The Insulin/IGF Signaling Regulator Cytohesin/GRP-1 Modulates Sensitivity to Excitotoxicity in C. elegans

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The Insulin/IGF Signaling Regulator Cytohesin/GRP-1 Modulates Sensitivity to Excitotoxicity in C. elegans

by

Nazila Tehrani

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract:
The Insulin/IGF Signaling Regulator Cytohesin/GRP-1 Modulates Sensitivity to Excitotoxicity in C. elegans

By
Nazila Tehrani

Advisor: Dr. Itzhak Mano

Excitotoxicity is a form of neurodegeneration that serves as the main underlying cause of brain damage in stroke/brain ischemia, and a contributing factor in a range of neurological diseases such as Epilepsy, ALS, Alzheimer, and Huntington’s disease. In excitotoxicity, over-activation of glutamate receptors causes necrotic neuronal cell death. In spite of intense study of excitotoxicity, the molecular mechanisms that lead from glutamate receptor activation to necrotic death remain a mystery. Aging neurons are known to be more vulnerable to excitotoxicity and less likely to recover, but the underlying reasons for the increased cellular vulnerability are unknown. To gain insight into the core process of excitotoxicity and factors that affect neuronal vulnerability, we turn to a model system that offers simplified but conserved signaling cascades and strong research tools. We therefore study excitotoxicity in the nematode C. elegans, in which a strong track record of studies of cell death mechanisms and signaling cascades suggests that this genetically accessible model system can help illuminate critical and conserved cellular processes. Our model combines a deletion of glutamate transporter 3 (glt-3) in a sensitized background (nuls5), resulting in glutamate-triggered necrotic cell death. Previously, other
investigators showed the role of the evolutionary conserved Insulin/IGF Signaling pathway (IIS) in longevity and cell stress resistance. Recently, we were able to show that the IIS cascade also controls vulnerability of postsynaptic neurons to excitotoxicity, by regulating the nuclear/cytoplasmic localization of the neuroprotective transcription factor FOXO3/DAF-16. Active IIS signaling causes FOXO/DAF-16 to be sequestered in the cytoplasm, while IIS inhibition allows FOXO/DAF-16 to enter the nucleus and activate protective genes. To gain further insight into the ability of this signaling cascade to regulate neurodegeneration, we study factors that are upstream and downstream of the IIS cascade and may be involved in excitotoxicity. As a preliminary step, I confirmed the involvement of IIS pathway in excitotoxicity. I worked towards preparing an excitotoxicity strain that is permissible for RNAi effect in neurons, to facilitate the ability to screen for the effect of FOXO/DAF-16 candidate target genes on neuroprotection. I then examined potential upstream modulators of IIS, and focused on the relationship between the Cytohesin/GRP-1 protein complex (previously shown to control insulin-regulated metabolism in mammals) and IIS in excitotoxicity. I observed that mutations that are expected to decrease the complex’s activity and its potentiation of IIS signaling reduced susceptibility to excitotoxicity, while an over-activation of this complex increased neuronal vulnerability. These results support that the Cytohesin/GRP-1 complex regulates the ability of the IIS cascade to modulate excitotoxicity. As a preliminary step to examine a possible direct communication between glutamate receptors and Cytohesin/GRP-1 IIS complexes, I examined their cellular localization. I observed a rough co-localization of the Cytohesin/GRP-1 complex components PPK-1 and GRP-1 and the glutamate receptor subunit GLR-1. Put together, these observations suggest that the Cytohesin/GRP-1 complex modulates IIS cascade’s ability to regulate the susceptibility to excitotoxic neurodegeneration, and that
glutamate might directly regulate this signaling cascade. We therefore provide novel insights into
conserved signaling cascades that control neuronal sensitivity in nematode excitotoxicity. We
hope that these new insights could inspire new research directions in the search for therapeutic
interventions in stroke and brain ischemia.
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Introduction:

a) **Excitotoxicity, a condition that involves over-stimulation by Glutamate, is a central process in neurodegenerative brain pathologies:**

Excitotoxicity is one of the main causes of lasting neuronal damage and neurodegenerative disease. Excitotoxicity is the result of excessive neurotransmitter signaling, in which the exposure of receptors to the excitatory amino acid Glutamate for prolonged time causes neuronal cell injury or necrotic cell death. Such a neurological cell death is the main cause of cell damage in stroke/brain ischemia, and a common down-spiral pathway for cell death in several diseases such as epilepsy, Huntington, ALS, and Alzheimer. Glutamate (Glu) is the major excitatory neurotransmitter in the brain. Under normal conditions, after Glu is released from the presynaptic cell, it binds to Glu receptors (GluRs) on the postsynaptic neurons and allows Na\(^+\) and Ca\(^{2+}\) to enter the cell, thus depolarizing the postsynaptic cell and activating several different pathways. To terminate the signaling pulse and clear the synapse in preparation for the next neuronal impulse, the released Glu is then taken up by Glu transporters (GluTs), which are located on the neurons and glia cells that surround the synapse. However, under pathological conditions, Glu clearance slows down and eventually fails. When there is a lack of energy in the brain (as happens during brain ischemia), Glu is not taken up, but rather accumulates in the synapse, and this causes over-excitation of the postsynaptic cell. The influx of ions causes exaggerated depolarization that, together with the excessive Ca\(^{2+}\) influx, causes swelling and lysis of the cell, leading to necrotic cell death. Since Glu is a common metabolite
present in high concentration in the cytoplasm of all cells, these lysed cells release more Glu and calcium, which causes excitation of neighboring cells and even more cell death \(^1,4,5,7,8\).

A.

Figure 1: Glutamate signaling. A) Normal glutamate signaling: Glu is released from the presynaptic cell into the synaptic cleft and binds to and activates glutamate receptor/channels. These receptor/channels open and Ca\(^{2+}\) and Na\(^+\) ions enter the postsynaptic cell. The released Glu is
simultaneously picked up by glutamate transporters (GluTs) located on the glia cells to terminate signaling. B) *Excitotoxicity condition*: There is an excess amount of glutamate in the synapse caused by GluT malfunction; therefore, the glutamate receptors are over-activated, excessive amount of Ca\textsuperscript{2+} enters the cells, eventually leading to necrotic cell death.

**b) Glutamate is the major excitatory neurotransmitter in the central nervous system**

Glutamate is the most important excitatory neurotransmitter in the central nervous system. Glutamate is involved in learning and memory and also plays role in central nervous system development\textsuperscript{7}. There are two ways to generate glutamate in central nervous system: *de novo* synthesis or recycling. While all cells can synthesize Glu *de novo* by basic metabolic pathways, neuronally released Glu can be recycled in what is called the glutamate-glutamine cycle: after release of glutamate to extracellular space, glutamate is taken up by the astrocyte. The astrocyte enzyme, glutamine synthase (GS) uses ATP to convert glutamate to glutamine. The astrocyte then releases the glutamine to extracellular space. Glutamine is consequently uptaken by the presynaptic neuron, which then uses the enzyme phosphate activated glutaminase (PAG) to convert glutamine to glutamate. Glutamate from either *de novo* synthesis or recycling is then pumped into glutamate-containing synaptic vesicles near the synaptic cleft, where it will be later release (Figure 2).
Figure 2: Glutamate-Glutamine cycle. Glutamate is taken up by glutamate transporters into glial cells, where GS converts glutamate to glutamine. Then the glutamine is released into extracellular area and is taken by the presynaptic neurons, where it is converted back to glutamate by using ATP and PAG enzyme.

Glutamate mediates its effect by activating glutamate-gated cation channels called ionotropic glutamate receptors (iGluRs), and glutamate-activated G-protein coupled receptors called metabotropic glutamate receptors (mGluRs). Some mGluRs inhibit cAMP signaling, while others activate Phospholipase C (PLC) and cause production of inositol-1,4,5-tri-sphosphate (IP3) and diacylglycerol (DAG) from L-3-phosphatidylmyo-inositol-4,5-bisphosphate (PIP2). DAG is then able to activate PKC and IP3 becomes an agonist for IP3 receptors, which leads to the release of Ca\textsuperscript{2+} from the endoplasmic reticulum \textsuperscript{7,11}. iGluRs (many times labeled just GluRs) are subdivided to three different groups according to their physiological properties and the
xenobiotic glutamate-like agonist that serves as their specific agonist: kainate, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and N-methyl-D-aspartate (NMDA). iGluRs are hetero-tetrameric complexes of similar subunits, and the identity of the subunits determines the specific properties of the complex. AMPA-Rs and NMDA-Rs are two main receptors that are involved in normal physiology and excitotoxicity. The different GluRs are usually co-localized in the postsynaptic membrane by their binding to scaffolding proteins that form the Post Synaptic Density, such as PSD95 (a protein that contains a protein-protein interaction domain called the PDZ domain). Most AMPA-Rs have high permeability for Na⁺ and K⁺ and low permeability for Ca²⁺; however, there are some AMPA receptors (because of the specific subunits that make up the tetrameric complex) are permeable to Ca²⁺. AMPA receptors open very fast, but they also desensitize very fast. On the other hand, NMDA receptors are permeable to Ca²⁺; but in resting polarized membrane potential, these receptors are blocked by a magnesium ion (Mg²⁺), which is expelled from the channel following sufficient depolarization. Thus, while weak depolarization activates only AMPA-Rs, strong depolarization due to robust AMPA-R activation causes the removal of Mg²⁺ from NMDA receptor so Ca²⁺ can enter the cell and activate several different pathways ¹¹. NMDA-Rs therefore have a special physiological role as detectors of concerted or enhanced stimulation. Moreover, under pathological conditions of even more intense Glu signaling, the NMDA receptor plays a role in both cell death and cell survival. A large body of evidence suggests that the localization of NMDA receptors determine their functions. NMDA receptors can be localized at synapses or on the dendritic shaft, a location referred to as extra-synaptic. When there is low level of excitation, Glu binds to NMDA receptors inside the synapse, open these receptor/channels, and lets the Ca²⁺/Na⁺ enter the cell; the Glu is taken up by GluTs before affecting surrounding cells. However, when there is over-
excitation, Glu spills out of the synapse and the extra-synaptic NMDA receptors also become activated \(^5,12\). While the view that assigns a special role for specific subunit combination in extrasynaptic NMDA-Rs is prominent in the field, others have challenged this view \(^{13}\).
**Figure 3: Release of glutamate to bind to glutamate receptors.** A) Under normal conditions, small amounts of glutamate are release and they bind to the synaptic AMPA and NMDA receptors. Activation of these receptors cause physiological signaling and supports the neuronal survival. B) Excess release of glutamate causes the activation of extrasynaptic NMDA in addition to synaptic receptors. The combined over-activation of synaptic and extrasynaptic receptors causes the neuronal death.

**c) Glutamate is removed from the synapse by specialized transporters:**

Under normal resting conditions the concentration of extracellular Glu in the synapses is 25 nM; however, during excitation the concentration of synaptic glutamate increases to 10 mM. Since the excess amount of glutamate is very dangerous for the cell, it is quickly cleared from synapses by glutamate transporters. There are five kinds of glutamate transporters (GluTs, in mammals called EAAT1-5)\(^7,9\). EAAT1-2 are located in the astrocytes, EAAT-3 and EAAT4 are located in postsynaptic cells, and the EAAT5 is mostly located in retina\(^7\). The crystal structure of GluTs showed that GluTs are organized as homotrimers. Each subunit functions independently and its structure contains eight transmembrane domains and two hairpins, one located between TM 6 and 7 and the other between TM 7 and 8, an area that makes up the catalytic core of the transporter\(^9\).
Figure 4. Glutamate transporter structure. GluTs contain eight TM and two hairpins domains (this figure is adopted from 9).

Although a full cycle of the transport reaction is slow relative to the speed of signaling, Glu binds to the GluTs at the same rate as it binds to NMDA receptors and AMPA receptors. The expression of high density of GluTs at the synapses helps ensure that in spite of the slow turnover rate of the transporter, all of the extra Glu in the extracellular space is space collected (first by mere binding to the transporters, and slightly later by actual transport)14. There are two different conformations observed in the GluTs: outward- and inward-facing conformation. Glu, accompanied by Na$^+$ ions, binds to the outward-facing conformation. Now the transporter can change the conformation to the inward-facing conformation and release the Glu and Na$^+$ ions to the cytoplasm. Then K$^+$ in the cytoplasm binds to the inward-facing conformation, allowing the transporter to change the conformation back to the outward-facing conformation14. Glu is found in high concentrations (~10 mM) in the cytoplasm of all cells, including the glia cells and neurons in charge of Glu clearance. Therefore, bringing synaptic concentrations of Glu back to 25 nM requires Glu transport into the cell against its concentration gradient. The driving force for Glu uptake is based on the electrochemical gradient of Na$^+$, H$^+$, and K$^+$. Together with the incoming glutamate, there is co-transport of 3 Na$^+$ and H$^+$ ions from the extra-cellular side and a counter-transport of K$^+$ from inside the cell, along their own concentration gradients. The translocation of these ions down their sharp concentration gradients provides enough driving force that enables the GluT to move Glu against its own concentration gradient7. However, when there is a problem with the electrochemical gradients of Na$^+$ and K$^+$, this causes the sharp decline in Glu removal from the synapse (Glu can even come out of the glial cell instead of going
inside so the transport is reversed). Since Glu transport is so dependent on the electrochemical gradients, it becomes extremely sensitive to any change, and will be the first system to shut down during a gradual decline in cellular energy levels. For example, in ischemic stroke, when there is energy failure, malfunction in Na\(^+\)/K\(^+\) pumps is induced. Though all cells in the body are affected by energy shortage and the disruption of ion gradients, brain cells are particularly sensitive because of the combined effect on GluTs and GluRs. The diminished electrochemical gradient very quickly leads to the malfunction of the glutamate transporters. Glu then has a catalytic effect on the neurons that express GluRs, causing widespread permeabilization to Na\(^+\) and Ca\(^{2+}\). In this case, accumulation of Na\(^+\) (coming through both AMPA and NMDA receptors) inside the cells causes the more water to enter the cell and, at the end, causes cell burst. In addition, the excessive activation of NMDA glutamate receptors and a toxic influx of Ca\(^{2+}\) into the postsynaptic cells, over-activates many Ca\(^{2+}\)-dependent enzymes and cascades, eventually causing necrotic cell death in these cells \(^{1,4-6,8}\). The molecular/biochemical nature of these cascades and the relative contribution of different suggested mechanisms to the core process of excitotoxicity is a matter of much debate.

d) **Energy plays a significant role in the glutamate metabolism**

As mentioned before, GluTs use an electrochemical gradient to clear the excess amount of Glu from synapses. The electrochemical gradient is maintained by Na\(^+\)/K\(^+\) pumps \(^7\). There are two ways that pumps get their energy: oxidative phosphorylation (mitochondria uses the oxygen to produce ATP) \(^15\) and glycolysis (using glucose to produce ATP) \(^16,17\). Therefore, blockage of generation of energy from each of these processes would cause the malfunction of Na\(^+\)/K\(^+\)-pumps and thus, cause the malfunction of GluTs \(^18,19\) (Figure 5). For example, ischemic stroke
happens as a result of blockage of arteries which causes deprivation of both glucose and oxygen and at the end excitotoxicity and cell death $^{20}$.

A.

B.

**Figure 5: Involvement of energy in the excitotoxicity.** A) *Normal Na$^+$/K$^+$ Pumping*. In this condition, enough ATP is generated, so the Na$^+$/K$^+$ pumps function normally and they maintain the electrochemical gradient. GluTs are able to use the electrochemical gradient and clear the
extra Glu from the synapses. B) Malfunction of Na⁺/K⁺ Pumping. When there is a depletion of ATP, the Na⁺/K⁺ pumps are not able to function correctly, which disturbs the maintenance of the electrochemical gradient around the cell. Thus, the GluTs’ function is disturbed are they are not able to function very well, leading to Glu accumulation in the synapse and excitotoxicity.

**e) Excessive glutamate stimulation and excitotoxicity may engage a number of cell death pathways**

Though acute cell swelling and death is prevalent at the core of the brain area affected by stroke, there is in fact a range of different kinds of cell death that are observed in excitotoxicity, depending of the intensity of the insult in each affected area. The cell swelling and burst described above is one of these kinds of cell death, and it is called necrotic cell death. Necrosis is result of severe insult which causes the sudden death of the cell. Previously, it was believed that necrosis is a disorganized, uncontrollable death (seen in both brain neurons and in other cell types throughout the body); however, recently researchers showed that necrosis is not necessarily uncontrollable, as it may result from a combination of several signaling pathways. For example, some forms of regulated necrotic cell death seen in both neurons and other cells became known as necroptosis or parthanatos, depending on the specific pathways used 21,22. The burst of neurons during necrosis further damages the surrounding neurons, for two reasons: A) there are high concentrations of Glu in the cytoplasm, as it is a common amino acid used in all cells for normal metabolism. The released Glu can now damage neighboring neurons. B) The release of cell content triggers an inflammatory response that exacerbates tissue damage. It is yet unclear what the contribution of these processes is to excitotoxicity, but so far clinical trials aimed at blocking these pathways failed to protect from ischemic damage 23.
Further away from the core of the affected area, where blood supply is partial and GluT malfunction is not as dramatic and acute, the neurons exposed to this less intense damage have more time to fight against these injuries or prepare themselves. To avoid the toxicity and propagating damage associated with necrosis, dying cells that have time to prepare for their demise will commit a well-orchestrated and less damaging cell suicide called program cell death or apoptosis. This kind of programmed cell death is coordinated, energy-dependent and works through a process involving a group of cysteine proteases called “caspases”. There are two major signaling pathways in apoptosis: extrinsic pathway and intrinsic pathway. The extrinsic pathway of apoptosis is initiated by activating cell death receptors such as the FAS receptor or Tumor necrosis factors (TNFs). After activation, this receptor binds to the Fas-Associated protein with Death Domain (FADD) and then recruits procaspase 8. This complex is called death-inducing signaling complex (DISC). At the DISC the caspase 8 is cleaved and activates pro-apoptotic proteins: it binds or it activates the executioner caspases 3, 6, and 7.

The intrinsic pathway of apoptosis is switched on by activating one of the pro-apoptotic BCL-2 family members in the outer member of mitochondria. By activation of these BCL-2 family members, members of two different groups of proteins (pro-apoptotic or anti-apoptotic) can move from the cytosol to the outer-member of mitochondria. After activation of pro-apoptotic proteins such as Bad, Bax, and Bid, these proteins oligomerizes, forming a pore in the mitochondria outer-membrane and thus release of cytochrome c. Later on, cytochrome c binds to Apaf-1, which oligomerizes together with pro-caspase -9 to produce the apoptosome. Following apoptosome formation, oligomerized caspase-9 is turned on, and it activates executioner caspases 3, 6 and 7.
Apoptosis usually happens during development to help control the number of specialized cells. The morphological characteristics of apoptosis include shrinkage of the cells, denser cytoplasm, packaged organelles, condensed DNA, and DNA laddering\textsuperscript{21,26}. There are several differences between necrotic cell death and apoptotic cell death. One of the important differences is that there is no spillage of cell contents and no inflammation that occurs during apoptosis.

A third kind of cell death is called autophagy. Autophagy has a dual function: it is involved in both cell survival and cell death. Autophagy usually helps cell survival in stressful conditions or starvation, by degrading or recycling some of the macromolecules such as ribosomes and endoplasmic reticulum fragments\textsuperscript{27}. However, in some cases, autophagy is used as a secondary mechanism of programmed cell death\textsuperscript{28}, in which it displays a distinct morphology, lack of condensed chromosomes, and production of vacuolization\textsuperscript{21}. Importantly, there is cross talk between autophagy and the other cell death programs (necrosis and apoptosis). For example, there is cross-talk between the apoptosis regulatory protein Bcl-2 and the autophagy regulator beclin\textsuperscript{28}. There is also cross-talk between autophagy and necrosis, as seen in studies that showed that inhibition of kinases receptor-interacting protein 1 (RIP1) and RIP3 (which are two proteins that involved in necroptosis), decrease the autophagy\textsuperscript{27}.

\textit{f) Excitotoxicity plays a major role in neuronal damage in stroke and a number of progressive neurodegenerative diseases:}

Stroke is the fifth leading cause of death and it is the major cause of disability in the United States\textsuperscript{29}. Each year, 80,000 of people have a stroke in the United States and majority of these patients are over 65 years old. The percentage of African Americans who die from stroke in mid-life is much higher than the Caucasian population; in addition, stroke is more prevalent in
women than men\textsuperscript{20,30,31}. There are two kinds of strokes: hemorrhagic and ischemic. The majority of stroke cases (87\%) come from ischemic stroke. Ischemic stroke is a result of blockage of blood vessels, typically due to a blood clot; thus the lack of blood flow causes an energy shortage in the brain areas that are normally nourished by the specific blood vessel\textsuperscript{20}. The center of the area that stroke occurs is called core area. The cells in this area go through acute, severe, and irreversible injury and at the end necrotic cell death. In contrast, damage in the surrounding area (Penumbra) is more moderate, and cells can survive from the insult a little longer, and then some of them die from apoptosis while others recover and achieve long-term survival\textsuperscript{4} (Figure 6). One of the most effective ways of therapy for ischemic stroke is thrombolysis therapy (opening of blood clot using a drug). However, the disadvantage of this therapy is that it can only be used in the first two hours after ischemic stroke onset. This is because ischemic stroke causes a gradual deterioration of the blood capillaries, so that anticoagulation/thrombolytic treatment at this later time causes hemorrhagic stroke, which also called hemorrhagic transformation, in the patient that initially presented with ischemic stroke\textsuperscript{32–34}. 

![Core area and Penumbra area](Figure 6)
Figure 6: The affected areas during and after the stroke. The red area represents the core area where the stroke initiated and the cells die by necrosis. The penumbra area is affected as a result of excess amount of glutamate that come from core area, so they have more time to sense the upcoming insult and they die from apoptosis (Figure is adopted from 4).

In addition to the direct involvement of excitotoxicity as the most prominent trigger of neurodegeneration in brain ischemia, excitotoxicity is also seen as a contributing factor to cell damage in many other neurodegenerative diseases that are triggered by other factors. Indeed, excitotoxicity is considered a common neurodegenerative process in many neurological diseases, as neuronal damage spiral down and out of control, at the advanced stages of the disease. Nonetheless, beyond these common factors seen in many neurological diseases, excitotoxicity seems to have a special contribution to certain neurological diseases.

I) Amyotrophic Lateral Sclerosis (ALS):

ALS is a fatal adult-onset neurodegenerative disease that causes death between 3-5 years after diagnosis. ALS is one of the main neurodegenerative diseases in which motor neurons degenerate in the brain stem, motor cortex, and spinal cord, leading to difficulties in speech, swallowing and breathing. ALS is seen more in men than women and highly prevalent in the West Pacific 35,36. There are two forms of ALS, a familial form (10% of cases) and a sporadic form (90% of cases) 37. ALS is a result of many factors such as oxidative stress, apoptosis, inflammation, proteins aggregation, and excitotoxicity. In both sporadic and familial ALS, as well as in a number of mutation triggered-ALS animal models, GluT expression in astrocytes declines dramatically relatively early in disease progression 38. As stated previously, the malfunction of glutamate transports causes the accumulation of glutamate in the extracellular
space and therefore, it causes over-activation of glutamate receptors and influx of Na$^+$ and Ca$^{2+}$ into the postsynaptic cell. The excess amount of Na$^+$ and Ca$^{2+}$ causes over-excitation of cells and in the end it causes necrotic cell death 36.

II) Alzheimer's disease:

Alzheimer’s disease (AD) is a neurological disease that involves dementia and memory lost. AD was first discovered by Alios Alzheimer in 1907 and it is the seventh cause of death in the United States 39. There are two hallmarks for recognizing the AD: extracellular accumulation of β-amyloid (Aβ), and intracellular neurofibrillary tangles of a microtubule binding protein called tau 40. Aβ is produced from APP precursor protein by shedding of an extracellular segment, a process mediated by the enzymatic activity of the β-secretase complex. Aβ causes cell damage in a number of ways that involve Glu signaling. Prolonged activation of the NMDA receptor causes a change in the relative activity level of α-secretase (which processes APP in a different way) to β-secretase, and more Aβ production. In return, part of the toxicity of Aβ and its oligomeric aggregates is mediated by over-activation of NMDA-Rs, so that Aβ makes the neuron more sensitive to glutamate. Aβ also binds to the lipid membrane and produces an oligomeric pore in the neuronal lipid membrane which increases permeability to Ca$^{2+}$ 41,42 and at the end causes excitotoxicity. Another toxicity mechanism is that Aβ might disturb the functions of ATPase and glutamate transporters. Ultimately, the over-activation of GluRs and influx of Ca$^{2+}$ activate many pathways such as PKA, MAPK, and AKT 43, resulting in excitotoxicity. Activation of Aβ and NMDA together causes the over-activation of ER and thus increase Ca$^{2+}$ release from intracellular stores and disrupt its homeostasis, which may later cause the
Another connection between Aβ and Glu signaling is through the cellular prion protein (PrP^C), which is an anchor protein that is able to bind to many extracellular proteins. Aβ binds to the NMDA receptors through the C terminal and affects the kinetic of glutamate receptor-mediated currents. The N-terminal site has a region that is able to bind to Aβ proteins. Therefore Aβ binds to the NMDA receptor through PrP^C, and it causes a decrease in the normal rate of desensitization of NMDA receptors; thus Aβ uses several mechanisms to enhance entrance of excess of amounts of Ca^{2+} into the cells and at the end, it causes excitotoxicity and cell death. \textsuperscript{43,45}

III) Huntington's disease:

Huntington’s disease (HD) is a dominant, autosomal genetic disease. HD appears often in middle age but it can appear at any age. \textsuperscript{46} The symptoms include irregular involuntary movement, and dementia. \textsuperscript{46-49} HD is result of mutation in the gene HTT which codes for the Huntington protein. The HTT gene contains repeated triple nucleotides (CAG) that in healthy individuals is repeated 35 times; however, when there is a mutation, the stretch of (CAG) expands, and can be repeated 120-150 times. \textsuperscript{49} CAG is a codon for glutamine; thus the trinucleotide repeat makes a chain of glutamine, denoted poly glutamine (poly Q). \textsuperscript{46} HTT is subjected to break down by a lot of proteases and one of these proteases is calpain. Over expression of HTT in mice increases release of Glu and over-activation of NMDA-R increases the Ca^{2+} concentration inside of the cell. Therefore, when the caplain is activated and begins to break down the HTT protein, it causes overstimulation of GluRs, and at the end excitotoxicity.
There are several lines of evidence that show the relationship between the HD and excitotoxicity. The first sign is an increase in the concentration of Glu as a result of malfunction of glutamate transporters. There is also evidence that shows increase in the activity of NMDA-R. The last thing that showed the involvement of excitotoxicity in HD is malfunction of mitochondria, which prevents the mitochondria from buffering the intracellular Ca$^{2+}$ so that it is not able to produce enough ATP$^{49,51}$.

**g) Susceptibility to excitotoxicity is modulated by several pathways, including cellular aging and insulin signaling**

The involvement of excitotoxicity in a range of acute and chronic neurological diseases led to an active search to find a way to prevent cell death as a result of excitotoxicity$^{52}$. A popular strategy in clinical trials, supported by early success in animal models, was based on wholesale inhibition of glutamate receptors with antagonists; however, inhibiting glutamate receptors, did not decrease the neuronal cell death; furthermore, inhibiting GluRs caused high rate of morality in patients. These disappointments led to the surprising conclusion that glutamate receptors are vital for neuronal survival and recovery$^{53}$. This conclusion implies that there are specific neuroprotective, anti-excitotoxic pathways, some of which might be triggered by glutamate.

Another form of neuroprotection is provided by the resilience of young neurons. It was shown that aging affects the rate of cell death after excitotoxicity. For example, when researchers compared the penumbra area of young patients and elderly patients who had similar damage from initial stroke, they observed that a larger area of the cells were able to recover in young
patients compared to the elderly patients. It was further shown that the difference in neuronal recovery rate between old and young animals can be attributed to the cellular level. When slices of animal brain from young or older animals were subject to stroke simulation in vitro (by oxygen and glucose deprivation), the rate of their recoveries were different. The neurons in the brain slice from older animals have the tendency to recover much less than the cells from tissue of brains of younger animals; therefore, the researchers conclude that cellular aging also plays a role in neuroprotection. The regulation of organismal aging by the insulin signaling pathway was first discovered in C. elegans, where it was shown that inhibition of this pathway caused the worms to live longer and show more resistance to stresses. Researchers have shown that there is a relationship between the Glutamate receptors and insulin signaling pathway. Some of the effects of insulin signaling involve excitotoxicity-triggered apoptosis, while others involve control of glutamate receptor surface expression. For example, previous research showed that insulin plays a significant role in the trafficking of AMPA receptors in dendrites, which effects long term depression (LTD). In another set of experiments, Man et al. were able to show that there is a functional and physical connection between PI3K and AMPA receptors and that cooperation of these two pathways effect the production of Long Term potentiation (LTP). It is therefore important to take a closer look at the molecular components of the insulin signaling pathway.

**h) The Insulin Signaling Pathway (IIS) is a conserved cascade that translates extracellular signals to cytoplasmic & nuclear response.**

Although insulin is best known for its ability to regulate sugar metabolism in the body, insulin also has a profound role in modulating neuronal activity in the CNS and the course of neurodegenerative diseases. Insulin can pass through the Blood Brain Barrier (BBB) through
specialized carriers, enter the brain, and bind to insulin receptors. The insulin receptors are concentrated selectively in specific areas in the brain such as hypothalamus and cerebral cortex. Hypothalamus is the part of brain that is responsible for several functions, such as endocrine regulation, metabolic system, reproduction, and circadian rhythms. Glutamate is the main excitatory neurotransmitter that middle hypothalamus neurons use to communicate with each other. Glutamatergic synaptic transmission in hypothalamus contributes to insulin-induced sympathoexcitation, demonstrating important interactions between these two signaling pathways. The insulin signaling pathway is involved in many neurological diseases such as AD, PD, and HD. The insulin signaling pathway also plays a major role in learning and memory and also aging.

Insulin signaling is mediated by a signaling cascade that is highly conserved from nematodes to humans. Briefly, Insulin/IGF binds to insulin receptors and allows the recruitment of insulin receptor substrate (IRS). IRS binds to phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) which then changes the signaling lipid PIP2 to PIP3. PIP3 is able to recruit several Ser/Thr kinases to the plasma membrane and helps to activate these kinases. PDK-1 is one of the kinases, which is brought up to plasma membrane. PDK-1 then activates AKT, which is also brought up to the membrane by PIP3. AKT phosphorylates FOXO transcription factor and inhibits the translocation of FOXO from cytoplasm to the nucleus. (Figure 7). Since the details of insulin signaling are important for this thesis, it is worth examining the steps of this cascade in more detail.
I) Insulin signaling starts with binding to its receptors:

Insulin receptors (IRs) are part of a family of Receptor Tyrosine Kinases (RTKs)\textsuperscript{71} The subfamily of RTKs to which insulin receptor belongs contain two different isomers (IRs) and also the IGF-1 receptor\textsuperscript{72}. Ligand binding to insulin receptors causes subunit dimerization, cross-subunit auto-phosphorylation, and recruitment of IRS, an adapter protein. IRS contains several domains, including a PH-PTB domain (important for binding phosphatidylinositol lipids), and a SH2 binding domain (responsible for binding to the phosphotyalted tyrosine residues such as the one in the autophosphorylation site of the IR) \textsuperscript{73}.

II) Phosphatidylinositol 3-kinase (PI3K) \(\rightarrow\) PDK-1 \(\rightarrow\) AKT \(\rightarrow\) FOXO:

PI3K Phosphatidylinositol 3-kinase (PI3K) adds a phosphate to phosphoinositides found in the cell membrane. The best studied phosphoinositide is PI\(_{(3,4)}\)P2, which can be broken down by G-protein activated phospholipase C (PL-C) into the second messengers IP3 and DAG. In addition to the ability of the cell to break down PIP2, the cell can alternatively add yet another phosphate to the inositol ring, and create PIP3. This function is mediated by the enzyme PI3K. There are three classes of PI3Ks (I, II, and III). Each of these classes produces different phosphoinositides and each of these classes also contains some subfamilies. Class I of PI3K is studied the most and there are four different subfamilies in vertebrates and only one kind in \textit{C. elegans} and \textit{Drosophila} \textsuperscript{74}. The class I PI3K is the only one that produces phosphotidylinositol-3, 4, 5-triphosphate (PIP3), which is also a second messenger that activates several pathways. PIP3 then serves as an anchor to recruit phosphoinositide dependent kinase-1 (PDK-1) and
Protein kinase B (AKT) to the membrane by binding to the PH domain of these proteins; PIP3 brings these proteins to the membrane and thus activates them. When PIP3 binds to the PH domain of AKT, it changes the conformation of AKT; thus the PDK-1 can phosphorylate and activate AKT. AKT phosphorylates many different proteins including Forkhead-type transcription factor (FOXO). There are four different kinds of FOXO in the mammals, FOXO1, FOXO3, FOXO4, and FOXO6. AKT is able to phosphorylate three different sites of FOXO and by doing so changes its conformation, allowing it to bind to the regulatory protein 14-3-3. Binding of FOXO to 14-3-3 prevents FOXO from entering the nucleolus (Figure 7). FOXOs have diverse functions in the cell. They are involved in apoptosis, cell cycle, cell stress, and also longevity. These specific functions are determined by post-translational modifications. These post-translational modifications that FOXO can undergo are phosphorylation, ubiquitination, and acetylation.
Figure 7: Insulin Signaling Pathway. This pathway was first discovered in C. elegans is involved in many processes such as in longevity, cell division, and metabolism and cell stress resistance.

1) The Cytohesin/GRP-1 complex is a regulator of Insulin/IGF Signaling pathway (IIS):

Given the critical importance of insulin, it is not surprising that this cascade is subjected to upstream regulation, but our understanding of this regulation and its possible effect on different modes of insulin signaling is incomplete. Previous investigation of the metabolic effects of insulin in mammalian liver cells and its growth hormone-like effect on fly body size showed that a protein complex that contains the protein Cytohesin is a key regulator of the IIS pathway. The Cytohesin complex contains three main proteins: Cytohesin, Arf and PIP5K. As a result of activation of this complex, PIP2 is produced, which is a substrate for PI3K. Two previous studies that were done in Drosophila and mammalian cells showed that Cytohesin affects the IIS. In one of the studies, Fuss et.al (2006) screened for mutations that phenocopy the effect of insulin signaling mutations in the fly, i.e., reduced body size. Using epistasis analysis they showed that a mutation in Steppke (found to encode a homolog of Cytohesin) in Drosophila acts upstream of the IIS’s PI3K to regulate body size. Furthermore, they find that this mutation causes Akt to be under-phosphorylated and FOXO to enter the nucleus; therefore, they conclude that the Cytohesin complex acts upstream of the IIS pathway. In addition, Lim et.al showed that activation of the Cytohesin complex enhances activation of PIP3 synthesis and the activation of IIS pathway.
I) Cytohesin:

Cytohesins are Guanine Exchange Factors (GEF), which are able to catalyze the exchange of GDP to GTP on the small G-proteins known as Arfs. Cytohesins are involved in many cellular processes such as cell adhesion, cytoskeleton dynamics, the IIS pathway in *Drosophila* and mammals, and endocytic trafficking. There are four different Cytohesins in the mammals (Cytohesin1-4) and there is one in *Drosophila* and in the nematode. Cytohesin consists of several domains, two of which are: a) Sec 7: responsible for exchanging GDP to GTP, and b) PH domain which binds to Phosphatidyl Inositol (PI). The other two domains of cytohesin are the C-terminal helix (CtH), which overlaps with the other domain, Polybasic domain (PBR) (Figure 8).

![Figure 8: Different domains of Cytohesin.](image)

Cytohesin contains four domains. A Sec7 domain plays a role in exchanging GDP to GTP in Arfs, a PH domain helps Cytohesin to bind to the membrane by binding to the PI, a Coil-Coil domain for interaction with scaffolding proteins, and a C-terminal polybasic domain that contains phosphorylation sites (This figure is adopted from Hayallah, *Indian J Pharm Sci.* 76(5): 387–400, 2014).

Cytohesin is regulated by two intra-molecular interactions that cause autoinhibition. There are several different ways that Cytohesin becomes autoinhibited (Figure 9). In the first intramolecular autoinhibitory interaction, the linker between the PH domain and Sec7 domain...
(CtH) plays a role as a pseudosubstrate to inhibit the Sec7. The second intramolecular interaction involves the coiled-coiled section of N-terminal of the protein, \(^{83}\). Basic activation of cytohesin is mediated by binding of GTP-bound Arf of a certain subtype (Arf6) to the PH domain, allowing cytohesin to expand the repertoire of active Arf subtypes, creating a positive feedback loop to activate Arfs \(^{84}\). The activation of Cytohesin is then further stabilized by two phosphorylation reactions: phosphorylation by PK-C mimics the activation by GTP-bound Arfs. Additionally AKT phosphorylates Cytohesin on a conserved residue, threonine 276 in the PH domain \(^{83}\). Therefore, Cytohesin activation further produces a positive feedback loop through AKT. However, if there is a deletion in the Sec 7 domain, the activation of ARF by Cytohesin is prevented and production of PIP2 is blocked. When there is decrease in the production of PIP2, the IIS pathway will be inhibited, which affects the activation of AKT and prevents the positive feedback loop.

![Figure 9: Autoinhibition of Cytohesin](image)

**Figure 9: Autoinhibition of Cytohesin.** AKT can phosphorylate threonine in PH domain and relieve the autoinhibition. The second part of autoinhibition is relieved by phosphorylation of CtH by PKC (the figure is adopted from \(^{83}\)).
Cytohesins are connected to a scaffold protein called tamalin/GRP-1 association scaffold protein (GRASP)\textsuperscript{85,87}. Tamalin contains several domains: a PDZ domain, a leucine zipper domain, and a proline-rich domain. By binding to such a multi-domain protein, Cytohesin is able to connect directly with the postsynaptic receptors such as mGluR1 through the PDZ domain and it also can be connected indirectly to the NMDA receptor and AMPA receptor through the post synaptic scaffolding protein PSD95\textsuperscript{88,89} (Figure 10). PSD95 plays a significant role in the AMPA trafficking and it was shown that over-activation PSD95 has a significant effect on the AMPA receptor response in hippocampus slices\textsuperscript{88,89}.

![Figure 10: Tamalin is a bridge between GluRs and Cytohesin/GRP-1. The scaffold protein tamalin (GRASP) connects the NMDA receptor and mGluR-1 to Cytohesins.](image)

**II) ADP Ribosylation Factor (ARF):**

ADP ribosylation factors (ARFs) are small G proteins that are members of the Ras family. There are three classes of ARFs. Class I contains ARFs 1-3, class II includes ARF4-5, and Class
III includes ARF6. ARFs are involved in cell migration, vesicular trafficking, and cell division. They are activated by Cytohesins and are able to activate lipid kinases, and phospholipase D, and recruit cargo proteins. One of major proteins that is activated by ARFs is PIP5K, which produces PIP2.

III) Phosphatidylinositol 4-phosphate 5-kinases (PIP5K):

PIP5K, which turns phosphatidylinositol 4-phosphate (PI(4)P) to phosphatidylinositol 4,5-bisphosphosphate PI(4,5)P$_2$ is another member of the Cytohesin complex. PIP5K has three different isomers: PIP5kα, PIP5kβ and the last PIP5kγ. Various cellular stimuli, which include neurotransmitters, hormones and growth factors, can activate PIP5K to produce PIP2; PIP2 has many functions in the cell. PIP2 is hydrolyzed by Phosphoinositide phospholipase C (PLC) to produce diacylglycerol (DAG, stays in the membrane) and inositol 1,4,5-trisphosphate (IP3, released to the cytoplasm). Cytoplasmic IP3 binds to IP3 receptor/channels on the surface of the endoplasmic reticulum (ER) and allows Ca$^{2+}$ release from the ER, causing an increase in intracellular Ca$^{2+}$ concentration. PIP2 is also the substrate for the previously mentioned type-I PI3K. PIP2 also plays a role in cell development and programmed cell death (apoptosis).

The previous sections provide detailed information on a number of critical signaling cascades that control excitotoxic neurodegeneration and insulin-regulated susceptibility to cell stress. However, this description also indicates an inherent difficulty in the analysis of these cascades: many of the critical mediators of signaling come in a number of redundant versions, so that knock-out (KO) of single of gene encoding derivatives of these key enzymes/factors is ineffective, while combination KO of all its forms is often lethal. Moreover, intricate lines of crosstalk connect different cascades into a web that is difficult to tease apart and determine what
is a primary core effect and what is a secondary or tertiary side effect. Therefore there are many obstacles to studying molecular and cellular signaling events that affect and regulate excitotoxicity in mammals. It is therefore beneficial to use a model organism that has simpler signaling systems, and is yet able to elucidate excitotoxicity. Thus, we decided to use the nematode *C. elegans* to elucidate IIS function and regulation in excitotoxicity.

**j) C. elegans research offers strong tools and simplified signaling cascades that at their core are comparable to mammals:**

There is extensive homology between human and *C. elegans* genomes, and basic mechanism of many of diseases and molecular processes were discovered in the *C. elegans* and found to be similar to their parallel process in mammals\(^{94-97}\). Diseases such as depression, Alzheimer’s disease, and diabetes can be studied in *C. elegans*, and many molecular details of critical cellular processes such as Insulin signaling pathway, apoptosis, and RNAi were first discovered in the *C. elegans*\(^{95-97}\).

There are many effective research tools that can be used to study *C. elegans*. For example, the full sequence was determined for the in *C. elegans* genome, and gene function can be easily studied by knock down (RNAi) or by gene deletion / knock out (many mutant strains are available for free from a common genetic bank that distributes KOs, point mutants, and transgenic strains). *C. elegans* can easily be cultured; they have a high reproduction rate and short lifespan; thus, many samples and different generations can be studied. Since these worms are transparent, different processes can be studied in intact live animals, using fluorescence markers *in vivo*, including processes such as neurite growth or degeneration. Even though this organism is very simple, it still includes important types of tissues and organs. Importantly for
us, *C. elegans* has a nervous system which contains 302 identifiable neurons that fall into all the major neuronal categories \(^94,98\). Animals can be put in different stress condition such as over-population, elevated temperature, and lack of food. During stress, their larva can go into a durable and resilient form called dauer, and return back to normal development when conditions improve (figure 11), making worm maintenance in lab very easy \(^99\)–\(^{101}\).

The simple life cycle and maintenance of *C. elegans* in the lab, together with its ability to self-fertilize, enable animals with strong mutations that would be fatal in other animals to be viable in nematodes. This resilience allows us to establish mutant strains that harbor critical mutations, but are viable and can be easily studied. Moreover, the genome of the nematode contains much less redundancy of genes encoding key factors in signal transduction (i.e., it does not contain *several* genes encoding slightly divergent versions of the same receptors, scaffold proteins, enzymes, and transcription factors, just one of each). Therefore, a mutation in a key enzyme or factor is likely to have a prominent effect without compensation from similar versions of the protein encoded by other genes. Furthermore, cross talk between signaling pathways is not as sensitive as in mammals, making the difference between a primary effect of a mutation and a secondary or tertiary side effect more clear. In spite of this reduced redundancy and complexity, the most important part of, or the core of a signaling cascade, is usually astonishingly well conserved from nematodes to humans. This observation allows us to make the reverse deduction that the conserved core of a signaling cascade can many times be detected by virtue of its conservation from nematodes to humans. In this notion we assume that non-core parts of the signaling pathway had the opportunity to diverge during evolution, while the essential core of the pathway remains conserved (similarly to the logic of alignment of protein sequences, where the
core active domains are highlighted by their conservation). Lastly, the large body of data and reagents (such as numerous widely-available mutant strains with carefully documented phenotypes) make the screening of candidate genes very effective. In sum, *C. elegans* offers a particularly strong set of tools and benefits that make it ideal for the analysis of complex signaling cascades. A specific cascade which is particularly important to our research and is well conserved in the nematode is that triggered by the neurotransmitter glutamate.

**Figure 11. Life-cycle of *C. elegans*.** From inside the eggs through adult. [http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm](http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm)

**k) Role of Glutamate Signaling Pathway in *C. elegans*:**

Although *C. elegans*, like other invertebrates, use glutamate as both an excitatory and inhibitory neurotransmitter, the prominent extent of use of glutamate in central excitatory synapses in nematodes is unusual for an invertebrate. Indeed excitatory glutamate signaling mediates most of the communication between the sensory neurons and their postsynaptic
partners the command (i.e., decision making) interneurons, and further downstream the neuronal circuits to mediate behavioral responses \(^{102,103}\). The molecular building blocks that make up excitatory signaling by the neurotransmitter glutamate are highly similar between mammals and \textit{C. elegans}. There are ten genes encoding ionotropic glutamate receptors in the \textit{C. elegans}; eight of them (GLR-1 to GLR-8) are similar to the mammalian AMPA receptors, and two are similar to mammalian NMDA receptors (NMR-1 and NMR-2) \(^{104}\). There are three metabotropic receptors (MGL-1 to MGL-3) in \textit{C. elegans} \(^{105}\). Glutamate is removed from synapses by glutamate transporters in \textit{C. elegans}. There are six Glutamate Transporters (GLTs) in the \textit{C. elegans} (GLT-1; GLT-3-GLT-7) \(^{106}\). Because GLT-1 and GLT-4 are expressed close to synapses, they may collect the Glu from the synaptic area; therefore, this group of GLT is called proximal GLTs. In contrast, GLT-3, GLT-6 and GLT-7 are expressed in the excretory canal cell and the assumption is that this group transfers the Glu from the body fluid to the excretory canal cell to be disposed; thus these GLTs are called distal GLTs (\textit{glt-5::gfp} expression is very weak and could not be determined with certainty). GLT-3 is one of distal Glutamate transporters that plays a significant role in clearance of Glu. Glutamate synthesis in the \textit{C. elegans} is not very well studied; however, it is shown that the \textit{C. elegans} use pyruvate carboxylase (\textit{pyr-1}) (it is an enzyme that produces oxaloacetate, which is an important component of the Krebs cycle) \(^{107}\) to produce Glu. Nematodes have four kinds of glutamine synthesis (\textit{gln-1-3} and \textit{gln-5}) and also they have three different kinds of glutaminase (\textit{glna-1-3}). In the same way as seen in mammals, in \textit{C. elegans}, Glu is packed in the glutamate vesicular by glutamate vesicular transporter, such as \textit{eat-4} to be transported to the active zone and released into the synapse. Then, as mentioned in above, glutamate is reuptaken by the glutamate transporters \(^{108,109}\).
**1) Excitotoxicity in C. elegans:**

Since glutamate signaling is similar in *C. elegans* and mammals and *C. elegans* also provides a very valuable tool to study the biological pathways, we decided to use *C. elegans* as our model to study excitotoxicity. Mutation in any of the six glt genes (individually and a few of the possible combinations) did not result in discernable neurodegeneration, prompting us to search for conditions of increased sensitivity that might be more susceptible to neurodegeneration. Previously, it was shown that the over-activation of $G_{\alpha}S$ (using a transgene labeled *nuIs5*) caused a low level of necrotic cell death in neurons, a condition which is not dependent on $[\text{Glu}].^{110}$ However, by combining the over-activation of $G_{\alpha}S$ with deletion of $glt-3$ ($\Delta glt-3$), we were able to generate an excitotoxicity model in *C. elegans* model (Figure 12). In our model, we were able to show stronger necrotic cell death, which is Glu dependent and it is very similar to the mammalian condition $^{106,111}$ (Figure 10 a,b). Interestingly, combining *nuIs5* with either $glt-1$ or $glt-4$ mutations did not result in enhanced neurodegeneration, underscoring the importance of the distal GluT $glt-3$ in maintaining glutamatergic signaling in the nematode. To confirm that $glt-3$ is responsible for the necrotic cell death, we reintroduced wild type $glt-3$ ($glt-3^+$) and we were able to observe that the number of necrotic cell death was reduced to the basal level. In contrast to our view that neurodegeneration in *nuIs5* is Glu independent, Berger *et al* initially suggested that over activation of $G_{\alpha}S$ ($G_{\alpha}S^+$) in postsynaptic neurons by the *nuIs5[P_{gir-1}::Gas^+;P_{gir-1}::GFP]* transgene causes the glutamate dependent neurodegeneration, because a mutation in *eat-4* (but not a mutation in *gir-1*) causes a small reduction in the number of neurodegenerating neurons $^{110,112}$. The contradiction between the small effect of *eat-4* and the lack of effect of *gir-1* was suggested by Berger *et al.* to be the result of the use of other GluRs in
the degenerating cells. However, later on, when we inhibited all three of the major GluRs in these cells (using a combined glr-1; glr-2; nmr-1 KO) we were not able to observe any difference in the number of dying neurons in comparing nul5 vs glr-1; glr-2;nmr-1;nul5 animals. Therefore, we conclude that the neurodegeneration mediated by nul5 alone is not Glu dependent, and the decrease that was observed by Berger et al. in the eat-4 mutants is likely to be the result of the feeding defect in these eat mutants (as starvation is known to partially protect the worm from necrotic cell death)\textsuperscript{111}. In contrast, the additional extensive necrosis seen in these neurons upon addition of glt-3 KO to the nul5 transgene is completely Glu dependent, as demonstrated by the observation that glr-1;glr-2;nmr-1 KO reduces neurodegeneration in glt-3;nul5 animals back to the background level observed in nul5 single mutants. Since the extended necrotic neurodegeneration seen in glt-3;nul5 animals is triggered by the KO of the GluT gene glt-3 and is eliminated by KO of the GluRs expressed in these neurons, we consider this condition as a \textit{bona fide} model of excitotoxicity.
Figure 12. A strain carrying a mutation in glt-3 and the nuIs5 transgene (which contains an integrated \[P_{glr-1}::\text{Gos}^*;P_{glr-1}::\text{GFP}\] construct) increases necrosis cell death. The neurons that normally express glr-1 are visualized with GFP because nuIs5 is transgenic for \[P_{glr-1}::\text{gfp}\]. a) DIC picture of \(\Delta\text{glt-3};\text{nuIs5}\) shows necrotic neurons in the head (marked by red arrows). b. Fluorescence picture of \(\Delta\text{glt-3};\text{nuIs5}\) shows the glr-1 expressing neurons labeled with GFP.

Using this model of nematode excitotoxicity we showed that excitotoxic neurodegeneration involves a number of Ca\(^{2+}\) sensitive proteins. Calreticulin is a Ca\(^{2+}\) “sponge” expressed in the ER and its presence is needed for cell death. Triggering neurodegeneration also requires calcineurin \(^{111}\). The dependence of nematode excitotoxicity on the atypical adenylyl cyclase (acy-1) is particularly interesting, since this is a type-9 adenylyl cyclase (AC9) that is inhibited by Ca\(^{2+}\) through the action of calcineurin (while most adenylyl cyclases are directly or indirectly stimulated by Ca\(^{2+}\)). Calcineurin is a calmodulin-dependent serine/threonine protein phosphatase that is involved in many different cellular processing in different cells. In mammalian neurons calcineurin is known to dephosphorylate GluRs, leading to reduced single
channel activity. As in mammals, in nematodes calcineurin is pluripotent, and its effects include changes in egg laying, behavior, and aging. Based on the mammalian studies on the effect of calcineurin on GluRs and the apparent synthetic lethality of cnb-1 mutations (affecting a regulatory subunit of calcineurin) in the excitotoxic background, we suggest that calcineurin has two major functions in our model: A) calcineurin inhibits adenylyl cyclase type 9/acy-1. B) calcineurin dephosphorylates and reduces the activity of AMPA receptors. In our model, over activation of Gαs overcomes these functions; it causes adenylyl cyclase type 9 to keep activating PKA, leading to continuous phosphorylation of the nematode’s Ca²⁺ permeable AMPA receptors and causing cells to be hyper-potentiated. The combination of potentiated GluRs (due to phosphorylation) and hyper-stimulation by excessive Glu (due to GluT KO) results in GluR hyperactivation and letting excessive Ca²⁺ enter the cells. A similar scenario could take place also in mammalian ischemia, since hypoxia/ischemia is regularly accompanied by mass secretion of AMP and Adenosine, causing hyper-activation of Gαs-linked A₂A adenosine receptors, possibly causing GluR potentiation. As we mentioned before, lack of energy causes malfunction of GluTs and thus increase of [Glu] in the synapses. Excess amount of Glu now gates the hyper-phosphorylated GluRs, causing hyper-activation of GluRs and influx of excess amount of Ca²⁺ and Na⁺ into the cells. The excess amount of Ca²⁺ inside the cell causes excitotoxicity and necrotic cell death (Figure 13).
Figure 13. A model for excitotoxicity in C. elegans. a) Normal signaling. In low concentration of Glu and Calcineurin-mediated inhibition of AC9, AMPA receptors mediate only minimal currents; thus the level of $\text{Ca}^{2+}$ stays low inside of the cell. b) During excitotoxicity. High levels of Glu combined with over activation of $G\alpha_s$ will overcome the activation of calcineurin. Hence, PK-A keeps the glutamate receptors phosphorylated and potentiated, and thus susceptible to hyperactivation. The combination of GluR potentiation with their overstimulation by GluT KO-mediated Glu surplus causes excessive $\text{Ca}^{2+}$ influx and excitotoxicity (Figure is adopted from reference 111).

These observations demonstrate that physiological and pathological Glu signaling is well conserved in C. elegans and can be studied with great efficiency in this model to provide new insights. But how about neuroprotective signaling cascades and especially the cascade that is at the center of this thesis, the insulin signaling cascade?
m) The Insulin/IGF-1 Signaling (IIS) pathway is well conserved in *C. elegans*:

IIS is particularly well conserved from the *C. elegans* to the human. In nematodes, the insulin-like ligand binds to insulin receptor/DAF-2 and activates PI3K/AGE-1. AGE-1 phosphorylates PIP2 to PIP3, which becomes an anchor for PDK-1 and AKT-1 and brings them to the membrane. AKT-1 phosphorylates FOXO/DAF-16 and that causes FOXO/DAF-16 to bind to 14-3-3/FFT-2 and prevents FOXO/DAF-16 from entering the nucleus. When IIS is inactivated, FOXO/DAF-16 enters the nucleus and triggers transcription of specific genes 69,114–116 (Figure 14).

Reduction of function mutation of the dauer-entry controlling genes *daf-2* and *age-1* also causes increase in longevity of non-dauer adult worms; therefore, the IIS pathway is also called the aging pathway 117,118. However, IIS pathway does not only play a role in aging but also plays a significant role in cell stress resistance. Researchers were able to show that inhibition of the IIS pathway makes the worm highly resistant to elevated heat, heavy metal, bacterial infection and oxidative stress 56,119,121. When the worms go through stress, DAF-16 translocates into the nucleus and activates the transcription of many stress resistance genes such as antioxidant SOD 120 or heavy-metal stress tolerance genes (*mtl*) 122. Many of these stress conditions are sensed by neurons, which express high levels of the insulin receptor DAF-2 123, and inhibition of the IIS pathway causes neuroprotection and survival of neurons. We therefore suspected that the IIS
pathway may also play a role in excitotoxicity. *C. elegans* is a very good model to study these stresses; therefore, we decided to study the excitotoxicity and IIS-mediated neuroprotection in this model system.\(^{124}\)

**Figure 14. The comparison of IIS pathway between *C. elegans* and Mammals.** The figure lists the major components of the IIS pathway, which is involved in metabolism, development, longevity, and cell stress resistance.

**n) Insulin signaling plays a neuroprotective role in excitotoxicity in both nematodes and mammalian neurons:**

We did several studies with our collaborator Dr. Kalb to investigate the role of IIS in excitotoxicity in nematodes and in primary cultures of mouse spinal cord neurons and observed similar effects in both systems. In one of the experiments, we genetically inhibited the IIS pathway by using a mutant PI3K/age-1 (using the *hx546* allele, carrying a reduction-of-function
mutation that does not affect dauer entry under normal growth conditions). We combined this mutation with the excitotoxicity strain and then compared the number of dying cells between the excitotoxicity strain Δglt3;nuIs5 and the Δglt;nuIs5;age-1(hx546). We were able to observe that the number of degenerating neurons in the Δglt;nuIs5;age-1(hx546) decreases significantly compared to the regular excitotoxicity strain. We further studied the role of the IIS pathway in the excitotoxicity by using a drug called Psammaphysene A (PA), which was isolated in a chemical screen and prevents FOXO-1 from exiting the nucleus. We were able to observe that by using PA, DAF-16 accumulates inside the nucleus (DAF-16::GFP was visualized by using a transgenic worm, Pmyo-3::daf-16::gfp). We were further able to see that as we increased the concentration of PA, the number of neurodegenerating neurons decreased. PA had a similar effect on FOXO localization and neuroprotection in mouse neurons exposed to excitotoxicity.

These observations demonstrate that the IIS cascade controls nuclear localization (and therefore, presumably activity) of FOXO/DAF-16 and protection from excitotoxicity, with a strong conservation from nematodes to mammals. The hypothesis that stands at the center of my thesis is that upstream regulators of IIS identified as controlling IIS activity in insulin-dependent metabolism are also at work in controlling susceptibility to excitotoxicity in the nematode. It is therefore worthwhile examining the state of our knowledge of these factors in the nematode.

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**o) Cytohesin complex in C. elegans: Cytohesin/Grp-1 → Arfs → PIP5K/PPK-1**

**I) GRP-1:**

GRP-1 is the closest homolog of Cytohesin in the nematodes. GRP-1 has the same structure as mammalian Cytohesin, including a coiled-coiled domain, sec-7 domain, a PH
domain, and a C-terminal domain. The roles of GRP-1 were examined in a couple of studies in nematodes and it was shown that GRP-1 plays a role in asymmetric cell division in the neurons and it also plays a role in the apoptosis\textsuperscript{126,127}

II) ARFS→PPK-1:

As mentioned previously, ARFs are small G proteins divided into three different classes. There is one representative from each class in \textit{C. elegans}. ARFs are also involved in vesicular trafficking\textsuperscript{128} and are able to activate PIP5K\textsuperscript{79}. There is only one PIP5K homolog in \textit{C. elegans}, encoded by the gene \textit{ppk-1}\textsuperscript{129,130}. PPK-1 is localized to the plasma membrane. PIP5K/PPK-1 produces PIP2 which plays a role in microtubule structure, protein localization, and cell signaling. \textit{C. elegans ppk-1} is expressed strongly in neurons. Overexpression of \textit{ppk-1} during development disrupted the cytoskeleton of neurons in adult worms\textsuperscript{130}.

In my thesis I am trying to provide evidence for the significance of the cytohesin complex in the regulation of nematode excitotoxicity. I also try to provide preliminary evidence to a possible communication between Glu and IIS signaling. This concept combines two notions appearing separately in the literature: 1) GluRs signaling complexes are associated with a protein called tamalin, which also associates with Cytohesin; and 2) Tamalin, known in that field as GRASP, associates with Cytohesin in insulin signaling\textsuperscript{77,88}. To study these hypotheses, we investigate the effect of Cytohesin/GRP-1, ARFs, and PPK-1 on the involvement of the `IIS pathway in \textit{C. elegans} excitotoxicity.
Experimental Procedures:

Summary of Strains:

These strains were obtained from the the *C. elegans* Genetics Center and the Japanese National Bioresource Project, or they were gifts from the producing labs. Strain used include:

- **wild type** (Bristol N2), *Excitotoxicity strain, glt-3;nuIs5*: ZB1102 Δglt-3(bz34) IV; nuIs5[glr-1::gfp-1;glr-1::Gαs(Q227L) V; lin 15(+)] V; zfp-1 KO: RB774: Δzfp-1 (ok554) III; *grp-1 KO*: otIs114 Is [lim-6::gfp; rol-6(d)] I; otIs220 Is [gye-5::mCherry; rol-6(d)] IV; Δgrp-1 (tm1956) III;

- **arf 1.2 KO**: VC567: Δarf-1.2 (ok796) III; *ppk-1 Over Expression*: EG3361 (lin-15(n765ts) X oxIs12 [unc-4;::GFP, lin-15+] X; ggIs25 [rab-3::ppk-1, lin-15+] I. (oxIs12 [unc-4;::GFP, lin-15+] X; ced-4: MT2551 ced-4(n1162) dpy-17(e164)III; rrf-3 GRP-1::GFP: zdIs5; grp-1(gm350) II; gmEx361 [grp-1::gfp::grp-1]; PPK-1::GFP: UF60: gqIs35 [rab-3::ppk-1::GFP lin-15(+)] IV; GLR-1::RFP: odls16 [glr-1::GLR-1::rfp]; rrf-3 KO: NL2099: Δarf3(pk1426); DAF-16::mCherry:IsBp_CR264[daf-16b::daf-16b::LAP(Spep-TEV-mCherry)::daf-16-3’UTR::Cb_unc119]/IsB_pCR264[daf-16-p::daf-16b::LAP(Spep-TEV-mCherry)::daf-16-3’UTR::Cb_unc119]III.

These *C. elegans* strain were grown and maintained on MYOB plates that contains OP50 *Escherichia coli* bacteria in 20°C. Most genotypes in crosses were followed by PCR, based on the identification of deletion as producing shorter PCR products when amplified from genomic DNA. nuIs5 was followed by the GFP marker coexpressed with the GαS* from P_{glr-1::GFP}. This GFP signal in the interneurons can be visualized with a fluorescent stereomicroscope. The
*ced-4(n1162)* mutation was followed with the closely linked dumpy phenotype and later the mutation was confirmed by sequencing of the DNA. The crosses for localization were followed using UV optical scope.

**Analysis of Neurodegeneration:**

To quantify the level of neurodegeneration, the Mano and Driscoll method was used. Thus, a chunk of agar with a specific strain was flipped over and placed on the cover slip and Nomarski Differential Interference Contrast (DIC) microscope was used to quantify the level of necrotic neurodegeneration. To that end, I screened through the animals on the chunk. For each animal, I would identify the developmental stage (based on the structure of its vulva), and count the number of the swollen necrotic cells (that are similar to “vacuoles”) located in the head near the nerve ring or the neck (where RIG neurons are located). To confirm that “vacuolated” necrotic cells correspond to postsynaptic neurons, we occasionally confirmed the existence of *P_glr-1::gfp-1*. A minimum of 30 worms (with much higher numbers usually used in critical data-points) in each developmental stage (L1-adult) were counted and the statistical significance between the control group and experimental group was calculated using *z*-test. To verify that phenotypes are derived from the mutations that we monitor and not from unknown background mutations, critical new crosses were repeated independently and analyzed independently to confirm our results.
**LY294002 Treatment:**

LY294002 (from LC labs) was dissolved in 100% ethanol to make the stock solution of 25mM. 20µM of ethanol as a control and 20 µM of the stock solution were added to the 12 well plates that contain MYObgar and OP50 to produce the final drug concentration of 0.2mM. After absorption of ethanol +/- drug, control and test worms were placed on the plates. After 3 days, the level of excitotoxicity was determined using DIC microscope. These experiments were done several times for optimum results. LY294002 treatment also done to determine the effect of LY294002 on AMPA receptor trafficking.

**Wortaminnin Treatments:**

Plates with different concentrations (500µM, 100µM, and 10µM) of Wortaminnin were prepared. Later on, worms of the excitotoxicity strain \((\Delta glt-3(bz34) IV; nuIs5[\(P_{glr-1::gfp-1;P_{glr-1::G\alpha_s (Q227L)} V])\) were placed there and then after 2 or 3 days the level of neurodegeneration were observed using DIC microscope.

**SecinH3 Treatment:**

In this experiment, we collaborated with Dr. Kalb (U. Penn). The drug was supplied by Dr. Kalb, dissolved in DMSO and then plates with the concentration of 50µM of SecinH3 was prepared. Excitotoxicity strain worms were placed and after two or three days the level of excitotoxicity was determined using DIC microscope.
**Statistical Calculations:**

The number of necrotic cell death fluctuates because of conditions of growth, such as running the strain by re-chunking the strain over a long period of time, which suppresses the number of necrotic cell death. Thus, we have tried to use the fresh worms that were isolated very recently. Using these fresh chuck animals and comparing them with control animals which grow in the same condition as the tested strains helps us to prevent the variants between the experiments. Each bar corresponds to at least 30 or more than 90 animals (L3). In the necrotic neurogedegneration field standard error is used to show error bars. The Z test score was used to measure the statistical significant differences between strains and when there is statistical significant. It showed in the graph. We have tried to use excitotoxicity strain, which re-isolated from the new cross; thus, the similarities between the control and tested strain increase. The significant crosses were done twice in two different independent crosses and number of necrotic cell death was scored, to verify the effect of the stains.

**Fluorescence Microscopy Analysis:**

We used fluorescence microscopy to study the co-localization between GLR-1 and GRP-1 (using *P*glr-1::GLR-1::RFP and *grp*-1(*gm350*) II; *gmEx361* 1 [*Pgrp*-1::gfp::grp-1]); and between GLR-1 and PPK-1 (using *P*glr-1GLR-1::RFP and *P*rab-3::PPK-1::GFP,*lin*-15(+))IV. For that reason we did crosses and produce strains:GLR-1::RFP; *gmEx361* 1 [*Pgrp*-1::gfp::grp-1] and GLR-1::RFP; *Prab*-3::ppk-1::GFP *lin*-15(+). We placed the adult worms on 2% agar pad that contained sodium azide (that is able to paralyze worms in few second) and used an inverted microscope that has Nomarski differential interference contrast (DIC) and epifluorescence optics to take different sets of pictures (red and green) of these worms. We used the GIMP program to
prepared and overlay these pictures for analysis. We used these technique to study the effect of LY294002 (the PI3K inhibitor) on AMPA receptor localization by comparing $Pglr-1GLR-1::RFP$ with $Pglr-1GLR-1::RFP + LY294002$.

**Synchronizing the worm populations:**

To do behavior assays, worms were synchronized. Approximately 20 adult worms were placed in a plate that has OP50 and left there to lay eggs. After that the P0 worms were removed from the plate and the plate was placed in 20°C, so the eggs could hatch and worms could develop.

**Behavior Assays:**

**Nose Touch:**

Nose touch sensitivity is controlled by glutamatergic activity of the avoidance circuit. L4-adults synchronized worms were washed three times with M9 solution. Then an eyebrow hair attached to a toothpick was placed in front of the worms so that their nose would collide with the hair as they move forward. Nose touch sensation causes the worm to pause or retract. The number of worms that paused or went backward was counted and the fraction of responsive animals was calculated in each session. This experiment was repeated several times, so we would test 30 samples in each group.
Spontaneous Forward Mobility:

The worm’s spontaneous mobility is typified by forward runs that are interrupted by short reversals, a process controlled by the forward and backward mobility circuits. The balance between forward and backward runs (or the duration of forward runs until they are interrupted by a reversal) is regulated by glutamatergic activity in these circuits. In this study, we used the Maricq lab protocol and determined the duration of forward movement of worms by measuring the time after which worms switched from forward to reverse movement\textsuperscript{131}.

RNA interference (RNAi):

This procedure is adopted from Dr. Savage-Dunn’s lab. The RNAi treatment was done by feeding. On the first day, a single clone of specific RNAi-expressing bacteria was picked up from the Ahringer library and was placed on Luria Broth medium (LB) agar plates that contain ampicillin and tetracycline. The plates were incubated overnight at 37\(^{0}\)C. On the next day, a single bacterial colony was picked up and transferred to the 1ml of LB liquid culture which contains 50 μg/ml of ampicillin (LB+Amp) and incubated with shaking in 37\(^{0}\)C overnight. On the third day, the prepared bacterial over-night (stationary growth) culture was added to the fresh 4ml of liquid LB+Amp and then was incubated in 37\(^{0}\)C for four hours to grow the bacteria to logarithmic growth phase. After that, 450 μL of the bacteria culture was spread to the prepared RNAi plates that contained 400 mM IPTG (to induce RNAi production) and 50 μg/ml of ampicillin. On the fourth day, 4 -6 L4 worms were placed in these plates. On the fifth day these...
worms were transferred to the new RNAi plates and after they lay eggs, the P0 animals were removed and the plate was placed in 20 °C, for further analyzes.
Results:

1) Using RNAi to study the downstream targets of DAF-16:

In our previous studies we demonstrated that the IIS cascade regulates neuroprotection in nematode excitotoxicity in correlation with nuclear localization (and hence activation) of FOXO/DAF-16. We are very interested to know what are the downstream transcriptional targets of DAF-16 that mediate this neuroprotection. Moreover, another PhD student in the lab (K. Genevieve Feldmann) has shown that another transcription factor, CREB/CRH-1, also provides neuroprotection in nematode excitotoxicity. Since these two transcription factors can cooperate on the activation of specific transcriptional programs \(^{132}\), it is therefore possible/likely that critical neuroprotective genes are also co-regulated by CREB/CRH-1 and FOXO/DAF-16 when they exert their pro-survival effects. Previous studies by a number of groups in the worm aging field have pointed out a large number of genes regulated by DAF-16 \(^{133}\) while other studies examined the genes targeted by CREB/CRH-1 \(^{134}\). As an initial lead on possible neuroprotective genes, we compiled a list of 55 genes co-regulated by CREB & DAF-16. Although these genes were not identified in the same studies and their involvement in excitotoxicity has not been determined, they do provide a possible starting point in the search for co-regulated anti-neurodegenerative genes. Testing such a number of genes by crossing their genetic KO strains with our excitotoxicity strain is highly labor intensive. As an alternative, we wanted to develop a system to test the effect of knock-down of these genes in nematode excitotoxicity by RNAi. Previously, it was shown that the *C. elegans*’ neurons are not sensitive to the RNAi, but certain mutations increase RNAi sensitivity in neurons. Therefore, we tried to generate a strain that would make our excitotoxicity strain more susceptible to RNAi. To that end, we crossed the
RNAi-sensitizing mutations *rrf-3(pk1426)*\(^{135}\) or *nre-1, lin15b*\(^{136}\) with the excitotoxicity strain to make transgene double mutants of (*RNAi-sensitizing-mutation; glt-3; nuIs5*. To assess the efficacy of this approach we tried to test if our new strain is sensitive to neuronal RNAi by monitoring the RNAi effect of a few genes that we previously showed by genetic mutation to be involved in excitotoxicity: *crt-1*, and *glr-1* and *glr-2*. Due to difficulties constructing some of the other RNA—sensitizing strain combinations, I only have results on the use of *rrf-3*.

The genes that we used to test the efficiency of the RNAi effect were *glr-1* & *glr-2*, which encode subunits of AMPA receptors. The second gene was *crt-1*, which is a gene encoding calreticulin, which is involved in the storage of Ca\(^{2+}\) inside the endoplasmic reticulum (ER). The experiment for the *glr1-1; glr-2* on *rrf-3; glt-3; nuIs5* was done only once. In that experiment we were not able to detect any significant changes in the level of neurodegeneration between the regular excitotoxicity strain treated with control RNAi and excitotoxicity animals treated with GluR RNAi (Figure 15a). Although we saw some effects in early developmental stages, we did not observe any changes in the number of dying cells when we inhibit the *crt-1* in excitotoxicity strain in the most informative developmental stages (L3-L4, when most of the neurodegeneration takes place) (Figure 15b). We also note an overall lower level of neurodegeneration observed in these experiments: while in our previous experiment, the level of neurodegeneration in L3-L4 in the excitotoxicity strain is between \(\sim 3.5-5\) \(^{111}\) in our RNAi experiment the level of excitotoxicity in the control condition is between \(\sim 2-2.5\). These effects may be result of many factors, either anecdotal (such as that the strain may not be fresh enough, making the mutation’s effect weaker), or substantive (such as that the mutation in *rrf-3* reduces the overall level of neurodegeneration in the excitotoxicity strain). Therefore, we concluded that for confirmation these results, these experiments should be repeated. However, at this point
(given that *crt-1* and GluR RNAi knockdown did not suppress excitotoxicity compared to control) the current data suggests that including the *rrf-3* in the excitotoxicity strain did not turn it into being RNAi sensitive. We are also trying to cross the excitotoxicity with *nre-1;lin15b* or neuronally expressed *sid-1* (other strains that make the worms’ neuron sensitive to RNAi).

Screening the broad range of potential DAF-16 targets for their effect on excitotoxicity by RNAi therefore faces significant challenges, and at the moment is not feasible. In a related project conducted by a new PhD student in the lab, we are using another path to identify these specific neuroprotective genes by performing a Cell-type –Specific Transcriptome analysis. We hope that the more precise identification of neuroprotective genes in this approach (which is focused on genes that are co-regulated by CREB/CRH-1 under the specific conditions of excitotoxicity) will reduce the number and increase the informative quality of candidate genes, so that their effect can be studied by genetic KOs.

a)
b) No significant changes were observed in number of dying neurons in L3 when \( glr-1 \& glr-2 \); and \( crt-1 \) were knocked down in excitotoxicity strain.

2) The Insulin/IGF Signaling (IIS) Pathway has a protective role in nematode excitotoxicity:

Previously, we used an \( age-1 \) mutation or a drug called Pammaphysene A (PA) to activate / prevent the exit of DAF-16 from the nucleus. We were able to observe a reduction in number of excitotoxicity-induced necrotic cells, an effect also seen in both mouse neuronal cell cultures and also in \( C. elegans \) neurons \(^{124}\). The observations that FOXO3/DAF-16 activation leads to cell survival is in contrast to reports from mammalian groups, who used other IIS-inhibiting pharmacological agents, who showed that inhibition of the IIS pathway increases cell death via apoptosis in both neurons and other cell types \(^{139}\), raising the possibility that FOXO/DAF-16 activation might have different effects on necrosis vs apoptosis. Therefore, we have tried to
reconfirm the role of the IIS/Cell Stress Resistance Pathway in neuroprotection from excitotoxic necrosis. To study the effect, we did two manipulations: first we inhibited the IIS/Cell Stress Resistance Pathway in the excitotoxicity strain glt-3;nuIs5 using the same pharmacological agent used in the mammalian studies, and secondly we over-activated the IIS/Cell Stress Resistance Pathway in the excitotoxicity strain glt-3;nuIs5 to test if we observe the opposite effect.

2a) Pharmacological Inhibition of the insulin signaling pathway using LY294002 shows that inhibiting AGE-1 signaling is neuroprotective.

We inhibited the IIS pathway using LY294002, a widely used PI3K/AGE-1 inhibitor, in our excitotoxicity strain\textsuperscript{75,140}. In addition to its wide use in mammalian studies, LY294002 was successfully used also in \textit{C. elegans} aging studies\textsuperscript{75}, it is cell permeable and its action is reversible\textsuperscript{80}. We placed animals from the excitotoxicity strain on a plate that contains 0.2mM of LY294002 (in accordance of previously determined effective concentrations) and observed the level of neurodegeneration (daily transferring the worm to fresh LY295002 or control plates and observing neurodegeneration levels after 3 days in the presence of the drug or solvent). Placing the excitotoxicity strain animals onto plates that contain Ethanol, the solvent in which the drug dissolved, caused a small (but significant and reproducible) reduction in the level of neurodegeneration; however, there was an additional reduction in neurodegeneration in plates that also included the drug itself (Figure 16 a,b), showing that inhibition of the IIS pathway caused a significant reduction in number of dying neurons in the head of \textit{C. elegans} compared to the sham-treated animal. Therefore, we were able to show that inhibition of the IIS with a widely
used anti-PI3K/AGE-3 drug reduces the extent of excitotoxic necrotic cell death (Figure 16 and Table 1).

a)
**Figure 16: Inhibition of IIS using LY294002.** Worm treated with LY294002 showed a significant reduction in the neurodegeneration in the head of L3 and L4 development stage compare to sham-treated worm. The experiment was done twice (months apart, with different stocks of the excitotoxicity strain). a is the first trial and b is the second trial. In all the bar graphs the standard Error showed by the bar and the *** represents p<0.01.

2b) Wortmannin is not an effective drug in nematode excitotoxicity.

Wortmannin is more potent than LY294002 when used in mammals, but unlike LY294002, there are no published reports of it being used in the *C. elegans*. In this experiment, we have used three different concentrations of Wortaminnin: 10µM, 100µM, and 500µM, to determine its effect on the excitotoxicity (Fig 17 a,b,c and Table1). First we tried 10µM and we did the experiment twice (Figure 17a). Then we tried to treat the worms with the 100µM of drug and we repeated this experiment three times (Figure 17b). In the last trial, we placed the worms in the 500 µM and we tried it only once (Figure 17c). Then, we counted the
number of necrotic cell deaths in the head of worms. Generally we did not observe any changes in the number of necrotic cells between the sham-treated worms and experimental worms.

The only effect that we observed was in the L4 in 10 μM (figure 17a); however, we are not certain that we are observing the real effect because the results from the first trial was different from the second trial and changes did not also appear in higher concentrations of the drug. Based on these current results, we have no reproducible evidence to suggest that wortaminnin affects excitotoxicity. Wortamannin may not be effective as a result of few factors, such as a wrong concentration, inability of the drug to pass through the cuticle, rapid drug metabolism in the worm, or lack of affinity of this inhibitor to the nematode version of the PI3K enzyme. Although we cannot determine the cause of lack of efficacy for wartmannin, the availability and effectiveness of the other PI3K inhibitor, LY294002, gives us a sufficient tool for our experiments.
c)

**Figure 17: Inhibition of IIS using Wortmannin.** The worms were treated with three different concentrations. **a.** Worms exposed to 10µM, some changes were observed in the level of cell death in L4 developmental stage. **b.** Worms treated with 100µM of the drug. C. Worms were placed in Wortmannin 500 µM. In b and c no changes were detected in level of excitotoxicity.

2c) **Over-activation of IIS/ Cell Stress Resistance Pathway by KO of zfp-1 causes excessive degeneration.**

Zinc finger protein 1 (ZFP-1) in *C. elegans* is a homolog of the mammalian protein AF10\(^{141}\). ZFP-1 is a regulatory protein that under stress conditions inhibits the expression of *pdk-1*\(^{142}\) protein in the IIS/Cell Stress Resistance pathway, allowing DAF-16 to translocate and stay in the nucleus. When DAF-16 goes to the nucleus, it promotes the transcription of ZFP-1, blocking *pdk-1* transcriptional activation and causing a negative feedback on IIS pathway\(^ {141}\). This normally-operative negative feedback loop can be broken by *zfp-1 KO*, resulting in over-
activity of the IIS cascade and under-activity of DAF-16. We crossed our excitotoxicity strain with zfp-1 (ok554). We observed (Figure 18) that the zfp-1 mutation caused a significant increase in neurodegeneration under excitotoxic conditions, demonstrating that indeed over activity of the IIS cascade reduces the neuroprotective effect of DAF-16. These data complement our previous and current observations on mutations and treatments that increase DAF-16 nuclear localization increase neuroprotection. Therefore, contrary to mammalian data on FOXO3’s involvement in apoptotic cell death, we reaffirm that suppression of the IIS cascade and activation of FOXO/DAF-16 plays a role in neuroprotection from excitotoxic necrosis.

![Figure 18](image)

**Figure 18:** Over-activation IIS increases neurodegeneration in excitotoxicity strain. A KO mutation in zfp-1 caused an increase in excitotoxic neurodegeneration. ** represents p<0.05, *** represents p<0.01.
3) The Cytohesin/GRP-1 Complex is involved in regulating excitotoxicity

3a) Using a pharmacological approach to inhibit Cytohesin/GRP-1

SecinH3 is an inhibitor of mammalian Cytohesin 1-3 and their fly homolog Steppke. SecinH3 binds to the Sec-7 domain of cytohesins and prevents it from binding to ARF\textsuperscript{77,78,81}. Researchers working on insulin-induced metabolism have previously shown that SecinH3 affects the Insulin/IGF signaling pathway in flies and mammals, and prevents the exit of FOXO from nucleus\textsuperscript{77,78,81}. We wondered if cytohesin is regulating IIS signaling in the context of nematode excitotoxicity. We therefore used SecinH3 to see its effect on excitotoxicity.

We placed the worms onto the plates that contained 50µM of SecinH3 for the experimental group, and 50µM of solvent for the control. Then we observed the level of excitotoxicity after two or three days. Treatment of worms with SecinH3 (which is supposed to inhibit GRP-1), showed no effect on the level of neurodegeneration (Fig 19 and Table1). We cannot be sure if the lack of effect on excitotoxicity reflects a successful drug effect but no involvement of Cytohesin/GRP-1 in excitotoxicity, or an unsuccessful effect of the drug on the nematode protein. For example, it is possible that we used an inadequate concentration, since it was previously shown that the drug’s IC\textsubscript{50} for mammals and flies is different from the IC\textsubscript{50} in yeasts\textsuperscript{78}. Alternatively, maybe the drug was not able to pass through the cuticle, making it ineffective.
Figure 19. Inhibition of Cytohesin/GRP-1 by SecinH3. Worms were placed in the plate that contains 50 µM SecinH3 and the level of neurodegeneration was determined. There were no significant changes in the level of neurodegeneration.

Table 1

<table>
<thead>
<tr>
<th>Treatment with drugs</th>
<th>Dosages</th>
<th>Number of necrotic cell deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002 (PI3K/AGE-1 Inhibitor)</td>
<td>0.2mM</td>
<td>Reduced</td>
</tr>
<tr>
<td>Wortmannin (PI3K/AGE-1 Inhibitor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10µM</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>100µM</td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>500µM</td>
<td>No effect</td>
</tr>
<tr>
<td>SecinH3 (GRP-1 Inhibitor)</td>
<td>50µM</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Treatment of excitotoxicity strain with different inhibitors
3b) Mutation of *grp-1* reduces excitotoxic neurodegeneration.

Since we were not able to get a clear result by pharmacological inhibition of Cytohesin/GRP-1, we decided to use a different approach, and study the effect of Cytohesin/GRP-1 in excitotoxicity using a genetic mutation. For that reason, we made a transgenic double mutants by crossing worms with a mutation in *grp-1(tm1956)* with the excitotoxicity strain *glt-3; nuls5*. We observed a significant reduction in neurodegeneration in L3 and L4 stages (Figure 20 a,b and Table2). Therefore, we conclude that Cytohesin/GRP-1 plays an important role in excitotoxicity. We repeated the experiment in two completely independent isolates of this strain (obtained by separate crosses, months apart) and were able to replicate the results.

a)
Figure 20: Mutation in *grp-1* decreases the number of necrotic cells in excitotoxic conditions. a) Number of dying neuronal cells was monitored and major reduction was observed in L3 and L4 when *grp-1* is eliminated. b) The second trial with a new isolate of the strain and a fresh stock of control showed a similar effect. *** represents p<0.01  c) Images of nematode excitotoxic necrosis. On the right, an atypical example of an unusually high level of necrotic cell death occasionally seen in the *glt-3;nuIs5*. On the right: A typical level of necrotic cell death in the transgenic double mutants *grp-1;glt-3;nuIs5*. 
3c) **Mutation of ARFs can affect neurodegeneration in the excitotoxicity strain.**

ARFs are also members of Cytohesin complex; they are small G-proteins that activate PIP5K/PPK-1, which in turn produces PIP2. Cytohesin/GRP-1 is a Guanine Exchange Factor (GEF) of ARF: Cytohesin/GRP-1 binds to ARFs through its sec-7 domain, causing it to bind GTP, stimulating ARF and resulting in PIP5K/PPK-1 activation and production of PIP2. PIP2 is a substrate for PI3K. Therefore, we wanted to study the involvement of ARFs in excitotoxicity. There are several subgroups of ARFs. Most of the mutations in ARFs in the *C. elegans* are lethal and can’t easily be tested in our system; therefore, we examined the effect of the *arf-1.2 (ok796)* *ko* mutation, which is not lethal. It was shown that ARF1.2 works the same pathway as Cytohesin/GRP-1 and its function overlap the ARF class III (ARF-6) \(^{127}\). The transgene double mutants, *arf-1.2;glt-3;nuIs5* exhibited a significant decrease in neurodegeneration in L3 animals, compared to *glt-3;nuIs5* (Figure 21). These data further suggest that the Cytohesin complex is involved in excitotoxicity (Table2).
Figure 21: Mutation in arf1.2 decreases the number of necrotic cells in excitotoxicity strain. arf-1.2 was crossed with glt-3;nuIs5. The mutant Δarf-1.2;Δglt-3;nuIs5 showed a decreased level of neurodegeneration in L3 ** represents p<0.01.

3d) Over-expression of ppk-1 exacerbates neurodegeneration in the excitotoxicity strain

PIP2 plays a critical role in different cellular processes, such as serving as a substrate for production of second messengers by Phospholipase C (PLC), serving as a membrane anchor for protein localization, regulation of actin in cytoskeleton, ion channel activity, vesicle trafficking, cell survival, and cell death. PIP2 is also a substrate for PI3K, a member of the IIS pathway143. PIP5K/PPK-1 is a member of the Cytohesin/GRP-1 complex that catalyzes the production of PIP2. Thus, over-activation of PPK-1 increases production of PIP2, causing the over-activation of IIS. After finding that loss-of-function mutations in the Cytohesin complex reduce excitotoxicity, we wanted to see if its over-activation has the opposite effect. In our experiment, we used a strain that causes ppk-1 over-expression under pan-neuronal promoter (Prab-3::PPK-
I) (previously shown to cause also over-activity of the enzyme\textsuperscript{130}) and crossed it with our excitotoxicity strain to determine its effect on neurodegeneration. We found a significant increase in neurodegeneration (Figure 22 and Table 2). Excitotoxicity begins to increase in L4, and increases even more in the adult; While this effect appears later in development than other effects we follow (which usually peak at L3) it correlates well with previous studies, which showed that \textit{ppk-1} is mostly expressed in L4 and adult stages\textsuperscript{130}.

![Figure 22: Over-expression of \textit{ppk-1} in the excitotoxicity strain.](image)

Over expression of \textit{ppk-1} increases of necrotic cell death in the excitotoxicity *** represents \textit{p}<0.01.
Table 2

<table>
<thead>
<tr>
<th>Tested Genes</th>
<th>Number of necrotic cells death</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>zf-1</em> (ok554)</td>
<td>Increased</td>
</tr>
<tr>
<td><em>grp-1</em> (tm1956)</td>
<td>Decreased</td>
</tr>
<tr>
<td><em>arf-1.2</em> (ok796)</td>
<td>Decreased</td>
</tr>
<tr>
<td><em>P_{nub.2}:PPK-1</em> (over-expression)</td>
<td>Increased</td>
</tr>
<tr>
<td><em>ced-4</em> (n1162)</td>
<td>No changes</td>
</tr>
</tbody>
</table>

Crossing these genes with the Excitotoxicity strain and testing their effects on the excitotoxicity

4) The Cytohesin/GRP-1 complex regulates the IIS pathway in nematode excitotoxicity.

In the previous sections, I provided evidence to support that the components of the cytohesin complex regulate the extent of neurodegeneration in excitotoxicity. I would now like to demonstrate that the cytohesin complex exerts this neuroprotective effect by acting in the same pathway as the IIS cascade. I do this by performing an epistasis assay, to demonstrate that the effects of the Cytohesin complex and IIS are not additives, so that blocking this pathway in one stage gives the same neuroprotective effect as blocking the same pathway in two different stages of the cascade. To do this, I blocked the proposed cascade at the Cytohesin/GRP-1 level by a KO mutation, and at the AGE-1 level by using the inhibitory drug LY295002. We exposed *grp-1; glu-3; nuIs5* worms to 0.2mM LY294002, and looked for additive effects on excitotoxicity (Figure 23a). We saw no changes in the level of neurodegeneration when comparing *grp-1; glu-3; nuIs5*+ LY294002 (where the proposed pathway is interrupted twice) to controls (*glu-3; nuIs5*
+ LY294002 or grp-1;glt-3;nus5 + solvent, where the proposed pathway is interrupted in one point or the other) (Figure 23b). Therefore, we conclude that Cytohesin/GRP-1 works in the same pathway as AGE-1 to regulate neuroprotection in nematode excitotoxicity.
Figure 23: Epitasis study. to determine if Cytohesin/GRP-1 and AGE-1 work in the same pathway to affect the excitotoxicity. a). A schematic of our proposed pathway, where the cytohesin complex is upstream of the IIS in regulating neuroprotection in excitotoxicity. b. There is no additive effect of age-1 and grp-1 on excitotoxicity *** represents p<0.01.

5) Components of the Cytohesin/IIS cascade partially co-localize with components of the Glu signaling cascade

Our longer-term goal is to address a model on which the signaling in the Cytohesin/IIS cascade is functionally associated with Glu signaling at the synaptic level. Previous mammalian studies have shown that GluRs can physically interact with PI3K but the significance of such an association, whether it is mediated by the Cytohesin complex, and its effect on excitotoxicity remains unclear. As a first step in that direction, we wanted to check if such an interaction is possible by the physical presence of these two signaling cascades in the same cellular compartment by comparing the expression patterns of GLR-1 with that of PPK-1 and GRP-1. We used a strain of red-fluorescent–labeled GLR-1 that is correctly targeted to the postsynaptic density and is sensitive to receptor trafficking signaling dynamics. We crossed the GLR-1::RFP: (odls16 [P_glr-1::GLR-1::rfp]) with GRP-1::GFP: (grp-1(gm350) II; gmEx361 [P_{grp-1 ::gfp::grp-1}]) (Figure 24) and also crossed GLR-1::RFP: (odls16 [P_{glr-1::GLR-1::rfp}]) with PPK-1::GFP: gqIs35 [P_{rab-3::ppk-1::GFP lin-15(+)}] (Figure 25). Although these experiments do not show the direct association between glutamate receptors and the Cytohesin/GRP-1 complex; they do however, suggest that PPK-1 and GRP-1 are both co-localized with GLR-1 in post synaptic neurons.
Figure 24: Partial Overlapping expression of the GLR-1 subunit of glutamate receptor and Cytohesin/GRP-1. Anterior is left in all panels. The set of top panels shows a lateral view (dorsal is up), the bottom set of panels show a dorsal view of another animal. Left panels: GLR-1::RFP: (odIs16[Plglr1::GLR-1::RFP]). Middle panels: GRP-1::GFP: (grp-1(gm350) II; gmEx361 [Pgrp-1::gfp::grp-1]). Right panels: Overlay of the images shows co-localization of the two labels.
Figure 25: Partial overlapping expression of GLR-1 and PPK-1. In all the pictures the anterior side is on the left. Up and bottom panels are dorsal pictures of two different worms. Left panels: GLR-1::RFP: *(odls16 [P_gr-1::GLR-1::rfp]*). Middle panels: PPK-1::GFP: *Prab-3::ppk-1::GFP lin-15(+)*. Right panels: Overlay of the images shows co-localization of the two labels.

6) Alternative explanations to the effect of Cytohesin/GRP-1 on excitotoxicity are not supported by our observations.

Our observations suggest that the Cytohesin/GRP-1 complex regulates susceptibility to excitotoxic death so that its removal results in decreased death. We propose that this effect is mediated by the neuroprotective effect of IIS cascade interruption and the resulting increased protective effect of DAF-16. However, analysis of existing literature might suggest two alternative explanations to our data. a) Based on the studies of the Garriga group on the Q
neuroblast lineage, it is possible that Cytohesin/GRP-1 normally triggers apoptotic cell death, and the anti-apoptotic effect of its removal is neuroprotective. b) Based on mammalian studies of GluR surface expression it is possible that Cytohesin/GRP-1 & IIS directly control trafficking of GluRs, so that modulating the activity of the IIS signaling changes the balance of GluR trafficking, reduces the level of functional GluRs at the synapse, and reduces excitotoxicity. In this section, I present experiments that address these two possible alternative explanations of Cytohesin/GRP-1’s effect.

6a) Canonical apoptosis, which could be modified by Cytohesin/GRP-1, does not have a detectable role in nematode excitotoxicity.

A few studies show that Cytohesin/GRP-1 plays a role in apoptosis following asymmetric cell divisions of post-embryonic neuroblasts, where the KO of grp-1 causes the inappropriate survival of some of the neuroblast sister cells. If apoptosis is part of the process of cell death in nematode excitotoxicity, an anti-apoptotic effect of grp-1 KO will suppress neurodegeneration. To address the role of canonical apoptosis in nematode excitotoxicity, one of our lab members (John Del Rosario) crossed the ced-4(n1162) mutation with the excitotoxicity strain to create combination mutants ced-4;glt-3;nuIs5 (Figure 26). We assumed that if apoptosis is involved as an alternative route for neurodegeneration, then by inhibiting apoptosis we would have more cell death with necrosis. In contrast, if canonical apoptotic mechanisms mediate some of the steps in what we see as excitotoxic necrosis, then blocking canonical apoptosis will reduce the appearance of necrotic corpses. However, there was no significant difference in the level of neurodegeneration, regardless of the presence or absence of the canonical apoptosis mediator ced-4. Therefore, we concluded that apoptosis does not play a role in nematode excitotoxicity.
(Figure 26, Table 2), and the effect of grp-1 KO cannot, therefore, be attributed to its potential effect on apoptosis.

![Graph showing number of dying head neurons per animal across developmental stages]

**Figure 26: Data by John Del Rosario: Apoptosis does not play a role in nematode excitotoxicity.** The level of neurodeneration was measured in Δced-4; Δglt-3; nuls5; however, there is no significant changes in level of excitotoxicity.

**6b) The Cytohesin/GRP-1 complex and IIS pathway do not grossly modify the balance of expression and internalization of AMPA Receptors to decrease the level of neurodegeneration:**

Several mammalian studies showed that insulin and PI3K regulate trafficking of AMPA receptors in LTD and LTP. Thus, there may be an alternative explanation to the neuroprotective effect of grp-1 ko on nematode excitotoxicity claiming that inhibition of IIS causes changes in AMPA receptor trafficking, and ultimately reduced neurodegeneration. To provide a preliminary visual test of this theory, we treated the worms GLR-1::RFP:
(odIs16[GLR-1::RFP]) with 0.2mM of LY294002 (the same drug used in some of the experiments with mammalian AMPA-R trafficking) and then we studied the AMPAR expression in the nerve ring. We were not able to observe gross visual changes in AMPA receptor fluorescence labeling in the nerve ring when comparing between drug treated worms and sham-treated worms (Figure 27a). AMPA-R functional synaptic expression level (which is affected by receptor trafficking) can also be tested using a more sensitive behavior assay.

Behaviors such as nose touch sensitivity and the duration of spontaneous forward runs (or frequency of reversals) are mediated by glutamatergic circuits that are highly sensitive to the functional strength of the synapses. Therefore even small changes in GluR trafficking have strong effects on these behaviors.

Such behavioral studies were recently performed by a new lab member who is now continuing my project, Ayesha Chowdhury. Ayesha conducted two nose touch behavior assays; in the first assays, she compared the grp-1 with the control (Figure 27b) and in the second assay, she studied the effects of mutation of age-1 and Tamalin/gras-1 and compared them with the control (Figure 27c). However, Ayesha was not able to observe any significant changes in the worms’ behavior. Ayesha also did a forward mobility assay and in that experiment she compared the age-1 mutants and Tamalin/gras-1 KO with controls; nevertheless, there were no major differences in mobility between age-1 or gras-1 mutant animals and WT, suggesting that these mutations do not modify the level of activity of glutamatergic synapses (Figure 27 d).
a) 

GLR-1::RFP (Control)  
GLR-1::RFP + LY294002

b) 

![Graph showing sensitivity of animals between Control and grp-1 strains.](chart.png)
c) Figure 27 Determination of the effect of IIS and Cytohesin complex members on behaviors that are sensitive to the strength of synaptic glutamatergic signaling (usually modified by regulating GluR trafficking).
Figure 27. Determination of the effect of IIS and Cytohesin complex members on behaviors that are sensitive to the strength of synaptic glutamatergic signaling (usually modified by regulating GluR trafficking). a) The inhibition of IIS (using LY294002) did not affect GLR-1 expression (the image shows two different animals for each of the two condition, upper panels in lateral view, lower panels in dorsal view). b,c). Two nose touch assays were conducted to study the effect of IIS on GluRs; however, no significant differences were detected. d) The forward mobility behavior assay did not show any signification differences in the duration of spontaneous forward mobility.

7) A preliminary assessment of the effect of a mutation in grp-1 on the nuclear translocation DAF-16 faced technical difficulties.

The observations described above, contradicting other alternative explanations for our observations, therefore lend further support for our model for the involvement of Cytohesin/GRP-1 in nematode excitotoxicity. These observations therefore support that Cytohesin/GRP-1 stimulates the IIS cascade to prevent activation of FOXO/DAF-16 by its nuclear translocation. In a previous study we have demonstrated that indeed a drug-mediated body-wide nuclear translocation of DAF-16 correlates with neuroprotection from excitotoxicity. We now want to test if this correlation can be seen also with the grp-1 mutation and specifically in the dying neurons. Two previous studies, using Drosophila and mammalian liver cell cultures, showed that the mutation or inhibition of Cytohesin causes FOXO to translocate to the nucleus. We wanted to extend these studies to the effect of grp-1 in nematode excitotoxicity. Therefore, we decided to combine a grp-1 ko, a DAF-16 sub-cellular reporter (labeled with mCherry) expressed from a native daf-16 neuronal promoter (gifted from Dr. Ruvkun’s lab), and a GFP marker of the postsynaptic glr-1 expressing neurons. We crossed pCR264 [daf-16-p::daf-16b::LAP(Spep-TEV-mCherry)::daf-16-3’UTR::Cb_ unc-119]III with Pglr-1::GFP to produce Pdaf-16b::mCherry::unc119;Pglr-1::GFP to observe the effect on DAF-16
localization. However, the background mCherry of this particular DAF-16 reporter strain was very strong, labeling many cells, and exhibiting “clumpy” subcellular distribution, making it very hard to separate between two individuals cells and detect cytoplasmic vs nuclear expression (Figure 27). These results are therefore inconclusive, and we decided later on to use another strain (with a better cytoplasmic vs nuclear expression labeling in neurons \(^{147}\), using GFP labeled DAF-16 and DsRed label in glr-1 expressing neurons) to determine if grp-1 ko has an effect on DAF-16 translocation in postsynaptic neurons.

Figure 28: Potential effect of grp-1 KO on DAF-16 nuclear vs cytoplasmic localization. DAF-16 and P\(_{glr-1}\) expression in WT (upper panels) and grp-1 KO (lower panels) worms (in both sets of images, the anterior sides of worms is left and dorsal is up). In each set, panels on the left shows postsynaptic neurons expressing glr-1 (using a transcriptional reporter P\(_{glr-1}\)::GFP; the panels in the middle show DAF-16 expression (using a full length translational reporter and the neuronal daf-16b variatio pCR264[daf-16-p::daf-16b::LAP(Spep-TEV-mCherry)::daf-16-3’UTR::Cb_unc-119]III). The right panels show the overlay of these two markers. In the upper
set these reporters are expressed in WT background, in bottom set we express them in the *grp-1* (*tm1956*) background. The complexity of the DAF-16 signal prevents clear identification of nuclear vs cytoplasmic labeling in *glr-1* expressing neurons.
Conclusions:

In this thesis I study the effect of the IIS cascade and its upstream regulators (assembled into the cytohesin complex) on necrotic neurodegeneration in a *C. elegans* model of excitotoxicity. Previously it was shown that excitotoxicity plays a significant role in many diseases, such as Alzheimer’s, ALS, Huntington’s disease and stroke. Research in mammals showed that the IIS pathway has several functions in the body: metabolism, aging, developmental, and cell arrest \(^{139}\). Mammalian studies also showed that activation of AKT (a member of the IIS pathway) increases neuroprotection \(^{148}\) and FOXO is involved in the activation of apoptosis by activating the expression of a pro-apoptotic protein called *bim* \(^{149}\). On the other hand, *C. elegans* studies showed that inhibition of IIS pathway and activation of FOXO/DAF-16 reduces cell stress and increases cell survival and neuroprotection in a number of scenarios \(^{150}\). Even though we were previously able to demonstrate that inhibition of IIS reduces the neurodegeneration in the *C. elegans* excitotoxic necrosis model\(^ {124}\), because of the superficial contradiction with mammalian apoptotic neurodegeneration studies we decided to reaffirm these results. Indeed, we were able to show that inhibition of IIS pathway using the same drug used in the mammalian apoptotic neurodegeneration studies (LY294002) reduced neurodegeneration in the *C. elegans* excitotoxic necrosis model. Furthermore, over-activation of IIS pathway (using *zfp-1 ko*) increases the level of neurodegeneration. These two experiments reaffirm the involvement of IIS pathway in necrotic neurodegeneration in the excitotoxicity model. These observations suggest that although FOXO/DAF-16 might have a pro-death effect in apoptotic neurodegeneration, it has a neuroprotective effect in excitotoxic necrosis. Since excitotoxic necrosis is the main mode of neurodegeneration at the core of the stroke area, activation of
FOXO/DAF-16–triggered neuroprotective programs might offer a new approach to addressing critical excitotoxic damage.

We next tried to look at upstream regulators of the IIS cascade. Since it is potentially linked to Glu signaling, we focused our study on the effect of a Cytohesin/GRP-1 complex. We first studied the effects of inhibition of the Cytohesin/GRP-1 complex; thus, we crossed the *grp-1 KO* and *arf-1.2 KO* with our excitotoxicity strain and determined the level of excitotoxicity in these strains. We were able to observe significant reduction in the level of neurodegeneration in the L3 developmental stage. We then studied the opposite effect, where the Cytohesin complex is hyperactive. To this end we crossed the over-expression strain of *ppk-1* with our excitotoxicity strain. Indeed, we saw that *ppk-1* overexpression increased the level of neurodegeneration. However, instead of observing changes in L3, we observed increases in the level of neurodegeneration in the developmental stage L4 and adult. This result is comparable with a pervious study that showed that the changes in *ppk-1* mutants begin in L4 and adult and that is result of expression of *ppk-1* in the L4 and adult developmental stages.

To demonstrate that the Cytohesin/GRP-1 works in the same pathway as IIS pathway we did an epistasis test. We were able to show that the Cytohesin/GRP-1 complex works in the same pathway as the IIS pathway to affect the neurodegeneration.

Studies from other researchers suggest that there may alternative explanations for observing these results. For example, there is evidence that the Cytohesin/GRP-1 plays a role in apoptosis and PI3K has effects on trafficking of AMPA receptors. However, we were able to show that these factors do not play effective role in an excitotoxicity model.
Since our long term goal is to be able to determine the connection between glutamate and the Cytohesin/GRP-1 complex and their effects on neurodegeneration, we tried to observe if there is physical co-localization of GRP-1 or PPK-1 with the glutamate receptor subunit GLR-1. We were able to make two strains: in one of them GRP-1::GFP expressed with GLR-1::RFP, and in the other one PPK-1::GFP is expressed with GLR-1::RFP. We were roughly able to show some co-localizations between GRP-1::GFP and GLR-1::RFP and PPK-1::GFP and GLR-1::RFP. These results are preliminary. Further analysis of such colocalization requires better image analysis. Moreover, the concept of GluR-IIS cross-complexing and functional cross-talk can be further established using co-immunoprecipitation (co-IP) and disruption of potential complex by overexpression of partial protein fragments.

In conclusion, based on our studies, we propose that Cytohesin/GRP-1 complex affects excitotoxicity through the IIS Pathway, by activating the IIS and preventing FOXO/DAF-16 from entering into the nucleus and activating the genes involved in neuroprotection. In the future we hope that we would be able to recognize a cross talk between glutamate signaling and the IIS cascade through a scaffold protein that connects the glutamate ionotropic receptor to the Cytohesin/GRP-1 complex. We also hope to identify the genes that are activated by FOXO/DAF-16 and are involved in neuroprotection, with special emphasis on genes activated by both CREB and FOXO (Figure 29)
Figure 29: Schematic picture for a model for regulation of susceptibility to excitotoxicity by the Cytohesin complex. In this model we show how Cytohesin/GRP-1 complex and IIS are regulating the transcriptional program activated by FOXO/DAF-16 to provide neuroprotection from excitotoxicity.

Our data provides novel insights to the mechanism that regulates susceptibility to excitotoxic neurodegeneration and suggest that the Cytohesin complex is an important regulator of IIS-mediated neuroprotection. We hope to continue investigating this process and provide new directions in the study of excitotoxic necrosis in both nematodes and mammals, which might eventually lead to novel avenues of intervention in brain ischemia and stroke.
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