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Caenorhabditis elegans glutamate transporter deletion induces AMPA-receptor/adenylyl cyclase 9-dependent excitotoxicity

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Abstract

In stroke and several neurodegenerative diseases, malfunction of glutamate (Glu) transporters causes Glu accumulation and triggers excitotoxicity. Many details on the cascade of events in the neurodegenerative process remain unclear. As molecular components of glutamatergic synapses are assembled in *Caenorhabditis elegans* and as many fundamental cellular processes are conserved from nematodes to humans, we studied Glu-induced necrosis in *C. elegans* and probed its genetic requirements. We combined Δ*glt-3*, a Glu transporter-null mutation, with expression of a constitutively active form of the alpha subunit of the G protein Gs. While neither Δ*glt-3* nor expression of the constitutively active form of the alpha subunit of the G protein Gs is severely toxic to *C. elegans* head interneurons, their combination induces extensive neurodegeneration. Δ*glt-3*-dependent neurodegeneration acts through Ca^{2+}-permeable Glu receptors of the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) subtype, requires calreticulin function, and is modulated by calcineurin and type-9 adenylyl cyclase (AC9). We further show that mammalian AC9 hyperactivates mammalian AMPA-receptors (AMPA-Rs) in a *Xenopus* oocyte expression system, supporting that the relationship between AMPA-Rs hyperactivation and AC9 might be conserved between nematodes and mammals. AMPA-Rs–AC9 synergism is thus critical for nematode excitotoxicity and could potentially be involved in some forms of mammalian neurodegeneration.

Keywords: *Caenorhabditis elegans*, calcineurin, cyclic AMP, glutamate transporters, ischemia, neurodegeneration.

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Glutamate (Glu) mediates most of the excitatory signaling in the brain by activating post-synaptic Glu-receptors/channels (GluRs) of the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA subtypes (Dingledine *et al.* 1999; Palmer *et al.* 2005). Exaggerated Glu signaling hyperactivates post-synaptic GluRs, leading to excessive depolarization, a buildup of toxic intracellular Ca^{2+} levels, dramatic cell swelling, and finally the necrotic death of post-synaptic neurons in a process termed excitotoxicity (Rothman and Olney 1986; Choi 1992; Arundine and Tymianski 2004). To prevent excitotoxicity, Glu is removed from the synapse by high affinity Glu-transporters (GluTs) (Danbolt 2001). GluT malfunction is a key event in acute human conditions such as stroke and traumatic brain injury (Rossi *et al.* 2000; Danbolt 2001), and in a range of progressive neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) (Cleveland and Rothstein 2001; Maragakis and Rothstein 2004). Despite the profound impact of excitotoxicity, the sequence of events leading from GluT malfunction and GluR hyperactivation to neuronal demise still remains a mystery. The failure of clinical trials testing GluR blockers as candidate anti-excitotoxic compounds (Birmingham 2002) emphasizes the need for a detailed understanding of the mechanisms regulating Glu balance, and the steps of the excitotoxic processes that follow its disruption, in a whole animal context.

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Abbreviations used: AC9, type 9 adenylyl cyclase; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid; AMPA-R, AMPA-receptor; CsA, cyclosporine A; ER, endoplasmic reticulum; GFP, Green Fluorescent Protein; Glu, L-glutamate; GluR, glutamate receptor; GluT, glutamate transporter; Gαs*, constitutively active alpha subunit of the G protein Gs; KA, kainate; NMDA-Rs, NMDA-receptors; PKA, protein kinase A; RNAi, RNA interference; vGluT, vesicular glutamate transporter.

Genetic analysis in small animal model systems has been repeatedly shown to provide novel and unbiased views on complex signaling pathways that are largely conserved from invertebrates to mammals. These studies have provided unexpected insights into the biology of critical signaling cascades even when these had been considered relatively well studied in other systems. Indeed, numerous genetic studies in *Caenorhabditis elegans*, including analyses of apoptotic cell death (Metzstein *et al.* 1998), synaptic function (Richmond *et al.* 1999; Rand *et al.* 2000), cell signaling (Sternberg and Han 1998), and RNA interference (RNAi) (Fire *et al.* 1998) have led to discovery of conserved molecules and mechanisms centrally relevant to human biology. Although there are differences between the nematode and mammalian nervous systems, there is a strong general conservation (in protein sequence and function) of many components of Glu synapses, including a vesicular Glu-transporter (vGluT) (encoded by the *eat-4* gene) (Lee *et al.* 1999), GluRs (*glr-1-8; nmr-1-2*) (Brockie and Maricq 2006), GluR anchoring proteins and auxiliary subunits (Rongo *et al.* 1998; Walker *et al.* 2006), and GluTs (*glt-1; glt-3-7*) (Kawano *et al.* 1997; Mano *et al.* 2007). Based on the precedent that basic principles of intracellular signaling pathways are well conserved from nematodes to mammals, we studied GluT knockout-induced necrosis in *C. elegans*, analyzing a critical form of neuropathology in the nematode that could also yield novel insights into analogous processes of aberrant GluT function and Glu-induced neurodegeneration in higher organisms.

We recently described the generation of likely null mutants for the six GluT genes in *C. elegans* and demonstrated that *glt-3* deletion results in excessive Glu signaling as assessed by genetic and behavioral criteria (Mano *et al.* 2007). Here we show that in combination with hyperactive constitutively active form of the alpha subunit of the G protein Gs (Gαs*) signaling, Δ*glt-3* can cause extensive necrotic neuronal death that is mediated by Ca^{2+}-permeable AMPA-receptors (AMPA-Rs) and a type 9 (calcineurin inhibited-) adenylyl cyclase, (AC9). We further show that a functional interaction between AMPA-Rs and AC9 is also observed when mammalian homologs are expressed in *Xenopus* oocytes. Our findings demonstrate that an excitotoxic-like condition can be triggered by the convergence of hyperstimulation of Glu and cAMP signaling pathways, defining a critical step in nematode excitotoxicity that may also contribute to neuronal death in other animals.

Materials and methods

Strains

The following *C. elegans* strains were obtained from the *C. elegans* Genetic Center (http://biosci.umn.edu/CGC) or from their original producers: **WT:** Bristol N2; **Δ*glt-3***: ZB1096 *glt-3(bz34) IV*; ***nuIs5***: KP742[*glr-1::gfp; glr-1::Gαs(Q227L) V; lin-15(+)*]; ***acy-1***: KP1182 *acy-1(nu329) III*; **Δ*nmr-1; Δglr-2 Δglr-1***: VM1268 Δ*nmr-1(ak4) II; Δglr-2(ak10) Δglr-1(ky176) III*; **Δ*cnb-1***: KJ300 *cnb-1(jh103) V*; **Δ*crt-1:*** RB1021 Δ*crt-1(ok948) V*; **Δ*clp-1***: Δ*clp-1(tm690) III* and Δ*clp-1(tm858) III*. Crosses to create mutant combinations were followed by PCR, except for *acy-1*, which was followed by sequencing. We constructed the following strains (two strain identification numbers indicate origin from previous independent crosses): **Δ*glt-3; nuIs5***: ZB1102 and ZB1103 *glt-3(bz34) IV; nuIs5 V*; ***acy-1;* Δ*glt-3; nuIs5***: ZB1104 and ZB1105 *acy-1(nu329) III*; *glt-3(bz34) IV; nuIs5 V*; **Δ*nmr-1; Δglt-3; nuIs5***: ZB1334 Δ*nmr-1(ak4) II; glt-3(bz34) IV; nuIs5 V*; **Δ*glr-2 Δglr-1; Δglt-3; nuIs5***: ZB1335 Δ*glr-2(ak10) Δglr-1(ky176) III; glt-3(bz34) IV; nuIs5 V*; **Δ*nmr-1; Δglr-2 Δglr-1; Δglt-3; nuIs5***: ZB1336 and ZB1337 Δ*nmr-1(ak4) II; Δglr-2(ak10) Δglr-1(ky176) III; glt-3(bz34) IV; nuIs5 V*; **Δ*clp-1; Δglt-3; nuIs5***: *clp-1(tm690* or *tm818) III; glt-3(bz34) IV; nuIs5 V.* **Δ*glt-3; nuIs5; Δcrt-1***: *glt-3(bz34) IV; nuIs5 V; Δcrt-1(ok948) V.*

Analysis of neurodegeneration

We monitored the extent of neurodegeneration in strains expressing Gαs* by observing free-moving animals with an inverted scope under Nomarski Differential Interference Contrast (DIC) optics with no anesthetics (by examining a chunk of agar from a freshly grown nematode culture flipped over on a slide). Swollen cells in the nerve-ring region were counted as head necrotic figures indicative of neurodegeneration (Fig. 1). In some animals, we verified the identity of these swollen cells as *glr-1*-expressing command interneurons by viewing the Green Fluorescent Protein (GFP) epifluorescence signal of these cells (in *nuIs5* both Gαs* and GFP are expressed from the *glr-1* promoter). To do this we mounted animals on an agar pad with M9 (no anesthetics) and observed them using a high power scope equipped with both Nomarski Differential Interference Contrast (DIC) and UV optics (Fig. 1a–c). We regularly obtained five to six independently crossed stains for each combination of mutations, verified that these strains presented the same phenotype in a medium size sample (20–30 animals of each developmental stage) and then quantitated the effect in depth in large samples of one of these strains (~80–100 animals of each developmental stage). Statistical significance of differences between groups was analyzed using *z* score.

Expression of GluR1 and AC9 in *Xenopus* oocytes

We used plasmids carrying cDNAs for rat GluR1 (pBTG-GluR1; Mano *et al.* 1996) and mouse AC9 [pBK-CMV-mAC9 (Premont *et al.* 1996), a gift from Dr R. Premont] to produce capped cRNA using the T3 mMessage mMachine from Ambion (Austin, TX, USA) and RNAeasy columns from Qiagen (Valencia, CA, USA); 4 ng GluR1 cRNA alone or with 4 ng AC9 cRNA were injected into *Xenopus* oocytes (ovaries purchased from NASCO; Fort Atkinson, WI, USA); defolliculation and injection as described previously) (Mano *et al.* 1996). Two electrode voltage clamp recordings were performed using the GeneClamp 500B amplifier and pCLAMP software from Axon CNS – Molecular Devices (Sunnyvale, CA, USA). Recording sessions started at least 4 days after cRNA injection. In some experiments, oocytes were tested for kainate (KA) responses and then incubated for 15 min with 2-deoxyadenine 3-monophosphate or cyclosporine A (CsA; 10 μM in oocyte Ringer's solution, OR2) before being tested again for KA responses (chemicals from Sigma, St Louis, MO, USA). Each experiment was repeated in at least two batches of oocytes.

Results

Deletion of glutamate transporter *glt-3* exacerbates necrotic neuronal death

As direct biochemical access to *C. elegans'* nervous system is particularly challenging, we chose to address the possibility of neurodegeneration mediated by excess Glu in the nematode using genetic manipulation. After showing that deletions in *C. elegans* GluT genes can elevate endogenous Glu signaling by increasing synaptic Glu concentrations (Mano *et al.* 2007) we asked if GluT gene disruption contributes to necrotic-like neuronal death in nematodes. In *C. elegans*, necrotic dying neurons associated with ion channel hyperactivation are readily visible in the transparent body and appear as swollen 'vacuolar' structures (Driscoll and Gerstbrein 2003). We examined the six GluT deletion strains (Δ*glt-1;* Δ*glt-3*–Δ*glt-7*) during development and young adulthood, but were unable to find evidence of consistent necrotic cell death in animals harboring either single GluT deletions or some double/triple mutant combinations of GluT deletions (e.g. the double mutant Δ*glt-1;* Δ*glt-3*, and the triple mutants Δ*glt-3;* Δ*glt-4;* Δ*glt-5*, Δ*glt-3;* Δ*glt-4;* Δ*glt-6*, and Δ*glt-3;* Δ*glt-4;* Δ*glt-7*). However, in a sensitized background in which a Gαs* is expressed in a subset of post-synaptic *C. elegans* neurons (Berger *et al.* 1998), we found that the Δ*glt-3* mutation (but not Δ*glt-1* or Δ*glt-4*) potently induces a Glu-dependent necrotic-like cell death. We conclude that deletion of GluT *glt-3*, which is known to elevate endogenous Glu signaling, can induce necrosis in a sensitized background.

In the transgenic strain *nuIs5* (Berger *et al.* 1998), Gαs* and a GFP reporter are both expressed from the promoter of the GluR gene *glr-1* (Brockie and Maricq 2006), fluorescently labeling post-synaptic neurons in glutamatergic synapses (including the command interneurons, which are a set of neurons critical for the processing of sensory information and the command of locomotion), and inducing necrotic swelling of some of these neurons (Fig. 1a). The baseline necrotic effect of Gαs* expression alone in this strain is weak, such that only a few of the neurons expressing the constructs die (usually one to two head neurons per animal), and death does not depend on AMPA-Rs (Berger *et al.* 1998).

To test whether the hyperstimulation of Glu synapses caused by *glt-3* deletion might increase the extent of necrotic-like cell death in the *nuIs5* background, we compared the number of dying head neurons (exhibiting swollen necrotic 'vacuole structures') in Δ*glt-3; nuIs5* double mutants to the number of dying head neurons in the parental *nuIs5* strain. As the appearance and disappearance of necrotic structures is a dynamic process (because of different times of onset, followed by the engulfment and elimination of cell corpses), we counted necrotic head neurons in a large number of animals at each stage of post-embryonic development (~100 animals of each strain in each stage). We found a large increase in the total number of necrotic neurons in Δ*glt-3; nuIs5* at all stages of post-embryonic development (Fig. 1b and d). At the L3 larval stage, we recorded three times more dying neurons in Δ*glt-3; nuIs5* animals than in *nuIs5* animals.

To quantitate the effect of Δ*glt-3* on necrosis of specific neurons, we monitored swelling of the RIG neurons – a specific pair of readily distinguishable neurons previously reported to exhibit necrotic-like vacuole structures in the heads of 7% of *nuIs5* animals (Berger *et al.* 1998). We observed that Δ*glt-3; nuIs5* animals can have more than six times as many necrotic RIG neurons as *nuIs5* animals do (Fig. 1b and e). We further confirmed that necrotic morphology corresponds to dying cells by counting surviving GFP-expressing RIG neurons in adult animals. We find that in the parental *nuIs5* strain, 81 ± 5% of the RIG neurons are present (as indicated by a strong GFP signal) and an additional 12 ± 4% of the RIG neurons are still present but appear abnormal (displaying a weak GFP signal, suggesting they might be compromised); 7% are missing and presumed dead. In Δ*glt-3; nuIs5*, only 7 ± 2% of RIG neurons appear healthy and an additional 30 ± 5% of the cells are present but appear abnormal. The remaining 63% of RIGs are missing and presumed dead.

To verify that the enhanced neurodegeneration seen in Δ*glt-3; nuIs5* animals is indeed mediated by the loss of *glt-3* function (and not by a non-related change in these mutant animals) we re-introduced a wild-type copy of *glt-3* (*glt-3*$^{+}$) and checked for rescue of the enhanced neurodegeneration. We crossed Δ*glt-3; nuIs5* animals with animals transgenic for an extra-chromosomal construct that carries the promoter region and full-length coding region of *glt-3* fused in-frame to *gfp* ($P_{glt\text{-}3}$ *glt-3::gfp*), a construct we have previously shown to rescue other phenotypic defects in Δ*glt-3* (Mano *et al.* 2007). We compared head neurodegeneration in cross progeny the carry the *glt-3*$^{+}$ transgene to cross progeny that do not, and saw that the presence of the *glt-3*$^{+}$ transgene reduced neurodegeneration to the background level seen in *nuIs5* animals (Fig. 1f), supporting that the enhanced neurodegeneration seen in Δ*glt-3; nuIs5* animals is caused by the loss of *glt-3* function.

Δglt-3-induced necrosis in the Gαs* background depends on AMPA type GluRs

To ask whether Δ*glt-3*-induced necrosis in the Gαs* background depends upon Glu signaling, we tested the genetic requirements for GluRs. We used GluR mutations present in strain VM1268 (Mellem *et al.* 2002), a strain that combines mutations in two AMPA-R-like subunit genes [Δ*glr-2(ak10)* Δ*glr-1(ky176)*] and a mutation in the NMDA-receptor (NMDA-R) NR1-like subunit gene [Δ*nmr-1(ak4)*], to create several compound strains combining multiple GluR

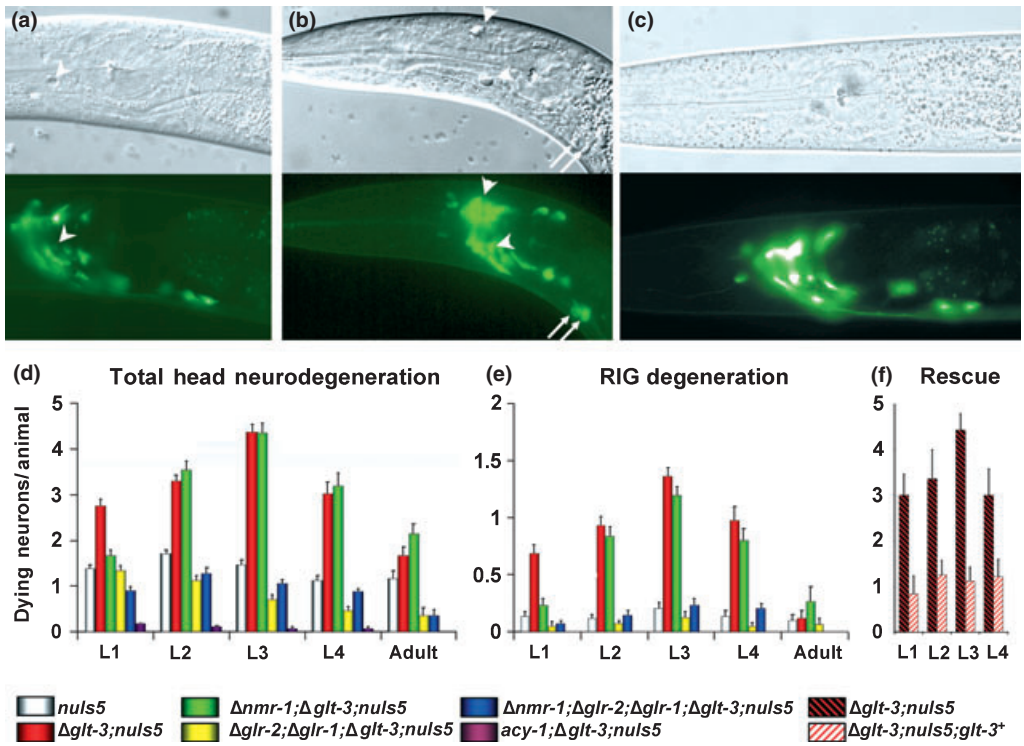

Fig. 1 *glt-3* glutamate transporter deletion dramatically exacerbates necrosis of head neurons in the Gαs* background. (a–c) Neurodegeneration phenotypes in different strain backgrounds. Nomarski DIC view (upper panels) and GFP-epifluorescence (lower panels) of heads of L2 larval stage animals. Animals were mounted on agar pads without anesthetics, anterior left, dorsal up. Arrowheads indicate necrotic neurons among nerve ring neurons. Full arrows indicate more posterior ventral RIG neurons with necrotic swellings of these in some backgrounds. (a) *nuIs5* ($P_{glr\text{-}1}$*::Gαs*; *$P_{glr\text{-}1}$*gfp*). Typically, we observed one necrotic figure in the nerve ring region, and necrotic figures in RIG neurons were rare (an average of 0.1–0.2 of a maximum of two RIG corpses per animal, counted in over 100 L2 animals.). (b) *Δglt-3; nuIs5*. Typically, we observed several necrotic neurons in the nerve ring region and one or both RIG neurons were swollen. (c) *Δglt-3; nuIs5; acy-1*. Necrotic figures in the head are completely absent in the background. (d–f) Quantitation of dying neurons (seen as necrotic figures) in the head area of *nuIs5* (white bars), in *Δglt-3; nuIs5* (red bars), in *Δnmr-1; Δglt-3; nuIs5* animals (no-NMDA-R animals, green bars), in *Δglr-2 Δglr-1; Δglt-3; nuIs5* animals (no-AMPA-Rs animals, yellow bars), in *Δnmr-1; Δglr-2 Δglr-1; Δglt-3; nuIs5* animals (no-GluRs animals, blue bars), and *acy-1; Δglt-3; nuIs5* animals (purple bars), at different developmental stages (each bar represents the average ± SEM, typically of 80–100 animals). (d) Total number of necrotic figures in the head. In L3, *Δglt-3; nuIs5* shows a threefold increase in the number of head necrotic figures, compared with *nuIs5* ($p < 0.001$). Eliminating NMDA-R (*nmr-1*) does not decrease cell death, whereas eliminating all functional GluRs ($p < 0.001$) or only AMPA-Rs (*glr-1* and *glr-2*; $p < 0.001$) suppresses necrosis. A mutation in the adenylyl cyclase *acy-1* blocks cell death altogether ($p < 0.001$). (e) Number of necrotic figures in RIG neurons. *Δglt-3* increases the number of RIG necrotic figures in L3 animals sixfold. NMDA-R is dispensable but AMPA-Rs and adenylyl cyclase are essential for necrosis as in (d). (f) Rescue of enhanced neurodegeneration by re-introduction of *glt-3*$^+$. *Δglt-3; nuIs5* animals were crossed with animal carrying $P_{glt\text{-}3}$ *glt-3::gfp* (an extra-chromosomal array that includes wild-type *glt-3*$^+$ fused to *gfp*, a construct previously shown to reverse the effects of *Δglt-3*; Mano *et al.* 2007). Black and red hatched bars represent head neurodegeneration in cross progeny animals that do not carry the *glt-3*$^+$ transgene, white and red hatched bars represent cross progeny animals that do carry the *glt-3*$^+$ transgene.

mutations with the *Δglt-3*/Gαs* necrosis-causing mutations. GLR-1 and GLR-2 are believed to form a heteromeric AMPA-gated receptor/channel that carries the large, fast-kinetics Glu-induced currents in the command interneurons (Mellem *et al.* 2002). NMR-1 is an obligatory subunit in the NMDA-R/channel complex that carries the slower and smaller NMDA-induced current in these cells. The compound mutant lacks all Glu-induced currents in the command interneurons (note that these are the neurons that express the toxic Gαs* construct in *nuIs5*).

We crossed the *Δglt-3; nuIs5* strain with the *Δnmr-1; Δglr-2 Δglr-1* strain, and generated compound mutants that either lack all of these GluRs, lack only the NMDA-like receptor, or lack only the AMPA type receptors. We quantitated the number of necrotic-like neurons in *Δnmr-1; Δglr-2 Δglr-1; Δglt-3; nuIs5* animals (no-GluR animals), in *Δnmr-1; Δglt-3; nuIs5* animals (no-NMDA-R-like animals), and in *Δglr-2 Δglr-1; Δglt-3; nuIs5* animals (no-AMPA-R-like animals). Elimination of all functional GluRs fully blocked the ability of *Δglt-3* to enhance cell death in the Gαs* animals,

supporting the involvement of a Glu-based toxicity (Fig. 1d and e). In the L2–L4 developmental stages, the extent of necrosis in the Δ*nmr-1; Δglt-3; nuIs5* (no NMDA-R-like) mutants, however, was similar to that of Δ*glt-3; nuIs5* mutants (where all GluRs are present), indicating that the NMR-1 NMDA-R-like subunit plays no essential role in the death mechanism. A small effect of Δ*nmr-1* early in development is also observed, suggesting a possible role of NMDA-R-like GluRs at this stage. However, given the absence of Δ*nmr-1* effect in the developmental stages that are most prone to neurodegeneration induced by Δ*glt-3* (i.e. L2–L4), the early Δ*nmr-1* effect might instead reflect other processes, such as the (possibly Glu-independent-) activity of *nmr-1* very early in development (Corrigan *et al.* 2005). By contrast, we found that the extent of death in the Δ*glr-2* Δ*glr-1; Δglt-3; nuIs5* (no-AMPA-R-like) mutants was reduced to a level similar to that seen in *nuIs5* and in Δ*nmr-1; Δglr-2* Δ*glr-1; Δglt-3; nuIs5* animals (with small variations that are expected given the dynamic nature of the appearance and removal of degenerating neurons). These observations indicate that it is the AMPA-R-like GluRs that have the central role in mediating Glu-dependent neurotoxicity. We conclude that the dramatic enhancement of necrosis seen in Δ*glt-3; nuIs5* mutants is mediated by elevated Glu and the AMPA-R-like channel complex made of *glr-1* and *glr-2*. These AMPA-Rs have been shown to be Ca^{2+}-permeable and are the principal mediators of Glu responses in command interneurons (Mellem *et al.* 2002). As the necrosis we quantitated is induced by Glu overstimulation and is mediated by specific post-synaptic Ca^{2+}-permeable GluRs, our results establish that this model of Glu-dependent neuronal death is highly analogous to the AMPA-R mediated form of mammalian excitotoxicity.

AC9 is critical for Glu-induced toxicity in the Gαs* background

We next addressed the mechanism by which Gαs* promotes Glu-dependent necrotic death. In the Gαs* strains, the basal toxic effect (which may be Glu independent, see Discussion) is mediated by ACY-1 (Berger *et al.* 1998), an AC9 homolog (Antoni *et al.* 1998). *acy-1* is one of four genes in *C. elegans* encoding different adenylyl cyclases. We asked whether the Glu-dependent enhanced necrosis observed in Δ*glt-3; nuIs5* animals also requires the specific adenylyl cyclase encoded by *acy-1* to be activated by Gαs*. To address this question we assessed the effect of the *acy-1(nu329)* mutation on necrosis induced by Δ*glt-3; nuIs5*. We found that *acy-1; Δglt-3; nuIs5* triple mutants exhibit a drastic decline in the number of necrotic figures in the head (Fig. 1c and d), elimination of necrosis in RIG neurons (Fig. 1c and e), and survival of all RIG neurons in adults (as assessed by counting GFP labeled neurons, data not shown). Thus, the *acy-1* mutation suppresses both the baseline and the Glu-dependent components of neuronal necrosis associated with Gαs* expression. We conclude that *acy-1* activity is required for excitotoxic effects in this model.

Calcineurin deficiency genetically interacts with *glt-3* to confer toxicity

Type 9 adenylyl cyclase is distinguished from other adenylyl cyclases by its biochemical inhibition by the protein phosphatase calcineurin (Antoni *et al.* 1998; Paterson *et al.* 2000). Given the defined requirement for *acy-1* in glutamate neurotoxicity (Fig. 1c–e), we hypothesized that calcineurin might normally limit neuronal death in the GluT excitotoxicity model by inhibiting ACY-1. We initiated studies to determine the role of calcineurin in Glu-dependent excitotoxicity by crossing Δ*glt-3* with a calcineurin mutant. Because of the extensive involvement of calcineurin in many cellular pathways, a complete suppression of calcineurin activity in the nematode [by a catalytic subunit mutation (Kuhara *et al.* 2002) or by an inhibitor (Gerstbrein and Driscoll, unpublished data)] is too toxic to be informative. A mutation in the regulatory calcineurin B subunit [Δ*cnb-1(jh103)*] (Bandyopadhyay *et al.* 2002) disrupts calcineurin function and causes pleiotropic defects, though animals are generally viable. In contrast, the Δ*glt-3; Δcnb-1* double mutant appears to be a highly penetrant embryonic lethal: the Δ*glt-3; Δcnb-1* double mutant could not be obtained in several independent crosses, and Δ*glt-3/glt-3*$^{+}$*; Δcnb-1/Δcnb-1* parents segregate only Δ*glt-3/glt-3*$^{+}$ or *glt-3*$^{+}$*/glt-3*$^{+}$ progeny with a large percentage of dead eggs generated (Fig. 2a and b). We found that dead embryos had highly distorted morphologies and we were unable to evaluate neuronal integrity or necrosis in these specimens. Thus, although we cannot follow toxicity of identified neurons, our data indicate that elevating Glu signaling concomitant with down-regulating calcineurin activity is toxic. Interestingly, our data also suggests Glu signaling may play an early and essential role in development.

Calreticulin is a molecular requirement for nematode Glu excitotoxicity

We have previously shown that null alleles of calreticulin, an endoplasmic reticulum (ER) chaperone that serves as a major Ca^{2+}-storing protein, prevent necrotic neurodegeneration induced by several triggers, including the basal Gαs*-induced neurodegeneration (Xu *et al.* 2001). To determine requirements for calreticulin function in Glu-dependent toxicity, we tested calreticulin deletion Δ*crt-1(ok948)*. We compared the level of neurodegeneration in Δ*glt-3; nuIs5* animals to that of Δ*crt-1; Δglt-3; nuIs5* animals (Fig. 2c). We found that Δ*crt-1; Δglt-3; nuIs5* animals exhibit virtually no necrotic figures, indicating that the *crt-1* mutation strongly suppresses Δ*glt-3; nuIs5* induced neurodegeneration. We conclude that calreticulin function (which includes regulation of ER calcium release and chaperone activity) is required for glutamate dependent-neurotoxicity in the Gαs* background.

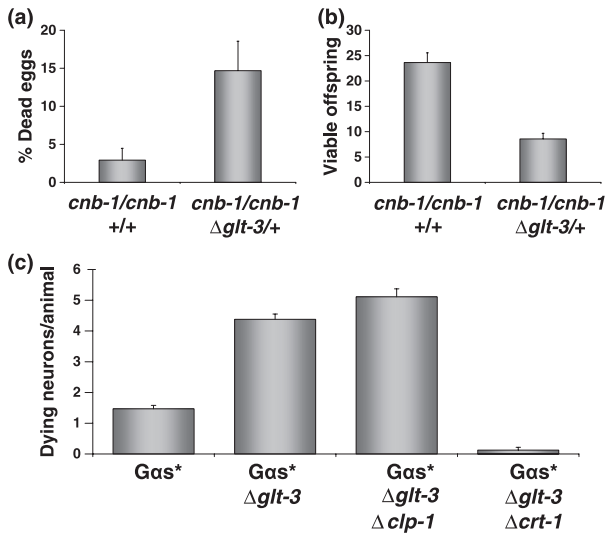

Fig. 2 Effect of calcineurin, calreticulin, and calpain deficiencies on toxicity in Δ*glt-3* animals. (a and b) Δ*glt-3;* Δ*cnb-1* animals are not viable. Several crosses between Δ*glt-3(bz34) IV* and Δ*cnb-1(jh103) V* strains did not yield double homozygote animals, even after seven rounds of sib-selection from Δ*glt-3/+;* Δ*cnb-1/*Δ*cnb-1* or Δ*glt-3/*Δ*glt-3;* Δ*cnb-1/+* parents (100 single animals picked at each round). We then compared egg viability (a) and the number of viable progeny per animal (b) from Δ*cnb-1* (n = 19) and Δ*glt-3/+;* Δ*cnb-1/*Δ*cnb-1* (n = 21) parents. Single animal parents were allowed to lay eggs for 24 h. The numbers of eggs that did not hatch (a) and the number of viable progeny were determined (b). Inspection of dead eggs revealed that embryos arrest early in development and feature many abnormalities that prevent cell identification and evaluation of the cause of lethality. (c) The calreticulin gene *crt-1*, but not the calpain gene *clp-1*, is required for neurodegeneration in Δ*glt-3; nuIs5* animals. Data show the number of dying head neurons in L3 animals. Data for *nuIs5* and Δ*glt-3; nuIs5* is from Fig. 1. Two alleles of Δ*clp-1* were used, and both Δ*clp-1;* Δ*glt-3; nuIs5* strains showed high level of neurodegeneration (n = 30). Δ*crt-1* suppresses neurodegeneration, as Δ*crt-1;* Δ*glt-3; nuIs5* animals show almost no dying neurons (n = 25).

Previous studies have suggested that some forms of *C. elegans* necrosis depend on the activity of the Ca^{2+}-dependent proteases of the calpain gene-family, and in particular the *clp-1* gene (Syntichaki *et al.* 2002). We tested for the involvement of *clp-1* in Glu excitotoxicity by constructing a strain with hyperactive Glu and Gαs* signaling and a reduced calpain activity. We used both the Δ*clp-1(tm690)* and the Δ*clp-1(tm858)* deletion alleles to test if *clp-1* is required for neurodegeneration in the Δ*glt-3; nuIs5* background (Fig. 2c). We found that the extent of necrosis in the Δ*clp-1;* Δ*glt-3; nuIs5* triple mutant is not significantly different from the Δ*glt-3; nuIs5* double mutant, suggesting that the *clp-1* calpain alone is not essential in the Glu-induced neurodegeneration model.

To summarize, in the *C. elegans* excitotoxic condition we study, several events take place in the interneurons that are post-synaptic to Glu synapses when deletion in the GluT gene *glt-3* causes these neurons to experience high Glu levels (Mano *et al.* 2007): an elevated Glu signal is transmitted by Ca^{2+}-permeable AMPA-Rs (Fig. 1) at the same time that a hyperactive cAMP signal is generated by the AC9, driven by the expression of Gαs* (Berger *et al.* 1998) (Fig. 1). Broadly expressed calcineurin and calreticulin also influence neurodegeneration, possibly by acting in the same post-synaptic neurons that express the hyperactive Glu signaling.

Mammalian AMPA-Rs can be hyperactivated by AC9 when expressed in *Xenopus* oocytes

Our data suggests that nematode excitotoxicity is mediated by a functional interaction between Ca^{2+}-permeable AMPA-Rs, AC9, and calcineurin – three proteins known to be co-expressed on the post-synaptic side of mammalian neurons (Sosunov *et al.* 2001; Tavalin *et al.* 2002). Protein kinase A (PKA) is a cAMP-activated kinase known to potentiate mammalian AMPA-R activity consequent to phosphorylation by increasing open-channel probability (P_o) by up to twofold; the protein phosphatase calcineurin acts to antagonize this potentiation (Banke *et al.* 2000; Tavalin *et al.* 2002). However, the involvement of AC9 in AMPA-R hyperactivity has not yet been considered for mammalian systems.

We therefore tested whether a functional interaction similar to the one we suggest for nematode excitotoxicity can take place between mammalian AMPA-Rs and AC9, at a post-transcriptional level in a single cell (i.e. cell autonomously). We co-expressed mammalian Ca^{2+}-permeable AMPA-Rs and AC9 in *Xenopus* oocytes using rat GluR1 and mouse AC9 cRNA (which when injected alone did not have an obvious effect on the oocytes). *Xenopus* oocytes are well documented to express a functional calcineurin (Czirjak *et al.* 2004), which we expected to be also present in the experimental model. We compared the macroscopic current response of GluR1 homomeric channels to application of KA (an AMPA-R agonist) in the absence or presence of AC9 under voltage clamp. We found that the current–voltage relations (Fig. 3a), the agonist apparent affinity (Fig. 3b), and the sensitivity of KA-responses to 2-deoxyadenine 3-monophosphate (a compound that inhibits all adenylyl cyclases except AC9; Fig. 3c) in oocytes expressing AMPA-Rs + AC9 were indistinguishable from those of oocytes expressing only AMPA-Rs. The overall activity level of AMPA-Rs (normalized at each recording session to the average response to application of 100 μM KA in oocytes expressing only AMPA-Rs) was slightly increased by the addition of AC9 (Fig. 3d).

We next tested the effect of blocking the endogenous calcineurin using the calcineurin inhibitor CsA. Blocking calcineurin is expected to increase the activity of AC9, and to subsequently potentiate the activity of AMPA-Rs. As 100 μM KA gives a response that is ~90% of the maximal response, a potentiation of this KA response by more than

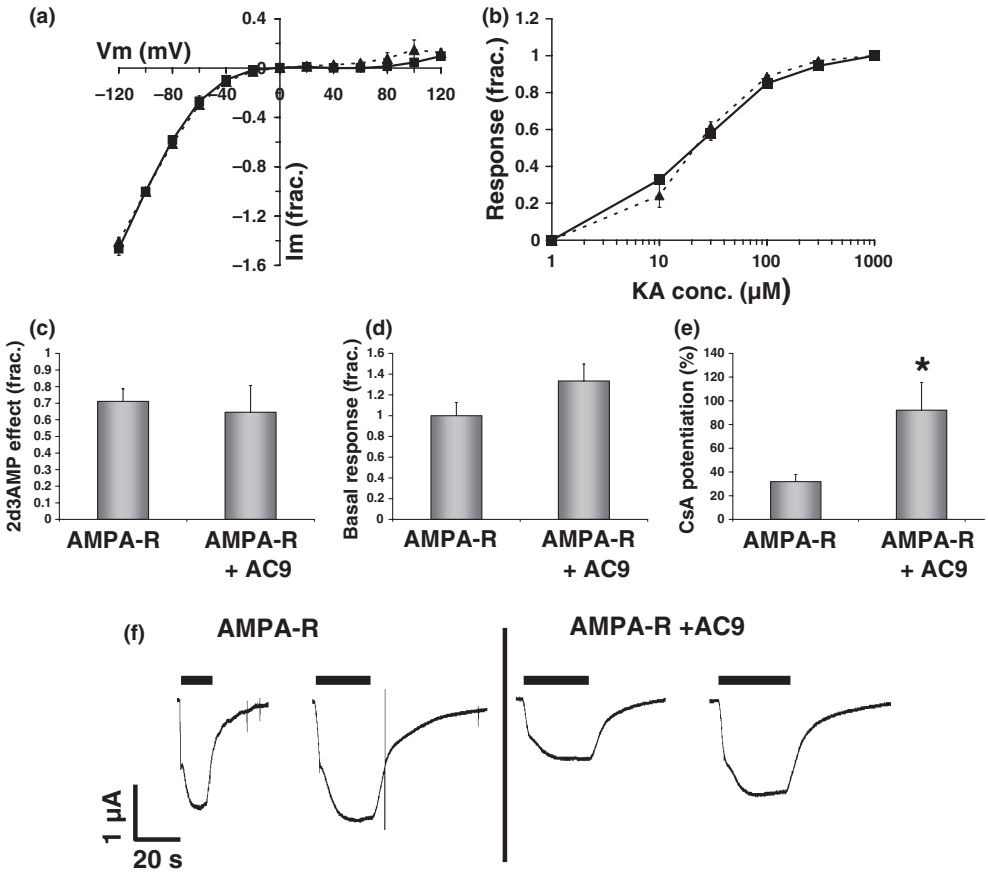

Fig. 3 Mammalian AMPA-Rs are hyperactivated by AC9 when calcineurin inhibition is removed. *Xenopus* oocytes expressing homomeric AMPA-Rs made of rat GluR1, with or without the addition of mouse AC9 (both from cRNA injection), show a specific change in the sensitivity to a calcineurin inhibitor. (a) Current–voltage relations in oocytes expressing GluR1 (squares, $n = 5$) are indistinguishable from those observed in oocytes expressing GluR1 + AC9 (triangles, $n = 3$). Current amplitudes were normalized to the response at −100 mV. (b) Dose–response relations of the responses to KA in oocytes (held at −60 mV). KA apparent affinity in oocytes expressing GluR1 (squares, $n = 5$) is indistinguishable from that observed in oocytes expressing GluR1 + AC9 (triangles, $n = 5$). Current amplitudes were normalized to the response at 1000 μM KA. (c) Effect of 2-deoxyadenine 3-monophosphate (2d3AMP), a general inhibitor of all adenylyl cyclases except AC9. For each oocyte, responses to 100 μM KA (at −60 mV) were compared before and after 15 min incubation with 2-d-3AMP (10 μM in oocyte Ringer solution). 2d3AMP sensitivity in oocytes expressing GluR1 ($n = 4$) is indistinguishable from that observed in oocytes expressing GluR1 + AC9 ($n = 3$). (d) Average response to application of 100 μM KA at −60 mV. For each oocyte, the average of three applications of KA was taken. To compensate for the daily changes in expression levels, the data were then normalized to the average GluR1 response at each recording session. Oocytes expressing GluR1 + AC9 ($n = 17$) show a 33% increase in average responses compared with oocytes expressing only GluR1 ($n = 21$) ($p = 0.053$). (e) Average potentiation of KA responses by the calcineurin inhibitor cyclosporin A (CsA). For each oocyte, the average of three KA responses before treatment was compared with the average of three KA responses after 10 min bath incubation in 10 μM CsA (in oocyte Ringer solution). Oocytes expressing GluR1 ($n = 11$) show 32% potentiation by CsA, compared with 92% potentiation in oocytes expressing GluR1 + AC9 ($n = 8$), a difference that is statistically significant ($^*p < 0.02$). (f) Typical recordings of KA-induced currents showing the potentiating effect of calcineurin inhibitor CsA. In each panel, the left trace is before treatment, and the right trace is after treatment with CsA. Horizontal bars above traces indicate application of the agonist. Differences in the basal response (before CsA) are in line with the daily changes in expression levels.

10% cannot be explained by a CsA-induced increased apparent affinity to KA, in line with a reported lack of change in apparent affinity in phosphorylated or CsA-treated AMPA-Rs (Yakel *et al.* 1995; Dingledine *et al.* 1999; Banke *et al.* 2000; Palmer *et al.* 2005). We treated AMPA-R-expressing oocytes (not injected with AC9 cRNA) with CsA and detected a 32% increase in the response to application of 100 μM KA (comparing responses before and after incubation with CsA in the same oocytes, Fig. 3e and f). This demonstrates calcineurin's moderate inhibitory capacity to regulate AMPA-R activity even in the absence of AC9, suggested previously to be mediated by a direct calcineurin–AMPA-Rs interaction (Tavalin *et al.* 2002; Dodge and Scott 2003).

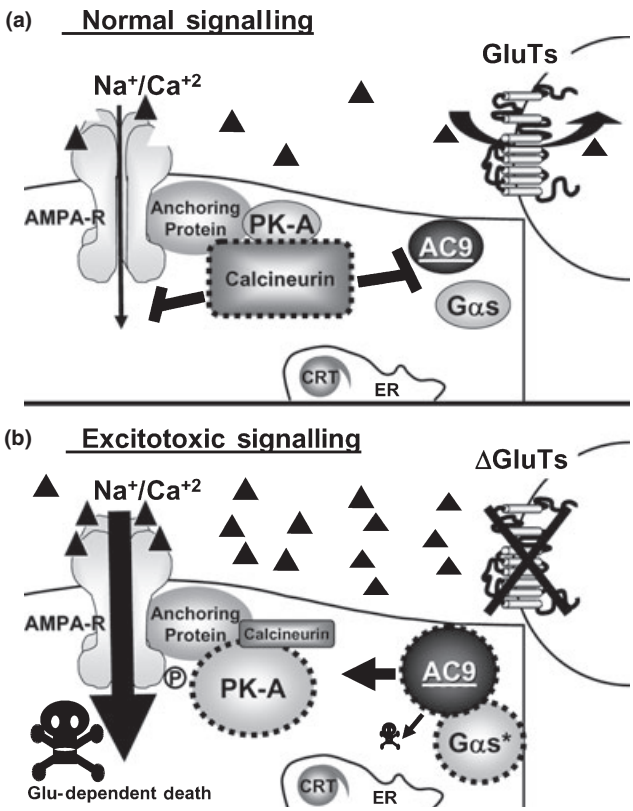

Fig. 4 A suggested mechanism for excitotoxic signaling in *Caenorhabditis elegans* – excess Glu and hyperactive cAMP signaling converge to cause Glu-dependent death. Depicted is our model for glutamatergic signaling (black triangles indicate glutamate) under normal and excitotoxic conditions in *C. elegans*. Gene products we confirmed to participate in nematode excitotoxicity are GluTs (cell surface glutamate transporter, *glt-3*), Gαs* (a constitutively active Gs protein α subunit, supplied from transgene *nuIs5*), AMPA-Rs (AMPA-type glutamate receptor/channel, *glr-1* and *glr-2*), calcineurin (a protein phosphatase), and AC9 (a type 9 adenylyl cyclase, *acy-1*). The function of the endoplasmic reticulum (ER) chaperone and Ca^{2+}-storing protein CRT (calreticulin, *crt-1*, previously shown to control Ca^{2+} release from intracellular stores in other forms of necrotic neurodegeneration in *C. elegans*; Xu *et al.* 2001) is also needed, though its mode of action in our present model is currently unclear. Proteins not directly tested here but proposed by analogy to mammals include PKA (cAMP-dependent protein kinase A) and an anchoring protein. (a) Normal signaling: During physiological Glu signaling, the post-synaptic neuron is stimulated by Glu-activated AMPA-Rs. The activity of non-neuronal GluTs clears Glu from the synapse. In the post-synaptic cell, Ca^{2+}-activated phosphatase calcineurin provides negative feedback, inhibiting the AC9 and shifting the phosphorylation/dephosphorylation balance of AMPA-Rs towards the under-phosphorylated, less active state. Although GluT deletion causes hyperstimulation of GluRs, it does not result in a runaway toxic response because of the inhibitory effect of calcineurin. (b) Excitotoxic signaling: Convergence of extracellular and intracellular overstimulated signaling pathways: expression of Gαs* overwhelms the negative effect of calcineurin and hyperstimulates the AC9. Several cAMP-effector pathways are activated, and produce a Glu-independent low level of cell death. In addition, the hyperactivation of PKA shifts the phosphorylation/dephosphorylation balance of AMPA-Rs towards the phosphorylated, more active state. When this condition is paired with GluT knockout and Glu overstimulation, the exaggerated response of AMPA-Rs results in robust Glu-dependent necrotic death. Similar combinatorial actions may potentiate excitotoxicity in mammals.

However, in oocytes expressing both AMPA-Rs and AC9, the potentiation of KA responses by blocking calcineurin was much larger and increased overall AMPA-R activity by 92%. This degree of potentiation correlates with the maximum possible potentiation of AMPA-Rs by phosphorylation reported previously (Banke *et al.* 2000).

Our observations indicate that mammalian AMPA-Rs have the potential to be post-transcriptionally and cell-autonomously regulated by AC9. In the presence of the inhibitory effects of calcineurin, AC9 does not exert strong effects on AMPA-R activity. However, if the double inhibition exerted by calcineurin (affecting both the AMPA-Rs and AC9) is relieved, Ca^{2+}-permeable AMPA-Rs can be driven to their maximal activity levels, a condition that could contribute to mammalian excitotoxicity.

Discussion

Pathological Glu signaling and excitotoxicity in *Caenorhabditis elegans*

Here we use the strong tools of genetic analysis to investigate the *in vivo* effects of multiple mediators of glutamate-induced neuronal necrosis, and report that elevated glutamate signaling induced by a GluT-knockout synergizes with exacerbated Gαs activity to result in neuronal necrosis in *C. elegans*. This Glu-dependent toxicity is mediated through Ca^{2+}-permeable GluRs and defines an excitotoxicity model in an invertebrate genetic system that shares many basic features of human excitotoxicity, a catastrophically damaging process in stroke, ischemia, and neurodegenerative diseases. We describe a novel mechanism for nematode excitotoxicity, where the exaggerated action of Ca^{2+}-permeable AMPA-Rs synergizes with overactive intracellular AC9/cAMP signaling to cause excitotoxic neurodegeneration. As aspects of this process are operable for mammalian proteins in a heterologous expression system, we consider the possibility that the mechanisms we describe in *C. elegans* may be operative in higher organisms.

An invertebrate excitotoxicity model with defined requirements for GluTs and GluRs

Several invertebrate models of neurodegenerative conditions have been developed by the expression of human disease genes in flies and nematodes (Driscoll and Gerstbrein 2003; Bilen and Bonini 2005), but less emphasis in the field has been given to modeling Glu-dependent excitotoxicity. By documenting a GluT-deletion-induced and GluR-mediated

neuronal necrosis, we describe a condition of nematode excitotoxicity that is significantly different from previously described neurotoxic conditions in invertebrates. In *Drosophila* (where Glu is not considered a major excitatory neurotransmitter of the CNS), RNAi-directed knockdown of a glial GluT is associated with neuropil degeneration (Rival *et al.* 2004), though this model remains to be genetically dissected in detail. The Gαs* toxicity in *C. elegans* has been suggested to involve Glu-dependent excitotoxicity. However, we and others have found that toxicity mediated by Gαs* alone (Korswagen *et al.* 1997; Berger *et al.* 1998) is not affected by GluRs. These studies include removal of *glr-1* (Berger *et al.* 1998) (the most important AMPA-R subunit in the command interneurons; Mellem *et al.* 2002), a combined *glr-1* and *glr-2* disruption, and disruption of *nmr-1*, the obligatory subunit of NMDA-R complexes (Fig. 1). Metabotropic GluRs are not expressed in the major command interneurons and are therefore unlikely to participate in Gαs* toxicity (see http://www.WormBase.org). Although mutations in the vGluT gene *eat-4* do partially reduce Gαs* neurotoxicity (Berger *et al.* 1998), the pleiotropic effects of *eat-4* include feeding defects and starvation, which can partially protect against necrosis induced by a variety of Glu-unrelated neuronal insults, including hyperactivity of channels of the Degenerin/Epithelial Na^+ Channels (DEG/ENaC) gene family (Syntichaki *et al.* 2002) and over-expression of defective, non-conducting channels (Aronoff *et al.* 2004). Thus, it is possible that neurotoxicity mechanism for Gαs* expression alone involves an *acy-1*-dependent process distinct from Glu excitotoxicity that results in the background-level necrosis of head neurons observed in the *nuIs5* (Gαs*-only) strain.

As the genetic disruption of GluT via *glt-3* elevates endogenous Glu signaling (Mano *et al.* 2007) and is toxic via a mechanism that requires action of identified GluRs, the combinatorial model we describe here appears to closely approximate forms of mammalian excitotoxicity. Genetic dissection of Glu excitotoxicity might be exploited to identify novel physiological strategies for limiting necrosis associated with Glu hypersignaling.

The exclusive dependence of Glu excitotoxicity on AMPA-Rs in *C. elegans* (Fig. 1) appears to contrast with classic forms of mammalian excitotoxicity, in which NMDA-Rs have been heavily implicated (Rothman and Olney 1987; Choi 1992; Arundine and Tymianski 2004). Studies of nematode GluR biology suggest some differences between mammals and *C. elegans* in the physiology and roles of AMPA-Rs and NMDA-Rs. Unlike mammalian NMDA-Rs, *C. elegans* NMDA-Rs are expressed in a fraction of the cells expressing AMPA-Rs (Brockie *et al.* 2001b) and, when co-expressed, are segregated to non-overlapping post-synaptic areas (Brockie *et al.* 2001a) (whereas in mammals the co-expression of NMDA-Rs and AMPA-Rs at the same dendritic spine is critical for Glu physiology; Malenka and Nicoll 1999). Furthermore, nematode NMDA-Rs do not show the Mg^{2+}-block characteristic of mammalian NMDA-Rs (Brockie *et al.* 2001a), and their NR2-like subunits (Brockie *et al.* 2001b) lack the large intracellular C-terminal domain that is functionally critical in mammalian NMDA-Rs (Kohr *et al.* 2003). Lastly, established learning and memory paradigms in *C. elegans* require AMPA-Rs, rather than NMDA-Rs (Rose *et al.* 2005). Our findings that AMPA-Rs mediate nematode excitotoxicity underscore the central importance of Ca^{2+}-permeable AMPA-Rs in *C. elegans* neurophysiology.

We find that calreticulin, an ER protein that can regulates calcium homeostasis, is required for nematode excitotoxicity, as was found for other forms of necrotic neurodegeneration in *C. elegans* (Xu *et al.* 2001). This requirement has been suggested to depend in part on calreticulin's impact on ER calcium storage and release (Driscoll and Gerstbrein 2003). However, calreticulin's other function as a protein folding chaperone might influence the expression of receptors, channels, and enzymes that are necessary for nematode excitotoxicity. Although we have found that mutations in one calpain gene (*clp-1*) do not impact Glu-dependent neurodegeneration, this does not exclude a possible involvement of calpains altogether, as there are multiple calpain genes encoded in the nematode genome.

A model for synergism between Glu and cAMP signaling in nematode excitotoxicity

We report here that elevated Glu signaling induced by GluT deletion in *C. elegans* can induce excitotoxic neuronal death if it is paired with elevated cAMP signaling. We find that this form of excitotoxicity depends upon a calcineurin-inhibited adenylyl cyclase, AC9 – a novel player in excitotoxic-like conditions. Several models can be proposed to link adenylyl cyclase and hyperactive Glu signaling in *C. elegans*. These include cAMP-induced pre-synaptic regulation of transcription as observed in *Drosophila* and *Aplysia* (Kandel 2001; Waddell and Quinn 2001). Alternatively, in a mammalian post-synaptic plasticity mechanism, AMPA-R activity is increased by changing its phosphorylation/dephosphorylation balance through the regulatory activity of an associated complex containing calcineurin and cAMP-activated PKA (Banke *et al.* 2000; Tavalin *et al.* 2002; Dodge and Scott 2003). Our data show that in *C. elegans* excitotoxicity both GluR and cAMP hypersignaling occur in the same post-synaptic neurons (as the activated Gαs* is expressed only in the post-synaptic neurons that express *glr-1*), and that mammalian proteins expressed in oocytes can reproduce AMPA-Rs hyperactivation by active AC9 at the post-transcriptional level in a cell-autonomous way. Based on the combination of previous studies in mammals and our data in *C. elegans*, we suggest a model for the convergence of hyperactive Glu and cAMP signaling in nematode excitotoxicity at the post-transcriptional level in the post-synaptic cell (Fig. 4). In this model, AMPA-Rs are expressed in post-

synaptic neurons together with calcineurin, which normally inhibits AC9 and reduces AMPA-R phosphorylation. If calcineurin inhibition is overcome by introduced Gαs*, AC9 is activated, cAMP is produced, PK-A is activated, and AMPA-Rs are phosphorylated to enhance their activity potential. When AMPA-R potentiation is paired with Glu overstimulation, the channel can be maximally hyperactivated, leading to Ca^{2+} influx that tips the balance to neurodegeneration.

Can a similar mechanism participate in mammalian excitotoxicity?

The *C. elegans* model we characterize is highly similar, though not identical, to classic forms of mammalian excitotoxicity (Rothman and Olney 1986; Choi 1992). As we have shown that a novel aspect of our model – the potentiation of Ca^{2+}-permeable AMPA-Rs by AC9 when the inhibitory effect of calcineurin is removed – can be reproduced (post-transcriptionaly and cell-autonomously) with mammalian proteins (Fig. 3), it is worth considering whether a similar mechanism might also play a part in some mammalian excitotoxic conditions. There is considerable evidence to suggest the involvement of Ca^{2+}-permeable AMPA-Rs in ischemia (Calderone *et al.* 2003; Liu *et al.* 2004) and ALS (Rao and Weiss 2004; Kwak and Kawahara 2005). Furthermore, PKA/cAMP and calcineurin signaling have been shown to control AMPA-R activity levels (Song and Huganir 2002; Tavalin *et al.* 2002; Groth *et al.* 2003). Although calcineurin has conflicting effects on a myriad of different signaling cascades (Shibasaki *et al.* 2002), some of the reported observations correlate with the model we propose here. Indeed, calcineurin activity drops in affected hippocampal areas after ischemia (Morioka *et al.* 1999), and in both sporadic and familial ALS (Ferri *et al.* 2004). Although these observations are in line with the model we propose, it is important to emphasize that our current study focuses on the analysis of nematode excitotoxicity, and provides only an initial glimpse into the possibility that a similar mechanism might be operative in AMPA-R hyperactivity in other animals. Further studies that include mammalian tissue cultures and higher animal disease models could address this question in the future.

In sum, we define a condition of glutamate-induced neuronal necrosis in *C. elegans* and describe some of the critical mediators of excitotoxicity in the nematode. Based on our observations, we suggest a novel model for *C. elegans* excitotoxicity, featuring a synergism between Ca^{2+}-permeable AMPA-Rs and AC9. We demonstrate that a key interaction in this cascade can also be operable when the corresponding mammalian proteins are co-expressed in a heterologous system. The landmark studies of apoptosis (Metzstein *et al.* 1998) and RNAi (Fire *et al.* 1998) in *C. elegans* elegantly demonstrated that basic molecular processes conserved from nematodes to mammals can be genetically dissected in nematodes to suggest critical directions for further analysis in higher organisms. Testing whether mechanisms similar to the one suggested here for nematode excitotoxicity might also be involved in some forms of mammalian excitotoxicity may be a future goal that could provide novel insights into critical steps in excitotoxic signaling in stroke and in chronic diseases like ALS.

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