Closed 7/1/08

NJ Commission on Spinal Cord Research Final Report

Genes Contributing to Necrotic Death on Injured Neurons: Molecular Mechanisms of Action and Roles in Neuronal Regeneration

Dr. Monica Driscoll
Rutgers, The State University of New Jersey
Department of Molecular Biology and Biochemistry
A232 Nelson Biological Laboratories
604 Allison Road
Piscataway, NJ 08904

Grant # 06-3053-SCR-E-O
Dates Covered: 12/15/06-12/30/07

Date of Report Submission: 4/23/08

A. Specific Aims

Necrotic cell death, often initiated by ion channel hyper-activation, plays a major role in the initial and prolonged death of neurons consequent to injury. Blocking or delaying such necrotic cell death would significantly limit this incapacitating neuronal damage and this goal is a clear priority for spinal cord research. We have identified genes critical for ion channel-induced neuronal necrosis using the powerful experimental model, the nematode *Caenorhabditis elegans.* Our work indicates that necrotic death mechanisms are conserved from nematodes to humans. Thus, when we molecularly identify genes that can mutate to block channel injury-induced necrosis, we expect to suggest novel approaches for therapeutic interventions in mammals. Likewise, defining genes that influence neuronal regeneration in this model are likely to provide insights that might be exploited for mammalian repair.

Aim 1: To molecularly identify loci that can mutate to suppress necrosis and determine the mechanisms by which such mutations block injury-induced death

We planned to clone 3 necrosis suppressor genes, identify the death-suppressing mutations, position gene function in the necrosis pathway, and consider mammalian homologs for similar function *in vivo*. This work was expected to extend molecular understanding of physiological necrosis mechanisms and identify novel targets that might be exploited to restrict 2° nerve damage following traumatic injury.

Aim 2: To test roles for cell death genes in ventral cord neuron regeneration after axotomy

Necrosis, apoptosis, and autophagy can influence the extent of neuronal loss consequent to SCI and the genes that regulate and execute these processes may likewise influence repair capacity. We planned to use new laser technology to sever individual motorneurons *in vivo* to address how cell death genes influence the capacity for regeneration to address molecular requirements for individual fiber regeneration in a physiological context.

B. Project Successes

Aim 1: To molecularly identify loci that can mutate to suppress necrosis and to determine the mechanisms by which such mutations block injury-induced death

1. Original plan

We proposed to clone 3 necrosis suppressor genes, identify the death-suppressing mutations, and position gene functions in the necrosis pathway.

Our strategy for non-biased selection of mutations that block or delay necrosis. *mec-4(d)* encodes a mutant ion channel that is hyperactive, conducting excess cations into neurons to initiate a necrotic cell death, similar in several respects to ion channel-mediated injury that accompanies spinal cord injury. To identify molecules required for *mec-4(d)*-induced necrotic neuronal death, we used a marked strain that expresses fluorescent GFP in neurons under the control of the *mec-4* promoter (designated $p_{mec\text{-}4}$GFP) in the toxic *mec-4(d)* background. In this parental strain, green fluorescent touch cells are missing—they have undergone necrotic neuronal death. After mutagenesis, we identify animals bearing rare suppressor mutations that allow the touch cells to live because they have several touch cells that fluoresce in a background of darkness. **We identified several loci that can mutate to block *mec-4(d)*-induced necrosis and we did molecularly identify three of them.**

2. Results

A. Death suppressor *des(bz178)* specifies a single amino acid change in highly conserved translocon SEC-61

Cloning the *bz178* locus. We had considerable interest in extragenic suppressor *bz178*, which was among the strongest death suppressors, with 5/6 neurons surviving over 95% of the time (compared to only 4% neurons surviving in the *mec-4(d)* background). *bz178* acts dominantly to suppress necrosis.

We mapped the *bz178* locus to a short interval and sequenced candidate mutant genes. Our sequence analysis identified *des(bz178)* as an allele of the *C. elegans* homolog of human SEC-61 and we have confirmed gene identity by transformation rescue. SEC-61 is a highly conserved member of the translocon complex that is positioned in the ER membrane, partially facing into the cytoplasm and partially facing into the ER lumen. A major job of the translocon is to allow misfolded proteins to be imported from the cytoplasm for refolding; conversely if proteins are not properly folded in the ER, they

are shipped back into the cytoplasm for degradation. The *bz178* allele encodes an R66C change in a cytoplasm-facing domain, an alteration in a highly conserved region of the protein. Over-expression of *sec-61(+)* appears toxic, loss of activity is lethal, and our GFP promoter fusion studies indicate that the *sec-61* gene is expressed in all cells.

Probing mechanism of action. ***Analysis of MEC-4(d) protein localization.*** One simple hypothesis is that translocon modification prevents *mec-4(d)* expression by limiting the amount of MEC-4(D) protein assembled into toxic channels. However, we found that the *bz178* mutation does not render animals touch insensitive (so the normal MEC-4 channel still appears to be made and assembled in the presence of the substitution R66C). Interestingly, however, whereas MEC-4::GFP is distributed to punctae along the neuronal process (thought to be the sites of assembled MEC-4 channel complexes) in the same way in wt and *bz178* backgrounds, things are different for a MEC-4(D)::GFP in a *sec-61(bz178)* background. In the presence of the SEC-61(R66C), the MEC-4(d)::GFP appears eliminated from the neuronal processes and instead appears diffuse in the cytoplasm. The suggestion is that the toxic MEC-4(d) channel (or at least the associated GFP label) is routed primarily to the cytoplasm as a consequence of SEC-61(R66C).

Testing how broadly sec-61(bz178) protects against necrosis. A critical question is whether the death suppression we observe is general, acting against multiple independent inducers of necrosis, or if it is specific to the *mec-4(d)* variant we used in the screen. To address this question, we examined several genetically-based necrosis inducers that are available in the *C. elegans* model. First we tested additional hyperactivated members of the DEG/ENaC family. We found that the two alleles of *deg-1* failed to be suppressed by *sec-61(bz178)*. One of these has the analogous substitution to *mec-4(u231)* (MEC-4A713V); the other effects an extracellular domain that appears to be required for efficient channel closing. We also found that muscle DEG/ENaC *unc-105(sd)* is not suppressed in the *unc-105/+* heterozygote; it appears that *unc-105; sec-61(bz178)* mutants are inviable, suggesting an interaction between the muscle DEG/ENaC and SEC-61 that is distinct from suppression. The bottom line is that *sec-61(bz178)* necrosis suppression appears fairly restricted to MEC-4(A713V).

We have hypothesized that multiple distinct insults to neurons converge to activate a central necrosis pathway and therefore we also tested distinct necrosis inducers for potential suppression by *sec-61(bz178)*. We tested hyperactivated acetylcholine receptor channel *deg-3(gf)*, transgenic poly-glutamine expansion models, and human Alzheimer's disease protein $A\beta_{1\text{-}42}$ expression, but did not observe changes in cell death or dysfunction phenotypes in these models. These data also support that that *sec-61(bz178)* necrosis suppression appears specific to MEC-4(A713V).

Impact of sec-61(bz178) on normal biology. We were curious what the SEC-61(R66C) translocon change does to normal biology of *C. elegans*. We find that *sec-61(bz178)* mutants are grossly normal in development, coordination, and fertility and do not exhibit any temperature-sensitive changes in overall biology. Since SEC-61 is an ER protein with critical roles in protein folding, we were interested in testing to see whether there were any phenotypes associated with ER stress. We challenged animals with tunicamycin, which prevents glycosylation and causes accumulation of proteins in the ER, inducing the unfolded protein response, a coordinated protective response against damage associated with protein unfolding. Wild type animals respond to tunicamycin by first becoming paralyzed and then recovering once they activate the UPR; *sec-61(bz178)* mutants exhibit a shortened paralysis phase, suggesting the UPR might be induced in this background. *sec-61(bz178)* mutants also exhibit better embryonic viability when exposed to tunicamycin than do wild type. We tested the hypothesis that *sec-61(bz178)* induces the UPR by introducing GFP reporter $P_{hsp4}GFP$, which is normally upregulated when the UPR is activated, but we find that this reporter is not induced in the *sec-61(bz178)* background (instead it appears diminished), suggesting the UPR is not directly induced by the SEC-61 change.

Addressing relationship between the unfolded protein response and necrosis progression. The implication of some regulators of protein folding homeostasis (*crt-1* and *sec-61*) in necrosis suppression led us to wonder about potential of the UPR to protect against necrosis. There are three activities induced in parallel in response to accumulation of unfolded proteins. The *pek-1* kinase is involved in translational and transcriptional control, *atf-6* encodes an ER-membrane-bound transcription factor activated by ER stress-induced proteolysis involved in translational control, and *ire-1* encodes a transmembrane serine/threonine kinase/endoribonuclease that senses unfolded protein levels within the ER in collaboration with the ER Hsp70-family member BiP/Kar2. We constructed double mutants for *pek-1, ire-1* or *atf-6* and *mec-4(d),* but we found that disruption of any individual gene did not suppress *mec-*

4(d)-induced necrosis. Thus, no major branch of the UPR is essential for *mec-4(d)*-induced necrosis. We also constructed triple mutants in which each UPR gene was tested in a *mec-4(d); sec-61(bz178)* mutant background. In the *pek-1* and *ire-1(lf); sec-61(bz178)* double mutants we observed an enhancement of *mec-4(d)* death suppression while the *sec-61(bz178); atf-6* double mutant does not change death suppression. These data supports the idea that two of the UPR three pathways may interact with mutant SEC61a in the mechanism of *mec-4(d)* death suppression.

Construction of a yeast model. Much understanding of translocon activity has come from analysis of the highly conserved yeast translocon and we hypothesize that this model might provide cell biological insight into how MEC-4(d) is differentially handed by the mutant translocon SEC-61(R66C). We therefore constructed a yeast strain with the R66C substitution harbored a single copy number CEN plasmid and we found it rescued the lethality of a sec61 null mutant, but conferred slow growth. We are using this model to test whether MEC-4::GFP is excluded from, or retained by, the ER in the presence of SEC-61(R66C).

Details on plans for continuation. We are actively finishing a manuscript on this work, which we plan to submit by July 1 (currently at advanced draft stage). We are conducting a few additional experiments that will define how MEC-4::GFP and MEC-4(d)::GFP are distributed in wt and *sec-61* mutant yeast. We have made many *mec-4* mutants with different amino acids encoded at position A713 to test how widely these are suppressed by *sec-61(bz178)* in nematodes, and we are using a RED-ER marker to help us distinguish where the GFP signal ends up when MEC-4(d)::GFP is expressed in the *sec-61(bz178)* mutant background. We note that Dr. Royal is pursuing these studies supported by the NJCSCR (08-3075-SCE-E-0).

3. Death suppressor locus *des(bz146)* encodes likely null alleles of UDP-glucose:glyco-protein glucosyltransferase

Cloning the *bz146* locus. Six death suppressor alleles on the X chromosome failed to complement each other (*bz91*, *bz121*, *bz125*, *bz130*, *bz146*, *bz156*) and were thus likely to affect the same gene. We narrowed down the location of the X-linked necrosis suppressor locus to a region covering 10 ORFs. We sequenced candidate genes to identify this locus as ORF F48E3.3. F48E3.3 (hereafter referred to as *egt-1*) encodes a secreted, soluble 1493 AA protein that has high homology to human UDP-glucose:glycoprotein glucosyltransferases. We identified the positions of the six mutations to confirm gene identity.

Probing mechanism of action. ***Impact on MEC-4 protein.*** UDP-glucose:glycoprotein glucosyltransferase recognizes suboptimally folded glycoproteins in the ER and adds a single glucose residue to a terminal mannose of their asparagine-linked oligosaccharides. The monoglucosylated glycoproteins serve then as substrate for the ER-resident lectins calnexin and calreticulin, which function as chaperones that help retaining such misfolded proteins in the ER until they are correctly folded. A logical potential death suppression mechanism could be that MEC-4(D) might not be folded efficiently in this background, which could account for death suppression. However, we do not detect a significant change in the distribution of MEC-4(+), nor do we find any defects in MEC-4-dependent touch sensitivity in the *egt-1* backgrounds. This indicates that *egt-1* action is not critical for functional expression of MEC-4. We are conducting more detailed studies on the MEC-4(D) variant.

Interaction with the UPR. The identification of two death suppressors implicated in protein folding homeostasis but not markedly changing apparent MEC-4 expression raises the possibility that the unfolded protein response plays a role in the transduction of the death suppressing effect in our mutant. Indeed, we have found that *egt-1(bz130)* and UPR genes *ire-1(zc14)* or *pek-1(ok275)* show reduced levels of *egt-1* suppression of *mec-4(d)*-induced death, suggesting that these genes may normally aid in death suppression. Consistent with this idea, a semi-dominant mutation (of unknown molecular origin) that activates the UPR, *upr-1(zc6),* is a mild *mec-4(d)* suppressor.

Impact on normal biology. *egt-1(bz130)* affect other important biological functions such as: reproductive capacity (diminished, with lower germ cell survival), trashing/swimming capacity (increased at 20°C), and life span (increased at 20°C). On the other hand, the extension in life span suggests that the mild activation of the UPR (about 3 times that of control strain at 20°C) could be beneficial to the differentiated soma.

One more related necrosis modulator. We have identified a mutation in another related UDP-glucose:glycoprotein glucosyltransferase, *egt-2(bz201)*, as a modulator of *mec-4(d)*-induced necrosis.

Plans for continuation: We are testing how *egt-1(bz130)* impacts other genetically induced necrosis models, we are testing pharmacological means by which to induce mild UPR induction, we are clarifying relative roles of *egt-1* and *egt-2*. We are actively compiling a paper on these data.

4. Work toward molecular cloning of 2 other death suppressors

bz200. 4 alleles (*bz200, bz100, bz180* & *bz181*) fail to complement each other, suggesting they are all alleles of the same locus. We have genetically mapped this death suppressor locus to Chromosome II and we are continuing fine SNP mapping in this region so we can complete cloning of the death suppressor gene.

bz199. *bz199* is a single mutation that we have mapped to Chromosome I between 3.05 and 4.79. Our data suggest *bz199* corresponds to a novel death suppressor locus in this region. We have recombined two genetic markers on either side of *bz199* and are introducing it into a *Pmec-4gfp mec-4(d)* Hawaii polymorphism strain so we can perform high resolution SNP mapping and define a more narrow genetic interval for cloning this locus.

Progress on Aim 2: To test roles for cell death genes in ventral cord neuron regeneration after axotomy

1. Original Plan

Necrosis, apoptosis and autophagy can influence the extent of neuronal loss consequent to SCI and the genes that regulate and execute these processes may likewise influence repair capacity. We planned to use new laser technology to sever individual motorneurons *in vivo* to address how cell death genes influence the capacity for regeneration. This is the first study to address molecular requirements for individual fiber regeneration in a physiological context and should provide new understanding of the basic biology of regeneration, an issue of central importance in SCI research.

2. Results

Development of the ALM model of axonal regeneration. We quickly learned we needed a model in which we could track individual nerve fibers in detail. Although we originally planned to look at ventral cord neurons, motorneuron processes overlap in the ventral cord, precluding us from unambiguously identifying single nerve fibers. We therefore optimized a neuronal regeneration model in the *C. elegans* focused on sensory neurons situated on either anterior side of the nematode. These ALM neurons have two processes: one long anterior axon that grows toward the nerve ring and one short posterior dendrite. When we laser axotomize the anterior axon of p_{mec-4}GFP-expressing ALM neurons 30 µm from the cell body, both processes outgrow within a few hours (Figure 1). Monitoring axon outgrowth length is easy as ALM processes grow in a very straight trajectory along the body. For regeneration analysis, we generally measure the average length of axon outgrowth 24 hours after axotomy on at least twenty animals for each strain. Animals of the wild type N2 reference strain display an axon outgrowth of 113+/-8 µm/24hrs. Note that our collaborators in the Samuel lab have pioneered new methods using microfluidic devices that now enable us to trap and immobilize nematode in "nano-cages" for laser axotomy with much enhanced efficiency.

Testing whether any of the major death programs play a role in neuronal regeneration mechanism. Our goal was to construct strains in which we inactivated each of the potential death programs (apoptosis, necrosis, autophagy) to ask how regeneration capacity was impacted. We constructed multiple strains for this purpose.

Autophagy genes impact neuronal outgrowth and cannot be unambiguously characterized for regeneration. Many genes known to regulate autophagy also impact vesicular trafficking within neurons and therefore impact axonal outgrowth. We have tested autophagy gene *unc-51*, but note that axon outgrowth is grossly abnormal in this mutant background, complicating that analysis. For the moment, we decided to focus on testing genes that do not normally alter axonal outgrowth and development.

Key apoptosis genes, including the *executor* caspase CED-3, are needed for efficient neuronal regeneration. *C. elegans* apoptosis transpires by a conserved mechanism that absolutely requires the function of the CED-3 executor caspase and the CED-4/Apaf homolog for cell death.

Initially, we wondered whether apoptotic machinery might be used in "local" cell death—getting rid of regions proximal to the injury site.

To test for a role of *ced-3* role in neuron regeneration, we first tested ALM regeneration in the canonical *ced-3* mutant *n717*. We found that this *ced-3* mutant displays markedly reduced regeneration compared to wild type N2 animals. We then studied regeneration of ALM neurons in animals bearing the *ced-3(n2433)* point mutation that alters the caspase active site and confers no detectable protease activity. We found that regeneration is strongly impaired in the *ced-3(n2433)* mutant background. Our data indicate that CED-3 caspase activity itself is unexpectedly required for efficient neuronal regeneration.

The regeneration activity of CED-3 requires CED-4 but occurs independently of death regulatory activities of CED-9 and EGL-1. We addressed whether CED-3 activity was controlled through the same activation cascade as during developmental apoptosis. During apoptosis, the binding of CED-3 by CED-4 triggers pro-CED-3 maturation as an active caspase. Usually, in non-apoptotic cells, CED-4 is sequestered and inhibited by CED-9. During apoptosis, EGL-1 expression inhibits CED-9, CED-4 is released and CED-3 caspase protease is activated. We thus studied regeneration in genetic backgrounds that impair CED-3 activation*: ced-4(n1162), ced-9(n1950)* and *egl-1(n1084n3082)*. We found that loss of function mutant of *ced-4* displays a regeneration phenotype similar to the *ced-3* caspase mutant. By contrast, mutations of the *ced-9* and *egl-1*apoptosis regulators do not affect regeneration. Our data suggest that CED-4 activates CED-3 during neuronal regeneration. However, it is clear that the standard regulatory network of CED-9 and EGL-1 is not used to control CED-3 activity in this circumstance--another regulatory pathway distinct from the canonical apoptotic pathway must recruit CED-3 during neuronal regeneration.

Necrosis gene calreticulin is also important for regeneration. Null mutants in ER calcium-storing chaperone calreticulin are strong suppressors of *mec-4(d)*-induced necrosis. We find that regeneration is impaired in a *crt-1* mutant. Exactly how calreticulin is involved is of considerable interest—crt-1 might regulate calcium in necrosis, might regulate calcium in the growth cone, or alternatively, calreticulin is known to be a substrate of CED-3 which moves to the cell surface to promote corpse removal in mammals—this role might be important.

Plans for continuation. Dr. Pinan-Lucarre is continuing this work supported by a postdoctoral fellowship from the NJCSCR. She has been highly productive in this effort, showing that: 1) other *C. elegans* caspases *csp-1*, *csp-2* and *csp-3*, also function in regeneration; 2) individual phagocytosis genes are not essential for neuronal regeneration; 3) CED-3 acts early in the regeneration mechanism; 4) CED-3 is required within the severed neuron for regeneration. She is finishing up some experiments addressing the relative roles of calreticulin and CED-3, and measuring calcium changes in injured neurons, and we are actively writing up this work, with submission intended for this summer.

C. Project Challenges

Overall, the projects have not presented huge challenges in that we made strong progress on all aims and that the work has resulted in continued funding and will constitute the basis of future proposals for NIH funding. We are collaboratively pursuing the role of caspases in the zebrafish model, which has progressed slower than we planned, but these experiments are ongoing.

D. Implications for Future Work and/or Clinical Treatment

Our work is "basic" in nature, but we underscore that the mammalian counterparts of the MEC-4 channel (ACIS channels) are hyperactivated and contribute significantly to neuronal death during ischemia in mouse models and that the CED-3 protease and CED-4 regulator have clear mammalian homologs that work analogously in apoptosis. Thus, we expect that the mechanistic information we can derive will be relevant to understanding of conserved neuronal injury and repair mechanisms. Our work highlights the importance of regulated functional assembly of ion channels that deliver necrotic insult, the potential beneficial effects of weak activation of the unfolded protein response in neuroprotection, and the roles of caspase proteases in early phases of neuronal regeneration. Our findings suggest specific followup experiments that should be pursued in mammalian models before move toward the clinic, but the implications are significant and should suggest rethinking in the field. For example, caspase inhibitors are delivered in some therapies to prevent apoptosis—our results suggest such treatments may deleteriously impact important initial outgrowth.

E. Plans to continue this research, including applications submitted to other sources for ongoing support.

I have outlined specific plans for research continuation in the results section above.

Both of the supported aims have resulted in continued funding for postdoctoral fellows training in the lab. Dr. Dewey Royal, who is quadrapalegic, earned an award from the NJCSCR to continue work on the necrosis suppressors, and he was promoted to the rank of an Assistant Research Professor in part as a consequence of this award. Dr. Pinan-Lucarre earned a postdoctoral award and has been producing groundbreaking results in her collaborative efforts on genetic influences on axonal regeneration after single cell level. I should also note that our collaborator at Harvard, Dr. Chris Gabel, is currently deciding on offers for his independent academic research position in which he will continue to use the *C. elegans* model for the analysis of neuronal regeneration.

In terms of supporting other funding opportunities, data from this support will constitute the basis for an NIH R01 proposal on neurodegeneration that we will submit to the NIH July 5.

F. Publications

As noted above, these projects have gone well, and we are "crossing all the t's" on three primary research articles describing this work (submission of all planned for this summer). One review article is in press, one in preparation.

Blum, E.S., Driscoll, M. and Shaham, S. (2008) Noncanonical death programs in the nematode *Caenorhabditis elegans*. Cell Death Diff. *in press*

Pinan-Lucarre, B., Gabel, C. Slone, R., Weisberg, S., Samuel, A. and Driscoll, M. (2008) *C. elegans* CED-3 apoptosis caspase activity is needed for neuronal regeneration. *in preparation*

Nunez, Y., Royal, D., Kamat, S., Royal, M. and Driscoll, M. (2008) *C. elegans* UDP-glucose:glycoprotein glucosyltransferases modulate DEG/ENaC-induced neuronal necrosis. *in preparation*

Royal, D., Royal, M., Lizzio, M., Nunez, Y, and Driscoll, M. (2008) A missense mutant in translocon subunit SEC61 blocks necrosis induced by a hyperactivated *C. elegans* DEG/ENaC channel. *in preparation*

Royal, D. (2008) Necrosis, different ways to a certain end. *in preparation*

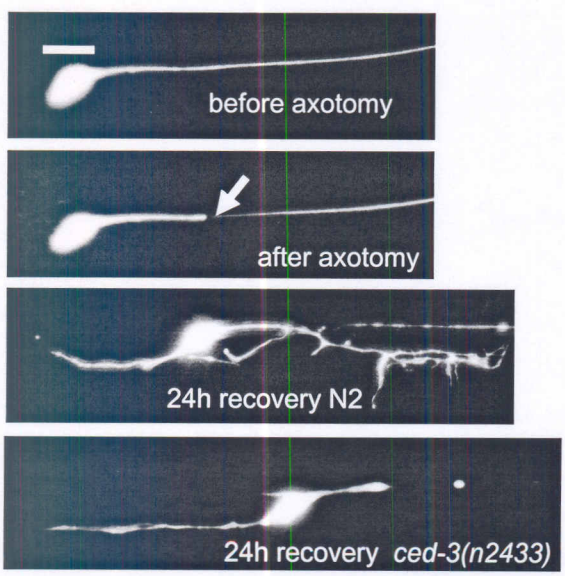

Figure 1. Regeneration of an ALM axon is impaired in a *ced-3* mutant.
ALM touch neuron P_{mec-4}::gfp labeled is shown before and after femtosecond laser axotomy of the anterior process. After 24 hours of recovery in the reference N2 strain, the anterior process regenerates and outgrowth is also triggered in the posterior process. Axonal regeneration is much less efficient in the *ced-3(n2433)* mutant strain.