A Non-Biased, *In vivo* Genetic Screen for Novel Genes That Protect Against Necrosis

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Grant Number: 04-2902-SCR-E-0
Grant Period covered: Jun. 15th, 2004~Jun. 30th, 2006
Date of Submission: Aug. 28,2006

Final Report

Overall Plan Summary and Rationale

Project rationale. Necrosis, or "unplanned" cell death, is a major contributor of the initial and prolonged death of neurons consequent to spinal cord injury (SCI). Blocking or delaying secondary neuronal necrosis would significantly limit debilitating consequences of injury. Detailed elaboration of the molecular mechanisms of neuronal cell death is essential for development of efficacious therapies for SCI.

We exploit the unique advantages of the *C. elegans* for genetic and molecular studies of cell death. The necrotic paradigm we study most involves initiation of cell death by hyperactivated ion channels expressed in six touch-sensory neurons and requires elevation of intracellular calcium via ER release, which activates calpain and cathepsin proteases (1). Like apoptotic cell death mechanisms, mechanisms of necrosis are conserved from nematodes to humans. Necrosis mechanisms deciphered using *C. elegans* will imply key molecules in necrosis in human and suggest novel strategies for therapeutic intervention of spinal cord injury.

The longterm goal of our research is to dissect the molecular mechanism of necrotic cell death. I have conducted the first classical forward genetic screen for mutations that enhance progression through necrotic cell death. The normal function of these genes should be to protect against necrosis in a native physiological context. Molecular pursuit of such genetically identified loci will enable us to decipher necrosis mechanisms and design novel intervention strategies.

Summary of originally proposed work. I proposed to construct a strain in which specific labeled neurons undergo inefficient necrosis, to mutagenize this strain and identify enhancers of necrosis, and to begin characterization and genetic mapping of enhancer loci.

Aim 1: To conduct a screen of the *C. elegans* genome to identify mutations that enhance progression through necrosis, the normal function of which could be neuroprotective.

Aim 2: To perform tests that characterize enhancer mutants to prioritize their future molecular cloning.

Progress Summary

Review of screen strategy (Figure 1). To identify the genes that protect against necrosis, we planned to construct a marked strain including a modest necrosis inducer. In this strain, neurons fluoresce green if alive—in the parental strain, most neurons live since necrotic death is rare. The idea is that consequent to mutagenesis, I would easily identify animals having necrosis enhancer mutations since the GFP signals in these animals would disappear or at least become significantly diminished. The powerful BIOSORT, an instrument that functions like FACS sorter for nematodes, allows automatic size-based and fluorescence-based screening and is sensitive enough to sort out even modest necrosis enhancer mutants.

Accomplishment 1: I constructed the starting strain for mutagenesis.

The two genetic features that my parental starting strain for mutagenesis needed to have were: 1) a weak inducer of necrosis--for this purpose I chose degenerin channel *mec-10(d),* which can be engineered to encode a large sidechain amino acid near the channel pore that weakly hyperactivates the channel (2); 2) a strong GFP signal in the cells that have the potential to undergo necrosis (i.e., those that express *mec-10(d)*).

I made gene fusions in which either the *mec-4* promoter (expressed in six neurons) or *mec-10* promoter (expressed in 10 neurons) drive expression of a GFP-tagged *mec-10(d)* genomic DNA (*mec-10*(A673V)GFP). I confirmed constructions by restriction mapping and DNA sequence analysis.

I made several transgenic lines by either biolistic transformation or microinjection followed by X-ray irradiation. I surveyed these transgenic lines for neuronal death and found the anticipated neuronal degeneration at very low rates. The strain that I selected for mutagenesis exhibits almost no degeneration at 20°C, enabling the BIOSORT to most effectively identify enhancer mutants against minimal background. I genetically mapped the transgene integration locus to Chromosome X in this strain.

I introduced our well characterized $P_{mec4}GFP$ integrated transgene (which strongly labels the six touch neurons) into the marginally-toxic *mec-10(d)* background. The strain is "green" as almost all the six neurons are alive at 20°C.

Accomplishment 2: I conducted a screen of 18,500 genomes to identify 18 interesting necrosis enhancers.

a. A screen of 18,500 mutagenized genomes. I conducted 4 independent screens for necrosis enhancers using the strategy I outlined above. In total, I have mutagenized and screened 18,500 genomes to date. Since the average loss-of-function mutation arises at a frequency of ~ 1/5000 (3), I expect that I have mutations in most average-sized gene targets that can mutate to exacerbate necrosis at this point.

b. Eliminating one unwanted class of potentially isolated suppressors. Of 120 candidate death enhancers first selected, I eliminated the unwanted class that simply lost the GFP reporter signal (array deletion or second site mutation that altered GFP stability, folding etc.) by PCR with primers that specifically amplify the $P_{mec4}GFP$ transgene and necrotic vacuoles checking. After eliminating these uninteresting mutants, I have 63 candidate necrosis enhancers that still include functional GFP reporters. I isolated 18 strong or intermediate necrosis enhancing alleles (*nen*).

Accomplishment 3: I conducted initial genetic characterization of several necrosis enhancers.

A. *nen(bz300)*

Dominant/recessive? I crossed *nen(bz300)* with a $P_{mec4}GFP$; *mec-10(d)* strain and checked the degeneration rate in F1 cross progeny to determine its dominancy. The F1 cross progeny should be heterozygous *nen(bz300).* Enhancement is lost in *nen(bz300)* heterozygous. *nen(bz300)* acts recessively.

Confirm that death effect depends upon *mec-10(d).* I want to be certain that the loss of neurons occurs through enhancement of *mec-10(d)* rather than some other process (perhaps, very rarely, via new generation of a *mec-4(d)* strong necrosis-inducing allele). To test this, I crossed *nen(bz300)* out of *mec-10(d)* background and checked

whether neuron degeneration happened or not. If degeneration still occurs, a *mec-10(d)*-independent necrosis inducer has been found. If not, it is more likely that a *mec-10(d)*-dependent necrosis enhancer has been found. I found that *nen(bz300)* depends on *mec-10(d)* to exert its cell death effect.

Mapping I used SNP mapping and found that *nen(bz300)* is not on Chromosome II, III, IV, V. I did linkage assignment analysis on Chromosome I and Chromosome X using P_{mec4}*GFP* and *mec-10(d)* as linkage markers separately and found *nen(bz300)* is on Chromosome I. I did 3 factor cross using *bli-4* which locates on position 0.95 of Chromosome I and found *nen(bz300)* is to the right of *bli-4.*

Epistasis study Epistasis analysis involves testing combinations of mutations of opposite phenotypes to determine the phenotype of the double, which suggests a likely order of action of tested genes. I constructed double mutants for *nen(bz300)* and *crt-1* (calreticulin null prevents toxic rise in intracellular calcium) and checked for the degeneration effect. I concluded that *crt-1* is epistatic to *nen(bz300)* (Figure 2). This means the *nen(bz300)* acts upstream of calcium rise and acts early in necrosis and is of most interest for further study.

B. *nen(bz301)*

Dominant/recessive? *nen(bz301)* acts dominantly after following the same procedure described above.

Molecular cloning of *nen(bz301)* and identification of the lesion that alter necrosis.

a. *nen(bz301)* is on Chromosome X. I used SNP mapping and determined that *nen(bz301)* is not on Chromosome II, III, IV, V. The marker transgene P_{mec4}*GFP* is located on Chromosome I. The *mec-10(d)* transgene is located on Chromosome X. I did linkage assignment analyses on Chromosome I and X using P_{mec4}*GFP* and *mec-10(d)* as linkage markers separately and found *nen(bz301)* is on Chromosome X.

b. *nen(bz301)* is a novel mutation on *mec-4* gene ----*mec-4(A149V).* Since *mec-4* gene is located on Chromosome X. I wanted to check whether *nen(bz301)* is a mutation on *mec-4* or not. I sequenced the *mec-4* gene and determined the lesion is *mec-4(A149V).* Like *mec-4(d), bz301* causes substitution of large sidechain amino acid for a small residue near the channel pore, but on the other side of the extracellular loop of MEC-4 protein.

Confirm that death effect depends upon *mec-10(d).* Is *nen(bz301)* is a cell death inducer or a cell death enhancer? To answer this question, I crossed out *mec-10(d)* transgene and checked for degeneration. No degeneration occurs in the *nen(bz301)* strain. While I crossed *nen(bz301)* back into *mec-10(d)* background and neuronal degeneration phenotype came back again. So, the death effect of *nen(bz301)* depends upon *mec-10(d)* (Figure. 3).

Touch sensitivity test. *mec-4* encodes a subunit of the DEG/ENaC channel family, believed to be the core subunit of a mechanically-gated ion channel that normally senses, and is activated by, touch. *mec-4* mutations usually disrupt touch sensitivity While *nen(bz301)* itself does not disrupt touch sensitivity at all (Figure 4).

Significance *nen(bz301)* affected the channel death-inducing stimulus rather than the death pathway, and was thus not useful for analysis of downstream necrosis mechanisms. We did, however, think it is useful for analysis the function of touch channel complex, especially, the function of MEC-10 protein.

C. *nen(bz302), nen(bz303)* and *nen(bz308)*

I mapped these 3 alleles to Chromosome X and determined they act dominantly. I also know that they are not *mec-4* mutations by sequencing. They are dominant, so it is hard to check whether they affect the same gene or not . Since *mec-10(d)* transgene is also on Chromosome X, I have to cross these alleles out of *mec-10(d) X* background and into another *mec-10(d)* background which located on Chromosome IV to enable the fine-structure genetic mapping. It is painful work since I have to select recombinants to get rid of *mec-10(d) X* transgene. I will continue this work in my next grant period from NJCSCR.

Plans to continue this research

I plan to continue molecular clone at least two loci that mutate to enhance necrosis and determine the molcular mechanisms of action of necrosis enhancers. I will test for effects on other weak necrosis inducers to confirm their general effects in necrosis. I will deternine if the necrosis enhancer loci have mammalian counterpart implicated in necrosis and spinal cord injury. This ongoing work will be supported by the graduate student fellowship grant from New Jersey Commission on Spinal Cord Research which starts from Jun. 15, 06 and ends on Jun. 30, 08.

Significance

Necrotic cell death is a major contributor to neuronal loss that accompanies spinal cord injury, yet effectiive tools to block the progression through necrosis are lacking. My work should identify currently unknown components of necrosis pathway that normally help protect the neuron from proceeding into necrotic cell death. I feel confident that my work can make fundamental contributions to the understanding of molecular mechanisms of injury-induced neuronal cell death and might ultimately suggest novel strategies for therapies in SCI.

This work constitutes basic research that is not yet published. Publication will be most appropriate when a given gene is cloned (actually work we expect will be completed beyond the two year funding period).

References:

1. Driscoll, M., Gerstbrein, B., Dying for a cause: invertebrate genetics takes on human neurodegeneration. Nat Rev Genet. 4,181-94.(2003)
2. Huang, M., Chalfie, M., Gene interactions affecting mechanosensory transduction in Caenorhabditis elegans.Nature. Nature 367, 467-70 (1994)
3. Brenner, S., The genetics of Caenorhabditis elegans. Genetics 77, 71-94. (1974).

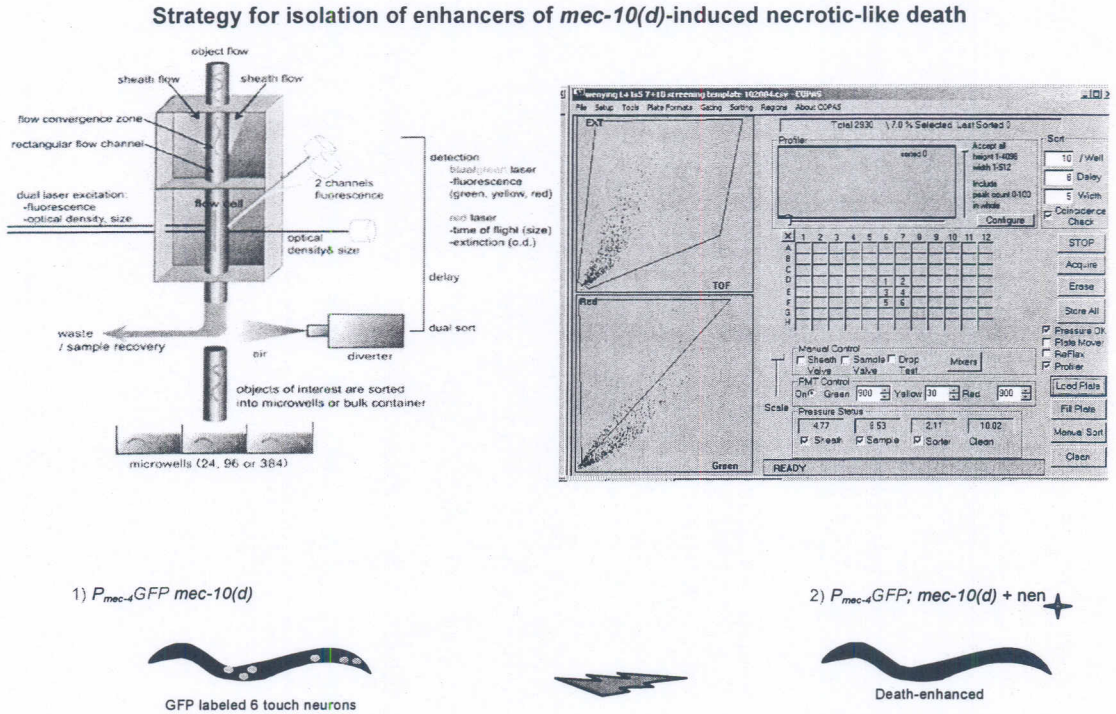

Figure 1. Strategy for isolation of enhancers of *mec-10(d)*-induced necrotic-like cell death.

The parental *mec-10(d)* worms induce very weak necrosis. Almost all the 6 touch neurons live and fluoresce green at 20°C. After mutagenesis, animals bearing a necrosis enhancer mutation will have less GFP signals since some of touch neurons undergo necrotic cell death. These worms can be sorted out by the powerful BIOSORT, which allows automatic size-based and fluorescence-based screening (left upper image). The image located at the upper right corner is the COPAS software that we use to set up parameters to sort out the interesting mutants. The worms that have strongest GFP signal are shown at the rightmost and lowest area on the lower-left panel. The worms that have a necrosis enhancer will have less GFP signal and are separately shown at the upper-left side of the same panel. By setting up the sorting parameters mutants can be automatically sorted out on to growth plates.

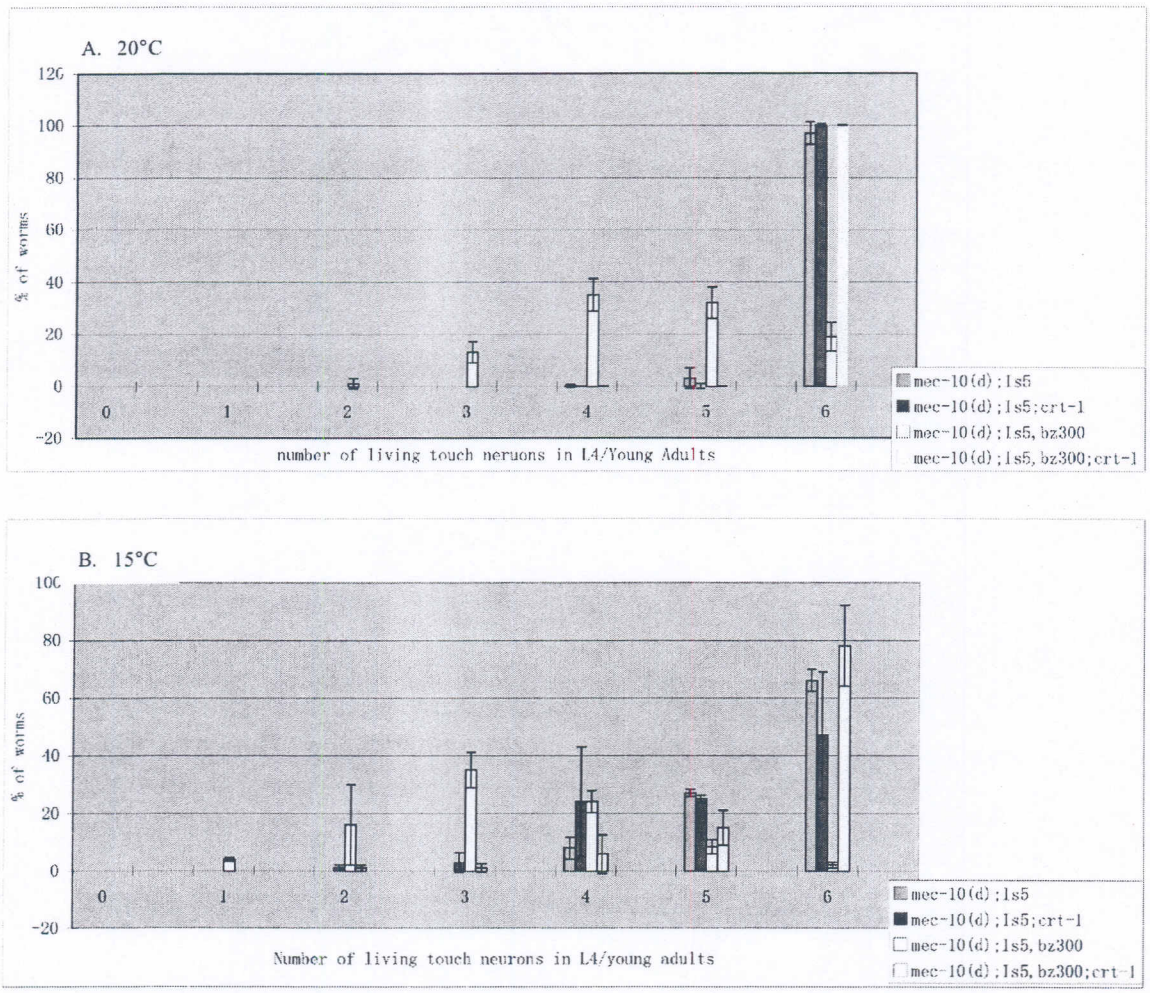

Figure 2. Epistasis studies of *crt-1* and *bz300*.
ls5 is P_{mec4}*GFP*. null mutation *crt-1(bz29)* prevents toxic rise in intracellular calcium and is a potent necrosis suppressor. The phenotype of double mutations—*crt-1*(death-protecting) and *bz300* (death-promoting) --- is death-protecting at both 20°C (A) and 15°C (B). This indicates that *crt-1* is epistatic to *bz300* and *bz300* acts upstream of *crt-1* in the necrosis pathway.

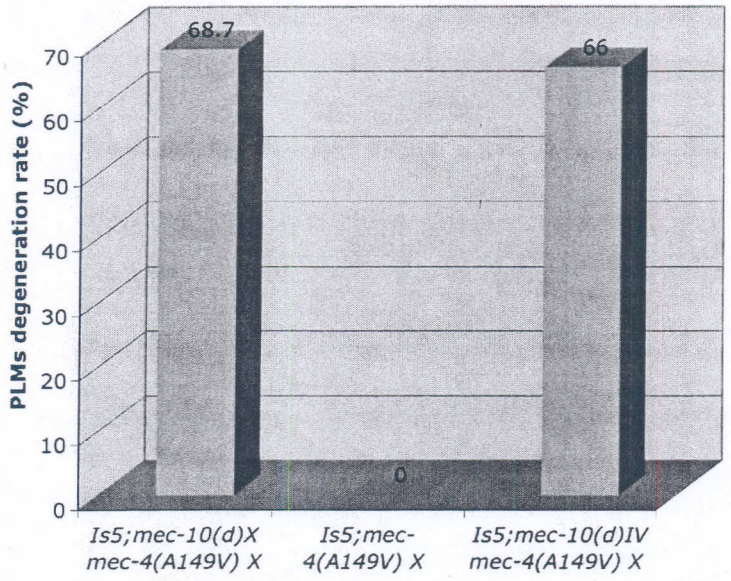

Figure 3. *mec-4(A149V)* needs *mec-10(d)* for its necrotic cell death effect.
I score the necrotic PLM neurons in early L1 stage (<6 hours after hatched), as indicated by swelling vacuoles. At least 200 worms were checked for each group.

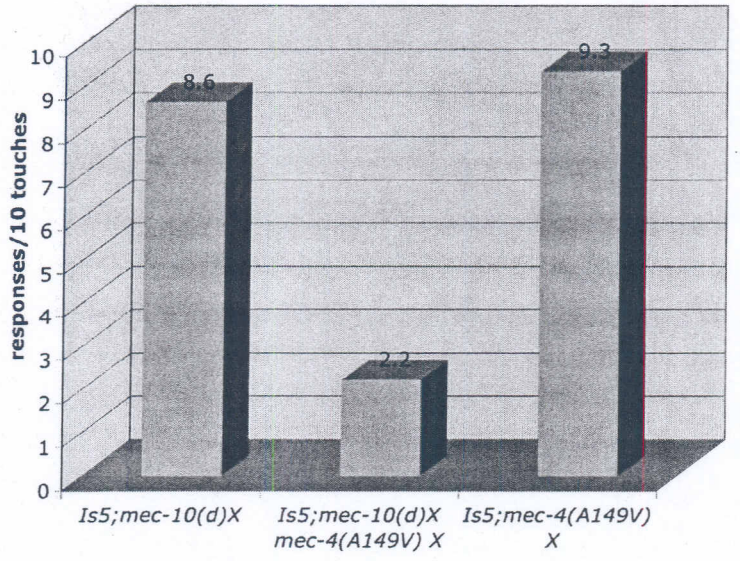

Figure 4. *mec-4(A149V)* interrupts touch sensitivity in conjunction with *mec-10(d).*
Animals were touched 10 times on the neck and the tail alternatively. The responses were recorded blind to the genotypes.