-7 cells, which had been transiently transfected with the *Drosophila* atypical sGC subunits, were incubated in the presence or absence of oxygen up to 50 fold more cGMP accumulated in the cells incubated in the absence of oxygen compared to normal atmospheric oxygen concentrations whereas cells transfected with the conventional subunits showed no change (Morton 2004b; Vermehren et al., 2006). This increase was graded over 21-0% oxygen, a characteristic that would be expected if the enzyme served as a molecular oxygen detector (Morton, 2004b). Incubation in 50% oxygen inhibited activity further suggesting that at 21% oxygen the enzyme was not fully saturated (Vermehren et al., 2006). Additionally, the activation in the absence of oxygen was blocked by the sGC inhibitor ODQ, suggesting that activation required the heme group (Morton 2004b). These data suggested that oxygen bound to the heme group in a manner analogous to NO binding to the heme group of conventional guanylyl cyclases, but caused inhibition of the enzyme rather than activation. This raised an apparent paradox: if NO and oxygen bind at the same site, how can one ligand stimulate the enzyme when the other is inhibitory when they are so chemically similar? Results of an experiment that exposed transfected cells to NO donors in the presence of difference concentrations of oxygen suggested a possible explanation (Vermehren et al., 2006). At 21% oxygen, NO was stimulatory – increasing cGMP levels about 2 fold – whereas NO added to cells incubated at 10% or 0% oxygen was inhibitory, reducing the cGMP levels to about the same levels as measured at 21% oxygen (Vermehren et al., 2006). One interpretation for this result is that although both NO and oxygen bound at the same site and both were inhibitory, NO was less effective as an inhibitor compared to oxygen. Thus, at 21% oxygen, NO displaced some of the oxygen bound to the heme group and relieved some of the oxygen inhibition, causing an apparent stimulation. This is comparable to both NO and CO binding to conventional guanylyl cyclases, but CO being less effective at stimulating enzyme activity compared to NO (Sharma and Magde, 1999). This hypothesis can be tested by examining the absorbance spectra of purified proteins. Soluble GCs, like other heme proteins, have a peak of absorbance at about 430nm (the Soret peak), which in the presence of a bound gas shifts to a lower wavelength (Stone and Marletta, 1994). Conventional sGCs do not bind oxygen and no shift in the Soret peak is observed when spectra are gathered under aerobic or anaerobic conditions. By contrast, the regulatory domain from a *C. elegans* atypical sGC (GCY-35) exhibited a shift from 430nm to 415nm when exposed to aerobic conditions (Gray et al., 2004). Interestingly, in the presence of NO, the Soret peak had a shoulder, suggesting that the heme group formed two stable nitrosyl complexes (Gray et al., 2004). It is not known whether GCY-35 is activated or inhibited by NO or oxygen, but the difference in the absorption spectra in the presence of NO and oxygen suggests that the enzyme activity could differ in the presence of the two gases.

Modeling and sequence comparison have also indicated the structural basis for the oxygen sensitivity of the atypical sGCs. Although the crystal structure of the heme domain has not been solved for any sGC, the structure of a chemotaxis protein of the obligate anaerobe *Thermoanaerobacter tengcongensis*, with a GC-like heme domain, has been solved (Pellicena et al., 2004; Nioche et al., 2004). This domain shares primary sequence similarity to sGC subunits and binds both O₂ and NO with high affinity (Karow et al., 2004). Recent studies have identified Y140 as a critical residue in determining NO/O₂ selectivity (Boon et al., 2005). These findings allow us to predict which sGC subunits will be able to form O₂ sensitive GCs. All three of the *Drosophila* (see Figure 1) and all 7 of the *C. elegans* atypical sGCs have a tyrosine

in the equivalent position. We have also analyzed additional sequences that have recently been deposited in data bases and these studies suggest that all insects probably have both oxygen-sensitive and oxygen-insensitive (conventional) sGCs (Vermehren et al., 2006).

BEHAVIORAL STUDIES INDICATING ROLES FOR ATYPICAL sGCs

Responses to hypoxia

The first evidence supporting a behavioral role for atypical sGCs in oxygen sensation was in *C. elegans*. When wild-type *C. elegans* were placed in a gradient of 0–21% oxygen concentrations, they congregated at intermediate concentrations of 5–12%, avoiding both high and low oxygen concentrations (Gray et al. 2004). Animals with null mutations in an atypical sGC, GCY-35, were evenly distributed across this gradient, failing to avoid both normal and anoxic conditions (Gray et al. 2004). Avoidance of high (21%) oxygen required the presence of a cGMP-gated ion channel in the neurons that expressed GCY-35 suggesting that the activity of GCY-35 was required for this behavior (Gray et al. 2004). As described above, the regulatory domain of GCY-35 bound oxygen via a heme group (Gray et al. 2004) suggesting that its activity was directly regulated by oxygen, but no direct evidence for this has been obtained (Morton 2004a).

The biochemical properties of the *Drosophila* atypical sGCs suggested that they might also function as molecular oxygen detectors (Morton 2004b). As the activity of the *Drosophila* atypical sGCs is activated by reduced oxygen concentrations we reasoned that they could mediate responses to hypoxia (Morton et al., 2005b). The *Drosophila* atypical sGCs are expressed in a subset of central and peripheral sensory neurons (Langlais et al., 2004) where they are ideally situated to respond to changing environmental oxygen concentrations and mediate behavioral responses to hypoxia (Morton, 2004b). We have recently begun to test this possibility (Morton et al., 2005b).

Drosophila larvae placed on a small pile of yeast paste, under normal atmospheric oxygen concentrations, will immediately burrow into the yeast and begin feeding with only their posterior spiracles above the surface of the yeast. When the larvae were exposed to low (1–10%) oxygen levels, they rapidly ceased feeding, withdrew from the food and began exploratory behaviors (Wingrove and O'Farrell, 1999). Several lines of evidence indicated that this behavior was mediated by increases in cGMP. Firstly, larvae that differ in the levels of cGMP-dependent protein kinase (G-kinase) due to a polymorphism in the *for* gene (Osborne et al., 1997) showed different response times to hypoxia: those with lower levels of kinase responded more slowly (Wingrove and O'Farrell, 1999). In addition, larvae fed NO synthase inhibitors also responded more slowly to hypoxia than control larvae while larvae over-expressing NO synthase responded faster (Wingrove and O'Farrell, 1999). This suggested that conventional sGCs mediated the behavioral response utilizing a NO/cGMP/G-kinase pathway (Wingrove and O'Farrell, 1999).

To determine whether the atypical sGCs were involved in this response, we specifically targeted the central and peripheral neurons that express the atypical sGC subunits by generating fly lines that expressed the yeast transcription factor, GAL4, under the control of the predicted promoter regions of the *Gyc-89Da* and *Gyc-89Db* genes (Vermehren et al., 2005). Using these fly lines we reduced cGMP levels specifically in these neurons by expressing a cGMP-specific PDE (bovine PDE5) using UAS-bPDE5 flies (Broderick et al., 2004). Larvae expressing bPDE5 in either *Gyc-89Da* or *Gyc-89Db* neurons showed much slower responses to hypoxia than larvae from either of the parental lines (Morton et al., 2005b).

The results of experiments with the p89Da-bPDE5 animals imply that an increase in cGMP in the *Gyc-89Da* neurons is necessary for the behavioral response to hypoxia. A major question

to resolve is the identity of the GC that is responsible for this increase in cGMP. It is very tempting to assume that it is the Gyc-88E/Gyc-89Da heterodimer within some of the sensory neurons that is directly responding to reduced oxygen levels with an increase in GC activity. Wingrove and O'Farrell (1999), however, suggested that a NO-sensitive conventional sGC is involved. It is not known whether the conventional sGC is co-expressed with any of the atypical subunits. If we assume that they are not co-expressed, it is still possible to reconcile the two sets of data. Wingrove and O'Farrell (1999) showed that larvae that over-expressed NO synthase were more sensitive to reduced oxygen than control larvae. As NO can displace oxygen bound to the atypical sGCs, increasing their activity (Vermehren et al., 2006), the increased NO would have a similar effect on the atypical sGCs as decreased oxygen stimulating the hypoxia escape response.

Chemotaxis to other chemicals

The atypical sGCs are also expressed in the ganglia that innervate the two main chemosensory organs in *Drosophila* larvae (Langlais et al., 2004). The main larval olfactory organ is the dorsal organ that is innervated by the dorsal ganglion and the main gustatory organ, the terminal organ is innervated by the terminal ganglion (Heimbeck et al., 1999). In situ hybridization experiments showed that all three atypical sGCs are expressed in a few cells in both the terminal and dorsal ganglia (Langlais et al., 2004; Morton et al., 2005a). We used the same promoter::GAL4 and UAS::bPDE5 fly lines described above to determine if there was a role for cGMP in the neurons that express the atypical sGCs in chemotaxis. *Drosophila* larvae are attracted to a wide range of volatile and non-volatile chemicals, which can easily be quantified by simple behavioral preference tests (Heimbeck et al., 1999). Using the larvae which expressed bPDE5 in neurons that expressed the atypical sGCs we showed that elevated levels of cGMP were required for chemotaxis to certain odorants and tastants (Vermehren et al., 2005).

While these data suggest a role for cGMP in the atypical sGC cells in chemotaxis, several questions remain unanswered. As with the hypoxia escape response, the data do not directly show that the atypical sGCs are the source of the cGMP required for the behavioral responses. The cellular basis for the chemotactic deficits is also undefined. In addition to the sensory neurons in the chemosensory organs, the atypical sGCs are also expressed in a large number of neurons in the CNS. It is not known whether the chemotactic deficits were due to reduced cGMP levels in the CNS or the sensory neurons or both. If the cGMP is required in the sensory neurons, it is also not known whether the cGMP is required in the primary signal transduction process of the cell, or whether it modulates the olfactory and gustatory signal transduction. Although many olfactory and gustatory receptors have now been identified in *Drosophila* and other insects (Robertson et al., 2003), recent studies have shown that they are quite distinct in their membrane topology and likely function from olfactory receptors in other species (Benton et al., 2006). The signal transduction that underlies these receptors is currently unknown and hence whether cGMP plays a direct or indirect role in the pathway is also unknown.

These findings also raise the question of why chemotaxis should utilize an oxygen-sensitive sGC. Studies with *C.elegans* shed some light on this issue. In addition to mediating oxygen sensitivity, GCY-35 also mediates feeding behavior (Cheung et al., 2004). In the presence of bacteria, some strains of *C. elegans* tend to aggregate when feeding, a behavior that requires the presence of GCY-35 (Cheung et al., 2004). This aggregation behavior is not seen when animals are exposed to reduced oxygen concentrations (Gray et al., 2004) and it has been suggested that one of the cues used by *C. elegans* to detect the presence of bacteria, is reduced oxygen concentrations (Gray et al., 2004).

Conclusions

We have provided here a brief overview of the properties and functions of sGCs in invertebrates. As in mammals, the conventional sGCs probably act as the major receptor for NO and mediate most of its actions. In addition, invertebrates express another class of sGC – the atypical sGCs, which are oxygen sensitive. There is also evidence for genes that code for similar enzymes are likely expressed by fish, birds and marsupials (Morton, Vermehren and Langlais, unpublished data). In vitro, the *Drosophila* atypical sGCs also respond, albeit weakly, to NO. Whether they are also receptors for NO in vivo and how the combined actions of NO and oxygen regulate their functions are important issues to resolve.

References

- Benton R, Saches S, Michnick SW, Vossall LB. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biology* 2006;4:240–257.
- Bicker G. STOP and GO and NO: nitric oxide as a regulator of cell motility in simple brains. *BioEssays* 2005;27:495–505. [PubMed: 15832386]
- Boon EM, Huang SH, Marletta MA. A molecular basis for NO selectivity in soluble guanylate cyclase. *Nature Chem. Biol* 2005;1:53–59. [PubMed: 16407994]
- Broderick KE, Kean L, Dow JA, Pyne NJ, Davies SA. Ectopic expression of bovine type 5 phosphodiesterase confers a renal phenotype in *Drosophila*. *J. Biol. Chem* 2004;279:8159–8168. [PubMed: 14662775]
- Caccone A, Garcia BA, Mathiopoulos KD, Min GS, Moriyama EN, Powell JR. Characterization of the soluble guanylyl cyclase beta-subunit gene in the mosquito *Anopheles gambiae*. *Insect Mol. Biol* 1999;8:23–30. [PubMed: 9927171]
- Cheung BHH, Arellano-Carbajal F, Rybicki I, de Bono M. Soluble guanylate cyclases act in neurons exposed to the body fluid to promote *C. elegans* aggregation behavior. *Curr. Biol* 2004;14:1105–1111. [PubMed: 15203005]
- Denninger JW, Marletta MA. Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim. Biophys. Acta* 1999;1411:334–350. [PubMed: 10320667]
- Fitzpatrick DA, O'Halloran DM, Burnell AM. Multiple lineage specific expansions within the guanylyl cyclase gene family. *BMC Evolutionary Biology* 2006;6:26. [PubMed: 16549024]
- Friebe A, Wedel B, Harteneck C, Foerster J, Schultz G, Koesling D. Functions of conserved cysteines of sGCs. *Biochem* 1997;36:1194–1198. [PubMed: 9063867]
- Gibb BJ, Garthwaite J. Subunits of the nitric oxide receptor, soluble guanylyl cyclase, expressed in rat brain. *Eur. J. Neurosci* 2001;13:539–544. [PubMed: 11168561]
- Gibb BJ, Wykes V, Garthwaite J. Properties of NO-activated guanylyl cyclases expressed in cells. *Br. J. Pharmacol* 2003;139:1032–1040. [PubMed: 12839878]
- Gibbs SM, Becker A, Hardy RW, Truman JW. Soluble guanylate cyclase is required during development for visual system function in *Drosophila*. *J. Neurosci* 2001;21:7705–7714. [PubMed: 11567060]
- Gibbs SM, Truman JW. Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of *Drosophila*. *Neuron* 1998;20:83–93. [PubMed: 9459444]
- Gibson NJ, Rössler W, Nighorn AJ, Oland LA, Hildebrand JG, Tolbert LP. Neuron-glial communication via nitric oxide is essential in establishing antennal-lobe structure in *Manduca sexta*. *Dev. Biol* 2001;240:326–339. [PubMed: 11784067]
- Gray JM, Karow DS, Lu H, Chang AJ, Chang JS, Ellis RE, Marletta MA, Bargmann CI. Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature* 2004;430:317–322. [PubMed: 15220933]
- Gupta G, Azam M, Yang L, Danziger RS. The $\beta 2$ subunit inhibits stimulation of the $\alpha 1/\beta 1$ form of soluble guanylyl cyclase by nitric oxide. Potential relevance to regulation of blood pressure. *J. Clin. Invest* 1997;100:1488–1492. [PubMed: 9294115]
- Heimbeck G, Bugnon V, Gendre N, Habelin C, Stocker RF. Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. *J. Neurosci* 1999;19:6599–6609. [PubMed: 10414987]

- Karow DS, Pan D, Tran R, Pellicena P, Presley A, Mathies RA, Marletta MA. Spectroscopic characterization of the soluble guanylate cyclase-like heme domains from *Vibrio cholerae* and *Thermoanaerobacter tengcongensis*. *Biochem* 2004;43:10203–10211. [PubMed: 15287748]
- Koglin M, Vehse K, Budaues L, Scholz H, Behrends S. Nitric oxide activates the $\beta 2$ subunit of soluble guanylyl cyclase in the absence of a second subunit. *J. Biol. Chem* 2001;276:30737–30743. [PubMed: 11406623]
- Langlais KK, Stewart JA, Morton DB. Preliminary characterization of two atypical sGC in the central and peripheral nervous system of *Drosophila melanogaster*. *J. Exp. Biol* 2004;207:2323–2338. [PubMed: 15159437]
- Lawson DM, Stephenson CEM, Andrew CR, Gerorge SJ, Eady RR. A two-faced molecule offers NO explanation: the proximal binding of nitric oxide to haem. *Biochem. Soc. Trans* 2003;31:553–557. [PubMed: 12773155]
- Liu Y, Ruoho AE, Rao VD, Hurley JH. Catalytic mechanism of the adenylyl and guanylyl cyclases: modeling and mutational analysis. *Proc. Natl. Acad. Sci. USA* 1997;94:13414–13419. [PubMed: 9391039]
- Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev* 2000;52:375–413. [PubMed: 10977868]
- Morton DB. Invertebrates yield a plethora of atypical guanylyl cyclases. *Mol. Neuro* 2004a;29:97–115.
- Morton DB. Atypical sGCs in *Drosophila* can function as molecular oxygen sensors. *J. Biol. Chem* 2004b;279:50651–50653. [PubMed: 15485853]
- Morton DB, Anderson E. MsGC- $\beta 3$ forms active homodimers and inactive heterodimers with NO-sensitive sGC subunits. *J. Exp. Biol* 2003;206:937–947. [PubMed: 12582136]
- Morton DB, Hudson ML. Cyclic GMP regulation and function in insects. *Adv. Insect Physiol* 2002;29:1–54.
- Morton DB, Hudson ML, Waters E, O'Shea M. Soluble guanylyl cyclases in *C. elegans* - NO is not the answer. *Current Biology* 1999;9:R546–R547. [PubMed: 10469574]
- Morton DB, Langlais KK, Stewart JA, Vermehren A. Comparison of the properties of the five sGC subunits in *Drosophila melanogaster*. *J. Insect Sci* 2005a;5:12. [PubMed: 16341244]
- Morton DB, Langlais KK, Stewart JA, Vermehren A. Atypical sGCs in *Drosophila* as possible oxygen sensors. 2005 abstract viewer/itinerary planner, program 613.7, Washington DC. Society for Neuroscience. 2005b
- Nighorn A, Byrnes KA, Morton DB. Identification and characterization of a novel beta subunit of sGC that is active in the absence of a second subunit and is relatively insensitive to nitric oxide. *J. Biol. Chem* 1999;274:2525–2531. [PubMed: 9891024]
- Nighorn A, Gibson NJ, Rivers DM, Hildebrand JG, Morton DB. The NO/cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of *Manduca sexta*. *J. Neurosci* 1998;18:7244–7255. [PubMed: 9736646]
- Nioche P, Berka V, Vipond J, Minton N, Tsai AL, Raman CS. Femtomolar sensitivity of a NO sensor from *Clostridium botulinum*. *Science* 2004;306:1550–1553. [PubMed: 15472039]
- Osborne K, Robichon A, Burgess E, Butland S, Shaw R, Coulthard A, Pereira H, Greenspan R, Sokolowski MB. Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* 1997;277:834–836. [PubMed: 9242616]
- Pellicena P, Karow D, Boon EM, Marletta MA, Kuriyan J. Crystal structure of an oxygen-binding heme domain related to soluble guanylate cyclases. *Proc. Natl. Acad. Sci* 2004;101:12854–12859. [PubMed: 15326296]
- Prabhakar S, Short DB, Scholz NL, Goy MF. Identification of nitric oxide-sensitive and -insensitive forms of cytoplasmic guanylate cyclase. *J. Neurochem* 1997;69:1650–1660. [PubMed: 9326294]
- Regulski M, Stasiv Y, Tully T, Enikolopov G. Essential function of nitric oxide synthase in *Drosophila*. *Curr. Biol* 2004;14:R881–R882. [PubMed: 15498477]
- Robertson HM, Warr CG, Carlson JR. Molecular evolution of the insect chemoreceptor gene molecular family in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 2003;100:14537–14542. [PubMed: 14608037]

- Russworm M, Behrends S, Harteneck C, Koesling D. Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. *Biochem. J* 1998;335:125–130. [PubMed: 9742221]
- Russworm M, Wittau N, Koesling D. Guanylyl cyclase/PSD95 interaction: targeting of the NO sensitive $\alpha 2/\beta 1$ guanylyl cyclase to synaptic membranes. *J. Biol. Chem* 2001;276:44647–44652. [PubMed: 11572861]
- Schaap P. Guanylyl cyclases across the tree of life. *Front Biosci* 2005;10:1485–1498. [PubMed: 15769639]
- Schmidt PM, Schramm M, Schröder H, Wunder F, Stasch JP. Identification of residues crucially involved in the binding of the heme moiety of sGC. *J. Biol. Chem* 2004;279:3025–3032. [PubMed: 14570894]
- Shah S, Hyde DR. Two *Drosophila* genes that encode the α and β subunits of the brain sGC. *J. Biol. Chem* 1995;270:15368–15376. [PubMed: 7797526]
- Sharma VS, Magde D. Activation of soluble guanylate cyclase by carbon monoxide and nitric oxide: a mechanistic model. *Methods* 1999;19:494–505. [PubMed: 10581149]
- Stone JR, Marletta MA. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochem* 1994;33:5636–5640. [PubMed: 7910035]
- Sweeney ST, Kendal B, Keane J, Niemann H, O'Kane CJ. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral effects. *Neuron* 1995;14:341–351. [PubMed: 7857643]
- Truman JW, De Vente J, Ball EE. Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects. *Development* 1996;122:3949–3958. [PubMed: 9012515]
- Vermehren A, Langlais KK, Steward JA, Morton DB. Atypical sGCs in *Drosophila* may be involved in taste responses. 2005 abstract viewer/itinerary planner, program 295.9, Washington DC. Society for Neuroscience. 2005
- Vermehren A, Langlais KK, Morton DB. Oxygen-sensitive guanylyl cyclases in insects and their potential roles in oxygen detection and in feeding behaviors. *J. Insect Physiol.* 2006(In Press).
- Wingrove JA, O'Farrell PH. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. *Cell* 1999;98:105–114. [PubMed: 10412985]
- Wong SK-F, Ma C-P, Foster DC, Chen A-Y, Garbers DL. The guanylyl cyclase-A receptor transduces an atrial natriuretic peptide/ATP activation signal in the absence of other proteins. *J. Biol. Chem* 1995;270:30818–30822. [PubMed: 8530525]
- Wright JW, Schwinof KM, Snyder MA, Copenhaver PF. A delayed role for nitric oxide-sensitive guanylate cyclases in a migratory population of embryonic neurons. *Dev. Biol* 1998;204:15–33. [PubMed: 9851840]
- Yuen PST, Potter LR, Garbers DL. A new form of guanylyl cyclase is preferentially expressed in rat kidney. *Biochem* 1990;29:10872–10878. [PubMed: 1980215]
- Zhao Y, Schelvis J, Babcock G, Marletta MA. Identification of the histidine 105 in the $\beta 1$ subunit of soluble guanylate cyclase as the heme proximal ligand. *Biochem* 1998;37:4502–4509. [PubMed: 9521770]

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Figure 1.

Schematic representation of the functional domains of select mammalian and *Drosophila* sGCs. All sGCs contain a regulatory and a catalytic domain whereas only Gyc-88E and the mammalian $\beta 2$ subunits contain an extended C-terminal domain. In the regulatory domain, *Drosophila* Gyc β -100B and Gyc-89Da/b contain additional sequences at the positions shown. Also shown are the locations of residues necessary for specific biochemical properties. In the regulatory domain these include His104 and the YXS/TXR motif in all β and β -like subunits; Cys78 and Cys214 in the conventional β subunits and mammalian $\beta 2$ and Y140 in the *Drosophila* atypical sGCs. In the catalytic domain the residues that interact with the GTP substrate are shown. Note there are a different set of residues in the conventional α and β subunits whereas the homodimeric subunits, mammalian $\beta 2$ and Gyc-88E, contain both sets and Gyc-89Da/b contains the same set as the α subunits.

Summary of the conventional and atypical sGCs in mammals, insects and *C. elegans*. Assignment as conventional or atypical sGCs is based on sequence similarity and biochemical properties (Morton 2004a). GCs in the same column represent orthologous sequences – the columns within the atypical sGC family are based on the phylogenetic analysis performed by Fitzpatrick et al. (2006).

Table 1

	Conventional sGCs			Atypical sGCs	
	$\alpha 1$	$\alpha 2$	$\beta 1$	$\beta 2$	
Mammals					
Insects					
<i>Manduca</i>	MsGC- $\alpha 1$		MsGC- $\beta 1$		MsGC- $\beta 3$
<i>Drosophila</i>	Gyc α -99B		Gyc β -100B		Gyc-88E Gyc-89Da Gyc-89Db
<i>C. elegans</i>				GCY-32 GCY-34 GCY-35 GCY-36 GCY-37	GCY-31 GCY-33