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Death- Associated Protein Kinase (DAPk); A Possible Modulator Of Neurodegeneration in A Caenorhabditis Elegans Model of Excittotoxicity

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Death- Associated Protein Kinase (DAPk): A Possible Modulator of Neurodegeneration in a *Caenorhabditis elegans* Model of Excitotoxicity.

A MS dissertation thesis

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Summary

Stroke is the third leading cause of death in the United States, exhibiting disproportional prevalence in minority communities. During stroke, restriction of blood supply to brain areas causes brain ischemia, generating a neurodegenerative cascade called excitotoxicity. Excitotoxicity is caused by the malfunction of glutamate (Glu) transporters (GluTs, a system that normally clears up the excess of excitatory neurotransmitter glutamate from the synaptic cleft). The malfunction of GluTs leads to a buildup of Glu in the synapse, thus overstimulating the Glu receptors (GluRs) located on the post-synaptic neuron. As a result, an overwhelming influx of Na^+ and Ca^{2+} causes toxicity in the post-synaptic neuron leading to necrotic cell death. Current treatments developed to ameliorate brain ischemia have very limited success and the molecular and cellular mechanisms that regulate the process of excitotoxicity remain largely unclear. Recent reports suggest that the process of excitotoxic cell death might be regulated by two related factors: Death-Associated Protein Kinase (DAPk, a critical master molecular cell death switch involved in several cell death pathways) and autophagy. However, the relationship between these two factors and the molecular basis for their potential role in excitotoxicity remain unknown. To understand the involvement of DAPk and autophagy in excitotoxicity, we simulate excitotoxic-like conditions in the nematode *C. elegans*, an effective and genetically accessible animal system. Glu-triggered neuronal necrosis in the nematode is generated in our lab by knocking out the GluT gene *glt-3* in combination with a sensitizing genetic background to cause an excitotoxic-like neurodegeneration. In my studies I have shown that absence of the nematode homolog of DAPk strongly reduces the levels of necrotic neurodegeneration while its overexpression exacerbates cell death. In contrast to the strong effect of DAPK, absence of autophagy only moderately affects neurodegeneration. These observations suggest that the lion's share of DAPk's effect on excitotoxicity is exerted by means other than autophagy. Of the many candidate pathways known to be regulated by DAPk I have selected a number of more promising ones (as they are known to regulate neuronal function) for further analysis. I am currently continuing our pursuit to elucidate the molecular mechanisms that lead to nematode excitotoxicity by looking at the possible DAPk-mediated signaling cascades and test their involvement in the process of cell death. Gaining insights into the molecular mechanisms by which DAPk is involved in nematode excitotoxicity might provide new clues to help us understand similar processes in mammalian brain ischemia, and might eventually suggest new strategies for therapeutic interventions in stroke.

Introduction

Stroke Pathology

Stroke is a major health concern in the United States. It is the third leading cause of long-term disability and hospitalization with a disproportional prevalence in minority communities[1,2]. In the United States, About 800,000 people suffer a stroke and ~18% of those people die from it every year[3]. During stroke, neurons are susceptible to massive neurodegeneration leading to tissue damage of different brain areas. Stroke-related brain damage is a progressively worsening process that causes gradual deterioration from the time of first symptoms, through patient admission to the hospital, to hours and even days later after stroke onset, giving us a window of opportunity for therapeutic treatments. However, the effectiveness of current treatments is very limited, and pharmacological trials to combat the neurodegenerative process were a great disappointment [4,5]. Typically, brain ischemia is initiated by a clot that obstructs the passage of blood to cerebral arteries, depriving brain areas of energy and causing extensive tissue damage. Neurons located at the core of stroke are acutely deprived of oxygen and glucose and suffer a harsh insult, causing them to die quickly by necrosis. In contrast, neurons located at penumbra (area located at the periphery of the damaged brain area) are stunned and vulnerable to die hours or days after stroke onset [6], [7]. Therefore, neurons located at these areas could potentially be protected from severe neuronal damage if adequate treatment is provided [8]. Elucidating the molecular and cellular mechanism during ischemia is still challenge, but understanding the events that triggers brain ischemia could help us to suggest strategies to diminish neuronal death.

Mechanism of Neurodegeneration by Excitotoxicity

During ischemia, one of the processes that seem to be more sensitive to the decrease in energy supply by glucose and oxygen depletion is the removal of the excitatory neurotransmitter Glu by GluTs. GluTs are secondary active transporters responsible for clearing the excess amount of Glu from synapses. Glu is mobilized across the membrane by co-transporting 3 Na^+ ions with one molecule of Glu and counter-transporting one K^+ ion[9,10,11,12]. GluTs depend on the $Na^+/K^+/ATPase$ pumps to provide Na^+ and K^+ gradients to bring Glu from the synapse into surrounding cells. Changes in Na^+/K^+ gradients can lead to malfunction of GluTs and increase the concentration of Glu in the synapses. The increase in Glu concentration in the synapses causes overstimulation of the three Ionotropic GluRs (ligand gated ion channels): N-methyl-D- aspartate (NMDA-R), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA-R) and kainate receptors (KA-Rs). The excessive depolarization of the post-synaptic cell by the over- stimulation of the AMPA-Rs and the unrestrained opening of the NMDA-Rs causes a large influx of Ca^{2+} and triggers a neurodegenerative process called excito-toxicity[13,14, 15] (Fig 1)

1)

Malfunction of the Mechanism of Glutamate Clearance Leads to Excitotoxicity
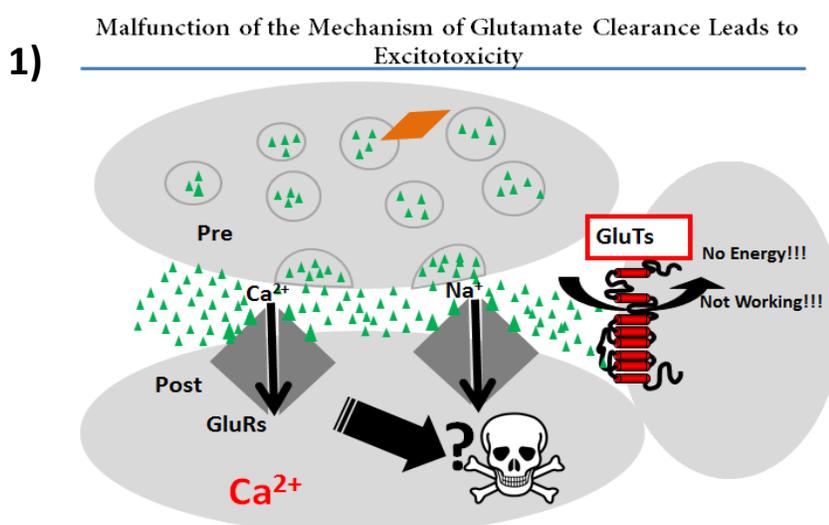
While neurons located at the core area of the stroke face a highly toxic Ca^{2+} overload, thus dying by necrosis, neurons located at the penumbra receive a smaller Ca^{2+} overload allowing them to die by apoptosis[16,17,18] (Fig 2) . The molecular and cellular mechanisms that govern neuronal cell death in excitotoxicity are still unknown. However, a number of research groups have recently reported that Death-Associated Protein Kinase (DAPk), a master regulatory molecular cell death switch, might be involved in promoting cell death decisions to enhance neurodegeneration.

2)
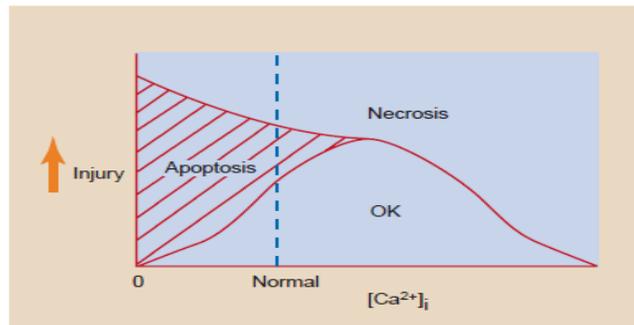
Lee and Choi, 1999

Death-Associated Protein Kinase: a master regulatory switch in cell death and other cell signaling events

Death-Associated Protein Kinase (DAPk) is a calcium/calmodulin (CaM)-regulated Ser/Thr protein kinase was initially identified as a tumor suppressor for its ability to promote cell death[19,20]. DAPk's -killing effect is activated by its dis-inhibition, which is regulated by several mechanisms, primarily a rise in intracellular Ca^{2+}. DAPk is activated by the binding of CaM to an inhibitory pseudo-substrate domain in DAPk, by desphosphorylation of an inhibitory autophosphorylation site within the CaM-binding domain (on Ser^{308}) [19,21-23], by ER stress[24], and by an intrinsic ROC domain responsible for dimerization and a RAS-like GTPase activity[21]. DAPk serves as a critical molecular cell death switch, due to its ability to select mechanisms of cell death such as apoptosis and autophagy; DAPk activation mediates the selective distinction between autophagy's pro –survival and pro-death roles [20,25,26]. In addition to DAPk's central role in autophagic cell death, DAPk also influences (by direct

phosphorylation or by protein-protein interaction) the activity of multiple critical signaling molecules[19,27,28]. These DAPk substrates/partners include regulators of autophagic and apoptotic cell death pathways (see below), and a number of proteins that can regulate glutamate signaling (Fig 3): *a)* Syntaxin-1[29] , a SNARE-complex protein regulating synaptic vesicle release[30,31]. DAPk phosphorylates Syntaxin 1 at Ser[188] , thus decreasing the binding of Syntaxin 1 to MUNC-18, a critical protein for synaptic vesicle docking[32]; b) CaMKK[33] , a CaM-activated Kinase Kinase that regulates the activity of cytoplasmic CaMK-I and nuclear CaMK-IV[34] (CaMKinases are known to be involved in Glu signaling). CaMKK activity is inhibited by DAPk ; *c)* ERK[35,36] , an extracellular-signaling pathway regulated MAPK[37] engaged in reciprocal inhibition with DAPk (MAPKs are known to regulate glutamatergic responses in the postsynaptic cell[38-41]) ; *d)* Pin1[42,43] , a parvulins-type propyl isomerase that changes the conformations of key phosphorylated proteins[44] and controls synaptic activity[45,46] and neurodegeneration[47-50] . The activity of Pin1 is inhibited by DAPK. Therefore, DAPk modulates the activation and function of a number of critical signaling cascades involved in cell death/cell survival pathways and neuronal activity.

Recent reports have also linked DAPK with brain ischemia and excitotoxicity[51]. During ischemia, the over-stimulation of post-synaptic Ca^{2+} -permeable GluRs and Ca^{2+}-sensitive phosphatase calcineurin[52] leads to the activation of DAPk. In contrast, inhibiting DAPk activation confers neuro-protection from excitotoxicity[53]. Some studies show that DAPk is a downstream effector of excitotoxicity and Ca^{2+} influx by its interaction with its inhibitor -DANGER/MAB-21(a protein that regulates the release of intracellular Ca^{2+} [54]through the IP3-receptor in the ER[55,56]). In contrast, other studies suggest that DAPk is an upstream regulator of Ca^{2+} influx by its interaction with the NR2B subunit of the NMDA receptor during excitotoxicity[57]. Therefore, although it is clear that DAPk

is a critical molecule for the modulation of excitotoxic neurodegeneration, its exact role and placement in the flowchart of the neurodegenerative cascade is still controversial.

DAPk Signaling Pathways that Can Regulate Excitotoxicity
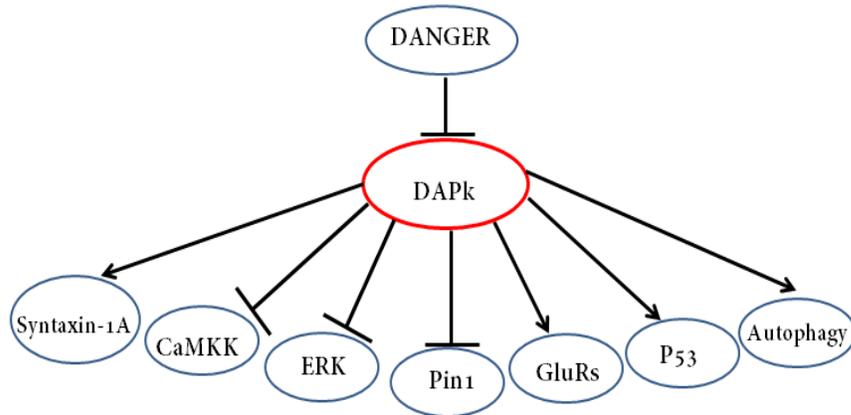
To start dissecting the possible role of DAPk in neurodegeneration by excitotoxicity, we selected DAPk-interacting partners that have a direct connection to known cell death pathways or can influence excitotoxic neurodegeneration by modulating glutamate signaling. One of the best studied pathways regulated by DAPk is the process of cellular degeneration during autophagy. DAPk directly modulates autophagic cell death by phosphorylating the BH3 domain of Beclin 1 (a key regulator of autophagy), thus promoting its dissociation from Bcl-2/X_L complex and activating the autophagy machinery [58,59] (Fig 4[25]). Since other lines of research independently suggest that autophagy might be involved in neurodegeneration, the autophagy pathway has the potential to be a point of investigation in neurodegenerative processes.

4)
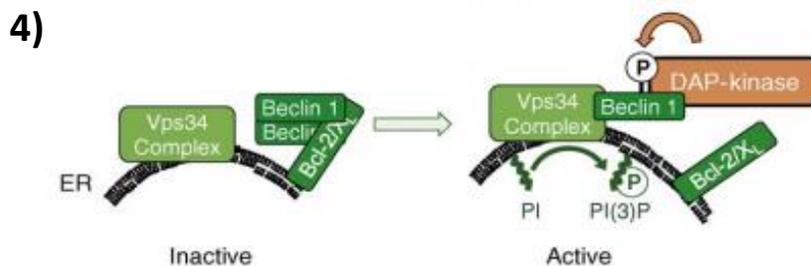
Autophagy: a Type-II Programmed Cell Death and A Possible Mediator of Excitotoxicity

Autophagy is an evolutionarily conserved "self-digestion" mechanism characterized by the digestion of intracellular organelles. During macroautophagy (the most studied and common type of autophagy) proteins and organelles subjected to degradation are engulfed by an inclusion membrane body called the autophagosome. The autophagosome is later fused to the lysosome where sequestered intracellular materials are degraded by lysosomal enzymes[60-66]. Autophagy was first described as a mechanism of cell survival during starvation to preserve the viability of the cell by sacrificing cellular components [67]. However, later on, emerging research described autophagy also as a mechanism of cell death (depending on the cellular circumstances) [68,69,70]. The autophagy pathway is carried out by three critical steps that are predominantly regulated by three specific proteins: 1) Induction step, regulated by Atg1/ULK1/UNC-51, initiates the formation of phagophores that triggers the establishment of the double membrane to sequester organelles. 2) Vesicle nucleation, regulated by Atg6/Beclin1/BEC-1, controls the recruitment of other proteins to start generating the inclusion membrane to form the phagophore. 3) Vesicle elongation involves the conjugation of lipid phosphatidylethanolamine (PE) with Atg8/LC3/LGG-1, allowing this protein to translocate from the cytoplasm to the growing autophagosome membrane. Atg8/LC3/LGG-1 then serves to anchor other proteins to the inclusion membrane and form a multiprotein complex (where proteins such as Atg5 and Atg16 play a critical role) for the completion of the autophagosome membrane (Fig 5[71]).

5)
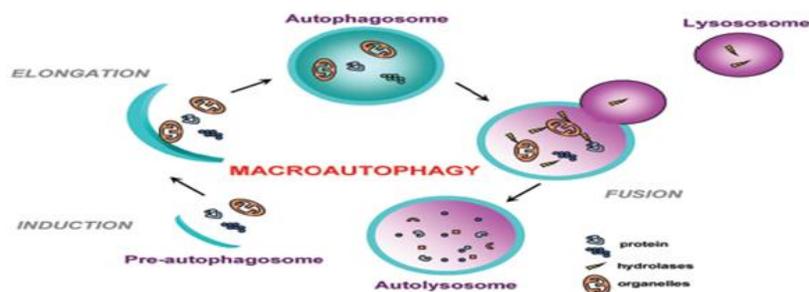
Autophagy has also been linked neurodegeneration during excitotoxic conditions. Some studies have shown that neurodegeneration triggered by brain ischemia can be accompanied by autophagy (indicating death *with* autophagy), while others point out that inhibiting autophagy could lead to a decrease in neuronal damage (suggesting death *by* autophagy)[72,71]. In Lurcher mice (mice that harbor a mutation that keeps certain GluRs constitutively active), an extensive depolarization in neurons leads to death of Purkinje cells by a process suggested to depend on autophagic cell death[73], though this suggestion has since met criticism[74,75] . It is therefore still unknown to what degree DAPk and autophagic cell death are (together or independently) involved in excitotoxicity. Facing many difficulties in trying to elucidate the sequence of molecular and cellular events that regulate the process of excitotoxicity in mammals, we instead focus our research on a genetically accessible simple animal system that exhibits a particularly strong track record in elucidating the correct sequence of cell-death processes and a reduced redundancy of signaling cascades.

Caenorhabditis elegans: A Powerful Animal System to Understand the Molecular and Cellular Events during Excitotoxicity

We use an invertebrate model system, *C. elegans*, that shows strong evolutionary conservation of a number of critical processes such as apoptosis, autophagy, aging, and RNAi [76-86]. Most importantly, *C. elegans* offers a unique model of excitotoxicity (see below) and is the only genetically accessible non-vertebrate to encode DAPk[19,87,88] (DAPk is not found in other invertebrates like *Drosophila*). Furthermore, unlike many invertebrates, *C. elegans* offers a unique opportunity to study excitotoxicity due to the high conservation of the components and the function of Glu synapses[89-98]. Indeed, in our lab we have previously developed a model of excitotoxicity in *C. elegans* by knocking out the GluT *glt-3* in a genetic background that renders neurons more susceptible to insult. This sensitivity is due to the presence of *nuIs5*[99], a transgene containing multiple copies of P$_{glr-1}$::*GαS** (expressing the constitutively active *GαS** in the post-synaptic interneuron under

the promoter of the *glr-1* gene) and P*glr-1*::*gfp* (expressing *gfp* in those post-synaptic neurons)[100]. We have shown that while animals carrying just a constitutively- active *GαS** in post-synaptic interneurons show necrosis of an average of ~1.5 head neurons/animal, Δ*glt-3*; *nuIs5* mutants show exacerbated neurodegeneration with an average necrosis of ~4.5 head neurons/animal (Fig 6). Based on the dependence of exacerbated necrosis on Glu accumulation (mediated by GluT KO) and on the presence of Ca^{2+}-permeable GluRs of post-synaptic interneurons we concluded that this exacerbated neuronal necrosis represents a *bona fide* form of excitotoxicity in nematodes. We further determined that this form of excitotoxicity probably requires the release of Ca^{2+} from intracellular stores / ER. Therefore, with the possible role of DAPk in cell death processes and its possible role in the release of Ca^{2+} from the ER, DAPk became the major focus on my studies.

6)

GαS*   Δglt-3; GαS*
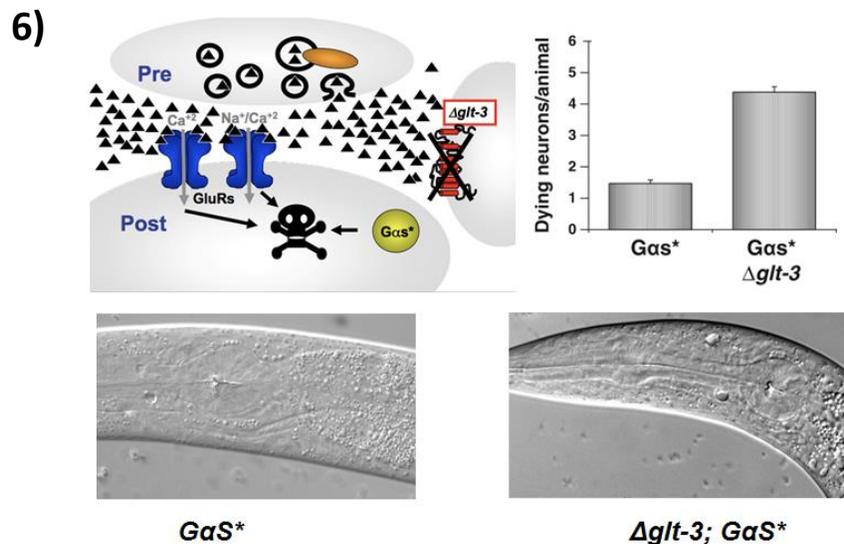
My current findings propose that although the mechanism of autophagy does not seem to be required for excitotoxic neurodegeneration, the level of neurodegeneration is dramatically linked to the activity of DAPk. The mechanism by which DAPk regulates necrotic cell death is still unknown, but my current focus is to clarify that issue by unraveling the mystery of DAPk signaling in nematode excitotoxicity.

Results

Death-Associated Protein Kinase (DAPk) Modulates Nematode Excitotoxicity

To test the involvement of DAPk in nematode excitotoxicity using a genetic approach, we combined our *C. elegans* model of excitotoxicity, *Δglt-3; nuIs5*, with a DAPk KO mutation - *dapk-1(gk219)*[101,102]. We looked at the extent of neurodegeneration in *dapk-1(gk219); Δglt-3;nuIs5* mutants and saw that *dapk-1 (gk219) KO* strongly reduces excitotoxic neuronal necrosis at all developmental stages, with an impressive ~50% reduction at the critical L3 stage (a stage previously shown to exhibit the highest levels of necrotic cell corpses, probably due to the maturation of Glu signaling and toxicity up to this developmental stage, and the removal of cell corpses in the following stages) (Fig 7a). In order to reaffirm the participation of DAPK in nematode excitotoxicity, we crossed *Δglt-3; nuIs5* mutants with the strain CZ9277 (*frIs7*[P_{nlp-29}::*GFP* ; P_{col-12}:*DsRed*] (*IV*) ; *juEx1933*[P_{hsp16}::*DAPK-1* ; P_{ttx-3}::*RFP*])[102]. This strain maintains DAPK constitutively active by overexpression, using the heat-shock-activated P_{hsp16}::*DAPK-1*. We generated a *Δglt-3; nuIs5; juEx1933*[P_{hsp16}::*DAPK-1* ; P_{ttx-3}::*RFP*] *(dapk-1^{wt})* strain and checked the level of neurodegeneration. We found the excitotoxic neurodegeneration induced by *Δglt-3; nuIs5* was exacerbated by ~60% in worms overexpressing DAPK-1 protein (Fig 7b), suggesting that DAPk indeed regulates the process of excitotoxicity.
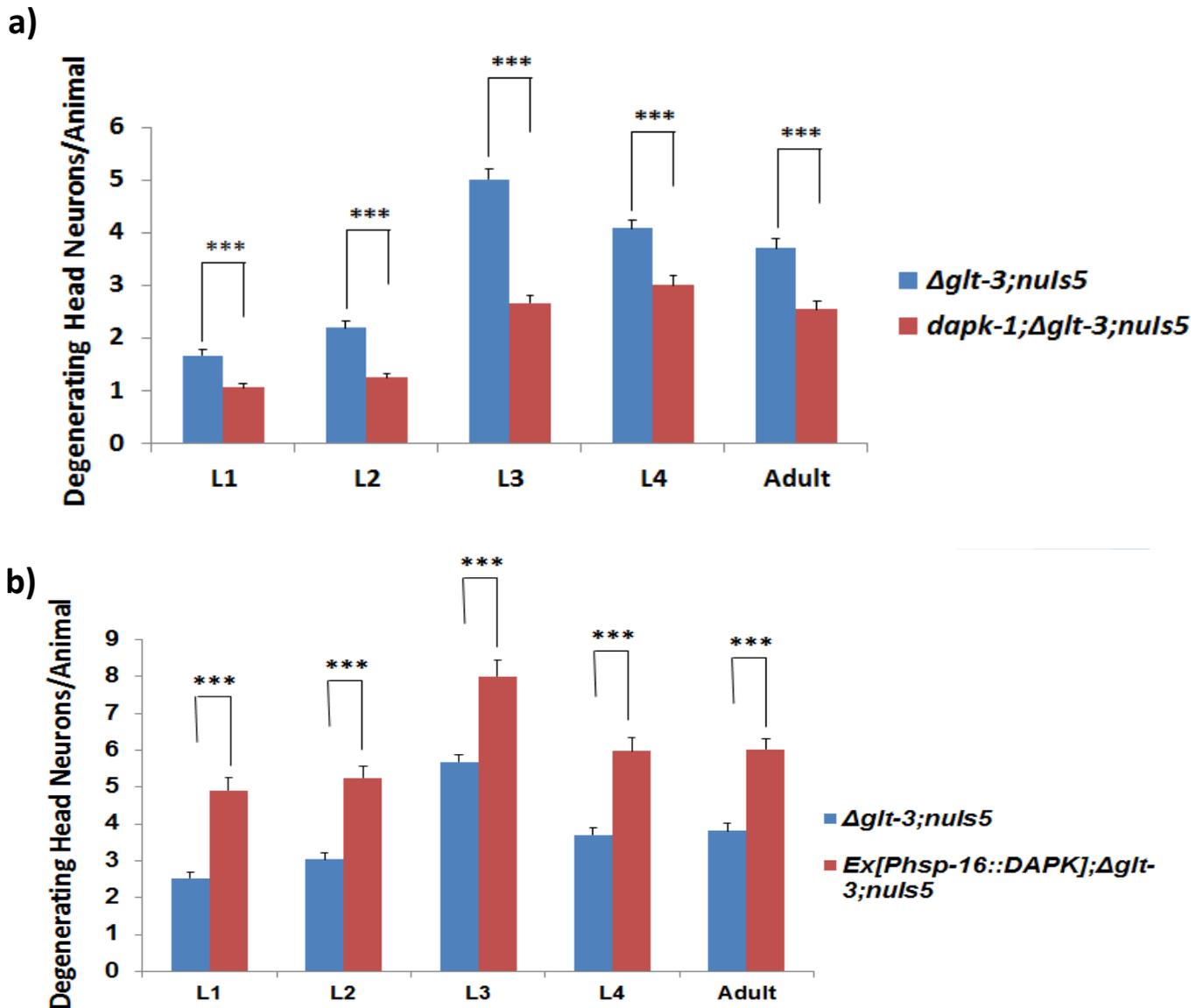
Figure 7

a)

b)

Figure 7 DAPk regulates neurodegeneration in nematode model of excitotoxicity. **a**, Average number of degenerating head neurons at all development stages (L1-Adult) per animal. Each bar represents ~90 animals carrying either *Δglt-3; nuIs5* or *dapk-1 (gk219); Δglt-3; nuIs5* mutations. Necrotic corpses were counted using Nomarkis/ DIC microscopy (in living animals without anesthesia). Result shows that blocking *dapk-1* strongly suppresses excitotoxic neurodegeneration from L1 trough Adult. ***, statistically significant difference using z test to compare our strain of excitotoxicity with our generated triple mutant strain, *p <0.001*. **b**, Number of degenerating neurons per animal. Each bar represents ~30 animals at each developmental stage. *Ex[P$_{hsp-16}$::DAPK]* animals carry an extrachromosomal construct triggering over-expression of DAPk following heat shock[102]. Control group are animals from the same plate (exposed to the same heat shock conditions) that do not have the extrachromosomal construct *juEx1933[P$_{hsp16}$::DAPK-1 ; P$_{ttx-3}$::RFP]* (as determined by the expression of red fluorescence in the AIY neuron from the *ttx-3* promoter) and are therefore only *Δglt-3;nuIs5*. Results show a higher level of necrotic cell death in *Δglt-3;nuIs5* animals when DAPk is over-active, confirming the role of DAPk in excitotoxic neurodegeneration.

Autophagy is not Required for Nematode Excitotoxicity

I next asked if DAPk regulation of excitotoxic neurodegeneration is correlated with a role for DAPk's most studied downstream pathway, autophagic cell death [103,104]. Independently of DAPk, autophagy was suggested to be a cell-death mechanism essential to the progression of other forms of neuronal necrosis in worms [105,106]. We tested the involvement of autophagy in our model of excitotoxicity by two different approaches. First, we combined our excitotoxicity model (double mutants *Δglt-3;nuIs5*), with *unc-51(e369)* to generate *unc-51(e369)*; *Δglt-3;nuIs5*. *unc-51* is one of the main regulatory genes in the autophagy pathway responsible for triggering the formation of phagophores to form the double membrane that sequester cellular organelles. Animals carrying the *unc-51(e369)* under the excitotoxicity background showed a very moderate though significant reduction in neurodegeneration in the L4 stage (but not in other stages) compared to control animals (Fig 8). Secondly, to further study the involvement of autophagy, we also monitored autophagy using a LGG-1/LC3 fluorescence flag. When autophagy takes place, LGG-1/LC3 aggregates in the cell in the form of puncta throughout the autophagosome membrane[107]. We used a DsRed version of the flag[108,109], *Ex[P$_{lgg-1}$:: DsRED::LGG-1 ; P$_{myo-2}$ ::GFP]*, where autophagy is reportedly marked by both puncta formation and overall increase in fluorescence intensity. Animals carrying *Ex[P$_{lgg-1}$:: DsRED::LGG-1 ; P$_{myo-2}$ ::GFP]* were crossed with *Δglt-3;nuIs5* mutants to generate *Ex [P$_{lgg-1}$:: DsRED::LGG-1 ; P$_{myo-2}$ ::GFP]; Δglt-3;nuIs5* as the experimental group, and *Ex[P$_{lgg-1}$:: DsRED::LGG-1 ; P$_{myo-2}$ ::GFP]; nuIs5* as the control group. If autophagy indeed mediates the 300% increase in neuronal cell death seen upon excitotoxic neurodegeneration, we expected an overall large increase in DsRed signal (in the form of intracellular puncta and overall fluorescence) in post-synaptic neurons undergoing excitotoxic necrosis. However, we observed only a slight, not statistically significant increase in DsRed intensity and in the number of neurons exhibiting puncta in *glr-1* expressing neurons when compared to the

control group (Fig 9a-c). These results suggest that autophagy is not required for nematode excitotoxicity, though low levels of autophagy may accompany excitotoxic neurodegeneration. Therefore, although DAPk is a major mediator of nematode excitotoxicity, autophagy, DAPk's most studied downstream signaling mechanism, is not tightly associated with Glu-dependent neuronal necrosis.

Figure 8
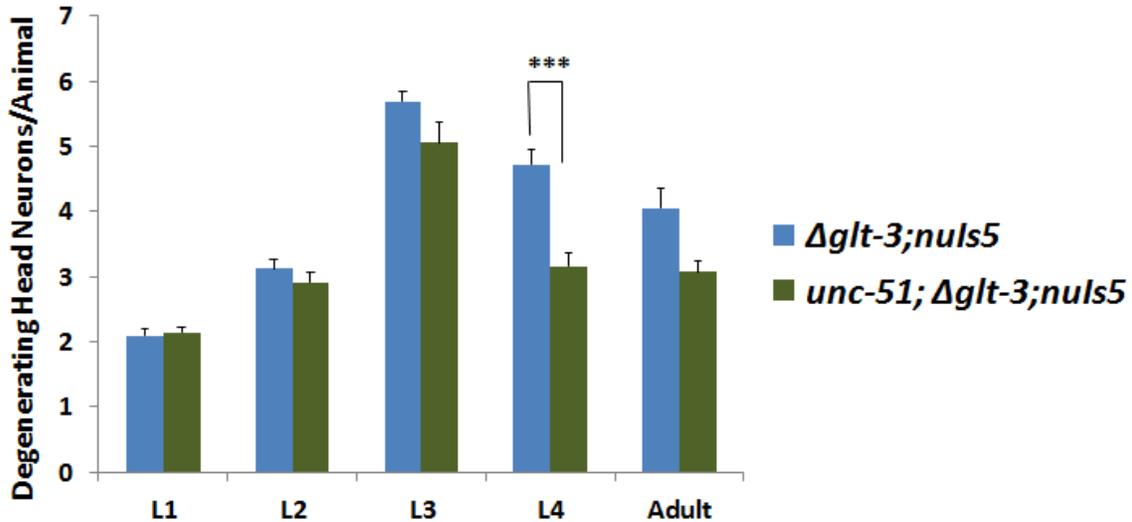
Figure 8, *unc-51* is not required for nematode excitotoxicity. More than 100 mutant animals (*Δglt-3;GaS* and unc-51; Δglt-3;GaS*)* at each developmental stage (L1- Adult) were evaluated on the extent of swollen degenerating neurons ("vacuolated" cells when using Nomarski/DIC microscopy). Results show that excitotoxic neurodegeneration is moderately reduced when autophagy is blocked by *unc-51* mutation only at the L4 stage. ***, statistically significant difference using z test to compare our strain of excitotoxicity with our generated triple mutant strain, p <0.001. N>100

Figure 9
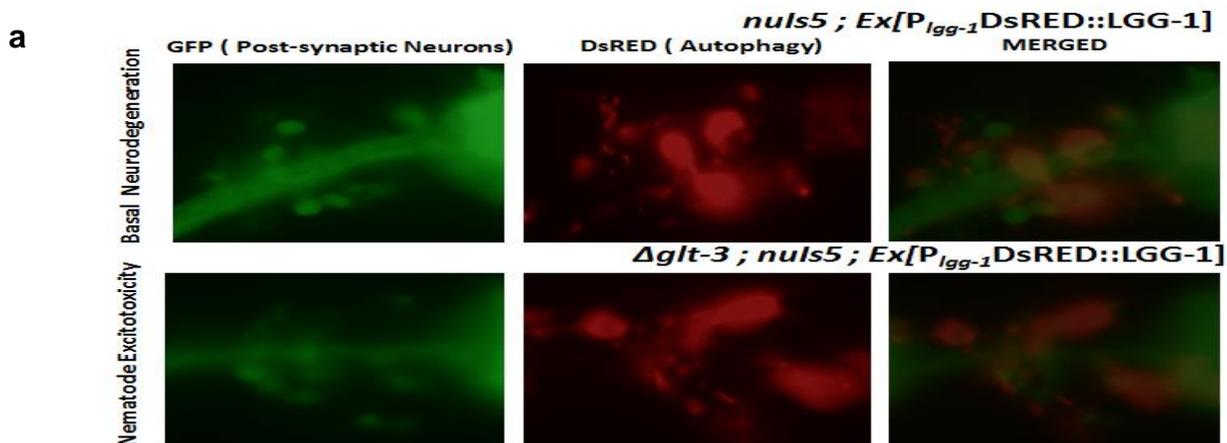
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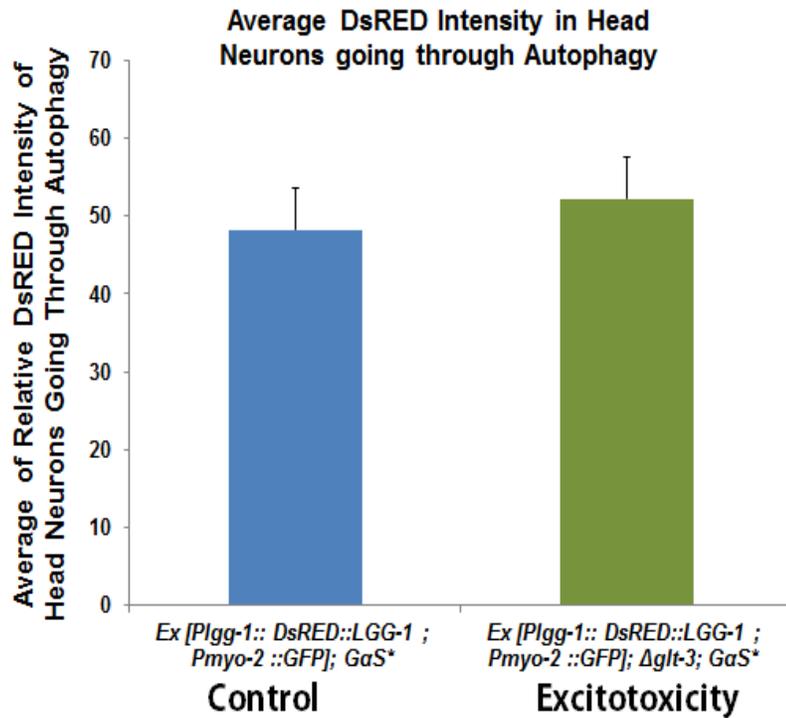
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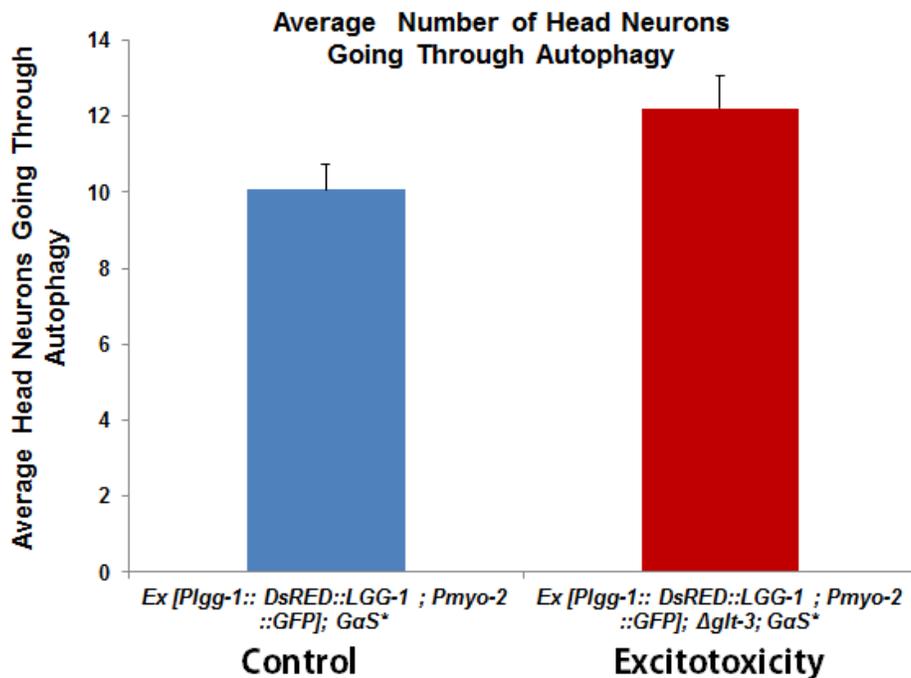
Figure 9, Autophagy is not a major feature of nematode excitotoxicity. **a**, LGG-1::DsRED expression on worms carrying either *nuIs5* (basal neurodegeneration) or *Δglt-3; nuIs5* (nematode excitotoxicity). DsRed expression was evaluated in P*glr-1*::*gfp* expressing neurons. **b-c**, ~25 L3-L4 worms from each generated strains (*nuIs5 vs Δglt-3; nuIs5*) were examined for the presence of autophagy by either determining the average DsRed intensity in post-synaptic neurons (**b**) or the average number of neurons showing puncta (**c**). No significant differences were found in the average DsRed intensity of control vs experimental animals, but there is a moderate increase in the number of neurons showing puncta.

DAPk does not modulate excitotoxicity by affecting synaptic vesicle release at the presynaptic cell and does not affect GluRs on the post-synaptic membrane.

Our current data shows that autophagy is not crucial for nematode excitotoxicity, suggesting that DAPk involvement in excitotoxic neurodegeneration is independent of autophagic cell death. However, DAPk is known to phosphorylate a considerable number of key signaling molecules, and a number of those are known to mediate neuronal signaling[19]. One of the proteins phosphorylated by DAPk is Syntaxin-1[29] , a SNARE-complex protein regulating synaptic vesicle release (although the functional consequences of Syntaxin-1 phosphorylation by DAPk remains unclear)[30,31]. Since DAPk-mediated regulation of synaptic vesicle release could control Glu accumulation in the synapse and increase post- synaptic activation of GluRs, DAPk KO could reduce the levels of excitotoxic neurodegeneration via its possible action on Syntaxin-1 in the pre-synaptic cell. In order to test this hypothesis, we used a behavioral / pharmacological approach, taking advantage of the observation that both DAPK-1 and Syntaxin-1 are broadly expressed in many neurons[102,110,111]. Therefore, a putative DAPk-Syntaxin interaction in the worm will affect synaptic vesicle release in all neurons, including cholinergic motor neurons. We therefore used WT and *dapk-1(gk219)* animals, and incubated them in the presence of aldicarb, a reversible acetylcholinerase inhibitor. Aldicarb causes worm paralysis over time by affecting the mechanism of acetylcholine breakdown, thus over-activating the acetylcholine receptors (AChRs) at the neuromuscular junction. Comparing Aldicarb sensitivity is a standard method of monitoring overall levels of synaptic release, because the more ACh is released the quicker the worms become paralyzed in the presence of aldicarb[112]. However, my results showed no significant differences between aldicarb sensitivity of dapk-*1(gk219)* mutants and N2 animals. Both mutants scored a similar time-course of aldicarb induced paralysis (Fig 10). Therefore, my data does not support the possibility of a DAPk phosphorylation-mediated effect on Syntaxin-1 and synaptic release in the worm.

As mentioned above, DAPk was found to regulate the Ca^{2+} entry through GluRs by interacting with the NR2B subunit of the NMDA receptors[57]. We reasoned that if by acting on GluRs DAPk is also an upstream regulator of excitotoxicity, DAPk is likely to be a regulator of normal GluR activity: DAPk might change the expression level, trafficking, or activity of GluRs at the membrane, thus increasing excitotoxicity in our model. We tested this hypothesis using a behavioral mobility assay, where we measured the time length of forward runs in spontaneous mobility (a movement regulated by glutamate signaling in AVA, AVD and AVE interneurons[113]). To perform this assay, we took L4 – young adult worms carrying the following mutations: *dapk-1(gk219)*, *nmr-1(ak4);glr-2(ak10),glr-1(ky176)* (animals known to be defective in backing response to tactile stimulation[89]) and N2 wild type animals. Our results show no difference in the length of spontaneous forward runs between dapk-*1(gk219)* mutants and N2 animals (Fig. 11). Therefore, my data do not support an effect of DAPk on the overall level of GluR activity in the worm.
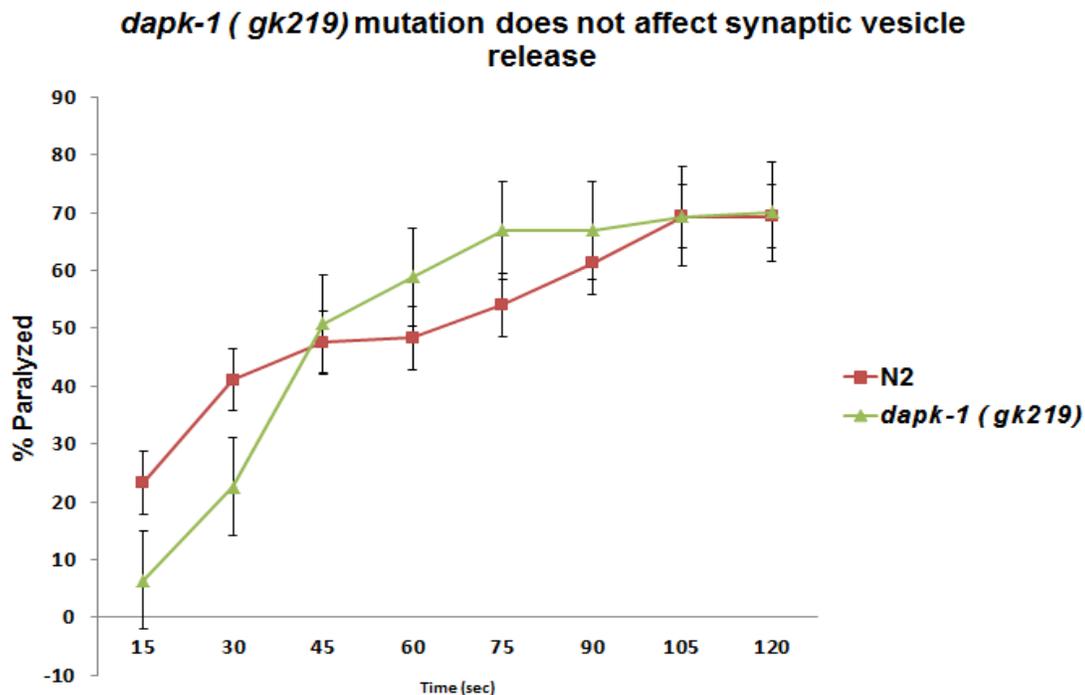
Figure 10

dapk-1 (gk219) mutation does not affect synaptic vesicle release
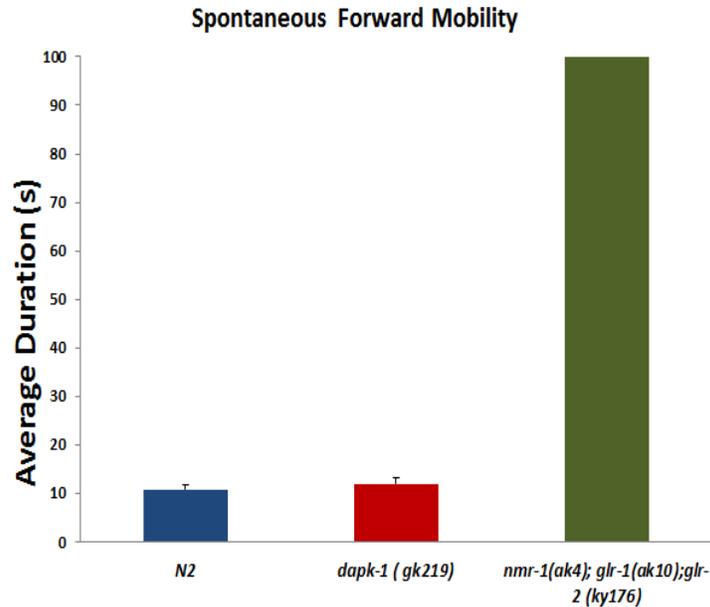
Figure 11

Spontaneous Forward Mobility

Figure 10-11, DAPk does not change synaptic vesicle releases or overall activity of Glutamate Receptors. **10)** ~30 L4- young adult worms from each corresponding strain were examined for possible changes in sensitivity to aldicarb. *dapk-1(gk219)* mutants behaved similarly to N2 wild type worms in their paralysis sensitivity to aldicarb (though *snt-1* worms showed high resistance to aldicarb, not shown).   **11)** At least 30 L4- young adult worms from animals mutant in *dapk-1(gk219)* or *nmr-1(ak4);glr-2(ak10),glr-1(ky176)* were compared to N2 wild type animals for changes in duration of forwards runs. No statistical significance was observed between *dapk-1(gk219)* mutants and N2 animals, but a large and strongly significant difference was observed when compared to animals carrying *nmr-1(ak4);glr-2(ak10),glr-1(ky176).*

CKK-1 does not Play a Role in Nematode Excitotoxicity

Recent findings using yeast two-hybrid experiments have revealed CaM- regulated protein kinase kinase (CaMKK) as a substrate with a high affinity phosphorylation site for DAPk interaction [33]. A possible inhibition by DAPk is especially significant because CaMKK phosphorylates CaMKI/IV, which activates the transcription factor CREB inside the nucleus to confer the activation of neuro-protective genes [114]. Another student in our lab (K. Genevieve Feldmann) has recently shown that CREB/*crh-1*KO confers neuroprotection in nematode excitotoxicity (unpublished data). We wanted to test whether a mutation in *ckk-1* (*C. elegans* ortholog of CaMKK) in *Δglt-3;nuIs5* or *dapk-1(gk219);Δglt-3;nuIs5* background could increase the level of nematode excitotoxicity . We crossed both mutants

and generated *ckk-1; Δglt-3;nuIs5,* and *dapk-1(gk219);ckk-1;Δglt-3;nuIs5* animals and compared their

level of excitotoxic neurodegeneration with animals carrying *Δglt-3; nuIs5* and *dapk-1 (gk219); Δglt-3;*

nuIs5 mutations serving as a control*.* Surprisingly, our results showed no difference in the levels of

necrotic neurodegeneration in the presence or absence of *ckk-1* (Fig.12). This implies that nematode

excitotoxicity may not be regulated at the level of CaMKK, and that DAPk does not regulate

nematode excitotoxicity through CKK-1.

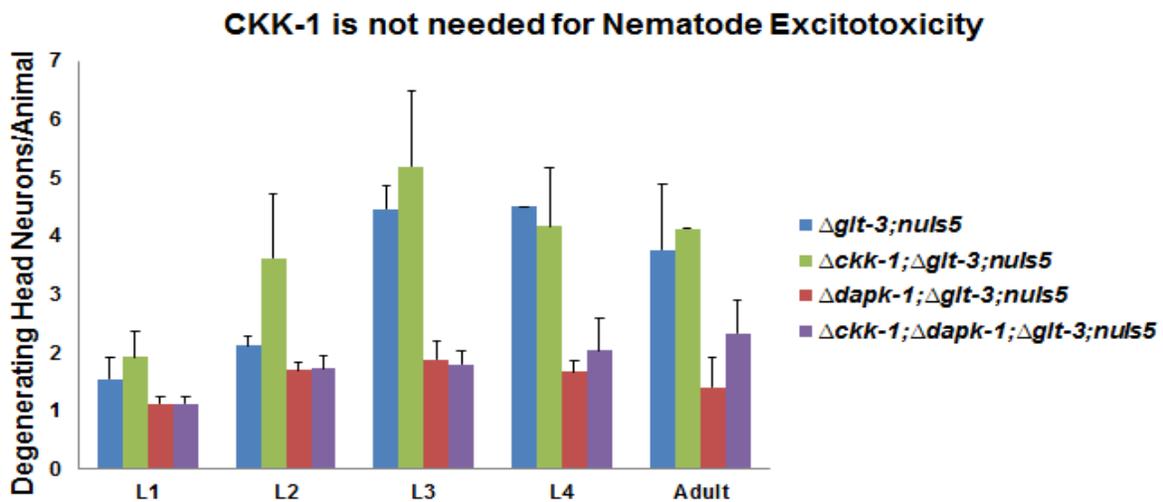**Figure 12**. *ckk-*1 does not modulate the level of excitotoxicity. About~40 mutant animals from each corresponding strain at each developmental stage (L1- Adult) were observed on the extend of swollen degenerating neurons ("vacuolated" cells when using Nomarski/DIC microscopy). Results show that excitotoxic neurodegeneration is not reduced nor increased by the inhibition of *ckk-*1 mutation.

Discussion/Ongoing Research

Death-Associated Protein Kinase (DAPk) is required for nematode excitotoxicity

Current mammalian research provides a relatively satisfying understanding of the initial synaptic events during brain ischemia, while offering much less information on the molecular events that lead to necrotic death following the over-stimulation of post-synaptic GluRs. Therefore, our focus is to elucidate the post synaptic events that regulate the mechanism of excitotoxic neurodegeneration using our novel, genetically-accessible model of nematode excitotoxicity. In the current studies we found out that inhibition and constitutive activation of DAPk modulates nematode excitotoxicity (Fig 7). Our data corroborates previous mammalian work, where DAPk is suggested to play a crucial role in excitotoxicity. However, we still do not know where DAPk is functioning to modulate neurodegeneration, nor do we understand the type of mechanism DAPk uses to regulate necrotic neurodegeneration.

An unexpected view of excitotoxic neurodegeneration

Since DAPk is known to control several signaling pathways, including pathways that promote cell death, we focused on the DAPk signaling pathways that could potentially modulate the levels of neurodegeneration, specifically neurodegeneration by excitotoxicity. Our first suspect in the list was autophagy. Previous reports from other groups suggest that in another *C. elegans* model of necrosis, where a *mec-4(d)* mutation is used to induce neurotoxicity, neurodegeneration is strongly reduced by blocking autophagy[105,106]. Unexpectedly, our current data suggest that autophagy is not needed for excitotoxicity, although it may moderately accompany neurodegeneration (Fig 8-9). Therefore, the mechanism of autophagic cell death is not the main pathway that regulates cell death by excitotoxicity. In order to further rule out the involvement of autophagy in excitotoxic

neurodegeneration, we have successfully crossed *glt-3*;*nuIs5* mutants with *atg-16.2*, a factor required for membrane elongation in the autophagy pathway. We are now in the process of confirming the homozygosity of the triple mutant (*atg-16.2; glt-3*; *nuIs5*). In addition, we are also taking a pharmacological approach, where we use 3-methyladenine (3-MA) to block the class III PI3K/hVps34 (a positive autophagy modulator at both the initiation and maturation stage of autophagosome). If autophagy plays a role in nematode excitotoxicity, we expect to see a significant reduction in neurodegeneration comparing our experimental groups (*atg-16.2; glt-3*; *nuIs5* or *glt-3*; *nuIs5* + (3-MA)) versus control groups (*glt-3*; *nuIs5* or *glt-3*; *nuIs5 , no drug*). However, the lack of effect of *unc-51* and the lack of evidence for autophagy as reported by DsRED::LGG-1 already argue strongly in against a significant role for autophagy in nematode excitotoxicity.

We initially thought that autophagy was a determinant factor for nematode excitotoxicity and that DAPk was the kinase responsible for activating the machinery to induce cell death. However, the involvement of DAPk in neurodegeneration can be independent of autophagy. Importantly, we started to dissect others pathways that are regulated by DAPk and can also change the overall activity of Glu signaling, thus changing the level of excitotoxicity.

Death-Associated Protein Kinase (DAPk) does not participate in early events of excitotoxicity

In the quest for identifying DAPk-interacting partners we eliminated two important early events that could be influenced by DAPk and potentially modulate the process of excitotoxicity. By using two different behavioral assays, we found out that DAPk does not seem to affect the mechanism of synaptic vesicle release by interacting with a critical SNARE complex molecule, Syntaxin- 1A, (Fig.10). In addition, DAPk is not associated with the overall activity level of post-synaptic GluRs (Fig.11). Therefore, we suggest that the involvement of DAPk in nematode excitotoxicity seems to be

mediated by one of the many intracellular events it might control at the postsynaptic-neuron. Ultimate clarification of the site of DAPk activity will require the postsynaptic-specific modification of its activity.

CaM Kinase Kinase/ CKK-1 does not participate in nematode excitotoxicity

Based on our current finding, we wanted to identify DAPk substrates that could collaborate or participate in excitotoxic neurodegeneration. CaMKK/CKK-1, a key protein for the activation of CREB's neuroprotective effect, became a suspect because DAPk inhibits its activity[28]. However, our current data implies that the absence of CKK-1 under our model of excitotoxicity does not change the level of neurodegeneration (Fig.12). A possible explanation for the lack of CKK-1 effect can be that the nematode protein has some functional differences from its mammaliam counterpart. For example, in spite of overall high sequence conservation, nematode CKK-1 does not have the C-terminal sequence that in mammals is phosphorylated by DAPk. These results suggested that we have to look for other avenues that could give us a clue to the signaling pathway DAPk is using to affect neurodegeneration.

Ongoing research/ Future Directions

We are currently testing other molecules that are downstream mediators of DAPk known to influence several cell death scenarios, including excitotoxicity. To that end, we have successfully crossed *dapk-1 (gk219); Δglt-3; nuIs5* mutants with the following mutants:

a) *ced-4* (encoding one of the critical proteins to regulate the process of apoptosis in worms[78,79]). CED-4 is highly conserved from nematode to mammals. Previous work in neuronal necrosis suggested that apoptosis does not seem to contribute much to necrotic neuronal cell death in the presence of DAPk[115]. However, DAPk could potentially divert some of the dying cells in the specific process of excitotoxic cell death from apoptosis to necrosis[116], causing *dapk-1 KO* animals to show less necrosis. Therefore, we want to generate *ced-4*; *dapk-1 (gk219); Δglt-3; nuIs5* and *ced-4; Δglt-3; nuIs5* to see if the process of neurodegeneration is regulated by that cell death mechanism.

b) DANGER/mab-21. The role of Ca^{2+} storage in nematode excitotoxicity[100], the function of DANGER/*mab-21* in regulating the activity of IP3-R[54], and the ability of DANGER/mab-21 to inhibit DAPk[117] (thus conferring neuroprotection) suggest that *mab-21* might be a good candidate molecule to study in nematode excitotoxicity. Therefore, we are currently crossing *mab-21(bx53)* mutants with our excitotoxicity strain.

c) Other downstream mediators of DAPk that play crucial role in Glu signaling at the post-synaptic neurons are also strong candidate mediators of DAPk's involvement in excitotoxicity. We have

prioritized the list of these mediators by focusing on the proteins that previous groups have reported exert an effect on post-synaptic neurons. At the top of this list we place:

1) The MAPK protein *mpk-1*[77,118-123] has been shown to be regulated by *dapk-1* in *C. elegans*[102] .MAPKs are known to control synaptic activity by regulating GluR trafficking[38,39], an effect seen also in worms[124].

2) the Pin1[42,45,46] is a propyl isomerase that is known to regulate the strength of postsynaptic Glu signaling and elevate GluRs expression. We are using its nematode homolog *pinn-1*[125], we have successfully crossed it with our excitotoxicity strain, and are currently screening for homozygous triple and tetra mutants.

Depending on the progress and results from these studies, we might also look at other known DAPk substrates/interacting proteins[28] or perform a genetic screen to suppress the extended neurodegeneration seen in our excitotoxicity strain in the absence of *dapk-1* (a similar genetic screen in the presence of *dapk-1* is currently carried out in the lab by A.O. Edokpolo). We hope that the combinations of these approaches will reveal the mechanism by which DAPk is controlling nematode excitotoxicity.

Conclusions

Brain ischemia is a major health concern in the United States while the effectiveness of the current treatments to ameliorate the neuronal damage is very limited. Therefore, we hope to discover critical cellular and molecular events that lead to excitotoxicity. Recent evidence demonstrates that DAPk (master regulatory cell death switch) is critical for excitotoxicity. However, the gaps in our understanding of its role still need to be filled. We aim to filll those gaps by understanding the signaling pathways through which DAPk mediates its neurodegenerative effect. We are taking a novel and powerful approach, where we are relying on the nematode *C. elegans* model of excitotoxicity due to its unique qualities, such as a genetic accessibility, the successful track record of using *C. elegans* to elucidate the conserved molecular basis of a number of cell death pathways, the availability of a powerful and informative model of excitotoxicity, and the high conservation of DAPk. We find that DAPk is a key regulator of excitotoxicity in the nematode, though probably not through its best studied effect, the promotion of autophagic cell death. We are currently using this system to further determine the mechanism for DAPk's involvement in excitotoxicity. Based on the current findings and the ability to answer questions relatively quickly with this model, we expect to shed light on the possible mechanism of DAPk involvement in excitotoxic neurodegeneration, a contribution that might ultimately promote suggestions on new therapeutic strategies to mitigate neuronal damage during stroke.

Materials and Methods

Strains

The following *C.elegans* strains were obtained from the *C.elegans* Genetic Center, from the Japanese National Bioresource Project for the Experimental Animal "Nematode C. elegans" , or from the original producers: **WT**: Bristol N2; **Nematode excitotoxicity model, *Δglt-3; nuIs5*:** ZB1102 *Δglt-3(bz34) IV; nuIs5 V; **Δdapk-1**: VC432 Δdapk-1(gk219) I; **dapk-1 over-expression:** CZ9277: frIs7[P$_{nlp-29}$::GFP ; P$_{col-12}$:DsRed] (IV) ; juEx1933[P$_{hsp16}$::DAPK-1 ; P$_{ttx-3}$::RFP] ; **Autophagy modulator unc-51**: CB369 unc-51(e369) V; **Autophagy label, LGG-1:DsRED:** Ex [P$_{lgg-1}$:: DsRED:: LGG-1; P$_{myo-2}$:: GFP]; **Glu-regulated mobility defective control Δnmr-1; Δglr-2 Δglr-1**: VM1268 Δnmr-1(ak4)II; Δglr-2(ak10) Δglr-1(ky176) III; **CaMKK KO Δckk-1**: Δckk-1(ok1033)III; PINN-1 KO **Δpinn-1:** Δpinn-1(tm2235)II; **mpk-1:** SD939 mpk-1(ga111)III.* All the crosses were followed by PCR analysis and by monitoring *nuIs5* GFP expression on *glr-1* expressing- interneurons under imaging UV optics scope, except *unc-51*, which was followed by uncoordinated phenotype and sequencing, LGG-1::DsRED, which was followed by DsRED expression in cells and *juEx1933*, which was followed by RFP expression on AIY neurons. We constructed the following crossed: ***Δdapk-1;Δglt-3; nuIs5:*** VC432 and ZB1102 *Δdapk-1(gk219) I; Δglt-3(bz34) IV; nuIs5 V*; ***Δglt-3; nuIs5; P$_{hsp16}$::DAPK-1:*** ZB1102 and *juEx1933* [P$_{hsp16}$::DAPK-1 ; P$_{ttx-3}$::RFP];*Δglt-3(bz34) IV; nuIs5 V*; ***Δglt-3; nuIs5;unc-51:*** ZB1102 and CB369 *Δglt-3(bz34) IV; nuIs5 V ; unc-51(e369) V*; **LGG-1:DsRED; *Δglt-3; nuIs5: Ex* [P$_{lgg-1}$:: DsRED:: LGG-1; P$_{myo-2}$:: GFP]; Δglt-3(bz34) IV; nuIs5 V; Δckk-1; Δglt-3; nuIs5:** Δckk-1(ok1033)III; Δglt-3(bz34) IV; nuIs5 V.

Neurodegeneration Analysis

The level of necrotic neurodegeneration in head neurons of animals carrying the over-expression of *nuIs5* was monitored using an inverted scope under Nomarski Differential Interference Contrast (DIC). Animals with no anesthetics were examining from a fresh chunk of agar of nematode culture and flipped upside down over a slide. Swollen "vacuolated-looking" cells located in the area of the nerve ring were counted as head neurons undergoing necrosis (suggested in previous protocol[100]) (we occasionally confirmed these dying neurons as neurons postsynaptic to Glu connections by verifying their labeling with the GFP co-expressed in *nuIs5* animals under the *glr-1* promoter). ~50-100 animals at each developmental stage (L1-adult) were scored for the extent of degenerating neurons. Animals carrying [P$_{hsp16}$::DAPK-1 ; P$_{ttx-3}$::RFP];*Δglt-3(bz34) IV; nuIs5 V* mutation were placed in a 35^{0}C incubator for 2 hours to induce activation of heat shock promoter, left to rest for about 30 minutes and scored regularly for two days for the extent of swollen degenerating neurons (protocol coordinated with the Chisholm group (UCSD) who developmed the *dapk-1* over-expression strain [102]). Statistical significance between control groups and experimental groups was analyzed using z-test score.

Fluorescence Microscopy Analysis

Animals carrying *Ex [P$_{lgg-1}$:: DsRED:: LGG-1; P$_{myo-2}$:: GFP]; Δglt-3(bz34) IV; nuIs5 V and Ex [P$_{lgg-1}$:: DsRED:: LGG-1; P$_{myo-2}$:: GFP]; nuIs5 V* were analyzed on the extent of *glr-1* expressing command interneuron undergoing autophagy as co-labeled with GFP & DsRed. Animals were mounted on a 2% agar pad with sodium azide (a compound that paralyzed the worms, worms are not scored here for vacuolar appearance) and observed using an inverted high power scope containing Nomarski Differential Interference Contrast (DIC) and epifluorescence optics. We quantified the amount of DsRED intensity in GFP-labeled (glr-1 expressing) neurons, by taking the neuron with the highest

DsRED intensity as 100 % and then relatively score the other neurons with smaller DsRED intensity percentage as proportion of the neuron with 100 % DsRED intensity (following the procedure used by the Tavernarakis lab, who developed this DsRed::LGG-1 marker). A total average of DsRED intensity from all neurons undergoing autophagy from single L3-L4 animals and then from the whole population was compared between control and experimental group and then analyzed for statistical significance of the difference. We also took an average of the number of neurons undergoing autophagy and compared between both populations of worms (control versus experimental).

Behavioral assays

Behavioral assays on nematode locomotion and worm paralysis were performed blindly following standard methods of behavioral analysis[98]. For Glu-regulated forward mobility we followed the protocol of the Maricq group (U Utah)[113]. For aldicarb assays we followed the Nonet lab protocol (Washington U)[126]. Briefly, we soaked worm plates with aldicarb to a final concentration of 0.5 mM and added food. We placed ~30 freshly growing young adult animal onto these plates. The percentages of paralyzed worms were recorded every 15 minutes for a period of 2 hours.

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