Chapter 2
Ligand-Gated Ion Channel

One of the problem of mathematical biology for physics is the study of structures of biological membranes and their functions among which the problem of charge transfer. There are various types of ion transport such as ion channels. Ion channels can be described as transmembrane proteins of excitable cells that allow a flux of ions to pass under defined circumstances. Channels may be either voltage-gated or ligand-gated. They tend to be relatively ion-specific and allow fluxes of typically ions.

Voltage-gated ion channel whose permeability of ions is sensitive to the transmembrane potential difference. However, in these dissertaton, mainly we are interested in the other one, Ligand-gated ion channel. Its permeability is increased by the binding of a specific ligand, typically a neurotransmitter at a chemical synapse. The permeability change is often severe. When no ligand bound, channels let through no ions. When a ligand is bound, it allow passage at up to 10^7 ions per second. It is also the potential-dependent ion channels. The functional organizations are a selective filter which imparting specificity of the channel with respect to various ions and a sensor of the membrane potential. Sometimes, it can be called gate mechanism functioning to open and close the ion channel in the potential's sign and its value.

2.1 Nonstationary Lyotropic Model

Ion channels locate in the lipid bilayer of the membrane, lyotropic model, and mediate the ion motion across the membrane by using its electrochemical po-

tential. The study of real ion channels allows the experimental data and some assumptions.

In ligand-gated ion channels, transmembrane curerents here will be calculated in a nonstationary. The model is lyotropic in the sense that dynamics become nonlinear functions of the reactant concentrations. The derived total currents fit recorded data significantly better than those derived from mass action, Ising, and other stationary type models.

Ion channels are excitable proteins that form hydrophilic pore across membranes of nerve, muscle, and other tissue. They produce and transduce electrical signals in living cells and are responsible for a large number of cell regulatory functions and network interactions such as in the immune and central nervous systems. The ion channel is therefore also a major target for drug therapy. The molecular basis for cooperative ligand-receptor interaction and various gating mechanisms of ion channels is hence a central issue in contemporary biological physics.

Many studies have considered the stochastic nature of gating mechanisms governing the single ion channel and concomitantly fluctuating current observed through patch clamp technique see more in Hamill, Marty, Neher, Sakmann and Sigworth (1981) and Hille (1992). The measurable quantities of interest to electrophysiologists are the mean current and the current variance due to random opening and closing of ion channels. Corresponding response dynamics and associated conformational transitions of transmembrane channel proteins have thus been described in Markov processes by Colquhoun and Hawkes (1981, 1982) and Horn (1984), other diffusion model by Millhauser, Salpeter and Oswald (1988) and Lauger (1988), fractal time theories by Sansom, Ball, Kerry, McGee, Ramsey and

Usherwood (1989), and ferroelectric model by Bystrov, Lakhno and Molchanov (1994). Cooperativity and superposition of many different or identical ion channels have also been studied in Ising and lattice type models in Kijima and Kijima (1980), Liu and Dilger (1993), and Ghosh and Muherjee (1993).

The response from a system of ion channels, the electric current, proportion to the open probability times the number of ion channels. It may be induced by a shift in the voltage-gated, the ligand-gated, or mechanical deformation of the channel.

The ligand-gated channel current is derived in stationary such as Hill (1913), Langmuir (1918) equations, also Ising and lattice models. However, the response and ligand concentration also depend on the concentration of receptors and both initail reactant concentrations.

Two different levels of interaction can give rise to molecular cooperativity in biological systems. The interaction between subunits of a macromolecular channel protein called long range cooperativity and the interaction between individual themselves called short range cooperativity.

For instance, in hemoglobin the first kind of interaction is apparent. Binding of oxygen O_2 at four receptor sites on a hemoglobin(Hb) molecule is coupled such that binding of the first oxygen increases the affinity of hemoglobin for binding of additional oxygen molecules. The binding could be seen as the chemical reaction,

$$nO_2 + Hb_n \rightarrow (HbO_2)_n$$

in which n is the number of binding sites per hemoglobin molecule ($n = 4$). The rate equation for binding of oxygen molecule to hemoglobin as

$$\frac{\partial \Psi}{\partial t} = k\ \rho^n\ r - k'\Psi \tag{2.1}$$

where Ψ is the concentration of oxygen-hemoglobin complexes, ρ is the concentration of oxygen, r is the concentration of hemoglobin, and also k' and k are the dissociation and the association constant, respectively which define the affinity K as

$$K = \frac{k'}{k} \tag{2.2}$$

If we assume that K is identical for all binding sites, that is the binding of one ligand does not influence the binding of others to binding site on membrane. However, it is common for ligand-binding interactions to display such influences. The binding of one ligand to receptor may encourage the binding of a second ligand to binding site on membrane. That is said to be cooperativity.

From Eq.(2.1), it can be considered in stationary model based on mass action law by condition

$$\frac{\partial \Psi}{\partial t} = 0 \tag{2.3}$$

Derived from Eq. (2.1) with condition in Eq. (2.3) by Eq. (2.2), then

$$K = \rho^n \frac{r}{\Psi} \tag{2.4}$$

Matsson(1996) assumed a response theory linearly proportional to the ratio of occupied to total number of receptors with boundary initial constrain $r_0 = r + \Psi$. The response(S) is given by

$$S = \frac{\Psi}{\Psi + r}\ 100\% \tag{2.5}$$

These quantity must be considered in percentage and we can be written in peak maximum comparing with the measured scale. Hill found the expression for fractional saturation S of hemoglobin as

$$S = S_{\max} \frac{\rho^n}{\rho^n + K} \tag{2.6}$$

where $S_{\max}$ is the peak of scale in measurement or maximum effect. This type of response predicts a scaling in the ligand concentration in units of $K^{1/n}$, which usually deviates from the half maximum response of Effective Concentration written EC_{50} assessed. In experiment, it is not always easy to measure the maximum effect because an error in it will affect all the data. The power n(sometimes written n_H), is also called Hill coefficient, has sometimes been called the slope of Eq. (2.6) although it determines the steepness of the curve this is perhaps misleading because the slope is not constant. However, the researcher are usually interested in the slope in the middle part of the curve, which is roughly linear and is the region used in bioassays.

This discrepancy, in ion channel systems, can be due to transport of ions through the channels, is induced long before a stationary state in the ligand-receptor interaction is developed. From the data assessed by Bevan, Oriowo and Bevan (1986), Mackey (1988), Bevan, Bevan, Kite and Oriowo (1988), Barlow and Blake (1989), and Kenakin (1989) show a response derived from stationary theories applied to nonstationary lyotropic biological systems may deviate by several orders of magnitude see in Fig. (2.1: (b)).

Kijima and Kijima (1980) found two-state model differencing between the response induced(cooperative interaction) by subunit protein on a lattice of receptors strongly interacting through short range forces (contact interaction) and systems of identical multisubunit proteins(oligomers) weakly interacting at a distance through long range forces. The model was indicated depending on the restricted relation between the Hill coefficient and cooperativity.

2.2 Short Range Cooperativity: Ising Model

The contribution from short range cooperativity to the Hill coefficient has also been evaluated in a nearest neighbour interaction, Ising model, by Liu and Dilger (1993). Ising model was first introduced to describe interactions between fermions which are arranged in an array and are placed in an external magnetic field. This model can be applied to the study of interactions between ligand-gated ion channel in a biological membrane. However there are only two distinct states as A is the unliganded state, and B is the full liganded state. These mean A with the nonconducting closed state and B with the conducting open state. The open channel probability will be a function of the ligand concentration entering into the expression for the chemical potential of the system.

Liu and Dilger (1993) consider an one-dimensional square lattice with n ligand binding sites on the surface membrane and a linear combination of the three interaction potential energies J, which is ε_{AB}, ε_{AA} and ε_{BB}, in units of k_BT , between nearest neighbouring closed/open (AB), closed/closed (AA), and open/open (BB) ion channel pairs. J defines as

$$J = \varepsilon_{AB}/2 - \varepsilon_{AA}/4 - \varepsilon_{BB}/4 \tag{2.7}$$

They obtained a Hill coefficient

$$n_H = n \exp[2J] \tag{2.8}$$

The energy of the system depending on n is

$$H = \frac{n\ (\varepsilon + \theta)}{2\ k_BT} + \frac{(\varepsilon_{AA} - \varepsilon_{BB})}{2} \tag{2.9}$$

where ε is the binding energy of one ligand molecule reduces the energy of the system and θ is the chemical potential energy of the ligand in solution which is defined as

$$\theta = \theta_0 + k_B T \ \ln [\rho] \tag{2.10}$$

where θ_0 is the standard chemical potential of the ligand and ρ is the ligand concentration. Then yields an open probability

$$P_0 = \frac{1}{2}\left(1 + \frac{\sinh [H]}{(\sinh^2 [H] + \exp [-4J] \ )^{1/2}} \right) \tag{2.11}$$

and total mean current of N identical ion channels and current i per channel are obtained

$$\langle I \rangle = N \ i \ P_0 \tag{2.12}$$

However, Eq. (2.11) scales in a constant related to the ligand binding equilibrium constant, which obscures a molecular physical interpretation much the same as for Eq. (2.6) and other stationary models applied in nonstationary systems.

Another serious problem with such models is that the Hill coefficient could be derived from quite different forms of local cooperativity and it is unclear which form is the correct one. In other words, without obtaining knowledge of previously molecular stated for the threshold for response, and how the slope of response depends on the short range interaction of molecules, then what is the value of a study in which the aims are reduced to find a curve that fits data, and what do we learn from experiments at a molecular level? What is the impact of such effects on firing of neurons and related biological functions, and the potency[1] and efficacy[2] of various humoral factors and drugs on organs with

[1]potency- It is closely related to the affinity of the ligand for the receptor. One is more potent than another because it has a smaller EC_{50}.

[2]efficacy- the maximum response that ligand can elicit.

large variations in the expression of vacant receptors? Without a correct physical treatment, discussions tend to become formal. These are some reasons for the subsequent physical, but unusual approach to what is usually considered to be familiar problems.

Contrary to the stationary type models, in a nonstationary, long range interaction, lyotropic model, which was derived previously by Matsson (1996) and Grzegorczyk, Jacobsson, Jardemark and Matsson (1998), we find that in a system of ion channels parameters like the threshold for response and EC_{50} depend markedly on the ligand and receptor concentrations and their start values.

Matsson (1996, 2001) studied proliferation of a leukemic gibbon ape cell line MLA-144 and DNA replication. In that case, $n_H = 1$, no net contribution due to short range cooperativity is expected. There is only one binding site per receptor. The derived response, its threshold and the scaling parameter, EC_{50} are in perfect agreement with assessed growth data from Smith (1982) compared in Fig. (2.1: (a)). Similarly, Grzegorczyk, Jacobsson, Jardemark and Matsson (1998), the derived response agrees well with the assessed whole cell ion channel current mediated by AMPA receptors expressed on *Xenopus* oocytes in Fig. (2.2). The derived slope of response, $n_H = 1$, agrees almost exactly with the values observed in both cases.

As will be demonstrated subsequently, in the suggested lyotropic model, signal firing of a neuron may occur as a result of coherence in the system of channel protomers, due to a long range interaction between the individual ion channels.

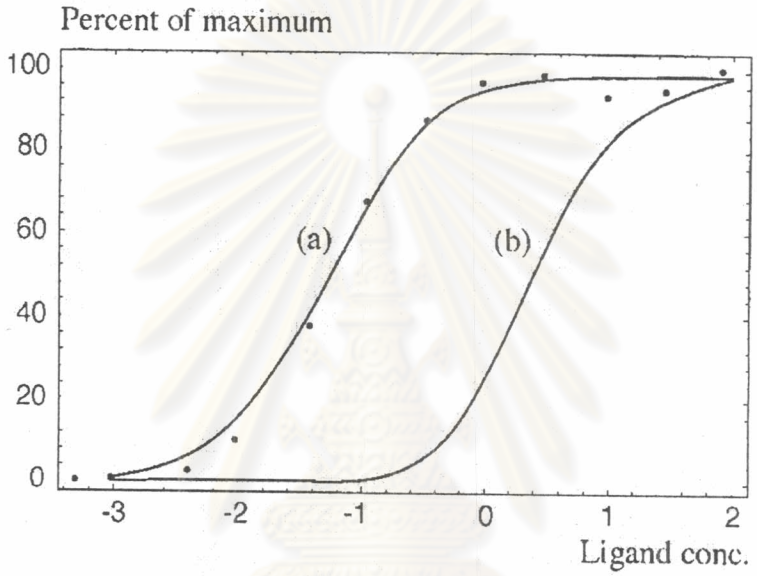

Figure 2.1: (a) Dose-response curve predicted by the derived nonstationary lyotropic model (solid line) compared with proliferation data (points) from the leukemic gibbon ape cell line MLA-144 with $EC_{50} = 0.055$ nM(b) Example of a dose-response curve derived from a mass-action type model with an assessed dissociation constant $K = 1.0 \pm 0.5$ nM

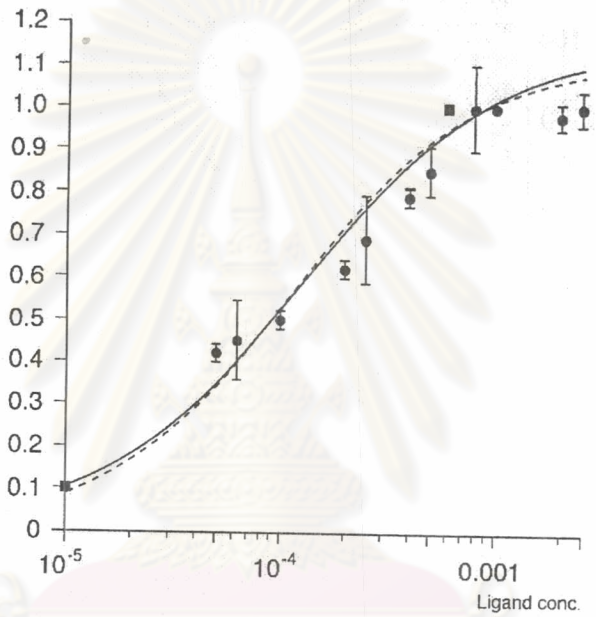

Figure 2.2: Comparison between recorded ligand-induced current data (points), Hill equation with $n_H = 1$ (solid line), and current derived from the derived nonstàtionary lyotropic model (dashed line). Response measured relative to peak response according to Celentano and Wong (1994).

2.3 Ligand Gating

Liu and Dilger (1993) assumed in the Ising type model, an ion-channel may have n different binding sites for a ligand, and a corresponding number of conformational substates. However, to demonstrate the nonstationary, lyotropic character of the scale and threshold for signal firing, for simplicity, each channel is here assumed to have only one binding site ($n = 1$) and two conformational states, liganded/open or unliganded/closed.

The rate of formation of ligand-receptor complexes, with concentration ψ, from Eq. (2.1), are obtained

$$\frac{\partial \Psi}{\partial t} = k\ \rho\ r - k'\Psi \tag{2.13}$$

through the initial constraints $\rho_0 = \rho + \Psi$ and $r_0 = r + \Psi$. Substituting into Eq. (2.13), the second order rate equation as

$$\frac{\partial \Psi}{\partial t} = k\,[\,\rho_K - \Psi]\,[\,r_K - \Psi] \tag{2.14}$$

where we use and define some expressions for compact form as

$$\rho_K = a + \sqrt{a^2 - b^2} \tag{2.15}$$

$$r_K = a - \sqrt{a^2 - b^2} \tag{2.16}$$

$$a = \frac{1}{2}\ (\ \rho_0 + r_0 + K) \tag{2.17}$$

$$b = \sqrt{\rho_0 r_0} \tag{2.18}$$

and also play the role of renormalized concentrations with

$$r' = r_K - \Psi \tag{2.19}$$

$$\rho' = \rho_K - \Psi \tag{2.20}$$

From Eq. (2.14), it can be integrated and carried out (see detail in appendix A)

$$\ln\left[\frac{\rho'}{r'}\frac{r_K}{\rho_K}\right] = 2\ k\ \sqrt{a^2 - b^2}\ t \tag{2.21}$$

Accordingly, in a high affinity system which $K = 0$, the dynamic concentrations in Eq. (2.21) can be replaced by $r_K = r_0$, $\rho_K = \rho_0$, $r' = r$ and $\rho' = \rho$ which will be used subsequently unless otherwise required. Then,

$$\ln\left[\frac{\rho}{r}\frac{r_0}{\rho_0}\right] = 2\ k\ \sqrt{a^2 - b^2}\ t \tag{2.22}$$

The time parameter t could be termed physiological time in the sense that it relates the usual time to the two continuous reactant concentrations.

Matsson (2001) previously demonstrated on DNA replication, many important biological functions. DNA replication are generated under nonstationary dynamical conditions. The response should be derived with Eq. (2.13) kept strictly nonzero. It implied a dependence of the entire system on the reactant concentrations. Apart from the threshold and scale, the numbers of nearest neighbours of liganded receptors, vacant receptors, and open and closed ion channels, also depend on the variable reactant concentrations. However, in stationary model these numbers are fixed and chosen ad hoc, thus obscuring the derived results. It is not just a question of modifying the standard chemical potential θ_0 by adding a term $k_B T\ \ln[\rho]$ to accommodate for the ligand concentration in the open probability and the energy of the system. The dynamics also depends on spare receptors and the initial reactant concentrations. Another problem is that depletion of the reactants, ligands and receptors, and the formation of ligand-receptor complexes in the actual nonstationary, chemically open system, require a modeling with all concentrations treated as probabilities.

2.4 Nonlocal Correlations

For instance, the definition of concentration of a ligand requires that each macroscopically small, but microscopically large, volume element about a receptor at a certain site should contains a large number of ligands and vice versa. However, at a critical ratio between the ligand and receptor concentrations, when ligands become almost depleted, the number of ligands in such a volume element decreases below one, and vice versa for the receptors. Both reactant concentrations should therefore be treated as probabilities.

Similarly, the concentration of ligand-receptor complexes, Ψ, plays the role of probability to find one ($\Psi = 1$), none ($\Psi = 0$), or any finite number of liganded channel complexes at a certain site x_υ, where $1 < \upsilon < n$, depending on the concentrations of the two reactants that form the complex. The probability to find a definite number of liganded receptors on ion channels (or excited synapses) at certain sites x_υ on a cell membrane should therefore be given by the infinite series of products

$$\begin{aligned} \varphi &= \mu \frac{\Psi(x_1)}{a}\frac{\Psi(x_2)}{a}\frac{\Psi(x_3)}{a}\cdots\frac{\Psi(x_n)}{a} \\ &= \mu \sum_{n=0}^{\infty}\left(\frac{1}{a}\right)^n \prod_{\upsilon=0}^{n}\Psi(x_\upsilon) \end{aligned} \tag{2.23}$$

where the surface membrane is approximated by an one dimensional aperiodic lattice. The term $n = 1$ in Eq. (2.23) accounts for the possibility of having a contribution already from the start. An arbitrary the n order product may represent $n - 1$ or a smaller number of particles depending on μ and the initial reactant concentrations, through the normalisation coefficient a . Similarly, any product with less than n factors may be proportional to the probability of finding n complexes.

In the slow interaction, long wavelength limit, where receptors and channels do not move or vibrate essentially relative to each other, Eq. (2.23) reduces to an infinite power series in Ψ , because, the spacing between receptors could then be neglected in comparison to the wavelength

$$\begin{aligned} \varphi &= \mu \sum_{n=0}^{\infty} \left(\frac{1}{a}\right)^n \Psi^n \\ &= \frac{\mu}{1-\Psi/a} \end{aligned} \tag{2.24}$$

The normalization parameters, μ and a, are to be determined such that firing of a neuron occurs at a definite number, N, of liganded ion channels, or excited synapses. This corresponds to a maximum electric current, $I_{\max} = N\, i$, induced across the membrane.

2.5 Total Current

Consider Eq. (2.13), the rate of ligandation of receptors on a patch of membrane or a whole cell, and rearrange term as the form

$$\frac{\partial \Psi}{\partial t} = k \left(1 - \frac{\Psi}{a}\right)^2 \left[a^2 - \frac{(a^2 - b^2)}{\left(1 - \frac{\Psi}{a}\right)^2} \right] \tag{2.25}$$

Rearranging form Eq.(2.24) as

$$\Psi = a \left(1 - \frac{\mu}{\varphi}\right) \tag{2.26}$$

and inserting Eq. (2.26) into Eq. (2.25), chosen the same normalization constant a in these two equations, it becomes

$$\frac{\partial \varphi}{\partial t} = \frac{k\, a}{\mu} \left(\mu^2 - \frac{(\, a^2 - b^2)}{a^2}\, \varphi^2 \right) \tag{2.27}$$

that is the rate for binding of ligands to ion channels with solution (see detail in appendix B)

$$\varphi = \frac{\mu\, a}{\sqrt{a^2 - b^2}} \left[1 + \tanh\left[k\sqrt{a^2 - b^2}\, t\right]\right] \tag{2.28}$$

The integration constant in Eq. (2.28) accounts for the possibility that some receptors / channels have been liganded / open already from the start. The total number of interacting membrane receptors / ion channels is given by

$$\begin{aligned} N &= \varphi(\infty) - \varphi(0) \\ &= \mu \frac{a}{\sqrt{a^2 - b^2}} \\ &= \mu \frac{\rho_0 + r_0}{\rho_0 - r_0} \end{aligned} \tag{2.29}$$

Depending on μ and the initial reactant concentrations, N can thus assume different values varied from one situation and subsystem to another. It is suggested that the neuron fires when it attains a threshold corresponding to a definite N value.

Combining the two solutions, Eqs. (2.22) and (2.28), the total probability for ligand binding to a patch of membrane or whole cell becomes

$$\varphi = N \left[1 + \tanh\left[\frac{1}{2} \ln\frac{\rho}{r}\frac{r_0}{\rho_0}\right]\right] \tag{2.30}$$

We use Eq.(2.5), response theory, we obtain

$$EC_{50} = \frac{r\ \rho_0}{E\ r_0} \tag{2.31}$$

which can be called a half-maximal response which becomes from Effective Concentration and E is an integration constant that could be related to efficacy (here $E = 1$) and r is the density of vacant (spare) receptors corresponding to closed ion channels. Then,

$$\varphi = N \left[1 + \tanh\left[\frac{1}{2} \ln\left[\frac{\rho}{EC_{50}}\right]\right]\right] \tag{2.32}$$

The current $I(\rho)$ with N liganded/open ion channels, is then proportional to

$$\begin{aligned} I(\rho) &= N\, i \left[1 + \tanh\left[\frac{1}{2}\ln\left[\frac{\rho}{EC_{50}}\right]\right]\right] \\ &= I_{\max}\frac{\rho}{\rho + EC_{50}} \end{aligned} \tag{2.33}$$

where

$$I_{\max} = 2\, N\, i \tag{2.34}$$

see more detail in appendix C.

2.6 One Binding Site per Channel

Cooperative effects, due to short range interaction between individual ion channel, may be well understood in Ising and lattice type models by Liu and Dilger (1993). However, with one binding site per ion channel, $n = 1$, in the limit of vanishing short range cooperativity, that is

$$J = 0$$

Then the energy of the system from Eqs. (2.9) and Eq.(2.10) becomes

$$H = \frac{(\varepsilon + \theta_0)}{2\, k_B T} + \frac{\ln[\rho]}{2} \tag{2.35}$$

and the total ion channel mean current approximate as

$$\langle I \rangle = N\, i\, \frac{1}{2}\,[1 + \tanh[H]] \tag{2.36}$$

By inserting Eq. (2.35) in Eq. (2.36) then

$$\langle I \rangle = N\, i\, \frac{\rho}{\rho + \exp[-(\varepsilon + \theta_0)/(k_B T)]} \tag{2.37}$$

and comparing with Eq. (2.33) one readily finds that the ligand concentration for half-maximal response, like in other stationary models, becomes a constant related to the equilibrium affinity constant,

$$\begin{aligned} EC_{50} &= \exp\left[-\frac{(\varepsilon+\theta_0)}{k_BT}\right] \\ &= K \end{aligned} \tag{2.38}$$

Corrections obviously due to short range cooperativity, $J \neq 0$, cannot account for the neglected nonstationary effects that makes the system depending on concentration.

Matsson (1996, 2001) studied for growth signaling in the leukemic gibbon ape cell line MLA-144, a direct relation between K and EC_{50} does not hold. In that cell line a half-maximal response was produced at $EC_{50} = 0.055\ nM$. Smith (1982) studied this binding and yield a value $K = 1 \pm 0.5\ nM$ for intermediate affinity IL-2 receptors. There are many reasons for this mismatch. One finds that the response is no longer linearly proportional to the receptor occupancy. Moreover, in high affinity systems the response is elicited predominantly at non-stationary boundary conditions in the ligand-receptor system. This can be shown in Fig. (2.1).

The same type of inconsistency is indicated in many ligand-gated ion channel systems with different agonists show EC_{50} and K, such as in glutamate (glu)-receptors of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) type see in Table (2.1). We have here selected examples of agonists [3] that induce a response without any net contribution to the slope coefficient due to short-range cooperativy, hence $n_H = 1$ is approximately valid. Bevan, Oriowo and

[3] agonist- This word use in phamacodynamic effect. Here is the ligand.

Agonist	EC_{50} (μM)	Binding K (μM)
S-sulfo-*L*-cysteine	59 ± 7.8	0.13
L-HCSA	546 ± 91	1.2
L-Cysteate	3310 ± 547	4.1
L-CSA	3981 ± 741	8.5

Table 2.1: Ligand-gated ion channel system in glu-receptors of AMPA type with different agonists representing EC_{50} and K values with $n_H = 1$. This data come from Patneau and Mayer (1990).

Bevan (1986), Mackay (1988) and Barlow and Blake (1989) observed that EC_{50} and $K^{1/n}$ values may differ by almost three orders of magnitude in the ligand concentration. Bevan, Bevan, Kite and Oriowo(1988) and Kenakin (1989) have speculated that the actual discrepancies could be due to transport proteins or a variable affinity. However, such effects cannot compensate for the lack of stationary (steady) state in the ligand-receptor dynamics.

O'Dell and Christensen (1989) studied voltage-clamp on native AMPA receptors in horizontal cells from stingray (*Dasyatis sabina*) represent an even more clear-cut example with a Hill coefficient $n_H = 1.06$. Patneaux and Mayer (1990) studied at $EC_{50} = 14.9\mu M$ upon exposure to quisqualate (QA), is here almost 3000 times larger than constant $K = 0.005\mu M$. Therefore, the mass-action based response with $n_H \approx 1$ hence $EC_{50} \cong K$ is displaced from assessed response data by more than three orders in the QA concentration.

Apart from this failure, mass action based response models have the unrealistic drawback of being independent of unoccupied receptors. In fact, both potency and response of a drug may vary appreciably from one tissue to another depending on large differences in receptor expression on different cells.

Celentano and Wong (1994) studied on the γ-aminobutyric acid (GABA) receptor function in patches from guinea pig hippocampal neurons. It is a ligand-

gate chloride ion channel found on the surface of virtually all vertebrate central neurons. They observed a triphasic response. Apart from a steady state current, exposure to GABA indicated fast, intermediate and slow components at values of 19 μM, 10.2 μM and 4.33 μM which are about 13, 7 and 3 times larger, respectively, than the separately assessed affinity constant $K = 1.47\ \mu M$. The corresponding Hill coefficients were $n_H = 0.93$, 1.06 and 1.00, respectively.