Structure-Function Studies

• Need a way to vary structure systematically

• Conventional site-directed mutagenesis is inadequate - the 20 natural amino acids provide limited structural diversity

• We vary structure using unnatural amino acid mutagenesis
Unnatural Amino Acid Incorporation into Ion Channels Expressed in Oocytes

1. gene for the protein with "stop" codon at site of interest
   - mRNA
   - STOP ("nonsense")
   - codon (UAG - amber)

2. a tRNA with:
   - a: the appropriate anticodon
   - b: an unnatural amino acid
   - unnatural amino acid chemically appended
   - "amber suppressing"
   - tRNA from Tetrahymena thermophila
   - appropriate anticodon

3. inject both into cell
   - Xenopus oocyte

4. folding; processing; assembly; transport to surface

5. electrophysiology

Wide range of unnaturals:
How do we do this?

tRNA Synthesis, Part A

- Prototype tRNA is 76 bases, always ending in CCA.
- First 74 bases are made by transcription from a synthetic gene (DNA); necessary anticodon is designed in.
The last two bases are chemically synthesized:
Synthesis of Unnatural Amino Acids

The unnatural amino acid

Also use 4-PO protecting group (previous slide); removed with I₂
Aminoacyl-tRNA Synthesis

Stored with amino group protected until just prior to injection
tRNA Design Issues

• the tRNA should be an efficient suppressor

• the tRNA must be *orthogonal* to the oocyte expression system

• not recognized by the endogenous aminoacyl-tRNA synthetases; i.e., not charge with a natural amino acid after it delivers the unnatural amino acid
tRNA DESIGN

yeast tRNA-Phe with amber suppressor anticodon effective in vitro - Schultz (1989)

reacylated in vivo

MN3 new mutations introduced to suppress reacylation effective at several sites of the nAChR in vivo

Nowak, et al. (1995)

THG73 utilize non-standard genetic code of Tetrahymena thermophila.
Natural amber suppressor (codes for Gln).
Much more efficient.
Nearly eliminates reacylation problems.

Saks, Sampson et al., JBC, (1996)
**Structure-Function Study**

*Flow of ions through a channel is equivalent to electrical current*

**Functional Probe: Electrophysiology**

Simply inject DNA or mRNA into oocyte, return 24 hours later.

Ligand-gated ion channel is expressed and it responds to neurotransmitter (e.g., ACh) by generating a current.

Physiology and pharmacology same as in natural environment.
Structure-Function Study

Single molecule recording via the patch clamp

Functional Probe: Electrophysiology

The Patch Clamp

Single molecule kinetics in real time
A Wide Range of Channels & Receptors

• Nicotinic Acetylcholine Receptor
  \(\alpha, \beta, \gamma, \delta\), of muscle; \(\alpha 4, \alpha 7\) of neuronal

• 5-HT\(_3\) Receptor

• NMDA Receptor

• GPCRs

• Transporter - GAT1

• K\(^+\) Channels
  voltage gated (Shaker, Shaker-IR)
  inward rectifying (Kir 1.1, 2.1, 3.1, 3.4)

• Na\(^+\) Channels

• CFTR

Any protein amenable to efficient heterologous expression