Cell Death and Survival II: Cell Death Signaling Pathways

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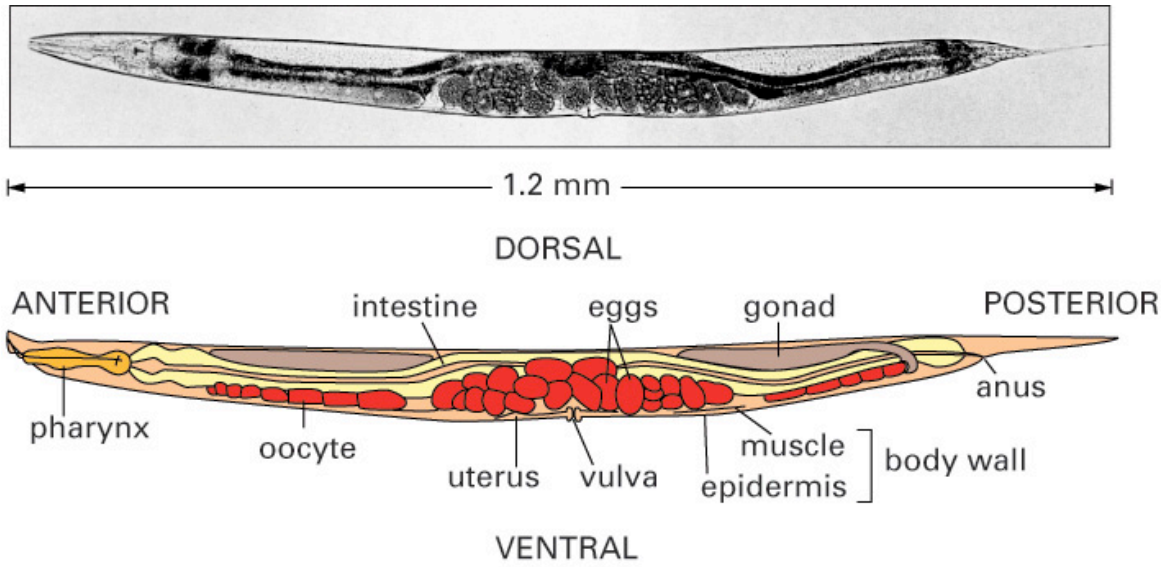
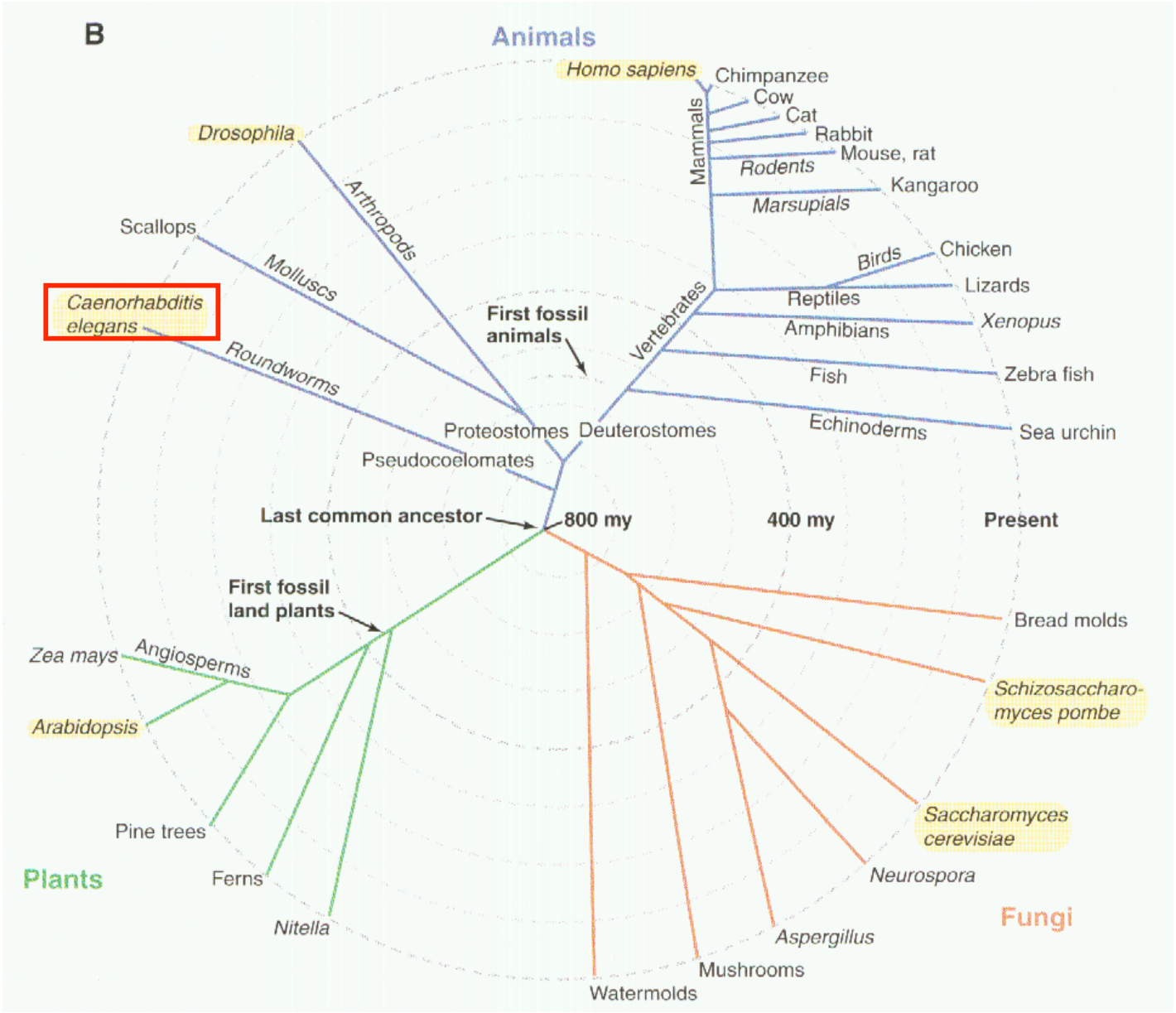
Discovery of molecular pathways that control cell death

- Recap:
  - Cell death is common during neuronal development
  - Cell death occurs in a programmed fashion
  - Cell death can be regulated by trophic signaling
  - Cell death occurs through an active process, not simply starvation
- Suggests there are specific molecular pathways that determine whether a cell will die and carry out the killing
- Discovery of these pathways pioneered by Brenner, Sulston, Horvitz and co-workers working in C. elegans

Caenorhabditis elegans

- **Free-living soil nematode (roundworm)**
- **3 day generation time, could be grown in quantity and superb for genetics**
- **Small genome (97 Mb; 2.6% humans); ≈19,000 protein-coding genes**
- **Few cells (≈1000)**
- **Transparent**

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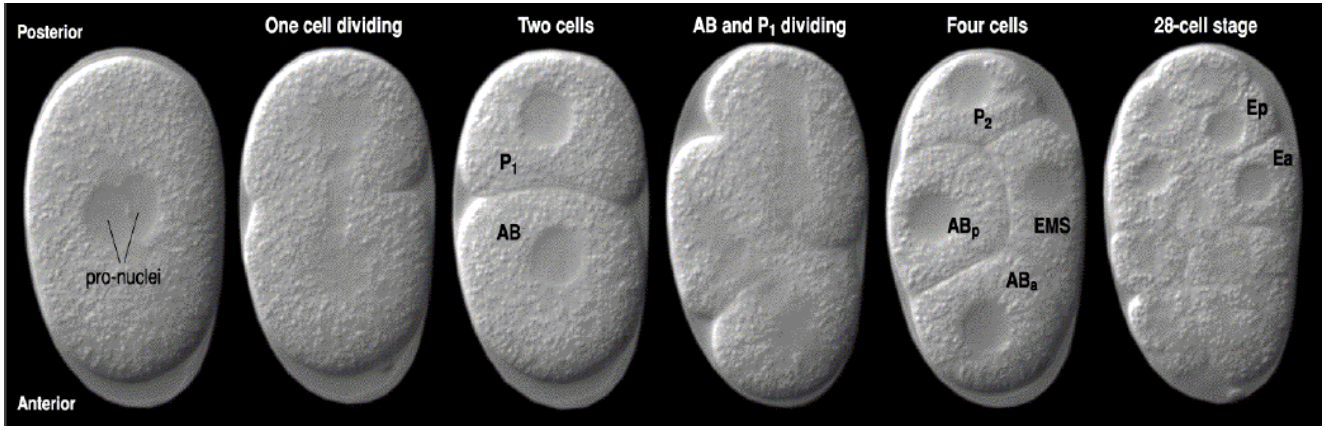
Animals
Homo sapiens
Chimpanzee
Cow
Cat
Rabbit
Mouse, rat
Rodents
Kangaroo
Marsupials
Mammals
Drosophila
Arthropods
Scallops
Molluscs
Caenorhabditis elegans
Roundworms
Birds
Chicken
Lizards
Reptiles
Xenopus
Amphibians
Vertebrates
First fossil animals
Fish
Zebra fish
Echinoderms
Sea urchin
Proteostomes
Deuterostomes
Pseudocoelomates
Last common ancestor
800 my
400 my
Present
First fossil land plants
Bread molds
Zea mays
Angiosperms
Schizosaccharo-myces pombe
Arabidopsis
Saccharomyces cerevisiae
Pine trees
Neurospora
Plants
Ferns
Fungi
Nitella
Aspergillus
Mushrooms
Watermolds

C. elegans as a model system

- **Sydney Brenner (1963): wanted a simple organism in which to understand animal development and behavior at the molecular and cellular level**
  - **"I would like to tame a small metazoan organism to study development directly… We propose to identify every cell in the worm and trace lineages. We shall also investigate the constancy of development and study its genetic control."**
  - **Master plan:**
    - **Complete cell lineage**
    - **Complete wiring diagram**
    - **Identify and study the function of the genes that build and are required for activity of nervous system**
      - **Complete genomic sequence**
- **John Sulston: cell lineage (1969)**

C. Elegans cell lineage

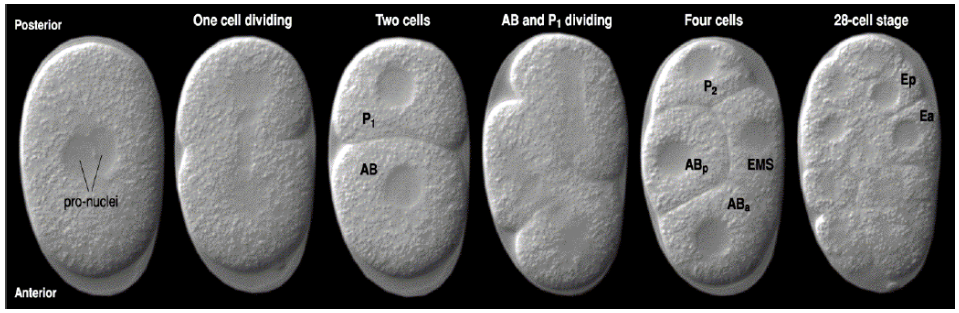
- *C. elegans* is transparent
- This property allowed John Sulston (joined by Bob Horvitz [1974] and others) to watch the animal develop under a microscope and follow the fate of every cell and all of its progeny : cell lineage analysis

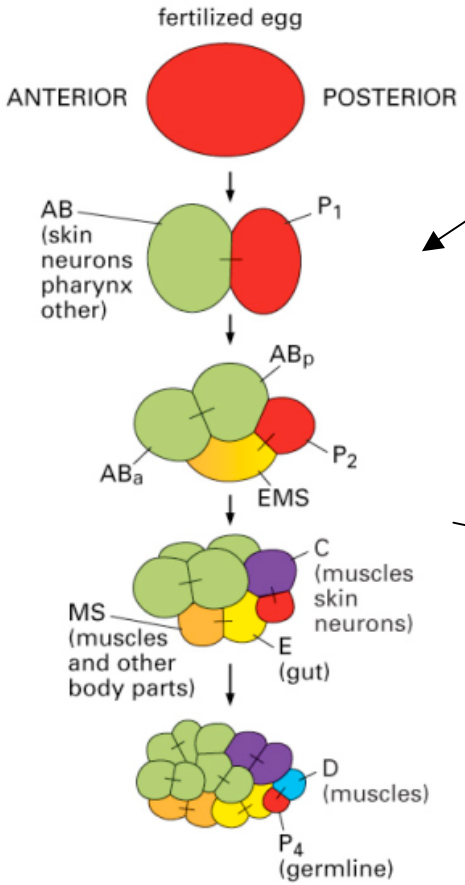


http://www.bio.unc.edu/faculty/goldstein/lab/celdev.mov

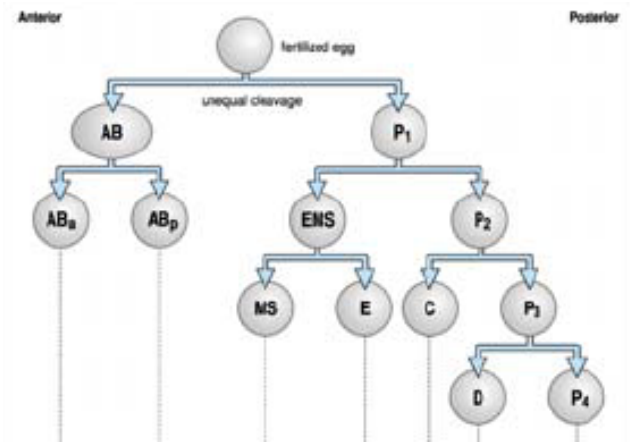
Cell lineage analysis

- ***C. elegans*** **embryo**



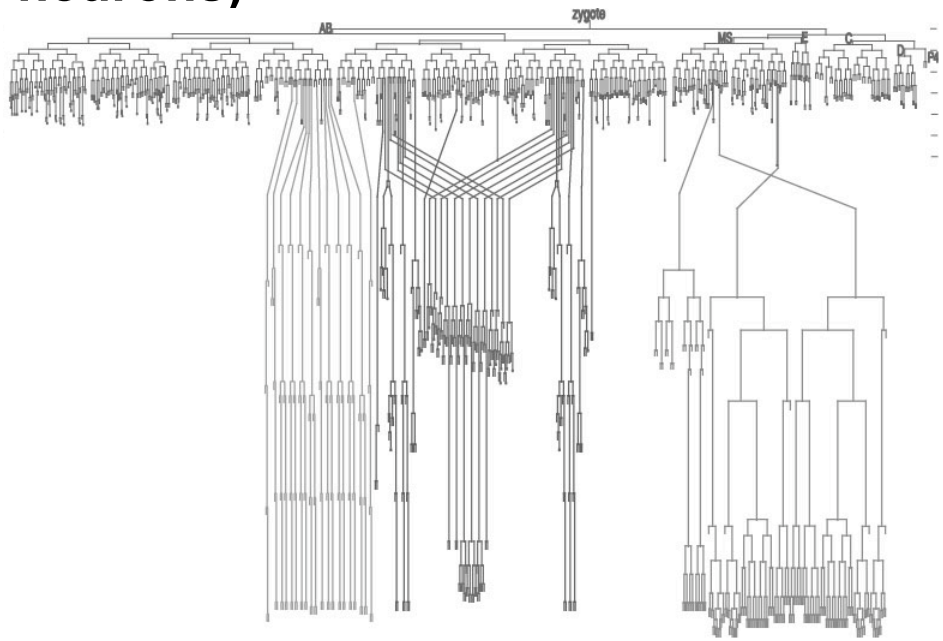


lineage diagram



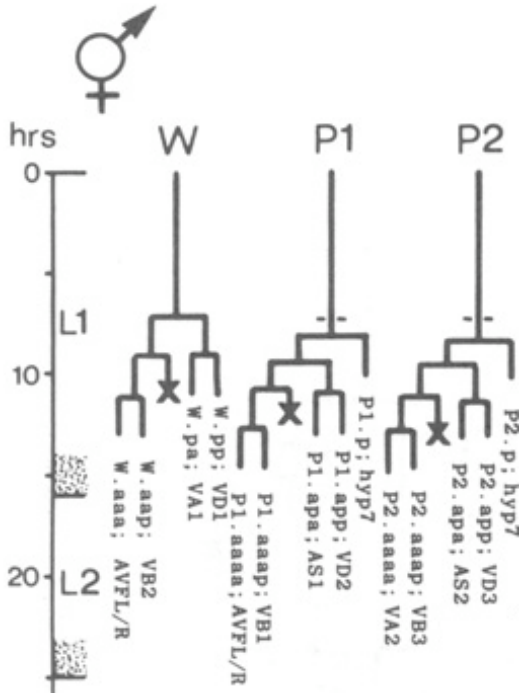
C. elegans lineage

- **First key observation: the cell lineage of every animal was identical**
- **Allowed them to make complete map of *C. elegans* cell lineage**
  - **C. elegans adult hermaphrodite contains exactly 959 somatic cells (302 neurons)**



Cell death in *C. elegans*

- **Key insight re cell death from the lineage:**
- **The 959 somatic cells of adult are derived from 1090 cells --- 131 cells die during development (including developing neurons)**
- **The same cells die in every animal : developmentally programmed cell death**
- **Led the way for the use of *C. elegans* genetics to study cell death**



Identification of ced-3

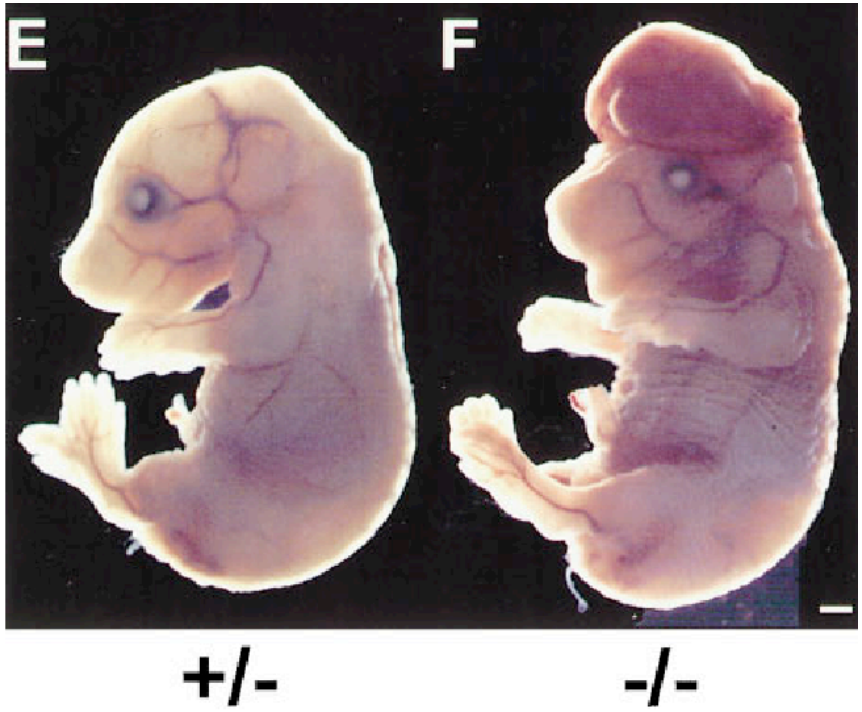
- Genetic screens performed to identify mutants animals in which the pattern of cell death was altered (cell death determining "ced" mutants)
  - Looked for animals in which altered numbers of cell corpses could be seen
- Horvitz lab: ced-3 mutants: no cells died; worm contained additional cells (including extra neurons) but was otherwise fine
- Ced-3: required for all cell deaths in *C. elegans*
- Ced-3 activity was required in the dying cell --- cells committed "suicide"
- Ced-3 gene encodes a protease required to trigger cell death

Caspases

- Ced-3 belongs to evolutionarily conserved family of proteases: Caspases (Cysteine in catalytic site Aspartate targeting proteases)
- Humans have 14 different Caspases (some involved in cytokine maturation, not death)
- Caspase overexpression in mammalian cells can trigger cell death

Caspases are important for vertebrate CNS development

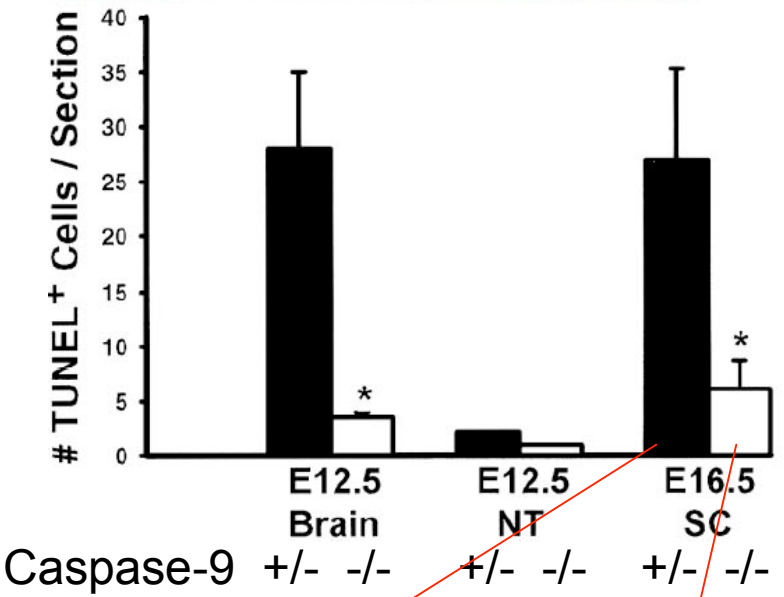
- **Caspase-9 knockout mouse: enormous expansion of midbrain**

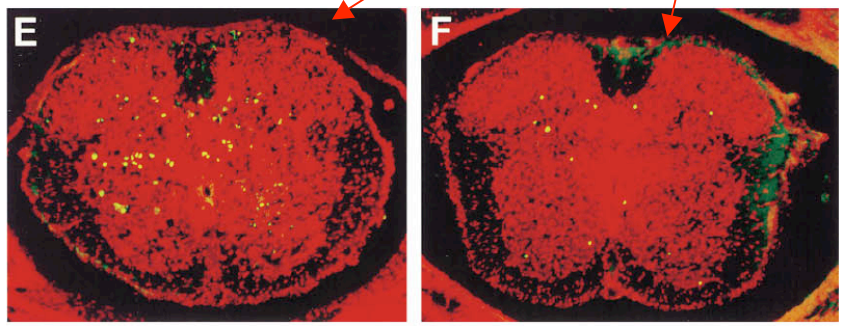


Caspase-9 mutant

Caspases are necessary for proper cell death in the vertebrate CNS

- Caspase-9 knockout mice have decreased amounts of cell death in the nervous system
- Some cell deaths still occur in these mutants: large number of caspases suggests substantial redundancy

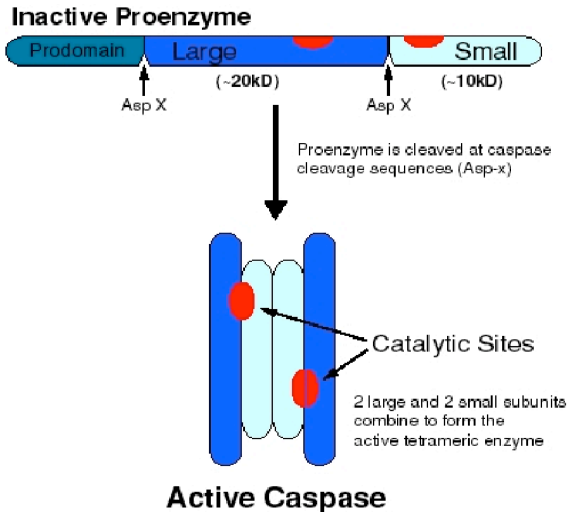




Caspase-9 +/- -/-

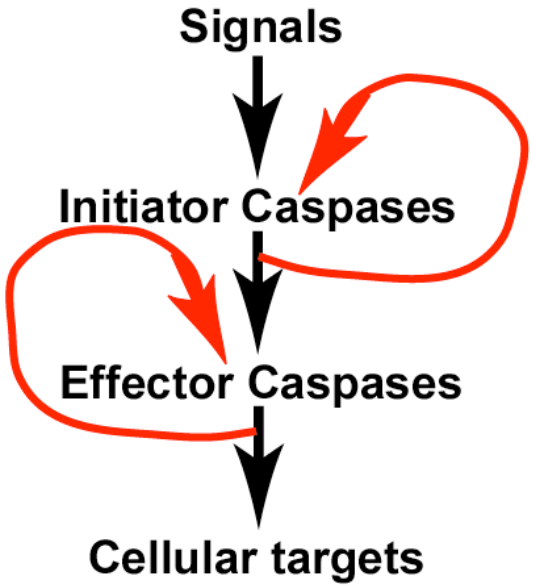
Caspases

- Mature caspases are tetrameric proteases
- Synthesized as inactive proenzymes
- Activated by proteolytic cleavage at Aspartate residues (capase-mediated)



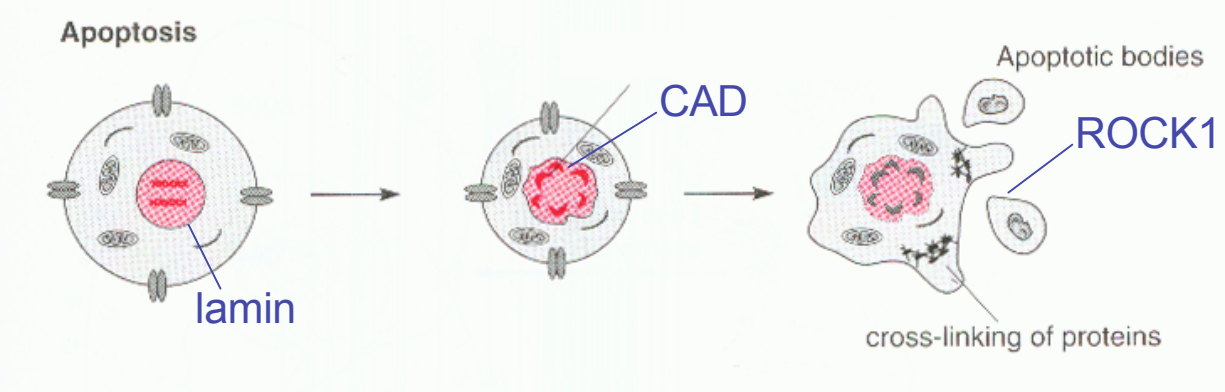
Caspase cascades

- Caspases fall into two classes:
- Effector caspases (eg caspase-3):
  - Lack intrinsic activity
  - Initially activated by upstream caspase cleavage
  - Responsible for cleaving downstream substrates in the cell to cause death
- Initiator caspases (eg caspase-9 and caspase-8)
  - Responsible for activating effector caspases
  - Transduce various signals into proteolytic activity
- Caspases also undergo autocatalytic activation --- positive feedback loop



How do effector caspases kill?

- Effector caspases are highly selective in target choice
- Cleave a variety of targets including:
  - iCAD--- inhibitor of CAD (Caspase Activated DNase)
    - » Caspase inactivates iCAD --- CAD is activated and cleaves genomic DNA
  - Nuclear lamins {NOT LAMININ}-- intermediate filament proteins in nuclear envelope
    - » Caspase cleaves lamins and destabilizes nuclear envelope
  - Rho-activated protein kinase ROCK1:
    - » Caspase activates ROCK1 (makes it rho-independent) -- ROCK1 phosphorylates myosin light chain and myosin is activated --- leads to membrane blebbing



How is caspase activity regulated?

- Caspases are broadly expressed
- How is their activity regulated? *C. elegans* genetics identified additional genes that promote or inhibit normally occurring cell death
- Ced-4 : animals with no ced-4 activity --- no cell deaths (like ced-3 mutants)
- Ced-9 : animals with no ced-9 activity --- all cells die
  - Not just that cells are sick: in animals with too much ced-9 activity --- no cell death

The core cell death pathway

- Ced-3, Ced-4: required for cell deaths
- Ced-9: inhibits cell deaths
- How might they act in a pathway?

The core cell death pathway

- Examine double mutants (like robo/comm)
- Ced-3 mutant: no cell death
- Ced-4 mutant: no cell death
- Ced-9 mutant: all cells die

- Ced-3/ced-9 double mutant: no cell death
- Ced-4/ced-9 double mutant: no cell death

The core cell death pathway

- Examine double mutants (like robo/comm)
- Ced-3 mutant: no cell death
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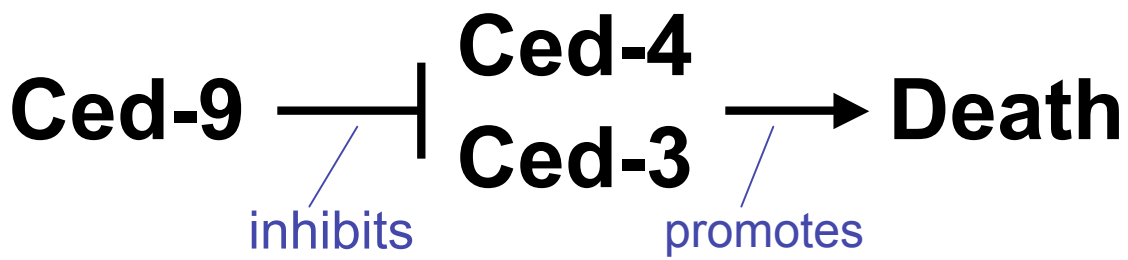
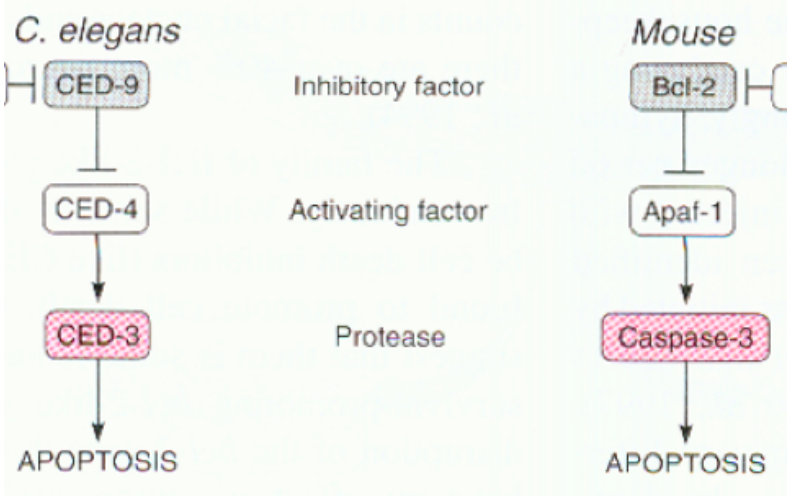
- Ced-3/ced-9 double mutant: no cell death
- Ced-4/ced-9 double mutant: no cell death

- Suggests:



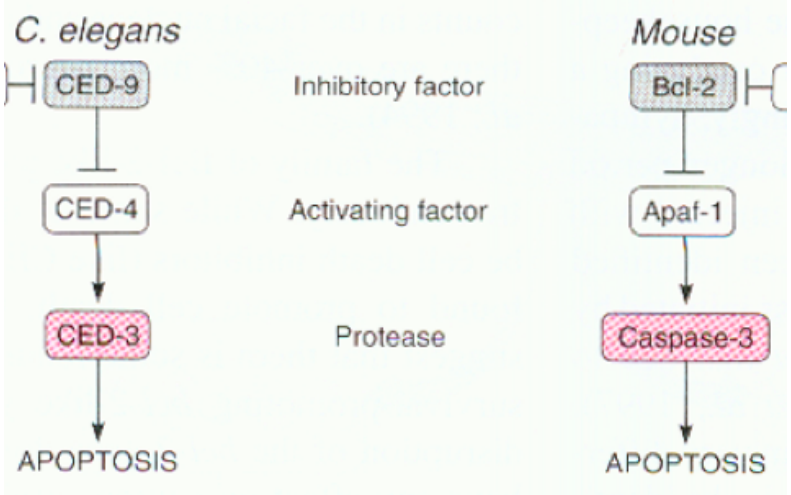
The core cell death pathway is evolutionarily conserved

- **Ced-9 is related to vertebrate Bcl-2**
- **Ced-4 is related to vertebrate Apaf-1**
- **Biochemical analysis indicates that ced-4/apaf-1 is required for caspase activation --- thus ced-4 acts upstream of ced-3**



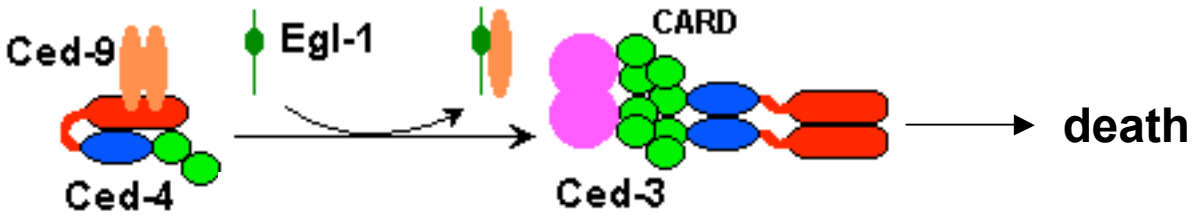
The core cell death pathway is involved in neuronal death

- Overexpression of Bcl-2 in transgenic mice inhibits cell death in the nervous system
  - Animals have larger brains, 40% more neurons in the retina
- Knockout of the Bcl-2 relative Bcl-x
  - massive neuronal death in brain, spinal cord
- Knockout of Apaf-1
  - Enlarged brain, decreased caspase activation and cell death (similar to caspase-9 and caspase-3 mutants)



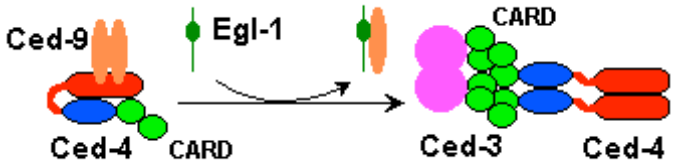
How is the core cell death pathway activated in *C. elegans*?

- In *C. elegans* Ced-9 binds Ced-4 and keeps Ced-4 inactive
- Death signals activate proteins that bind and sequester Ced-9 (eg. Egl-1)
- Ced-4 is now free; free Ced-4 complexes with and activates Ced-3



How can the core cell death pathway be activated in vertebrates?

- In vertebrates things are a bit different:
- Bcl-2 (ced-9) does not appear to exert its main anti-apoptotic function by binding Apaf-1 (ced-4)
- Vertebrates contain multiple Bcl-2-like proteins --- some of which actually promote rather than inhibit death



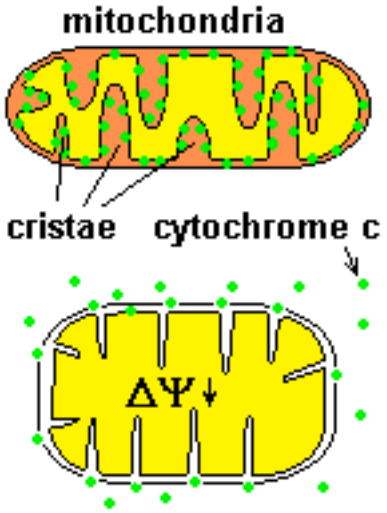
C. elegans mechanism

How can the core cell death pathway be activated in vertebrates?

- Xiadong Wang (1996)
- Cell-free system for Caspase activation
- Monitored activation by Caspase self-cleavage
- Biochemically purified activating agent
  - Turned out to be a big surprise: Cytochrome c
  - Mitochondrial protein involved in energy production
- How does this work?

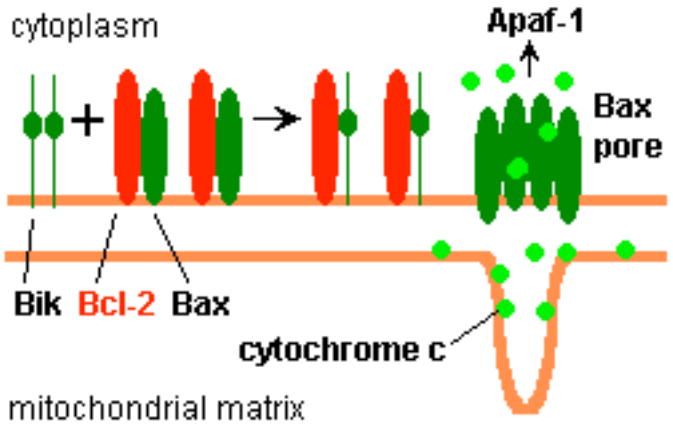
Cell death induction may involve Cytochrome c release in vertebrates

- Cytochrome c is diffusible subunit of mitochondrial electron transport chain
- Apoptotic activation leads to an increase in mitochondrial membrane permeability and cytochrome c release
- Release may involve Bcl-2 (ced-9) family members



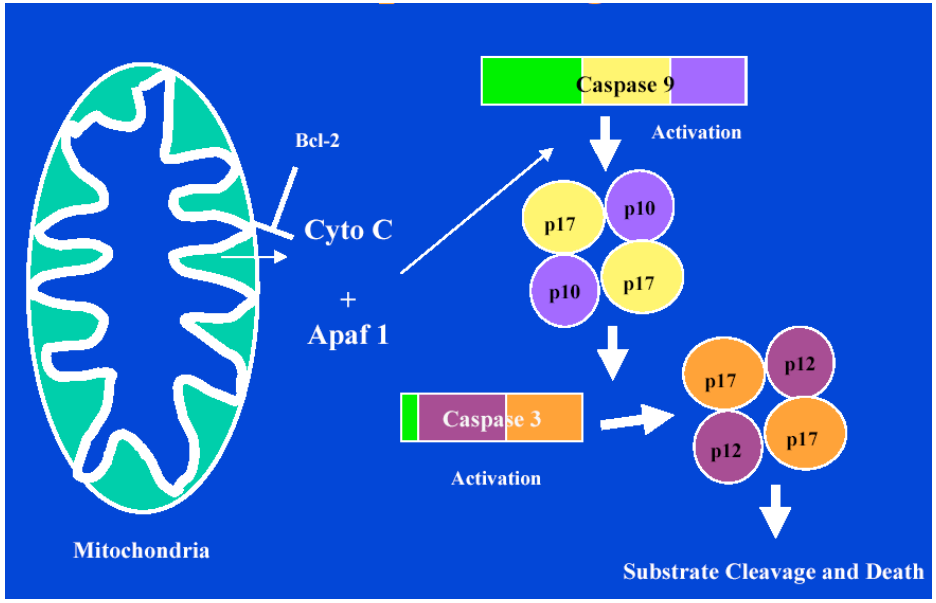
Bcl-2 family members in cytochrome c release (still controversial)

- There are multiple Bcl-2-like proteins in vertebrates
- Some promote, some inhibit death
- Bcl-2 inhibits death; Bax promotes death
- Bax forms pores in outer membrane of mitochondrion permitting Cytochrome c release
- Bcl-2 inhibits Bax from forming pores (mechanism involves physical interaction --- could stop Bax from binding mitochondrial membrane)
- Bik (egl-1 relative) binds to Bcl-2, thereby releasing Bax to form pores



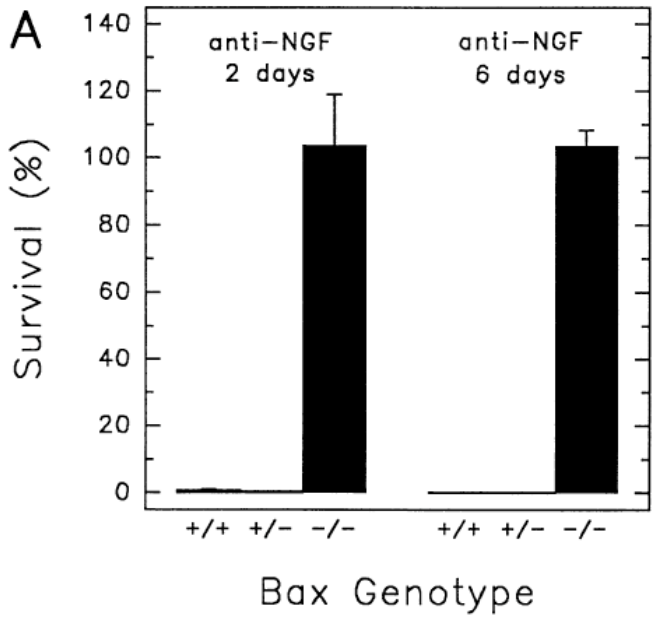
Current model for mitochondrial pathway of caspase activation

- In response to apoptotic activation, cytochrome C is released from mitochodrion (release is inhibited by Bcl-2)
- Cytochrome C binds and activates Apaf-1
- Apaf-1 oligomerizes and binds “initiator caspase” (Caspase-9)
- In this complex (called “apoptosome”), Caspase-9 is activated
- Caspase-9 cleaves Casapse-3 and cell death ensues



Neurotrophic factors and the core cell death pathway

- Bax: promotes cytochrome c release -- activates Apaf-1
- Bax knockout mice:
  - Sympathetic neurons no longer require NGF for survival in culture



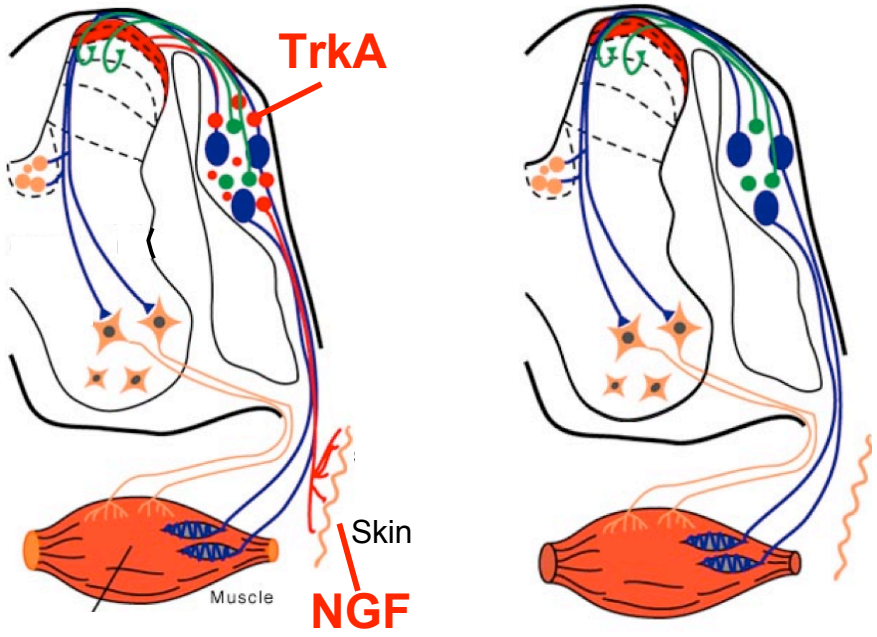
Model for neurotrophin action

- **The loss of NGF requirement in Bax knockout neurons suggested the following model:**

- **Test the model *in vivo* using genetics**

Loss of neurotrophin signaling leads to neuronal loss

- **Knockout of NGF or TrkA causes selective loss of temperature and pain sensing neurons**

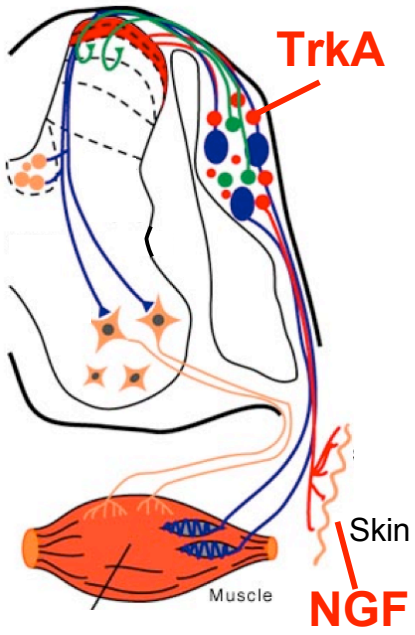


Wild type

NGF$^{-/-}$ or TrkA$^{-/-}$

Knockout of core cell death molecule removes need for survival signaling

- **Bax/NGF and Bax/TrkA double mutants have larger than normal numbers of temperature and pain sensing neurons (≈160%)**



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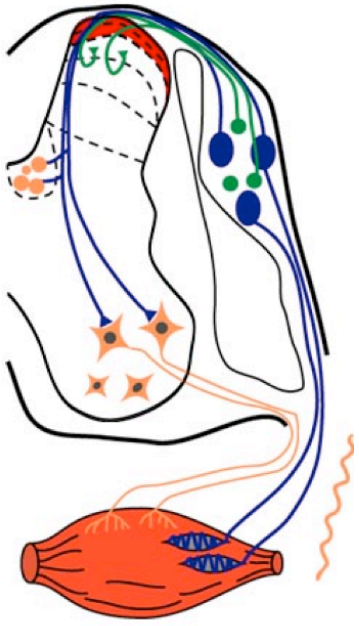
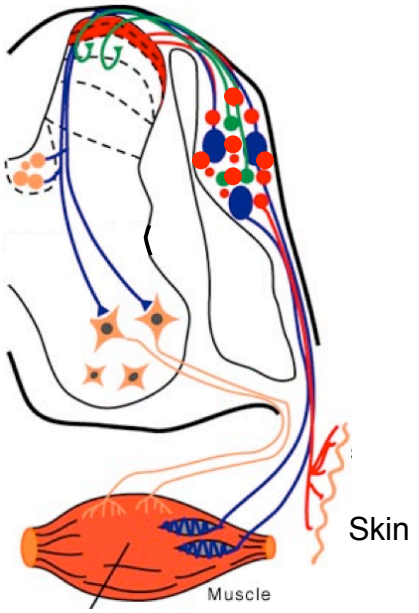


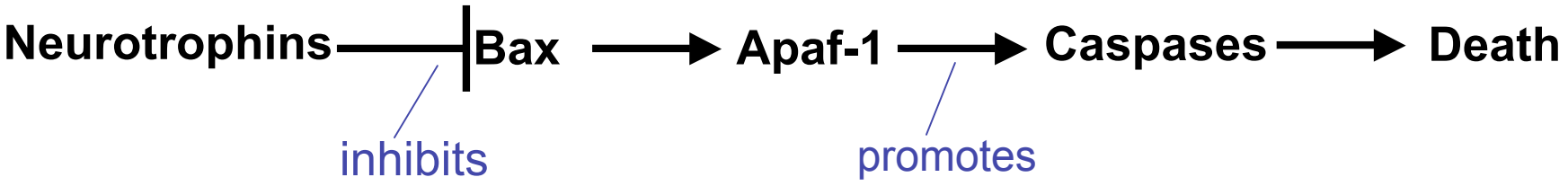
$NGF^{-/-}$ or $TrkA^{-/-}$



$Bax^{-/-}/NGF^{-/-}$ or $Bax^{-/-}/TrkA^{-/-}$

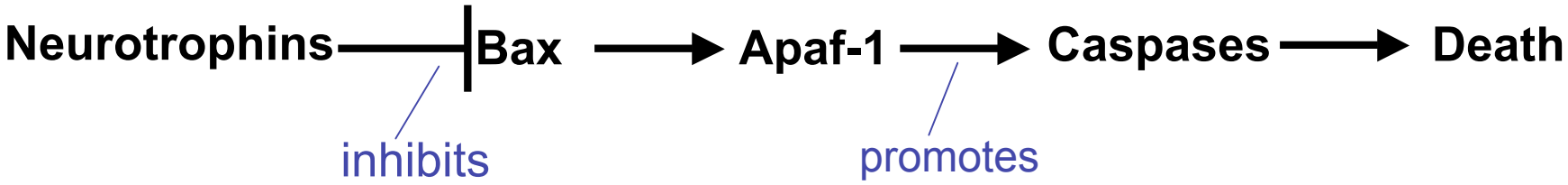
Neurotrophins needed to inhibit core cell death pathway

- Bax knockout eliminates requirement for NGF/TrkA
- Caspase inhibitors also block cell death upon withdrawal of trophic factors
- Thus: inhibition of core cell death pathway makes neurons NGF-independent (true for other trophic factors as well)



Do Neurotrophins directly regulate cell death pathway?

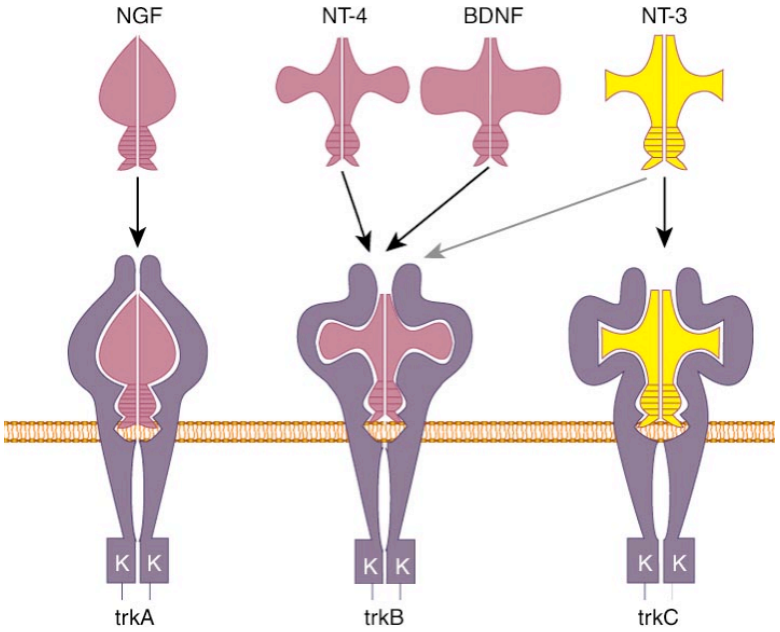
- **Data suggest:**



- **Key question: how do trophic factors such as neurotrophins inhibit the cell death machinery?**

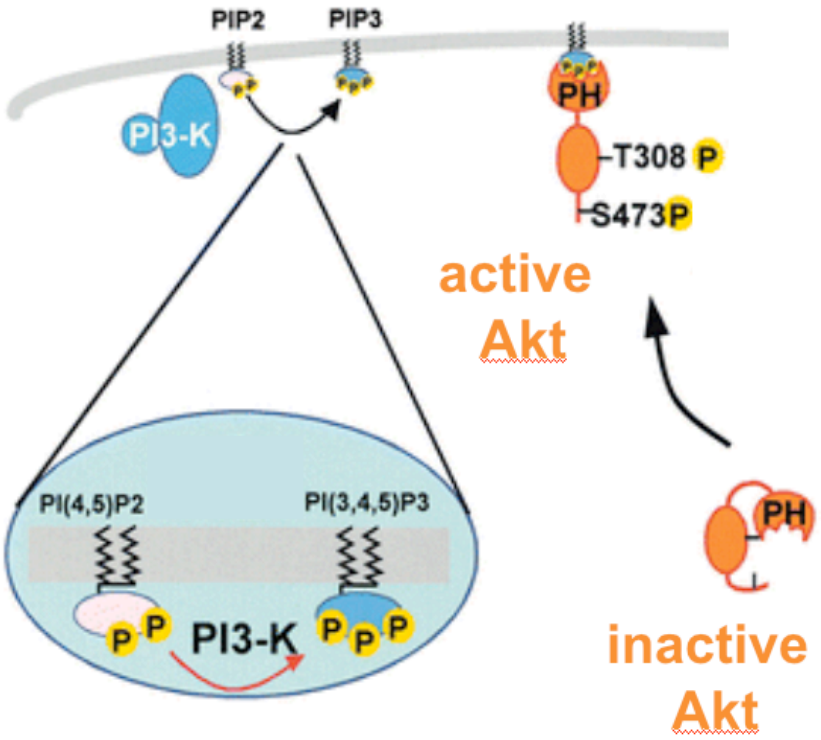
Neurotrophin receptor signal transduction via Trk receptors

- Trks: family of neurotrophin-binding receptor tyrosine kinases
- Each neurotrophin binds subset of Trk family members
- Neurotrophins form dimers --- can bring together two receptor molecules and permit activation by cross-phosphorylation



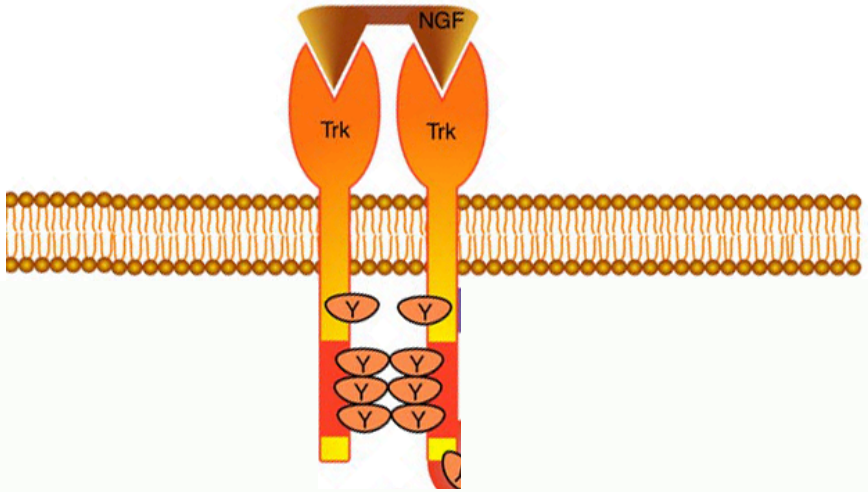
PI-3 kinase and Akt: key signal transducers activated by Trk receptors

- PI-3 kinase: lipid kinase: phosphorylates the membrane lipid phosphotidyl inositol
- These phospholipids activate and localize a key regulator of cell survival: the protein kinase Akt
- PI-3 kinase inhibitors block the survival effect of NGF
- Dominant-active Akt supports survival without neurotrophins
- Dominant-negative Akt blocks effect of survival factors



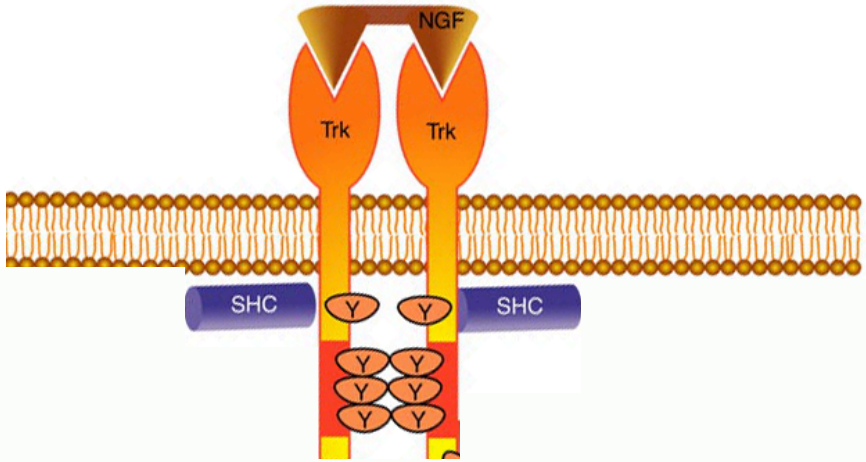
Activation of PI-3K via Trk signaling: step1 Trk activation

- NGF dimer binds to Trks
- Trks cross-phosphorylate and become highly active tyrosine kinases
- Trks phosphorylate themselves on many residues



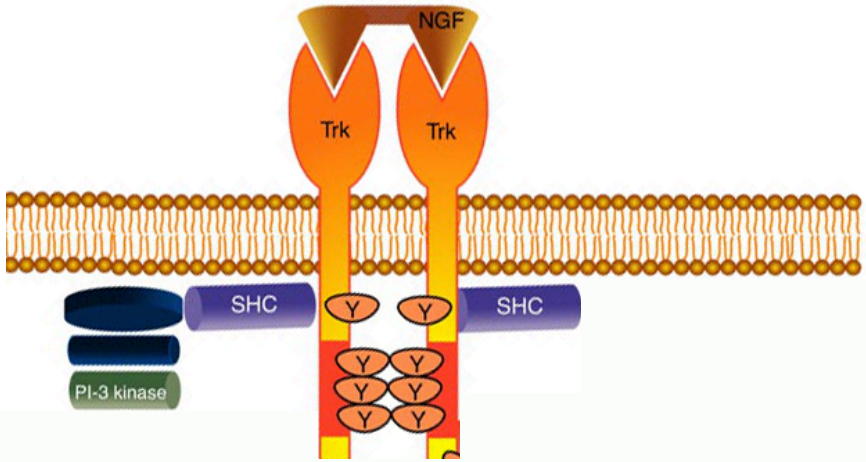
TrkA signaling: step 2
SHC recruitment

- Phosphorylated Trk receptor recruits and activates specific signaling proteins including:
  - SHC
- SHC contains a protein domain that recognizes a site on the receptor
  - SH2 domain: binds to phosphorylated tyrosines in a sequence-specific manner



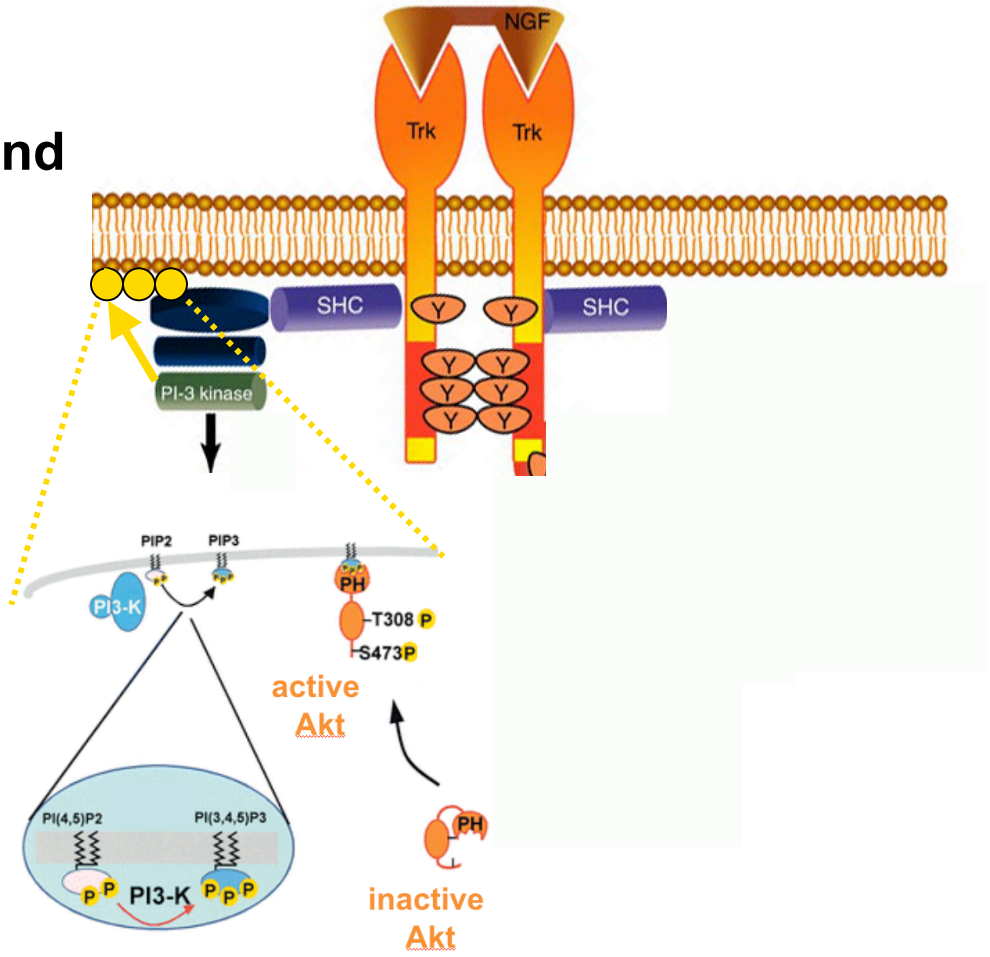
TrkA signaling: step 3: PI-3 kinase activation

- SHC contains additional protein domains that bind to other proteins: including PI-3 kinase
- SHC recruits PI-3 kinase to the membrane and assists in its activation



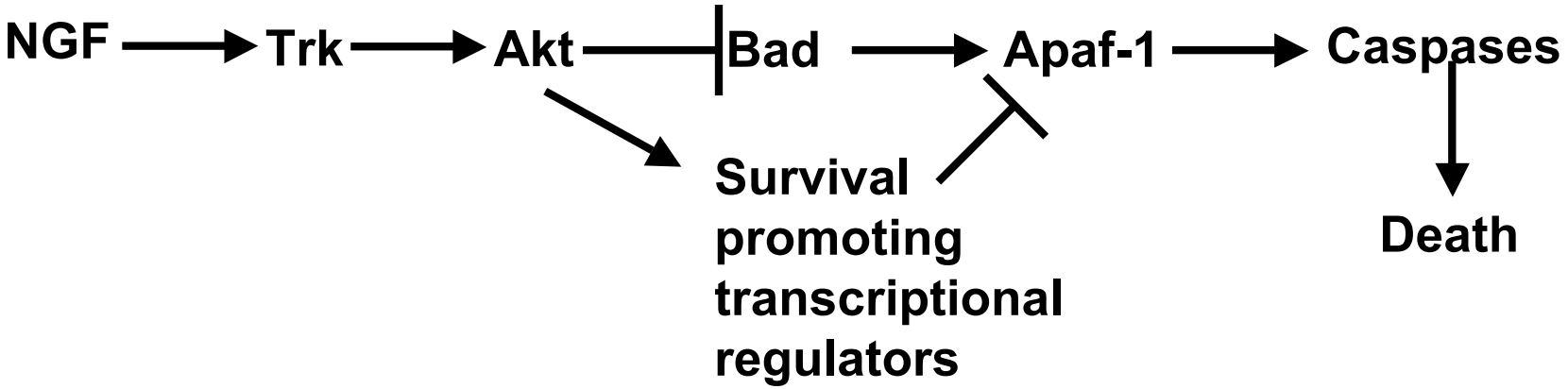
TrkA signaling: step 4
Akt activation

- **PI-3 kinase phosphorylate plasma membrane lipids and activates Akt**



How does Akt act?

- Akt phosphorylates Bad (Bax-like protein)
- Phosphorylated Bad binds to a chaperone which sequesters and inhibits its activity
- Akt also phosphorylates transcription factors that promote survival



Additional pathways activated by Trk receptors

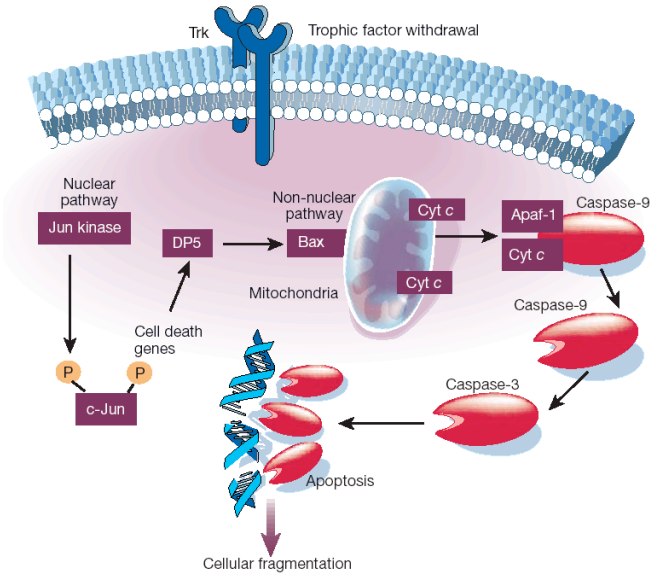
- Trk receptors also activate a second set of kinases (the MAP kinases) which target many of the same factors as the Akt pathway (including Bad)

Death without neurotrophins

- NGF withdrawal causes decrease in PI-3 kinase and MAP kinase signaling
- Recall: death requires transcription and translation so NGF is not simply suppressing a default death program

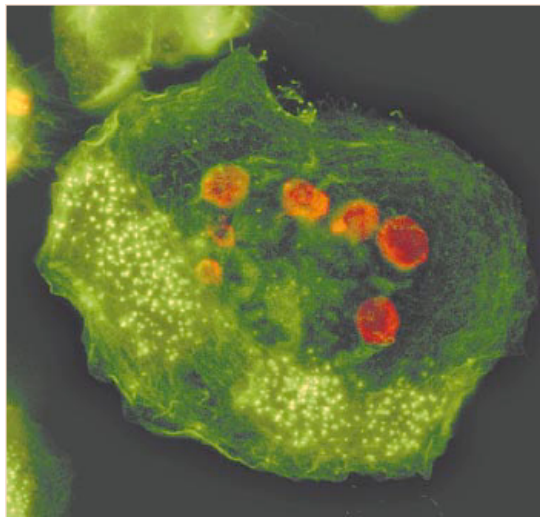
Death without neurotrophins

- Upon NGF withdrawal see:
  - activation of a different kinase cascade (the Jun kinase cascade)
  - transcriptional induction of death promoters upon NGF withdrawal: including DP5 which can activate Bax



The fate of a dying cell: engulfment (corpse removal)

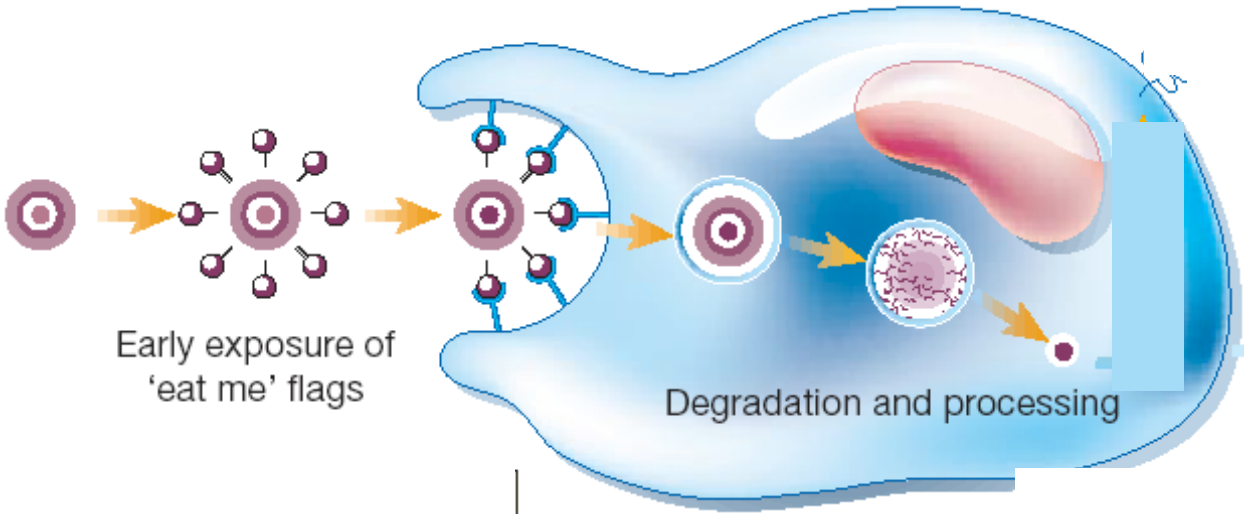
- Dying cells are actively removed by surrounding cell --- often by dedicated engulfing cells (phagocytes) such as “macrophages” (big eaters)
- Waste disposal (remove cell corpses, prevent leakage of contents from dying cells)
- Can also suppress inflammation at site of death



Macrophage containing multiple apoptotic bodies

Cell engulfment (corpse removal)

- **Dying cells produce “eat me” signals (such as phosphatidyl serine)**
- **These signals are recognized by receptors on phagocytes**



Use of PCD in development: matching axons and targets

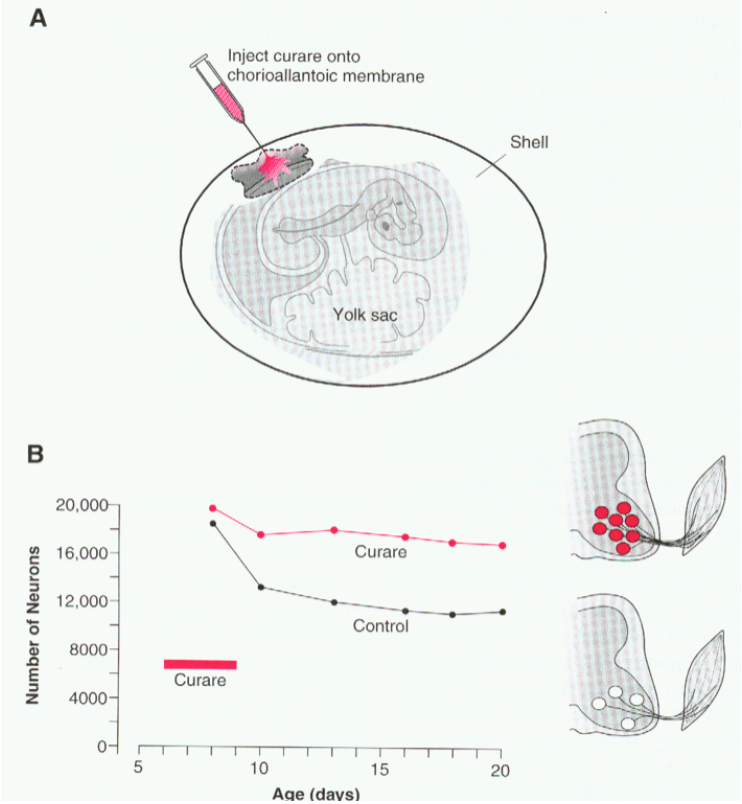
- Approximately twice as many spinal motor neurons are generated in the chick as there are primary myotubes
- However, in the adult spinal motor neurons have a 1:1 relationship with number of primary myotubes present during the period of PCD
- If change number of myotubes later in development, don't change number of spinal motor neurons
- Period of PCD is the critical period for matching
- Suggests that targets provide limiting quantities of essential factors required by the axons to avoid PCD

Use of PCD in neuronal developemnt: PCD of motor neurons is regulated by electrical activity

- **Blockade of neuronal activity during period of motor neuron PCD prevents nearly all motor neuron death**

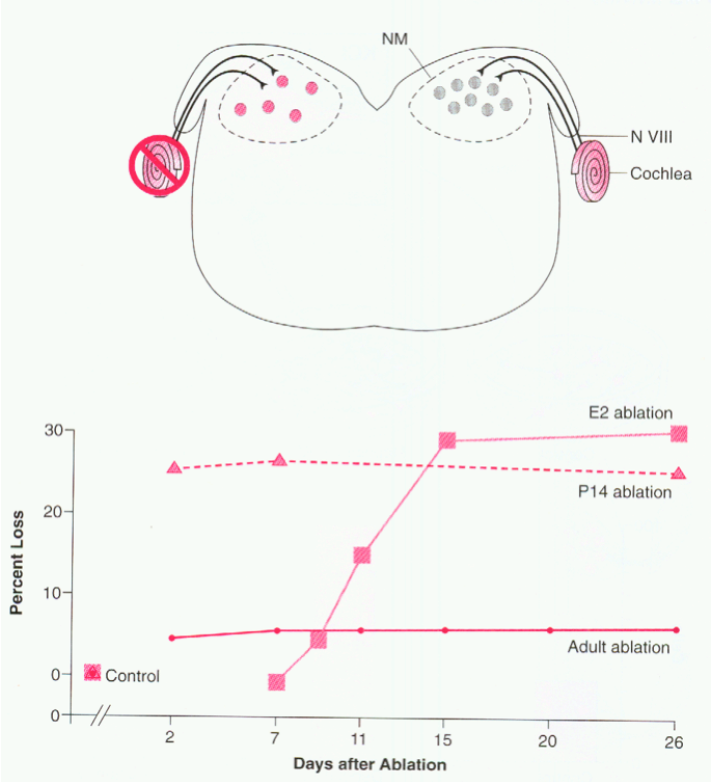
However....

- **There is some evidence that activity stimulates production of neurotrophic factors such as BDNF**



Neurons often rely on synaptic target cells for survival

- Levi-Montalcini: removal of the cochlea (target) caused significant loss of auditory nerve fibers
- Only happened during a critical period: the period of normally occurring PCD
  - If ablate before this time: death took place later
  - If ablate after this period: no significant death



Putting it together: how PCD is used in the nervous system

- PCD serves multiple functions:
  - Removing cells that have no function (sexually dimorphic example) or are no longer needed (Rohon-Beard cells)
  - Pattern formation and morphogenesis (localized cell death sculpts the folds in the fly brain)
  - Systems matching: creation of optimal levels of innervation between neurons and targets (Hamburger/Levi-Montalcini work; motor neuron system)
  - Error correction: axons that lose their way die (floor plate is a source of trophic factors for commissural axons)
  - Removal of defective or harmful cells (many eyeless fly mutants have defects in retinal differentiation --- defective cells commit suicide --- “death by frustration”)
  - Evolutionary change

PCD in disease

- There is substantial neuronal loss in many human diseases --- suggests that inappropriate activation of PCD pathways may be important
- Example: Spinal muscular atrophy:
  - Type I SMA: mutations in an inhibitor of caspases and in a Bcl-2 interacting protein gene

Using understanding of PCD to treat disease

- Davidson and Steller examined fly mutants that suffer retinal degeneration
- Contain same mutations found in humans with retinitis pigmentosum: photoreceptors lost over time
- Expressed a caspase inhibitor in the mutant flies
- Rescued the retinas from degeneration and created eyes that were perfectly functional
- Core cell death pathways and their regulators: potentially important drug targets