Axon guidance I

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Neuronal Wiring: Functional Framework of the Nervous System

Stretch Refl ex
sensory neuron →
motor neuron

Stretch reflex circuit
Early theories of axonogenesis

- Schwann: many neurons link to form a chain
- Hensen: axon forms around preexisting threads between cells
- Wilhelm His (1886) and Santiago Ramon y Cajal (1890): Proposed that axon is an outgrowth from a neuron
Axon outgrowth

• Ross Harrison (1907): Invented tissue culture to demonstrate axon extension
  – Isolated piece of neural tube from tadpole
  – Placed neuroblasts in drop of frog lymph on coverslip inverted over depression slide
  – Watched axons emerge from differentiating neurons in his “hanging drop” prep at 56 microns/hour

Adapted from Harrison (1908)
The growth cone

- At leading edge of the axon (and dendrite) is a motile structure where much of the control of axon navigation takes place: the growth cone
Growth cones are highly dynamic

- Growth cones crawl over a variety of surfaces to reach their targets and have a wide variety of shapes *in vivo*

Ramon y Cajal 1890
Movies of axon guidance in vivo

- Xenopus spinal cord
    » 5 min/frame; 5 hours;

- Xenopus visual system
  - [http://www.anat.cam.ac.uk/pages/staff/academic/holt/large.mov](http://www.anat.cam.ac.uk/pages/staff/academic/holt/large.mov)
    » 3 min/frame; 6 hours
Neurons show cell-type specific axon projection patterns.
An axon’s complex journey can be broken into discrete segments

• Axons navigate using a series of intermediate targets
  – Example: Ti1 neuron in grasshopper limb bud
Intermediate targets play essential role in navigation

- Ablate Cx1 cell -- axon halts
How are axons guided?

- Ramon y Cajal (1892): Chemotropism
  - Axons guided by diffusible cues from target cells: based on anatomical observation

Cajal (1890), day 4 chick spinal cord
Are axons guided by chemotropism?

- **YES: 1980’s:** Target tissues shown to attract appropriate axons at a distance *in vitro*
- **Example:** vertebrate spinal cord
- **Floor plate explant attracts commissural axons**
What is the floor plate chemoattractant?

- Marc Tessier-Lavigne’s lab (1994)
  - 20,000 chick embryonic brains
  - Fractionated protein extracts
  - Followed outgrowth promoting activity
  - Isolated two major proteins
  - Sequenced the proteins
  - Cloned two related proteins: netrin-1 and netrin-2
Netrins

- Secreted proteins
- Related to a portion of the extracellular matrix protein laminin
- Associated with cell surfaces and the extracellular matrix
Netrin-1

- Netrin-1 is an important floor plate chemoattractant
  - Expressed by floor plate cells
  - Netrin-1 mutant mice have commissural axon guidance defects
- And....
Netrin-1 is 50% identical to C. elegans UNC-6

- Unc-6: found as a mutation that affects the nematode’s movement (uncoordinated)
- *unc-6* mutations disrupt axon guidance along DV axis
- Netrin-1/unc-6: evolutionarily conserved midline chemotropic factor
- Attractant and repellent?
Netrin-1 can also function as a chemorepellent

dorsal

ventral

trochlear motor axons

floor plate (netrin-1)

COS cells

tissue explant

netrin-1 expressing COS cells

tissue explant
What determines whether Netrin/Unc-6 attracts or repels?

- 1) The Netrin receptors an axon expresses
  - Two Netrin receptors known:
    » Unc-40/DCC: necessary for attraction and repulsion
    » Unc-5: necessary for repulsion
Distinct functions of netrin receptors

- **Unc-40/DCC**: necessary for attraction and repulsion
- **Unc-5**: necessary for repulsion
Ectopic expression of Unc-5 can change an axon’s trajectory

- Expression of the Unc-5 receptor in neurons that normally do not express Unc-5 (“ectopic expression”) can redirect axons away from the ventral midline
- Depends on Unc-6 (Netrin) and Unc-40 (co-receptor)
Relationship between Unc-5 and Unc-40

- Simply antagonistic? No: Unc-5 often depends on Unc-40 for its repulsive function (long-range)
- Appear to collaborate: Unc-5 and Unc-40 proteins bind to one another in Netrin-dependent fashion
- Important questions:
  - How does unc-5 convert attraction to repulsion?
  - How does unc-40 mediate attraction?
  - What are the downstream signaling pathways?
What determines whether netrin/Unc-6 attracts or repels?

- 2) The cues that are received in combination with Netrin-1

- Laminin: extracellular matrix protein
  - Laminin alone neither attractant nor repellent
  - Common substrate for axons to encounter
  - Growth cones express transmembrane receptors for laminin (integrins)

- Suggests complex integration of multiple signals within growth cone
How does laminin convert attraction to repulsion?

- Laminin alters levels of cAMP in the growth cone
- Is this important?
  - Artificially elevate cAMP levels with cAMP analog, laminin no longer has effect: Netrin remains attractive
  - If inhibit cAMP signaling with drugs, Netrin repels without added laminin
- How cAMP regulates this is unknown
Mechanisms for determining how a growth cone responds to Netrin:

• 1) Repertoire of Netrin receptors present (unc-40, unc-5)
• 2) Identity of other guidance cues received in combination (eg., laminin)
Lessons from Netrins

- A single guidance cue can mediate attraction and repulsion.
- Related proteins regulate axon guidance at the midline in worms, flies and vertebrates. Evolutionarily ancient.
- A single cue can regulate multiple steps in growth cone navigation. (In flies, Netrin is required for the RP3 motor neuron to synapse on its target muscle.)
- Axon guidance cues/receptors also cell migration. Regulate fundamental cellular constituents that affect cell motility.
Axon guidance at the midline:

- In bilaterally symmetric animals it is important to coordinate both sides.
- Netrins attract axons to the midline: what then?
- Two classes of axons:
  - Ipsilateral: don’t cross midline, but grow along it.
  - Contralateral: Cross midline once, then grow along it.
Axons at the CNS midline

• Why do axons grow along rather than within the midline?
• Why do some axons cross and others not cross?
• Why do axons only cross once?
• Partial answer: midline makes not just long-range attractant (Netrin), but also a short-range repellent.
Control of axon guidance at the CNS midline

- Key insights from *Drosophila*:
  - Robo and Slit:
  - Slit is a secreted protein expressed by glial cells at the CNS midline
  - All CNS axons express Robo: a transmembrane receptor protein that binds to Slit
  - Slit repels Robo-expressing axons *in vitro*
Slit and Robo at the CNS midline

- In Slit mutants and Robo mutants axons enter the midline and don’t leave
- Slit acts as midline repellent for axons expressing Robo receptor
- Why is the Robo mutant defect less severe than the Slit mutant defect?
Robo belongs to a family of receptors

- Robo2 is closely related to Robo
- Robo and Robo2 are partially redundant
- Thus Slit/Robo signaling repels axons from the midline
- However: If Slit repels axons from the midline, how can axons ever cross?
Midline signaling part 2: how do some axons cross the midline?

- Commissureless (Comm): novel intracellular transmembrane protein
- Comm mutants have strong axon guidance defects: the opposite of Slit and Robo mutants
The interaction between Comm and Robo

- Test how Robo and Comm functionally interact by a genetic interaction test (epistasis).
- Way to order gene function
- Sample genetic pathway:
  - Gene A inhibits the function of Gene B
  - Mutant in gene A has opposite phenotype of mutant in gene B
  - Make an AB double mutant: what kind of phenotype do you get?
The interaction between Comm and Robo

• Test how Robo and Comm functionally interact by a genetic interaction test (epistasis).
• Way to order gene function
• Sample genetic pathway:
  – Gene A inhibits the function of Gene B
  – Mutant in gene A has opposite phenotype of mutant in gene B
  – Make an AB double mutant: what kind of phenotype do you get?
  – What would be the result if Gene B inhibited the function of Gene A instead?
The interaction between Comm and Robo

- Robo and Comm have opposite phenotypes: consistent with Robo inhibiting Comm or Comm inhibiting Robo function
- Make comm;robo double mutant
- Double mutant resembles Robo
The interaction between Comm and Robo

- Robo and Comm have opposite phenotypes: consistent with Robo inhibiting Comm or Comm inhibiting Robo function.
- Make comm;robo double mutant.
- Double mutant resembles Robo.
- Suggests: Comm negatively regulates Robo function.
How does Comm regulate Robo?

- Comm is expressed in neurons
- Comm binds to Robo and targets it for degradation
- Thus neurons expressing Comm do not put Robo on their growth cones
- Comm-expressing neurons are not repelled from the midline
How is Comm regulated?

• The story so far:
  – Slit inhibits midline crossing by repelling Robo-expressing axons
  – Comm inhibits Robo-expression on axons permitting midline crossing
  – How are these two counteracting forces combined to generate regulated midline crossing?
Comm regulation is key for midline guidance decisions

- How do axons enter the midline?
  - Comm expression is tightly regulated at the transcriptional level.
    » It is only expressed in contralaterally projecting neurons transiently--- just prior to their midline crossing.
    » It is never turned on in ipsilaterally projecting neurons.
      - Comm ON--Robo OFF -- axon enters midline (Panel B)

- How do axons ever leave the midline?
  » Comm expression is turned off during crossing.
    - Comm OFF--Robo ON -- axon leaves midline (Panel C)
Control of midline crossing

- Combination of attractive and repulsive interactions control where axons project near the CNS midline
  - Ipsilateral axons: Never cross midline, grow along it
    » 1) Attracted toward the midline by Netrin
    » 2) At midline repelled by Slit
  - Contralateral axons: Cross midline once, grow along it
    » 1) Attracted toward the midline by Netrin
    » 2a) As approach midline, express Comm
    » 2b) Comm inhibits Robo expression, axon ignores Slit and enters midline
    » 3a) Neuron turns off Comm, Robo protein reaches the growth cone
    » 3b) As Robo expression is restored, axon becomes repelled by Slit and leaves midline
Axon guidance cues and receptors
Navigation within the target region

- Axon reaches target region: still many possible target cells: How does axon choose correct one?
  - Topographic map formation (reach appropriate location within target field)
  - Post-synaptic target cell selection
Topographic maps

- Orderly anatomical representations of a physical property of the world (visual space, sound frequency, odor)
- Basic types:
  - Ordered by anatomical position (e.g. visual system)
    » adjacent neurons project to adjacent targets
  - Ordered by neuron type (e.g. olfactory system)
    » neuron expressing same odorant receptor (detecting same odor) project to same place
Retinotectal system

• Ordered by anatomical position
  – Adjacent neurons project to adjacent targets
• Thousands of retinal ganglion cells projecting to thousands of tectal targets:
  – How establish precise map?
Construction of retinotectal map

- Roger Sperry (1950’s): studied frog retinotectal system
  - Cut/crush optic nerve
    » Rotated eye 180 degrees
    » Nerve allowed to regenerate
    » Frog acted as if visual world upside down
  - Axons regrow to original target even if functionally inappropriate: suggested map not purely formed by neuronal activity
Construction of retinotectal map

• Sperry regeneration experiments (pt 2):
  – Cut/crush optic nerve
  – Remove half the eye
  – Axons from remaining half of eye grew to their appropriate part of tectum; rest of tectum empty (if wait a longer time, it’s more complicated)
  – Suggests: Recognition between axon and target
• 1963: Sperry proposed Chemoaffinity hypothesis
  – Axon and target cells have selective chemical affinities for one another
Sperry chemoaffinity model

- Thousands of axons and targets: will each axon/target pair have its own unique, complementary label?
- No --- Sperry predicted existence of complementary gradients of signaling molecules on axons and targets:
  - Rationale:
    - Economical: only a few molecules needed
    - Act over large region: Gradients could be sensed throughout target. If axon is in wrong place could tell which way to go toward target. It would not be just a random search for a match.
- Are there actually such gradients? If so, gradients of what?
Assaying for gradients of chemoaffinity molecules

- 70’s and 80’s: *In vitro* assays demonstrated activities consistent with chemoaffinity
- Bonhoeffer devised tectal membrane “stripe assay”

Stripe Assay: Prepare membranes from anterior and posterior tectum

Posterior axons avoid posterior tectal membranes

Anterior axons don’t care
1995: first “chemoaffinity” molecules identified

- Eph receptors: transmembrane receptor tyrosine kinases
- Ephrins: bind Eph receptors
  - Class A: GPI-anchored
  - Class B: transmembrane
Eph/Ephrin

- Basic rules:
  - EphA bind EphrinA
  - EphB bind EphrinB
- Eph’s are unusual receptor tyrosine kinases: require clustering of ligand
  - Likely to require cell-cell contact, hence highly localized signaling
- Known to mediate axon repulsion
Eph and Ephrins in the Retinotectal System

- Expressed in complementary gradients on axons/targets.
Ephrins can mediate topographically specific repulsion

- **In vitro**: ephrins repels P but not A axons.
- **In vivo**: ectopic Ephrin A2 causes posterior but not anterior axons to stop short.
Ephs and Ephrins in map formation

- Gradients of Ephs and Ephrins found in many brain regions where topographic maps form
- How do they determine placement of axon within a map?
  - One model: $[\text{Eph}] \times [\text{Ephrin}] = \text{repulsive force}$: axons move until hit threshold of repulsion
  - However: Knockouts seem to randomize map
    » don’t cause all axons to go to one extreme
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  - However: Knockouts seem to randomize map
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  - Second model: Relative level of signaling compared to other axons is key
Evidence for relative level model

- Put EphA3 receptor into locus expressed in ≈50% of retinal ganglion cells (Isl2)
- If absolute signaling is key: Isl2- axons target normally
- If relative level of signaling is key: Isl2- axons shift and two separate maps form: one for Isl2- and one for Isl2+ axons
Result: two maps form

- Isl2⁻ axons form map over posterior half of target
- Isl2⁺ axons form map over anterior half of target
- Suggests relative level of Eph signaling is key
Ephrins and Ephs: Who is the ligand and who is the receptor?


- However... EphB2 mutant where extracellular domain present, but no kinase => No Defect!!

- Look at expression:
  Ephrin Bs are expressed on axons
  EphB2 expressed on SUBSTRATE!

Is EphB2 a ligand for Ephrin Bs?
Ephrins and Ephs: Who is the ligand and who is the receptor?

- Can EphB2 act as a ligand for Ephrin Bs?

  • Binding of EphB2 to EphrinB1 causes the intracellular domain of Ephrin B1 to become tyrosine phosphorylated.

=> EphBs and Ephrin Bs can mediate bidirectional signals

  • Provides a way for coordinating the response of two interacting cell populations.
The Eph/Ephrin Interaction:
How can the interaction between membrane-associated proteins result in repulsion?

- EphA3 binding to Ephrin A2 promotes proteolytic cleavage of Ephrin A2.
The Eph/Ephrin Interaction:
How can the interaction between two membrane-associated proteins result in repulsion?

- **Uncleavable form of EphrinA2**: Growth cone collapses but doesn’t withdraw.
- **Proteolysis permits withdrawal (and redirection).**
Next time:
Target selection (continued)
Cell biology of growth cone navigation