Cell Death and Survival I: Neurotrophic Hypothesis, Survival Factors/Receptors

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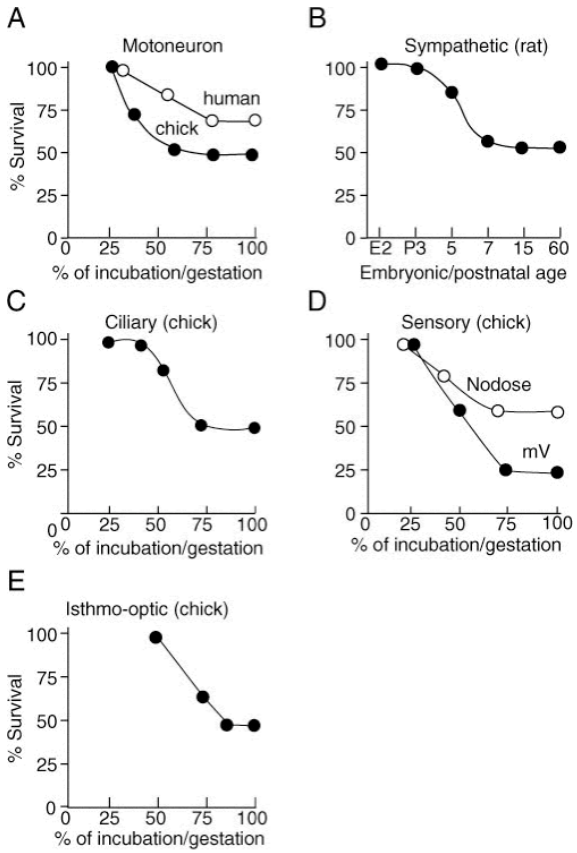
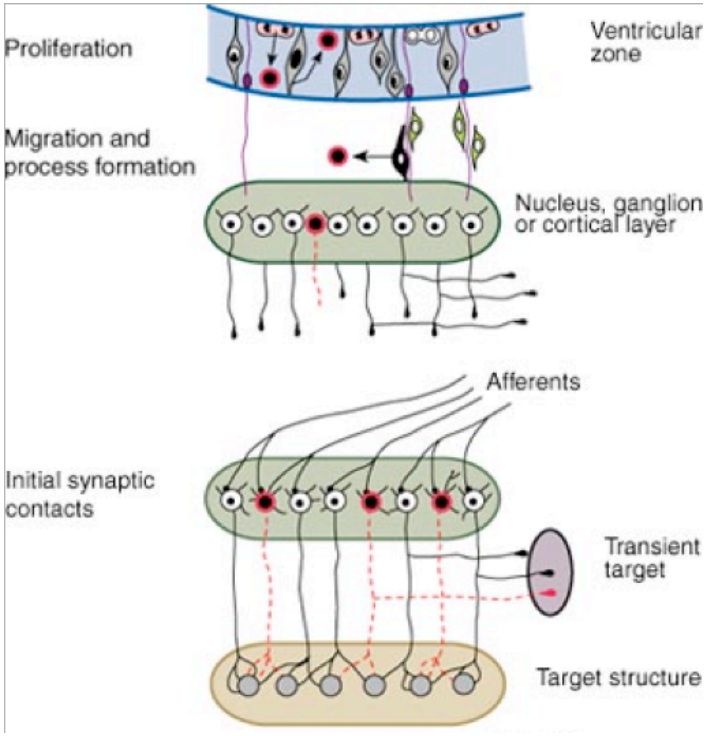
Neuron loss is a normal part of development

- A significant fraction of all neurons generated die
- Relative balance of neuron production and loss determines final numbers of neurons
- Important in disease, also, perhaps, in evolutionary change

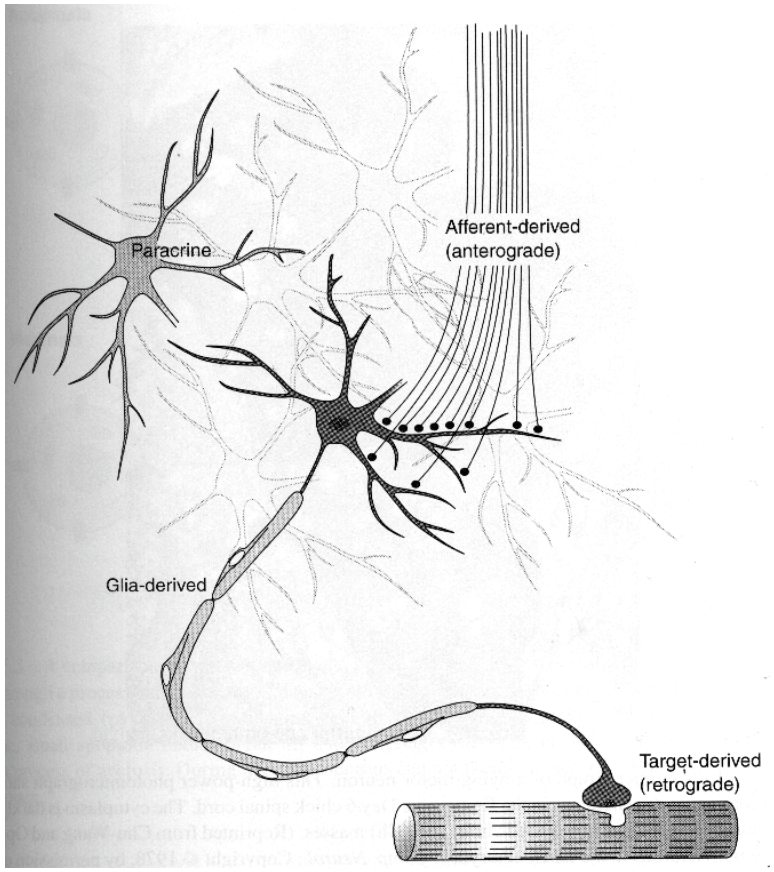


Neurons die at multiple stages in development



Neurons depend on survival signals

- Survival often depends on receiving appropriate survival signals
- Neurons can receive survival signals from a variety of sources
  - Afferents (inputs) (anterograde)
  - Targets (retrograde)
  - Glia (glial-derived)
  - Distant sources (paracrine)

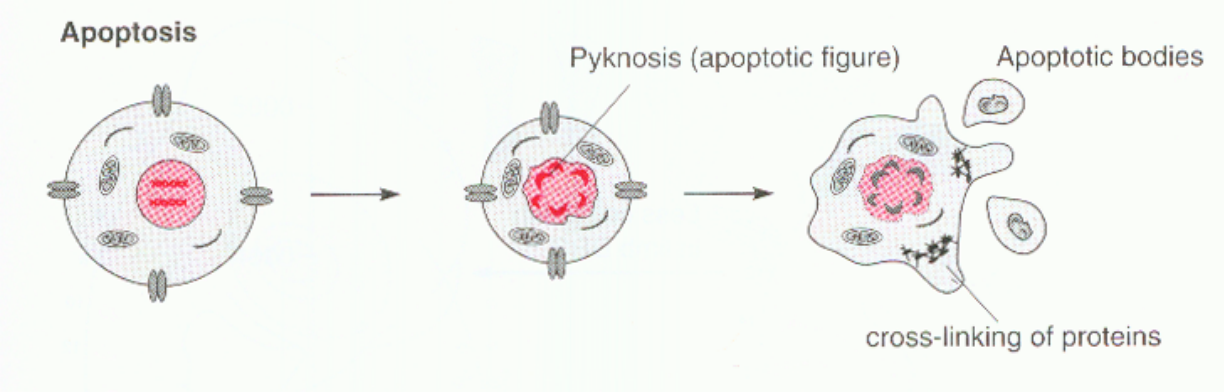


Discovery of Neuronal Cell Death

- Hooke (1665) : first cells described from cork were actually cell corpses
- Carl Vogt (1842) : saw dying cells in developing toad nervous system and at metamorphosis
- John Beard (1896) --
  - Followed fate of large sensory neurons in skate spinal cord (Rohon-Beard cells)
  - Saw these neurons differentiate and send out processes to ectoderm in embryo
  - These neurons then degenerated (functionally replaced by larval DRG neurons)
- Suggested that cell death can occur in a “programmed”, predictable fashion

Programmed Cell Death (PCD)

- PCD (aka Apoptosis): Controlled cell deletion
  - Dying cell has distinct morphological features
    - » Condensed cytoplasm and nucleus
    - » Nuclear fragmentation, membrane blebbing, organelles intact
    - » Condensed chromatin, DNA fragmentation

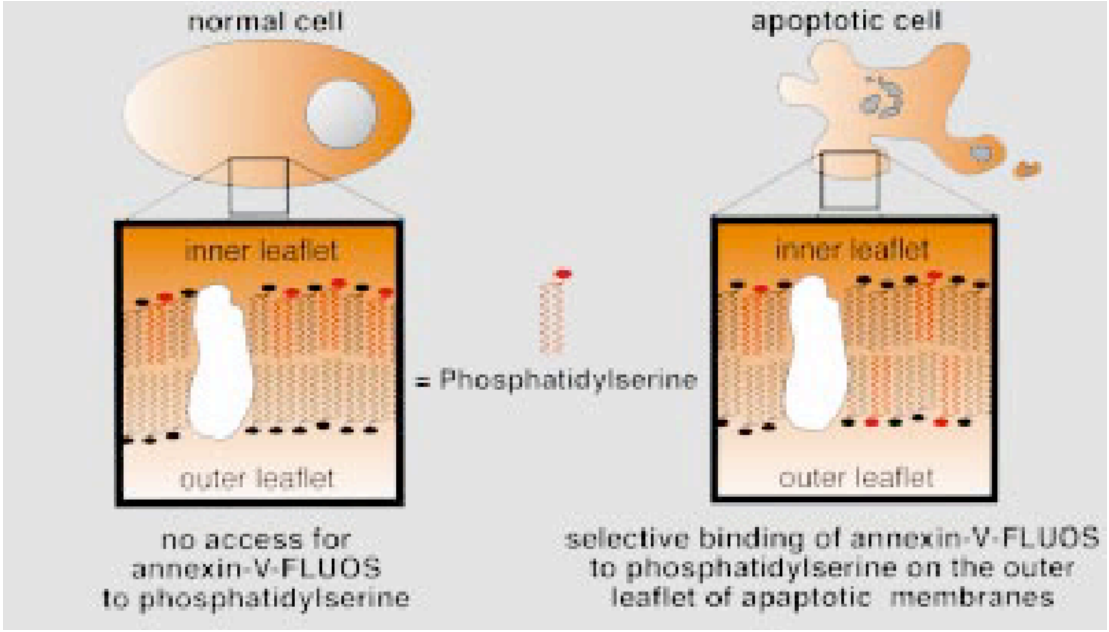


Common methods for measuring PCD

- Take advantage of properties of dying cells:
  - flipping of phospholipids in plasma membrane (annexin staining)
  - DNA fragmentation of DNA (TUNEL)
  - At late stages: holes in membrane (acridine orange)

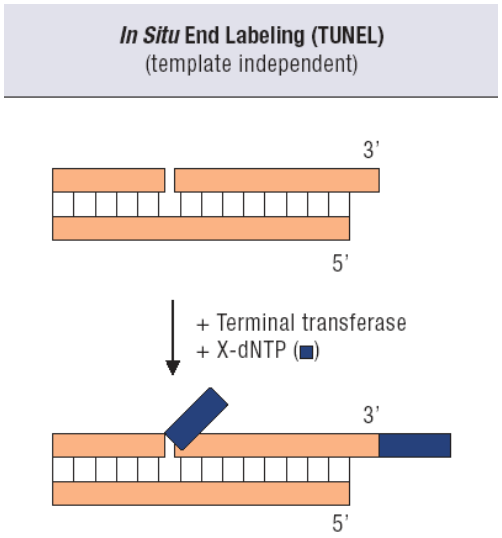
Annexin V staining measures changes in membrane lipid location

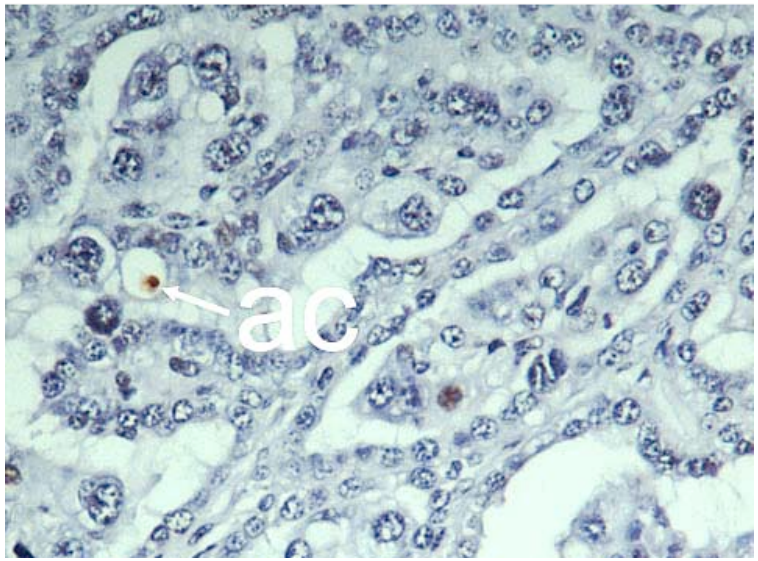
- Phosphatidylserine is a phospholipid normally found only on inner leaflet of plasma membrane
- When cells undergo PCD lipids flip (flipases activated)
- Annexin-V binds phosphatidylserine
- Annexin-V only binds to unpermeabilized cell if lipid has flipped



TUNEL measures DNA fragmentation

- **TUNEL labeling: TUNEL (Terminal transferase UTP Nick End Labeling)**
  - **Terminal transferase: DNA/RNA polymerase that extends free 3'-OH ends of DNA**
  - **DNA fragmentation greatly increases number of 3'-OH ends**
  - **Use terminal transferase to add labeled UTP to free 3'OH ends**

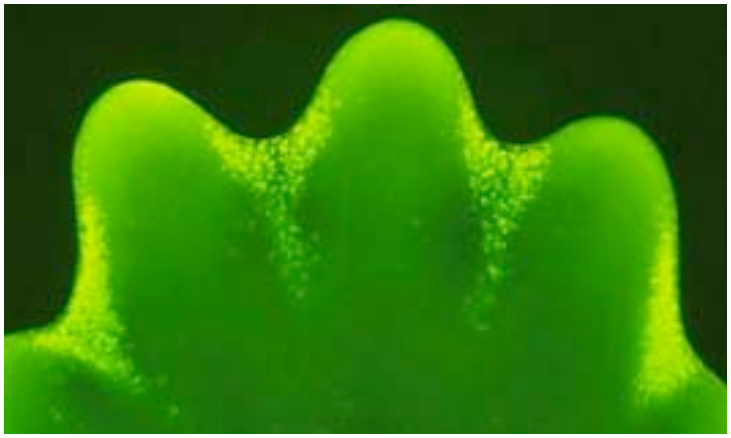




TUNEL staining
Bovine placenta

Acridine orange measures membrane integrity

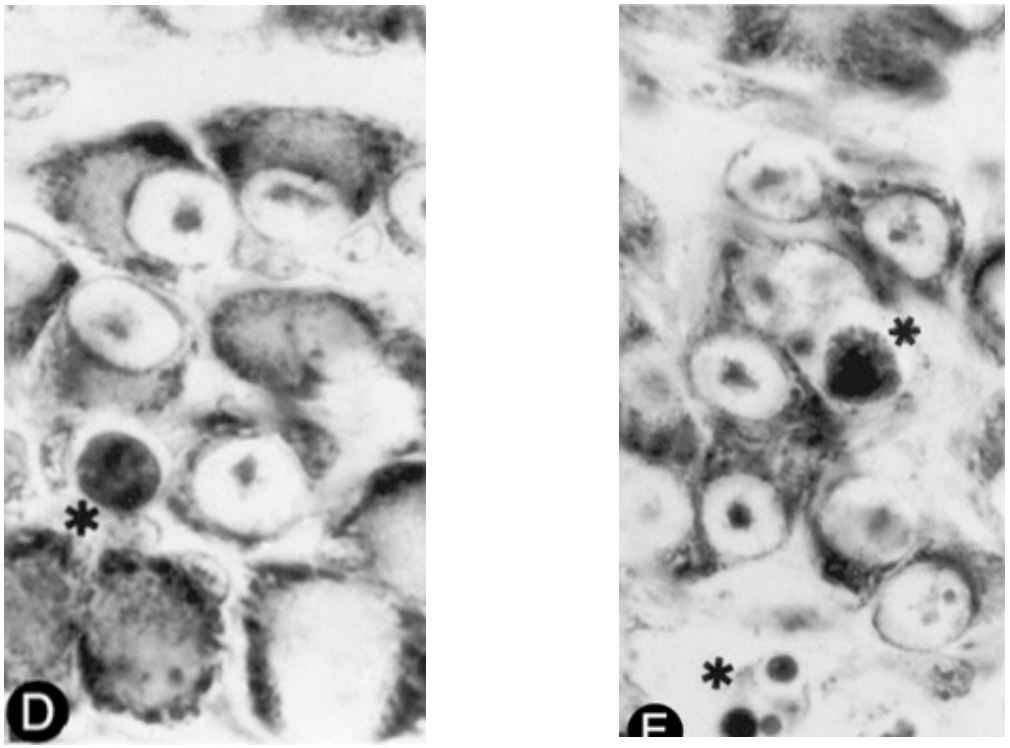
- **Acridine orange (AO): a dye that binds nucleic acid and becomes fluorescent**
- **AO can't cross intact plasma membrane**
- **Dying cells eventually develop holes in their membranes**
- **AO gains access to intracellular compartment -- binds DNA/RNA --- cells fluoresce**



AO staining: footplate mouse embryo E13.5

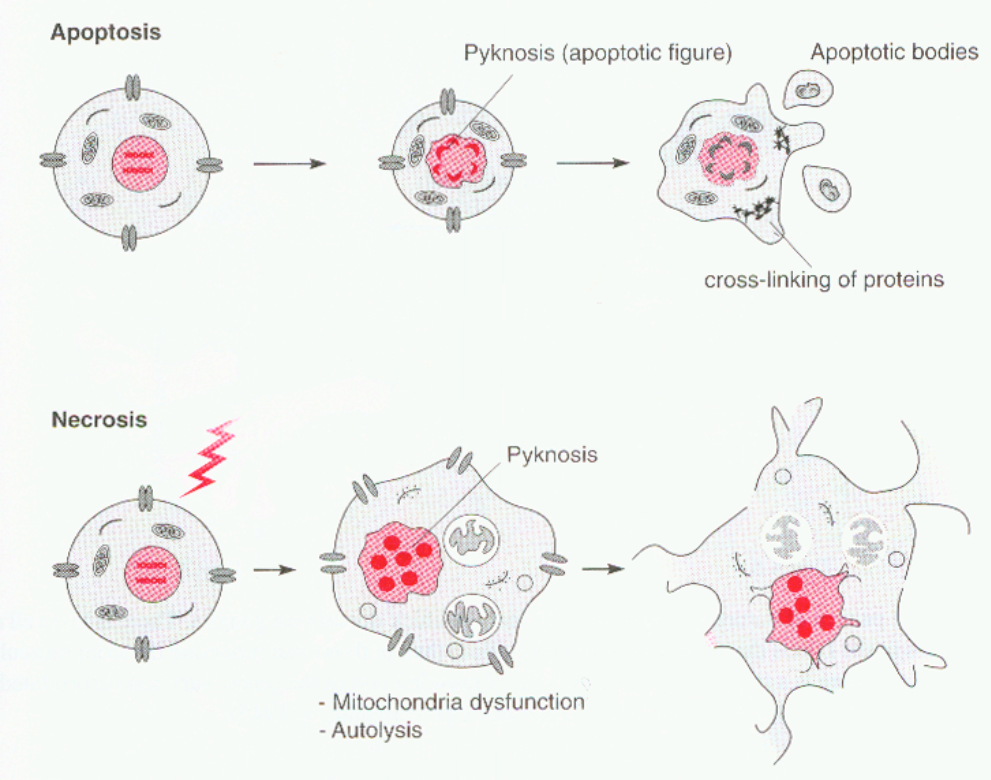
Morphology of neurons undergoing PCD

- **Apoptotic chick sensory and motor neurons**



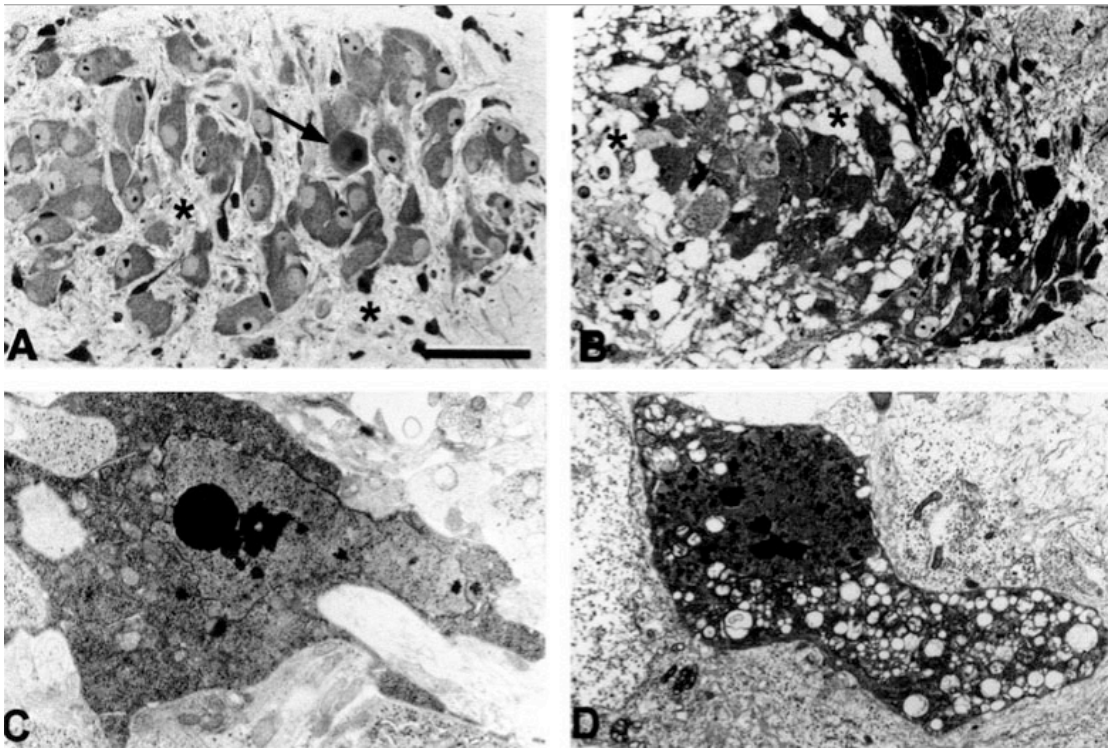
Cell death can also result from damage

- **Necrosis: death in response to traumatic injury (eg, glutamate excitotoxicity)**
- **Necrotic cells have different appearance from apoptotic cells: how distinct these deaths really are at a mechanistic level is unclear**



Ultrastructure (electron microscropic examination) of dying cells

Ventral horn chick embryo (motor neurons)



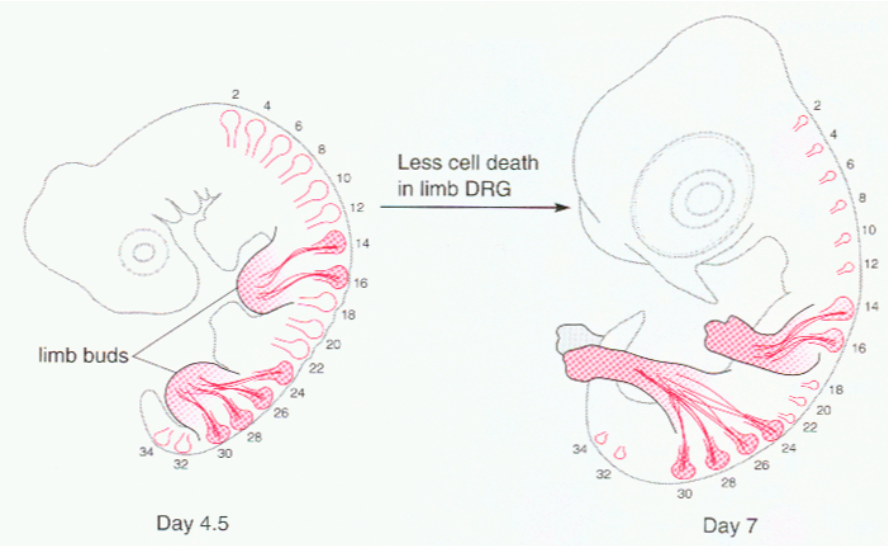
apoptotic neuron necrotic neuron

How is neuronal death regulated?

- Removal of peripheral target was known to prevent proper development of innervating neurons
- Viktor Hamburger/Rita Levi-Montalcini (30'-50's): showed that this was due to death of differentiated neurons and showed that the target could regulate neuronal death

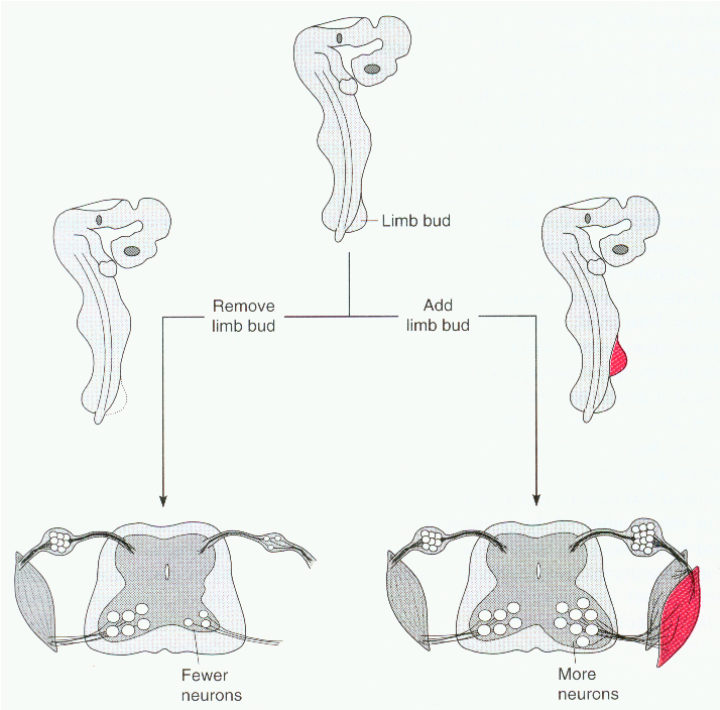
Neuron death is common during normal development

- Hamburger and Levi-Montalcini: demonstrated that large numbers of neurons die in normal animals (in DRGs ≈30%)
- Degree of death correlates with size of target
  - Less death in DRGs that innervate limbs



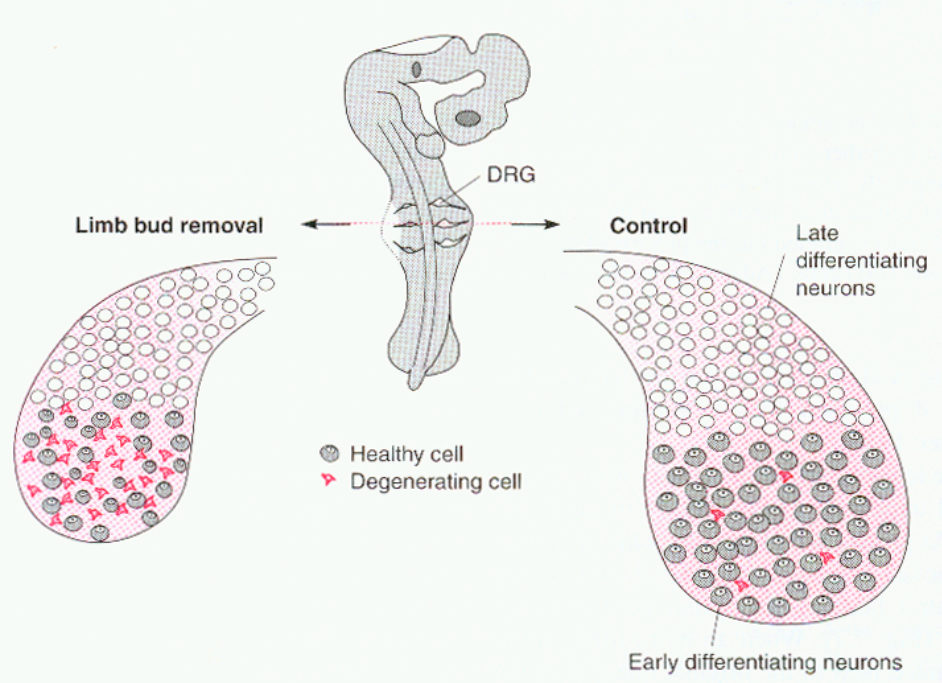
Amount of target tissue affects neuron number

- Number of neurons present affected by changing target target size
  - Remove limb bud --- fewer neurons
  - Add extra limb bud-- more neurons



Target influences survival

- **Removal of limb bud did not affect proliferation or generation of neurons**
- **Increased number of degenerating neurons**



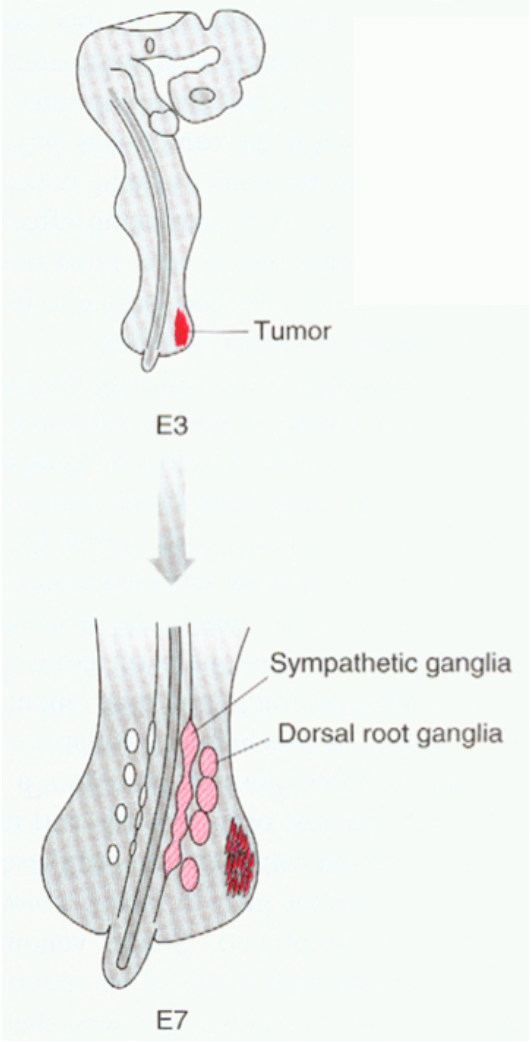
The Neurotrophic Hypothesis

- Dependence of neuron survival on their targets suggested that the target cells produce signals that promote neuronal survival
- Neurotrophin hypothesis:
  - Immature neurons compete for target-derived trophic factors that are in limited supply
  - Only neurons that establish correct synaptic connections survive
  - Predicted existence of neurotrophic (nerve feeding) factors aka “neurotrophins”

Discovery of the first neurotrophin

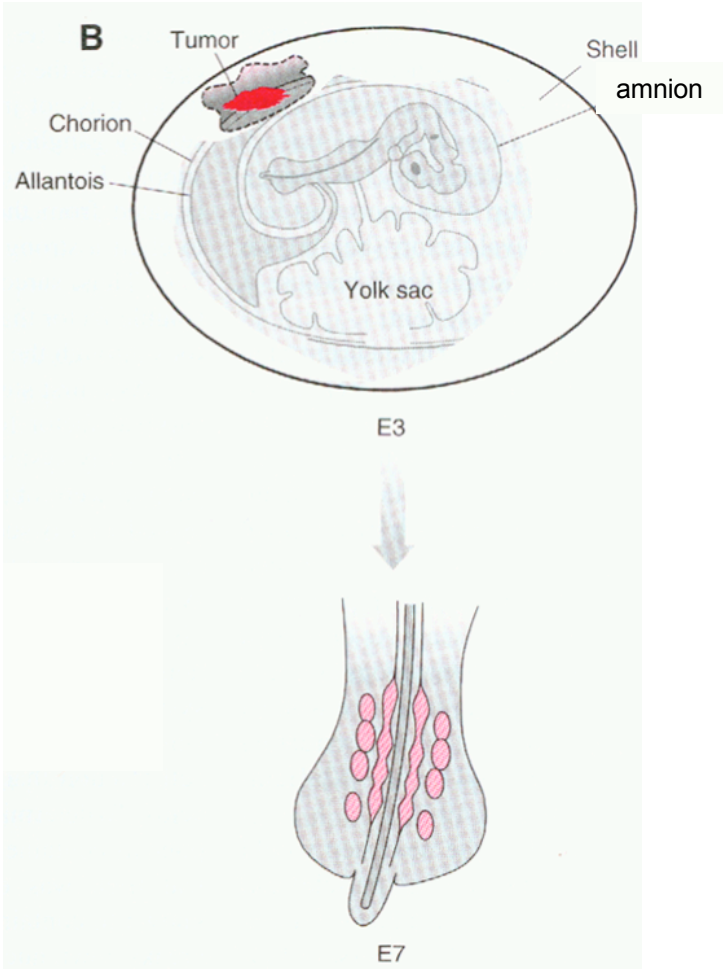
- First step to identification of a neurotrophin
  - Elmer Bueker: 1948: grafted a mouse tumour into the body wall of a chick embryo --- saw sympathetic nerve fibers enter the tumour

- Hamburger/Levi-Montalcini:
  - tumour cells increased size of multiple ganglia
  - tumour also promoted sympathetic fibers to enter many abnormal regions --- including blood vessels



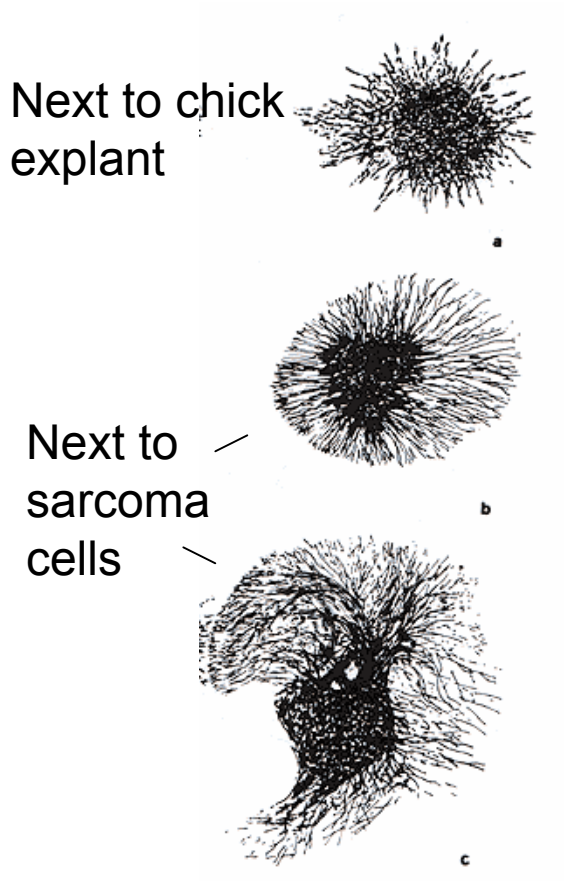
The neurotrophin could act at a distance

- **Hamburger/Levi-Montalcini:**
  - **Got similar results when put tumour cells on embryonic surface--- diffusible factor**



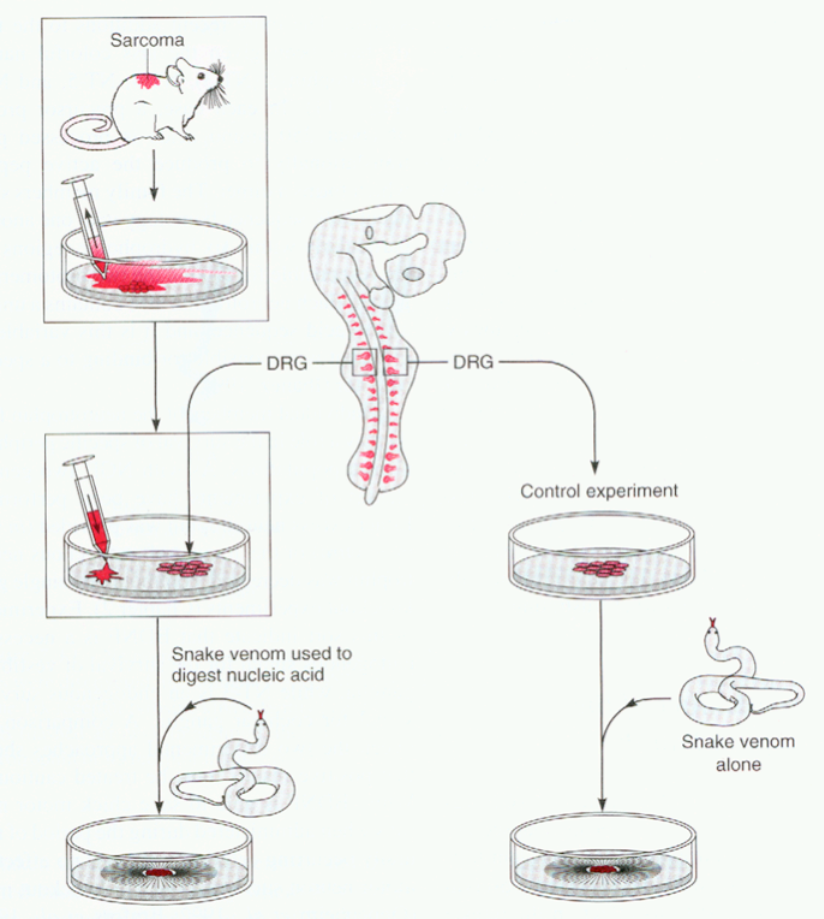
Demonstrating neurotrophic activity in vitro

- **Levi-Montalcini placed chick sympathetic ganglia next to chick tissue or sarcoma cells**
- **Waited 24 hours**
- **Sarcoma cells promoted axon outgrowth**
  - **Also appeared to orient axon extension**
- **Argued the factor acted directly on neurons**



Purifying Nerve Growth Factor (NGF)

- Levi-Montalcini joined by biochemist Stanley Cohen (1956)
- Fractionated extracts from sarcoma cells --- identified neurotrophin-enriched fraction : called it NGF
- To show NGF was a protein (not nucleic acid) used snake venom (contains high levels of phosphodiesterase)
- Snake venom super-concentrated source of NGF!



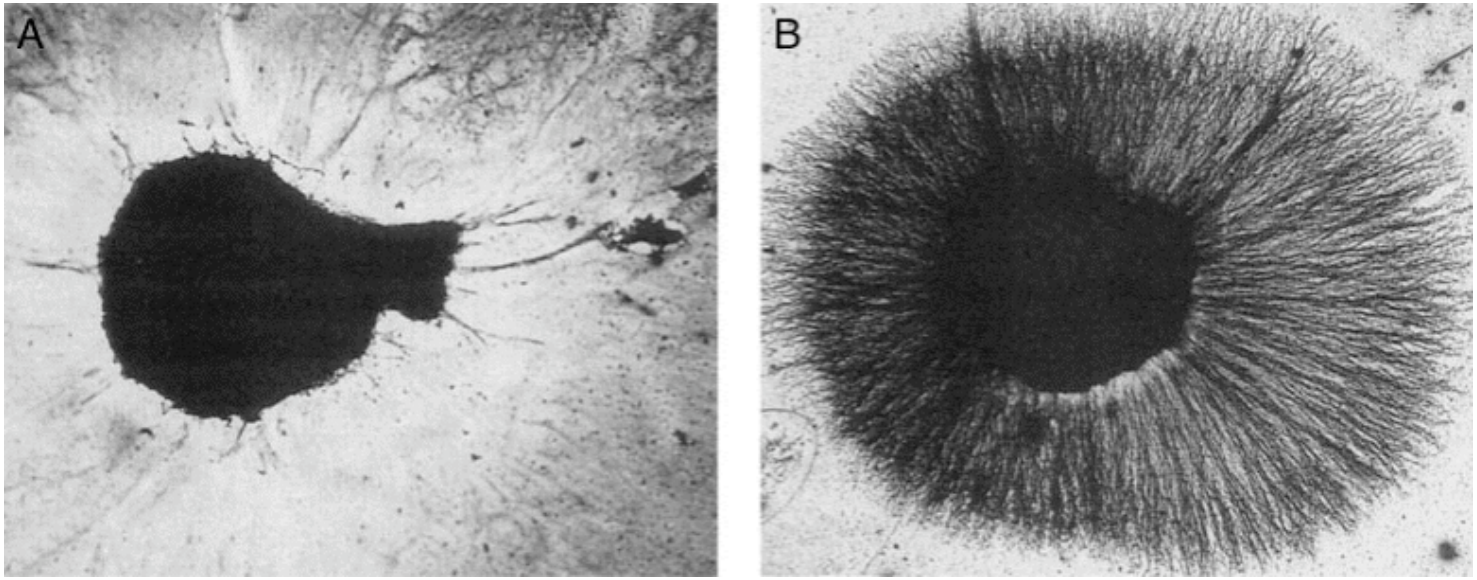
Purifying NGF

- Presence of NGF in snake venom suggested might be present in mouse salivary glands
- Abundant source --- used for large-scale isolation (1956) … eventually protein sequencing (1971) and molecular cloning (1983) of NGF

Is NGF sufficient to keep neurons alive?

- **Now had purified NGF (1956)**
- **Added NGF to explanted sympathetic ganglia**
- **Promoted strong survival and outgrowth response**

chick sensory ganglia: 24 hour in culture

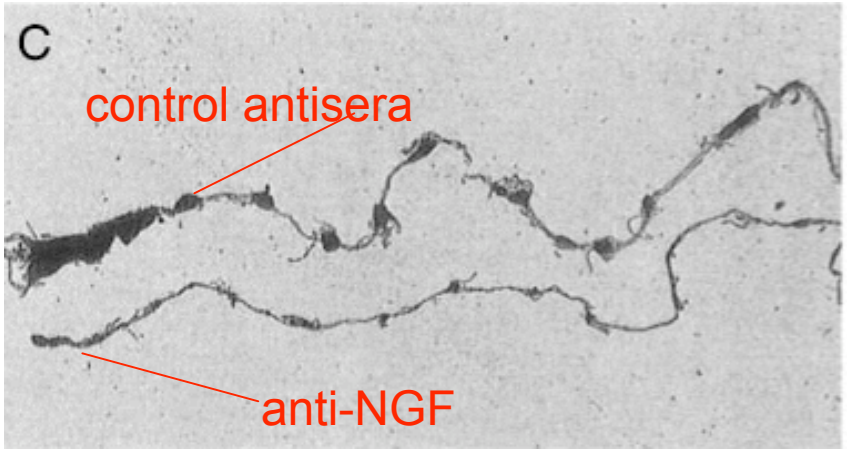


- NGF

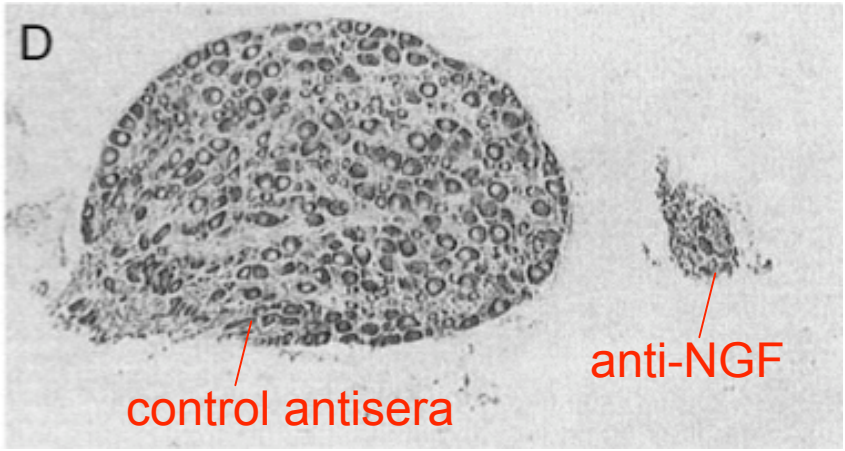
+ NGF

Is NGF normally necessary for survival?

- **Made antisera against NGF (1960)**
- **Injected antisera into newborn mice**
- **Sympathetic ganglion neurons lost**



Chains of sympathetic ganglia



Individual sympathetic ganglia

NGF isn't the only Neurotrophin

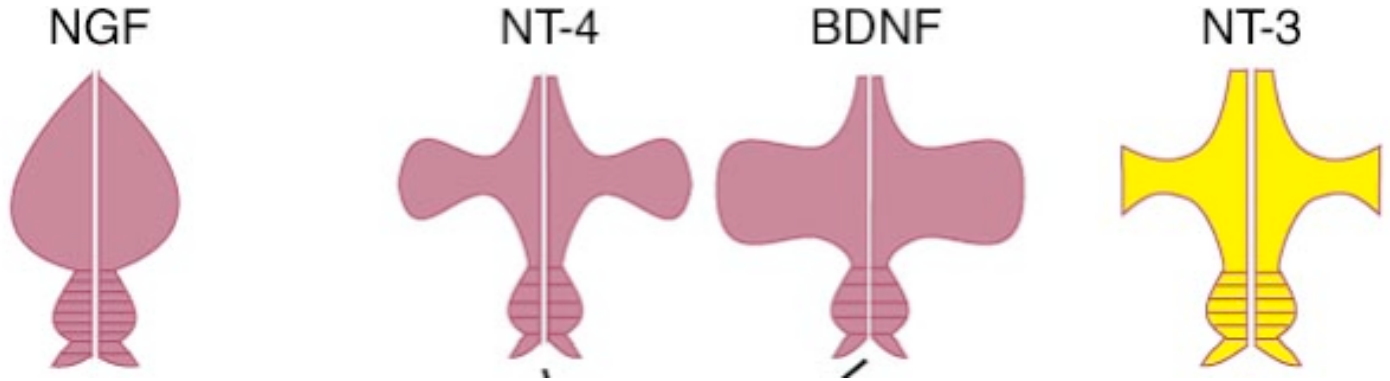
- Many neurons didn't respond to NGF
- These neurons did respond to factors present in tissue/cell line extracts
- Suggested the existence of additional neurotrophins

Discovery of Brain-Derived Neurotrophic Factor (BDNF)

- Yves Barde (1980's) --- saw that NGF did not promote neurite outgrowth from cultured rat retina
- Found that extract from pig brain promoted outgrowth
- Purified 1 microgram from 1.5 kg of pig brain --- microsequenced protein
- Cloned BDNF
- What did it look like?

The neurotrophin family

- BDNF and NGF ≈50% identical in amino acid sequence
- Additional relatives identified by sequence
- All neurotrophins can promote neuronal survival: each has different spectrum of target neurons

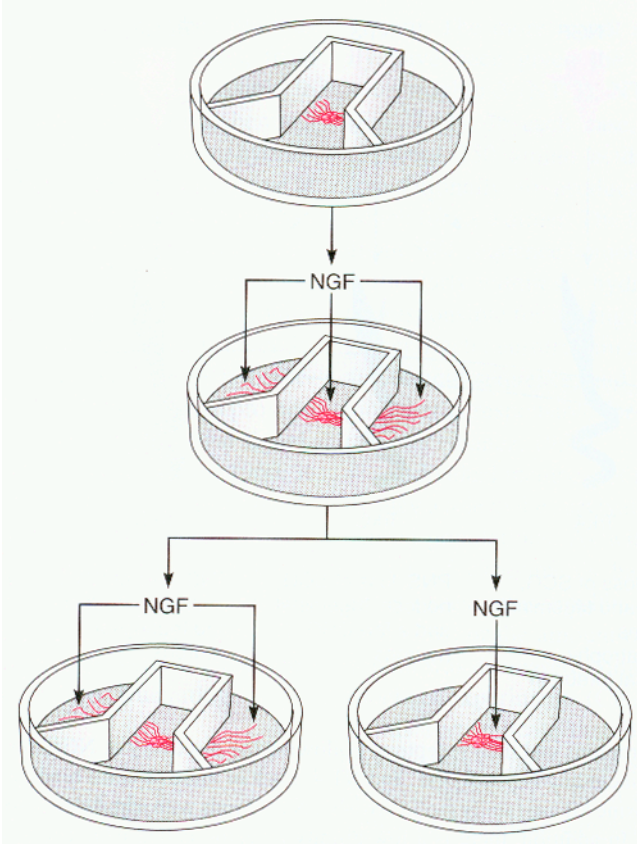


Neurotrophin receptors

- Neurotrophic hypothesis: targets produce signal that promote neuronal survival
- Neurons predicted to express neurotrophin receptors
  - NGF bound with high affinity to sympathetic and sensory axons

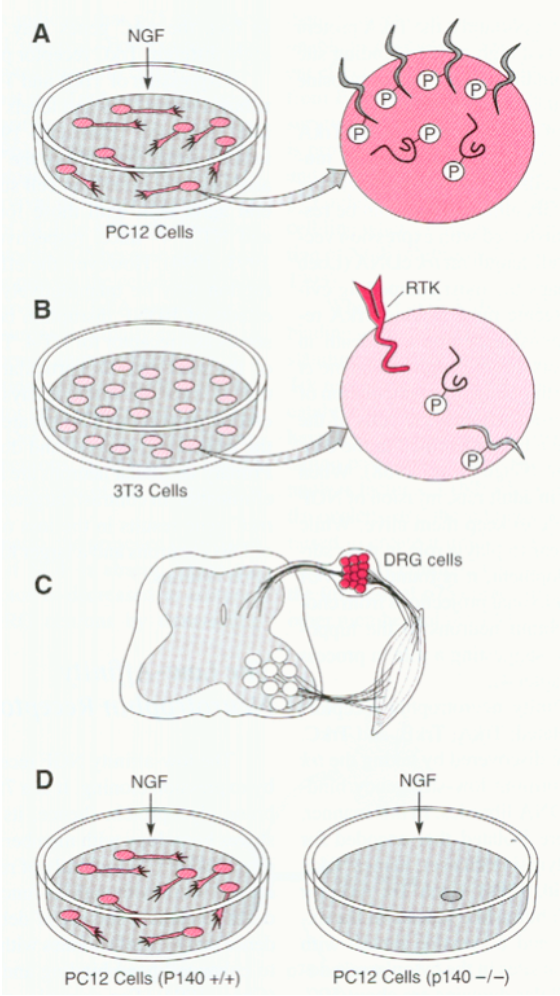
NGF at axon tip prevents death

- Expose neurites and cell body of sympathetic neurons to different media
- Put NGF in either chamber--- sufficient to rescue neuron from death (acts globally)
- However: only promote and retain outgrowth of neurites in direct contact with NGF (acts locally)



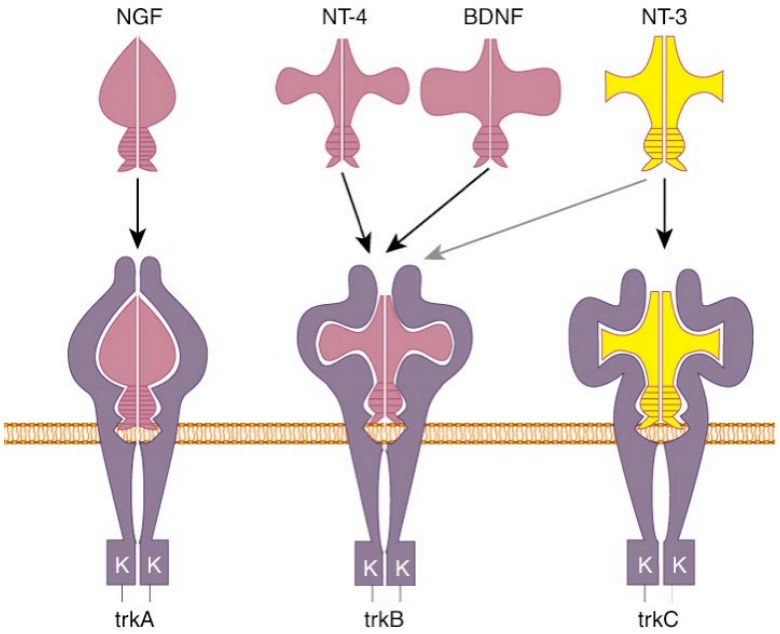
Identification of a receptor for NGF

- NGF promotes tyrosine phosphorylation of proteins
- The proto-oncogene TrkA was found to be a receptor tyrosine kinase
- TrkA expressed in DRG neurons
- Eliminate TrkA from PC12 cells --- no longer respond to NGF
- TrkA is a receptor for NGF



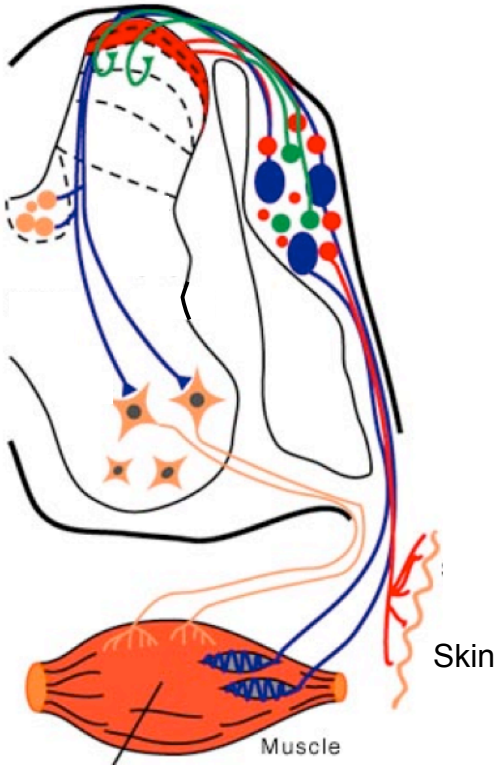
Trk family of Neurotrophin receptors

- TrkA belongs to a 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
- Truncated forms of these receptors that lack the kinase domain are also made --- often by glia --- may act as ligand sinks or dominant-negative Trks



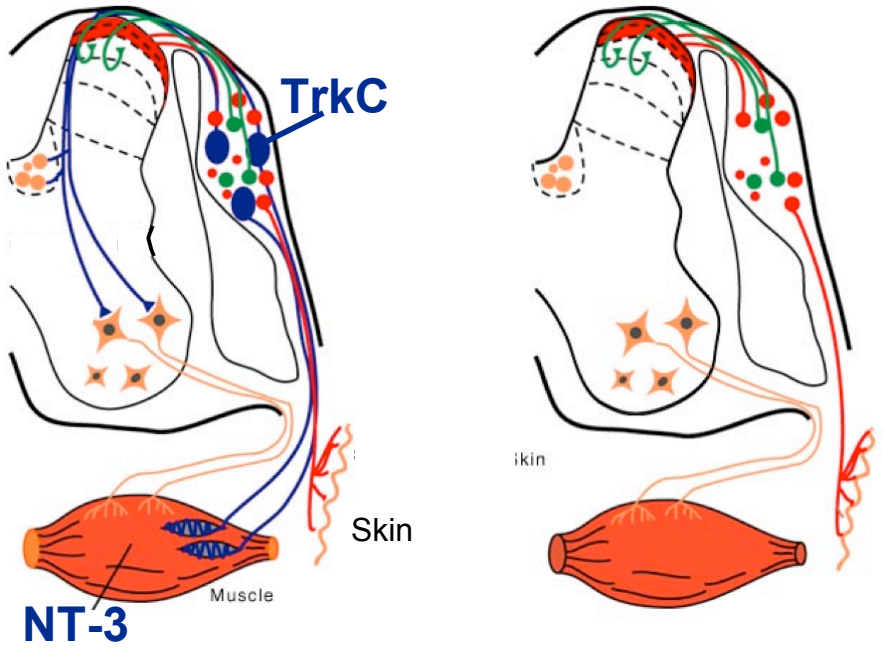
Neurotrophins and Trk receptors play important roles in neuronal survival

- Different subsets of sensory neurons express different Trk receptors
- Different targets produce different neurotrophins
- Mouse spinal cord:
  - Muscle produces NT-3, sensory neurons innervating spindle express TrkC
  - Skin cells produce NGF, thermo and pain-sensing neurons express TrkA



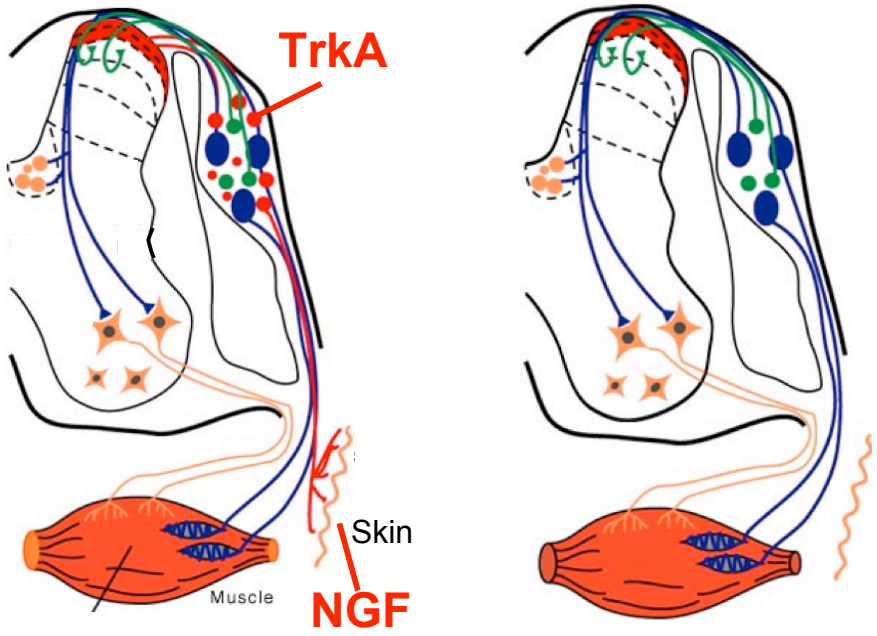
Loss of neurotrophin signaling leads to neuronal loss

- **Knockout of NT-3 or TrkC causes selective loss of spindle sensory neurons**



Loss of neurotrophin signaling leads to neuronal loss

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

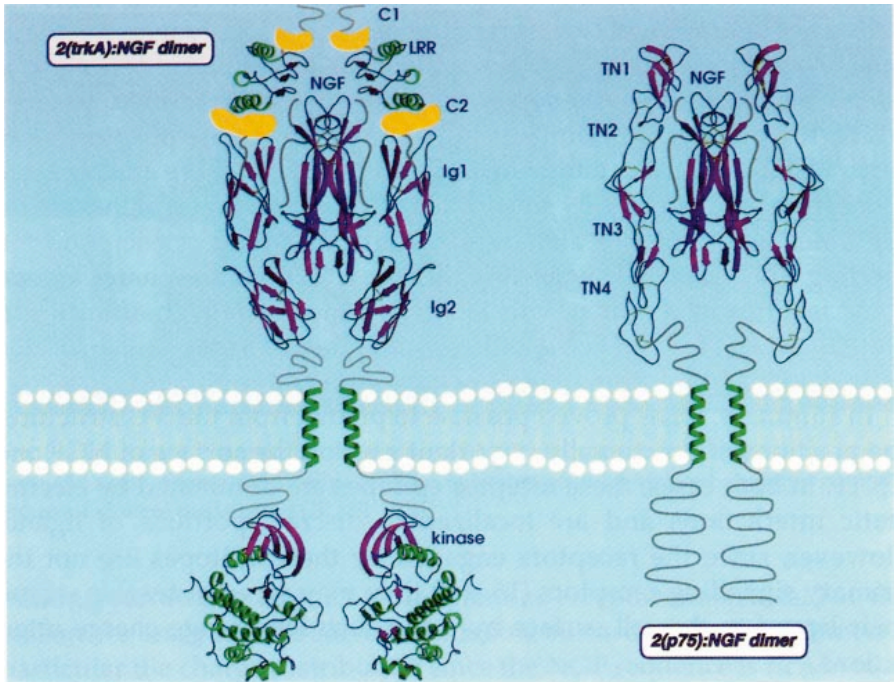


Wild type

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

$p75^{NTR}$: second class of neurotrophin receptor

- $p75^{NTR}$ Neurotrophin Receptor : binds NGF, BDNF, NT-3 and NT-4
- Not a receptor tyrosine kinase, but a member of the TNF receptor family
- TNF receptors are activated by binding of ligand -- recruit host of cytoplasmic signaling proteins
- $p75^{NTR}$ and Trks activate distinct signaling pathways

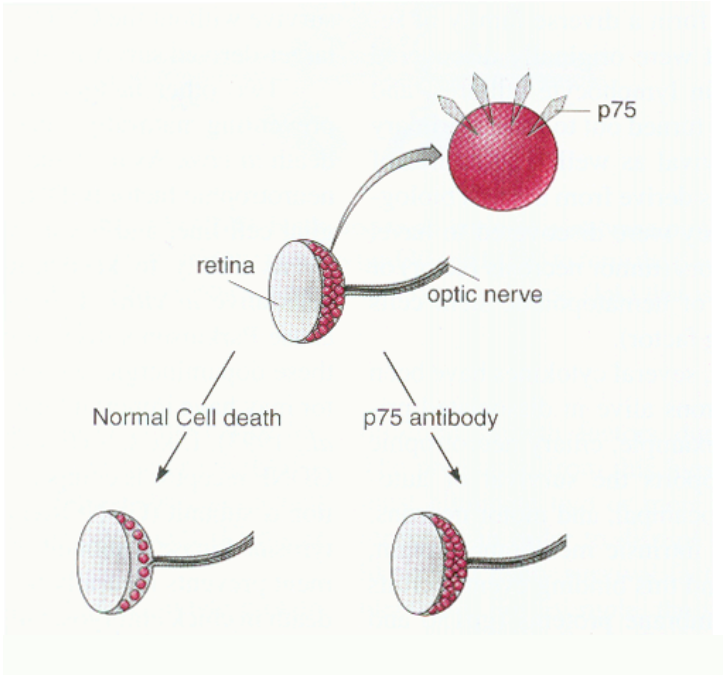


$p75^{NTR}$ combine with Trks to generate diverse set of neurotrophin receptors

- $p75^{NTR}$ originally called “low-affinity” receptor, Trks “high-affinity” receptors --- misnomer
- Both p75 and Trks bind neurotrophins on their own with similar affinity
  - p75 or Trks alone K_d’s $\approx 10^{-9}/10^{-10}$M
- p75 and Trks can associate to form receptors with higher affinity
  - p75+Trk $K_d \approx 10^{-11}$M

$p75^{NTR}$ has bi-functional role in neurotrophin signaling

- $p75^{NTR}$ can inhibit death (acting with Trks)
  - $p75^{NTR}$ knockout mice show some minor sensory neuron loss
    - » not essential for Trk signaling
    - » neurons need higher doses of neurotrophins to survive
- $p75^{NTR}$ can also promote death (acting alone)
  - In cells that don't express Trks, $p75^{NTR}$ can promote neurotrophin-dependent death
    - » Antibodies against p75 can inhibit retinal ganglion cell death

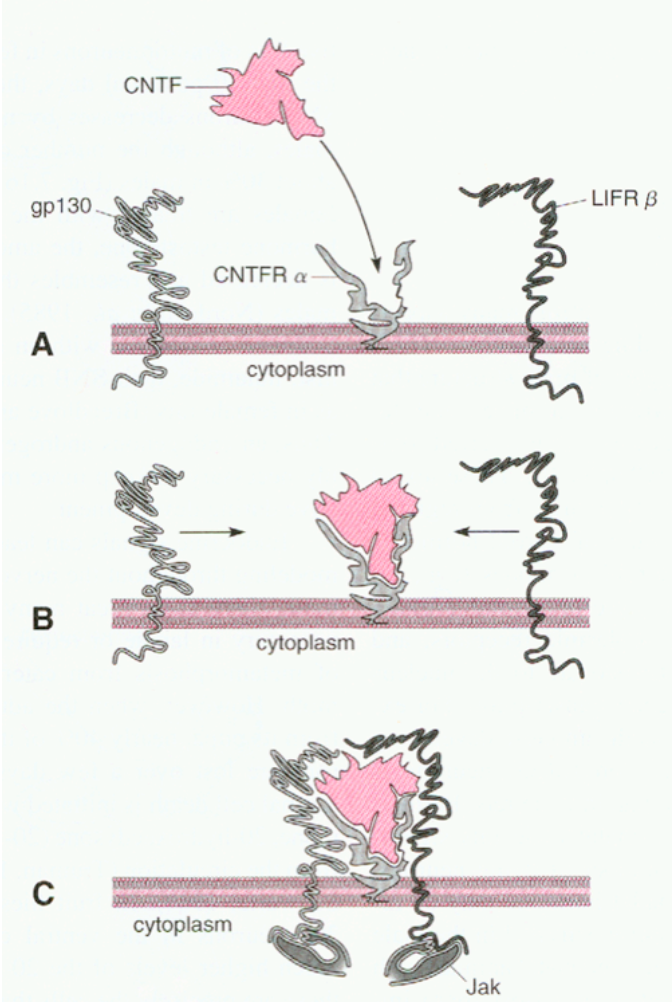


Additional classes of signaling molecules also regulate survival

- Neurotrophins: NGF, BDNF, NT-3, NT-4
- Cytokines: CNTF, LIF, CT-1
- Growth Factors: EGF, PDGF, Insulins, FGFs, GDNF
- Interleukins (ILs)
- Tumour Necrosis Factors (TNFs)
- Colony Stimulating Factors (CSFs)
- Interferons (IFNs)

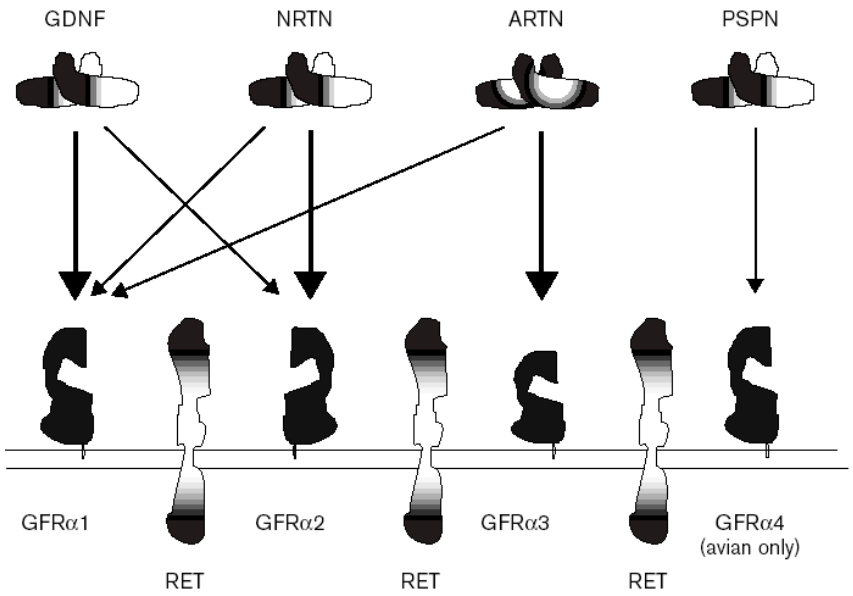
Cytokine-mediated survival

- Cytokines: originally described as growth factors for lymphocytes -- also act as neuronal survival factors
  - Ciliary Neurotrophic Factor (CNTF): promotes survival of autonomic, DRG, hippocampal and motor neurons
- Cytokines associate with a cell-surface receptor complex that can activate the JAK/STAT pathway and modulate transcription
- Knockout of CNTFR causes increased motor neuron death



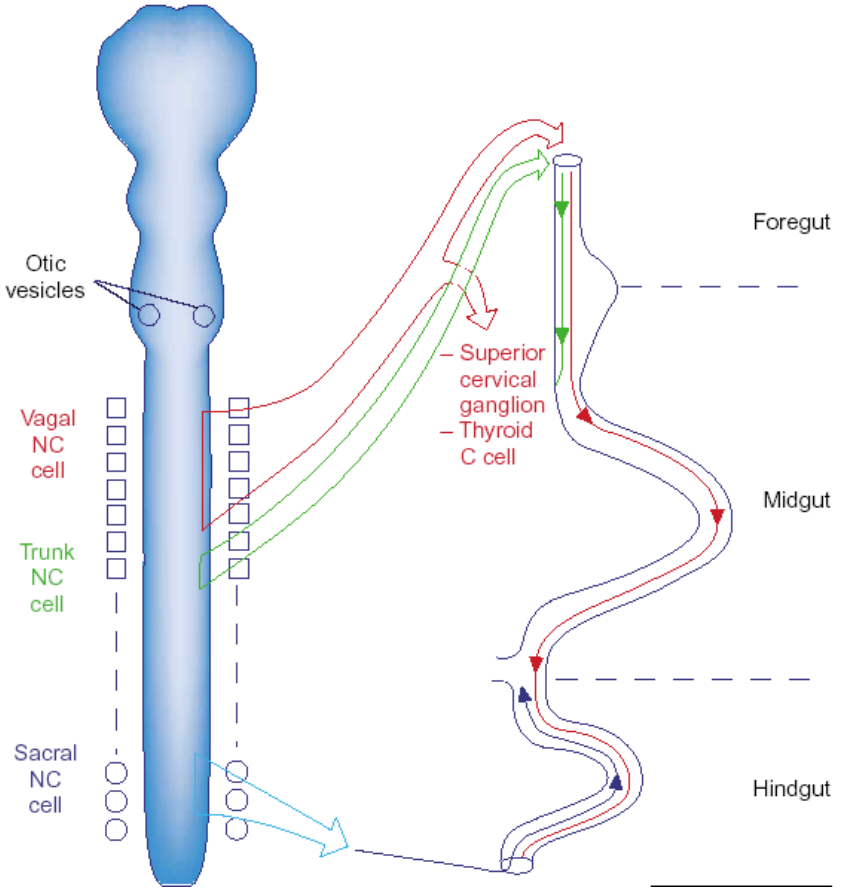
GDNF-family of survival factors

- **Glial-Derived Neurotrophic Factor (GDNF):**
  - **Belongs to family of four factors**
- **Each binds to particular GFR subunits**
- **Signal through Ret receptor tyrosine kinase**



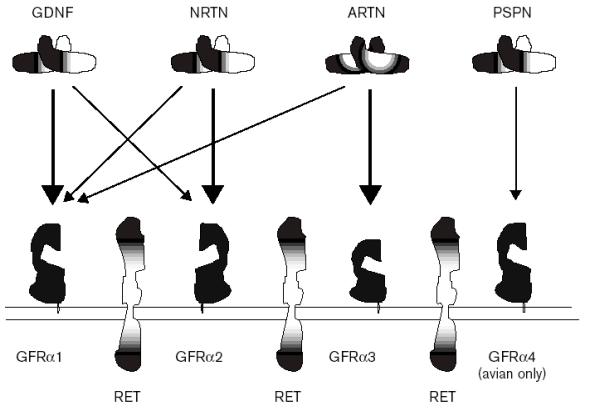
GDNF-family signaling in enteric neurons

- **Enteric nervous system:**
  - **Derived from neural crest cells**
  - **Control digestive processes (motility, secretion)**
- **Enteric neuron precursors express Ret, GFR□1**
- **GDNF produced by the GI mesenchyme**

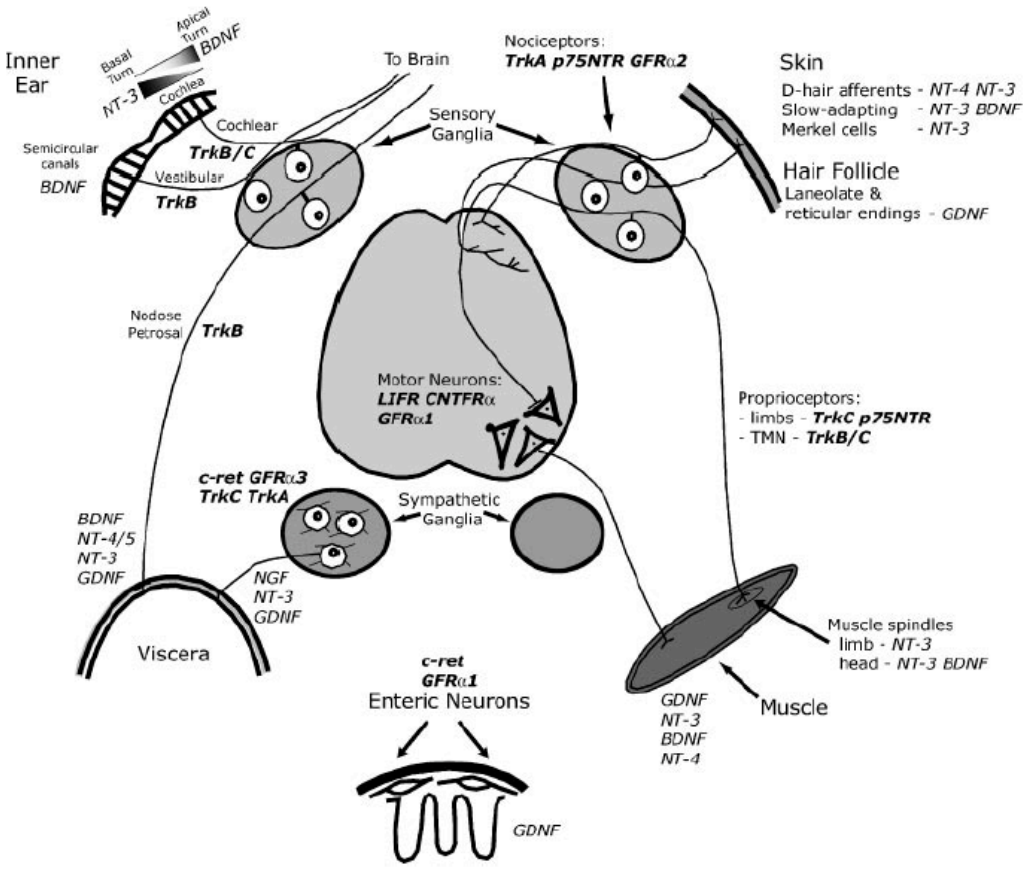


GDNF-family signaling in enteric neurons

- **Ret$^{-/-}$ mice lack all enteric sympathetic neurons**
  - **see massive apoptosis among precursor population**
- **See partial loss in GDNF $^{-/-}$ and GFRα3 $^{-/-}$ -- likely redundancy among GDNF-family members**
- **Ret loss-of-function in humans causes Hirschprung's disease**
  - **congenital absence of parasympathetic innervation in the lower intestinal tract**

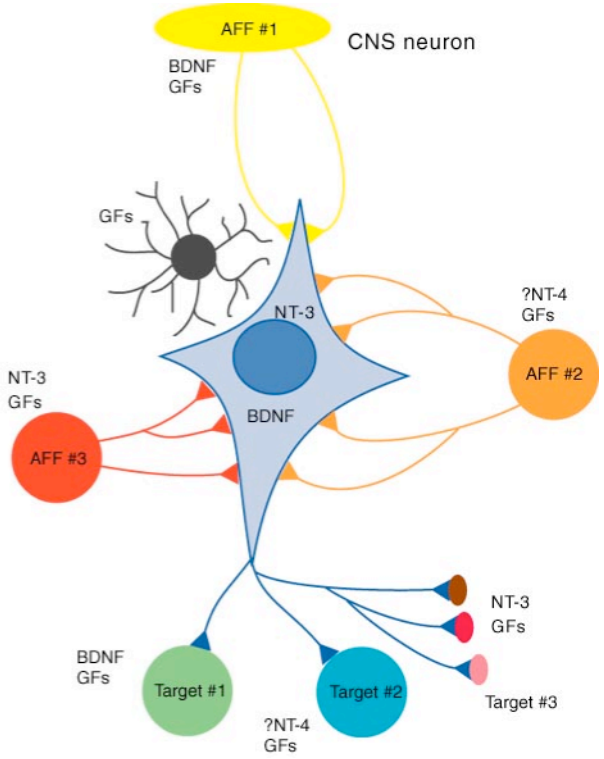


A diversity of trophic signals function throughout the PNS



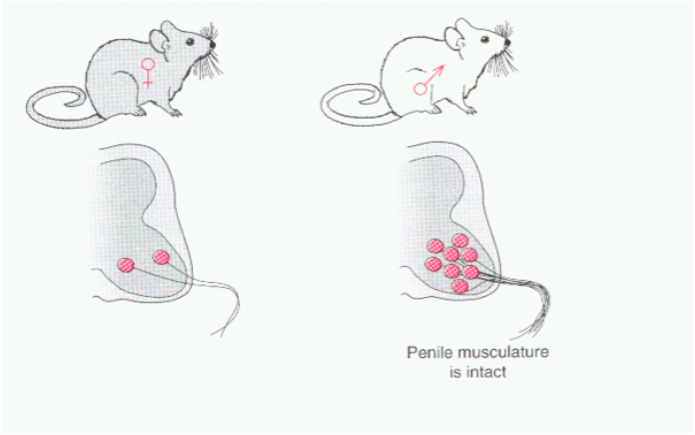
Survival factors in the CNS

- CNS neurons produce and respond *in vitro* to neurotrophins and other survival factors
- Effects of knockouts much less pronounced than in PNS (see elevated apoptosis in hippocampal and cerebellar granule cells in TrkB$^{-/-}$ mice)
- May reflect greater diversity of possible sources in CNS vs PNS
  - Multiplicity of inputs, targets, glia etc…



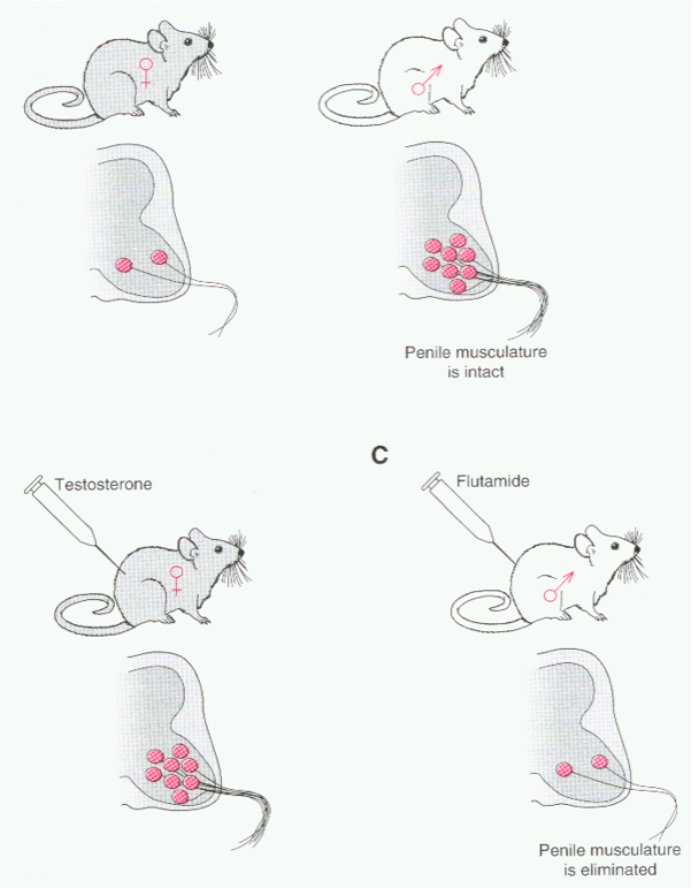
Endocrine control of neuronal survival

- Hormonal signals, including sex hormones, also influence patterns of neuronal survival
- Rat spinal cord contains motor nuclei housing motor neurons that innervate muscles in penis
  - Present in males
  - Nearly absent in females
- Sexual dimorphism due to death of these neurons in females



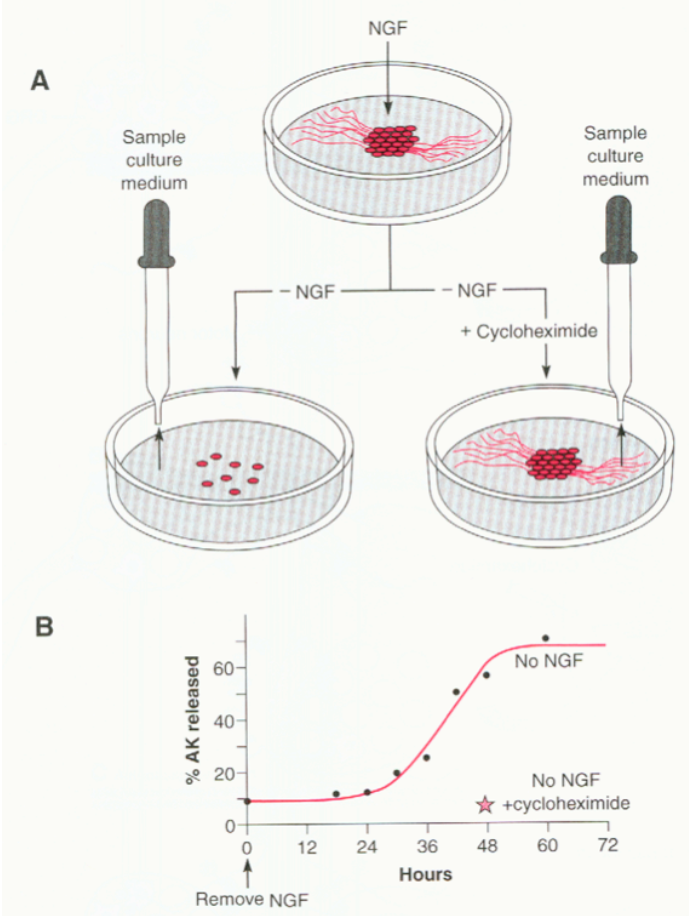
Endocrine control of neuronal survival

- This sexual dimorphism is under hormonal control
- Treat females with testosterone: cell death decreases in these nuclei and motor neurons survive
- Castrate males and treat with testosterone antagonist: cell death increases and motor neurons don't survive



Cell death is an active process

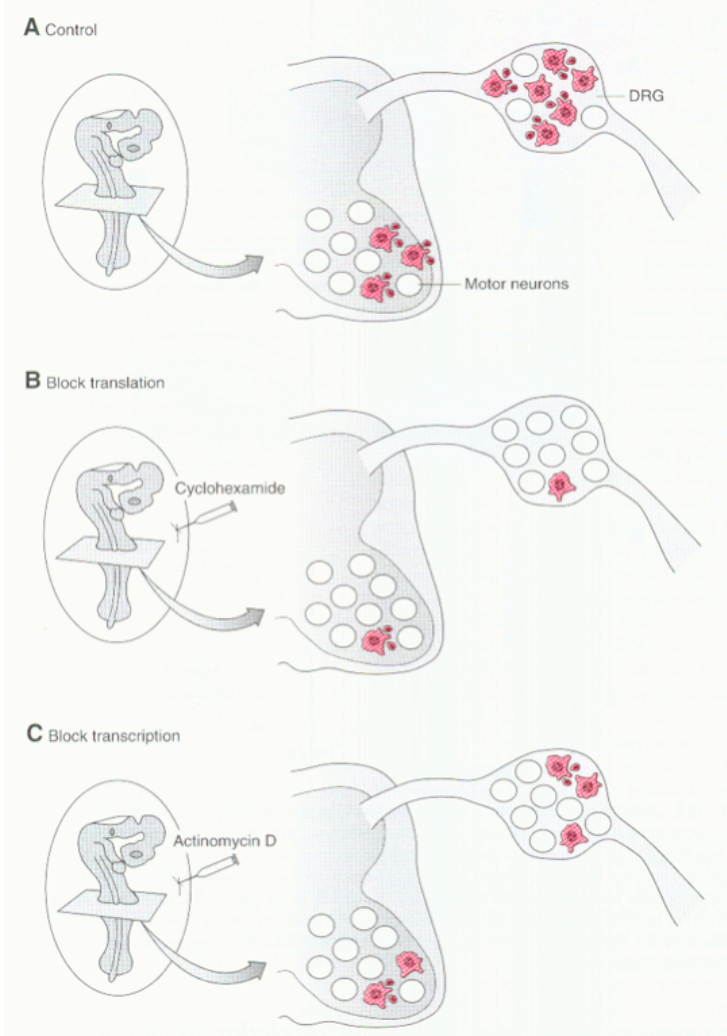
- Originally thought that neurons die of passive starvation in absence of trophic factors
- 1988: Eugene Johnson's group found that neuronal cell death can be delayed by blocking protein synthesis *in vitro*
- Sympathetic neurons die within 48 hours in culture without NGF
- If block translation, survive
- Thus: Cell death requires protein synthesis -- death is not just starvation



AK : adenylate kinase: cytoplasmic enzyme

Cell death is an active process *in vivo*

- Treat chick embryos for 10-12 hours with inhibitors of transcription or translation during peak time of motor neuron and DRG cell death
- In each case, see increase in number of neurons and decrease in number of neurons undergoing cell death



Regulation of cell death

- Cell death is abundant during neuronal development
- Neurons rely on a host of trophic factors to survive
- Trophic factors initiate signal transduction events in the receiving cell
- Cell death is an active process -- cells activate a death program
- Next time:
  - The core cell death machinery
  - Positive and negative regulation of the cell death machinery --- including, how trophic factors interface with the cell death machinery

Significance of K_d

- $K_d = k_{on}/k_{off}$

  K_d = equilibrium dissociation constant

  $K_a = 1/K_d$ = association constant

  k_{on}= association rate constant

  k_{off}= dissociation rate constant

- K_d can be used to estimate lower limit to lifetime of complex --- because k_{on} can't exceed $\approx 10^9$ M^{-1} sec^{-1}

Two types of binding sites for NGF

- Put labeled NGF on chick sensory neurons
- See two sets of binding sites
  - Low affinity (Kd ≈ 10^{-9} M)
  - High affinity (Kd ≈ 10^{-11} M)
  - Low affinity sites ≈ 10X more abundant than high affinity sites

Primer on protein binding

- Affinity --- strength of binding
- Specificity --- preference of binding to target versus non-target sites
  - High affinity, high specificity: growth factor/receptor
  - High affinity, low specificity: MHC-peptide
  - Low affinity, high specificity: T cell receptor to MHC-peptide

Significance of relative affinities

- Two classes of NGF receptors:
  - Low affinity (Kd ≈ 10^{-9} M)
  - High affinity (Kd ≈ 10^{-11} M)
- High affinity NGF sites will be largely occupied at NGF concentrations that will fill only a few percent of low affinity sites