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Genes Contributing to Necrotic Death of Injured :*Driscoll Major Project Funded by NJCSCR*
les in Neuronal RegenerationRo Molecular Mechanisms of Action and :Neurons
12/15/05-12/14/07

:*Predoctoral Award ,Wenying Zhang*Identifying & Characterizing Novel Genes That Protect Against Neuronal Necrosis *in vivo* 6/15/06-6/14/08

Necrotic neuronal death initiated by ion channel hyperactivation plays a major role in both the initial and the--Necrosis consequent to injury occurs in two phases .prolonged death of neurons consequent to injury the second wave of death occurs in response to the necrosis of the neurons ,physcial injury first due to Blocking or delaying such secondary necrotic cell death would significantly limit .directly injured nding of molecular necrosis mechanisms isundersta ,however ,Regretably .incapacitating neuronal damage .induced necrosis-and no truly effective therapeutics are available to block injury ,poor

induced neuronal-Our overall goal is to identify genes to promote or that protect against ion channel In this simple .*elegans Caenorhabditis* the nematode--rful experimental modelnecrosis using a powe that are genetically (channel (d)4-the MEC ,for example)we have identified mutant ion channels ,animal ,Interestingly .of the neurons in which they are expressed hyperactivated and induce necrosis have recently been (a1ASIC)hyperactivation of mammalian counterparts of these nematode channels .in mice and neuronal loss shown to make a huge contribution to ischemic brain damage

has revealed *elegans .C* ion of death mechanisms associated with ion channel hyperactivation inDissect mediated toxicity share a common mechanism requiring-that nematode and mammalian models of channel An important outcome of this .hepsin proteasesand activation of calpain and cat $^{+2}$a rise in intracellular Ca executed by the/like cell death can be regulated-apoptotic necrotic-work is the appreciation that non products of specific genes that are conserved and therefore may be modulated for purposes of to *elegans .C* we can exploit genetic approaches uniquely applied in ,Thus .on in humansneuroprotecti induced-channel injury ,alternatively to promote ,genes that can mutate to block or (if not all)identify most We .ules that act in a neuronal necrosis pathwayThe outcome is a genetic description of molec .necrosis .can build on this molecular information to suggest novel therapeutic intervention strategies

the potential for major scientific advance in this ,this work is clearly basic in nature oughWe note that alth genetics were absolutely instrumental in deciphering mammalian *elegans .C*--model has strong precedent ding of regulatednan accomplishment that revolutionized molecular understa ,apoptotic death mechanisms this year ,Moreover .Prize in Physiology and Medicine Nobel 2002cell death and was awarded part of the researchers who *elegans .C* Nobel Prize in Physiology and Medicine was awarded to two 2006the .gene inactivation *in vivo* stranded RNA interference mechanism for-discovered the conserved double model for providing invaluable molecular *elegans .C* here is little question about the potential of thet ,Thus .nt biological processesainsight into import

Objectives and General Rationale

red for efficient necrosis if we are to defineWe are convinced that it is critical that we identify all genes requi model offers *elegans .C* The .molecular death mechanisms and design effective intervention strategies conceived bias about what such-without any pre–unique advantages for accomplishing this important task induced cell death and-injury t a mutant nematode gene block or promotetha "demand"we can ,are genes .determine the molecular mechanisms by which this is accomplished we can then

sequent to cationcon **necrotic cell death *block* to identify mutations that** Our major project seeks The .channel hyperactivation and to then exploit powerful genetic tools to molecularly identify these genes The general idea is that .normal function of the product of such genes is to promote or facilitate necrosis We also have a fantastic graduate student in .onal deathuriens can limit devastating netargeting these prot proteins that are these mutations identify—**necrosis *promote* identifying mutations that** the lab who is producing or activating may-is that over class this The general idea regarding .normally neuroprotective we then define how they ,are identified sOnce necrosis suppressors and enhancer .protect against necrosis and collaborate with colleagues who study models of ,suppressing variants-are altered to become death There are two .ian SCI to test for importance of the homologous proteins in mammalian necrosismammal identifying and characterizing death (1 :fundmentally important anticipated outcomes of this work

our study will identify (2 ;rotic cell deathmodulators will teach us about the molecular mechanisms of nec for novel therapeutic intervention strategies that (proven to be active in a physiological context)key targets .limit secondary nerve damage following traumatic injury

We are .**study how cell death genes impact regeneration** to The second major objective of our work is to ***in vivo*** **sever individual neurons** art femtosecond laser technology to-the-of-collaborating to use state quirements foraddress how cell death genes influence the capacity for regeneration to address molecular re we can directly follow ,In this transparent organism .individual fiber regeneration in a physiological context the injury and regrowth of severed neurons within the living animal and we can test specific genes define The signficance of the basic genetic definition of requirements for .regeneration their contributions to .neruonal regeneration for the spinal cord injury problem is considerable

Accomplishments on Genetic Modulators of Necrosis

(Driscoll grant 1 Aim) Analysis of necrosis suppressor mutants (1

biased screen for mutations that block or delay necrosis identified novel necrosis suppressor-A non into $^{2+}$and Ca $^{+}$conducting excess Na ,encodes a mutant ion channel that is hyperactive *(d)4-mec* .**loci** mediated injury that-similar in several respects to ion channel ,I deathneurons to initiate a necrotic cel accompanies spinal cord injury and directly parallel to toxic action of hyperactivated homologus mammalian we ,in the living animal Taking advantage of fluorescent labelling of viable neurons .a1channel ASIC This .mutagenized genomes for mutations that suppress necrotic cell death 56,000previously screened and we planned to molecularly ,new death suppressor genes 5work identified an absolute minimum of and have precisely ,We have cloned two genes to date .CIR fundingclone three of these with NJCS .mapped two others

.61-change in conserved translocon SEC Ie AAspecifies a sing *(178bz)des* Death suppressor sequence ,Our mapping .dominantly acting death suppressor ,is a strong *178bz* Extragenic suppressor homolog of human *elegans .C* as an allele of the *(178bz)des* and transformation rescue identified ,analysis ch allows misfoldedwhi ,is a highly conserved member of the ER translocon complex 61-SEC *.61-SEC* conversely if proteins are not properly folded in the ;proteins to be imported from the cytoplasm for refolding allele encodes an *178bz* The .61they are shipped back into the cytoplasm for degradation via SEC ,ER One .an alteration in a highly conserved region of the protein ,facing domain-asmC change in a cytopl66R expression by limiting the amount of *(d)4-mec* simple hypothesis is that translocon modification prevents GFP in the:(D)4-of MEC istributionwe do not detect a change in the d ,However .protein (D)4-toxic MEC may induce the protective "unfolded 61-We have indirect evidence the lack of SEC .mutant background We also wonder whether ER calcium regulation dynamics are altered in .protein response" to limit necrosis We expect to submit a .mechanistic possiblities we are currently testing ,suppress death the mutant to .publication on this work in the upcoming year

protein-glyco:glucose-encodes null alleles of UDP *(146bz)des* Death suppressor locus .glucosyltransferase

Six death suppressor alleles on the X chromosome have mutations in *egt-1*, which encodes a secreted, soluble 1493 AA protein that has high homology to human UDP-glucose:glycoprotein glucosyltransferases. 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.

The identification of two death suppressors implicated in ER folding issues but not markedly changing apparent MEC-4 expression is interesting, especially given that we know another ER chaperone calreticulin

is a strong death suppressor. We are currently testing hypotheses on what they have in common and expect to bring this work to publication by the end of the funding period.

alleles that fail to complement each 4 **.other death suppressors 2We are close to molecular cloning of** an interval ,2.12and 86.map between position (*181bz* & *180bz ,100bz ,200bz*) other on Chromosome II is a *199bz* .we can now test candidates to confirm gene identification ,Thus .candidate ORFs 18including PWe are continuing fine SN .3.29and 0.0single mutation that we have mapped to Chromosome I between .Our goal is to molecularly clone these locus in the upcoming grant period .mapping for these genes

The analysis of the outcome of a saturation genetic screen for death suppressors defines ***:Significance*** and provides initial information on their potentcy e modelth how many genes can mutate to block necrosis in t have notidentified by our effor the genes The importance of this work is that .action and mechanisms of arly identified suppressorEach molecul .context *in vivo* to influence necrosis in an been previously identified Our .a new target for therapeutic intervention sunderstanding of necrosis mechanisms and suggest sextend work to date has identified multiple genes than can be mechanistically linked in protien chaperoning and mechanistic We are well positioned to figure out what .which is quite exciting ,scalcium homeostasi e processes and might be exploited to regulatesknown drugs interfere with the—functions are relevant .necrosis

The .ecial circumstances applybut sp ,Our budget from NJCSCI is fairly large ***:Budget Note*** who is striken with multiple (Dewey Royal .Dr)necrosis suppressor work is that of a postdoctoral associate Royal .Dr ,Consequently .he works in a wheelchair and cannot perform fine motor functions—sclerosis technican to conduct bench experiments and a "reader" to enable him to write and keep up must rely on a We are deeply grateful to .salaries to the effort 2.5e need to devote The reality is that w .the literature with of SCI hich is not unlike the challengesw ,stancethe NJCSCIR for their support of this special circum .victims

(Predoctoral award for Wenying Zhang)Necrosis enhancer project (2
enhance genetic screen for mutations that *the first* Graduate student Wenying Zhang is conducting the ,Because the normal function of such genes is normally to prevent or delay necrosis .necrotic cell death *normally exert a protective function against necrosis in a* genes identified by enhancer mutations should .cation of such genes has clear therapeutic potentialIdentifi *.physiological context ,native*

.C constructed a marked previously We **.enhancers in this model-We identified the first necrosis** a channel is 10-MEC *.(d)10-mec* called transgene strain that harbors a modest necrosis inducer *elegans* mutant subunits lead to very (d)10-into an ion channel but MEC 4-assemble with MEC-thought to co bunitsu We screened .just on the edge of toxicity neurons in this strain are—little neurodegeration on their own Our characterization of enhancer .enhancers of necrosis genetic mutagenized genomes to identify 18,500 alleles that act to 61mutations to date has eliminated uninteresting enhancer classes and leaves us with .either strongly or partially ,enhance necrosis

a potent death ,*301bz* We found that **.channel subunit 4-One necrosis enhancer encodes a novel MEC** but ,that does not induce necrosis or even dysfunction on its own ,4-is a novel change in MEC ,inducer rsuingThis is somewhat unexpected and we are pu .induces necrosis ,variant (d)10-together with the MEC .electrophysioloigcal analysis of this aberrant channel to gain insight into necrosis induction

We have mapped the next strongest extragenic ***.300bz*** **We are genetically pursuing strong enhancer** We continue genetic .NP mapping to enable cloningand are pursuing high resolution S 1 .enhancer to Chr alleles to define the number of genes identified and to progress toward their k on the other enhancerwor .molecular cloning

in this This work provides the first identifcation of mutations that enhance necrotic potential ***.Significance*** regulated to limit neuronal death in spinal cord-the activities encoded by these genes might be up—model edly advance understading of basic necrosisdoubtthe genes affected will un Identification of .injury .mechanisms in a native environment

,Driscoll grant .Addressing molecular requirements for cell death genes in neuronal regeneration (3 (2Aim

through-recent break a ,In our field .njury recoveryFunctional regeneration is a critical goal for spinal cord i .neurons can functionally regenerate consequent to axotomy *elegans .C* paper unexpectedly showed that influence the extent of neuronal loss consequent to apoptosis and autophagy death programs ,isNecros we wondered whether ,Since regeneration appears to require limited degeneration of affected neurons .SCI Aravi .Dr are collaborating with the group of We .repair capacity neuronal influence cell death genes might to address *in vivo* neurons nology to sever individuallaser tech the art-of-Samuel at Harvard to use state by doing laser nanosurgery in mutants how cell death genes influence the capacity for regeneration .work has already provided exciting novel data in the field This .defective in specific death types

We have now conducted time lapse ***.elegans .C* Time lapse analysis of neuronal regeneration in** alConsequent to a femtosecond laser pulse that severs neuron .photography on severed sensory neurons does nothing ,the cell body proximal neuron retracts a bit ,axons but does not damage surrounding tissue The distal .hours and then sends a dramatic growth cone that searches for the original target 7for about Reconnection is fairly .like degeneration-rade by Walleriansevered nerve end appears to begin to deg .of wild type neurons have reconnected 65% ,hours of axotomy 24such that within ,efficient

Our goal was to construct **!is required for neuronal regeneration 3-Key death protease caspase CED** to ask (autophagy ,necrosis ,apoptosis)ins in which we inactivated each of the potential death programs stra severed ,We constructed multiple strains for this purpose .how regeneration capacity was impacted lysing various death types but we have repeated oneWe are still ana .and tracked regeneration ,neurons of 15%at best —nerationeare defective in reg mutants *3-ced*—nd to statistical significancebackgrou revealed a role for This data unexpectedly !rotease is missingconserved apoptosis p recover if this euronsn and we will test how extensively apoptosis genes are required in the ,nerationeprotease in reg *3-ced* .upcoming funding period

in a This is the first study to address molecular requirements for individual fiber regeneration **.Significance** physiological context and develops a model that should provide new understanding of the basic biology of our preliminary data strongly ,Unexpectedly .ral importance in SCI researchan issue of cent ,regeneration This is an exciting finding that .caspase is critical for regeneration apoptosis 3-suggest that the killer CED how a gene of known killer regarding but that raises fascinating questions ,we have not yet published implications for therapeutic Our data already holds strong .function can actually function in neuronal repair !may actually impair regeneration capacity but caspase inhibitors are being used to limit cell death—design .ed work in a high profile journaldIn the upcoming year we expect to publish our exten

Advance r ClinicalSummary and Implications fo

for the understanding of neuronal injury *hold truly profound potential* but can ,in nature "basic"Our work is The devastating consequences of spinal cord injury are .mechanisms that appear to be conserved attributed in large part to neuronal damage that occurs soon after the incident and there are no efficacious considerable the We exploit .ccompanies injurylike cell death that a-treatments that limit the necrotic model system to identify genetic loci that can mutate to significantly suppress *elegans .C* advantages of the We have already cloned .activation-like neuronal death induced by ion channel hyper-or enhance necrotic As .s and expect to clone two additional ones in the timeframe of this fundingtwo of these death suppressor we may be able to suggest new strategies for ,we evaluate their mechanisms of death suppression .therapeutic intervention

d neuronsventral cor *elegans .C* that logy to confirmthe art laser techno-of-used state we have ,Moreover ssand we have begun to addre and sensory neurons can regenerate consequent to single axon axotomy trala cen ,caspse *3-ced* that at least dWe have foun .potential roles for cell death genes in neuronal repair This work changes thinking on .is needed for efficient regeneration ,n apoptosisexecutor killer protease i Melitta .roles of death genes in injury and has pushed us to establish collaborations with the lab of Dr otease in zebrafish spinal cord injury to test for conserved effects in apr 3-Schachner to test the role of CED .vertebrate model

We thank the NJCSCR for giving us the opportunity to develop these exciting new research areas and we

.look forward to a productive upcoming year