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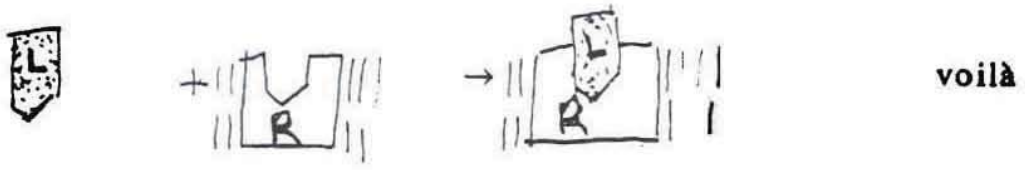
Cellular Neurobiology
Class Handout - Chip Quinn

Receptor Binding Made ~~Thrilling~~ Bearable

Neurotransmitters (ligands) usually act by binding with high affinity (i.e., tightly) to protein receptors on the postsynaptic membrane.



voilà

The binding affinity is usually high enough that one can detect specific receptor-ligand interactions even in membrane preparations from whole brain. Such binding studies are crude but easy and are often informative. Here's how to do it:

First: one buys or makes a radioactively labeled ligand -- either the neurotransmitter itself or a chemical relative of the neurotransmitter that acts as an agonist or antagonist. If several alternative ligands are available, one tries to choose the most potent -- potency of action usually correlates with tightness of binding.

Second: one prepares a (fairly crude) membrane fraction from whole brain or from some chosen part of the brain -- such as the hippocampus or cerebellum. One does this by homogenizing the brain or brain structure, removing crud with a brief, low speed centrifugation, then collecting the membrane fraction by centrifuging at higher speed. One can recover the membrane in the pellet at the bottom of the centrifuge tube, but ordinarily one collects it on a "shelf" over a layer of high-density, concentrated sucrose solution. One resuspends the membrane fraction, measures its protein content, then dilutes it to some standard concentration.

Third: in a series of test tubes one mixes aliquots (equal portions) of membrane with various concentrations of the radiolabelled ligand -- typically a serial dilution series from [L] = 10 μM to [L] = 0.1 nM. One allows a few minutes for the ligand and receptor to interact. Loud rock

music played during this waiting period allows one both to avoid boredom and to annoy one's neighbors. One then dumps each tube onto a glass filter in a vacuum filtration apparatus, and quickly washes the filters with ligand-free buffer solution. Unbound ligand goes through the filter and can be collected separately in the filtrate solution. Ligand which has bound to membrane receptors is retained with the membrane on the glass filters.

So we have rapidly done a series of experiments measuring bound ligand as a function of ligand concentration. What do we do with this information?

Intermission: A Brief Romp Through Theory Land

From the *law of mass action* (Chemistry), the rate of association between a receptor and a ligand molecule to form a complex is proportional to the concentration of each reagent. In other words, the rate of new R-L complex formation = [R] × [L] × a constant, call it k_{on}

Similarly the rate of R-L complex dissociation is the molar concentration of the complex, [RL] × another constant, k_{off}

Here [R] denotes the molar concentration of unbound (free) receptor; [L] is the concentration of free ligand; and [RL] is the concentration of ligand-receptor complex.

At equilibrium, the association rate = the dissociation rate and

$$\frac{k_{off}}{k_{on}} = K_D = \frac{[R][L]}{[RL]} \qquad (1)$$

K_D has the units of concentration (e.g., M, μM, nM). Note that the *tighter* the binding of a ligand to a receptor, the *smaller* the K_D for the interaction. Moderately loose receptor-ligand interactions typically have K_D's around 1μM. Very tight interactions have K_D's around 0.1 nM.

Act II Back To The Measurements

Now, kiddies, to understand our filter binding measurements in terms of equation (1) and K_D's we need to make a substitution, viz:

Total receptor = [R] + [RL]. This value, which corresponds to the maximum binding capacity of membrane fraction, we will call B_{MAX}. In other words, some of the receptor is bound and some is free, but the total is a constant, B_{MAX}.

If:

$$B_{MAX} = [R] + [RL]$$

then:

$$[R] = B_{MAX} - [RL]$$

Now we want to solve equation (1) for [RL] which corresponds to the counts bound to the filter. Clearing the denominator we get

$$K_D [RL] = [R][L]$$

Substituting the expression for [R] in equation (2) into equation (3):

$$K_D [RL] = [L] (B_{MAX} - [RL])$$

$$K_D [RL] = B_{MAX} [L] - [RL][L]$$

Solving for [RL]:

$$K_D [RL] + [L] [RL] = B_{MAX} [L]$$

$$[RL] = \frac{B_{MAX} [L]}{K_D + [L]} \qquad (5)$$

$$[RL] = \frac{B_{MAX}}{1 + \frac{K_D}{[L]}}$$

Equations (5) and (6) are alternate, equivalent forms of the equation which gives binding curve (i.e., the equation which gives [RL] the counts bound to the filter as a function of [L], the concentration of ligand). Textbooks tend to present equation (5) because it is a simple fraction and is easier to print. The pros tend to use equation (6) because it is easier on the intuition. That is, it is easier in equation (6) to see the relationship of K_D to [L] as a term in the denominator -- i.e., as a reducer of binding.

[Note that equation (6) looks *exactly* like the Michaelis-Menton equation for enzyme activity, V as a function of substrate concentration [S]

$$V = \frac{V_{MAX}}{1 + \frac{K_m}{S}}$$

This is because enzyme and substrate must associate to form a *complex* before catalysis occurs and a product is formed.

The figure below indicates how the equation (6) plots out -- in other words how ligand concentration influences binding.

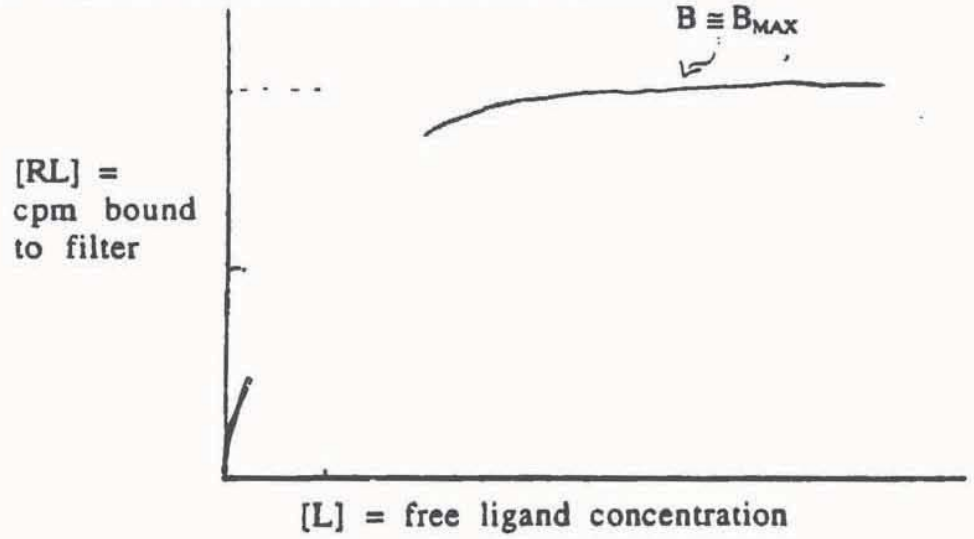


Figure 1

Note two things: (i) binding *saturates* at high ligand concentration. This is a characteristic of binding to molecularly defined receptors; (ii) half-maximal binding occurs when [L] = K_D. This follows from equation (6). It is a quick and easy way to find K_D from this series of filter-binding measurements.

Nonspecific Binding. Frequently the search for high-affinity receptors for a ligand is impeded by non-specific interactions of the ligand with other components (crud) in the membrane preparation. These non-specific interactions (e.g., general stickiness) are typically *low-affinity* and *non-saturable*. In theoretical terms, K_D and B_{MAX} for these non-specific interactions are both very large. The one convenient property of such interactions is that they tend to be *linear with ligand concentration over the range of interest*. This means one can subtract

them graphically. In figure 3, one notes the slope, m, of the linear part of curve A, (at high ligand concentration). One then plots the line B corresponding to the equation $y = mx$ through the origin, and subtracts off the nonspecific binding ($B_{non} = m\,[L]$) from the total binding to obtain curve C. Finally, one uses data from curve C to estimate K_D and B_{MAX} for the *specific* binding interactions. One does this estimation of these parameters either rough-and-ready, as indicated from figure 2...

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...or via a Scatchard plot, as described below.

A Scatchard Plot

In figure 2 we used only one or two binding measurements (those in the region of $B_{MAX}/2$) to estimate K_D. We would like to use all our measurements. Furthermore, in figure 2 we have no objective way to determine whether the saturable binding results from one type of receptor with one K_D, or from several types of receptors with slightly different K_D's. The easiest way to deal with these issues is to use a

different combination of variables (different x and y axes), so that binding data from a single K_D will give a straight-line plot. The current champeen of such plots is the Scatchard plot. This plot looks screwy and is in many ways difficult to intuit; but it is easy to derive.

In our algebraic manipulations to get the expression for binding (Equation 6 and Figure 2) from the definition of dissociation constant (Equation 1) we had the intermediate equation

$$K_D [RL] = B_{MAX} [L] - [RL] [L]$$

If we divide both sides of this equation by K_D and [L] we get:

$$\frac{[RL]}{[L]} = \frac{B_{MAX}}{K_D} - \frac{1}{K_D} [RL]$$

Hotcha! The Scatchard-plot equation. This equation is of the form of a linear equation

$$y = mx + b$$

with

$$b = \frac{B_{MAX}}{K_D}$$

and

$$m = \frac{-1}{K_D}$$

Except that the axes, x and y are unusual.

$$y = \frac{[RL]}{[L]} = \frac{[\text{Bound Ligand}]}{[\text{Free Ligand}]}$$

and

$$x = [RL] = [\text{Bound Ligand}]$$

Note that [RL] = molar concentration of ligand-receptor complex. [RL] is proportional to the counts bound on the filter. [L] = the molar concentration of the free ligand in the mixture to be filtered. Ordinarily one arranges the experiments so that a negligible fraction of the ligand

is actually bound, so that (free ligand concentration) _ (total ligand concentration). [If this approximation is inaccurate, one can easily determine [L] from the counts in the flow-through filtrate.]

Here is a typical Scatchard plot for high-affinity receptor binding

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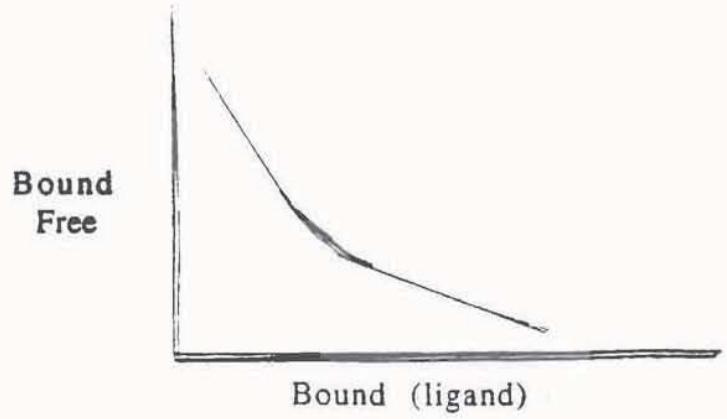
From this we can infer the total molar concentration of receptor [B_{MAX}] from the x-intercept and K_D from the inverse of the downward slope. For a Scatchard plot one uses only the *specific* binding activity (see figure 3).

There are several alternative coordinate transformations to equation (6) that give linear plots. The reason the Scatchard plot is the current champeen is because (empirically) it is the best at resolving multiple K_D's in the binding activity. Suppose one has two forms of opiate receptor, one with a K_D of 10 mM, one with a K_D of 1 mM. One would get a curve something like this:



which resolves into two relatively linear segments with different slopes.

[*Important*: If you ever turn into an experimental pharmacologist, and you do binding studies, *don't* take out a ruler and infer K_D's and B_{MAX}'s from the line segments. A complex Scatchard plot is *extremely* hostile to intuitive interpretation. What you would need to do, you pharmacologist you, is to optimize the fit of the curve using a special computer program, of which there are many.]

Competition (Inhibition-of-Binding) Assays

Given a radioactive ligand and its receptor (e.g., opium and the δ opiate receptor), one can search for and assay other ligands that bind to the same receptor. These might be organically synthesized compounds or natural products with neurotropic activity (e.g., morphine, Δ^9 tetrahydrocannabinol). Alternatively, they might be partially purified brain fractions which contain endogenous ligands (e.g., met-eukephalin, endorphin).

The new ligand candidates to be tested need not be radioactively labelled if one uses a *competition* or inhibition assay. To do this one obtains a radio-labelled ligand (L*) and a brain membrane fraction with binding activity as outlined above. One prepares tubes with aliquots of receptor. To this one adds labelled ligand to the concentration $[L^*] = K_D$ and various concentrations of the new candidate compound [C] (for competitor). One dumps these tubes onto glass filters and as before measures counts bound to each filter. If the new compound, C, binds to the same receptor as does L*, it will compete with L* for sites on the receptor and will decrease the amount of L* bound (e.g., radioactive counts-per-minute retained on the filter).

The idea behind this assay is simple to the point of boredom and indirect to the point of befuddlement. To repeat: the chief advantage of a competition assay is that it enables one to assay candidate ligands *without labelling them*. One can even purify new ligands from a crude gemisch.

The theory behind such competitive assays is that the competitor, C, forms a complex with its receptor. The receptor competitor interaction has its own characteristic dissociation constant, K_C.

$$K_C = \frac{[R]\,[C]}{[RC]} \qquad (9)$$

Globally,

$$RL^* + C \overset{K_D}{\rightleftharpoons} R + L^* + C \overset{K_C}{\rightleftharpoons} RC + L^*$$

Only this entity corresponds to radioactive counts retained on the filter

Since we are dealing with an *equilibrium* situation, it doesn't matter in which order we consider the receptor's interactions with its ligands. We will consider R-C interactions first. Note that the free R after competition (R′) will be diminished by the fraction γ where:

$$\gamma = \frac{R'}{R} = \frac{[R]}{[R] + [RC]} = \frac{}{1 + \frac{[RC]}{[R]}}$$

now from equation (9)

$$\frac{[RC]}{[R]} = \frac{[C]}{K_C}$$

so

$$\gamma = \frac{1}{1 + \frac{[C]}{K_C}}$$

Plugging R' ($= \gamma R$ into equation (3) we get

$$K_D[RL] = \gamma[R][L]$$

or

$$\frac{K_D}{\gamma}[RL] = [R][L]$$

carrying this derivation through as before, we get

$$[RL] = \frac{B_{MAX}}{1 + \frac{(K_D/\gamma)}{S}}$$

or

$$[RL] = \frac{B_{MAX}}{1 + \frac{K_D}{[L]}\left(1 + \frac{[C]}{K_C}\right)} \qquad (10)$$

If $[L] = K_D$ as we specified, this equation becomes

$$[RL] = \frac{B_{MAX}}{2 + \frac{[C]}{K_C}}$$

In practice, pharmacologists tune [L] to about K_D and measure IC_{50}, the inhibitor concentration that gives half the uncompeted displacement of the labelled radioligand. This is less quantitatviely spiffy, but it's close enought for quantitative comparisons. Shown below is a typical competition curve for two drugs which bind to a brain receptor for opiate compounds (see below).

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Applications:

This all is a bit of a megillah, and for what: how do receptor-binding studies help anyone really? Well for one thing, drug companies use 'em to find new drugs. For another thing, scientists use 'em to isolate receptors to high-potency psychoactive drugs (e.g., opiates), to understand their mechanism of action on the brain. Two examples:

) Pert and Snyder noted (Science 179: 1011, 1973) that opiates (morphine, heroine, codeine, etc.) had psychoactive effects at low dosage. They reasoned that the opiates must interact at these concentrations with some molecular entity in the brain. Therefore they labeled an opiate binding ligand, prepared membrane fractions from brain tissue, and looked for (and found) high affinity. (They were not the first individuals to conceive this idea, but they were the first to get it to work). An incisive control in their experiments was the demonstration of tight binding by the biologically active stereoisomer but not its inactive enantiomer.

Pert and Snyder's studies quickly spawned a cottage industry when they and other researchers noted that different opiate compounds had different spectra of physiological and psychological effects. Researchers eventually isolated, defined and cloned three major subtypes of opiate

receptor -- delta, kappa and mu. See Nichol's et al

The next step in this process was the insight by several researcgers that people probably didn't evolve opiat receptors just to get addicted to plant compounds -- there must be some natural, endogenous compound in the brain that bound to such receptors. Several groups purified small peptides from the brain based on their ability to compete with and inhibit the binding of radio-labeled opiate liquids to the previously characterized membrane receptors. When genes for encoding such peptides (endorphins, dynorphins and ekephalins) were cloned, they turned out to encode large peptides (Nichols et al, 274). The classification and anatomical localization of opiate receptors, together with the classification and localization of endogenous opioid legands, puts the treatment of pain and the treatment of addiction on a much more scientific basis than previously. It also facilitates the search for new and more specific ligands -- opioid agovests to use as analgesics on the one hand and opiate antagonists (such as methadone or naloxone) to use in addiction treatment on the other.

Work along exactly similar lines had been done to characterize receptors and natural ligands for Δ^9THC, the active ingredient in marijuana. Brain receptors have also been found for benzodiazepines (e.g. valium) and PCP (angel dust -- yum).

(2) The other stunning application of receptor-binding studies is in the partial understanding of schizophrenia. Schizophrenia is a disease with both genetic and environmental components to its origin. It affects about 1 percent of humans without evident discrimination by gender, race or level of cultural industrialization. Most affected individuals are moderately to severely incapacitated in their social dealings and competence; however the most severe symptoms of the disease can be ameliorated with compounds such as thorazine (p-chlorpromazine) which are variously termed major tranquilizers, neuroleptics, or antipsychotics.

The only solid information on the possible mechanism of the disease is summarized in the graph in figure 5 below.

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D_2 receptors are type-2 receptors to the neurotransmitter dopamine. Type-2 receptors are coupled to G_i (adenylate-cyclase-inhibitory) proteins in second-messenger cascades. They were originally distinguished from the majority D_1 dopamine receptors pharmacologically -- because D_2 receptors were bound tightly to a particular subclass of antipsychotic drugs (the butyrophenones) whereas D_1 receptors did not. Figure five shows a relatively tight correlation between the clinical efficacy of antipsychotic drugs (smallness of dose needed to alleviate symptoms) and their binding affinity to D_2 receptors. Antipsychotic drugs are *antagonists* to dopamine receptors.

Actually, I lied a little in the paragraph above. Once the D_2 receptor is purified it can be sequenced and the gene specifying it can be cloned. Researchers can then look through the human cDNAs for similar genes. By this method, two new members of the dopamine-receptor gene family D_3 and D_4 were identified, based on sequence similarity to the D_2 receptor gene. The dopamine binding activity of the D_3 and D_4 was confirmed by expressing them in cultured cells or *Xenopus* oocytes; their binding specificity is similar to that of D_2 receptors. Will the real receptor involved in schizophrenia please stand up? Very recent reports indicate that *D_4 receptors are overexpressed* in schizophrenic patients but not in control individuals. The method of measuring D4-receptor abundance was indirect (as described in the handout Seeman et al, 1993 -- and the accompanying, explanatory blurb by L. Iversen). Nevertheless the effect looks large and relatively well-controlled for the principal confounding variable -- the possibility of misleading changes in schizophrenic brains induced by the patients' chronic treatment with antipsychotic drugs. Stay tuned.

Various pages removed due to copyright considerations. Please see:

Iversen, L. L. "Dopamine receptors. The D4 and schizophrenia." *Nature* 365, no. 6445 (Sep 30, 1993): 393.

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