Chapter 1

An Introduction to Ligand-Gated Ion Channels and Summary of Dissertation Work

1.1 Synaptic Transmission

The basis of information processing in the nervous system involves both electrical and chemical signaling. Neurons function by propagation of electrical signals across their membranes, called an action potential, traveling down a neuron's axon. Each neuron connects with one- to ten-thousand (10^3–10^4) other neurons through specialized junctions, called synapses. To communicate with another neuron, the signal from the first neuron, called the pre-synaptic neuron, must move towards an axon terminal that has formed a synapse with the dendrite of the second neuron, called the post-synaptic neuron. Communication between neurons at synapses primarily involves a chemical signal — the information is encoded as small molecules called neurotransmitters. When the electrical signal in a pre-synaptic neuron reaches a synapse, the neurotransmitter is released. The binding of neurotransmitters to their specific receptors on the post-synaptic neuron causes ion channels to open. Ion conduction through the channels consequently alters the electrical potential across the membrane of the post-synaptic neuron, regenerating an electrical signal.

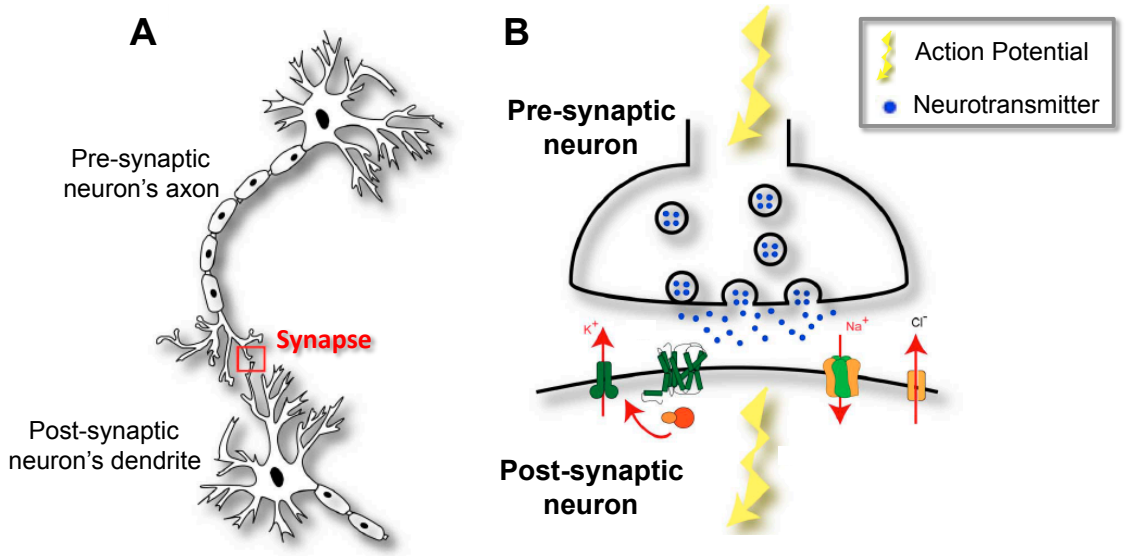


Figure 1.1. Synaptic transmission (**A)** Two neurons connect to form a synapse. **(B)** Propagation of information in the nervous system involves both electrical signal (an action potential) and chemical signal (a release of neurotransmitters)

Activated neuroreceptors either directly or indirectly produce electrical signals in the post-synaptic cell. The majority of neuroreceptors fall into two main classes, the metabotropic receptors and the ionotropic receptors. Metabotropic receptors couple to intracellular second-messenger systems through heterotrimeric G-proteins, and as such, they are known as G-Protein coupled receptors (GPCRs). In contrast, ionotropic receptors, also known as ligand-gated ion channels (LGICs), contain intrinsic pores that switch conformation from closed to open upon neurotransmitter binding, allowing ions to flow. GPCRs mediate slow synaptic transmission, acting through second-messenger pathways, whereas LGICs mediate fast synaptic transmission. In LGICs, binding of the neurotransmitter induces a conformational change in the protein that opens an ion-permeable pore that spans the cell membrane. Ion flow

upon channel opening either encourages or discourages the firing of an action potential in the post-synaptic neuron, depending on whether the LGIC is excitatory (cation selective) or inhibitory (anion selective).

1.2 Ligand-Gated Ion Channels (LGICs)

In vertebrates, the term LGICs specifically refers to three families of ionotropic receptors: Cys-loop receptors, ionotropic glutamate receptors (iGluRs), and P2X receptors (P2XRs). The Cys-loop family constitutes the largest class of LGICs. This family includes the nicotinic acetylcholine receptor (nAChR), 5-hydroxytryptamine-3 receptor (5-HT_3R), γ-aminobutyric acid receptor type A and C ($GABA_{A/C}$R), and glycine receptor (GlyR). The nAChR and 5-HT_3R are excitatory while the $GABA_{A/C}$R and GlyR are inhibitory. iGluRs are activated by the neurotransmitter glutamate, mediating most fast excitatory transmission in the central nervous system (CNS). Only in the case of NMDA receptors, glycine or *D*-serine is also required for activation. Finally, the most recently discovered ionotropic P2X receptors are activated by ATP.

All three families of LGICs are multimeric integral membrane proteins that incorporate extracellular ligand-binding sites and a transmembrane ion-permeable channel. A conformational change directly and very rapidly couples the binding of neurotransmitters to the opening of the channel, which activates within a few microseconds. The process that links neurotransmitter binding to the open conductance state of the receptor is termed "gating." The result is an

excitatory or inhibitory change in the electrical properties of the membrane, and in the case of channels that conduct Ca^{2+}, the entry of a second messenger. The structural rearrangement associated with activation of the ion channel poses important concerns in drug-receptor interactions and molecular recognition.

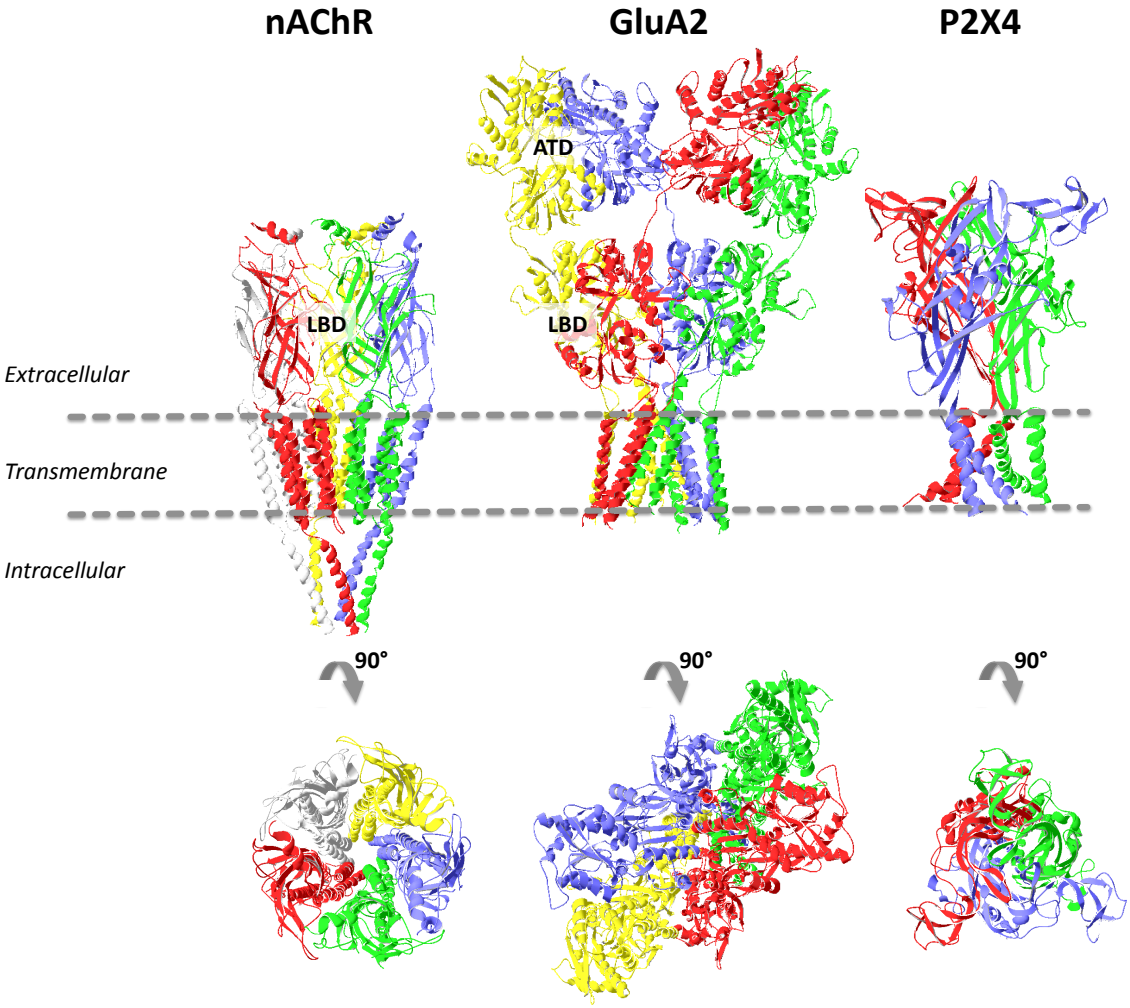


Figure 1.2. Examples of structures for the three families of LGICs: *Torpedo* nAChR (Protein Data Bank code 2BG9) for Cys-loop receptors, rat homomeric GluA2 receptor (Protein Data Bank code 3KG2) for iGluRs, and zebrafish P2X4 receptor (Protein Data Bank code 4DW0) for P2X receptors

An agonist of a receptor is a ligand that mimics the endogenous neurotransmitter by producing the same conformational change and the same biological response upon binding to the receptor. Receptors can be activated by endogenous agonists, such as neurotransmitters, or exogenous agonists, such as drugs. *Efficacy* of an agonist refers to the relative ability of the agonist-receptor complex to produce a maximum functional response. *Full agonists* bind and activate a receptor, displaying full efficacy at that receptor, while *partial agonists* also bind and activate a given receptor, but have only partial efficacy at the receptor relative to a full agonist. Receptor binding to an *antagonist* results in the inhibition of a biological response. An *antagonist* is a ligand that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

1.2.1 Cys-loop superfamily

The Cys-loop receptors are pentamers composed of five subunits arranged around a central ion-conducting pore (Figure 1.2, *left*). Subunits share a common structure consisting of a large, *N*-terminal extracellular domain that contains the agonist-binding site and also the signature disulfide loop, four transmembrane α-helices (M1–M4) that line the ion pore, and a short extracellular *C*-terminus. Nicotinic acetylcholine receptors (nAChRs) are the best-characterized members of the family and are therefore generally considered the prototypical Cys-loop receptor (*1*–*3*). The works described in this dissertation primarily focus on nAChRs.

The nAChRs mediate rapid synaptic transmission in the central and peripheral nervous systems (*1*, *4*, *5*). They are activated endogenously by the neurotransmitter acetylcholine. Nicotine, the active compound of tobacco, coincidentally activates these receptors. To date, seventeen nAChR subunits have been cloned: α1–α10, β1–β4, γ, δ, and ε (*3*). These subunits arrange as homo- or hetero-pentamers to form more than 20 active and pharmacologically distinct nAChR subtypes in humans. Of these subtypes, the *muscle-type* $(\alpha1)_2\beta1\gamma\delta$ is the best studied owing to its precise subunit stoichiometry.

No high-resolution structure of a nAChR exists, but a significant amount of relevant structural information is currently available. The identification and structural characterization of a family of snail acetylcholine-binding proteins (AChBPs) was the major advance in the early 2000s (*6*–*11*). The AChBPs are soluble, pentameric proteins that share 20–25% sequence identity with the extracellular ligand-binding domain of the nAChRs. Their high-resolution x-ray crystallography structures have served as structural templates for many functional studies of the residues involved in ligand binding in the nAChRs, including agonists, antagonists, and allosteric modulators. However, the AChBPs are simply soluble proteins that evolved to contain a binding site and do *not* contain an ion channel. As such, they offer little information about the activation/gating pathway of the nAChRs.

A structure of the full-length receptor at medium 4.0 Å resolution has been achieved by electron microscopy (EM) studies of the nAChR from *Torpedo* electric ray (*12*–*14*). Many amino acid side chains cannot be resolved in this cryo-

EM structure, but it does provide a general picture of the overall topology and secondary structures of a full-length protein. In 2007, a crystal structure of the extracellular domain of the nAChR α1 solved at 1.94 Å resolution was published (*15*). The glycosylation patterns are well resolved at this resolution, but the key agonist-binding Trp residue is missing from this structure.

The most recent advances in Cys-loop receptor research are the publications of x-ray crystal structures of orthologous pentameric receptors from bacteria and archaea, which belong to the same extended family as the vertebrate Cys-loop receptors called the pentameric ligand-gated ion channel (pLGIC). The x-ray structure of a prokaryotic pLGIC from the bacterium *Erwinia chrysanthemi* (ELIC) at 3.3 Å resolution in the presumed close conformation was published in 2008 (*16*). A year later in 2009, two x-ray crystal structures of a proton-gated pLGIC from the bacterial *Gloeobacter violaceus* (GLIC) appeared at 2.9 Å and 3.1 Å resolutions, and both are believed to be in the open conformation (*17*, *18*). The expression of these bacterial channels yielded functional cationic ion channels (*19*, *20*). The first structure of a eukaryotic member of pLGIC, the *anionic* glutamate receptor from *C. elegans* (GluCl), was recently solved at 3.3Å resolution (*21*).

From the available structural information, it is now well accepted that agonists bind at the interface of adjacent subunits in the nAChR pentamer (*1*, *4*, *5*). The agonist-binding site is a compact pocket comprised of amino acids from several noncontiguous regions from the *principal* (always an α subunit) and *complementary* subunits (such as the γ, δ, or ε subunits in the muscle subtype and

the β2 or β4 subunits in the neuronal nAChRs). Five conserved aromatic residues form what is known as the *aromatic binding box*; e.g., α1Y93 (loop A), α1W149 (loop B), α1Y190 (loop C), α1Y198 (loop C), and ϒW55/δW57 (loop D) of the muscle type. There are two agonist-binding sites in a receptor, and it is known that both must be occupied to optimally activate the receptor. The fifth subunit that is not involved in the binding site formation is termed the *accessory subunit*; e.g., the β1 subunit of the muscle nAChR.

The ion channel pore is lined by the M2 helix from each subunit of the pentamer. Each M2 helix contributes several highly conserved hydrophobic residues that constitute the channel gate. The leucine-9′ residue (where 9′ represents the ninth residue from the cytoplasmic end of the transmembrane helix) comprises the narrowest constriction point in the *Torpedo* cryo-EM structure and is located at the approximate midpoint of the M2 helix (*14*). This residue has been shown to play a critical role in channel gating, and when mutated to a more polar amino acid, the pore is stabilized in an open, ion-conducting conformation (*22*, *23*).

1.2.2 Glutamate-gated ion channels (iGluRs)

Tetrameric iGluRs are widely expressed in the central nervous system where they mediate fast excitatory synaptic transmission in the brain of vertebrates. Eighteen human iGluR genes were cloned, and 4 major classes of iGluRs have been identified to date. The NMDA receptors that play key roles in

synaptic plasticity are obligate heteromers formed by co-assembly of different combinations of the GluN1, GluN2A–GluN2D, GluN3A and GluN3B subunits. Their ion channels are Ca^{2+} permeable ion and are blocked by extracellular Mg^{2+}. Other subfamilies of iGluRs are not sensitive to Mg^{2+} block. GluA1–GluA4 are co-assembled to form homomeric or heteromeric AMPA receptors that mediate fast excitatory synaptic transmission at the majority of central synapses. Kainate receptors regulate neuronal excitability and are formed by co-assembly of GluK1–GluK5. GluD1 and GluD2 have not been shown to form functional channels.

The first crystal structure for a *full-length* iGluR is the structure of the GluA2 homotetramer, solved at a resolution of 3.6 Å (*24*). The crystal structure of the full-length AMPA receptor reveals that each AMPA receptor subunit is organized into four discrete regions: the amino terminal domain (ATD), the ligand-binding domain (LBD), the transmembrane ion channel pore domain (TMD), and the cytoplasmic domain (Figure 1.2, *middle*). The transmembrane segments are organized in the expected 4-fold symmetry found in voltage-gated ion channels, but with inverted topology. Each subunit has 3 membrane-spanning helices plus a pore helix. The third transmembrane helix from the four subunits forms a bundle crossing, which acts as a barrier to ion permeation and forms the lining of the pore. The extracellular domains form 85% of the mass of an iGluR core. The extracellular domains are loosely packed assemblies with two clearly distinct layers of ATDs and LBDs, each of which has both local and global 2-fold axes of symmetry. Different subunit pairs form dimer assemblies in

the ATD and LBD layers — this subunit crossover was entirely unexpected prior to the publication of this crystal structure.

In addition to the full-length structure, it is possible to express the extracellular domains of iGluRs, both ATD and LBD, as soluble proteins genetically excised form the ion channels. Several x-ray crystal structures of the ATD and LBD are currently available at high resolution. The LBD appears to have a clamshell-like shape that is formed by two domains, D1 and D2, and the ligands bind in the cleft between the two domains (*25*). Because of the difficulty of expression and crystallization of a full-length receptor, the crystal structures derived from these soluble proteins continue to provide valuable structural insights into binding of ligands, mechanism of activation, as well as allosteric modulation by drugs and endogenous ions.

1.2.3 P2X receptor family

P2X receptors are non-selective cation channels gated by extracellular ATP. They are widely expressed in many tissues and are believed to play key roles in various physiological processes such as nerve transmission, pain sensation, and the response to inflammation (*26*–*31*). There are seven P2X receptor subunits in mammals: P2X1–P2X7. They co-assemble into a homomeric or heteromeric trimer (Figure 1.2, *right*). The ATP sensitivity and functional properties of P2X receptors vary widely, including the ATP affinity, ion permeability, and desensitization kinetics (*32*).

Crystal structures of P2X receptors only became available very recently. The first publication of the x-ray crystal structure of zebrafish P2X4 in 2009 represents the greatest breakthrough (*33*). Very recently, a structure for P2X4 in the ATP-bound form was published (*34*). The structures confirm several findings from previous mutational studies. The ATP-binding site is identified to be interfacial between two subunits (*35*–*40*), and the channel gate is located at the external portion of the second transmembrane helix (*41*, *42*).

No P2X-receptor related protein has been identified in the genomes of *D. melanogaster*, *C. elegans*, yeast or prokaryotes (*43*), unlike the other two families of LGICs, which makes their evolutionary origins a mystery.

1.2.4 Crosstalk between LGICs

Fast neurotransmitters such as GABA–Glycine (*44*), ATP–GABA (*45*, *46*), and ATP–acetylcholine (*47*–*49*) are co-released during synaptic transmission. Interaction between their respective receptor channels is likely to play a critical role in shaping the synaptic responses. In fact, cross interaction between two structurally and functionally different LGICs has been demonstrated in the form of non-independent receptor function, both in cultured neurons and heterologous expression systems. Co-activation of both receptors in an interacting pair typically leads to a cross-inhibitory interaction that translates into non-additivity of the recorded currents. For example, a number of P2XR subtypes were shown to interact with members of the Cys-loop receptor family,

including nAChRs, 5-HT_3R, and $GABA_{A/C}$ receptors (*50*–*63*). Furthermore, interactions between GlyR and $GABA_A$ receptors (*64*, *65*), as well as between AMPA receptors and NMDA receptors (*66*), have been reported. Evidence supporting physical association between these receptors is also available (*52*–*54*).

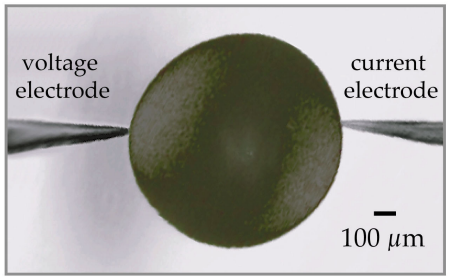
1.3 Methods for Investigation of Ion Channel Function

1.3.1 Two-electrode voltage-clamp recordings on *Xenopus* oocytes

We used *Xenopus* oocytes, egg cell precursors from an African frog, for expressing and investigating the function of LGICs. These cell are very large in size, ~ 1 mm in diameter, which allows a physical injection of RNA and other materials into the cells. Upon mRNA injection, the cell synthesizes, folds, assembles, and transports the protein to the surface of the cell membrane.

When an agonist is applied to an oocyte expressing an LGIC, ion pores open, allowing current to flow across the cell membrane. Current recordings on oocytes are conducted in a whole cell two-electrode voltage-clamp mode (Figure 1.3). In this setup, one electrode measures the voltage difference across the cell membrane, and the other electrode injects current into the cell to hold it at a particular voltage, typically at −60 mV. A feedback circuit connected to the voltage electrode is used to determine the current required to maintain this potential. The current electrode must inject current equal to that passing through the open channels, and therefore, the required current is a direct measure of the sum of all ion channel gating in the cell.

Functions of wild-type and mutant LGICs are evaluated in this setup by measuring the current response to agonists or antagonists applied to the cell. Increasing concentrations of agonists induce increasing current magnitudes (up to saturation) because more ion channels are open. Increasing concentrations of antagonists produce the opposite effect. Across wild-type and mutant receptors, we use EC_{50} as a convenient metric to compare ion channel functions and IC_{50} to compare receptor sensitivity to antagonists. Agonist EC_{50} and antagonist IC_{50} are determined by fitting the dose-response data to the Hill equation, and these values are the effective concentrations at the midpoint of the dose-response curves.



Xenopus oocyte

Figure 1.3. Current recording from a *Xenopus* oocyte on a whole-cell two-electrode voltage-clamp setup

1.3.2 Unnatural amino acid mutagenesis

Unnatural amino acids (UAAs) are synthetic amino acids that are not found in nature. Site-specific incorporation of unnatural amino acids permits systematic probing for structure-function correlations at the chemical scale. This technique offers much greater precision compared to the conventional

mutagenesis technique, which is limited by the natural twenty amino acids, because one has a rational control over the modification introduced into the protein of interest.

Several methods are available for incorporation of unnatural amino acids into proteins. In the present work, we employed the *in vivo* nonsense-suppression methodology (*67–70*). This method allows for the site-specific incorporation of a synthetic amino acid into a protein expressed in a living *Xenopus* oocyte. The procedure begins with replacing the amino acid position of interest with a non-coding codon (nonsense codon), usually a stop codon, by conventional site-directed mutagenesis (Figure 1.4). This would typically result in the premature termination of the protein's biosynthesis, but it is not the case here because we supply the translation system with a suppressor tRNA, a tRNA whose anticodon recognizes the nonsense codon. We pre-couple the suppressor tRNA with a desired unnatural amino acid enzymatically. The suppressor tRNA is designed to be orthogonal, which means it is not recognized by any of the endogenous aminoacyl-tRNA synthetases.

Once we inject both the mRNA and the tRNA-UAA into *Xenopus* oocytes, the translation then proceeds with the unnatural amino acid incorporated into the protein at the site of interest. Two different stop codons are utilized for site-specific incorporation of two unnatural amino acids into a receptor, as demonstrated in **Chapter 2** of this thesis. Alternatively, one may employ a closely related strategy for unnatural amino acid incorporation in *Xenopus*

oocytes, which is known as *frameshift suppression* (*71*, *72*). This technique utilizes a four-base codon (GGGT or CGGG) instead of a nonsense codon.

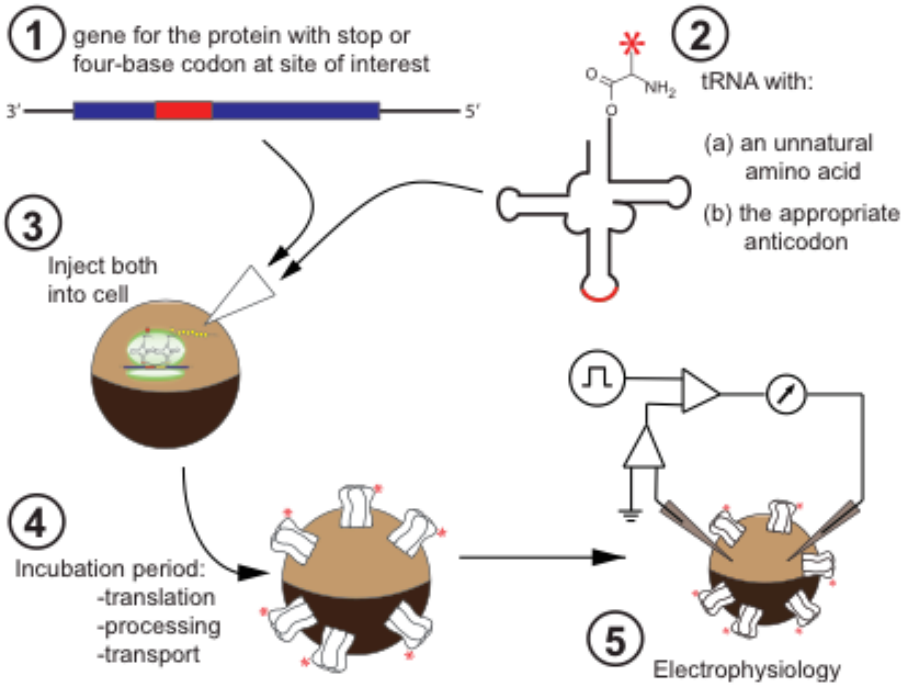


Figure 1.4. The nonsense-suppression methodology for incorporating unnatural amino acids into ligand-gated ion channels expressed in *Xenopus* oocytes

1.4 Summary of Dissertation Work

This dissertation describes three studies involving all three families of the LGICs. We demonstrated that the intrinsic receptor structures, drug-receptor interactions, and receptor-receptor crosstalk are determinants of receptor function and ion channel activities.

Chapter 2 describes a detailed structure-function investigation of the conserved Phe-Pro motif in the Cys loop of the muscle-type nAChR. This motif is universally conserved among the pentameric receptor channels. Both residues were substituted with natural and unnatural amino acids. In the receptor, a strong interaction between the Phe and Pro residues is evident, as is a strong preference for aromaticity and hydrophobicity at the Phe site. A similar influence of hydrophobicity is observed at the proline site. We also observed a correlation between receptor function and *cis* bias at the proline backbone across a simple homologous series of proline analogs, which could suggest a significant role for the *cis* proline conformer at this site in receptor function.

Chapter 3 concerns the key binding interaction of memantine, a prescribed drug for Alzheimer's disease, on the NMDA receptor. The data suggest that the special property of memantine as a potent NMDA receptor blocker stems from the presence of the two methyl groups and a proper shape-matching to the binding site. Comparing affinities of memantine and amantadine, a structurally related drug but lacking the methyl groups, in response to pore mutations enables us to identify the methyl group binding pockets for memantine on the NMDA channel pore.

Chapter 4 describes an investigation of the inhibitory crosstalk between α6β4-containing nAChRs of the Cys-loop superfamily and P2X2, P2X3, and P2X2/3 receptors. When the two distinct receptors are co-expressed in *Xenopus* oocytes, their biophysical properties are modulated from their normal behavior when expressed alone. The effect is constitutive and does not require channel

activation. When they are co-activated by their respective agonists, the observed current is smaller than the sum of the currents evoked by individual application of their agonists. Proposed molecular mechanisms for the cross interaction are also discussed.

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