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$G\alpha_s$-Induced Neurodegeneration in *Caenorhabditis elegans*

Allison J. Berger, Anne C. Hart, and Joshua M. Kaplan

Department of Genetics, Harvard Medical School, Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

We describe a genetic model for neurodegeneration in the nematode *Caenorhabditis elegans*. Constitutive activation of the GTP-binding protein $G\alpha_s$ induces neurodegeneration. Neuron loss occurs in two phases whereby affected cells undergo a swelling response in young larvae and subsequently die sometime during larval development. Different neural cell types vary greatly in their susceptibility to $G\alpha_s$-induced cytotoxicity, ranging from 0 to 88% of cells affected. Mutations that prevent programmed cell death do not prevent $G\alpha_s$-induced killing, suggesting that these deaths do not occur by apoptosis. Mutations in three genes protect against $G\alpha_s$-induced cell deaths. The *acy-1* gene is absolutely required for neurodegeneration, and the predicted ACY-1 protein is highly similar (40% identical) to mammalian adenylyl cyclases. Thus, G_s-induced neurodegeneration is mediated by the second messenger cAMP. Mutations in the *unc-36* and *eat-4* genes are partially neuroprotective, which indicates that endogenous signaling modulates the severity of the neurotoxic effects of $G\alpha_s$. These experiments define an intracellular signaling cascade that triggers a necrotic form of neurodegeneration.

Key words: cell death; neurodegeneration; necrosis; signal transduction; G-protein; cAMP; mutant; Caenorhabditis elegans

Neuronal cell death is a prominent feature both of normal brain development and of particular pathological states. Neuron cell deaths can be placed into two general categories (apoptotic and necrotic) on the basis of a variety of criteria. Although a great deal is known about the molecular pathways leading to apoptotic deaths, the pathways leading to necrotic cell deaths are less well understood. Most examples of necrotic neurodegenerative deaths occur in pathological states, e.g., stroke (or other cerebrovascular injury) or neurological disorders. One hallmark of these neurodegenerative disorders is that in each case specific classes of neurons are targeted for degeneration. For example, dopaminergic neurons of the substantia nigra are lost in Parkinson's disease, whereas in epilepsy and focal ischemia CA1 and CA3 neurons of the hippocampus are killed selectively (Pulsinelli et al., 1982; Ben-Ari, 1985).

Genes involved in inherited neurodegenerative disorders have been characterized in humans as well as in a several model organisms. Nine genes involved in inherited human neurodegenerative disorders have been cloned, including huntingtin, ataxin, and SOD. Genetic models for neurodegeneration also have been described in mice (Mullen et al., 1976; Herrup and Wilczynski, 1982; Norman et al., 1995), flies (Grether et al., 1995; Hay et al., 1995; Rangnathanan et al., 1995; Chen et al., 1996; White et al., 1996), and *Caenorhabditis elegans* (Driscoll and Kaplan, 1997; Hengartner, 1997). From these model systems several genes have been identified that apparently control neurodegenerative cell deaths. For example, four genes leading to necrotic forms of neuron death in worms have been described, each encoding proteins that are similar to mammalian ion channel subunits—the epithelial sodium channel homologs (MEC-4, MEC-10, and DEG-1) and a neuronal acetylcholine receptor homolog (DEG-3) (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Treinin and Chalfie, 1995). In these cases neuron death is thought to occur by exaggerated or toxic influx of ions, perhaps akin to excitotoxicity in mammals.

Although these genetic studies successfully have identified the many genes involved in neurodegenerative deaths, in most cases the identity of these genes has not implicated directly a defined signal transduction pathway in neurodegeneration. Alterations in G-protein-coupled phospholipase C signaling lead to retinal degeneration in *Drosophila* (for review, see Rangnathanan et al., 1995). A second example of G-protein-induced neurodegeneration has been reported recently. Constitutive activation of the heterotrimeric G-protein G_s causes neuronal degeneration in *C. elegans* (Korswagen et al., 1997). Here we show that cells differ greatly in their susceptibility to $G\alpha_s$-induced killing, that the neurotoxic effects of $G\alpha_s$ are mediated by *acy-1* (which encodes a protein that is 40% identical to mammalian adenylyl cyclases), and that endogenous neural signaling modulates the severity of $G\alpha_s$-induced killing. These results define an intracellular signaling pathway by which G_s triggers a necrotic form of neuron death.

MATERIALS AND METHODS

Plasmid construction and transgene expression. The $G\alpha_s$ expression vector (KP#20) was constructed by ligating a 1.5 kb *Nco*I–*Xho*I fragment encoding a GTPase defective (Q227L) mutant rat $G\alpha_s$ cDNA containing the hemagglutinin (HA) epitope into the *glr-1* expression vector CX#1 (Hart et al., 1995; Maricq et al., 1995). A *mec-7::*α_s*(gf)* expression plasmid (KP#7) was constructed by ligating the 1.5 kb *Nco*I–*Xho*I

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Correspondence should be addressed to Dr. Kaplan at his present address: Department of Molecular and Cell Biology, 361 Life Sciences Addition, University of California, Berkeley, CA 94720-3200.

Dr. Hart's present address: Department of Pathology, Harvard Medical School and Massachusetts General Hospital Cancer Center, 149-7202 13th Street, Charlestown, MA 02129.

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$G\alpha_s$(Q227L) into the *mec-7* expression vector pPD52.102. Transgenic animals were prepared by microinjecting various expression constructs together with a *glr-1::gfp* plasmid (KP#6), using *lin-15* as a transformation marker (Huang et al., 1994). Two stable lines carrying *glr-1* expression constructs for both green fluorescent protein (GFP) and the GTPase defective $G\alpha_s$ (*nuIs3* and *nuIs5*) were isolated after γ-irradiation. Unless otherwise noted, data reported in the text refer to *nuIs5*, which is referred to as α_s*(gf)*. Integrated lines typically greatly overexpress the transgenes. Thus, it is likely that the *nuIs5*-encoded $G\alpha_s$ is more abundant than the endogenous *C. elegans* $G\alpha_s$.

Characterization of the neurodegeneration phenotype. Swollen or missing cells were identified by examining the morphology of GFP-expressing cells. The *glr-1*-expressing cells were identified previously (Hart et al., 1995; Maricq et al., 1995). In α_s*(gf)* animals ~90% of the PVC neurons degenerate. Other cells degenerate at lower frequencies, including AVA, AVD, AVE, AVG, PVQ, RIG, and SMD. Cell deaths with similar morphology and similar cell type specificity were observed in both *nuIs3* and *nuIs5* animals. $G\alpha_s$-induced neurodegeneration in various genetic backgrounds was quantitated as the number of swollen PVC neurons in L1 larvae and as the percentage of PVC neurons that are missing or are swollen in adult hermaphrodites. Statistical differences between genotypes was determined by the method of attributable risk (Devore, 1987). We compensated for multiple comparisons by setting $p < 0.005$ as the threshold for significance. Confidence intervals of 95% were calculated as 1.96* (SEM).

Antibody staining. $G\alpha_s$ expression in transgenic animals was monitored by staining fixed animals with anti-HA antibodies. Fixation and antibody staining were done according to a protocol devised by M. Nonet. Briefly, worms were fixed and their cuticles reduced in Bouin's solution with β-mercaptoethanol (BME). Worms were washed sequentially in BTB [1× borate buffer, pH 9.2 (20 mM H_3BO_3 and 10 mM NaOH), 0.5% Triton X-100, and 2% BME], BT (1× borate buffer and 0.5% Triton X-100), and finally in AbA solution (1× PBS, 1% BSA, 0.5% Triton X-100, 0.05% sodium azide, and 1 mM EDTA). The anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim, Indianapolis, IN) and goat anti-mouse rhodamine-conjugated antibody (Cappel, Cochranville, PA) were used at a 1:100 dilution, and incubations were done in AbA solution overnight at room temperature.

Isolation of acy-1 *mutations.* Mutations that restore normal locomotion rates to α_s*(gf)* homozygotes were isolated from the F2 self-progeny of EMS mutagenized hermaphrodites. Candidate suppressor mutants subsequently were screened for the reduction of $G\alpha_s$-induced swelling in L1 larvae. In a screen of 7500 haploid genomes, the *nu327*, *nu329*, and *nu343* alleles were isolated. Other suppressor mutations isolated in this screen will be described elsewhere.

Positional cloning of acy-1. The *acy-1* mutations *nu327*, *nu329*, and *nu343* are all linked to *dpy-17* in two-factor mapping experiments. Three-factor mapping placed *acy-1* between *emb-5* and *dpy-17*: (*nu327 dpy-17*) 37/37 *unc-32*; (*nu329 dpy-17*) 16/16 *unc-32*; (*nu343 dpy-17*) 4/4 *unc-32*; *unc-79* (6/14) MJ#NEC2 (5/14) *nu329* (3/14) *dpy-17*; *emb-5* (1/16) *nu327* (15/16) *dpy-17*. The cosmid F17C8 (which carries ACY-1) was microinjected into *acy-1(nu327); nuIs5* animals, and transgenic lines were isolated by using *goa-1::gfp* (KP#13) as a transformation marker (Ségalat et al., 1995). Four independent lines were obtained, two of which were rescued for the *acy-1* phenotype, i.e., they had increased degeneration of the PVC neurons. Sequences spanning the GENEFINDER-predicted exons of ACY-1 (accession number Z35719) were amplified from the mutants *nu327*, *nu329*, and *nu343*, and the resulting fragments were sequenced directly by cycle sequencing. The GENEFINDER prediction for the first exon was confirmed by isolating partial cDNA clones from the Barstead RB1 cDNA library by PCR amplification.

Analysis of acy-1 *expression.* A deleted derivative (KP#106) of the cosmid F17C8 was isolated by digestion with *Afl*II and religation. KP#106 contains the entire 8.35 kb of the *acy-1* genomic region, together with 5.2 kb 5′ and 4.9 kb 3′ flanking sequences. An *acy-1::gfp* expression vector (KP#107) was constructed by PCR-amplifying a 1.7 kb fragment containing the GFP coding region and the *unc-54* transcription terminator from pPD95.75 and ligating this fragment into the unique *Asp*718 site in KP#106, creating a fusion protein containing the first six exons of *acy-1* fused to GFP. The ACY-1::GFP fusion protein contains six predicted transmembrane domains of ACY-1 and hence is membrane-localized. Transgenic animals carrying KP#107 were isolated by microinjection, using *lin-15* as a transformation marker (Huang et al., 1994). Expressing cells were identified on the basis of their morphology and nuclear positions.

Isolation and responsiveness of eat-4 *mutations.* We screened 11,000 mutagenized haploid genomes for animals that failed to respond to nose touch. Mutants isolated were subjected to a series of secondary screens, including dye filling of the amphid sensory neurons and responsiveness to osmotic shock and volatile repellents. Six alleles of *eat-4* (*n2458*, *n2474*, *nu2*, *nu142*, *nu143*, and *nu146*) were isolated in this screen. A seventh allele, *eat-4(ky5)*, was isolated by C. Bargmann (University of California at San Francisco, San Francisco, CA) as a chemotaxis defective mutant. All seven *eat-4* alleles are normal for dye filling but are defective for all three ASH sensory behaviors.

Behavioral assays. ASH sensory responses were assayed as previously described (Kaplan and Horvitz, 1993; Hart et al., 1995; Maricq et al., 1995; Troemel et al., 1995). For nose touch, animals were tested 10 times each, with a positive response being scored when animals either halted forward movement or initiated backward movement after the stimulus. Osmotic avoidance assays were performed with either of two protocols. Assays in Table 3 were done as described previously (Hart et al., 1995). Assays in Table 2 were done with a modified protocol described by C. de Vries and R. Plasterk (personal communication). Briefly, worms were washed twice with S-basal and once with water and placed inside a semicircle of 60% glycerol with bromphenol blue. A microliter of diacetyl at a 1:100 dilution was placed outside the semicircle at the opposite edge of the plate. The percentage of adult worms that crossed the osmotic barrier after 10 min was calculated. For ASH-mediated volatile avoidance, an eyelash was dipped in 1-octanol and held near an animal's nose; responses were quantitated by recording the length of time that elapsed before an animal reversed locomotion.

Chemotaxis assays were performed as previously described (Bargmann et al., 1990). Assay plates contained 2% Difco-agar, 5 mM potassium phosphate, pH 6.0, 1 mM calcium chloride, and 1 mM magnesium sulfate. Animals were placed at the center of the plate, with 1 ml of diluted attractant and 1 ml of 1 M sodium azide at one edge of the plate; 1 ml of ethanol and 1 ml of sodium azide were placed at the opposite edge. Dilutions in ethanol were as follows: 1:1000 for diacetyl, 1:100 for isoamyl alcohol, and 1:200 for benzaldehyde. The chemotaxis index was calculated after 1 hr as the (number of worms at attractant − number of worms at control solvent)/total number of animals.

RESULTS

$G\alpha_s$-induced neurodegeneration

While studying signaling by the G-protein G_s, we made the observation that expression of a constitutively active rat $G\alpha_s$ cDNA caused neurodegeneration in *C. elegans*. Mutations that diminish the GTPase activity of $G\alpha_s$ have been shown to cause constitutive, agonist-independent signaling (Landis et al., 1989; Lyons et al., 1990; Wong et al., 1991). We expressed a rat cDNA encoding a GTPase-defective (Q227L) $G\alpha_s$ subunit, hereafter referred to as α_s*(gf)*, in *C. elegans* neurons by using the *glr-1* glutamate receptor (GluR) promoter. We chose the *glr-1* promoter because glutamate-responsive cells might be prone to neurodegeneration, because it is highly expressed, and because *glr-1*-expressing cells control locomotion, an easily assayed behavior. The *glr-1* promoter is expressed in 17 classes of neurons, including interneurons required for locomotion (Hart et al., 1995; Maricq et al., 1995). $G\alpha_s$ was coexpressed with the GFP protein of *Aequorea victoria* (Chalfie et al., 1994), which allowed us to examine the morphology of $G\alpha_s$-expressing cells. Transgenic *glr-1::*α_s*(gf)* animals were paralyzed and a subset of the expressing neurons swelled to several times their normal diameter and eventually disappeared, presumably because they died (Fig. 1, Table 1). These results suggest that exaggerated $G\alpha_s$ signaling kills neurons.

$G\alpha_s$-expressing neurons differed greatly in their susceptibility to $G\alpha_s$-induced toxicity. In first-stage (L1) *glr-1::*α_s*(gf)* larvae, the swelling of different cell types occurred at very different frequencies, and these differences were seen in two independent α_s*(gf)* transgenes: in *nuIs5*, PVC 88%, AVD 34%, and RIG 7%; in *nuIs3*, PVC 77%, AVD 44%, and RIG 6%. One potential mech-

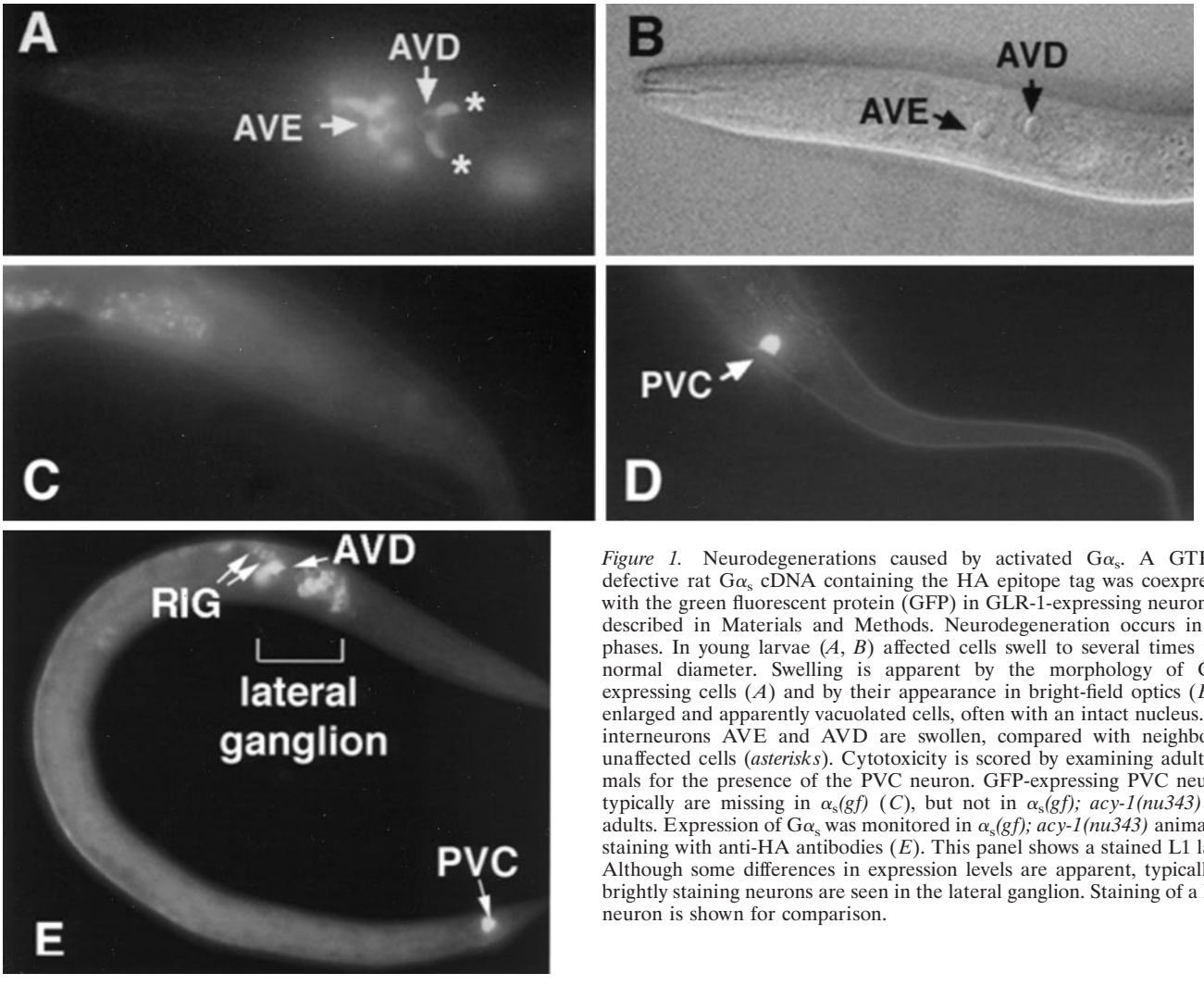


Figure 1. Neurodegenerations caused by activated $G\alpha_s$. A GTPase-defective rat $G\alpha_s$ cDNA containing the HA epitope tag was coexpressed with the green fluorescent protein (GFP) in GLR-1-expressing neurons, as described in Materials and Methods. Neurodegeneration occurs in two phases. In young larvae (*A*, *B*) affected cells swell to several times their normal diameter. Swelling is apparent by the morphology of GFP-expressing cells (*A*) and by their appearance in bright-field optics (*B*) as enlarged and apparently vacuolated cells, often with an intact nucleus. The interneurons AVE and AVD are swollen, compared with neighboring unaffected cells (*asterisks*). Cytotoxicity is scored by examining adult animals for the presence of the PVC neuron. GFP-expressing PVC neurons typically are missing in $\alpha_s(gf)$ (*C*), but not in $\alpha_s(gf)$; *acy-1(nu343)* (*D*) adults. Expression of $G\alpha_s$ was monitored in $\alpha_s(gf)$; *acy-1(nu343)* animals by staining with anti-HA antibodies (*E*). This panel shows a stained L1 larva. Although some differences in expression levels are apparent, typically 10 brightly staining neurons are seen in the lateral ganglion. Staining of a PVC neuron is shown for comparison.

anism for this apparent cell type specificity would be that cells differ substantially in their levels of $G\alpha_s$ expression. Because the rat $G\alpha_s$ cDNA contains the HA epitope, we were able to test this possibility by staining $\alpha_s(gf)$ animals with anti-HA antibodies (Fig. 1*E*). Differences in $G\alpha_s$ expression correlated well with differences in toxicity for some cells, but not for others. For example, RIG neurons expressed much less $G\alpha_s$ and swelled much less frequently than PVC neurons, whereas AVD and PVC neurons expressed equivalent amounts of $G\alpha_s$ but swelled at significantly different frequencies. In general, 10 neurons in the lateral ganglion expressed levels of $G\alpha_s$ similar to those seen in the PVC, whereas most $\alpha_s(gf)$ animals have only one or two dying cells in the lateral ganglion (Fig. 1*A*). Thus, many more cells express $G\alpha_s$ than are found to die, and differences in $G\alpha_s$-induced toxicity do not always correlate with differences in $G\alpha_s$ expression. To demonstrate further the specificity of $G\alpha_s$-induced neurodegeneration, we expressed $\alpha_s(gf)$ by using the *mec-7* promoter. MEC-7 tubulin is expressed abundantly in five neurons that sense light touch to the worm's body, which are called touch cells (Savage et al., 1989; Hamelin et al., 1992; Mitani et al., 1993). Animals expressing the *mec-7::*$\alpha_s(gf)$ transgene are indistinguishable from wild-type animals, having no obvious defect in touch sensitivity nor in the morphology of the touch cells (data not shown). Therefore, both the *glr-1* and the *mec-7* expression constructs support the notion that the effects of $G\alpha_s$ on neural activity and on neurodegeneration are cell type-specific.

The pattern of cell deaths that was observed does not explain the severity of the locomotion defect in $\alpha_s(gf)$ animals. The GLR-1-expressing interneurons AVA, AVB, AVD, and PVC play an important role in locomotion; hence these cell deaths could, in principle, explain the locomotion defects (Chalfie et al., 1985). However, the sluggish locomotion defect is apparent in 100% of the $\alpha_s(gf)$ animals, although cell deaths are found in only a subset of animals (see above). For example, a significant fraction of uncoordinated animals can be found in which only the PVC neurons have died (A. Berger and J. Kaplan, unpublished observations). Because killing the PVC neurons with a laser microbeam is not sufficient to cause uncoordinated locomotion (Chalfie et al., 1985), these results suggest that the $\alpha_s(gf)$ transgene inhibits the function of these interneurons in addition to causing a subset of these cells to die.

ACY-1 mediates $G\alpha_s$-induced neurodegeneration

To identify the targets of $G\alpha_s$, we isolated mutations that block $G\alpha_s$-induced paralysis and neurodegeneration. In a screen of 7500 haploid genomes, we isolated three semidominant mutations that map to the cluster of chromosome III (Fig. 2, Table 1). Via a series of experiments we showed that these mutations occur in

Table 1. Role of cAMP and neural activity in G_s-induced neurodegeneration

mut: $\alpha_s(gf)$ genotype (MUT gene product)	PVC swelling (%)	PVC degeneration (%)
+	88 ± 7	89 ± 6
Adenylyl cyclase:		
acy-1(nu327)	19 ± 8*	0 ± 0*
acy-1(nu327)/+	63 ± 11*	ND
acy-1(nu329)	0 ± 0*	0 ± 0*
acy-1(nu329)/+	27 ± 9*	ND
acy-1(nu343)	4 ± 4*	4 ± 4*
acy-l(nu343)/+	24 ± 9*	ND
Degeneration:		
deg-1(u506u550) (ENaC)	83 ± 7	97 ± 3
mec-6(e1342)	84 ± 6	91 ± 5
unc-8(n491n1192) (ENaC)	91 ± 5	90 ± 5
Calcium channels:		
eg1-19(n582) ($\alpha 1$ subunit)	90 ± 7	92 ± 5
unc-2(e55) ($\alpha 1$ subunit)	86 ± 5	82 ± 7
unc-36(e251) ($\alpha 2$ subunit)	79 ± 7	68 ± 5*
unc-36(e873) ($\alpha 2$ subunit)	85 ± 4	74 ± 6*
Glutamate signaling:		
glr-1(n2461) (GluR A)	82 ± 5	95 ± 4
eat-4(ky5)	78 ± 7	58 ± 6*
eat-4(n2474)	89 ± 6	62 ± 7*
Apoptosis:		
ced-3(n717) (ICE)	94 ± 5	85 ± 6
ced-4(n1162)	87 ± 6	95 ± 3
Exocytosis:		
unc-104(e1265) (Kinesin)	95 ± 4	99 ± 3
snt-1(n2665) (Synaptotagmin)	89 ± 6	92 ± 5

Swelling and cytotoxicity of PVC neurons (mean ± 95% confidence interval) caused by the $\alpha_s(gf)$ transgene were quantitated in various genetic backgrounds, as described in Materials and Methods. For each data point 30–80 animals were analyzed. *Indicates significantly ($p < 0.005$) different from $\alpha_s(gf)$ single mutants.

an adenylyl cyclase gene, which we have named *acy-1*. First, two of these mutations were mapped to a 1.5 cm genetic interval between MJ#NEC2 and *dpy-17* (Fig. 2*A*). Second, a transgene carrying a cosmid from this interval (F17C8) corrected the mutant phenotype of *acy-1(nu327)* animals (Fig. 2*B*). Third, all three alleles corresponded to mutations in the predicted exons of the gene F17C8.1 (Fig. 2*C,D*), one of two predicted adenylyl cyclase genes in the *C. elegans* genome database. It is unclear why these mutations are partially dominant. The molecular nature of the mutations, together with the fact that the mutant phenotype is rescued by a wild-type copy of the F17C8 cosmid, suggests that these mutations reduce ACY-1 activity. For example, *nu329* and *nu343* are predicted to disrupt pre-mRNA splicing. Thus, it is possible that $\alpha_s(gf)$ animals are highly sensitive to changes in cAMP levels; however, because none of the genetic deficiencies in this region uncovers the *acy-1* gene, we cannot test directly whether *acy-1* is haplo-insufficient. These results suggest that $G\alpha_s$ neurodegeneration is mediated by changes in intracellular cAMP.

The expression pattern of *acy-1* was determined by analyzing a GFP reporter construct. The *acy-1::gfp* fusion protein is expressed in virtually all neurons and body muscles; however, it does not appear to be expressed in other tissues (Fig. 3). Therefore, the cell type specificity of $G\alpha_s$-induced neurodegeneration cannot be explained by the expression pattern of the *acy-1::gfp* reporter. One caveat to this conclusion is that the expression of reporter genes can differ from that of the endogenous genes if, for example, some critical regulatory elements are missing in the reporter constructs.

Role of ACY-1 in sensory behaviors

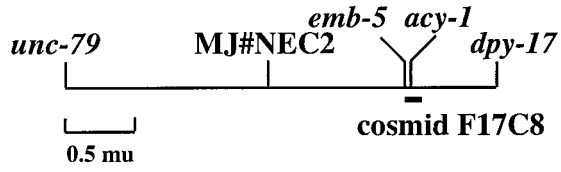
cAMP has been implicated in a wide variety of signaling pathways, and ACY-1 is expressed in most if not all neurons and muscles in the worm. Therefore, we would expect that *acy-1* mutants would have defects in behavior or development. In particular, the *C. elegans* $G\alpha_s$ subunit was shown previously to be essential for viability as well as for regulating locomotion and egg laying (Korswagen et al., 1997); hence we would expect to find similar defects in *acy-1* mutants. Surprisingly, *acy-1* mutants are overtly normal, with no obvious defects in development, fertility, egg laying, or male mating behaviors (data not shown). Because the deaths of GLR-1-expressing cells are prevented by *acy-1* mutations, we tested whether other behaviors mediated by GLR-1-expressing cells are impaired by *acy-1* mutations. GLR-1-expressing cells are required for response to body touch and for locomotion (Chalfie et al., 1985); however, these behaviors are not affected in *acy-1* mutants (see Table 4) (our unpublished observations). Three sensory behaviors mediated by the ASH neurons (nose touch, osmotic shock, and volatile repellent responses) are also likely to be mediated by the GLR-1-expressing cells (Bargmann et al., 1990; Hart et al., 1995; Maricq et al., 1995; Troemel et al., 1995), yet none of these behaviors is impaired in *acy-1* mutants (Table 2).

Recently, two putative cyclic nucleotide-gated ion channels (TAX-2 and TAX-4) were shown to be involved in axon morphogenesis, thermotaxis, and olfaction (Coburn and Bargmann, 1996; Komatsu et al., 1996). Because these channels in principle could be activated by the cAMP produced by ACY-1, we examined *acy-1* mutants for defects in these processes (Table 2). Mutations in *tax-2* and *tax-4* cause certain chemosensory neurons to grow out supernumerary axonal processes, which can be visualized by staining the animals with the fluorescent dye DiI (Coburn and Bargmann, 1996; Komatsu et al., 1996). We found no abnormal sensory axon morphologies in DiI-stained *acy-1* mutants (data not shown). Chemotaxis toward isoamyl alcohol and benzaldehyde requires TAX-2 and TAX-4 channels (Coburn and Bargmann, 1996; Komatsu et al., 1996). We found that the response of *acy-1* mutants to benzaldehyde was normal, whereas their response to isoamyl alcohol was impaired slightly. Finally, the thermotactic behavior of *acy-1* mutants was also normal (A. Berger and O. Hobert, unpublished observations). Because *acy-1* mutants lack the axon and olfactory defects seen in *tax-2* and *tax-4* mutants, it is unlikely that ACY-1 is the sole source of cyclic nucleotides required for the activation of TAX-2 and TAX-4. These results are consistent with the fact that TAX-4 channels are activated selectively by cGMP (Komatsu et al., 1996). Furthermore, these results do not exclude the possibility that cAMP is required for these behaviors, because there is at least one other adenylyl cyclase in the worm genome.

Cytotoxic targets of cAMP

Several previously identified genes were considered good candidates for mediating the toxic effects of $G\alpha_s$ (see Table 1). The putative cyclic nucleotide-gated ion channels TAX-2 and TAX-4 are not expressed in *glr-1*-expressing cells (Coburn and Bargmann, 1996; Komatsu et al., 1996) and hence are unlikely targets in this case. The *mec-6*, *unc-8*, and *deg-1* genes were implicated previously in neurodegeneration (Chalfie and Wolinsky, 1990;

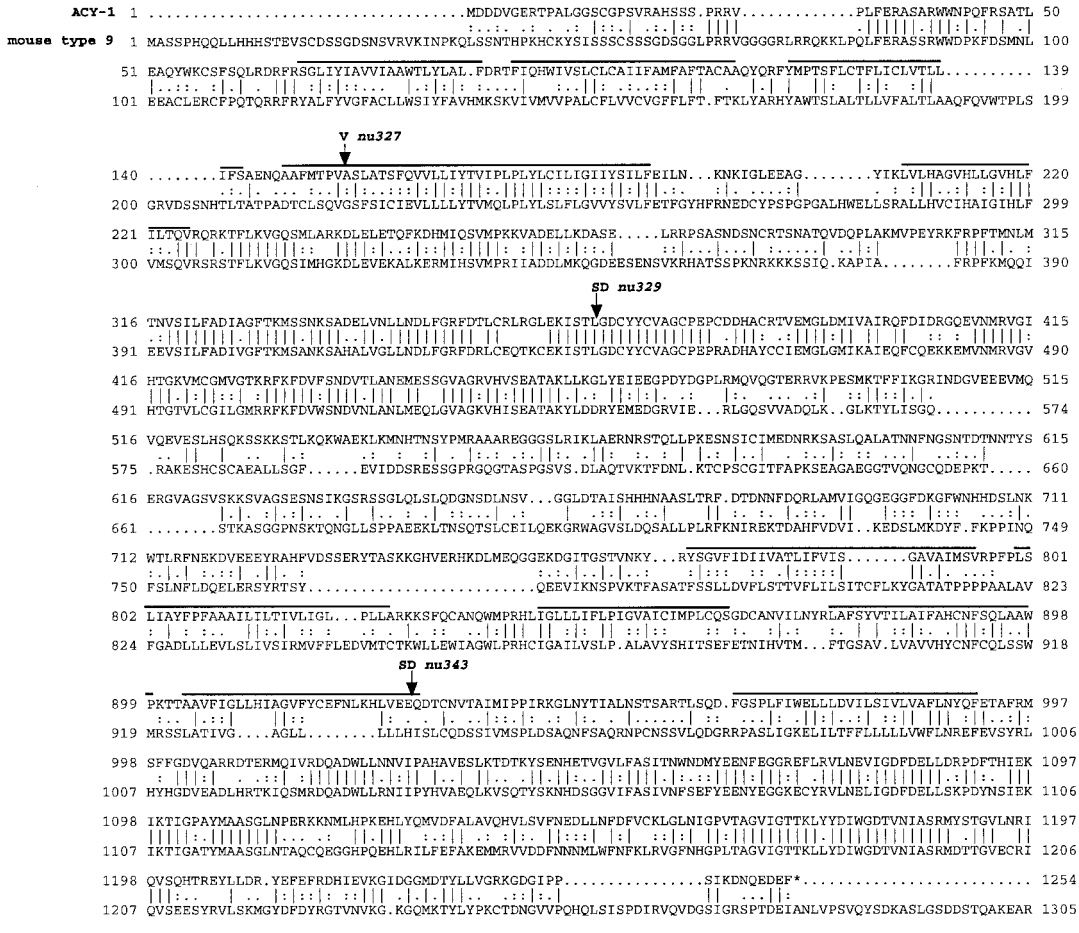
A



B

genotype	% PVC degeneration
acy-1(nu327); α_s*(gf)*	**12**
acy-1(nu327); α_S*(gf); nuEx(F17C8)*	**75**
α_S*(gf)*	**88**

C



D

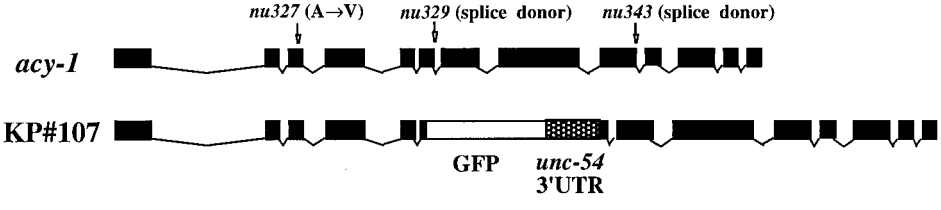


Figure 2. ACY-1 encodes an adenylyl cyclase. *A*, Genetic and physical map position of *acy-1*. *B*, Transgenes containing the cosmid F17C8 rescue the *acy-1(nu327)* mutant phenotype. *C*, The predicted amino acid sequence, as predicted by GENEFINDER (accession number Z35719) and confirmed by our RT-PCR analysis, of ACY-1 (*top*) is shown aligned to mouse adenylyl cyclase type 9 (*bottom*), the most highly related sequence (40% identical) in the database. *Underlined* sequences indicate predicted transmembrane domains. *D*, Predicted structure of the *acy-1* gene (using the GENEFINDER algorithm) and of the GFP fusion construct (KP#107) are shown. Positions of the *acy-1* mutations *nu327*, *nu343*, and *nu329* are indicated (*C*, *D*).

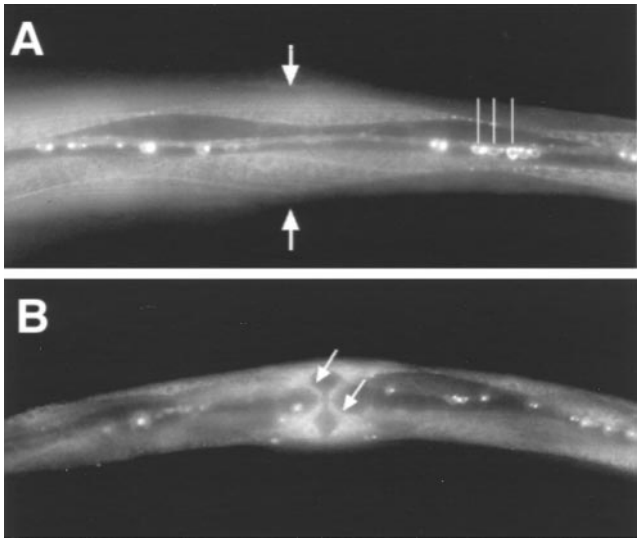


Figure 3. ACY-1 is expressed in neurons and muscles. The KP#107 *acy-1::gfp* fusion gene was expressed in transgenic animals, as described in Materials and Methods. ACY-1 appears to be expressed in most or all muscles and neurons. *A*, Expression in the two ventral rows of body muscles (*arrows*) and in the ventral cord neurons and neuropile (*lines*). *B*, Expression in the vulva muscles (*arrowheads*). Nearly all of the 302 neurons in the adult appear to express ACY-1. Cell bodies can be identified on the basis of the bright fluorescence in intracellular membranes (presumably the endoplasmic reticulum or Golgi apparatus). ACY-1 does not appear to be expressed in non-neural tissues, nor is it expressed in the pharynx.

Driscoll and Chalfie, 1991; Shreffler et al., 1995; Tavernarakis et al., 1997), and the DEG-1 and UNC-8 proteins are similar to mammalian epithelial sodium channel subunits (ENaC), which are activated potently by cAMP-dependent protein kinase (PKA) (Sariban-Sohraby et al., 1988; Oh et al., 1993; Bubien et al., 1994). The *unc-2*, *unc-36*, and *egl-19* genes encode subunits of voltage-dependent Ca^{2+} channels (Schafer and Kenyon, 1995; Lee et al., 1997) that are likely to be regulated by PKA (Curtis and Catterall, 1985) and also have been implicated in neurodegeneration. The *glr-1* gene encodes an ionotropic GluR (Hart et al., 1995; Maricq et al., 1995), GluRs have been implicated in neurodegeneration in mammals (Olney, 1986; Choi, 1988), and PKA augments the response of mammalian neurons to glutamatergic agonists (Greengard et al., 1991; Wang et al., 1991; Colwell and Levine, 1995).

Of these candidate genes the *unc-36* mutations (*e251* and *e837*) conferred a slight but significant reduction in $G\alpha_s$-induced cytotoxicity (Table 1). Interestingly, the *unc-36* mutations had no effect on cell swelling. These results suggest that either Ca^{2+} influx or depolarization of the affected cells modulates susceptibility to $G\alpha_s$ cytotoxicity. All other candidate genes had no effect on either neuron swelling or deaths in *glr-1::*α_s*(gf)* animals (Table 1). Our results do not exclude the possibility that these other candidate PKA targets play a role in $G\alpha_s$-induced toxicity. For example, more than one type of channel may be capable of mediating the toxic effects of $G\alpha_s$, in which case neurodegeneration would be prevented only in multiply mutant animals.

Role of apoptosis and necrosis in $G\alpha_s$ neurodegeneration

Whether neurodegeneration occurs by apoptosis or by necrosis has remained controversial (Choi, 1996). The $G\alpha_s$-induced deaths appear to be necrotic (i.e., undergoing cell swelling and delayed cytotoxicity) rather than apoptotic. We directly tested the role of apoptosis in these deaths by testing the effects of mutations in cell death genes (Hengartner, 1997). We found that mutations in the cell death genes *ced-3* and *ced-4*, both of which are required for apoptosis (Ellis and Horvitz, 1986), had no effect on $G\alpha_s$-induced swelling or killing (Table 1). Thus, apoptosis is not required for $G\alpha_s$-induced killing.

Table 2. Analysis of sensory behaviors in *acy-1* mutants

Nose touch	Responding (%)	Worms tested (number)
Wild type	76 ± 3	10
glr-1(n2461)	14 ± 6	6
acy-1(nu329)	79 ± 3	12
Osmotic avoidance	**Escapers (%)**	**Trials (number)**
Wild type	0 ± 0	6
osm-3(p802)	70 ± 3	5
acy-1(nu329)	3 ± 2	5
Volatile avoidance	**Seconds**	**Worms tested (number)**
Wild type	2.4 ± 0.3	19
eat-4(ky5)	10.6 ± 1.6	17
acy-1(nu329)	4 ± 0.6	18
Chemotaxis		
Benzaldehyde	**Chemotaxis index**	**Trials (number)**
Wild type	91 ± 2	9
odr-1(n1936)	4 ± 7	9
acy-1(nu329)	73 ± 6	9
Isoamyl alcohol		
Wild type	84 ± 2	15
odr-1(n1936)	6 ± 5	17
acy-1(nu329)	48 ± 7	17
Diacetyl		
Wild type	83 ± 5	5
odr-7(ky4)	15 ± 7	5
acy-1(nu329)	79 ± 4	5

Behavioral assays were performed as described in Materials and Methods. For nose touch, each worm was tested 10 times. For osmotic avoidance and chemotaxis, each trial tested between 50 and 150 animals. Errors represent SEM. Chemotactic responses to isoamyl alcohol were somewhat variable, with six assays showing a severe defect (14 ± 4) and 11 assays showing a subtle defect (mean = 67 ± 3). This variability does not reflect changes in temperature during growth or assays.

Role of endogenous neural signaling in $G\alpha_s$-induced neurodegeneration

Because $G\alpha_s$ often couples to neurotransmitter receptors thereby producing or altering synaptic transmission, we wondered whether endogenous neural activity would regulate $G\alpha_s$-induced neurodegeneration. The weakly neuroprotective effect of *unc-36* mutations is consistent with this hypothesis. Decreased calcium influx or decreased cell excitability in *unc-36* mutants could explain this neuroprotective effect. In addition, *unc-36* mutations have been shown to decrease endogenous synaptic transmission, albeit by an uncharacterized mechanism (Nguyen et al., 1995). Because UNC-36 channels play a role in many different aspects of

Table 3. Role of *eat-4* in ASH sensory responses

Genotype	Nose touch (% respond)	Osmotic avoidance (% escape)	Volatile avoidance (seconds)
Wild-type	86 ± 3	2 ± 1	2.9 ± 0.9
eat-4(ky5)	1 ± 1	75 ± 6	9.9 ± 1.6
eat-4(n2474)	2 ± 1	54 ± 6	9.6 ± 1.5

ASH-mediated sensory responses to nose touch, osmotic shock, and volatile repellents were compared in wild-type and *eat-4* mutants, as described in Materials and Methods. Errors indicate SEM in all cases. The number of animals and trials for each genotype were as follows: for nose touch, 10 animals and 100 trials; for osmotic avoidance, 60 animals and 5 trials; and for volatile avoidance, 25 animals and 25 trials.

Table 4. Role of cAMP and neural activity in $G\alpha_s$-induced paralysis

Genotype:	Spontaneous locomotion (% moving)
Wild type	98 ± 2
$\alpha_s(gf)$	15 ± 6
acy-1(nu329); $\alpha_s(gf)$	49 ± 7
acy-1(nu329)	97 ± 3
eat-4(ky5)	100 ± 0
eat-4(ky5); $\alpha_s(gf)$	48 ± 8

Locomotion behavior of $\alpha_s(gf)$ single and double mutants was quantitated as the percentage of animals undergoing spontaneous locomotion on plates without food. For each genotype, the number reported is the mean ± SE for three independent experiments.

neural activity, we reasoned that mutations that more specifically perturb the cytotoxic mechanism might produce a more profound neuroprotective effect.

To test directly the role of synaptic activity on these cell deaths, we tested the effect of mutations in the *unc-104* and *snt-1* genes, which encode phylogenetically conserved proteins (kinesin heavy chain and synaptotagmin) that are required for synaptic vesicle transport and exocytosis (Otsuka et al., 1991; Nonet et al., 1993). We found that neither the *unc-104* nor *snt-1* mutations reduced $G\alpha_s$-induced cell killing. This result suggests that the overall levels of synaptic input are not required for killing, per se.

Given its role in excitotoxicity in mammals, we wondered whether endogenous glutamate signaling is required for $G\alpha_s$ neurodegeneration. We found that a loss-of-function *glr-1* mutation did not reduce $G\alpha_s$-induced cell swelling or cytotoxicity (Table 1). This result does not exclude the possibility that glutamate neurotransmission mediates cAMP-induced cytotoxicity. The *C. elegans* genome sequence (currently ~85% complete) predicts eight additional ionotropic GluR subunits; therefore, the *glr-1* mutation is unlikely to eliminate glutamate signaling *in vivo*.

eat-4 mutations are neuroprotective

Because GLR-1 receptors are required for several mechanosensory behaviors (Hart et al., 1995; Maricq et al., 1995), we reasoned that other mutations affecting these behaviors might be neuroprotective. Previous work has shown that ASH sensory neurons mediate an aversive response to three distinct stimuli (nose touch, osmotic shock, and volatile repellents) and that the ASH-mediated touch response requires functional GLR-1 glutamate receptors in synaptic targets of ASH (Hart et al., 1995; Maricq et al., 1995; Troemel et al., 1995). We isolated seven alleles of the *eat-4* gene, originally identified because of its function in pharyngeal pumping (Avery, 1993), in a screen for mutations that eliminate ASH-mediated touch sensitivity. All seven *eat-4* strains have similar behavioral defects. In particular, they have severe defects in the ASH-mediated touch, osmosensory, and volatile repellent responses (Table 3). We found that *eat-4* mutations significantly reduced $G\alpha_s$-induced cytotoxicity but had no apparent effect on cell swelling (see Table 1). PVC cytotoxicity in *eat-4 unc-36;* $\alpha_s(gf)$ triple mutants (55 ± 5%) was not significantly different from that seen in *unc-36;* $\alpha_s(gf)$ double mutants, suggesting that *eat-4* and *unc-36* act in a single pathway regulating $G\alpha_s$-induced killing. In addition, *eat-4* mutations dramatically improved the locomotion rate of $\alpha_s(gf)$ animals (Table 4). Thus, the *eat-4* gene plays a significant role in $G\alpha_s$-induced effects on neurodegeneration (reducing cytotoxicity) and on neural activity (reducing paralysis).

DISCUSSION

We and others (Korswagen et al., 1997) have shown that the expression of a constitutively active form of $G\alpha_s$ induces a form of neurodegeneration in the nematode *C. elegans*. The $G\alpha_s$-induced deaths appear to be necrotic, because the affected cells swell and subsequently lyse and because these deaths are not prevented by mutations in the cell death genes *ced-3* and *ced-4*. We provide here a detailed analysis of the $G\alpha_s$ killing pathway. Neurons differ greatly in their susceptibility to $G\alpha_s$-induced neurodegeneration, ranging from 0 to 88% killed. Three genes (*acy-1*, *eat-4*, and *unc-36*) that contribute to $G\alpha_s$-induced neurodegeneration are identified. $G\alpha_s$ killing appears to be mediated by the second messenger cAMP, because *acy-1* mutations block killing. Our data also suggest that the two phases of $G\alpha_s$ neurodegeneration can be distinguished genetically, because *acy-1* mutations block both swelling and cytotoxicity, whereas other mutations (i.e., *unc-36* and *eat-4*) reduce cytotoxicity but have no effect on swelling. However, because we have not quantitated the extent or duration of cell swelling, it remains possible that there is a more subtle correlation between the extent of swelling and cytotoxicity. Finally, killing is not dependent on synaptic transmission, but it is modulated by endogenous neural signaling.

Function of ACY-1

We have isolated mutants that lack ACY-1, an adenylyl cyclase. ACY-1 appears to be expressed in nearly all neurons and muscles, but not in other tissues. These results suggest that ACY-1 adenylyl cyclase is likely to participate in many neural signaling pathways. Therefore, we would expect that *acy-1* mutants would have defects in behavior or development. Consistent with this notion is that mutations that inactivate the *C. elegans* $G\alpha_s$ subunit (GSA-1) are homozygous lethal (Korswagen et al., 1997). Surprisingly, the behavior and morphology of *acy-1* homozygotes are nearly indistinguishable from wild-type animals. Several behaviors were examined in greater detail, including behaviors mediated by GLR-1-expressing cells, behaviors regulated by GSA-1, and those that require the cyclic nucleotide-gated channels TAX-2 and TAX-4. We found that *acy-1* mutants were proficient in all of these behaviors. These results suggest that *acy-1* alleles do not eliminate ACY-1 activity, that the function of GSA-1 required for viability and for regulating behaviors is mediated by another adenylyl cyclase, or that ACY-1 acts redundantly with other forms of adenylyl cyclase. The genome sequence predicts at least one other adenylyl cyclase gene, which could account for the discrepancy between the *gsa-1* and *acy-1* mutant phenotypes. Our results also suggest that ACY-1 is not the sole source of cyclic nucleotides required for the activation of TAX-2 and TAX-4,

which is consistent with previous studies suggesting that TAX-4 channels are activated selectively by cGMP.

Role of cAMP in neurodegeneration

Several possible mechanisms could explain the toxic effects of activated $G\alpha_s$. We believe that the most likely explanation is that cAMP regulation of ion channels or ion transporters grossly alters membrane permeability, leading to cell swelling and death. In other systems many ion channels have been shown to be potently regulated by cAMP. We tested the *C. elegans* homologs of several of these potential cAMP targets, finding that mutations reducing the activity of UNC-36 calcium channels had a modest but significant neuroprotective effect. Because voltage-dependent calcium channels correspond to heteromultimers of several types of subunits, it is difficult to predict the extent to which alteration of the UNC-36 α2 subunit reduces the overall calcium permeability of cells *in vivo*. Although other candidate mutations were not neuroprotective, these results do not exclude a role for these genes, e.g., if several genes play redundant roles in inducing neurodegeneration.

An alternative explanation for $G\alpha_s$-induced neurodegeneration is that cells expressing the $G\alpha_s$ transgene have taken on the fates of cells that undergo developmentally programmed cell deaths. Several facts argue against this model. First, all of the cell deaths that occur during normal *C. elegans* development are apoptotic, by both morphological and genetic criteria (Hengartner, 1997). Therefore, the necrotic cell deaths produced by $G\alpha_s$ are not seen in normal development. Second, the *glr-1* promoter used to express $G\alpha_s$ is expressed starting in the threefold embryo (Hart et al., 1995; Maricq et al., 1995) after most cell fate choices (and in fact most cell deaths) already have occurred (Sulston et al., 1983). Third, $G\alpha_s$-expressing cells continue to express the *glr-1* promoter, indicating that at least some aspects of cell fate have not been altered.

It is also possible that overproduction of cAMP diminishes ATP levels, leading to a metabolic crisis and cell death. We think that this model is unlikely for three reasons. First, $G\alpha_s$-induced cell deaths are cell type-specific, which would not be predicted by this model. Second, suppression by *acy-1* mutations is semidominant, which implies that cells are sensitive to subtle changes in cAMP levels. Third, mutations that alter endogenous neural signaling (i.e., *unc-36* and *eat-4*) modulate $G\alpha_s$ toxicity, implying that the toxic signal is mediated by normal signaling pathways. These results suggest that, rather than creating a catastrophic metabolic event, $G\alpha_s$ kills via endogenous signaling pathways. On the other hand, the terminal phases of cytotoxicity must include the gross alteration of cellular metabolism and cellular integrity. Thus, in addition to inducing a cytotoxic signal, overproduction of cAMP also might hasten death by acting as a metabolic sink.

cAMP has been implicated in growth control and cell death in other systems. For example, elevated cAMP levels are cytotoxic in S49 lymphoma cells (Coffino et al., 1975), and these deaths subsequently have been shown to occur by apoptosis (Lanotte et al., 1991; Duprez et al., 1993). The molecular target of cAMP in the induction of lymphoid apoptosis has not been determined. In other cell culture systems cAMP induces growth arrest in G1 of the cell cycle (Khan et al., 1996). To our knowledge this is the first report of cAMP-induced neurodegeneration. In fact, in several other models of neurodegeneration cAMP has been shown to be neuroprotective (D'Mello et al., 1993; Dockwerth and Johnson, 1993; Kawakami et al., 1996; Michel and Agid, 1996).

Role of *eat-4* and *unc-36*

We found that *eat-4* and *unc-36* mutations are partially protective against the cytotoxic and paralytic effects of the $G\alpha_s$ transgene. Although these protective effects are admittedly subtle, we believe that they are real for several reasons. First, all of our data were compared by statistical methods, using a relatively strict threshold for significance ($p < 0.005$). Second, we have examined many other genetic backgrounds and found no similar protective effects, which indicates that effects of this magnitude are uncommon. Third, for both *eat-4* and *unc-36* we found the neuroprotective effect in two unrelated strains carrying different alleles of these genes. Therefore, it is highly likely that these protective effects are caused by the mutations in *unc-36* and *eat-4* rather than by some uncharacterized mutation in the genetic background. By all of these measures the effects of the *eat-4* and *unc-36* mutations are real; therefore, we conclude that these genes play some role in determining the severity of $G\alpha_s$-induced killing.

The *eat-4* gene was identified initially in screens for mutations that disrupt eating behavior (Avery, 1993). The *eat-4* eating defect is caused by the elimination of a glutamate-induced inhibitory synaptic signal (mediated by the M3 motor neuron), which can be observed in extracellular recordings of pharyngeal muscle activity (Raizen and Avery, 1994). The defect in pharyngeal neurotransmission is likely to be caused by a presynaptic defect in M3, because pharyngeal muscles isolated from *eat-4* mutants are responsive to glutamate iontophoresis (Dent et al., 1997). We have shown that *eat-4* mutants are defective for three ASH-mediated sensory behaviors, one of which (nose touch) is mediated by GLR-1 GluRs. Thus, both the eating defects and the sensory defects observed in *eat-4* mutants could be explained by an underlying defect in glutamate neurotransmission. The *eat-4* gene has been cloned (R. Lee, E. Sawin, M. Chalfie, H. R. Horvitz, and L. Avery, personal communication); however, the molecular identity of EAT-4 does not reveal what role it plays in neuronal signaling.

Two sorts of models could explain the neuroprotective effects of *eat-4* and *unc-36* mutations. First, these mutations could be neuroprotective, because $G\alpha_s$-induced deaths are mediated in part by endogenous glutamate neurotransmission. Alternatively, EAT-4 and UNC-36 could act in the dying cells, directly or indirectly mediating the cytotoxic effects of cAMP. $G\alpha_s$-induced killing is not diminished by mutations that impair synaptic transmission, which would favor the model that EAT-4 and UNC-36 act in the dying cells. However, EAT-4 is not expressed in PVC neurons (R. Lee, E. Sawin, M. Chalfie, H. R. Horvitz, and L. Avery, personal communication), which favors the idea that EAT-4 acts in the presynaptic partner. Neither of these results conclusively tests these models. The reported EAT-4 expression pattern may be incomplete. Moreover, several results indicate that residual synaptic transmission occurs in *unc-104* and *snt-1* mutants. The *unc-104* allele used in this study is a partial loss of function, because null alleles are homozygous lethal (Hall and Hedgecock, 1991), and residual synaptic transmission has been documented in synaptotagmin null mutants in worms, flies, and mice (DiAntonio et al., 1993; Littleton et al., 1993; Nonet et al., 1993; Geppert et al., 1994). Moreover, some glutamate may be released by a nonvesicular mechanism (Attwell et al., 1993). In fact, cell swelling might stimulate glutamate efflux through volume-sensitive osmolyte channels (Jackson and Strange, 1993). Finally, it is also possible that defects in exocytosis make cells more susceptible to necrosis, for example by preventing the ad-

dition of new membranes to swelling cells. Further experiments will be required to distinguish among these models.

Similarities to other forms of neurodegeneration

Several other *C. elegans* mutations have been described that cause a necrotic form of neurodegeneration (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; Treinin and Chalfie, 1995). In particular, mutations in the *deg-1* gene also cause a specific subset of neurons to undergo necrosis, including the PVC neuron (Chalfie and Wolinsky, 1990; Garcia-Anoveros et al., 1995). Our results suggest that the *deg-1*-induced and $G\alpha_s$-induced cell deaths occur by distinct mechanisms because *mec-6* mutations block *deg-1*-induced deaths, but not $G\alpha_s$-induced cell deaths (Chalfie and Wolinsky, 1990); however, it remains possible that ACY-1 is required for *deg-1*-induced deaths.

$G\alpha_s$-induced cell deaths also share some properties with glutamate-induced excitotoxicity in mammals. Neurodegeneration occurs in two phases (swelling and killing), and killing is apparently cell type-specific. Reducing the activity of voltage-dependent calcium channels is neuroprotective in both cases; however, the protective effect of *unc-36* mutations is modest (albeit statistically significant). Finally, the neuroprotective effects of *eat-4* mutations imply that glutamate may play a role in $G\alpha_s$-induced neurodegeneration. Further evidence will be required to determine whether $G\alpha_s$-induced cell deaths and excitotoxicity are related mechanistically.

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