Methodological Approaches To Study Receptors

Lecture #3

Determining The Structure of Receptors

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Determining the Structure of Receptors

Overview

Step 1: *Membrane Topology*

* Hydropathy Plots e.g. nAChR & iGluRs
* Engineered Reporter Sites e.g. iGluRs

Step 2: *3-Dimensional/Static Structure*

* Electron Cryomicroscopy e.g. nAChR
* X-Ray Crystallography

Step 3: *Conformational Events*

SCAM Analysis
Voltage clamp fluoremetry

Determining the Structure of Receptors

Membrane Topology: Hydropathy Plots

Things To Be Considered

Objective:
Identify membrane spanning, cytoplasmic or extracellular regions of the protein

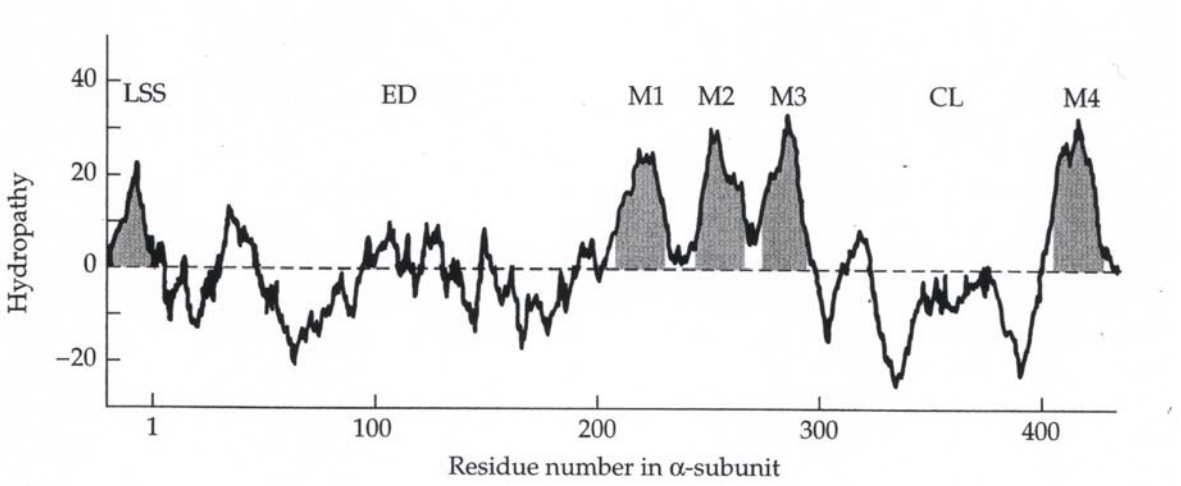
Assumptions:
1. Transmembrane segments are α-helical in nature
2. Membrane spanning amino acids are hydrophobic in nature

Practicality:
1. Assign each amino acid a numerical rating of hydrophobicity
2. Identify 20 or more amino acids that are hydrophobic in nature

Determining the Structure of Receptors

Membrane Topology: Hydropathy Plots

Typical Example



e.g. calf α-subunit of nAChR

- 5 Hydrophobic Peaks
- 1st Peak is Leading Signal Sequence
- 2nd – 5th Peaks Correspond to M1 – M4 regions

Determining the Structure of Receptors

Membrane Topology: Hydropathy Plots

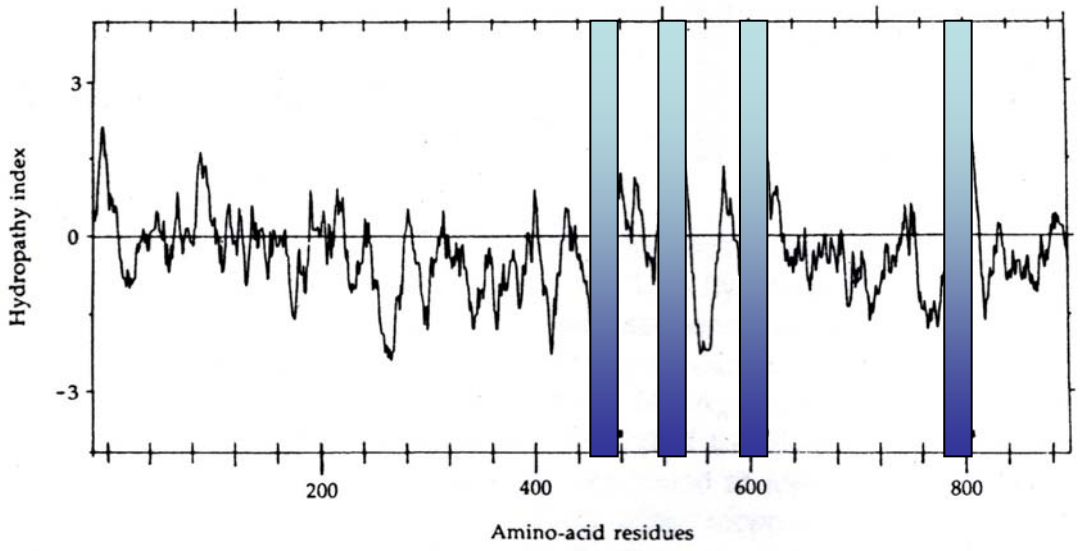
Benefits & Disadvantages

- Simple, based on assignment of amino acid hydrophobicity
- Limited, represents a topological “hypothesis”
- Topology prediction should be supported by experimentation

Determining the Structure of Receptors

Membrane Topology: Engineered Reporter Sites

Why Topology Should Be Confirmed by Experimentation

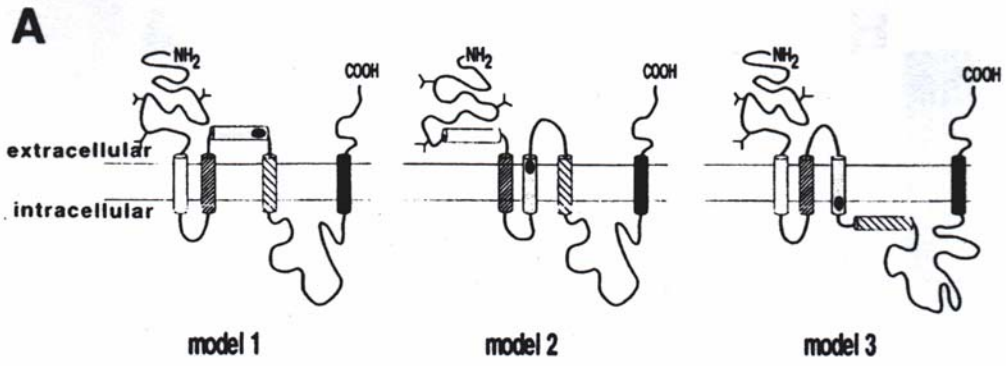


see Hollmann et al (1989) Nature, 342, 643-648.

- 4 Hydrophobic Peaks
- Suggest 4 Membrane Spanning Domains
- Actual Protein Has Only 3 Membrane Spanning Domains

Determining the Structure of Receptors

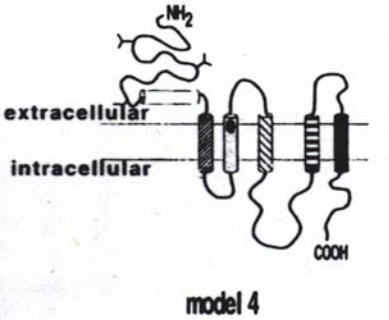
Membrane Topology: Engineered Reporter Sites



Models suggested from hydropathy plots and single point mutations

4 TMDs

N and C terminals extracellular



Model suggested from immunocytochemical data

4 TMDs

N terminal extracellular

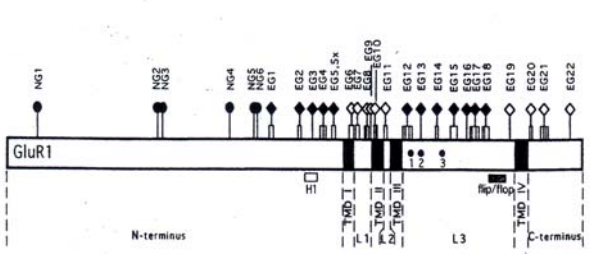
C terminal intracellular

see Hollmann et al (1994) Neuron, 13, 1331-1343.

Determining the Structure of Receptors

Membrane Topology: Engineered Reporter Sites

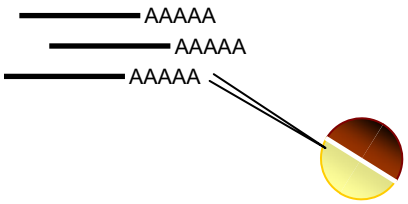
Developing An Experimental Strategy



Engineered N-glycosylation sites

Strategy

- N-glycosylation enzymes are compartmentalized
- N-Glycosylation restricted to lumenal face of ER
- Only extracellular sites can be glycosylated



Inject oocyte with mutant receptor

Result

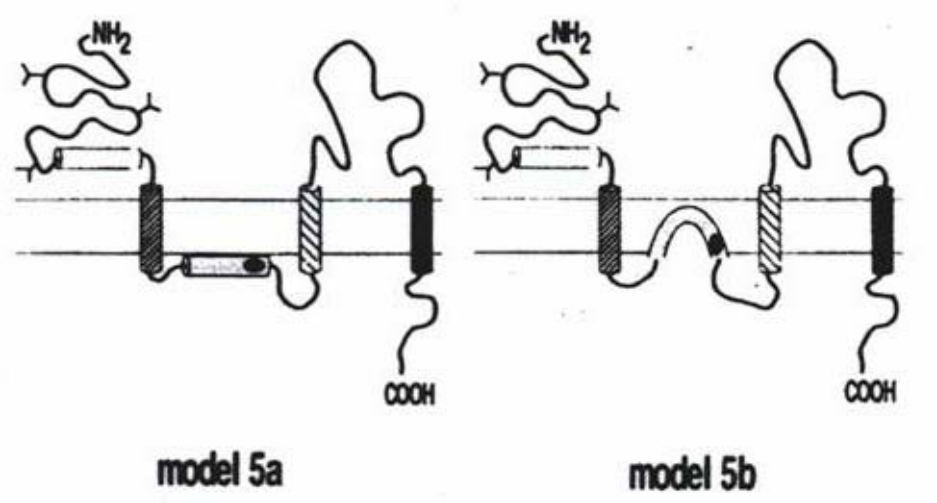
- Purify protein
- Label C-terminal with antibody
- Look for gel shift as indicator of glycosylation

see Hollmann et al (1994) Neuron, 13, 1331-1343.

Determining the Structure of Receptors

Membrane Topology: Engineered Reporter Sites

Revised Topology For Ionotropic Glutamate Receptors



see Hollmann et al (1994) Neuron, 13, 1331-1343.

- Consists of 3 Transmembrane Domains and NOT 4
- Pore region is a re-entrant loop (model 5b)
- N and C termini are extra- and intracellular respectively

Determining the Structure of Receptors

3D/Static Structure: Electron Cryomicroscopy

Things To Be Considered

Ideal:

Obtain receptor structure at atomic level by X-ray crystallography

Problem:

1. Eukaryotic membrane proteins are difficult to crystallize
2. Require hydrophobic girdle for stability
3. Eukaryotic proteins are glycosylated

Outcome:

These factors render surface of protein fuzzy, variable and weakly interacting

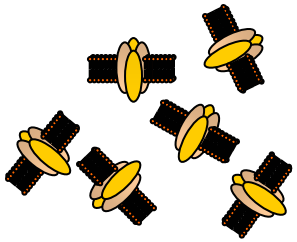
Solution:

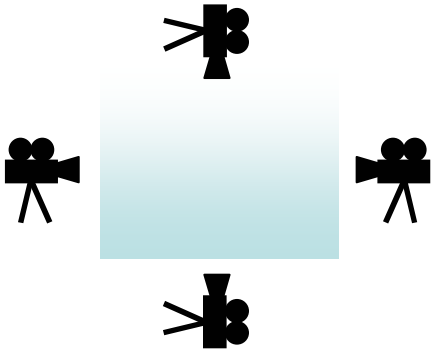
Obtain structural information using alternative approach, Electron Cyromicroscopy, pioneered by Henderson and Unwin

Determining the Structure of Receptors

3D/Static Structure: Electron Cryomicroscopy

Procedure



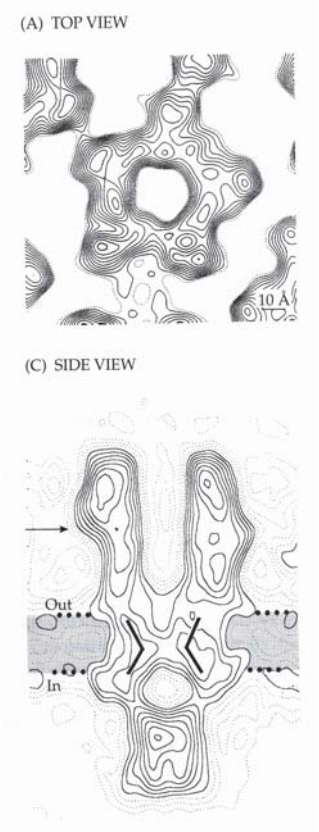


- Purify protein from source rich in it
- Quickly freeze with protein embedded in membrane
- Shoot many micrographs at different angles & focal planes
- Extensive digital processing to obtain single averaged image
- Successful for:
  - Bacteriorhodopsin
  - Gap junctions
  - Ryanodine receptor
  - Aquaporins
  - nAChR
  - Shaker K channel

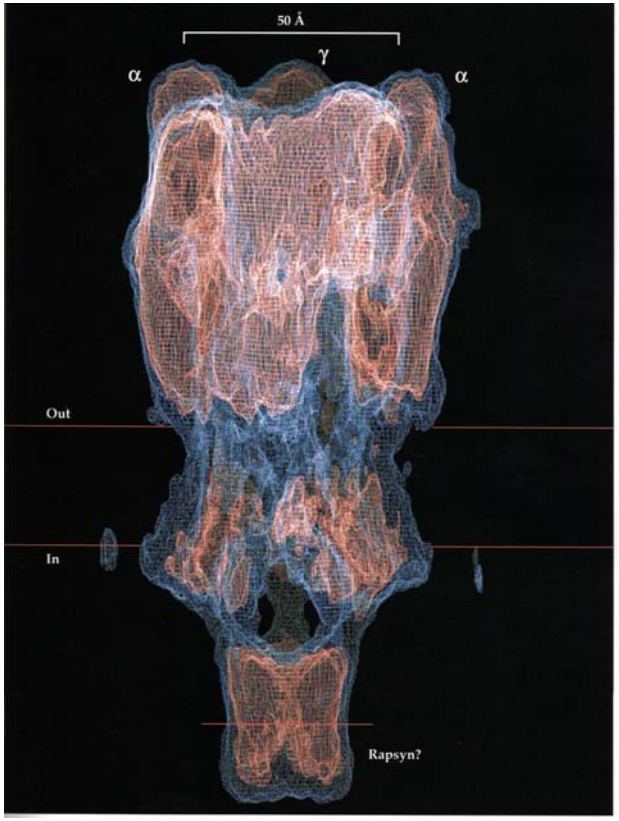
Determining the Structure of Receptors

3-D/Static Structure: Electron Cryomicroscopy

nAChR is a tall hourglass



9 Å
Resolution



4.6 Å
Resolution

See Unwin, 2000

Determining the Structure of Receptors

3-D/Static Structure: Electron Cryomicroscopy

Benefits & Disadvantages

- Challenging, requires high expertise level
- Limited, requires source rich in protein of interest
- Constrained, atomic resolution not achieved so far though possible theoretically
- Successful, given insight into protein structure in the absence of X-ray crystallography

Determining the Structure of Receptors

3D/Static Structure: X-Ray Crystallography

Things To Be Considered

Ideal:

Direct approach to obtain receptor structure at atomic level

Problem:

1. Need source rich in protein of interest
2. Find solutions to grow crystals
3. As described above for section on “electron cryomicroscopy”

Solution:

Identification of ion-channel genes in bacteria permitted large scale purification
Protein fortunately is not glycosylated

Two examples, K-channel, KcsA from *Streptomyces lividans*
Mechanosensitive channel, MscL from *Mycocaterium tuberculosis*

Determining the Structure of Receptors

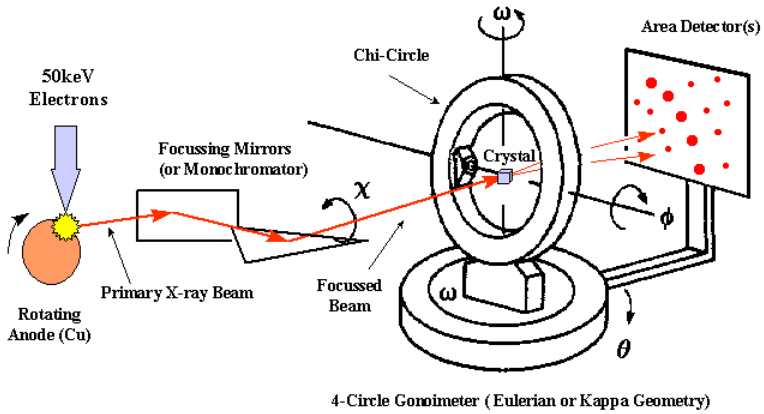
3D/Static Structure: X-Ray Crystallography

Procedure

Theory

Technique that exploits the fact that X-rays are diffracted by crystals. Diffraction pattern obtained from X-ray scattering permits reconstruction of the electron density map.

Practicality



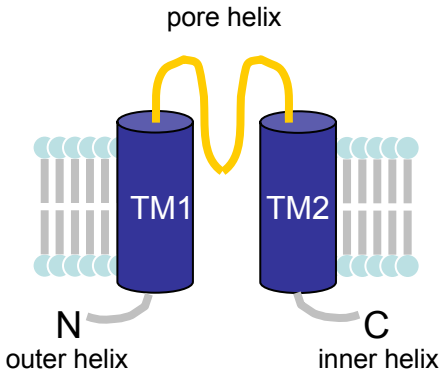
4-Circle Goniometer (Eulerian or Kappa Geometry)

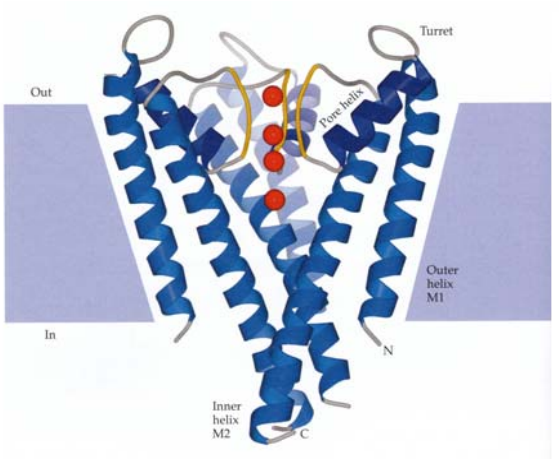
1. Obtain protein crystals
2. Collect diffraction spots
3. Known amino acids are positioned in electron density map
4. Optimize match with diffraction pattern
5. Experimental electron density map gives accurate molecular structure

Determining the Structure of Receptors

3D/Static Structure: X-Ray Crystallography

KcsA K^+ channel is an inverted tepee





Subunit Structure

- Subunit of 2 transmembrane
- Consists of 160 residues
- Mature protein is a tetramer

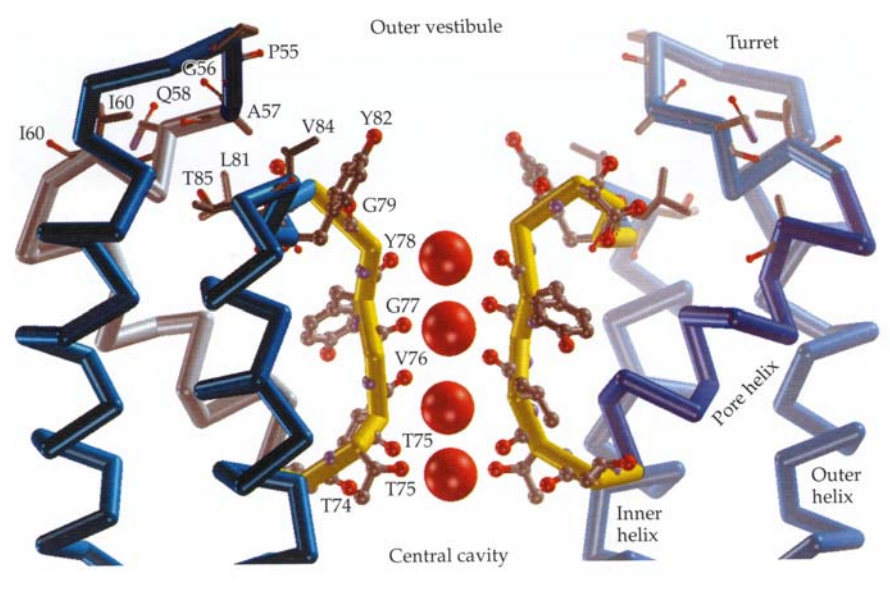
Mature Protein

- TM1 & TM2 are α-helices: outer & inner helix
- TM1 & TM2 form an inverted tepee
- Pore helix forms the selectivity filter of pore

Determining the Structure of Receptors

3D/Static Structure: X-Ray Crystallography

Structure explains transport of K^+ ions through the pore



- Narrow aqueous pore, 3Å diameter
- K^+ ions are dehydrated and move in a single file
- Close proximity establishes electrostatic repulsion

Determining the Structure of Receptors

3D/Static Structure: X-Ray Crystallography

Benefits & Disadvantages

- Extremely successful, direct approach to obtaining atomic resolution
- Challenging, requires high expertise level
- Limited, ion-channels are ubiquitous NOT abundant

What Have We Learned?

1. Determining the molecular identity of receptors

Getting the first clone

- Classical approach
- Expression cloning

Isoforms & Evolutionary Precursors

- PCR

2. Determining the stoichiometry of receptors

Selective Tagging

Constraining Stoichiometry

3. Determining the structure of receptors

Membrane Topology

- Hydropathy Plots
- Engineered Reporter Sites

3-Dimensional Structure

- Electron Cyromicrocopy
- X-ray crystallography

Determining the Structure of Receptors

Further Reading

1. Hille, B. (1996) Ion channels of excitable membranes; Structure of channel proteins Chapter 13, 3rd Edition, Sinauer Associates, Inc.

2. Hollmann *et al* (1989) Cloning by functional expression of a member of the glutamate receptor family. Nature, 342, 643-648.

3. Hollmann *et al* (1994) N-glycosylation site tagging suggests a three trans-membrane domain topology for the glutamate receptor GluR1. Neuron, 13, 1331-1343.

4. Unwin, N. (2000) The Croonian Lecture 2000. Nicotinic acetylcholine receptor and the structural basis of fast synaptic transmission. Phil. Trans. R. Soc. Lond. B Biol. Sci., 355, 1813-1829