OBJECTIVES:

1. Review the concepts of partial agonism, competitive and non-competitive antagonism and interpret data sets relating to these concepts.

2. Reinforce the concepts associated with ligand - receptor interactions and interpret data sets relevant to receptor analysis.

3. Investigate how the rank order of binding of different drugs to a receptor preparation can be used to determine the type of receptor.

4. Explore how the Schild equation can be used to determine important information about an antagonist.

5. Discuss the spare receptor concept and how one demonstrates this kinetically.

INTRODUCTION:

There is a distinction between binding curves and dose - response curves. The endpoint for binding curves is the number receptor sites or the binding affinity while a dose-response relates information concerning the effect of the drug. Both kinds of data follow similar kinetics and there are several ways of plotting data from each kind of study. The Kd on a binding curve is the point at which receptor occupancy is half maximal and is similar to the EC_{50} or ED_{50} of dose-response curves. The maximum response is called Bound max or B max and is compared with Effect max. It is important to learn to interpret any graph displaying such data and derive whatever information is possible from the relationships plotted. In addition to the dose-response and log-dose-response curves which we studied in an earlier laboratory, data also can be plotted with a Scatchard plot or with a Lineweaver-Burk (double reciprocal) plot. In Scatchard analysis the y axis is Bound / Free and the x axis is Bound, both referring to the molar concentration of ligand. The slope of the line which is defined by the data is the binding affinity (K_a) and its reciprocal is the K_d, or dissociation constant (equal to conc at 1/2 saturation). The x intercept is the number of binding sites and the y intercept is Bound / K_d. In the Lineweaver-Burk analysis, the y intercept is 1 / Bound, the x intercept is 1 / K_d and the slope is K_d / Bound. One could substitute EC_{50} and Effect max in these same graphs.
Receptor Identification by Rank Order of Binding:

In ligand-receptor interactions both the concentration of drug at the receptor and the affinity of the receptor for the drug are important in drug action. A receptor is usually defined by the rank order of binding for a number of different ligands. For example, catecholamine receptors are separated into types by the rank order of binding of different catecholamines (first drug binds better than the second, etc.):

- α1-adrenergic receptor: EPI > NE > DA >> ISO
  - EPI = epinephrine
  - NE = norepinephrine
  - DA = dopamine
  - ISO = isoproterenol
- α2-adrenergic receptor: CLO > EPI > NE > ISO
  - CLO = clonidine
- β1-adrenergic receptor: ISO > EPI > NE > DA
  - ISO = isoproterenol
  - DA = dopamine
- β2-adrenergic receptor: ISO > EPI >> NE >> DA
  - CLO = clonidine
- dopamine receptor: BRO > APO > DA > NE > EPI
  - BRO = bromocriptine
  - APO = apomorphine

Binding Parameters and Biological Response Systems:

From binding studies, it is possible to determine the binding affinity of the receptor for different ligands and the binding site number for a particular tissue or cell type. As will be discussed below, one can also determine whether there are spare receptors and how many receptors must be occupied before a response is seen. The interaction of a ligand with a receptor in binding studies does not define possible antagonism. Some of the ligands which interact with receptors antagonize the action of other ligands at the receptor and this is determined in relation to a biological effect. To determine agonistic and antagonistic effects for drugs, an experimental system is used in which one can analyze both the binding parameters and a certain biological response or endpoint after treatment with different ligands. This is most easily accomplished in cell culture systems and a variety of different endpoints can be measured. For example, a cell line that responds to treatment with β-adrenergic agonists by secreting a particular protein would be useful in defining whether new experimental drugs have β-agonist or β-antagonist activity.

Receptor Antagonism:

Antagonists bind to the receptor but do not result in a biological response. This binding can be competitive or non-competitive. Non-competitive agents bind irreversibly to the receptor and new receptor must be synthesized for the cell to recover the ability to respond. With a competitive antagonist, increased concentrations of an agonist can eventually overcome the inhibition due to the antagonist by competing away the effect. An antagonist can also be a partial agonist. The appearance of graphs depicting these important relationships are below. Which of the graphs represents competitive and which represents non-competitive antagonism?

A useful equation for determining the binding affinity of antagonists is the Schild equation:

\[ \frac{C'}{C} = 1 + \left( \frac{[I]}{K_i} \right) \]

Where:
- \( C \) = concentration of agonist to give an effect in absence of antagonist
- \( C' \) = concentration of agonist to give an effect in presence of antagonist
- \([I]\) = concentration of antagonist
- \( K_i = K_d \) of the inhibitor, or conc at which inhibition is 50%

Note that if \( C' = 2C \), then \( K_i = [I] \)
Curve A reflects agonist binding to a constant amount of receptor in the absence of antagonist. In B, C, D an irreversible antagonist is added stepwise to the same amount of receptor preparation and the x axis reflects how much agonist must be added to achieve the same effect. Note that as receptors are occupied, eventually the effect is reduced as the concentration of agonist approximates the $K_d$. 

Upregulation and Downregulation of receptors: In some physiological situations, such as when a particular receptor is under constant, high dose stimulation, the receptor concentration will be downregulated. This can occur because of receptor internalization. The result is that the cell may become less sensitive to stimulation. In some situations the there might be an up-regulation of the same receptor if one treats with an antagonist. This is thought to be due to the prevention of internalization or down-regulating mechanisms. The changes in receptor number can have important consequences relating to the responsiveness of the system. When one stops treatment, it may be important to wean or slowly taper the dose to allow the receptors to recover.

Spared Receptor Theory: When a maximal response can be achieved in a biological system at only partial occupancy of the receptors which drive that response, a cell is said to have spared receptors. The consequence of this is that the cells are able to respond at much lower concentrations of ligand (increased sensitivity) and do not lose their responsiveness to endogenous ligand as easily because of receptor depletion. Furthermore, spared receptors allow cells to respond to agonists that have lower binding affinity. Normally such ligands dissociate rapidly, terminating the response. An irreversible antagonist can be used to demonstrate spared receptors by a stepwise elimination of receptors until the response disappears. This is accomplished by monitoring the biological response in the absence of the irreversible competitor and comparing it to the response in the presence of increasing concentrations of irreversible competitor. As one approaches the concentration of competitor that begins to inhibit the biological response, one has eliminated spare receptors. At this point the $EC_{50}$ approximates the $K_d$ for the receptor (see below).

If $e$ is defined as the factor expressing how many receptors must be occupied to get an effect, the formula $e \times [DR] / [R_{total}] = \text{Effect} / \text{Effect}_{max}$ or $e = (\text{Effect} / \text{Effect}_{max}) / ([DR] / [R_{total}])$ defines whether there are spared receptors. This relationship could also be expressed $e = EC_{50} / K_d$. If $e < 1$, there are spared receptors; if $e > 1$, the interaction is that of partial agonism; if $e = 0$, there is antagonism; if $e = 1$, all receptors must be occupied for maximal effect.

General Requirements for Establishing a Binding Assay:

Choice of receptor source: Specific binding is enhanced by increasing the purity of the preparation. However, if quantitation is the goal, the variable recovery with receptor purification may complicate the interpretation of results.

Choice of radioligand: The ideal ligand is of high affinity and reasonably high specific activity. Ideally, it would bind only to the receptor of interest. In biological systems it is unlikely that the chosen ligand will bind only to the receptor of interest. Especially in relatively impure systems, the ligand will bind to multiple sites. The problem facing the investigator is to define the binding site in which he is interested by experiments or calculations that eliminate non-specific binding. There are two approaches, either identifying highest affinity binding or identification of binding which is preventable by agents.
more selective than the chosen ligand.

Technique for Identifying the Bound Ligand:

a. Methods disturbing equilibrium: filter, absorbant
   Advantage: can measure bound at low receptor number
   Disadvantage: may give falsely low values for bound if equilibrium is disrupted

b. Methods that do not disturb equilibrium:
   Advantage: can be used with rapidly reversible binding interactions
   Disadvantage: mathematical determination requires sufficient conc of receptor

Specific Experiments to establish the system:

Define and minimize non-specific binding: Use 100 - fold excess of ligand or competitor
Choose receptor concentration: Determine range of receptor conc across which Bound is a linear function of receptor concentration
Establish Equilibrium Time: Examine binding at lowest possible conc of ligand

Definitive Experiments:

Titration of saturation: use convenient amounts of receptor, radiolabelled ligand
Determine receptor conc: mathematically determine via kinetic analysis
Define non-specific binding: 100 - fold excess

PROBLEMS:

Problem #1: You are a world-class scientist working on identifying estrogen receptors in various tissues. The animal model which you are working on is supersensitive to low doses of estrogen and a high percentage of animals treated with low doses of estrogen develop liver tumors. This is a unique example of estrogen inducing tumors rather than acting as a tumor promoter. You have collected livers from these animals, prepared a liver homogenate, and pipetted the appropriate tubes to contain radioactive ligand and excess unlabelled ligand and a fixed aliquot of the receptor. Your results will reveal whether these animals have normal hepatic estrogen receptors.

Assay setup:

The following set up is used to determine binding parameters: Three sets of tubes:

Total count tubes: to determine total radioactive ligand in companion tubes these tubes have no receptor in them

Total binding tubes: contain fixed receptor conc and different amounts of radioligand

Non-specific binding tubes: contain fix receptor conc, different amounts of radioligand AND 100 - fold excess unlabelled ligand

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<th>TOTAL COUNTS</th>
<th>FIXED RECEPTOR</th>
<th>100-FOLD EXCESS</th>
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### Results:

#### STEROID RECEPTOR BINDING DATA

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<th>Total Added (nM)</th>
<th>Total Bound (CPM)</th>
<th>Total Bound (nM)</th>
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Plot the data on a Scatchard Plot and a Double Reciprocal plot.

What is the $K_d$? __________________________________________________________________________

What is the binding site number? ________________________________________________________________________
Problem #2:

You have developed a neuronal cell line which secretes a protein called medstudin when it is stimulated with either Drug A or Drug B. Both drugs belong to a class of pharmaceuticals that are psychoactive. You are interested in identifying the type of receptor which populates the membrane of your cell line and would like to know what role spare receptors play in the responsiveness which you have observed. There are several receptors which have been characterized for this class of drugs. Their rank order of binding is as follows:

Receptor X: Drug A > Drug B > Drug C > Drug D
Receptor Y: Drug D > Drug C > Drug B > Drug A
Receptor Z: Drug D = Drug C = Drug B = Drug A

After performing a binding study, you graph the following results:

What kind of receptors do your cells have? ______________________________
What is the Kd for each of the Drugs: Drug A: _____________ Drug B: __________
Drug C: _____________ Drug D: _____________

You conduct a dose - response study to determine the EC₅₀ values for the drugs for which you have binding data in addition to some new drugs which you just received from a colleague.
Is there any evidence of spare receptors in the data you have analyzed thus far? __________

What is the evidence and what assumptions do you make:
__________________________________________ ________________________

What can you say about Drug E? __________________________________________

You conduct an additional study in the presence of various amounts of Drug D in the absence and presence of the two new drugs you have acquired, Drug E and Drug F. Your results are plotted in the following graph:

What can you say about Drug E?_________________________________ _______
What can you say about Drug F? __________________________________ ________
Can you calculate a Kd for either of these drugs? _______________________________
How would you do an experiment to determine the presence of spare receptors?