COVER PAGE

Principal investigator: Saumya Pant (Dr Barth Grant's Lab)

Address: A307, 604 Allison Road, Piscataway NJ 08855,
Telephone Number: 412-915-7971, Lab: 732-445-7340

Name of Organization/Institution: Rutgers, The State University of New Jersey

Grant Title: Rab-10 and its Effectors in Glutamate receptor recycling
Grant Number: NJCSCR Grant # 06-2915-SCR-E-0

Grant Period Covered by the Report: August 2006-June 2008 (Entire length of Grant)
Date of Submission of the Report: March 2nd, 2010

RECEIVED

MAR - 2 2010

NJ COMMISSION ON
SPINAL CORD RESEARCH

My work funded by the New Jersey Commission on Spinal Cord Research predoctoral fellowship focused on elucidating the mechanisms of endocytic recycling that control glutamate receptor localization at the synapse. Initial injury and ichemia within the spinal cord leads to an uncontrolled release of glutamate from intracellular pools. This is thought to lead to a depletion of ATP and further depolarization of surrounding neurons, and release of additional glutamate at the synapse, ultimately leading to excitotoxic death to a wide domain surrounding the original injury. Prevention of the secondary excitotoxic effects should greatly ameliorate the debilitating effects and leads to better prognosis and recovery. A better understanding of how the cells control the cell surface pool of glutamate receptors is a first step toward interrupting this cycle.

Our lab utilizes the nematode worm *Caenorhabditis elegans* as a model system to study endocytic recycling. Our lab established for the first time the role of the RAB-10 protein in endocytic recycling [1] . Collaborative studies with the Rongo Lab (Rutgers) helped us to establish that the *rab-10* null animals also show defects in the localization of glutamate receptors [2](Figure 1). Our lab found that in the *rab-10* null animals glutamate receptors are unable to recycle to the synaptic membrane and remain trapped in endosomes. In order to elucidate the mechanism of RAB-10 function in endocytic recycling, our lab undertook a yeast two hybrid screen to find RAB-10 interacting proteins (Chen and Grant, unpublished[3]). Among the novel RAB-10 effectors identified from this screen were HUM-2, a type V myosin motor, GCK-2, a homolog of a mammalian Serine/ Threonine MAP4 kinase, and EHBP-1, a predicted actin bundling protein (Figure 2). All of these proteins have been implicated in endocytosis in other systems either directly or through interacting proteins. The Grant lab previously identified another gene, RME-1(receptor mediated endocytosis defective-1) as crucial for endocytic recycling and delivery of transmembrane cargo to the plasma membrane[4]. RME-1 is a homo-oligomerizing ATPase and EH-domain protein. Although initially identified as a factor required to recycle receptors in non-neuronal tissues, RME-1 is now known to also mediate recycling in the nervous system, and in particular has been shown to mediate recycling of postsynaptic glutamate receptors in mammals.

I sought to investigate the molecular mechanisms whereby RME-1, RAB-10 and RAB-10 interacting partners HUM-2, GCK-2 and EHBP-1 may affect glutamate receptor recycling at the synapse, and determining if these proteins play a role in excitotoxic neuronal cell death.

AIMS, PROGRESS and CHALLENGES

AIM 1: To determine how the RAB-10 GTPase regulates the recycling of glutamate receptors in the *C. elegans* nervous system. Yeast two hybrid revealed candidate effectors for the RAB-10 protein (proteins that only bound the active GTP-bound RAB-10 form RAB-10 Q67L but not the inactive GDP bound form, RAB-10 T22N). RAB effectors perform the various functions that are attributed to a given RAB. Thus, it was logical to explore if these candidate RAB-10 effectors contribute to the GLR-1 recycling function of RAB-10.

a)To determine if the RAB-10 interacting proteins are expressed in glutamate receptor expressing neurons .We cloned the various RAB-10 interactors and introduced them into GFP fusion vectors. These transgenes include GFP translational fusions that are driven by the specific gene's own promoter. *C. elegans* transgenic strains for these constructs were obtained by biolistic transformation which is an extremely time consuming and labour intensive process. We were able to determine that all three proteins are broadly expressed in the nervous system (Figure 3). We next cloned hum-2, gck-2 and ehbp-1 as GFP-fusions specifically targeted to the GLR-1 expressing interneurons. These constructs are translational fusions driven by glr-1 promoter driven GFP. The biolistic transformation failed to give any transgenic lines even after two biolistic transformation attempts.

b) **Developing antibodies to HUM-2 and GCK-2 to visualize the endogenous proteins in the nervous system.** Having failed to obtain glr-1 promoter driven GFP lines, we focused on

obtaining antibodies to endogenous GCK-2 and HUM-2 protein that we could utilize this for colocalization, analysis in mutant background and immuno-precipitation experiments. We cloned fragments of HUM-2 including the central coiled-coil region which is a method of choice for unconventional myosins and several fragments of GCK-2 (including the carboxy terminal 100 amino acids, the kinase domain, the C-terminal region excluding the kinase domain, the carboxy terminal 200 amino acid fragment , etc.) into the pGEX-2T expression vector, which is a standard vector of choice for the purpose of expressing glutathione sepharose (GST)-tagged fusion proteins. We utilized these constructs to express and purify GST fusion proteins and worked extensively on standardizing our protein expression system to obtain high yield of proteins through pilot experiments. Unfortunately, we failed to obtain any soluble protein even under these conditions. In fact, most GCK-2 expression plasmids even proved to be lethal/ harmful for harboring cells. Several vector systems including GST fusion backbone of pGEX-2T and pMAL-C4X (for Maltose binding protein fusion) and cell systems such as BL21 magic and Arctic Express cells (Stratagene) which are known to aid the yield of soluble protein by allowing low temperature protein expression in the presence of low temperature expressed chaperone proteins were tried. Facing these issues with solubility of proteins, we finally requested a peptide antibody directed to GCK-2. We affinity purified the serum from two rabbits utilizing standard protocol and purification utilizing the same peptide utilized for immunization. Unfortunately, we found that the antibody was not specific for GCK-2 utilizing immunofluorescence on dissected and fixed worm intestine and gonad tissue as well as by western blot analysis on lysates from WT and *gck-2(-)* worm lysates. Both approaches revealed the presence of non-specific signal in the *gck-2(-)* worms similar to WT worms.

c) ***hum-2* and *gck-2* were established as bonafide regulators of endocytic recycling.**
In our past studies with *rab-10*[1], the mutant caused recycling defects in polarized epithelium with cargo accumulation in early endosomes. In neurons which are similarly highly polarized cells, the GLR-1 protein was found to accumulate in early endosomes in the *rab-10* mutant background. We therefore began our investigation by determining whether similar to *rab-10* mutant, the interacting proteins also similarly affect glutamate receptor localization. We obtained mutants for each of the interacting genes viz. *gck-2, hum-2, cnt-1* and *ehbp-1. cnt-1* and *ehbp-1* related projects became part of another graduate student's thesis and submitted manuscript and their discussion will not be included in this report. The *hum-2(ok596)* mutant strain was obtained from the *C. elegans* gene knockout group at Oklahoma while the *gck-2(tm2537)* deletion mutants was obtained from the Japanese *C. elegans* knockout consortium (courtesy Dr Shohei Mitani). (Figure 3b and 3d). The mutants were obtained after the sib-screening stage and we determined the exact mutation in the gene by PCR analysis and sequencing of the deletion product. Mutagenesis screen based knockouts often harbor background mutations. To counter for this possibility we backcrossed the mutant strains. The backcrosses are performed to re-isolate the mutant through segregation after crossing into the wild type strain. The backcross process was completed to a satisfactory 3X backcrosses. Since our proposal aimed at determining whether recycling of GLR-1 would be affected in mutants for the RAB-10 effectors, we immediately performed pilot studies to determine whether the mutants could affect endocytic recycling in the worm's intestinal polarized epithelium which tissue is our lab's specialty in terms of marker analysis. We were able to conclusively establish that in the *C. elegans* intestine similar to the effect of the *rab-10(-)*, mutants for both interacting proteins, *gck-2(-)* and *hum-2(-),* behave as regulators of endocytic recycling, causing accumulation of GFP labeled recycling cargo such as clathrin independent cargo hTaC (human interleukin receptor chain alpha) and clathrin dependent cargo, hTfR(human Trasferrin receptor) (Figure 4). Therefore, we were able establish these RAB-10 interacting proteins as novel regulators of endocytic recycling.

d) **GLR-1 localization is altered slightly in both *hum-2* and *gck-2* mutant backgrounds.** The glr-1p-glr-1-GFP strain, nuIs25 was introduced into the *hum-2* and *gck-2* mutants to score for glutamate receptor localization. To introduce the GLR-1-GFP fusion reporter into these mutants

we developed intermediate balancer strains to follow the mutation through crosses. These are *dpy-11(-);glr-1p-glr-1-GFP* for *hum-2(-)* and *gck-2(-)* which both map within Chromosome V. We established conditions for analysis of the *rab-10(-);glr-1p-glr-1-GFP* strain to set up imaging and quantitation according to our labs imaging system(at the stage of the proposal, imaging and results for *rab-10(-);glr-1p-glr-1-GFP* were from our collaborating lab and on a different microscope and deconvolution system). Upon careful analysis of the mutant strains and extensive measurement and morphometry analysis performed with the Metamorph software for image analysis and measurement, we found that both *gck-2(-)* and *hum-2(-)* cause a very slight decrease in the size of GLR-1-GFP labeling in interneurons(Figure 5). This is in fact the opposite of results obtained with the *rab-10* mutant which causes an aggregation of glutamate receptors. We were surprised by this result and to follow up on this, we carried out a parallel analysis of the *hum-2* and *gck-2* mutants in the worm's polarized intestinal tissue. We found that similar to our results in glr-1 interneurons, in the intestinal cells also, *gck-2(-)* and *hum-2(-)* caused an accumulation of GFP-RME-1 which is the opposite of the diffusion of RME-1 observed in the *rab-10(-)*(Figure 6). This analysis led us to further dissect changes in endocytic markers in the worm's intestine and we found that *gck-2* and *hum-2* both cause a mislocalization of RAB-10-GFP labeled structures. Additionally, certain other recycling markers are also mislocalized in the *gck-2(-)* and *hum-2(-)* backgrounds (Pant, Grant Manuscript in preparation, included in my Thesis submitted to Rutgers University). We conclude that HUM-2 and GCK-2 are not major players in RAB-10's function as a regulator of Glutamate receptor localization in terms of absence of a GLR-1 aggregation effect in mutants. That said, we still wanted to investigate whether the slight decrease in size of GLR-1-GFP puncta observed in the *hum-2* and *gck-2* mutants correlated with defects in the ability of the mutants to show correct functionality of GLR-1 interneurons. These findings are discussed in the following section.

e) **Behavioral assays to score for defects in GLR-1 Recycling**. **Behavioral assays to score for defects in GLR-1 Recycling**: All the mutants were analyzed by behavioral assays for sensation and effective GLR-1 mediated response. Assays included (i) Spontaneous reversal of worms over five minutes on food free plates, (ii) Backward avoiding motion on gentle nose touch(performed on thinly seeded bacterial lawn), (iii) Chemotaxis assay for avoidance of 200 mM Copper sulfate, (iv) Maze test to reach chemoattractant Diacetyl(1:1000) through a maze of chemo-repellant Copper sulfate.

i) **Spontaneous reversal of animals**. Wild type worms will spontaneously reverse their direction of motion periodically by involving a characteristic bending of the tail and slight curling of the posterior body. This function is a read out for GLR-1 activity in the interneurons. Defects in spontaneous reversal indicate defects in interneuron GLR-1 function e.g. *glr-1(-)* animals and *rab-10(-)* mutants also show a decrease in spontaneous reversal. We found that *hum-2(-), gck-2(-), ehbp-1(-) and cnt-1* mutants show these defects. (Figure 7)

ii)**Response to gentle nose touch to respond by backing motion**. If an eyelash is placed in the path of a forward moving worm and it collides nose on with the eyelash, the spontaneous avoiding/backing movement is a function of GLR-1 activity in the interneurons. *glr-1(-)* and *rab-10(-)* animals show a decreased avoidance response and this was true for *hum-2(-), gck-2(-), ehbp-1(-) and cnt-1* mutants also. (Figure 8)

(iii) **Chemotaxis assay for avoidance of 200 mM Copper sulfate**. Worms respond to high osmotic strength solutions by avoidance response. WT worms do not cross a 200 mM $CuSO_4$ barrier. *glr-1 (-),hum-2(-)and cnt-1* mutants showed a decreased response(Figure 9)

(iv) **Maze test to reach chemo-attractant Diacetyl(1:1000) through a maze of chemo-repellant Copper sulfate.** Worms are repelled by 200 mM copper sulfate and strongly attracted by 1:1000 diluted Diacetyl. If a maze is created such that worms are placed diametrically opposite to Diacetyl but with a maze of copper sulfate lines separting the worms from diacetyl, then the movement of worms to diacetyl through the maze involves complex neuronal circuits and neuron-muscle coordination. We tested for such complex reflex functions utilizing this maze

assay. We similarly scored for this response in *glr-1 (-), rab-10(-), rme-1(-), hum-2(-), gck-2(-), ehbp-1(-) and cnt-1* mutants.(Figure 10)The given results are the average of two experiments performed by me. The data presented in Figure 7 and Figure 8 reveals that both *hum-2* and *gck-2* mutants do exhibit defects in both spontaneous reversal as well as avoidance response to gentle nose touch. This leads us to the conclusion that HUM-2 and GCK-2 contribute to the GLR-1 function in the interneuron circuitry by means of the small alteration in size of GLR-1 positive organelles or perhaps by regulating and fine tuning the rate of GLR-1 recycling. It is also interesting that *ehbp-1(-)* worms are highly compromised in coordinated movement and are very sluggish. In fact, they were not scorable on movement based assays. Additionally, *ehbp-1(-)* worms are lethal beyond adulthood therefore L4 larvae were scored in these assays instead of young adults used for scoring the other strains. The *ehbp-1(-)* phenotype is more severe than the *glr-1(-)* phenotype which may indicate its absolute requirement for other facets of nervous function as well.

f)**GST Pulldown experiments to confirm the RAB-10 interactions of GCK-2 and HUM-2.** We confirmed the yeast two hybrid interaction *in vitro* by performing GST pulldown studies. We generated soluble RAB-10Q67L GTP locked protein since our efforts to generate soluble GCK-22 and HUM-2 had been unsuccessful. Generally soluble RAB proteins are difficult to generate but we tried several different conditions and were able to optimize expression utilizing Arctic express cell protein expression system to obtain average yield of soluble RAB-10 protein. We demonstrated that HA-tagged in vitro transcribed and translated GCK-2 binds strongly to RAB-10 Q67L even in the GST pulldown system.

g) **Narrowing down the RAB-10 binding region of HUM-2 and GCK-2.** Since this project ultimately envisioned elucidating how the RAB-10 and interactor association is important for glutamate receptor recycling, we narrowed down the RAB-10 and interactor binding region by creating systematic deletions from both ends of the clones pulled out from the Yeast Two hybrid screen. We narrowed down the GCK-2 binding region to a 350 amino acid stretch and established that the terminal 25 amino acids of GCK-2 are essential for binding to RAB-10. We also established that the GCK-2 kinase domain may be auto-regulated.(Figure 11)

h) **Elucidating the mechanism of action of RAB-10 and its effectors in receptor recycling.**GCK-2 is a Serine/ Threonine kinase and we wondered if RAB-10 could be a potential substrate for GCK-2. We probed GFP: RAB-10 strains in the background of *gck-2* mutant, GCK-2 over expression and compared these to the wild type GFP- RAB-10 protein expression by Western Blot using anti-GFP antibody. Under the limit of resolution of a SDS-PAGE gel we failed to observe any difference in GFP-RAB-10 specific bands. We wanted to rigorously test this hypothesis by performing an *in vitro* kinase assay testing RAB-10 or HUM-2 or EHBP-1 phosphorylation by GCK-2. We were unable to complete this part of the proposal due to absence of soluble GCK-2 protein for kinase assays.

AIM II:

Excitotoxic cell death is a major contributor to the spread of primary spinal cord injury. We proposed to determine whether loss of endocytic recycling factors can alter the frequency of excitotoxic cell death in neurons through regulation of glutamate receptor recycling. We are testing the possibility that RME-1and RAB-10 may represent therapeutic targets to reduce excitotoxic damage immediately after spinal cord injury. Our hypothesis is that in the absence of these recycling factors glutamate receptors will accumulate intracellularly where they are not active, ameliorating the excitotoxic effects.

a) **Investigation of excitotoxicity (caused by hyper activated Gαs) in the background of reduced endocytic recycling.**nuIs5[Gαs] contains the hyperactivated GTPase defective heterotrimeric G-protein G_s which promotes excitotoxicity and is a well studied model for excitotoxic cell death in neurons. This construct carries a GFP tag for easy visualization of neurons. Upon necrosis cells swell up and later die. The process can be scored by observing necrotic neurons which appear as vacuoles under Normarski microscope. Dead neurons are

phagocytosed and the loss of neurons by excitotoxic cell death can be confirmed using epifluorescent microscopy to count the number of GFP marked neurons surviving to adulthood, and also by loss of GFP marked neurons in the adult relative to control nuIs5[Gαs] animals[5]. We constructed strains carrying *rab-10(-)* and *rme-1(-)* mutations in the background of nuIs5[Gαs]. We scored these strains for excitotoxic cell death effects intrinsic to *rab-10* and *rme-1* mutants. Analysis was performed in both L1 larvae and adult hermaphrodite worms by scoring for death of neurons utilizing the absence of GFP-labeled neurons under the fluorescent microscope as well as vacuolated cell body corpses under Nomarski. We focused on the best characterized assay for the tail PVC neurons (Figure 12). Utilizing both sets of neurons we were able to conclude that neither *rab-10* nor *rme-1* mutant affect cytotoxicity (Figure 13) caused by hyperactivated Gα subunit. Therefore, affecting endocytic recycling does not alter excitotoxic cell death by affecting GLR-1 recycling and reducing GLR-1 at the cell surface.

AIM III:

Our lab has been actively investigating how actin cytoskeleton regulation affects endocytosis and endocytic recycling in particular. In mammals, actin regulatory proteins Syndapin and Amphiphysin are important regulators of synaptic function by affecting endocytosis at the neuronal synapse. We wanted to investigate glutamate receptor function in the background of mutations in these genes. Behavioral assays for GLR-1 functionality were performed in the *amph-1(-)* and *sdpn-1(-)* strains.

a) **Behavioral assays to score for defects in GLR-1 Recycling**. The *amph-1(-)* and *sdpn-1(-)* mutants were analyzed by behavioral assays for sensation and effective GLR-1 mediated response. Assays included (i) Spontaneous reversal of worms over five minutes on food free plates, (ii) Backward avoiding motion on gentle nose touch(performed on thinly seeded bacterial lawn), (iii) Chemotaxis assay for avoidance of 200 mM Copper sulfate, (iv) Maze test to reach chemo attractant Diacetyl (1:1000) through a maze of chemorepellant Copper sulfate. We performed the tests on wild type N2, *amph-1(-)* and *sdpn-1(-)* mutants. (Figures 14-17)

We compared the results for wild type (N2), *amph-1(-)* and *sdpn-1(-)* animals. Analysis was performed twice and the given results are the average of two experiments. The data presented in Figures 14 and Figure 15 reveal that neither *amph-1* nor *sdpn-1* mutants exhibit defects in spontaneous reversal or avoidance response to gentle nose touch. This leads us to the conclusion that AMPH-1 and SDPN-1 do not contribute to GLR-1 function in the interneuron.

b)**Analysis of AMPH-1 function in the worm's polarized epithelial tissue.** Though we were unable to detect defects in GLR-1 localization in interneurons we proceeded with analysis of AMPH-1 function in the worm's polarized intestinal tissue. We were able to establish AMPH-1 as a novel regulator of endocytic recycling in the worm's intestine. These results were recently published in Nature Cell Biology in the December 2009 issue[6] and the manuscript is included as an attachment to this narrative.

CONCLUSIONS.

We have been able to establish that novel RAB-10 interacting proteins, the Serine/Threonine MAP4K GCK-2(germinal center kinase) as well as a myosin type V motor HUM-2 can alter the GLR-1 mediated sensory response in the *C. elegans* interneuron. We found that in the GLR-1 interneuron as well as polarized intestinal epithelia, *gck-2(-)* and *hum-2(-)* animals alter the endocytic recycling markers and GLR-1 localization differently from RAB-10. While *rab-10(-)* causes an aggregation of GLR-1, *gck-2* and *hum-2(-)* animals both cause a reduction in size of GLR-1 puncta. This implicates GCK-2 and HUM-2 along with RAB-10 as potential therapeutic targets in the spinal cord regeneration pathway. Future studies might involve a genomics based investigation on the level of these genes upon ischemic injury. We also narrowed down the RAB-10 binding regions of GCK-2 and HUM-2. Furthermore, we found that affecting endocytic recycling does not alter the rate of excitotoxic cell death by altering cell surface levels of GLR-1.

PERSPECTIVE.

The NJCSCR Grant enabled us to start a brand new field of neuronal studies in our lab which had otherwise concentrated mainly on non-neuronal tissues. As a student investigator, with the help and encouragement from my mentor Dr Barth Grant and his facilitation of my collaboration with various labs at Rutgers, I was able to learn first hand the complex behavioral assays shown here. In turn, I was able to train an undergraduate in my lab and we plan to pursue these studies going forward. As we pursue novel endocytic proteins in the lab, we will also investigate the GLR-1 profile utilizing these same genetic and sensory assays as part of the mutant analysis. Interestingly, while we were pursuing these results with the behavioral chemotaxis assays we also became increasingly aware of the role of cilia and membrane traffic in the cilia as an inter-related topic and this gave rise to a whole new project dealing with cilia membrane traffic regulators as a collaboration with another lab here at Rutgers. We are especially very grateful to the support from this grant which aided that part of the Amphiphysin project performed while this grant was active. Accordingly, NJCSCR was acknowledged in the publication as well as all the talks and posters resulting from this work. In all, I delivered two talks on this research and presented four posters relating to AMPH-1's recycling function. These are listed at the end of this narrative.

PUBLICATIONS.

Saumya Pant, Mahak Sharma, Kruti Patel, Steve Caplan, Chavela Carr, Barth D. Grant. **Physical and functional association of RME-1/Ehd and AMPH-1/Amphiphysin/Bin1 in endocytic recycling.** Nature Cell Biology **11**, 1399 - 1410 (2009).

First author manuscript #2 detailing results on the RAB-10 GCK-2 interaction and its role in recycling is in preparation.

CONFERENCE ABSTRACTS.

Gordon Research Conference on Molecular Membrane Traffic (2009) Amp (h) ing Up Recycling: AMPH-1 is a Novel Regulator of RME-1 mediated Endocytic Recycling. *Saumya Pant*, Mahak Sharma, Kruti Patel, Steve Caplan, Chavela Carr, Barth D. Grant.

***Talk at International Worm Meeting (2009)** Amp (h) ing Up Recycling: AMPH-1 is a Novel Regulator of RME-1 mediated Endocytic Recycling. *Saumya Pant*, Mahak Sharma, Kruti Patel, Steve Caplan, Chavela Carr, Barth D. Grant.

Rutgers Joint Molecular Biosciences Graduate Students' Association Annual Research Presentation(2009). AMPH-1 is a Novel Regulator of RME-1 mediated Endocytic Recycling. *Saumya Pant*, Mahak Sharma, Kruti Patel, Steve Caplan, Chavela Carr, Barth D. Grant. (* Second Prize in Poster Category)

American Society for Cell Biology Meeting (2008). AMPH-1 is a Novel Regulator of RME-1 mediated Endocytic Recycling. *Saumya Pant*, Mahak Sharma, Kruti Patel, Steve Caplan, Chavela Carr, Barth D. Grant.

***Talk at Greater New York Area Worm Meeting (2008)** AMPH-1 is a Novel Regulator of RME-1 mediated Endocytic Recycling. *Saumya Pant*, Barth D. Grant.

Figure 1. Glutamate receptor localization is disrupted in *rab-10(-)* animals. GLR-1 accumulates in enlarged endosomes in rab-10 mutants. GLR-1::GFP fluorescence in wildtype and rab-10 mutant backgrounds is shown. Data was recorded on a Zeiss axiovert laser scanning confocal microscope.

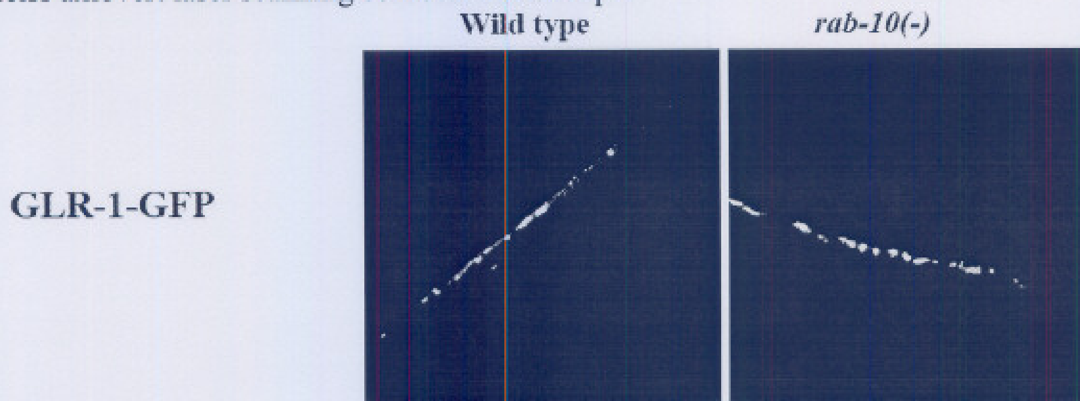
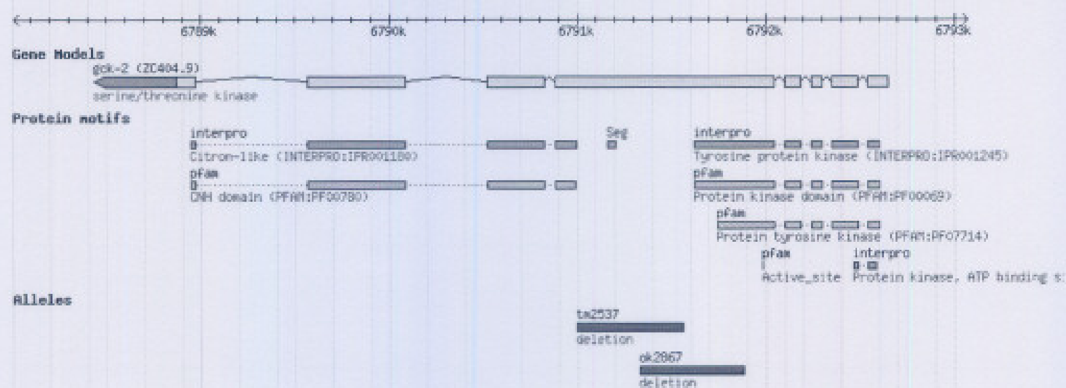


Figure 2. A domain diagram for *C. elegans* GCK-2 and HUM-2 depicting the various domains identified in the proteins by homology searches.

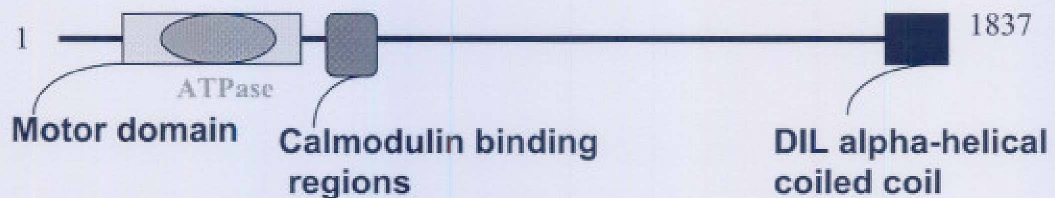
(a) The full length GCK-2 protein(AA 1-829) is shown as a cartoon with various domains demarcated. GCK-2 belongs to the germinal center kinase protein family (GCK). It is a subgroup of the Ste20 protein family. GCK family proteins have roles as MAP4K pathway proteins often serving as MAP4Kkinase3 kinases. The GCK protein family is classified into 8 sub-families and C. elegans GCK-2 is a member of Group 1 GCK proteins. This subfamily is characterized by a highly variable intermediate region with several proline-rich motifs (with potential SH3-binding sites), a citron homology domain (CNH) and a conserved C-terminal extension which has been implicated in membrane insertion and protein dimerization. (b) Gene diagram for gck-2 gene depicting the exon-intron boundaries and position of the 565 base pair deletion in allele tm2537 at the end of the kinase domain(please note that the direction of the gene diagram runs opposite to the protein domain diagram shown in a) (Adapted from Wormbase).

a

b.



c. **The full length HUM-2 protein is shown as a cartoon with various domains demarcated.**



d. **Gene diagram for hum-2 gene depicting the exon-intron boundaries and position of the deletion in allele *ok596*.**

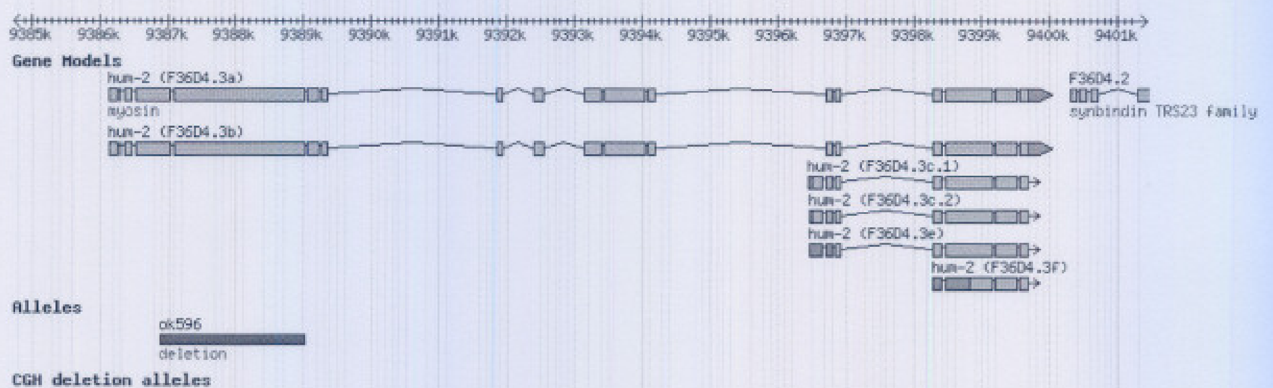


Figure 3: GCK-2, HUM-2 and EHBP-1 are Broadly Expressed in *C. elegans* Nervous Tissue. GFP tagged translational fusions of each effector are highly expressed in the nervous tissue(p refers to promoter eg gck-2p =gck-2's promoter)

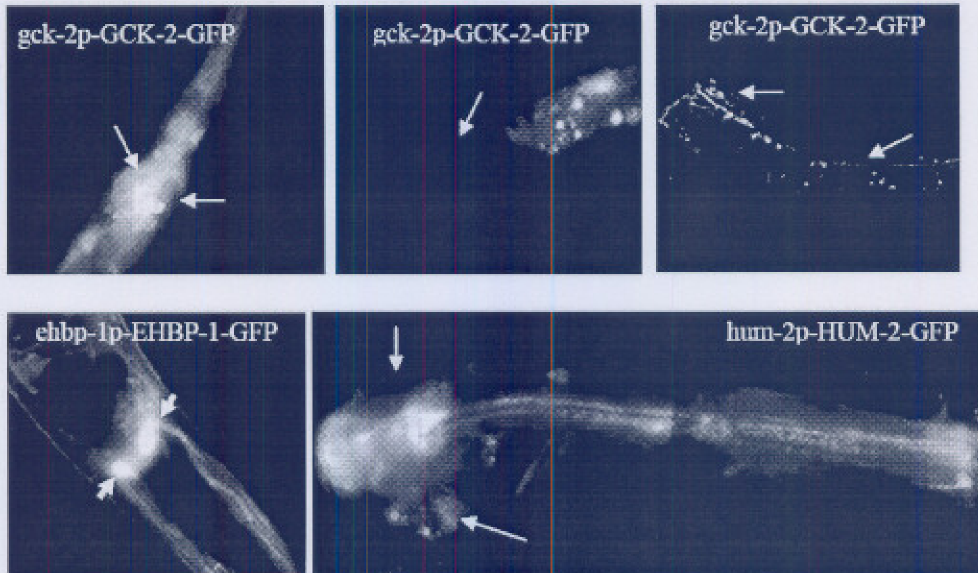


Figure 4: *C. elegans gck-2* and *hum-2* mutants display abnormal trafficking of recycling cargo.

(**a-b**) hTAC-GFP, a cargo protein internalized independently of clathrin, accumulates intracellularly in *gck-2* mutants. Arrowheads indicate punctate and tubular hTAC-GFP signal in the intestine.(**c-d**) hTAC-GFP, accumulates intracellularly in *hum-2* mutants. (**e**)Quantification of hTAC-GFP signal in the intestine of living wild-type and *hum-2* mutant animals with respect to average number of labeled structures per unit area.

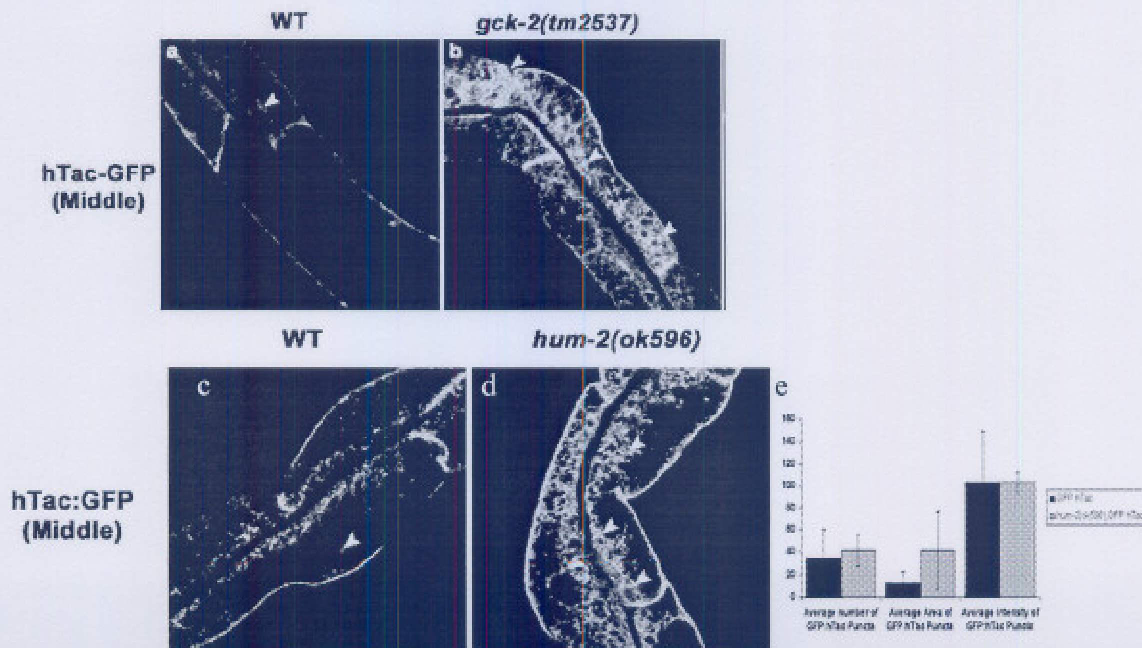
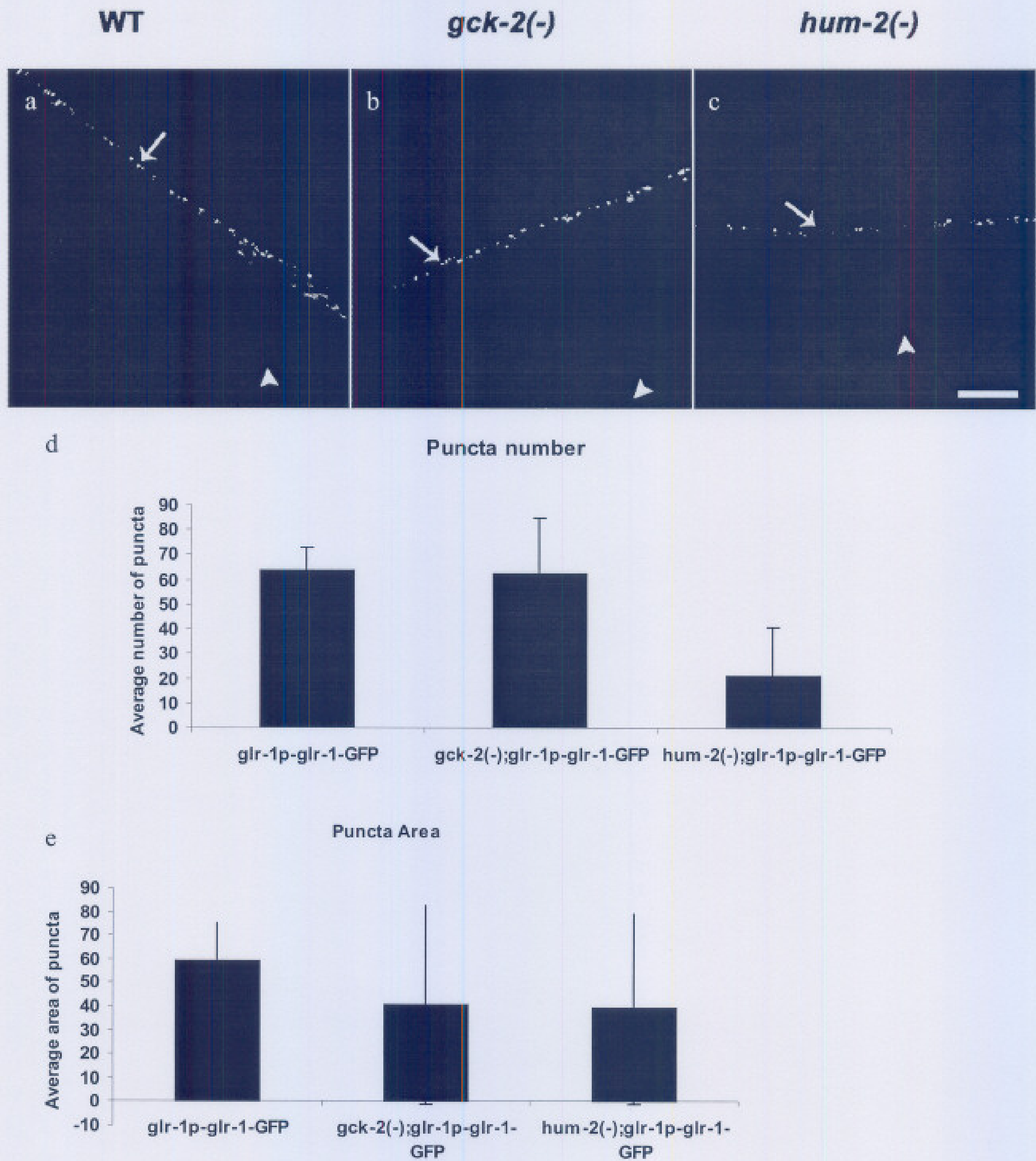


Figure 5: Mutation of the Rab-10 Effectors GCK-2 and HUM-2 cause a slight mislocalization of GLR-1 GFP in the ventral nerve cord. (a-c)In *gck-2(-)* and *hum-2(-)* GLR-1-GFP(white arrows) localizes to slightly smaller puncta over the nerve cord. (Arrowheads indicate worm body autofluorescence.)(d-f)Quantification of glr-1p-GLR-1-GFP in terms of puncta characteristics.(n=6 for each genotype).



f

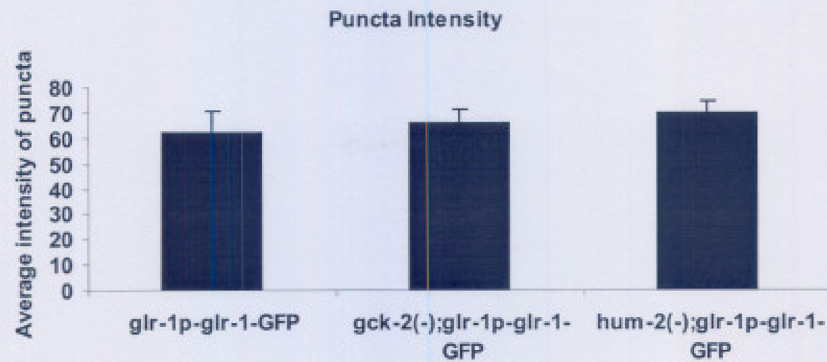


Figure 6. Rab-10 Effectors Disrupt the RME-1 marked Recycling Endosome in Polarized tissue. Confocal micrographs of GFP-RME-1 in wild type, *gck-2(-)* and *hum-2(-)* backgrounds. GFP-RME-1 accumulates in enlarged structures in both *gck-2(-)* and *hum-2(-)*.(n~10 worms for each group).

GFP-RME-1 in polarized cells

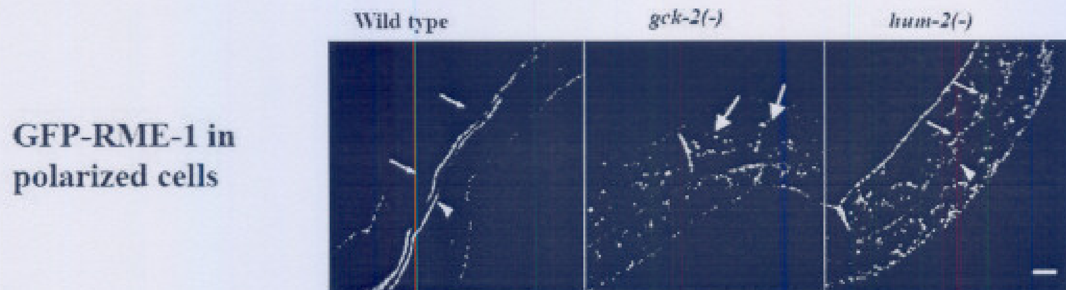


Figure 7:Classical Behavioral Assays to define sensory response defects in RAB-10 effector mutants. *hum-2(-), gck-2(-), cnt-1(-)* each display Glutamate receptor activity defective characteristics such as decrease of spontaneous reversal per minute. (*ehbp-1* mutants are extremely slow and uncoordinated which made locomotion based assays difficult to score. These worms were excluded from all the current assays). Worms were allowed to adjust to a food free agar plate for five minutes and then their spontaneous reversal motions were counted over five minutes. The data is the average of two experiments and error bars depict $\pm$ standard deviation.

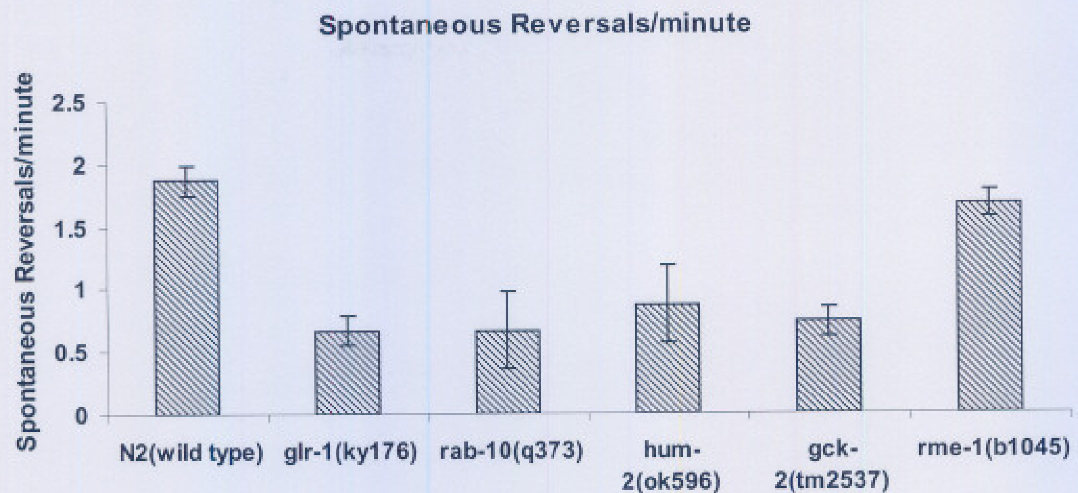


Figure 8:Classical Behavioral Assays to define sensory response defects in RAB-10 effector mutants. *hum-2(-), gck-2(-), cnt-1(-)* each display Glutamate receptor activity defective characteristics such as decreased response to gentle nose touch. (ehbp-1 mutants are extremely slow and uncoordinated which made locomotion based assays difficult to score. These worms were excluded from this assay). Worms were placed on a plate thinly seeded with bacteria. Backward avoiding response was counted over 10 consecutive gentle touches on the nose. A fine hair was placed in front of the worm moving forward so it collided head on with it.(n=10)

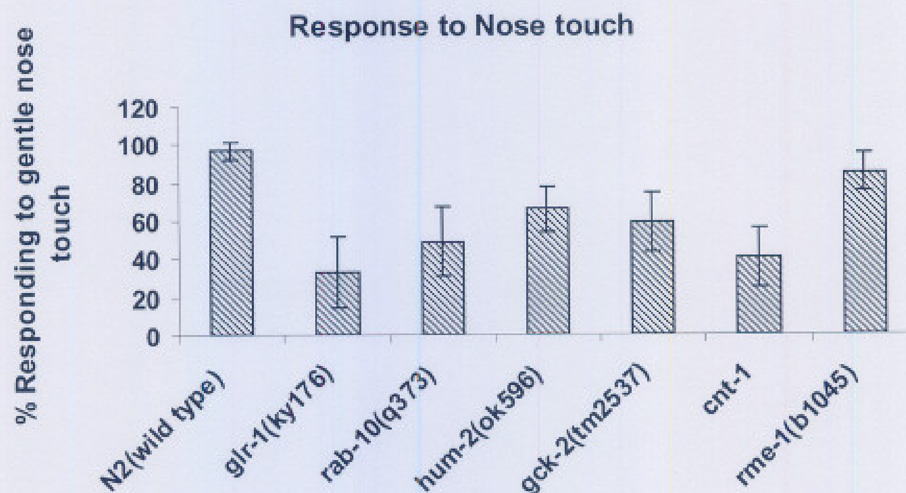


Figure 9:Classical Behavioral Assays to define sensory response defects in RAB-10 effector mutants. We wanted to assay if *rab-10(-)* and its effector protein mutants such as *hum-2(-)* and *gck-2(-)* worms are also defective in the perception of sensory stimuli which is a feature that can affect GLR-1 defect assays.(ehbp-1 mutants are extremely slow and uncoordinated which made locomotion based assays difficult to score. These worms were excluded from the current assay). Worms were placed in the center of a 2 cm diameter ring of 200mM Copper sulfate on a 10 cm food free agar plate. After one hour it was scored how many worms were repelled by the CuSO4 and stayed within the ring instead of crossing the repellant.(n=10)

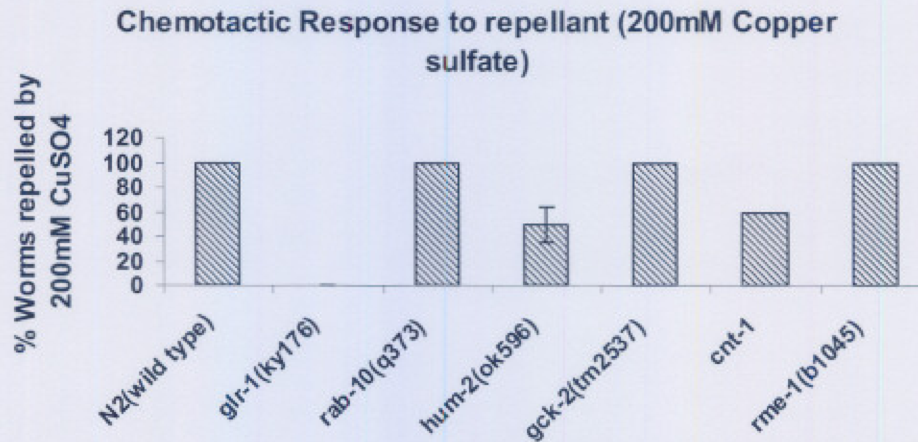
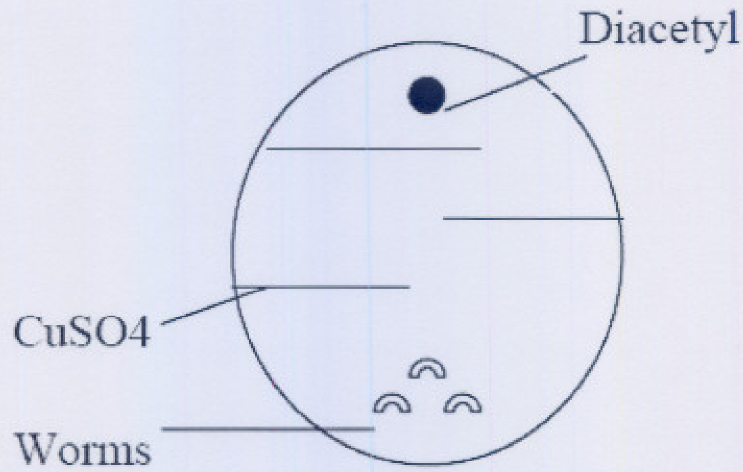


Figure 10:Classical Behavioral Assays to define sensory response defects in RAB-10 effector mutants. We wanted to assay if *rab-10(-)* and its effector protein mutants such as *hum-2(-)* and *gck-2(-)* worms are also defective in the complex locomotory behavior which is a feature that can affect GLR-1 defect assays. (*ehbp-1* mutants are extremely slow and uncoordinated which made locomotion based assays difficult to score. These worms were excluded from the current assay). A maze was created as shown below by staggering 3 lines of repellant Copper sulfate and placing attractant Diacetyl(diluted 1:1000) at one pole of a food free 10 cm agar plate. Worms were placed opposite pole to attractant. Worms reaching the diacetyl after 2 hours were scored. (n~15)

Design of the Maze assay



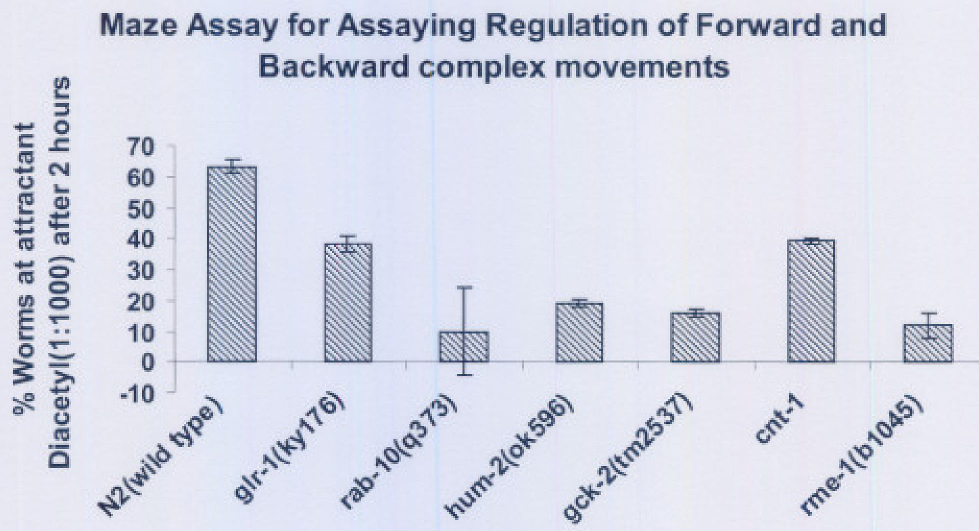


FIGURE 11: Narrowing down the RAB-10 binding region of GCK-2 and HUM-2 utilizing yeast two hybrid.

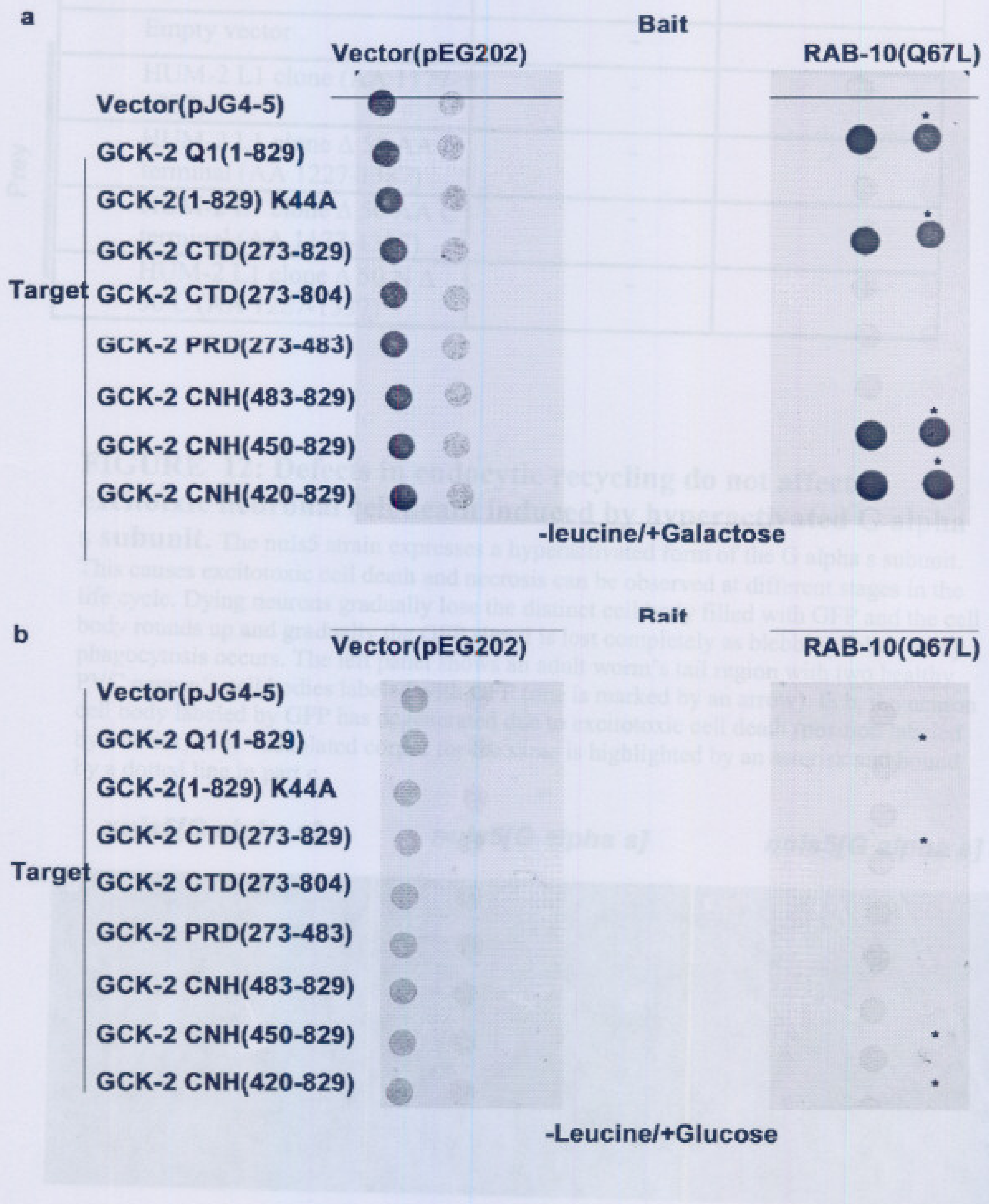


FIGURE 13:GαS induced cytotoxicity in L1 larvae and young adult worms is not altered significantly by blocking endocytic recycling of glutamate receptors.

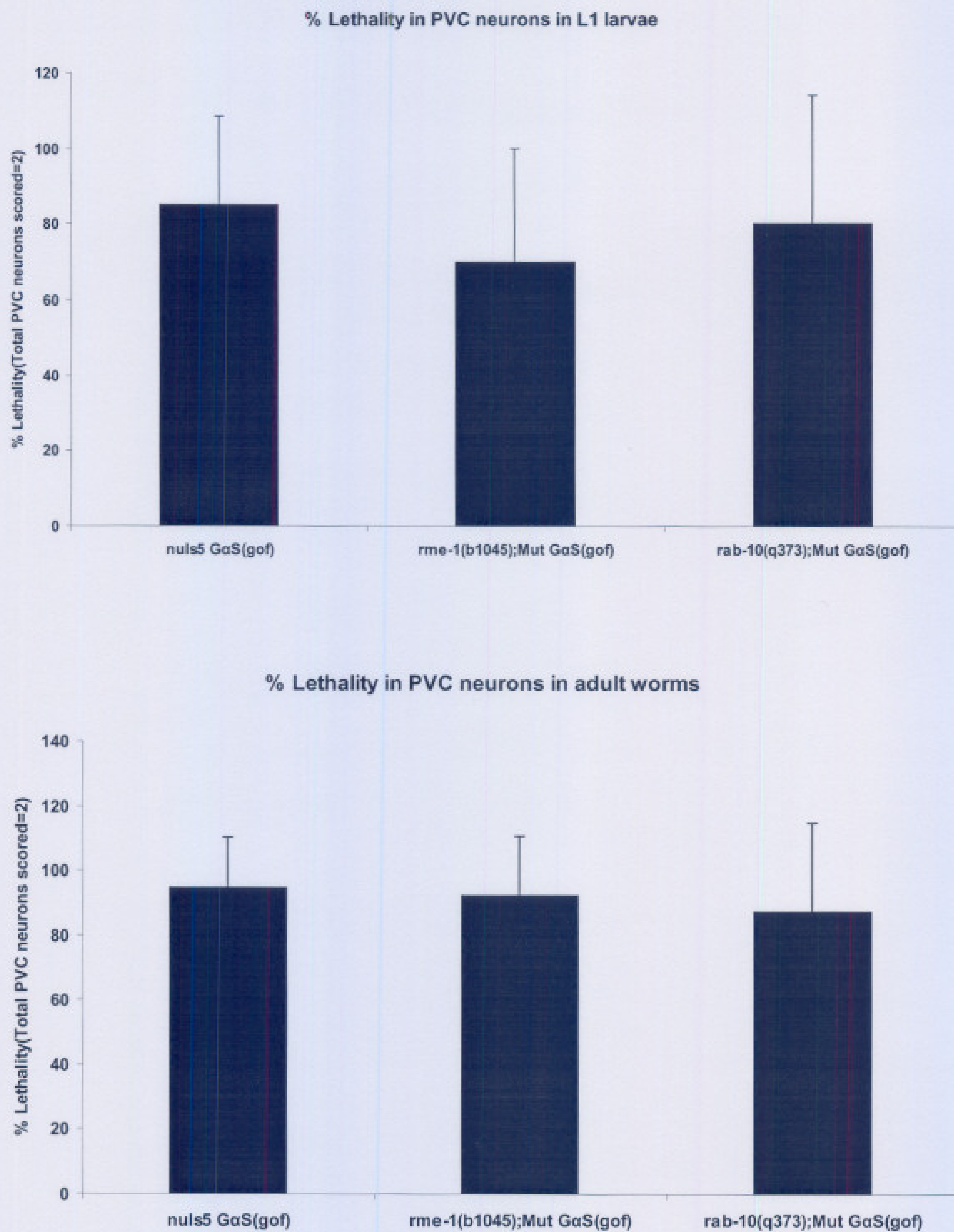


FIGURE 14: Classical Behavioral Assays for sensory response defects in Actin regulatory proteins AMPH-1 and SDPN-1.

amph-1(-) and *sdpn-1(-)* do not display Glutamate receptor activity defective characteristics such as decrease of spontaneous reversal per minute. Worms were allowed to adjust to a food free agar plate for five minutes and then their spontaneous reversal motions were counted over five minutes. The data is the average of two experiments and error bars depict ± standard deviation.

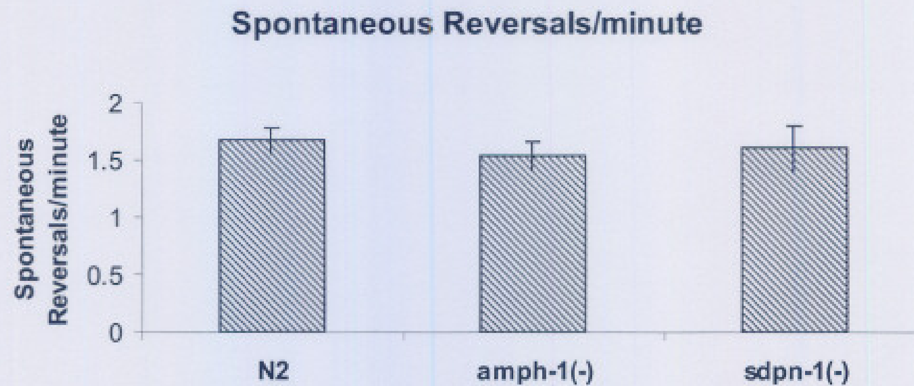


Figure 15:Classical Behavioral Assays to define sensory response defects in Actin regulatory proteins AMPH-1 and SDPN-1. *amph-1(-)* and *sdpn-1(-)* do not display Glutamate receptor activity defective characteristics such as decreased response to gentle nose touch. Worms were placed on a plate thinly seeded with bacteria. Backward avoiding response was counted over 10 consecutive gentle touches on the nose. A fine hair was placed in front of the worm moving forward so it collided head on with it.(n=10) The data is the average of two experiments and error bars depict ± standard deviation.

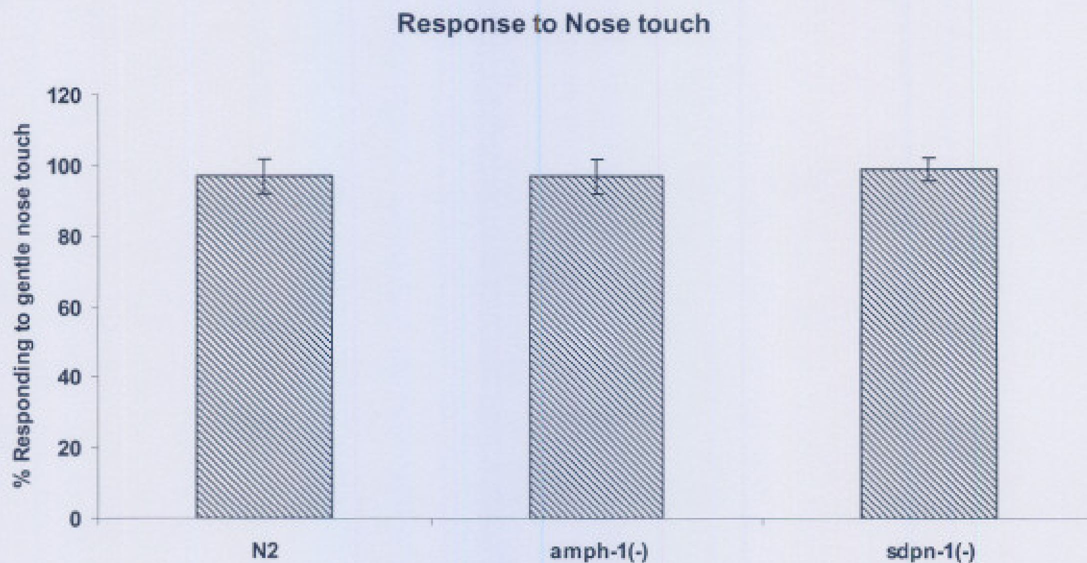


Figure 16:Classical Behavioral Assays to define sensory response defects in Actin regulatory proteins AMPH-1 and SDPN-1. We wanted to assay if *amph-1(-)* and *sdpn-1(-)* worms are defective in the perception of sensory stimuli. Worms were placed in the center of a 2 cm diameter ring of 200mM Copper sulfate on a 10 cm food free agar plate. After one hour it was scored how many worms were repelled by the CuSO4 and stayed within the ring instead of crossing the repellant.(n=10)

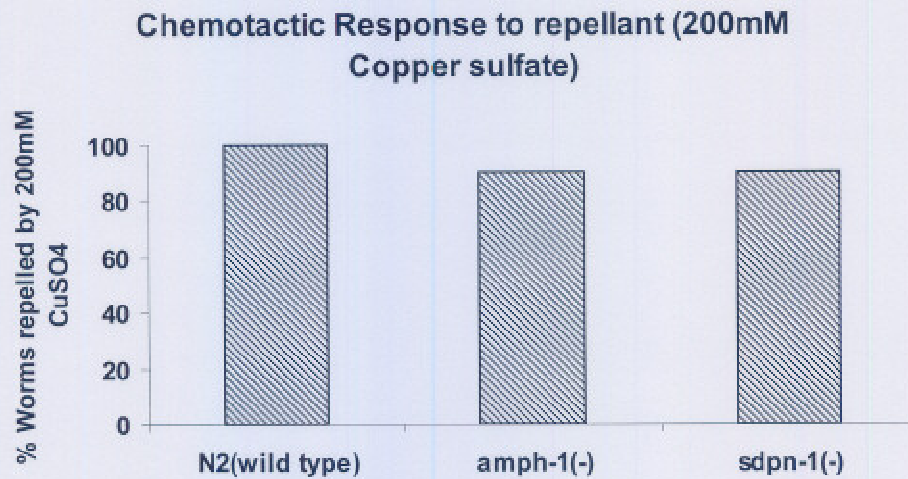
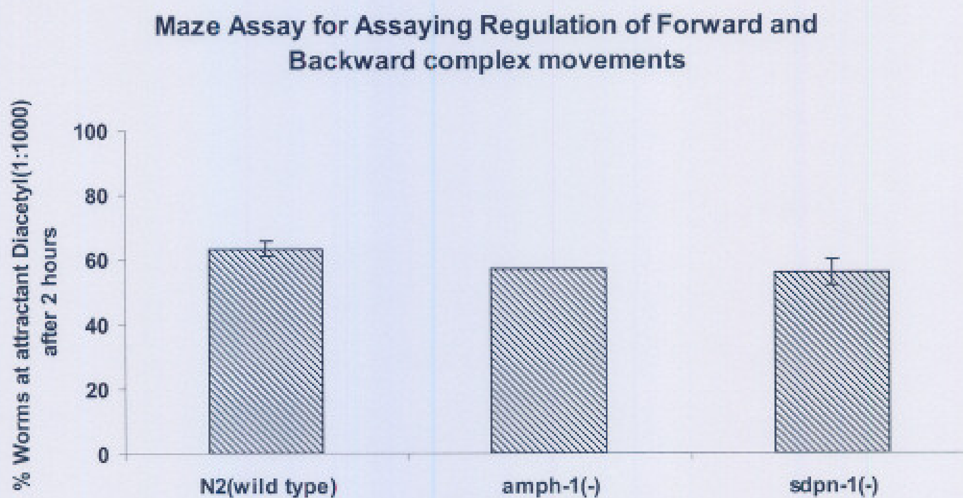


Figure 17:Classical Behavioral Assays to define sensory response defects in Actin regulatory proteins AMPH-1 and SDPN-1. We wanted to assay if *amph-1(-)* and *sdpn-1(-)* worms defective in complex locomotory behavior which is a feature of a functional reflex circuit. A maze was created as shown below by staggering 3 lines of repellant Copper sulfate and placing attractant Diacetyl(diluted 1:1000) at one pole of a food free 10 cm agar plate. Worms were placed opposite pole to attractant. Worms reaching the diacetyl after 2 hours were scored.(n~15)



BIBLIOGRAPHY:

1. Chen, C.C. *et al.* RAB-10 is required for endocytic recycling in the Caenorhabditis elegans intestine. *Molecular biology of the cell* **17**, 1286-1297 (2006).
2. Glodowski, D.R., Chen, C.C., Schaefer, H., Grant, B.D. & Rongo, C. RAB-10 regulates glutamate receptor recycling in a cholesterol-dependent endocytosis pathway. *Molecular biology of the cell* **18**, 4387-4396 (2007).
3. Chen, C.C.-H. in Department of Molecular Biology and Biochemistry, Vol. Ph D (Rutgers University NJ, USA, Piscataway; 2007).
4. Grant, B. *et al.* Evidence that RME-1, a conserved C. elegans EH-domain protein, functions in endocytic recycling. *Nature cell biology* **3**, 573-579 (2001).
5. Berger, A.J., Hart, A.C. & Kaplan, J.M. G alphas-induced neurodegeneration in Caenorhabditis elegans. *J Neurosci* **18**, 2871-2880 (1998).
6. Pant, S. *et al.* AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling. *Nature cell biology* **11**, 1399-1410 (2009).