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

![Diagram of TUNEL labeling]

**in Situ End Labeling (TUNEL)**
(template independent)

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 (e.g., 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
p75<sup>NTR</sup>: second class of neurotrophin receptor

- **p75<sup>NTR</sup>** 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<sup>NTR</sup> and Trks activate distinct signaling pathways
p75\textsuperscript{NTR} combine with Trks to generate diverse set of neurotrophin receptors

- p75\textsuperscript{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\textsuperscript{NTR} has bi-functional role in neurotrophin signaling

- p75\textsuperscript{NTR} can inhibit death (acting with Trks)
  - p75\textsuperscript{NTR} knockout mice show some minor sensory neuron loss
    » not essential for Trk signaling
    » neurons need higher doses of neurotrophins to survive
- p75\textsuperscript{NTR} can also promote death (acting alone)
  - In cells that don’t express Trks, p75\textsuperscript{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 GFRα 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, GFRα1
- GDNF produced by the GI mesenchyme
GDNF-family signaling in enteric neurons

- Ret<sup>-/-</sup> mice lack all enteric sympathetic neurons
  - see massive apoptosis among precursor population
- See partial loss in GDNF<sup>-/-</sup> and GFRα3<sup>-/-</sup> -- likely redundancy among GDNF-family members
- Ret loss-of-function in humans causes Hirschsprung’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 \textit{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 $K_d$

- $K_d = \frac{k_{on}}{k_{off}}$
  - $K_d =$ 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