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Study of cGMP regulation in Drosophila melanogaster and its importance in identification of novel druggable targets of Diarrhea in humans

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Abstract— **This article puts forward a detailed study of the various genes that are upregulated or downregulated under specific stress conditions in the Drosophila gut. Drosophila melanogaster, the fruit fly, has been used as an experimental model for elucidation of many novel drug delivery pathways in humans due to 70% similarity between the Drosophila and human genome, as shown by BLAST results. The unique localization of the mammalian membrane or receptor guanylyl cyclase, GC-C at the luminal face of the intestine and its physiological roles recently revealed this receptor as a novel druggable target for the treatment of diarrhea, chronic constipation, and maybe in the prevention and therapy of colorectal cancer. Diarrhea is the third leading cause of childhood mortality in India. Detailed study of molecular mechanisms and pathways functional during diarrheal diseases, the genes responsible for it and preventive and control strategies need to be reviewed for better planning and organization of health services.**

Index Terms— **guanylyl cyclase, Drosophila melanogaster, diarrhea, colorectal cancer.**

I. INTRODUCTION

The concept of secondary messengers was established in 1957 by Sutherland, Rall and Berthet, that led to the discovery of intracellular messengers, such as guanosine 3'5' cyclic monophosphate (cGMP) (Ashman *et al*., 1963). Since that time many studies in a wide variety of tissues and organisms have demonstrated the crucial role that cGMP plays in many physiological processes. A majority of studies in vertebrate models have elucidated the physiological roles of cyclic GM such as changes in cGMP levels affect olfaction (Breer and Shepherd 1993; Kroner et al. 1996), taste (Rosenzweig et al. 1999; Krizhanovsky et al. 2000), the regulation of gene expression, and the activation of immediate early genes (Haby *et al*., 1994). Quite a few studies like the first identification of a cGMP-dependent protein kinase (PKG) (Kuo and Greengard, 1970), isolation of the first nitric oxide (NO)-insensitive soluble guanylyl cyclases (GCs) (Nighorm *et al*., 1999; Simpson *et al*., 1999), and the identification that a naturally occurring polymorphism responsible for a foraging phenotype was due to mutations in a PKG gene (Osborne *et al*., 1997), were all done in the model organism *Drosophila melanogaster*. All these studies have provided information about the various genes that are regulated in response to cGMP and the downstream effects on signaling cascades induced upon stress, injury and other biological processes that are regulated by cyclic nucleotides.

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1. cGMP Regulation

1. 1. Source of Intracellular cGMP:

The enzyme guanylyl cyclase (GC) catalyzes the synthesis of cGMP from guanosine triphosphate (GTP) and the enzyme phosphodiesterases (PDEs) hydrolyses cGMP to guanosine monophosphate (GMP) to maintain the balance of intracellular cGMP (Morton *et al*., 2002).

1. 2.Cellular components that regulate cGMP concentration:

1.2.1. Guanylyl Cyclases:

Among the various classes of GCs, the two major classes identified in mammals are receptor GCs (membrane-bound) and soluble GCs (cytoplasmic) (Tremblay *et al.,* 1988).

1.2.2. Receptor GCs:

Sequence analysis of the *Drosophila* genome using BLAST has revealed five genetic loci that are predicted to code for receptor GCs, as compared to vertebrates where seven isoforms have been identified (Morton *et al*., 2004). In rats, they have been named as GC-A to GC-G (Garbers and Lowe, 1994; Füllet *et al*., 1995; Lowe *et al*., 1995; Schulz *et al*., 1998).

Morton *et al*., 2004 study showed that compared to the amino acid sequences of other insect GCs, *Drosophila* receptor GCs possess the same molecular features like-a variable extracellular domain, a single transmembrane domain, an ATP binding/protein kinase-like domain, a highly conserved dimerization and catalytic domains. This probably indicated that these rGCs could be functionally equivalent to mammalian rGCs.

The expression pattern of two *Drosophila* receptor GCs has been reported. Gyc32E is primarily expressed during oogenesis in the ovarioles (Gigliotti *et al*., 1993), whereas the expression of Gyc76C is observed in various tissues, including the optic lobe, the central brain, the thoracic ganglia, the digestive tract, oocytes and muscles (Liu *et al*., 1995; McNeil *et al*., 1995). It would be interesting to explore the expression pattern of the remaining three rGCs in flies.

1.2.3. Receptor like GCs:

The expression of receptor-like guanylyl cyclases was first studied in *Manduca sexta* (Simpson *et al*., 1999). CG5719 and CG9783 are receptor like GCs identified in the *Drosophila* genome that have only a catalytic domain and a dimerization domain as compared to receptor GCs, but lack the variable extracellular domain, the single transmembrane domain and an ATP binding/protein kinase-like domain. As they lack the key domains for cGMP production, these GCs might be regulatory cytoplasmic proteins that may participate in the cGMP pathway.

In the Morton *et al*., 2004 study, a phylogenetic dendogram revealed that the *Drosophila* Gyc32E gene is closely related

to the Rat GC-B gene and also has an evolutionary relationship with the Rat GC-A, both of which are a receptors for the peptide hormones called the atrial natriuretic peptide (ANP). Similarly, the *Drosophila* CG4224 and CG3216 are evolutionarily related to the Rat GC-C that binds the ligands guanylin, uroguanylin and heat stable enterotoxin receptors. The endogenous ligands for the *Drosophila* GC-C homologs remain unknown.

1.2.4 Ligands and Activators:

The crustacean hyperglycemic hormone (CHH), an invertebrate peptide is related to a family of peptides in insects that can activate rGCs. CLUSTALW analysis of CG13586 from *Drosophila* with the insect CHH-B revealed similarity at the nucleotide level. In the predicted *Drosophila* sequence, there is an insertion of 39 residues that is absent in the insect CHH-B sequence.

The other known activators are intracellular calcium regulated GC-activating proteins (GCAPs) that activate the mammalian retinal GCs, GC-E and GC-F (Palczewski *et al*., 2000) under low concentrations of calcium. Certain genes identified in the *Drosophila* genome include frequenin (Frq) (Pongs *el al*., 1993), neurocalcin (Nca) (Teng *et al*., 1994) and three other genes CG5744, CG7646 and CG5890 encode peptides that are similar to GCAP in other insect and GCAP-I like proteins of mouse.

1.2.5 Phosphodiesterases-

cGMP hydrolyzing phosphodiesterases (PDE) encoding genes discovered in *Drosophila* are CG1627 andCG3765 that are most similar to PDE9 family members of mammals. Also, the genes CG14940 (related to the PDE1family (calcium/calmodulin activated PDEs), CG8729 (which appears to be a PDE11 family member) and CG10231 (related to PDE5) hydrolyze both cGMP and cAMP as these have a dual specificity for these cyclic nucleotides.

1.2.5.1. GAF regulatory domains of PDE5

In general, many PDE families contain a GAF regulatory domain (named after the cGMP-binding PDEs, the *Anabaena* adenylyl cyclase and the fhlA gene), which forms allosteric binding sites for cGMP (Soderling and Beavo, 2000).These domains have been studied in most detail for PDE5, where they appear to be involved in a negative feedback loop regulating intracellular cGMP levels. PDE5 also contains two cGMP binding sites that are required for its phosphorylation by both cAMP-dependent protein kinases (PKA) and cGMP-dependent protein kinases (PKG) (Turko *et al.,* 1998b), and this positively increases the catalytic activity of PDE5 (Corbin *et al.,* 2000).

In the Morton *et al*. 2004 study, PROSITE analysis revealed that both CG10231 and CG8279 contain two predicted GAF domains. In addition, a serine in a similar position to the ser92 is phosphorylated in PDE5 is conserved in both these *Drosophila* genes.

1.3. Regulators of cGMP Functions:

1.3.1. Molecular Targets

There are three primary protein families that can act as cGMP binding proteins within cells.

- cGMP regulated PDEs
- cGMP-dependent protein kinases (PKG), and
- cGMP-gated ion channels.

All three families are characterized by containing cyclic nucleotide binding sites. The protein kinases PKA and PKG are also activated by cyclic nucleotides (Morton *et al*., 2002)

1.3.2. Protein kinases and substrates

The cGMP- dependent protein kinase of mammals is expressed in smooth muscle (Schultz *et al.,* 1977; Winquist *et al.,* 1984; Kuno *et al.,* 1986; Paul *et al.,* 1987), epithelial cells (de Jonge and van Dommelen, 1981), blood platelets (Waldmann *et al.,* 1987), pericytes (Joyce *et al.,* 1983)and Purkinje cells of the cerebellum (Lohmann *et al.,* 1981; DeCamilli *et al.,* 1984)and comprises of a cyclic nucleotide binding regulatory domain and a kinase domain (Takio *et al.,* 1984). This feature is also possessed by the *Drosophila* cGMP dependent protein kinases.

Kalderon *et al*., 1989studystated that isolation of genes for cGMP- dependent protein kinase in *Drosophila* would not only provide information about the various isoforms of these proteins and their distribution, but also would elucidate the biological functions of these protein kinases in a whole organism when the activity of these enzymes will be genetically regulated. Thus, they reported the characterization of two *Drosophila* cGMP-dependent protein kinase sequences that have similarity to the mammalian bovine lung cGMP- dependent protein kinase (cGK).

The first gene identified in *Drosophila*, DG1 codes for a single protein product that has sequence similarity to the regulatory domain, cGMP binding domain, kinase domain and carboxyl terminal domain of its mammalian bovine homolog with only a difference in the amino terminal dimerization domain (Kalderon *et al*., 1989).

The second gene, DG2, which is also responsible for the food searching behavior in *Drosophila*, after transcription produces three major and several minor gene products. They are DG2;T1, DG2;T3a, DG2;T3b, DG2;cD4, DG2;T2 and DG2;cD5. Kalderon and Rubin 1989 study established a relationship between the gene products of DG2. Sequence homology clearly shows DG2;T2a and DG2;T3b are truncated products of DG2;T1. Also, DG2;cD5 is identical to DG2;T2. Primer extension studies have revealed that DG2;T1, DG2;T2 and DG2;T3b are the 3 major transcripts of the DG2 gene (Kalderon and Rubin, 1989).

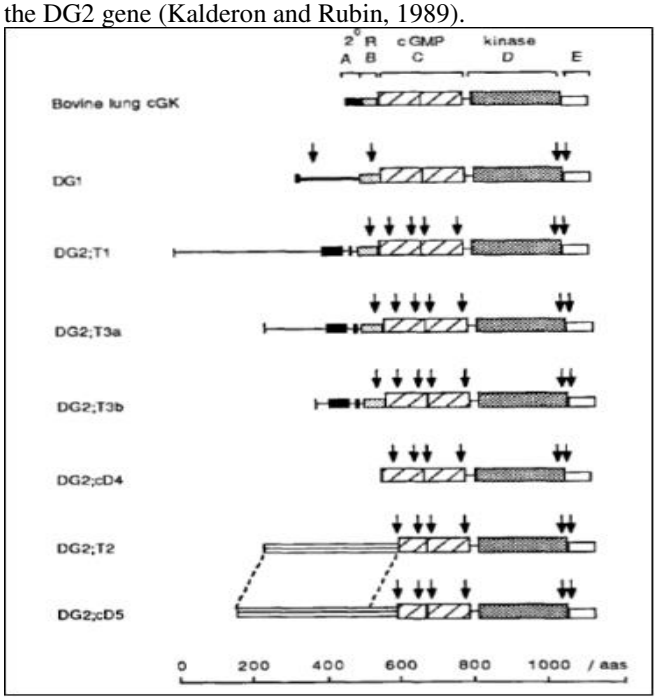

Figure 1.Drosophila cGMP-dependent protein kinases (cGMP-dependent protein kinases in Drosophila (Kalderon et al., Rubin et al., 1989)

The deduced protein product of the Drosophila DG1 gene and the six putative products of the DG2 gene (DG2;T1, DG2;T3a, DG2;T3b,
DG2;cD4, DG2;T2, DG2;cD5) are represented underneath the prototypical
bovine lung cGMP-dependent protein kinase, denoted cGK (Takio et al., 1984) which has been divided into
(A) dimerization(p),
(B) regulatory (R, believed responsible for inhibition of the kinase
domain in the inactive holoenzyme dimer), (C) cGMP binding,
(D)Kinase, and
(E) carboxyl-terminal domains. Segments of similar
amino acid sequence are shaded equivalently.
At the bottom is a scale in amino acids (aa).
Comparison of sequences of the bovine homolog in mammals and *Drosophila* DG1 and DG2 genes reveals that DG1 has 51% and DG2 has 64% similarity to bovine cGMP-binding domain sequences. Similarly, DG1 has 70% and DG2 has 75% sequence identity to the kinase domain of their bovine homolog (Kalderon and Rubin, 1989). Thus, an observed 55% of overall identity between *Drosophila* and mammalian cGKs establishes the possibility of a similar mode of action and overlapping substrate specificity that needs to be explored.
DG1 and DG2 gene products were detected at all stages of *Drosophila* development from embryo to adult, with low DG2 expression in early embryos and high DG2 expression in ring neuron R3 (Chen *et al*., 2012, Renn *et al*., 1999, Martín-Peña *et al*., 2014) ellipsoid body (Zars *et al*., 2000) segmental nerve (Renn *et al*., 1999) mushroom body beta-lobe (Zars *et al*., 2000) and pars intercerebralis of adult heads. In *Drosophila,* DG2 is highly expressed in brain, kidney and intestinal mucosa (Macpherson et al., 20014) and DG1 expression was observed in cell bodies of optic lamina in adult heads, in Sf9 cells of the brain and in the body of adult *Drosophila*, with lower levels of expression in embryos (Foster et al., 1996). In the *Drosophila* MT principal cell, DG1 is expressed in the cytoplasm and DG2 is expressed in the basolateral membrane.

A BLAST analysis reported by Morton *et al*., 2004 shows that the-

- two cloned PKG genes, Pkg21D (also known as DG1) *and foraging (for,* also known as DG2), are most similar to mammalian PKG type I
- The third, CG4839, has similarity to mammalian PKG type II.

The orthologues of PKG substrates have been identified in Drosophila. These include-

- the IP3R(inositol 3-phosphate receptor)- CG1063
- L-type calcium channels- Ca-β(CG42403) and Ca-α1D (CG4894)
- calcium sensitive potassium channels(*Irk1, Irk2, Irk3*,CG10706)
- Phospholipase C and the cystic fibrosis transmembrane conductance regulator(*Mrp4*)

1.3.3. Cyclic nucleotide gated channels

The *Drosophila* genome contains predicted genes for three classes of ion channels that have intracellular cNMP-binding domains (Littleton and Ganetski, 2000). These classes are-

- The cyclic-nucleotide gated ion channel , *CngA*(CG42701*)* that are orthologues of the mammalian retinal cGMP-gated ion channels(Zagotta and Siegelbaum, 1996),
- The I_h channels (CG8585) that are activated by hyper polarization and contain a cNMP-binding site (Ludwig *et al.,* 1998), and expressed in sensory tissues like eye, antennae and auditory organs.
- The *eag* class of voltage activated potassium channels that also contain a cNMP-binding site(Briiggeman *et al.,* 1993).*eag* family of ion channels contains three members eag (CG10952),*eag-like* K^+ *channel (elk*)) (CG5076) and *seizure (sei*)(CG3182) (Littleton and Ganetsky, 2000).

There are four genes in the *Drosophila* genome that appear to code for cyclic nucleotide-gated ion channels (CNGs) (Littleton and Ganetski, 2000). Two have been cloned and partially characterized, which are the *cyclic nucleotide-gated ion channel protein (cng)* (Baumann *et al.,* 1994) and *cng-like (cngl)* (Miyazu *et al*., 2000), while two additional genes, CG3536 and CG17922, have been identified from sequencing data of the *Drosophila* genome.
The *cng* channel is expressed in the antennae and eyes (Baulnann *et al.,* 1994). *cngl* is expressed in antennal lobes, mushroom bodies, neurons in the thoracic ganglia and in muscle fibers (Miyazu *et al*., 2000).

2. Role of cGMP in biological processes of *Drosophila*:

2.1. Sensory physiology in *Drosophila*
The genetic analysis of phototransduction in *Drosophila* has described the signal transduction pathways in great detail and shows that inositol 3-phosphate formation is the primary signal (Montell *et al*., 1999). Certain studies also demonstrated the involvement of cGMP in phototransduction. *Drosophila cng* and I_h channels are expressed in photoreceptors (Baumann *el ell.,* 1994; Marx *et al.,* 1999). Application of exogenous cGMP resulted in light-induced currents in *Drosophila* photoreceptors (Bacigalupo *et al.,* 1995).*Drosophila* also has a large family of G protein-coupled receptors that are expressed in olfactory and gustatory neurons which are receivers of cGMP signals (Clyne *et al.,* 1999; Vosshall *et al*., 1999).

2.2 Neuronal Development
Studies have shown that cGMP also plays a role in neuronal development, particularly in axonal pathfinding and synapse formation (Tessier- Lavigne and Goodman, 1996).
A study by Gibbs and Truman in 1998 has also shown that NOS, NO sensitive soluble GCs and NO-stimulated cGMP immunoreactivity are localized to *Drosophila* photoreceptors.

2.3. Ecdysis
At the end of each molt, insects need to escape from the cuticle of the previous instar. This is achieved by a sequential behavior called ecdysis or eclosion for adult ecdysis (Reynolds, 1980). Cyclic GMP has a major role in this behavior of *Drosophila*. During eclosion in *Drosophila* an increase in cGMP was occasionally observed in tracheae,

which correlated with the release of the eclosion hormone (Baker *et al*., 1999).

2.4 Food- search Behaviour

A genetic analysis of food searching behavior in *Drosophila* has shown that cGMP plays a regulatory role in this behavior (Sokolowski *et al.*, 1998). Two naturally occurring mutations in the DG2 gene are responsible for different alleles of the *foraging (for)* gene that exhibit different behaviors. In the presence of food, larvae with the *sitter* allele stayed somewhat stationary and remained in a single patch of food whereas flies with the rover allele continued to search for additional food by moving between patches of food (Sokolowski *et al*., 1980).The levels of cGMP and hence the PKG activity in the heads of naturally occurring adult sitter flies were lower as compared to naturally occurring rover flies (Osborne *et al.,* 1997).

2.5 Response to Environmental Stresses:

The role of multiple cell-specific signaling pathways that include second messengers such as cyclic GMP (cGMP) and calcium, are capable of modulating tissue, and hence, organismal responses to stress that can be studied in a fluid transporting epithelium, i.e. the Malphigian tubule (MT) of the *Drosophila melanogaster,* which is a fluid-secreting tissue (Dow, 2013). *Drosophila melanogaster* tubules emerge from the hindgut, just behind the junction with the midgut, and constitute a pair of anterior and posterior tubules (Beyenbach *et al*., 2010). The MT consists of two major cell types- principal cells and stellate cells (Dow, 2009). Therefore, insects can occupy diverse environmental niches due to their ability to overcome several environmental stressors like temperature, desiccation, xenobiotic, osmotic and infection stress**.**Morphologically the components involved in cGMP and Calcium signaling are distributed as follows in MT principal cells-

A. The **basolateral membrane** has-

- Receptors for Guanylyl cyclase enzymes called receptor guanylyl cyclase (rGC) (eg. Gyc76c), and
- Ca2+ channels [L-type transient receptor potential (TRP) and TRP-like (TRPL) and cyclic nucleotide-gated (CNG)] on the basolateral membrane.

B. In the **cytosol**-

- The nitric oxide synthase (NOS) gene on stimulation by calcium synthesizes Nitric Oxide (NO), which further stimulates soluble guanylyl cyclases (sGCs) to release cGMP whose downstream effect is the activation of the *Drosophila* phophokinases DG1 and DG2 ((Dow *et al*., 1994a).
- Mitochondria, Golgi complex, Endoplasmic reticulum and peroxisomes play a role in calcium signaling.
- Cytosolic phosphodiesterases like PDE4 and mitochondrial phosphodiesterases PDE8 are present.

C. The **apical membrane** has the following components-

- The vacuolar holoenzyme H+-ATPase (V-ATPase)
- Membrane-bound phosphokinase DG2
- Membrane-bound phosphodiesterases like PDE1, PDE11 and PDE5/6**.**

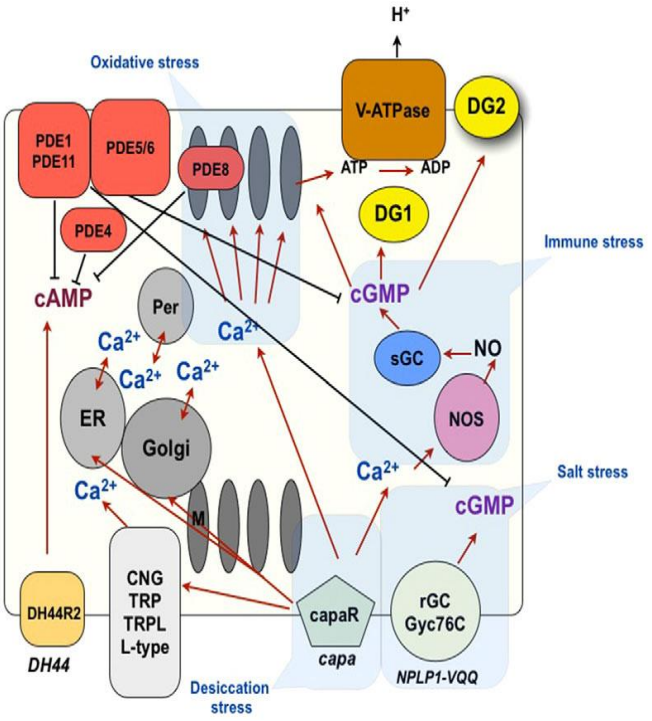

Figure 2: Signaling pathways and components in the *Drosophila melanogaster* Malpighian tubule principal cell in response to specific stresses.
(Cell signaling mechanisms for insect stress tolerance by Shireen A. Davies*, Davies *et al*., Dow *et al*., 2014)

2.5.1. cGMP signaling in response to specific stresses:

Cyclic GMP, produced in response to nitric oxide and natriuretic peptides, is a key regulator of vascular smooth muscle cell contractility, growth, and differentiation (Murthy *et al.*, 2001) and helps in combating hypertension, cardiac hypertrophy, atherosclerosis, and vascular injury/restenosis in mammals, especially humans. cGMP regulates gene expression both positively and negatively at transcriptional as well as at posttranscriptional levels (Pilz *et al*., Casteel *et al*., 2002). In *D. melanogaster* tubules, fluid secretion into the tubule lumen is stimulated by the V-ATPase located on the tubule principal cell apical membrane (Allan *et al*., 2005; Davies *et al*., 1996; Dow, 1999). Transepithelial fluid secretion rates in the tubule main segment are stimulated by cAMP or cGMP (Dow *et al*., 1994b) and the V-ATPase is also thought to be the ultimate target of cyclic nucleotide signaling in the tubule principal cell, because of increased transepithelial potential difference in intact tubules treated with either cAMP or cGMP (Bijelic and O'Donnell, 2005; Davies *et al*., 1995). Recent evidence shows thatcGMP directly increases ATP concentration in tubules. cGMP is transported into principal, but not stellate, cells via ABC transporters (Riegel *et al*., 1999), and application of exogenous cGMP to intact tubules results in increased ATP concentration (Davies *et al*., 2013).

Also, cGMP signaling has been implicated in an increasing a number of physiological processes. Recent work on several cell and tissue systems suggest that the phosphodiesterases that regulate the breakdown of cGMP, as opposed to its synthesis, are pivotal in maintaining the role of cGMP in cellular function (Allershausen *et al*., 2003)

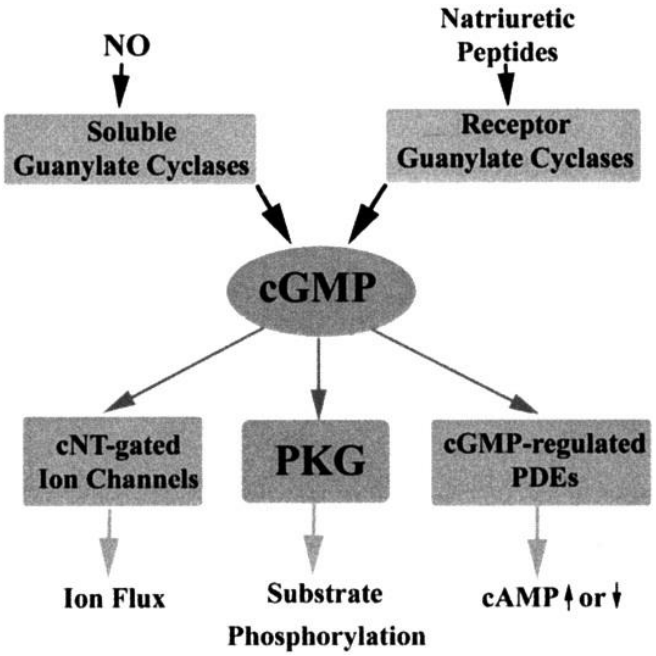

Figure 3. Cyclic GMP signaling pathway Cyclic GMP is synthesized by soluble guanylate cyclases in response to NO or by receptor guanylate cyclases, which are activated by natriuretic peptides. Depending on the cell type, cGMP has several intracellular targets in addition to cGMP-dependent protein kinases (PKG) (Beavo et al., 1995)

2.5.1.1. Immune Stress

MT tissues play a role in immunity and express all components of the Imd (McGettigan *et al*., 2005) and Toll (Chintapalli *et al*., 2007, Robinson *et al*., 2013).Gram negative bacteria induces Imd pathway (Lemaitre *et al*., 1995)and gram positive bacteria or fungi induces Toll immunity pathways in *Drosophila* (Lemaitre *et al*., Reichhart *et al*., Hoffmann *et al*., 1997). Antimicrobial peptides include Diptericin**,** Attacin**,** Cecropin**,** Metchnikowin**,** Defensin and Drosomycin, which may be secreted into either the haemolymph or by the gut (McGettigan *et al*., 2005; Tzou *et al*., 2000). They are produced by the tubules when the *Drosophila* orthologue of the transcription factor Nf-kβ, that is, Relish is activated in a downstream process upon activation of both the Imd and Toll pathway.

C42-Gal4 driver fly lines were generated by inserting Gal4 (a yeast transcription factor) into c42 cells (tubule principal cells) that express GAL4 under the control of nearby genomic enhancers. These flies were crossed with UAS-Relish-His6 transgenic flies (flies in which an upstream activation sequence (UAS) was inserted with the gene encoding Relish, tagged with 6-Histidine). The F1 generation flies were called c42>UAS-Relish-His6 reporter flies. The MTs dissected from these transgenic (both, normal and infected) flies were stained with DAPI (a red dye) and Relish was tagged with FITC (fluorescein isothiocyanate, green dye) (Hedengren *et al*., 1999). It was observed that in the absence of an immune stress, Relish was localized to the basolateral membrane of tubule principal cells. When these cells were treated with exogenous peptidoglycan (PGN) from Gram-negative bacteria, it was observed that Relish localized to the nucleus of tubule principal cells. Treatment with nanomolar concentrations of cGMP before PGN treatment showed reduced nuclear localization of Relish. On increasing the concentration of cGMP gradually in the presence of 5µg/ml PGN, Relish localization increased to the basolateral membrane. These experiments show thatcGMP acts to modulate the expression of the Imd pathway in a dose-dependent manner; whereby low nanomolar concentrations are shown to stimulate activation of Imd pathway and higher micromolar concentrations of cGMP are shown to inhibit the immune stress even in the presence of Gram-negative PGN.

2.5.1.2 Salt stress

The endogenous *D. melanogaster* neuropeptide NPLP1-VQQ is almost exclusively expressed in the brain and thoracico-abdominal ganglion in adult *Drosophila* and in the CNS of *Drosophila* larvae (Baggerman *et al*., 2005). NPLP1-VQQ was shown to be a ligand for the tubule-enriched Gyc76c rGC in Drosophila S2 cells (Overend *et al*., 2012). This ligand-receptor binding stimulated cGMP signaling in the cytosol thus increasing mitochondrial ATP production. Availability of high amounts of ATP substrate stimulated V-ATPase activity that led to increased fluid secretion rates from the tubule principal cells.

NPLP1-VQQ/Gyc76c activation also results in Relish nuclear translocation and increased diptericin expression in tubule principal cells. As Gyc76c/cGMP is a modulator of the Imd pathway (Overend *et al*., 2012), it was likely that NPLP1-VQQ/Gyc76c activation would enhance survival to an immune challenge. However, targeted knockdown of Gyc76c in tubule principal cells showed that flies are not susceptible to immune stress, but are rather when subjected to salt stress (Overend *et al*., 2012). This means that the NPLP-VQQ/Gyc76c axis is important for flies to resist salt stress and not pathogens.

2.5.1. 3 Oxidative stress

Production of reactive oxygen species (ROS) as a byproduct of ATP production by the very metabolically active MT tissue that is packed with mitochondria means that the tubule must be able to detect and cope with excessive ROS and oxidative stress (Terhzaz *et al*., 2006). A neuropeptide stimulus, i.e. Dromecapa-1 and Dromecapa-2 activates the mitochondria of the principal cell that is present in the apical membrane that produce ATP in response to oxidative stress (Terhzaz *et al*., 2006). The hydrolysis of ATP to ADP in the cytosol activates the V-ATPase that causes fluid secretion from the tubules.

Shireen Davies and Julian Dow., 2014, through their combined study discovered that modulation of principal cell inositol 1, 4, 5 triphosphate 3-kinase (IP3K) signaling, using the GAL4/UAS system has been shown to increase ROS production. Therefore, flies in which IP3K-1 is overexpressed in only tubule principal cells are significantly more susceptible to oxidative stress (Davies *et al*., 2014).

D.melanogaster insulin like peptides (DILPs) also plays a major role in tolerating oxidative stress, which have previously been shown to play important roles in neurobiology, nutritional status and ageing (Birse *et al*., 2011; Nassel, 2012; Partridge *et al*., 2011; Söderberg *et al*., 2012). There are 7 DILPs, among which DILP-5 along with the single Insulin Receptor (dINR) was shown to be expressed in tubule principal cells (Söderberg *et al*., 2011). DILP-5 signaling possibly has an inhibitory effect on the activity of the mitochondrial enzyme, Mn^{2+} superoxide dismutase (SOD) since it has been seen that knockdown of SOD decreases oxidative stress tolerance, but knockdown of DILP-5 from

tubule principal cells increases the ability of the cell to tolerate oxidative stress (Davies *et al*., 2014)

2.5.1.4 Desiccation Stress

Drosophila melanogaster has been used effectively in studies of insect desiccation tolerance: excreted water loss rates are reduced in desiccated *D. melanogaster* (Folk and Bradley, 2003). The *D. melanogaster* capa receptor, capaR, is a G-protein coupled receptor and is encoded by gene CG14575 (Iversen *et al*., 2002; Park *et al*., 2002; Terhzaz *et al*., 2012). Capa peptides like Drome Capa-1 (a neuropeptide) stimulate cGMP and Ca^{2+} signaling, (Davies *et al*., 2013) on binding to G-protein coupled capa peptide receptor, CapaR on the basolateral membrane of the tubule principal cell. The intracellular calcium concentrations are regulated by the Golgi complex and ER and the entry of exogenous calcium into the cell is controlled by the various calcium channels expressed on the basolateral membrane. The hike in intracellular calcium concentration stimulates the mitochondria to release ATP. The hydrolysis of ATP to ADP supplies energy to V-ATPase in the apical membrane of the MT principal cells and stimulates the release of a hydrogen ion from these cells. This results in a transepithelial potential difference that causes increased fluid secretion from the principal cells of MT.

Tubules from transgenic principal cell-specific RNAicapaR knockdowns were assessed against wild-type parental flies. In the transgenic flies, in the absence of CapaR, intracellular calcium signaling was absent. This led to decrease in fluid secretion from the cells. Thus, the transgenic flies showed more tolerance to desiccation stress and greater survival rates as compared to wild type parental flies. This means that CapaR negatively regulates resistance to desiccation stress.

II. CONCLUSION

There are certain aspects of intracellular messengers that have not been studied and experimentally proved. Firstly, the expression of 3 out of 5 isoforms of the receptor GCs in *Drosophila* has not been characterized. There are candidate intracellular calcium regulated GC-activating proteins (GCAPs) in *Drosophila*, but their respective receptor GCs, and whether they are positively or negatively regulated by increasing calcium levels, remains to be determined. Two of the predicted *Drosophila* PDE encoding genes, CG1627 and CG3765 have incomplete catalytic domains. This could mean that these genes do not code for PDEs or that the sequence prediction software has incorrectly predicted the intron/exon structure of the gene, and additional coding sequences need to be identified.

There is still much to be learned about the regulation of cell signaling pathways by individual signaling components in insect epithelia. For cGMP signaling, in particular, even in mammalian systems, these are recently discovered processes like role of PDEs in determining cellular levels of cyclic nucleotides (cN), the actions of cN-signaling pathways, regulation of catalytic efficiencies of PDEs in catalyzing the breakdown of cAMP and/or cGMP by various processes including phosphorylation, cN binding to allosteric GAF domains, changes in expression levels, interaction with regulatory or anchoring proteins, and reversible translocation among subcellular compartments(Francis *et al*., 2011), and so further understanding of cGMP signaling in insect stress responses is required that will have new and wide-ranging implications. The cyclic nucleotide (cGMP and cAMP) signaling in response to various stresses has only been studied in *Drosophila* malpighian tubules, but the biological question as to what role these guanylyl cyclases play on infection or other stresses in the rest of the fly needs to be explored. Performing such fundamental work in insects will also reveal new mechanisms in human stress signaling (Becker *et al*., 2010; Jaiswal *et al*., 2012).

III. DISCUSSIONS AND FUTURE PROSPECTS

The unique localization of the mammalian membrane or receptor guanylyl cyclase, GC-C at the luminal face of the intestine and its physiological roles recently revealed this receptor as a novel druggable target for the treatment of diarrhea, chronic constipation, and maybe in the prevention and therapy of colorectal cancer (Hodges *et al*., 2010).Biological assays in a T84 cells, a human colon carcinoma- derived cell line led to the discovery of the ligands of GC-C (Currie *et al*., 1992). Guanylin was purified from the rat jejunum and uroguanylin from urine(Hamra *et al*., 1993; Kita *et al*., 1994). In humans, GC-C is primarily expressed in epithelial cells from the duodenum to the rectum, but is absent in esophagus and stomach. It is located within apical membranes of epithelial cells populating the crypt-villus axis of the small intestine, as well as crypt and surface epithelia of the colon (Birbe *et al.,* 2005).In humans, the intestine plays a role in systemic fluid and electrolyte homeostasis, thus helping to mediate acute conditions of diarrhea. Guanylins regulate intestinal electrolyte and fluid transport and epithelial renewal bybinding to GC-C located in the apical membrane of the epithelial cells. The other roles that cGMP plays in the mammalian small intestine are:increased epithelial cGMP levels stimulate cGK type II, which mediates an activating phosphorylation of CFTR channels. Intriguingly, cGMP can also reduce visceral pain in intestinal diseases.

In *Drosophila,* the role of cGMP signaling in the gut has not yet been studied but there is a lot of similarity in terms of the expression of mammalian GC-C homologs in *Drosophila,* like the Gyc32E and also the regulators of GC-C, for eg: *Mrp4* (mammalian homolog of the CFTR channel). Hence, the main aim of the project is to study the role of cGMP signaling and the various functions of the rGCs in regulating phenotypes, i.e, fluid balance, inhibition of stem cell proliferation and stimulation of innate immunity pathways in *Drosophila*.

The function of receptor guanylyl cyclases (rGCs), especially Gyc76C and its role in cGMP signaling in response to mediating immune stress has been studied in detail in the malphigian tubule of the *Drosophila melanogaster*, but no such effective study has been done in the *Drosophila* gut. Therefore, another study could be to look sat the role of rGCs and their function in cGMP signaling in response to immune stress in the *Drosophila* gut, which could potentially elucidate important cell signaling pathways that would play a role in intestinal homeostasis in humans.

IV. ABBREVIATIONS

- ADP: adenosine diphosphate
- AMP: anti- microbial peptides
- ANP: atrial natriuretic peptide

- ATP: adenosine triphosphate
- cAMP: cyclic adenosine monophosphate
- cGMP: cyclic guanosine monophosphate
- CHH: crustacean hyperglycemic hormone
- CNGs: cyclic nucleotide-gated channels
- cNMP: cyclic nucleotide monophosphate
- CNS: Central nervous system
- DILPs: *Drosophila* insulin- like peptides
- dINR: *Drosophila* insulin- like peptide receptor
- EDTA: Ethylene diamine tetraacetic acid
- GC: guanylyl cyclase
- GCAPs: guanylyl cyclase activating proteins
- IP3: Inositol-3-phosphate
- LB: Luria Bertani broth
- MT: Malphigian tubule
- NO: Nitric oxide
- PCR: Polymerase chain reaction
- PGN: peptidoglycan
- PDEs: phodphodiesterases
- PKA: cAMP- dependent protein kinase
- PKG: cGMP- dependent protein kinase
- rGC: receptor guanylyl cyclase
- ROS: reactive oxygen species
- sGC: soluble guanylyl cyclase
- SI: Septic injury
- UAS: upstream activation sequence
- UC: Unchallenged
- V-ATPase: Vacuolar ATPase

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