Graduate Research Lab Rotation Report - 2

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Lab – Dr. Robert C. Speth

Duration- 1/07/13 - 2/01/13

Laboratory Research area:

During my lab rotation, I observed the experiments of research study on ^{125}I-SI Ang II radioligand binding assay and angiotensin II receptor subtype quantification. Applying different ligand-receptor binding assay techniques on the membrane preparations from rat/mouse brain/adrenal glands/pancreas/kidney and Ang II receptor autoradiography, this lab finds out the subtype of Ang II receptors present there and their binding affinity as well as specificity.

Ongoing experiments and learned techniques:

During my rotation in the lab I observed and learned the micropipette calibration, lab swipe and minimization of radioactivity in working lab, buffer solution preparation, preparation of AM5 solution, ^{125}I-SI Ang II radioligand binding assay, tissue sectioning, dissection of rat and application of graph-pad Prism software in ligand-receptor binding assay.

Buffer solution: The recipe for the 0.5L assay buffer (Assay Medium or AM) is:

NaCl	4.38g
Na2EDTA	930mg
Bacitracin	70.5mg
500mM dibasic NaPO4	50ml
Distilled deionized water	450ml

pH is adjusted to 7.1-7.2 with HCl

Hypotonic buffer solution: The recipe for the 0.5L hypotonic buffer solution is:

500mM NaH2PO4	12ml
200mM Na2HPO4	70ml
Distilled deionized water	918ml

pH was checked to be 7.15

^{125}*I-SI Ang II radioligand binding assay:*

The radioligand was prepared in Georgetown University and in my first observation I wanted to see whether it gets bound to mouse pancreas. After that, binding assay was planned to be done with adrenal, brain & mouse pancreas membrane preparation for (0.2-2nM) six different gradual concentration of radioligand.

Principle:

Radioligand binding assays have greatly facilitated the characterization of receptors and the ligands (substrates) that interact with them. Radioligand binding is used to: (1) characterize receptors in their natural environment as well as those transfected into cell lines; (2) study receptor dynamics and localization; (3) identify novel chemical structures that interact with receptors; and (4) define ligand activity and selectivity in normal and diseased tissues.(McKinney & Raddatz, 2006) The most frequently used assay based on this technique is the membrane filtration receptor assay. A variation on the membrane assay, the intact cell radioligand binding assay has specific advantages in some circumstances such as screening large numbers of small cell samples and studies of receptor internalization(Bylund & Toews, 1993). For many binding assays, a suitable radioligand and a crude homogenate of a tissue known to contain the receptor are required. The homogenate and the radioligand are mixed, and at an appropriate time (empirically determined), the unbound radioligand (L*, or free) is rapidly separated from the ligand bound to the receptor (L*R, or bound), usually by filtration. Tissue sources for radioligand binding include tissue slices, subcellular fractions, or intact cellular preparations including native, immortalized, or transfected cells (McKinney & Raddatz, 2006).

The criteria established for validating a binding assay are as follows (Cuatrecasas and Hollenberg, 1976):

1. Specific binding must be saturable, indicating a finite number of receptor sites, although in some instances, nonspecific binding can appear saturable as well.
2. The binding affinity, defined as the dissociation constant (K_D), should be consistent with physiological values established for the receptors (e.g., 100 pM to 10 nM).
3. Binding should be reversible, consistent with a physiological mechanism for terminating the effect of a ligand at the receptor.
4. The tissue and subcellular distribution of the specific binding should be consistent with what is known about the proposed physiological effects of the endogenous ligand, and with what is known about the localization of the receptor.

5. The substrate selectivity of binding for both agonists and antagonists should be consistent with the pharmacology of the natural ligand in functional and whole-animal tests. Conversely, ligands known to be inactive at the targeted receptor should not affect radioligand binding.
6. There should be a correlation between the binding and concentration-response data in identical tissue preparations.
7. Activity in a binding assay should be predictive of activity in an established animal model of receptor function.

Radioligand binding assays only measure the affinity and density of a ligand binding site. The efficacy, pharmacodynamic, and pharmacokinetic properties of the ligand are not revealed in a binding assay, but rather, must be assessed using functional in vivo and in vitro analyses(McKinney & Raddatz, 2006).

The parameters measured in radioligand binding assay are K_D and B_{max} derived from the bimolecular reaction according to the law of mass action.

$$K_D = \frac{k_{-1}}{k_1} = \frac{[L^*] \times [R]}{[L^* R]}$$

$$B_{\max} = [R]_{TOT}$$

The K_D is expressed in molar units of concentration (e.g., nanomolar or picomolar). The binding affinity of a receptor for a ligand is a molecular consequence of its structure, with the K_D used to identify and classify receptors based on this affinity. Therefore, the determination of K_D is a primary goal in developing a binding assay once the optimal conditions for specific binding are established.

In the assay, the species measured is the bound ligand (i.e., the L*R complex). The receptor-ligand complex, which is embedded in the plasma membrane, is readily isolated from the aqueous reaction mixture by filtration. By quantifying the radioactivity recovered on the filter, the amount of radioligand bound to the tissue is measured. The radioligand attached to the receptor is considered the specific binding component. Some radioligand will be nonspecifically trapped with the lipid membrane or other constituents of the assay mixture. Nonspecific binding is defined as radioactivity detected in the tissue sample that is not bound to the receptor of interest and is quantified by measuring the amount of radioligand associated with the tissue in the presence of very high concentrations of an unlabeled ligand, whereas specific binding is the radioactivity displaced by saturating concentrations of an unlabeled ligand selective for the receptor being studied. In equilibrium binding assays, unbound and bound ligands are separated from each other after the

forward and reverse binding reactions reach equilibrium. With a saturation binding experiment, assays are performed using a series of radioligand concentrations, ranging up to a concentration at which virtually all of the receptors are occupied with ligand. An example is shown in Figure 1.1, which describes the binding of [3H]N-methylscopolamine ([3H]NMS), a muscarinic cholinergic receptor antagonist, to muscarinic receptors in N1E-115 mouse neuroblastoma cells. The concentration of [3H]NMS is plotted on the abscissa, with the amount of radioligand bound to the filters at each concentration plotted on the ordinate. Displayed in this figure are total, specific, and nonspecific binding, where total binding is the sum of specific and nonspecific binding components.

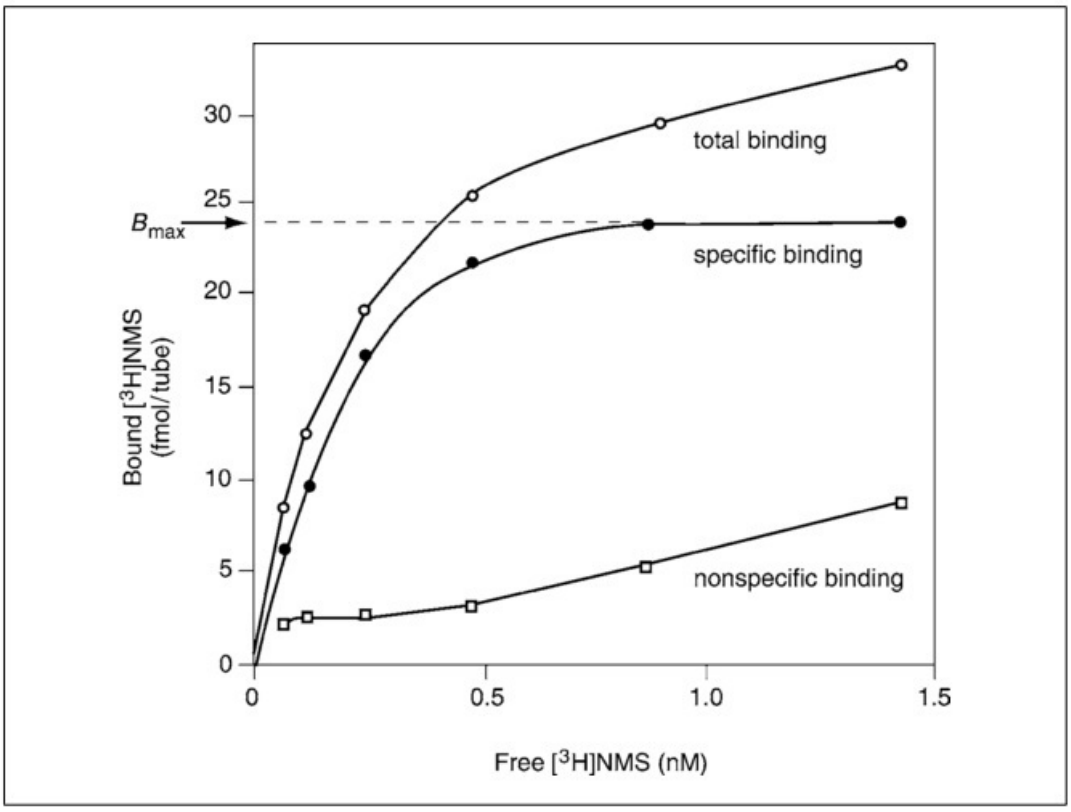


Figure 1.1 Saturation binding to muscarinic receptors on N1E-115 mouse neuroblastoma cells. Six concentrations of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS), with or without 10 μM unlabeled NMS, were incubated with ~300,000 intact cells/tube for 45 min at 15°C before rapid filtration was performed to separate bound from free. The total binding is the sum of the specific and nonspecific binding. Nonspecific binding is defined as the amount of binding found in the tube containing both the radioligand and unlabeled NMS.

Because there are a finite number of physiologically relevant receptors in the tissue, specific binding becomes maximal (i.e., approaches B_{max}) as the concentration of radioligand increases (Fig. 1.1). B_{max}, therefore, is a measure of the number of receptor sites in the preparation. However, by its nature, nonspecific binding does not saturate with increasing concentrations of radioligand. The concentration

of radioligand at which the amount of specific binding is one half B_{max} approximates the equilibrium binding dissociation constant (K_D). However, to obtain the K_D and B_{max} from a plot it is preferable to fit the specific binding data with a mathematical model in a computer program to determine K_D and B_{max} (McKinney & Raddatz, 2006).

Radioligand:

Antagonist radioligand is preferred because it is known to interact selectively with the target site as it will usually bind with a high affinity whether the receptor is coupled to membrane-associated signal transduction elements (e.g., G proteins) or is in a desensitized state. In general, antagonists tend to bind to receptors with much higher affinity than agonists. Additionally, agonists induce conformational changes in receptor-effector complexes that can cause ligand-receptor complexes to exist in multiple states with different binding characteristics. Another advantage of antagonist radioligands is that they do not activate the receptor which, in the case of binding with metabolically active cells, can result in the desensitization, and reduction in affinity, of the site. Moreover, agonists tend to label only a portion of the actual receptor present and, as most receptors are present at concentrations of <10 pmol/mg protein, specific binding for agonists can be quite low relative to antagonists. For these reasons, radiolabeled antagonists are the preferred ligands for most binding assays. In general, compounds with a specific activity <20 Ci/mmol do not make good radioligands (McKinney & Raddatz, 2006). Table-1 outlines the characteristics of the radioisotopes commonly employed for receptor identification.

The radioligand we used in the lab experiment was ^{125}I-SI Ang II. The reference data and purification process is as follows:

Reference Date: 1/10/2013 (Preparation date)

Amount: 512 µCi (2*256 µCi)

Volume: 1.0 ml (2*0.5ml) in 1.5ml microcentrifuge tubes

Concentration: 512 µCi/ml 235nM

Specific activity: 2175 Ci/mmol (theoretical specific activity of monoradioiodinated ligand)

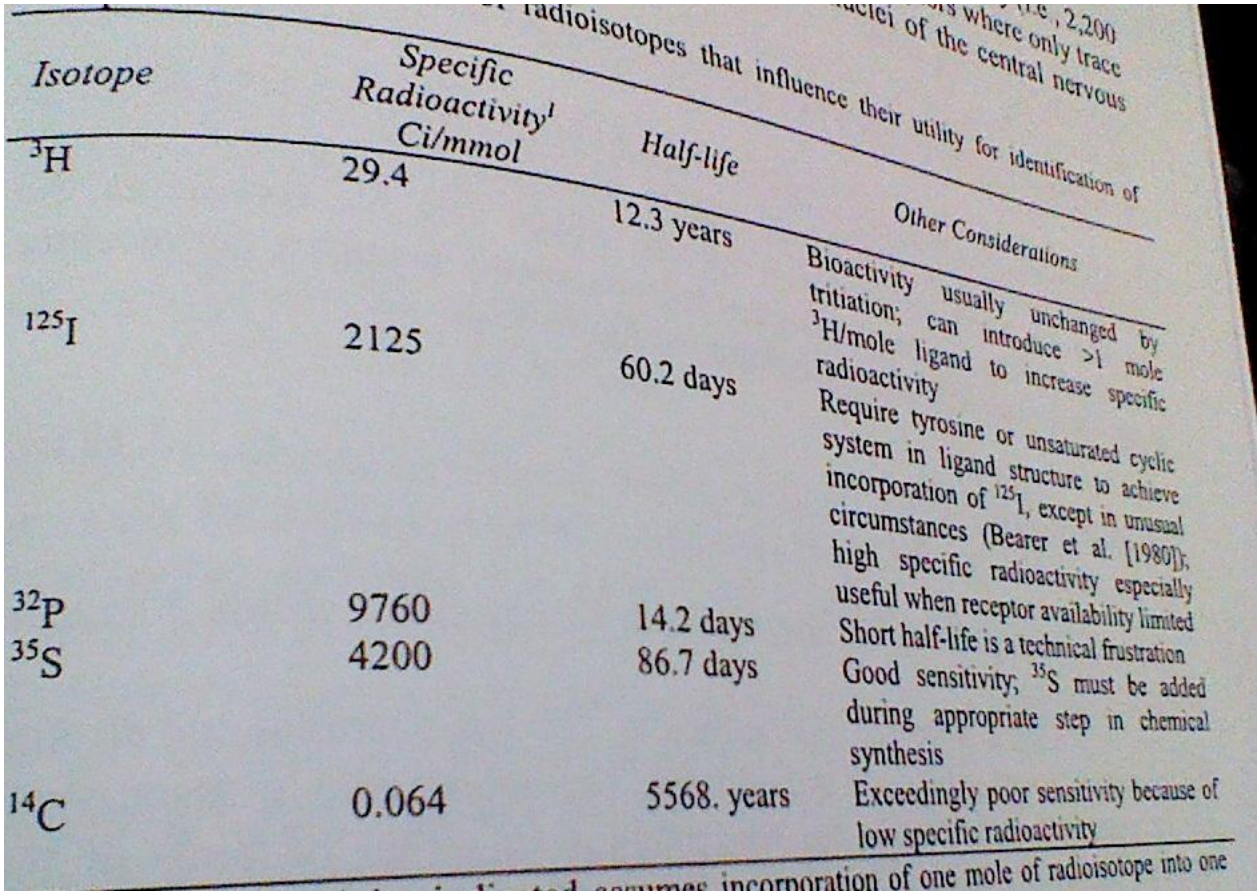
Solvent: 1 part (18% acetonitrile: 82% Triethylamine phosphate, pH 3.0) 1 part distilled water

Stabilizers: contains 2mg/ml bovine albumin

Lot number: 130110B

Prepared by Robert Speth, Ph.D. FAAAAS at Georgetown University

Table-1: Characteristics of radioisotopes that influence their utility for identification of receptors (Limbrid, 2004).



Isotope	Specific Radioactivity, Ci/mmol	Half-life	Other Considerations
^{3}H	29.4	12.3 years	Bioactivity usually unchanged by tritiation; can introduce >1 mole ^{3}H/mole ligand to increase specific radioactivity
^{125}I	2125	60.2 days	Require tyrosine or unsaturated cyclic system in ligand structure to achieve incorporation of ^{125}I, except in unusual circumstances (Bearer et al. [1980]); high specific radioactivity especially useful when receptor availability limited
^{32}P	9760	14.2 days	Short half-life is a technical frustration
^{35}S	4200	86.7 days	Good sensitivity; ^{35}S must be added during appropriate step in chemical synthesis
^{14}C	0.064	5568. years	Exceedingly poor sensitivity because of low specific radioactivity

Preparation and purification procedures:

Peptide is radioiodinated with $Na^{125}I$ using the chloramines T procedure (Hunter et.al., 1962) (Hunter, W.M. and Greenwood, F.C., Nature 1962, 194: 495-6). Monoradioiodinated peptide is resolved from uniodinated peptide and diradioiodinated peptide by HPLC using C_{18} reverse phase column eluted isocratically with acetonitrile: triethylamin phosphate (pH 3.0) mobile phase. The column eluate containing monoradioiodinated peptide (peak labeled as mono ^{125}I-SI Ang II on the accompanying chromatogram) is diluted with water to lower the concentration of iodine-125 to reduce autolysis.

A concentrated solution of bovine albumin is added to give a final concentration of 2mg/ml to also reduce autolysis. In some cases, the radioligand requires special storage procedures, such as addition of

antioxidants or protease inhibitors in the case of a peptide radioligand, to slow or prevent degradation. Similar preservation procedures maybe needed to prevent the degradation of receptors in the tissue preparation(McKinney & Raddatz, 2006).

Based upon the decay catastrophe hypothesis, disintegration of an iodine-125 molecule destroys the peptide to which it is bound (Catt & Baukal, 1973). Based upon this concept, the specific activity of the radioligand does not change. However, the concentration of ^{125}I-Sar^{1},Ile^{8} Angiotensin II will decrease with a half-life of 60.14 days. Radioiodinated ligand is frozen at -20^{0}C in a lead container.

Affinity and pharmacological selectivity of radioligand: A high affinity radioligand is desirable because it allows for separation of bound from free ligand by filtration since the rate of dissociation is directly related to affinity. The primary consideration in selecting a radioligand is its selectivity for the receptor of interest (McKinney & Raddatz, 2006).

Nonspecific binding: The physicochemical properties of a ligand determine its level of nonspecific binding due to interactions with lipid membranes and/or filter or scintillation bead material in the assay (McKinney & Raddatz, 2006).

Tissue preparation:

The tissue (rat kidney, liver and pancreas) to be analyzed was disrupted to increase access of the radioligand to the receptor population. A tissue homogenizer was employed to disrupt tissue for use in a binding assay. Typically, the tissue is kept cold in ice bucket until it is disrupted in the homogenizing vessel and buffer. The temperature was not to rise, or homogenizer was not run for too long, because this can result in significant loss of receptor binding because of heat denaturation or because of proteolytic activity.

The standard procedure is to homogenize the tissue or cells of interest in a hypotonic buffer using either Polytron (Brinkman) or a Tissumizer (Tekmar). Remarkably, most receptors are reasonably stable (generally for hours) following death, although it is wise to remove the tissue to ice reasonably quickly (Bylund & Toews, 1993).

After that the tissue is centrifuged at high speed (20,000rpm) carried out at 4^{0}C. Following centrifugation the supernatant is decanted and the pellet is re-homogenized after suspending it into 25ml AM5 solution. Inhibitors (o-phenathroline & PMSF) can be used (500:1 proportion) before recentrifugation. O-phenanthroline is a chelating agent ptotease inhibitor which prevents the peptide degradation of radioligand (Karamyan, Gadepalli, Rimoldi, & Speth, 2009) and PMSF is a pure serine

protease inhibitor which protects the receptors and ligands from proteolytic degradation (Wangler et al., 2012). Both o-phenanthroline and PMSF increases the specific binding of ^{125}I-Ang II (Karamyan et al., 2009). The purpose of two centrifugation steps is to remove any soluble interfering substances, such as endogenous neurotransmitters and guanine nucleotides, which may interfere with the radioligand binding assay (Bylund & Toews, 1993).

Endogenous ligands for the targeted receptor can interfere with radioligand binding. This is minimized by using a series of washing steps and incubations, with or without degradative enzymes, to remove or destroy the endogenous ligand. Extensive washing entails repeated pelleting by centrifugation followed by resuspension in fresh buffer. For example, incubation of monoamines at 37◦C allows endogenous enzymes to degrade the natural ligand, such as norepinephrine or dopamine(McKinney & Raddatz, 2006).

Buffer selection:

In most cases, a homogenization/assay buffer is selected that yields the highest ratio of specific versus nonspecific binding (McKinney & Raddatz, 2006). In the lab experiments we used the hypotonic buffer solution of pH around 7.15.

Binding assay conditions

Equations used to analyze binding data are based on two assumptions: that the receptor concentration is low and that the free radioligand concentration (F) does not change during the assay. If the receptor concentration is increased such that F changes significantly because a significant amount of ligand is receptor bound, affinity will be underestimated. To avoid this, the receptor concentration should be <10% of the radioligand K_D (McKinney & Raddatz, 2006).

Binding assays can be performed at a variety of temperatures, depending on the requirements of the experiment. Room temperature (25◦C) is convenient, but it must be tightly controlled to obtain reproducible results. The choice of assay temperature and the duration of incubation must be determined empirically to identify the conditions that yield the best signal-to- noise ratio for the particular receptor and ligand combination (Bylund & Toews, 1993),(McKinney & Raddatz, 2006).

Separation of the Receptor-Ligand Complex (L*R) from Unbound (Free) Ligand:

Once the tissue-bound radioligand has been separated from the free radioligand in the assay mixture, usually by vacuum filtration, the former is quantified using liquid scintillation spectroscopy. The radioactivity thus determined is considered total binding, from which specific binding (defined as the binding displaced in the presence of a saturating concentration of unlabeled ligand) and nonspecific binding, the amount of total binding that remains associated with the tissue in the presence of the saturating concentration of unlabeled ligand can be determined (McKinney & Raddatz, 2006). Filtration is the most efficient and convenient method of separating free from bound radioligand because it requires minimal handling of samples as compared to centrifugation. (Limbird, 2004) After incubation of the radioligand with the tissue preparation, the contents of the assay tubes are aspirated onto filters where the tissue and bound radioligand are trapped, while the unbound radioligand passes into the effluent. For the vacuum filtration, in lab, we used cell harvester with BSA solution-wet filter and buffer as rinsing solution. The filter was labeled at right hand side and circular wet portion containing membrane and radioligand was collected in 12*75mm tube and appropriately labeled for gamma counter. Gamma counter (Cobra gamma counter) counts filter circles for 1 minute each.

Analysis of Saturation Binding Curves:

Using nonlinear regression to determine B_{max} and K_D these steps were followed to analyze the data with nonlinear regression:

1. Specific binding was calculated at each concentration of ligand (from total binding-nonspecific binding).

2. The specific binding data was converted from counts per minute to more useful units such as fmol/mg protein or sites per cell.

3. x was defined as the radioligand concentrations in nM. y is the specific binding in fmol/mg or sites per cell.

4. Data was fitted to the non linear one site saturation binding assay to Graph pad Prism software.

5. K_D and B_{max} were found from result data in Prism file.

Laboratory Experiment:

Effect of protease inhibitor on ^{125}I-SI Ang II binding to AT_1 receptor of rat kidney tissue membrane.

Procedures:

1. The kidney tissue was weighed in a tared centrifuge tube. It was 644mg.
2. Tissue was homogenized in a centrifuge tube for 5-10 seconds adding 25ml hypotonic buffer solution.
3. Another centrifuge tube was weighed filling up with DD water to balance the tissue tube and centrifuged at maximum speed (20,000 rpm) for 20 minutes with minimum temperature 4^0C.
4. Supernatant was decanted.
5. Tissue pellets were suspended in 25ml AM5 solution and homogenized completely putting homogenizer tip all the way down into the tube and all of pellets were re-suspended.
6. Homogenate was splitted into two aliquots of 12.8ml which is around 12.43g.
7. Inhibitor was added in proportion of 500:1 which was 12.3µL in the aliquot 1 and marked with inhibitor (rack 2).
8. PD123319 (an AT_2 angiotensin receptor antagonist) was added in both the aliquots.
9. Hot dilution of radioligand was prepared according to the attached tabulation sheet. (next page)
10. Diluted hot solution of gradual concentration was added in the two (with inhibitor & without inhibitor rack) racks as Rack-1: tube11, 12 and 23, 24 contains 0.2µl hot solution, and then tube9, 10 & 21, 22 contains 0.5 µl hot solution and so forth. Rack-2 was also titrated with hot solution of gradual concentration like the rack-1.
11. 10 µl Ang II, the AT1 receptor occupying agonist, was added to rack-1: tube 1-12 and rack-2:tube1-12.
12. 10 µl AM5 was added to the rest of the tubes of rack-1 and rack-2 (to which Ang II was not added).
13. 2% tissue homogenate was added to each rack and vortexed to mix well.
14. Direct count was taken in gamma counter for 1 minute each to get free ligand data.
15. Tubes were put into the rack from gamma counter in such a way that it will be in two rows and tube 13, 14 will follow tube 1, 2 and so forth.

16. Cell harvester was washed with DD water and wet filter with cold BSA solution was placed on cell harvester. After checking the cell harvester that all the tubes are aspirating and buffers are delivered correctly the rack-1 and rack-2 was run consecutively with fresh filter for each rack.
17. The filter with circle wet portion was collected and put into gamma counter rack.
18. Filter circles were counted in gamma counter for 1 minute each. First two samples will give non-specific binding data followed by total binding data and so forth.
19. The data were placed in the excel spread sheet which was pre-formulated with calculation for specific binding in fmoles/gm.
20. The bound data and free ligand concentration was analyzed to calculate K_D and B_{max} from Prism software.

Calculation: Radioligand hot solution dilution

Species and tissue		rat	forebrain			
Assay Date	1/31/2013	184.3772602	nM			
Ref Date	1/10/2013	235	nM			
	dilution in tubes		2.5			
	stock (nM)	working conc. (nM)	final conc. (nM)	µl of hot (Radioligand)	volume to be (µL)	AM5 solution needed
SI Ang II	184.3772602					
Preparing mother hot solution from 5nM hot solution		5	2	32.4063824	1195	1162.593618
Gradual solution from 2nM mother solution		3	1.2	282	470	188
		2	0.8	188	470	282
		1.25	0.5	117.5	470	352.5
		0.75	0.3	70.5	470	399.5
		0.5	0.2	47	470	423
				705		

repetitions	no of racks	desired vol (µL)	tot vol required	adjusted vol to make (µL)
4	2	40	320	470
receipt date	1/17/2013	216.7443044		

	ref date	assay date
µCi used	16.56371221	12.995625

Use fresh buffer made with DD H2O

Rack	DC Free	DC cpm	DC fmol	DC nM	DC Bound	Spec fmol	Free nM	Bound/free uL	Nsp /Free	Free nM	Spec fmol	Total Bound fmol		TBd/Free	NSP bound fmol		NSBd/ Free	spec/nsp
	831990.3				7581.4													
	829494.3		25	100	7419.5					2.472	2.4069		3.303	1.336		0.8965	0.2714	2.6848
#	825095.1				27067.3					1.446	1.6759		2.198	1.52		0.5222	0.2376	3.2092
1	838658.8	831310	253.2	2.532	26865.6	6.017	2.472	2.434	0.087	1.017	1.1268	8.259	1.512	1.487	2.241	0.3857	0.255	2.9214
	483811.5				4190.8					0.622	0.7105		0.949	1.525		0.2384	0.2512	2.9804
w	494929.2				4256.1					0.328	0.3823		0.509	1.551		0.1262	0.2482	3.029
i	486111.5				17035.5					0.262	0.302		0.399	1.522		0.0971	0.2434	3.1088
t	489374.8	488557	148.8	1.488	18518.8	4.19	1.446	2.897	0.081			5.495			1.306			
h	352379.1				3190.2													
o	341658.4				3048.3													
u	337418.4				11880.7													
t	341393.6	343212	104.5	1.045	12583.1	2.817	1.017	2.769	0.084			3.781			0.964			
	210510.9				1864													
i	211414.7				1992													
n	208387.9				7510.9													
h	210115.6	210107	63.99	0.64	7837.5	1.776	0.622	2.855	0.08			2.372			0.596			
i	110826.3				1006.5													
b	111721.7				1035													
i	110061				4228.6													
t	110611.7	110805	33.74	0.337	3996.7	0.956	0.328	2.915	0.07			1.271			0.316			
o	88227				778.8													
r	88142.2				792.3													
	89430.3				3097.5													
	88600.9	88600.1	26.98	0.27	3357.8	0.755	0.262	2.879	0.06			0.998			0.243			

Table-2: Radioligand binding data for the rack #1- without inhibitor

Rack	DC Free	DC cpm	DC fmol	DC nM	DC Bound	Spec fmol	Free nM	Bound/free uL	Nsp/ Free	Free nM	Spec fmol	Total Bound fmol		TBd/Free	NSP bound fmol		NSBd/ Free	spec/nsp
	834273.4				5299.4													
	833339.7				5963.7					2.375	7.318		8.014	3.375		0.6963	0.0869	10.509
	840714.3				60795.2					1.362	5.116		5.544	4.072		0.4282	0.0772	11.948
#	850501.6	839707.3	255.77	2.56	68837.4	18.295	2.375	7.704	0.06	0.923	4.164	20.036	4.521	4.897	1.741	0.3567	0.0789	11.675
	492403.6				3233.7					0.564	3.014		3.219	5.712		0.2055	0.0638	14.667
2	491733.4				3692.7					0.292	1.905		2.025	6.928		0.1198	0.0591	15.908
	488363.3				45076.2					0.236	1.392		1.475	6.246		0.0834	0.0566	16.676
w	483558	489014.6	148.95	1.49	44604.4	12.79	1.362	9.394	0.07			13.861			1.071			
i	338390.8				2684.6													
t	341394.8				3084.3													
h	332224.3				36207													
	337131.1	337285.3	102.73	1.03	36914.6	10.41	0.923	11.28	0.08			11.302			0.892			
i	207549.7				1612.9													
n	211820.8				1710.8													
h	208895.3				26276.4													
i	210946.5	209803.1	63.898	0.64	25797.4	7.5348	0.564	13.37	0.07			8.0485			0.514			
b	112145.6				999													
i	113051.1				938.3													
t	111151.7				17261.7													
o	110148	111624.1	33.993	0.34	15494.6	4.7634	0.292	16.3	0.06			5.0628			0.299			
r	89161.4				693.3													
	89245.2				656.5													
	88862.9				12178.4													
	88656	88981.38	27.096	0.27	11681	3.4791	0.236	14.73	0.05			3.6877			0.209			

Table-3: Radioligand binding data for the rack #2- with inhibitor

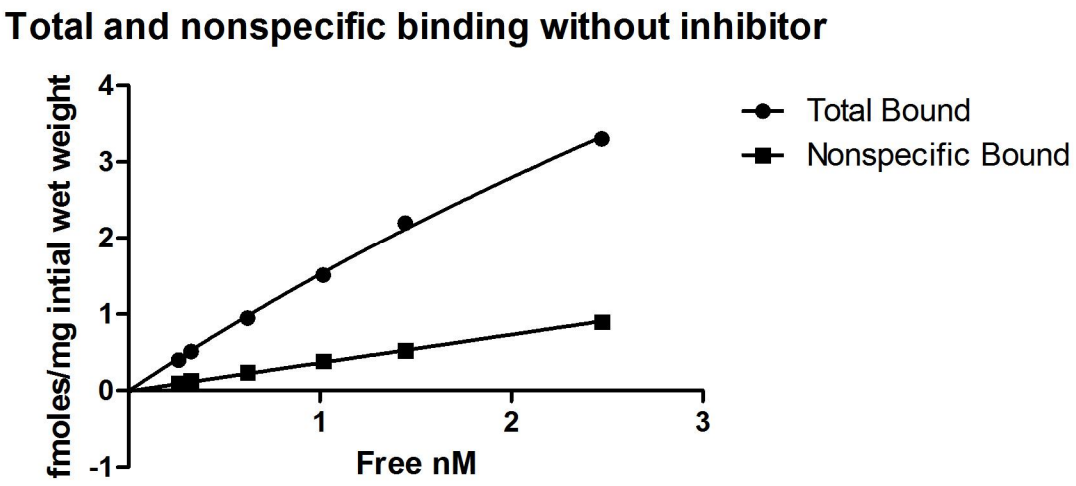


Fig 1: Total and nonspecific binding without inhibitor. For total binding, K_D = 7.096 and B_{max} = 9.372

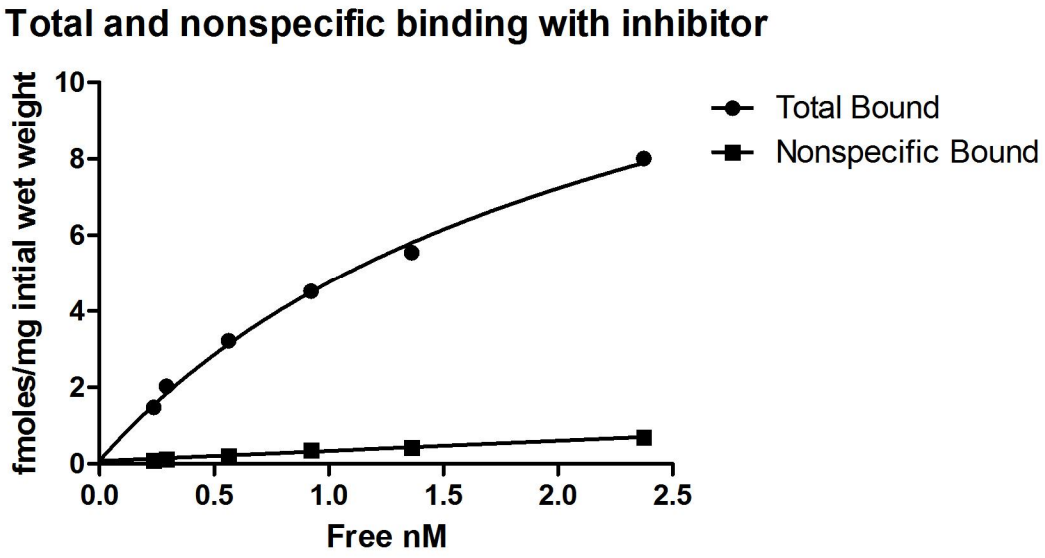


Fig 2: Total and nonspecific binding with inhibitor. For total binding, K_D = 2.01 and B_{max} = 13.29

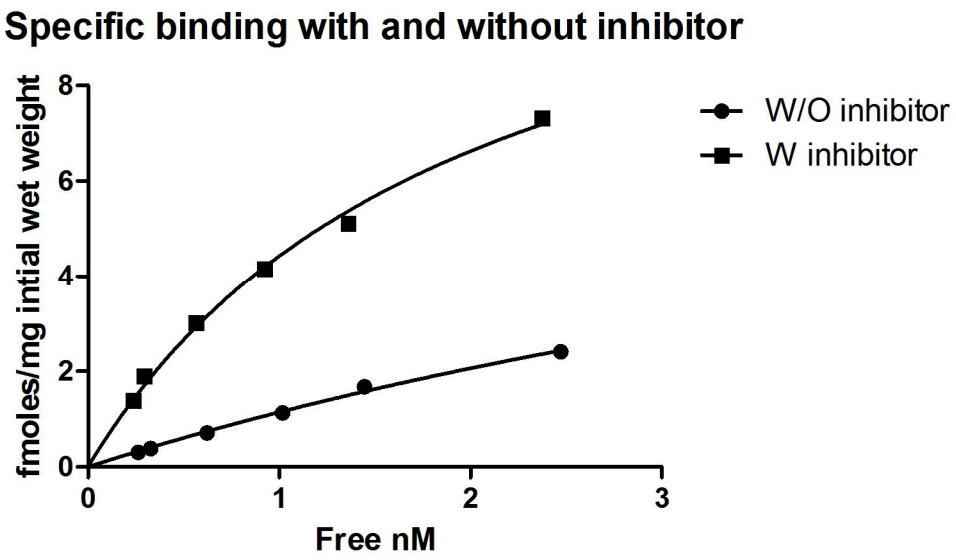


Fig 3: Representative saturation binding analysis of ^{125}I-SI Ang II to AT_1 receptors in kidney membrane in assay buffer with 100mM o-phenanthroline and 100mM PMSF. For specific binding without inhibitors the K_D is 8.055±2.441 nM and B_{max} is 10.37±2.565 fmole/mg wet wt. and with inhibitors the K_D is 1.972±0.2729 nM and B_{max} is 13.16± 1.061 fmole/mg wet wt.

Discussion:

Binding experiment was carried with ^{125}I-SI Ang II in rat kidney membranes to find out the effect of inhibitors on ^{125}I-SI Ang II binding with kidney AT_1 receptors. Under the experimental conditions, the inhibitors (o-phenanthroline & PMSF) significantly decrease the K_D value which means the affinity to AT_1 receptors of the radioligand was increased. These protease inhibitors might block the receptor metabolism or prevent the Radioiodinated Ang II degradation in the experimental condition. The increase in B_{max} value is also significant which means more receptors are available to bind with the free ligand. This indicates the receptor unmasking by the protease inhibitors. Optimization of the function of these protease inhibitors for specific radioligand or specific receptor under specific experimental condition could be next study.

Cryocut sectioning of rat kidney & adrenal:

During the lab rotation in this lab I learned to section the rat kidney & adrenal gland by cryocut machine. The section I cut was 20µ. The section was cut and examined whether it's getting proper shape to mount on the slides. It the temp is warm the section get coiled and cannot be mounted on slides. So temperature was reduced to an optimized level. After attaching the tissue on the tip of coiled surface of holder through some gum adhesion the blade was ran through the tissue and first few sections were not uniform thickness. After a while the section was getting uniform thickness. The section was thaw mounted onto the slides. The slides were numbered as 1-1, 1-2, 1-3, 1-4 and date was written over it with pencil. The sequence of mounting the sections on the slides were 1-1, 1-2, 1-3, 1-4 and cyclically again 1-1, 1-2, 1-3, 1-4. For kidney I got 6 sections per slide and adrenal I got 11 sections per slide.

The knife blade is very sharp. So, I had to be extra careful handling and cleaning the blade if needed. And mounting the small thin sections on the slides was done carefully so that the section doesn't get squeezed.

Dissecting rat forebrain and collecting internal organ:

I have observed the sacrificing of rat for dissecting its forebrain and collecting internal organs. The rat's cage and required information for approval was noted. Then the rat was put into CO_2 jar and kept until it became unconscious. After that the unconscious rat was decapitated.

The scissor's sharp edge was entered into upper crevices and cut through the joint of skull. The lobes of forebrain and pituitary glands were collected carefully. The collected brain and pituitary was placed on aluminum foil and kept refrigerated.

For the collection of internal organs the body of the rat was lay flat on napkin and the skin was cut to get internal organs. The heart and lungs was separated first and collected on aluminum foil. Then the liver and lymph system was separated carefully and collected. After that reproductive system of the female rats were collected including the ovary and uterine.

Lab Swipe:

The radioactive materials were constrained within one room of the lab. After the radioligand binding assay the working surface was examined with survey meter every day. If there is any hot surface it was swiped with wet tissue and examined again. The predefined places those are suspected to be radioactive within the radioactive lab are swiped once in a week. The tissue paper cut in around 2.5 by 2.5 inches was used for lab swipe. The 15 different places were swiped and the swiped tissue paper was pressed to make bead size to put inside tube to count in gamma counter. To have a background radioactivity count an unswiped tissue is taken. If any surface/ place is more than 100count compared to background count it is swiped very well with clean wet napkin and after that the surface is swiped again to check the radioactivity.

Experimental precautions:

1. The experiments and laboratory materials may contain radioactivity which are hazardous to health. Care should be taken according to radioactive safety plan to avoid contamination.
2. The radioactive materials should be disposed of with utmost care and caution.

Conclusion:

Lab rotation at Dr. Robert Speth's laboratory was an interesting and learning experience for me. Getting the opportunity to have experiments on radioligand binding assay, some partial procedures of autoradiography and rat organ sectioning greatly enhanced my laboratory experiences and learning. The ongoing experiments and techniques gave me a very good idea about the research of the lab.

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