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March 7, 2008

New Jersey Commission on Spinal Cord Research
Annual Narrative Report

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Address:	Waksman Institute 190 Frelinghuysen Rd. Piscataway, NJ 08854
Grant Title:	The Role of Notch/LIN-12 Glutamate Receptor Signaling and Excitotoxic Neuronal Damage
Grant Number:	05-3038-SCR-E-0
Grant Report Period:	12/15/04 – 12/30/07

Signature of Principal Investigator:

New Jersey Commission on Spinal Cord Research
FINAL NARRATIVE REPORT

Principal Investigator: Christopher Rongo

1. ORIGINAL AIMS OF THE PROJECT

Spinal cord injury to neurons, which can result from mechanical damage or ischemia induced by vascular damage, results in the release of the neurotransmitter glutamate at the initial damage site. Extracellular accumulation of glutamate in turn overactivates glutamate receptors on secondary site neurons, resulting in the excessive depolarization of these cells, calcium influx, and subsequent excitotoxic death of the neurons. The identification of agents that block or delay glutamate receptor activation would significantly limit receptor-induced damage that results from spinal cord injury. One of the primary ways by which neurons regulate glutamate receptor activity is by regulating the amount of these receptors that reach the synaptic membrane surface. This proposal aims to identify important factors that regulate the trafficking of glutamate receptors to the synaptic membrane using a genetic approach in the nematode *C. elegans*.

C. elegans uses AMPA-type glutamate receptors in a mechanosensory circuit. One of these receptors, called GLR-1, is localized to synaptic membranes via PDZ proteins and in an activity dependent manner. As in mammals, the overactivation of glutamate receptors in *C. elegans* can lead to excitotoxic neuronal death. Thus, to better understand glutamate receptor function and malfunction, we used a genetic approach to study the localization and function of the glutamate receptor GLR-1 in *C. elegans*. We found that the presenilin protein SEL-12 and the Notch-like protein LIN-12 can regulate the levels of GLR-1 receptor at synapses. We were intrigued by this finding for several reasons. First, presenilins like SEL-12 have been implicated in neurodegeneration in Alzheimers disease, suggesting a common link between glutamate receptor regulation and neurodegeneration. Second, mammalian presenilin proteins interact with proteins called Mints, and LIN-10, the *C. elegans* homolog of Mints, is required for proper localization of GLR-1 receptors. Thus, SEL-12 and LIN-10 might be working together to regulate glutamate receptors. Third, the Notch-like protein LIN-12 is a transmembrane protein that had previously been implicated in cell-cell communication during development. Thus, our findings suggested that this developmental signaling molecule might be reused during adulthood to regulate neuron physiology – a very novel finding. We proposed three aims to characterize the LIN-12/SEL-12 signal transduction pathway in *C. elegans* neurons with respect to its role in GLR-1 localization and in glutamate-mediated neuronal death.

Our first aim was to determine which components of the LIN-12 pathway are required for GLR-1 localization. By testing mutants for different ligands and effectors of the signaling pathway, we reasoned that we would have a better understanding of which LIN-12/SEL-12 signaling components participate in the regulation of glutamate receptors.

Our second aim was to perform a detailed analysis of the different domains of LIN-12 protein to determine which domains of this protein were involved in glutamate receptor regulation. As the different domains of LIN-12 can interact with different binding partner proteins to conduct signaling, we reasoned that our analysis could narrow down which of these binding partners is critical for LIN-12 regulation of glutamate receptors.

Our third aim was to determine whether defects in LIN-12 signaling can influence the excitotoxic death of neurons. We reasoned that the subcellular location of glutamate receptors within a neuron could affect the ability of the receptors to kill that neuron. Thus, by finding ways of modulating receptor localization, we could suppress neurodegeneration.

2. PROJECT SUCCESSES

Aim 1. To determine which components of the LIN-12 pathway are required for GLR-1 localization.

Rationale: The LIN-12 pathway in *C. elegans* contains multiple proteins for each signaling step in the pathway. Moreover, while the canonical output of LIN-12 signaling is through the LAG-1 effector, there are other non-canonical outputs that utilize other effectors. By testing mutants for all of the known Notch pathway components, we planned to determine which components were specific for LIN-12's role in GLR-1 localization.

Progress: We tested GLR-1 localization in mutants for 4 different ligands and 7 different effectors for LIN-12. Loss of function mutations in *lag-2* result in a similar phenotype to loss of function mutations in *lin-12* and *sel-12*: a decrease in the amount of synaptic GLR-1 receptors (Fig. 1). We have not detected a GLR-1

localization phenotype for the other ligand mutants. Our results indicate that LAG-2 is the relevant LIN-12 pathway ligand regulating GLR-1 localization.

One simple explanation for the role of the LIN-12 pathway is that it might regulate the transcription of GLR-1. We tested this in two ways. First, we fused an RFP reporter gene to the *glr-1* promoter, and introduced this reporter into wild-type nematodes, *lin-12* loss of function mutants, and *lin-12* gain of function mutants. We found that similar levels of RFP were detected in all three strains. Second, we isolated mRNA from wild type, *lin-12* loss of function mutants, and *lin-12* gain of function mutants. Using reverse transcription and real time PCR, we measured the levels of *glr-1* transcript in these three strains. We found similar levels of *glr-1* mRNA in all three. Taken together, our results suggest that the LIN-12 pathway does not directly regulate *glr-1* transcription and/or mRNA levels. Rather, our work suggests that LIN-12 signaling regulates the transcription of yet unknown localization factors, which in turn regulate GLR-1 synaptic levels post-translationally.

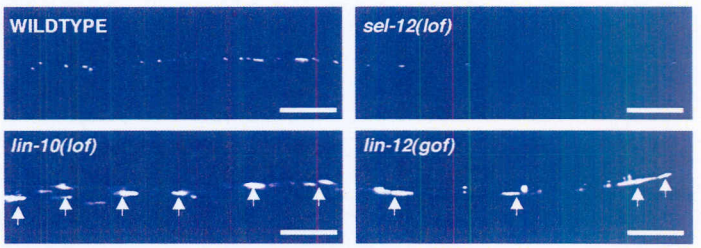

Figure 1. GLR-1::GFP Localization Is Regulated by LIN-12 signaling. Images of postsynaptic clusters of GLR-1::GFP fluorescence in the ventral cord bundle of dendrites are shown. All images are of living animals of the indicated genotype. Wild type nemtodes expressing GLR-1::GFP localize receptors to small clusters along the ventral cord dendritic bundle. Both *lin-10(lof)* and *lin-12(gof)* mutants accumulate large structures of unlocalized receptor (arrows). Both *sel-12(lof)* and *lin-12(lof)* [not shown] have fewer and dimmer GLR-1 puncta. Scale bar is 10 microns.

Aim 2. To Determine By Structure/Function Analysis The Domains Of LIN-12 Required For GLR-1 Localization.

Rationale: Many of the noncanonical LIN-12 effectors interact with disparate domains within the LIN-12 intracellular region, and hence their function might be discernable by making specific mutations within this domain. Thus, we proposed to generate and to test LIN-12 transgenes that contain mutations and/or deletions in different regions of the LIN-12 intracellular region. We also proposed to use different cell-type specific promoters to determine whether expressing LIN-12 cDNA cell-autonomously can rescue the GLR-1 localization phenotype of *lin-12* mutants.

Progress: We generated several transgenes containing the *lin-12* cDNA under the control of either the *glr-1* promoter (specific to GLR-1-expressing interneurons), the *unc-119* promoter (specific to all neurons), the *dlg-1* promoter (specific to all hypodermal cells), the *lin-12* promoter (a positive control), and the heat shock promoter (ubiquitous and inducible). We introduced these transgenes into *lin-12* mutants and tested for their ability to rescue GLR-1 localization defects. Surprisingly, we were not able to obtain consistent rescue using the *glr-1* promoter. Moreover, were unable to confirm that LIN-12 is expressed in interneurons. It remains possible that LIN-12 has a non-autonomous role in glutamate receptor trafficking, perhaps by regulating the function of support cells or glia.

During our genetic analysis of the LIN-12 pathway, we also examined the dynamics of GLR-1 localization using photoactivatable GFP

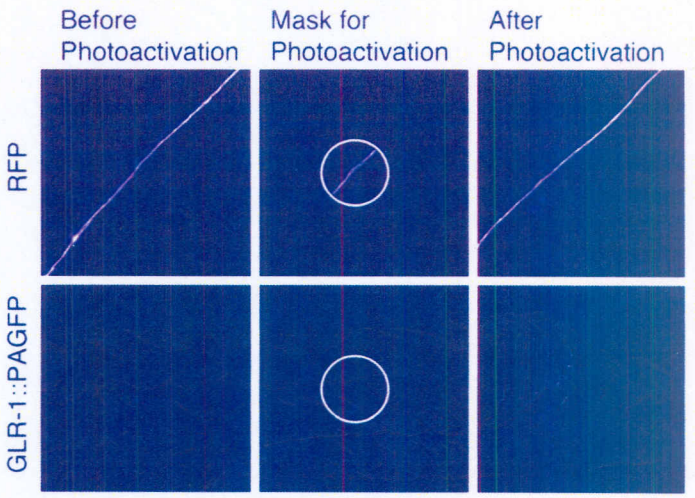

Figure 2. Photoactivation of specific clusters of GLR-1::PAGFP. The *glr-1::pagfp* transgene was introduced into the germline of nematodes along with $P_{glr\text{-}1}$*::RFP*, a transgene the expresses RFP in the same neurons. Neurites containing unlocalized RFP (visualized with TRITC filters) are shown in the top three panels. The same neurites containing GLR-1::PAGFP (visualized using FITC filters) are shown in the bottom three panels. Prior to activation, the neurites can be clearly visualized with RFP, but no GLR-1::PAGFP can be detected. We applied a mask that blocked excitation of the specimen in all regions except within the circle. We captured this image prior to activation (middle column). We then exposed the masked region to 420nm illumination for 10 seconds. We removed the mask and captured full images of the nematode immediately after the photoactivation using the same exposure settings as in the left column of images. GLR-1::PAGFP clusters that have become fluorescent after photoactivation are only seen in the exposed area. Note that the animal is alive but anesthetized, and has moved slightly during the experiment, which can be controlled by following morphological and fluorescent cues that can be clearly identified outside the experimental region.

(PAGFP), a version of GFP that does not fluoresce until activated by high frequency light (Fig. 2). We tagged GLR-1 with PAGFP, and used the resulting GLR-1::PAGFP transgene to do pulse-chase analysis in living animals. Using this technique, we showed that the turnover rates of GLR-1 are faster in a *lin-10* mutant compared to wild-type animals (Fig. 3). We therefore began to explore the dynamics of GLR-1 trafficking, including endocytosis and membrane recycling.

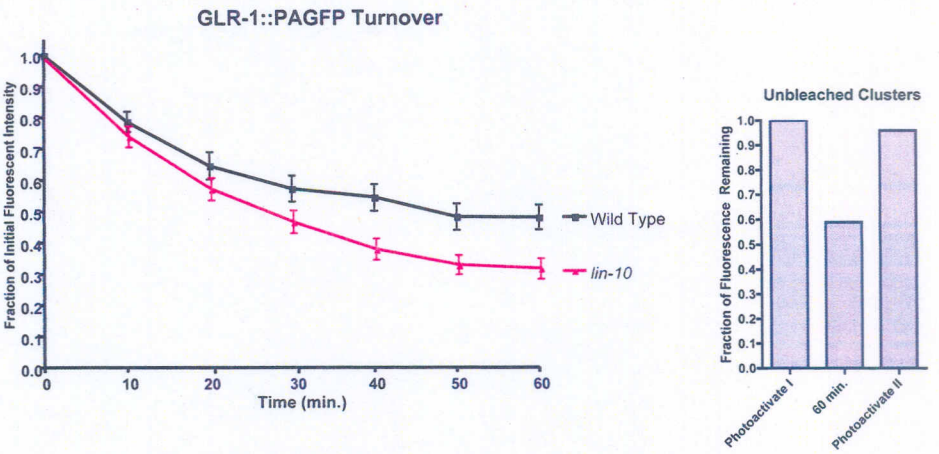

Figure 3. GLR-1 receptors turn over faster and to a greater extent in *lin-10* mutants. Synapses from animals expressing GLR-1::PAGFP (black squares for wild type, red triangles for *lin-10* mutants) were photoactivated and monitored for fluorescent intensity every 10 minutes. A bleached standard curve was generated and used to account for photobleaching during image acquisition. For each synapse, remaining activity was quantified as a fraction of the starting fluorescence. The average fraction for 25-30 synapses is shown. To control for fluorescent run-down during the experiment, animals were photoactivated for a second round (Photoactivate II on the graph). The near full recovery of fluorescent signal indicates that nearly all of the original photoacticated receptors were replaced with yet unactivated receptors.

Regulated endocytosis of AMPA-type glutamate receptors was of particular interest, as it is critical for synaptic plasticity. However, the specific combination of clathrin-dependent and independent mechanisms that mediate AMPAR trafficking *in vivo* had not been fully characterized. Thus, we set out to identify factors that regulate GLR-1 endocytosis and recycling. We found that animals lacking RAB-10, a small GTPase required for endocytic recycling of intestinal cargo, are similar in phenotype to animals lacking LIN-10: GLR-1 accumulates in large accretions and animals display a decreased frequency of reversals (figures are in enclosed manuscript). Mutations in *unc-11* (AP180) or *itsn-1* (Intersectin 1), which reduce clathrin-dependent endocytosis, suppress the *lin-10* but not *rab-10* mutant phenotype, suggesting that LIN-10 functions after clathrin-mediated endocytosis. By contrast, cholesterol depletion, which impairs lipid raft formation and clathrin-independent endocytosis, suppresses the *rab-10* but not the *lin-10* phenotype, suggesting that RAB-10 functions after clathrin-independent endocytosis. Animals lacking both genes display additive GLR-1 trafficking defects. We proposed that RAB-10 and LIN-10 recycle AMPARs from intracellular endosomal compartments to synapses along distinct pathways, each with distinct sensitivities to cholesterol and the clathrin-mediated endocytosis machinery. We published these findings in *Molecular Biology of the Cell*.

Aim 3. To Determine Whether Defects In LIN-12 Signaling Can Influence The Excitotoxic Death Of Neurons.

***Rationale*:** The expression of activated $G\alpha_s$ results in the excitotoxic death of neurons in *C. elegans*, which is suppressed by mutations that decrease glutamate signaling, and enhanced by mutations that increase glutamate signaling. The regulated trafficking of glutamate receptors modulates glutamate signaling; thus, we hypothesized that mutations in the genes that regulate glutamate receptor trafficking would influence excitotoxicity. We previously identified several genes that regulate glutamate receptor abundance at synaptic membranes. Here we test whether loss of function mutations in those genes affect excitotoxic death caused by $G\alpha_s$.

Progress: We tested several mutations in LIN-12 signaling components, including LIN-12 itself, and found that these mutations did not significantly impact excitotoxic death. Next, we tested several genes known to regulate GLR-1 trafficking, including *kel-8* (BTB-Kelch 8), *unc-11* (AP180), *lin-10* (Mint2), *rab-10*, *unc-43* (CaMKII), and *xbp-1*. We found that mutations in *kel-8*, *lin-10,* and *rab-10* suppressed excitotoxic death induced by $G\alpha_s$. Excitotoxic death in *C. elegans* neurons, like in mammalian neurons, proceeds via an initial cell swelling phase, followed by a necrosis phase. We found that mutations in *lin-10* and *rab-10* have little

effect on the number of neurons that swell, but significantly reduce the number of swollen neurons that go on to necrose. Mutations in *kel-8* reduces both swelling and eventual necrosis. As describe earlier, LIN-10 is a PDZ domain protein that mediates the recycling of GLR-1 receptors that were previously endocytosed by clathrin-dependent pathways. RAB-10 is a small GTPase that regulates the recycling of GLR-1 receptors that were previously endocytosed by clathrin-independent pathways. Mutants for *lin-10* and *rab-10* accumulate their glutamate receptors inside early endosomes rather than at synaptic membranes. KEL-8 is a ubiquitin ligase, and GLR-1 receptors might be accumulating in an internal compartment of unknown origin in *kel-8* mutants (our future experiments will address the more precise role of KEL-8 in this neuroprotective process). Taken together, our results demonstrate that factors that regulate glutamate receptor trafficking can also modulate neuron killing during excitotoxicity.

3. PROJECT CHALLENGES

We encountered two major challenges. First, mutations in *lin-12* resulted in significant variation in phenotype. There are two major Notch homologs in *C. elegans*: *lin-12* and *glp-1*. We hypothesized that the reason *lin-12* might produce variable results is that it is partially redundant with *glp-1*; indeed, this is the case for their role in embryogenesis. We examined GLR-1 localization in *glp-1* single mutants, but did not find a significant phenotype. The *lin-12 glp-1* double mutants are lethal, which precluded any analysis of GLR-1 in these double mutants.

The second major challenge was that we were not able to rescue *lin-12* mutant with a wild-type *lin-12* transgene. This precluded us from performing an *in vivo* structure/function analysis, as described in Aim 2. Aim 2 is geared towards a mechanistic understanding of glutamate receptor trafficking. Thus, we initiated a new series of mechanistic experiments to replace the original proposed experiments. We examined the dynamics of glutamate receptors *in vivo*, and found two regulators of glutamate receptor membrane trafficking: RAB-10 and LIN-10. Our findings from this alternative approach resulted in a published manuscript in *Molecular Biology of the Cell*.

4. IMPLICATIONS FOR FUTURE RESEARCH AND/OR CLINICAL TREATMENT

Glutamate receptor activation remains one of the primary, damage-inducing factors during ischemia and neuronal injury. Can the location of the receptors within or on neurons influence the ability of these receptors to kill the cell? Our results suggest that the sequestration of glutamate receptors into endosomes and other internal membrane-bound trafficking compartments in these mutants helps to protect neurons from excitotoxic death. Current approaches for minimizing neuronal damage have focused on blocking the channels directly. Our findings support the idea that regulators of glutamate receptor trafficking might also be promising pharmacological targets to minimize brain damage after stroke or traumatic injury.

5. FUTURE PLANS

Mutations in KEL-8 result in mistrafficking of GLR-1 receptors, and also reduce excitotoxicity. We have recently found that KEL-8 can interact with two different p38 MAP Kinase proteins. Mutations in these kinases (called PMK-1 and PMK-3) can modulate the effects of excitoxicity. Thus, KEL-8 might regulate excitotoxic death by ubiquitinating these kinases. We are currently testing this hypothesis, and, based on our preliminary data, will be applying for NIH funding for this project.

Our findings that GLR-1 receptor trafficking is regulated by RAB-10 has led us to investigate whether other small GTPases regulate glutamate receptor trafficking. The preliminary experiments from our analysis of RAB-10 and LIN-10 were used to obtain an NIH R01 grant. Thus, we were able to leverage our results from this NJCSCR project to obtain a $1.5 million R01 grant from NIH to further understand glutamate receptor function and malfunction.

6. RESEARCH PUBLICATIONS

Glodowski, D.R., Chen, C. C.-H., Schaefer, H., Grant, B.D., and Rongo, C. RAB-10 regulates glutamate receptor recycling in a cholesterol-dependent endocytosis pathway. *Molecular Biology of the Cell* 18(11):4387-96.