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SPINAL CORD RESEARCH

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Project Title: Identification of Early-Acting Genes Critical for Injury-induced Neuronal Cell Death

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1. Original Aims of the Project

Traumatic spinal cord injury (SCI) is a devastating event associated with loss of sensory, motor and autonomic neural function, often accompanied by chronic neurogenic pain. The catastrophic impact for affected individuals and their families cannot be overstated. Tragically, currently applied clinical interventions offer at best only modest protection against the axonal and neuronal degeneration that underlies impairment. A more detailed understanding of the molecular mechanisms of neuronal injury and death is required to design novel and effective therapies.

Necrotic neuronal death initiated by ion channel hyper-activation plays a major role in both the initial and prolonged death of neurons consequent to injury. Necrosis consequent to injury occurs in two phases--the first due to physical injury, the second wave of death occurs in response to the necrosis of the neurons directly injured. Blocking or delaying such secondary necrotic cell death would significantly limit incapacitating neuronal damage. Regrettably, however, understanding of molecular necrosis mechanisms is quite limited and the mammalian genes that are most critical to necrosis remain poorly defined.

Our goal is to identify genes essential for the progression through ion channel-induced neuronal necrosis using a powerful experimental model--the nematode *Caenorhabditis elegans*. In this simple animal, we have identified mutant ion channels (for example, the *mec-4(d)* channel) that are genetically hyper-activated and induce necrosis of the neurons in which they are expressed. Interestingly, hyper-activation of mammalian counterparts of these nematode channels (ASIC channels) have recently been shown to make a huge contribution to ischemic brain damage in mice.

Dissection of death mechanisms associated with ion channel hyper-activation in *C. elegans* has revealed that nematode and mammalian models of channel-mediated toxicity share a common mechanism requiring a rise in intracellular Ca^{2+} and activation of calpain and cathepsin proteases. An important outcome of this work is the appreciation that non-apoptotic necrotic-like cell death can be regulated/executed by the products of specific genes that are conserved and therefore may be modulated for purposes of neuroprotection in humans. Thus, we can exploit genetic approaches uniquely applied in *C. elegans* to identify most (if not all) genes that can mutate to block or delay channel injury-induced necrosis. We can build on this molecular information to suggest novel therapeutic intervention strategies. We note that although this work is clearly basic in nature, the potential for major scientific advance in this model is not without precedent--*C. elegans* genetics were absolutely instrumental in deciphering mammalian apoptotic death mechanisms, an accomplishment that revolutionized molecular understanding of regulated cell death and was awarded part of the 2002 Nobel Prize in Physiology and Medicine.

The original specific aims of our NICSCR-supported research were:

Aim I: To conduct a high-throughput saturation genetic screen to identify mutations that block necrotic cell death consequent to Na^+ channel hyper-activation and to identify and genetically map mutations capable of reversing the death process. This aim exploited a newly available automated mutational screening capacity based on sorting of fluorescent signals or neuronal viability. We sought (with considerable success) to ask how many genes could mutate to block channel-induced necrosis, to identify these genes by mutation, and to determine with fairly high precision where these are situated in the genome—accomplishing all of this would facilitate the future molecular cloning of genes critical to the progression through necrosis. Aim 1, then, was to set the genetic groundwork for an investigation that will identify key targets for novel therapeutic intervention strategies that limit secondary nerve damage following traumatic injury.

Aim II: To clone novel death suppressor locus *des(bz2)*, which encodes a semi-dominantly acting mutation that blocks efficient progression through necrosis and significantly rescues neurons from injury-induced cell death. We planned to identify the gene, define the death-suppressing mutation, determine how this protein influences necrosis in the *C. elegans* model, and later collaborate to determine if similar alteration of the mammalian homolog can be neuroprotective in SCI models.

2. Project Successes

Aim 1—Identification of genes that can mutate to suppress necrotic cell death

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. **(Figure 1).**

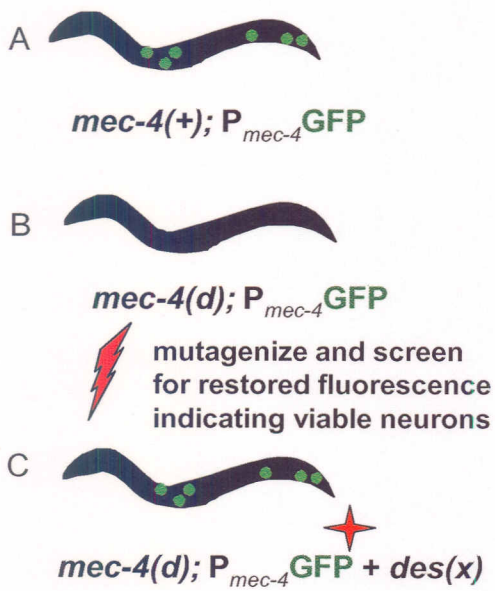

Figure 1. Strategy for Screen for Necrosis Suppressors

(A) Pmec-4GFP labels six touch sensory neuron but **(B)** these are killed by the hyperactive mutant channel *mec-4(d)*. After mutagenesis **(C)**, animals harboring necrosis suppressor genes are identified by the restoration of green touch neurons.

Identification of novel necrosis-suppressors. We screened 56,000 mutagenized genomes for mutations that fully or partially suppress necrotic cell death. This work identified 16

novel loci, which define an absolute minimum of 5 new death suppressor genes (two are on the X chromosome, 3 are on autosomes). We also determined the identified mutations do not affect normal touch sensation (which argues they effect the downstream death mechanism rather than the death initiating hyper-activated ion channel). We are refining the genetic map positions of these 5 strongest death suppressor loci and have narrowed down the genetic intervals for three of them to regions of ~20 genes, which leaves us very well poised for cloning the loci and making definitive gene identifications. A tremendous amount of work on this screen, a major goal of our NJCSCR grant is summarized simply in **Table 1.**

Significance: Our NJCSCR-funded genetic screen was highly successful, in that it identified previously unknown genetic loci and set the stage for a complete molecular description of events that occur during injury-induced neurodegeneration. Importantly, there is NO OTHER experimental system in which we can so rapidly and cleanly address the genetic requirements for necrosis and there is considerable precedent to argue that results will be relevant to unraveling analogous mechanisms in humans.

The analysis of the outcome of a saturation genetic screen for death suppressors defines how many genes can mutate to block necrosis, establishes their genetic map positions, and provides initial information on their potency and mechanisms of action that helps prioritize loci for future molecular analysis. The importance of this work is that the several genes likely to be identified by our effort have not been previously known to influence necrosis in an *in vivo* context. Each molecularly identified suppressor will extend understanding of necrosis mechanisms and suggest a new target for therapeutic intervention.

Table 1: Summary of Novel Necrosis Supressor loci

Reference Allele	Number of Alleles Isolated	Recessive	Map Position
des(bz130)	**6**	**Yes**	**X: between -1.7 and -1.6 (19 predicted gene candidates)**
des(bz178)	**1**	**No**	**IV: between 127,624 and 241,788 on YAC Y57G11C (20 gene candidates)**
des(bz200)	**2**	**Yes**	**II: between 1.82 and 2.15 (covered by 5 cosmid clones)**
des(bz199)	**1**	**Yes**	**I**
des(bz181)	**1**	**Yes**	**II**

Aim 1--Extending a molecular model for necrosis in *C. elegans*

Channel hyper-activation provokes catastrophic ER Ca^{+2} release. Previously, we found that null alleles of ER Ca^{2+}-binding chaperone calreticulin, which plays a major role in maintenance of intracellular Ca^{2+} stores[48], are *strong* suppressors of *mec-4(d)*-induced necrosis[49]. Mutations in ER Ca^{2+} release channels IP3 receptor ITR-1 and ryanodine receptor UNC-68 also significantly suppress death, as can dantrolene treatment, which blocks ER Ca^{2+} release. The implication is that for *mec-4(d)*-induced cell death, the intracellular Ca^{2+} concentration must rise to critical levels for progression through necrosis, similar to what occurs in mammalian excitotoxicity. Moreover, our findings implicate the ER as an important source of the extreme Ca^{2+} elevations required for necrotic cell death, evidence for which in mammals has been recently reviewed. A fascinating finding is that both dantrolene and ryanodine (inhibitors of the ryanodine-sensitive receptor RyR) and 2APB (an inhibitor of ER Ca^{2+} release IP3 receptor)

are neuroprotective in a mammalian model of compressive SCI, suggesting a critical role for ER Ca^{2+} stores in secondary traumatic SCI.

Documentation of an unexpected capacity for calcium import for the MEC-4(d) channel. Our initial studies on necrosis initiation brought a central mechanistic question into focus: how does a hyper-activated ion channel function induce the ER to release excess calcium??? To make a long story short, we postulated that initial action of the MEC-4(d) Na+ channel (documented to conduct Na+), induced some inappropriate Ca^{2+} current that in turn signaled for ER Ca^{2+} release (a calcium-induced calcium release mechanism). However, our extensive tests of potential plasma membrane Ca^{2+} sources all failed to identify the offending channel or transporter. We then realized that no one had characterized the possible Ca^{2+} permeability of the MEC-4(d) channel (which was presumed to be Na+ selective by analogy to some mammalian family members). We conducted genetic and electrophysiological studies, some of which relied on intragenic *mec-4* alleles isolated in our genetic screen, to show that this channel did conduct Ca^{2+}, both in oocytes and *in vivo*. Our data also established that calreticulin death suppression does not involve lowering intracellular Ca^{+2}; rather, data support an induced catastrophic ER Ca^{+2} release as required for necrosis. What was particularly interesting about our finding is that colleagues studying the neuronally-expressed mammalian ASIC1a channel was unexpectedly found to be calcium permeable and to contribute significantly to neuronal loss in brain ischemia.

Significance: These studies added details to working models for molecular mechanisms of necrosis, in defining an unexpected calcium permeability for the MEC-4(d) channel, showing that Na^{+} and Ca^{+2} influx are tightly linked, and supporting a catastrophic ER calcium release event in toxicity. Results from other labs on the mammalian counterpart of MEC-4(d)—hyper-activated ASIC1a made the strong case that toxicity mechanisms involving this channel class are hugely physiologically important. Our work was published in the high–profile journal *Nature Neuroscience* in 2004.

Aim II: To clone novel death suppressor locus *des(bz2),* which encodes a semi-dominantly acting mutation that blocks efficient progression through necrosis and significantly rescues neurons from injury-induced cell death.

***des(bz2*) is an allele of mec-4 that allows temperature-dependent induction of necrosis.** Necrosis suppressor *bz2* had an interesting property—necrosis appeared to be induced with normal efficiency (as evidenced by neuronal swelling) but many neurons could recover to live. We genetically mapped the *bz2* locus to a region on the X chromosome close to *mec-4*. We found that *bz2* encodes a A745T substitution for a residue in the intracellular C. terminus of the MEC-4 protein. We confirmed that the bz2 mutation is causative for phenotypes by engineering the same change into a wild type *mec-4* gene and showing it confers the same genetic properties as *bz2* when the transgene is introduced into wt animals. Interestingly, we found that *mec-4(bz2)* is a strongly temperature sensitive mutation such that necrosis can be induced by a temperature shift. Our electrophysiological analysis confirmed that the MEC-4A745T variant channel has normal properties, but suggested that the subunit does not traffic to the surface efficiently. Indeed, we documented a trafficking/stability defect at the restrictive temperature in *in vivo* tests in *C. elegans*. We are finishing up a manuscript describing these results, and expect to submit 9/05.

Significance. Our data established that low channel activity is correlated with low toxicity, defined the first trafficking defect for MEC-4, and identified the C-terminal MEC-4 domain as important in this trafficking, a potential domain for interference in vivo. Unexpectedly, we documented a strong temperature-sensitive induction for necrosis in this mutant. What this means is that we can delay necrosis to the adult stage by changing the temperature—this is critically important for design of high throughput drug assays which are hard to implement in the embryo.

3. Project Challenges

We normally expect multiple challenges in research as we progress that often force us to revise protocols or change approaches. This project, however, went unusually smoothly and we were able to accomplish all the major objectives outlined in our original proposal.

Technical challenges that we encountered but overcame included optimization of GFP screening protocols using an automated BIOSORT that was relatively new to the lab and adopting new state-of the art gene mapping techniques. We did originally expect that death suppressor allele *bz2* affected a novel gene rather then *mec-4*. However, when we established that *bz2* affected MEC-4 itself in a strongly temperature-dependent manner, we recognized that we had characterized a reagent we had long sought with the intention of use in high-throughput drug screens! In short, by characterizing a temperature-inducible death that we could delay until adulthood, we could more easily screen for drugs that interfere with necrosis. We also defined a potential disruption strategy for hyper-activated DEG/ENaC channels—interference with the trafficking to the surface by targeting the C terminal-domain.

4. Implications for Future Research and/or Clinical Treatment

The devastating consequences of spinal cord injury are attributed in large part to neuronal damage that occurs soon after the incident and there are no efficacious treatments that limit the necrotic-like cell death that accompanies injury. We exploited the considerable advantages of the *C. elegans* model system to identify genetic loci that can mutate to significantly suppress or enhance necrotic-like neuronal death induced by ion channel hyper-activation. In the two year time-frame of previous funding, we were able to successfully set the stage for molecular identification of novel death suppressors, exactly as planned. In the future, we will molecularly identify these loci and define how they act to regulate progression through necrosis.

Our work is "basic" in nature, but *holds truly profound potential* for the understanding of neuronal injury mechanisms that appear to be conserved. As we advance understanding of mechanism, and define specific molecules that are required, we can suggest plausible molecular targets for therapies. Indeed, our work has converged with findings on mammalian counterparts of MEC-4, to implicate ASIC 1a in spinal cord injury—a hypothesis we are now testing with ASIC1a knockout mice in collaboration with colleagues at the Keck Center for Collaborative Neuroscience Research at Rutgers. In addition, we have established a reagent (*mec-4(bz2)*) that induces necrosis with a change in temperature, which will facilitate high throughput drug screens for compounds that block or delay necrosis in collaboration with Cambria Biosciences, MA. In sum, our NJCSCI research has provided several novel necrosis suppressor loci to clone and has generated important new research directions that hold implications for the clinic.

5. Plans for Continuation of Research (include application to other sources)

We have two major projects for continuation of research: 1) molecular identification of the necrosis suppressor genes we identified in this study, and 2) high throughput screens for pharmacological reagents that can block or delay necrosis. Since survival of only 10% of spinal axons might enable injury victims to retain significant function, strategies for blocking/limiting neuronal death are of central importance.

Our plans for funding these continuation studies are as follows:

1) Molecular cloning and characterization of necrosis suppressor loci. In the near future we plan to apply for NIH funding for this project. However, NIH funding is highly highly competitive at present, and it seems likely we will need to succeed at 1-2 molecular clonings in order to get a fundable priority score (I sit on the NIH study section that reviews such projects). For this reason, we have applied for NJCSCR for further advancement of this project, with a focus on molecular identification of death suppressors.

Genes Contributing to Necrotic Death of Injured Neurons: Molecular Mechanisms of Action and Roles in Neuronal Regeneration, M. Driscoll, PI, 12/05-12/07.

2) High throughput screens to identify candidate anti-necrosis drugs. We have collaborated with Cambria Biosciences to submit an NIH SBIR grant (Phase I SBIR (1 R43 NS049688-01A2) to fund drug screen pilots. We got a priority score of 189, but will not make the highly competitive payline this time, so we will resubmit. We also plan to submit this plan to ISOA, a private foundation, for November 7, 2005.

6. Publications

***Nature Neuroscience* (2004) 7:1337.** Some death suppressor mutations we isolated were intragenic and altered the hyperactivated MEC-4(D) channel that initiates necrosis. Study of some intragenic mutations led us to the realization that the death initiation signal included BOTH excess Na^{+} influx and excess Ca^{+2} influx, which was unexpected. Our data also established that death suppression does not involve lowering intracellular Ca^{+2}; rather, data support an induced catastrophic ER Ca^{+2} release as required for necrosis. We attach this publication.

Royal et al, in prep. Our work identified a suppressor mutation (*bz2*) that induces neuronal swelling but suppresses the progression through necrosis. We identified this mutation as affecting the normal trafficking of the MEC-4(D) channel. The paper (**to be submitted in 9/05**) shows that lowering ion influx increases survival and defines a potential strategy to diminish channel activity. It also shows that neuronal swelling is not a "point of no-return" or death commitment—rather, neurons can recover from this injury. Interestingly, *bz2* proved to be a strong temperature sensitive mutation—which can be used to specifically turn on necrosis in the adult. This is a breakthrough for drug screen applications. Our data also provide new insight into trafficking of potentially toxic DEG/ENaC channels. We prefer to submit this manuscript to NJCSCR when it is ready for submission.