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SPINAL CORD RESEARCH

**Identifying & Characterizing Novel Genes
That
Protect Against Neuronal Necrosis *in vivo***

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Final Report

Overall Plan Summary and Rationale

Project rationale. Necrosis, or “unplanned” cell death, is a major contributor to the devastating consequences of spinal cord injury (SCI), stroke, and ischemia. 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.

One of our experimental goals is to identify the molecular modulators of neuronal necrotic death using the unique advantages of the *C. elegans* for genetic and molecular studies. The necrotic paradigm we study most involves initiation of cell death by hyperactivated ion channels (MEC-4(d) and MEC10-(d)) 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 implicate key molecules in necrosis in human and suggest novel strategies for therapeutic intervention in spinal cord injury.

The longterm goal of our research is to dissect the molecular mechanism of necrotic cell death. Previously, our lab has conducted successful genetic screens for suppressors of *mec-4(d)*-induced necrosis—work that identified genes normally needed for progression through necrosis. I conducted the first genetic screen for **enhancers** of a mild necrosis-inducing stimulus conferred by hyperactivated MEC channels (MEC-10(d)) (2) to identify 17 medium-strong necrosis enhancer alleles (*nen*) (Figure 1). 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

Aim 1: To molecularly clone two loci that can mutate to enhance necrosis, the normal function of which should be neuroprotective.

I planned to position at least 2 necrosis enhancer genes on the genetic map, molecularly identify the enhancer genes and describe the death-enhancing mutations.

Aim 2: To determine the molecular mechanisms of action of necrosis enhancers.

I planned to characterize properties of 2 necrosis enhancer genes, determine where these act in the genetic pathway for necrosis, and ask if mammalian homologs might function similarly in necrosis.

Progress Summary

Review of screen strategy (Figure 1). To identify the genes that protect against necrosis, I constructed a marked strain including a modest necrosis inducer-*lsmec-10(d)*. MEC-10 is a channel subunit thought to co-assemble with MEC-4 into an ion channel but MEC-10(d) mutant subunits lead to very little neurodegeneration on their own—neurons in this strain are just on the edge of toxicity. I screened 18,500 mutagenized genomes to identify genetic enhancers of necrosis, using a touch neuron-specific fluorescent GFP to identify death enhancement accompanied by signal loss. 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 strength necrosis enhancer mutants.

Accomplishment 1: One necrosis enhancer (*bz301*) encodes a novel MEC-4 channel subunit.

I found that *bz301*, a potent *mec-10(d)* necrosis inducer, is a novel amino acid change in channel subunit MEC-4(A149V). MEC-4(A149V) executes normal MEC-4 function in touch sensation and does not induce necrosis on its own, but rather combines with the MEC-10(d) variant to create a strongly neurotoxic channel. The MEC-4(A149V) + MEC-10(d) channel conducts elevated Na⁺ and Ca²⁺ currents (with a disproportionate increase in Ca²⁺ current) in the *Xenopus* oocyte expression system, and exhibits altered binding of the channel inhibitor amiloride. This result documents the first example of synergistically toxic inter-subunit interactions in the DEG/ENaC channel class and provides evidence that Ca²⁺ current levels may be decisive factors in the tipping the balance between neuronal survival and necrosis.

Details: a) *bz301* is a semi-dominant enhancer of *Ismec-10(d)* that exacerbates necrosis in a calreticulin-dependent mechanism- In the *Ismec-10(d)* *bz301* mutant (20°C), 49% of young L1 larvae have 1-2 necrotic PLMs (Figure 2 A), and yet 84% lack 1-2 PLMs by the L4 stage (Figure 2 B). The fact that more neurons are dead by the L4 stage than appear dying at the L1 stage suggests that necrosis onset can be after the L1 stage in the *Ismec-10(d)* *bz301* strain, and we confirmed this by visual inspection of L2 and L3 larvae that were devoid of swollen necrotic neurons as L1s (data not shown). Although *bz301* is a strong necrosis enhancer, its effects are less potent than observed for *mec-4(d)*--in *mec-4(d)* mutants, 95% of L1 larvae have 1-2 necrotic PLM neurons and 100% have either 1 or 2 neurons dead by the L4 stage. We find a significant amount of death in the *bz301/+* heterozygotes, which approaches that in *bz301/bz301* homozygotes (Figure 2 C). These results establish the genetically semi-dominant action of allele *bz301* in necrosis enhancement.

We also tested whether enhancer *bz301* activates necrosis with similar genetic requirements to *mec-4(d)*. In *mec-4(d)*-induced death, progression through necrosis requires calreticulin, a calcium-storing ER chaperone, which we propose is needed for release of ER calcium stores and amplification of toxic Ca²⁺ overload¹⁵. We constructed the triple mutant *crt-1; Ismec-10(d) bz301* to ask if the enhanced death is blocked by the *crt-1* null mutation. We find that calreticulin deficiency fully blocks necrosis-enhancing effects of *bz301* (Figure 2 A, B). We conclude that *bz301*-induced necrosis involves a mechanism similar to that induced by the MEC-4(d) channel.

b) Necrosis enhancer *bz301* encodes MEC-4(A149V), adjacent to a conserved extracellular domain- One class of necrosis enhancer that could have been identified in our screen could include *mec-4* mutations that themselves induce necrosis^{8,16}. Since our genetic mapping placed *bz301* on the X chromosome near *mec-4*, we sequenced the *mec-4* coding sequence in this mutant background. We found that *bz301* does encode a *mec-4* mutation, but one distinct from previously sequenced *mec-4* alleles^{17,18}, specifying amino acid change A149V (Figure 3). A149 is located 19 amino acids from MEC-4 membrane-spanning domain I (MSDI) on the extracellular side of the protein, adjacent to a conserved DEG/ENaC domain of unknown function (Figure 3 A, B). This residue is commonly a nonpolar residue or an Ala (in 32 out of 40 family members (Figure 3 B and Supplemental Figure 1), and four DEG/ENaC family members (snail FaNaC, *C. elegans* DEG-1, and two uncharacterized *C. elegans* family members) normally encode Val at this position (Supplemental Figure 1). Interestingly, this region

was found to be a site of interaction between two adjacent subunits within the trimeric channel complex in the recently solved ASIC1a structure³.

To confirm that the *bz301*-encoded *mec-4* mutation is causative for necrosis enhancement, we constructed a *mec-4(bz301)* allele by site-directed mutagenesis and introduced the *pmec-4(bz301)* allele into the *lsmec-10(d)* background (Figure 3 C). We find that *pmec-4(bz301)* induces necrosis. We conclude that the MEC-4(A149V) change is responsible for necrosis-enhancer activity.

c) *mec-4(bz301)* requires *lsmec-10(d)*, but not *mec-10(+)*, to induce necrosis- *mec-4(bz301)* might potentiate neurotoxicity of *lsmec-10(d)* or, alternatively, *mec-4(bz301)* could encode a novel mutation that causes necrosis on its own. To distinguish between these two possibilities, we crossed *mec-4(bz301)* away from *lsmec-10(d)* and we scored for touch neuron viability in the *mec-4(bz301)*-only background. We found that *mec-4(bz301)* does not confer neurotoxicity when present in an otherwise wild-type background (Figure 4 A). Importantly, when we reintroduced a *mec-10(d)* integrated transgene array different from the one used to generate the parental *lsmec-10(d)* strain into the *bz301* strain, we found that the neurodegeneration phenotype was restored (Figure 4 A). These experiments demonstrate that *mec-4(bz301)* must act together with *mec-10(d)* to enhance necrosis and that the neurodegeneration phenotype does not depend on any unusual features of the *mec-10(d)* transgene array that might have been introduced into the original enhancer mutant background by mutagenesis.

In the combinatorial toxicity situation we characterize for *mec-4(bz301)+mec-10(d)*, the strains we initially tested for neurodegeneration also have two wild type genomic copies of *mec-10*. To address whether *mec-10(+)* activity is required for synthetic neurotoxicity, we replaced the genomic *mec-10(+)* copies with *mec-10* null deletion allele *tm1552*, while leaving the *mec-10(d)* gene array in place. We found that eliminating *mec-10(+)* alters neither *lsmec-10(d)*- nor *lsmec-10(d);mec-4(bz301)*-mediated neuronal degeneration (Supplemental Figure 2A). We conclude that synthetic neurotoxicity requires only mutant MEC-4(A149V) and MEC-10(d) subunits, presumably as components of a hyperactivated heteromeric DEG/ENaC channel.

d) MEC-4(A149V) functions normally in touch sensation- *mec-4(+)* is required for sensitivity to gentle touch⁸ and contributes to the pore of the mechanotransducing complex¹⁹. We wondered whether mutant subunit MEC-4(A149V), which does not kill touch neurons on its own, might still possess functional MEC-4 activity. We compared touch sensitivity of wild-type and *mec-4(bz301)* mutants to show that touch responses are, in fact, normal in the *mec-4(bz301)* mutant (Figure 4B, dark grey bar). In the *lsmec-10(d);mec-4(bz301)* double mutant, the touch response is impaired (Figure 4B, white bar), likely the consequence of touch receptor degeneration (*lsmec-10(d)* does not disrupt function on its own Supplemental Figure 2 B). Thus, not only is MEC-4(A149V) non-neurotoxic on its own, MEC-4(A149V) also serves as a functional MEC-4 channel subunit.

Overall we conclude that *mec-4(bz301)* encodes MEC-4 amino acid change A149V, which is neither neurotoxic nor channel-disrupting on its own *in vivo*, but can combine with the *lsmec-10(d)*-encoded MEC-10(A673V) mutant subunit to generate a strongly neurotoxic channel.

This work has been published in a first-author paper, acknowledging NJCSCR:

Zhang W, Bianchi L, Lee WH, Wang Y, Israel S, Driscoll M. Intersubunit interactions between mutant DEG/ENaCs induce synthetic neurotoxicity. *Cell Death Differ.* 2008 Aug 1. [Epub ahead of print]

Accomplishment 2: *nen(bz300)* is a strong recessive necrosis enhancer that enhances *crt-1*-dependent necrosis.

a) *nen(bz300)* is mapped to the right arm of Chromosome I. *nen(bz300)* is a strong recessive necrosis enhancer of *mec-10(d)*. I mapped *nen(bz300)* to the right arm of Chromosome I (right to *bli-4*, which is at +.95), apart from known *mec* genes. I crossed *unc-75* (I: 9.30) with a *bli-4 nen(bz300) Is5* strain (the *mec-10(d)* weak death inducer is homozygous all the time). Out of 39 *unc-75 Is5* strains I got 13 *unc-75 nen(bz300) Is5* strains. This indicates that *nen(bz300)* is located right to *unc-75* (I:9.30). I crossed *unc-101* (I:13.23) with the *bli-4 nen(bz300) Is5* strain. Out of 50 *unc-101 Is5* recombinant animals I did not get any that were genotypically *unc-101 nen(bz300) Is5*. Out of 159 *bli-4 Is5* recombinant animals I did not get any *bli-4 nen(bz300) Is5* animals. This map data suggested that *nen(bz300)* might be fairly tightly linked to *unc-101*.

b) *nen(bz300)* depends on the presence of *mec-10(d)* to be neurotoxic. I crossed *nen(bz300)* away from *mec-10(d)* to show that *nen(bz300)* does not induce necrosis on its own—it depends on the presence of *mec-10(d)* to be neurotoxic and thus it is a new necrosis enhancer rather than inducer.

c) *nen(bz300)* is a strong enhancer that appears to act upstream of ER Ca^{+2} release. To ask whether *nen(bz300)* enhances necrosis with genetic requirements established for other DEG/ENaC-mediated necrosis, we tested whether calreticulin is needed for the death enhancement. We find that the *crt-1* null mutation blocks all necrosis in *nen(bz300) Ismec-10(d)* (Figure 5). This observation suggests *bz300* acts early in the necrosis pathway, upstream of catastrophic ER Ca^{+2} release—a critical part of the necrosis induction mechanism because of its potential value in therapeutic design.

Continuing work. We expect to use powerful modern bulk sequencing for later gene identification, fairly rough mapping is likely to be sufficient. I will check the roles of *bz300* in multiple death-inducing paradigms, such as glutamate excitotoxicity models, the *deg-3(u662)* acetylcholine receptor hyperactivation model, and the Alzheimer disease *Isp_{unc-54}Ab₁₋₄₂* model. I will determine if these necrosis enhancer loci have mammalian counterparts implicated in necrosis and spinal cord injury.

Significance

Necrotic cell death is a major contributor to neuronal loss that accompanies spinal cord injury, yet effective 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.

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)

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

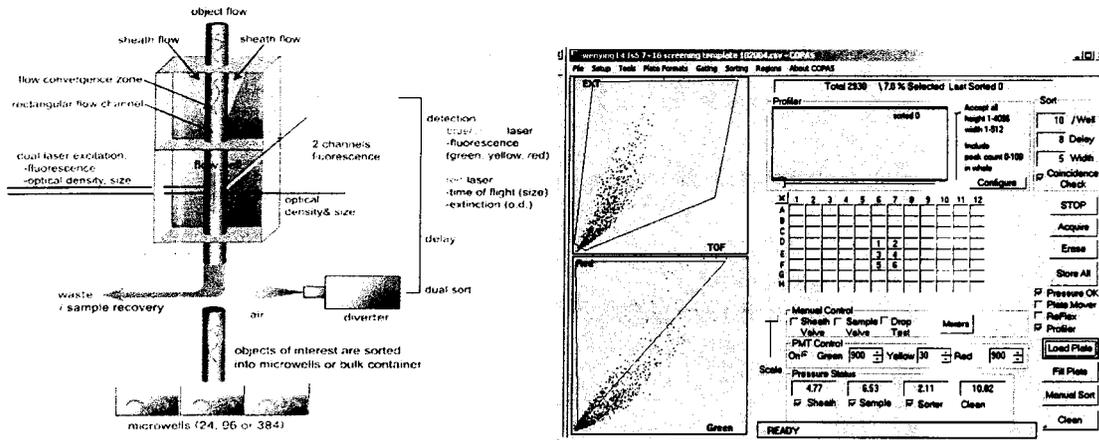


Figure 1. Strategy for isolation of enhancers of *mec-10(d)*-induced necrotic-like cell death. The parental *mec-10(d)* worms induce very weak necrosis. Almost all the 6 touch neurons live and fluoresce green at 20°C. After mutagenesis, animals bearing a necrosis enhancer mutation will have less GFP 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.

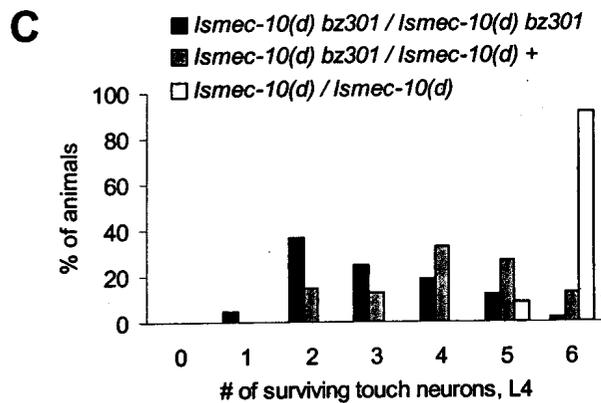
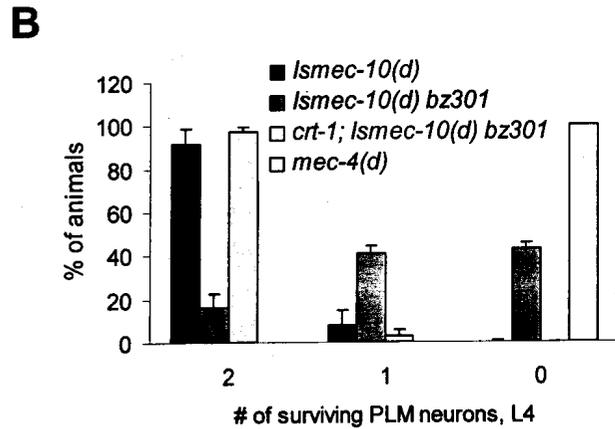
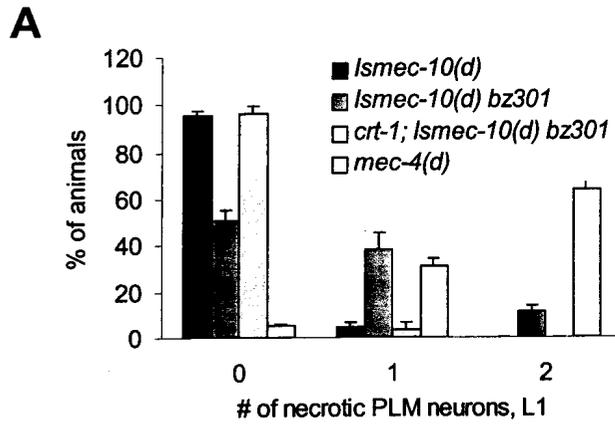
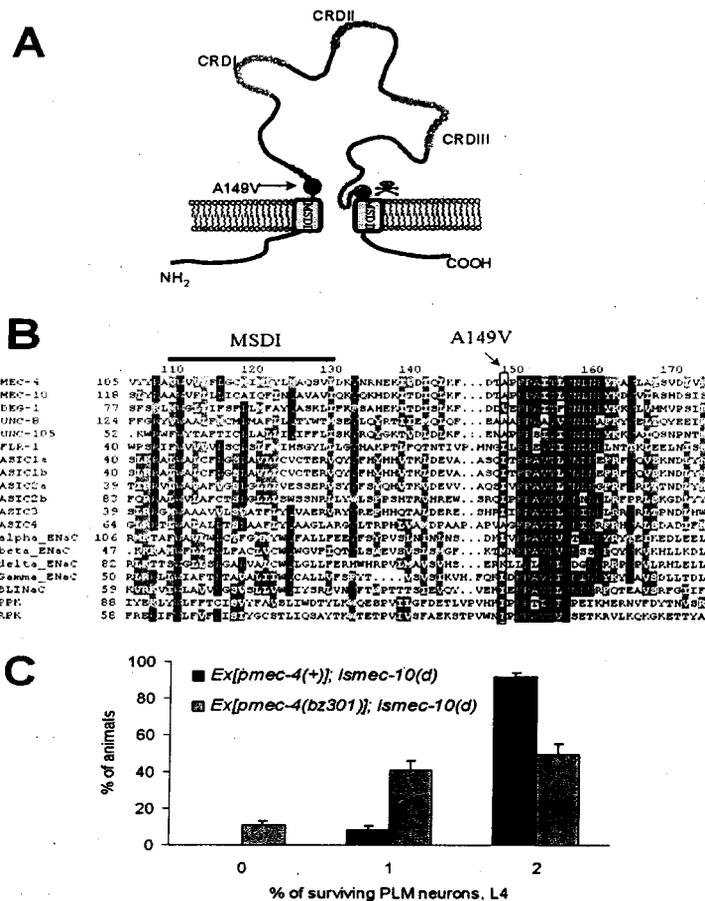


Figure 2. *bz301* acts semi-dominantly to enhance neuronal loss in *Ismec-10(d)* via a calreticulin-dependent mechanism. A: Quantitation of swollen necrotic PLM touch neurons during the early L1 stage (within 4 hours after hatching) in *Ismec-10(d)* (black), *Ismec-10(d) bz301* (dark grey), *crt-1(bz29); Ismec-10(d) bz301* (light grey) and *mec-4(d)* (white) animals; $n \geq 230$ in at least 3 independent trails, 20°C. **B:** Quantitation of surviving fluorescent PLM touch neurons at the L4 stage for *Ismec-10(d)* (black), *Ismec-10(d) bz301* (dark grey), *crt-1(bz29); Ismec-10(d) bz301* (light grey) and *mec-4(d)* (white) animals. Comparison of the extent of PLM swelling and PLM death for *Ismec-10(d) bz301* reveals that more PLMs die than appear as swollen necrotic figures in L1, suggesting that neurodegeneration can have late larval onset, and we confirmed this by visual inspection of older larvae (data not shown); $n \geq 170$ in at least 3 independent trails, 20°C. The *crt-1* null mutation suppresses cell death induced in *Ismec-10(d) bz301*. **C:** *bz301* acts semi-dominantly. We counted the number of surviving touch receptor neurons (of 6 total) in *bz301* homozygotes (black) or heterozygotes (grey). *Ismec-10(d)* is homozygous in all lines. *bz301* heterozygotes and homozygotes have extensive neuronal loss, but nearly all *Ismec-10(d)* homozygotes (white) have 6 touch cells surviving.



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Figure 3. *bz301* is a *mec-4* allele that encodes substitution A149V adjacent to a highly conserved extracellular domain. **A:** Cartoon representing the transmembrane topology of the MEC-4 subunit and the position of the A149V substitution. Relatively short N- and C-terminal MEC-4 domains project into the cell and a single large central loop containing 3 conserved cysteine-rich domains (CRDI, II, III) extends extracellularly. Position A149 is indicated by a light grey dot and an arrow. The highly conserved region right after A149 is indicated in dark grey. A short loop preceding membrane-spanning domain II (MSDII) is thought to participate in pore formation (grey) and MSDII contributes to the channel pore. The highly conserved *d* position MEC-4(A713) at which large side chain AA substitution hyperactivates the channel is indicated by a black dot. Not all domains are drawn to scale. **B:** Amino acid sequence alignment of MEC-4 and several DEG/ENaC family members in the region corresponding to MEC-4(A149). The amino acid change specified by *mec-4* allele *bz301* is noted at the position indicated by an arrow and a box, amino acid numbers correspond to MEC-4 primary sequence, position of membrane-spanning domain I is indicated. Included in the alignment are some better-studied *C. elegans* family members, human ASICs and ENaCs, fly PPK-1 and RPK-1. Residues common to all DEG/ENaCs are boxed in black; similar residues are boxed in grey. **C:** Confirmation that *bz301* is the causative mutation for neurodegeneration. The necrosis-enhancer property is also observed for an engineered transgene *bzEx170[pmec-4(bz301)]* (grey) introduced into the *Ismec-10(d)* background but not *bzEx177[pmec-4(+)]* (black), supporting that the necrosis-enhancer property is conferred by the *bz301* mutation.

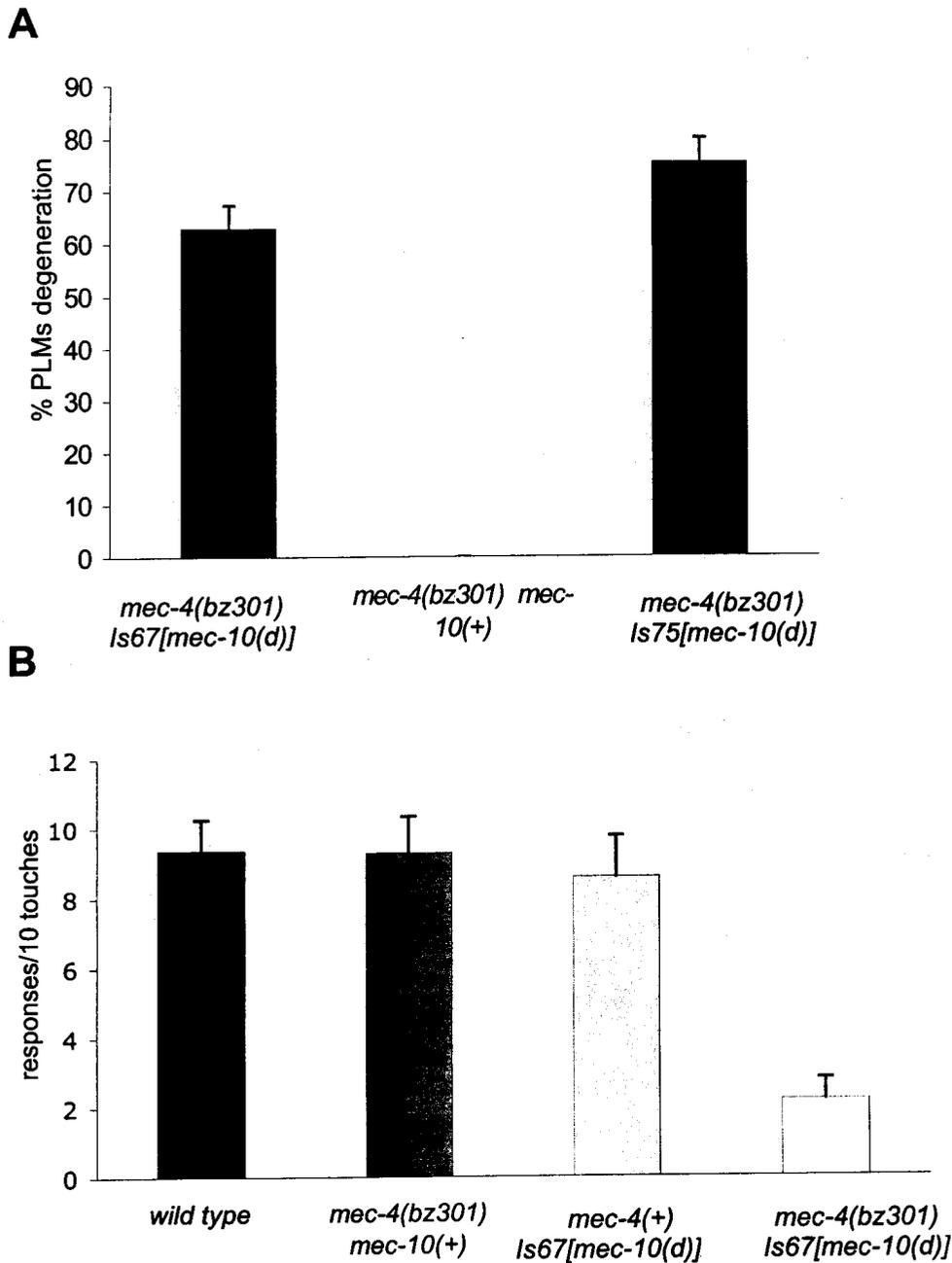


Figure 4. *mec-4(bz301)* encodes a functional MEC-4 subunit that requires *lsmec-10(d)* for neurotoxicity. **A.** *mec-4(bz301)* is neurotoxic only in conjunction with *lsmec-10(d)*. *bzls67 X* and *bzls75 IV* are two independently isolated integrated arrays of *mec-10(d)*, and both are neurotoxic when combined with *mec-4(bz301)*. Y axis indicates the percentage of PLM neurons that undergo degeneration by the L4 stage; $n \geq 170$ in at least 3 independent trials, 20°C. All lines have the GFP transgene labeling the 6 touch neurons (not shown). MEC-4(A149V) needs MEC-10(d) for its necrotic effect, but cannot induce cell death on its own. **B.** The MEC-4(A149V) subunit is a functional MEC-4 subunit. We assayed touch sensitivity of wild type (black), *mec-4(bz301)* (dark grey), *bzls67[mec-10(d)]* (light grey) and *mec-4(bz301) bzls67[mec-10(d)]* (white) at the L4 stage. We touched animals 10 times and recorded the number of avoidance responses for each animal, $n = 30$, 3 independent trials performed, 20°C. Since *mec-4(bz301)* exhibits normal touch sensitivity, the MEC-4(A149V) subunit is functional *in vivo*. Note that the combinatorial touch insensitivity of *mec-4(bz301)* and *mec-10(d)* is consistent with their combinatorial degeneration action.

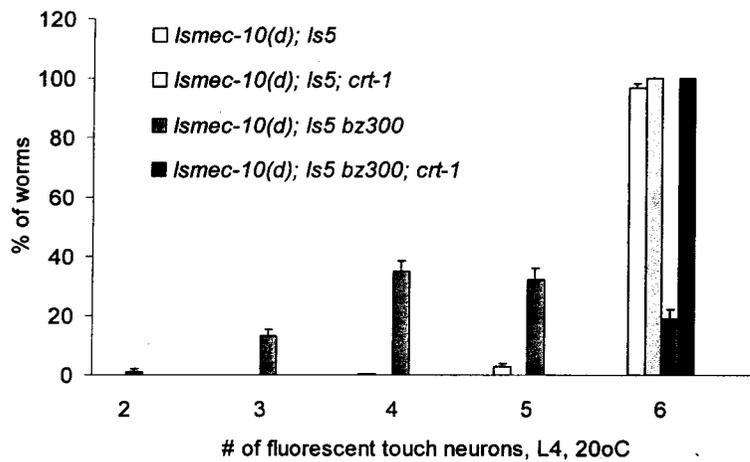


Figure 5. *nen(bz300)* enhances necrosis in an *Ismec-10(d)* background, via a *crt-1*-dependent mechanism. *Is5* is a GFP reporter expressed in touch neurons use to determine how many of 6 possible touch neurons are viable

