

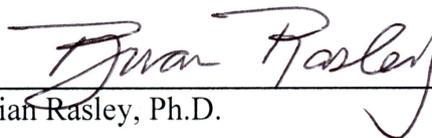
CHARACTERIZATION OF THE ADENOSINE A1 RECEPTOR IN SUMMER AND  
WINTER ARCTIC GROUND SQUIRRELS

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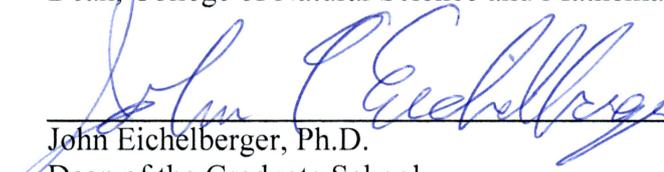
  
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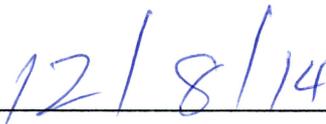
  
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CHARACTERIZATION OF THE ADENOSINE A1 RECEPTOR IN SUMMER AND WINTER  
ARCTIC GROUND SQUIRRELS

A  
THESIS

Presented to the Faculty  
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By

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## Abstract

Hibernation is an adaptation that allows the Arctic ground squirrel (*Urocitellus parryii*) to survive the harsh arctic winter. Recently the activation of the Adenosine A<sub>1</sub> receptor (A<sub>1</sub>AR) has been shown to be necessary for entrance into hibernation during the winter but not summer season. In the current study we characterize the A<sub>1</sub>AR in the forebrain, hippocampus and hypothalamus of summer and winter AGS. We also tested the hypothesis that increased A<sub>1</sub>AR agonist efficacy is responsible for increased sensitization of the A<sub>1</sub>AR during the winter season. The resulting <sup>35</sup>S-GTPγS binding data indicated an increase in agonist potency during the winter season in all three brain regions. A plausible explanation of our results is that increased potency in the forebrain during the winter season is due to an increase in efficacy as indicated by a greater number of receptors in the high affinity state. In addition <sup>35</sup>S-GTPγS binding, [<sup>3</sup>H] DPCPX saturation and competition assays establish for the first time pharmacological characteristics such as EC<sub>50</sub>, K<sub>d</sub>, K<sub>i10</sub> and K<sub>ihi</sub> in AGS brain.



Dedication

*To my family and friends.*



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## Introduction

Hibernation is an adaptation that allows an animal to survive times of low resource availability by going into a suspended animation like state. During hibernation, hibernators such as the Arctic ground squirrel (AGS; *Urocitellus parryii*) drastically decrease core body temperature and enter a hypometabolic state (Williams et al., 2012, Buck and Barnes 2000). Several studies have been conducted to ascertain the mechanisms that facilitate the hibernation state without a definitive answer (Dawe and Spurrier, 1972, Kondo and Kondo, 1992, Oeltgen et al., 1987). Despite these studies, it is still largely unknown what mechanisms are responsible for entrance into hibernation. Here past studies on hibernation induction triggers will be reviewed.

In the 1970's it was found that blood serum from hibernating 13-lined ground squirrel (*Citellus tridecemlineatus*) triggered hibernation in summer 13-lined ground squirrels two to twenty-two days post injection (Dawe et al., 1970). This unknown factor in the hibernator's serum was deemed the hibernation initiation trigger (HIT). Further experimentation showed that the timing of blood collection from the hibernators influenced how fast the summer euthermic animals entered hibernation (Dawe and Spurrier, 1972). It was determined that serum from a woodchuck (*Marmota monax*) in a long torpor bout caused summer 13-lined ground squirrels to enter hibernation in less time than serum from the same squirrel in a short torpor bout. In addition to this discovery it was determined that blood from a 13-lined ground squirrel in intermediate arousal did not induce torpor. This indicates that the HIT increases in concentration during deeper hibernation bouts and is not active during arousal. The authors also found that serum from winter animals that underwent dialysis and mixed with summer euthermic blood was not able to induce torpor. When the hibernation serum dialysate was diluted and then injected the summer animals went into hibernation. The authors conclude that there is a factor in the summer

animal blood that causes the deactivation of the HIT. This discovery leads to the question of why the summer euthermic animals underwent hibernation when the factor that deactivates the HIT should be expressed. One possible explanation is that the HIT induces a cascade of events that leads to either an increased expression of the HIT or a decrease in the inactivation component.

Analysis of Asian Chipmunk (*Tamias sibiricus*) serum proteins using gel permeation high-performance liquid chromatography revealed a 140-kDa complex of four proteins called hibernation protein (HP), HP-20, -25, -27 and -55 (Kondo and Kondo 1992). These proteins disappear when the hibernation season starts. In addition to Asian Chipmunks, these proteins were found in 13-lined ground squirrels but were not evident in non-hibernating species. This would indicate that control over the hibernation is due to a complex that inactivates several proteins. The protein complex could break apart due to an unidentified enzyme making its constitutive parts active. Interestingly, the authors of this paper find that the HPs do not reappear in the blood during inter-bout arousals. This indicates that plasma HPs are not required for arousal. Since the HPs were not found in the blood of hibernating animals, it seems unlikely that the blood would cause entrance into hibernation during the winter. A possible explanation for this is that 140-kDa HP complex could act as an inhibitory control over the HIT, allowing the animal to enter hibernation without changing the concentration of the HIT protein. This would have enabled the HIT to go unnoticed due to the authors' interest in the changes in protein expression between hibernation and summer animals. Follow-up studies indicated that the HIT complex was migrating to the brain during the entrance into hibernation (Kondo et al., 2006). The authors also found that administration of an antibody against HP-20 during hibernation shortened the hibernation bout. This indicates that HPs are important for entering into the hibernation state. Woodchuck albumin was also analyzed for the existence of the HIT protein

using 2-d gels and column chromatography (Horton et al., 1998). Two different proteins were found that had differential expression in regard to season, an 88kDa winter specific protein and a 77kDa summer specific protein. Partial sequencing of the 88kDa protein showed an alpha 1-glycoprotein like structure, with low affinity for the delta opioid receptor ( $K_i = 15813 \pm 2216\text{nM}$ ). The authors do not test other receptor populations for affinity. It is interesting that Asian Chipmunks have an inhibitory 140kDa protein complex and Woodchucks have unique proteins. This discrepancy could be due to the use of different techniques or that the strategy for entering hibernation is species specific.

The discovery of unique proteins expressed in hibernation lead to several studies focused on determining the mechanism of action of the HIT. The opioid system became of interest due the fact that the injection of high doses of opiates causes a depression of body temperature (reviewed in Clark 1979). It was determined that 13-lined ground squirrels that received the opioid receptor antagonist naloxone delivered through an osmotic mini-pump over 28 days showed a decreased number of hibernation bouts when injected with blood serum from bears (Bruce et al., 1987). Upon removal of naloxone the 13-lined ground squirrels started to show hibernation bouts similar to control animals. This data indicates that the opioid system is involved in the entrance into hibernation. However, it is interesting to note that the animals were still able to undergo hibernation. Naloxone has high affinity for the delta opioid receptors and lower affinity for the Mu and Kappa receptors. This could mean that the delta opioid receptor is only partly involved and that the actions of HIT are through either the Mu or the Kappa opioid receptors. Further study into the opioid system revealed that hibernation was not inducible in the summer through the kappa opioid receptor using woodchuck serum (Oeltgen et al., 1987). Subsequent studies into the opioid system revealed that stimulating the delta opioid receptor with D-Ala<sup>2</sup>-D-Leu<sup>5</sup>

enkephalin (DADLE) induced hibernation in a similar degree as woodchuck serum (Oeltgen et al., 1988). This response was slightly different for the mu and kappa opioid agonists, which induced hibernation to a much lesser degree than DADLE. Interestingly, upon injection of woodchuck serum both kappa and mu opioids decreased the hibernation bouts when compared to saline and woodchuck serum. Although this work points to the idea that hibernation is induced by the delta opioid receptors, there are several topics to consider. First, the authors indicate that the 13-lined ground squirrels were trapped in the wild in the summer, but do not indicate when the animals were used in their experiment. Knowing the timing of the experiment is important because it took the animals a minimum of five days and a maximum of nineteen days, which could put the animals into the hibernation season. If this is the case then it would be difficult to separate normal from DADLE induced hibernation bouts. Another point to consider is the authors did not reverse the effects of DADLE or HIT using an antagonist. This leads to the question of whether or not the effects seen are specific to the opioid receptors.

Recently, the metabolite adenosine has been implicated in the entrance into hibernation during the hibernation season in Arctic ground squirrels (Jinka et al., 2011). The researchers in this study find that intracerebroventricular administration of the A<sub>1</sub>AR agonist N<sup>6</sup>-Cyclohexyladenosine (CHA) induced hibernation reliably during the mid-season of hibernation. CHA did not induce hibernation in the summer season and unreliably in the early hibernation season where only one third of the animals entered hibernation. The authors were also able to reverse entrance into hibernation using an A<sub>1</sub> receptor (A<sub>1</sub>AR) antagonist, providing further support that the A<sub>1</sub>AR is important for the entrance into torpor. One noteworthy aspect of this experiment is that CHA did not induce hibernation after the first hibernation bout, which is odd considering the system for entrance into hibernation should already be expressed. An

explanation for these results is that the A<sub>1</sub>AR could be acting in parallel of another receptor system that is inducing hibernation. That would mean that as the season progresses, the A<sub>1</sub>AR could be up regulated to have a greater effect on the organism. Conversely, a down regulation of a receptor that has inhibitory action on the A<sub>1</sub>AR would have the same effect. The second explanation is supported by research done on the distribution of A<sub>1</sub>AR in the Columbian ground squirrel (Lee et al., 1993). The authors used autoradiographic binding studies to determine the A<sub>1</sub>AR concentration in several brain regions including the midbrain and hypothalamus. The hypothalamus showed no difference in concentration of A<sub>1</sub>AR or adenosine. Due to the static nature of the A<sub>1</sub>AR and adenosine concentration it is likely that the sensitization is due to the decoupling of another receptor type. Although the authors found no difference in the hypothalamus they did show a difference in the midbrain. Note that the autoradiographic binding data was conducted in a different species than the AGS, which could have a different mechanism of entrance into hibernation. This is unlikely however since A<sub>1</sub>AR regulates onset of torpor in hamsters and AGS suggesting a universal A<sub>1</sub>AR mechanism (Tamura et al., 2005). Another research group found increased expression of c-Fos, an indicator of neuronal activity in the medial preoptic area of the hypothalamus during the entry phase of the hibernation bout in 13-lined ground squirrels (Bratincsak et al., 2007). This is interesting due to the medial preoptic area of the hypothalamus being implicated in the control of thermoregulation. Additional information about the induction of hibernation through an A<sub>1</sub>AR mechanism is scarce.

In the current study, the A<sub>1</sub>AR was characterized in summer and winter season AGS to investigate the mechanism of A<sub>1</sub>AR seasonal sensitization. To achieve our goal, A<sub>1</sub>AR agonist affinity and potency was determined. In order to interpret the results it is necessary to review how an agonist binds to the A<sub>1</sub>AR and the effects of synergistic binding.

A<sub>1</sub>AR is part of the seven trans-membrane G-protein coupled receptor family (GPCR). GPCRs are characterized by an N-terminal extracellular end, a C-terminal intracellular tail and a peptide chain that snakes through the membrane and forms the seven trans-membrane domains. In order to propagate the external signal, the C-terminal tail binds with a G-protein complex made up of a G<sub>α</sub>, G<sub>β</sub>, and G<sub>γ</sub> subunit. In the absence of stimulation the G<sub>α</sub> subunit of the G-protein complex is bound to guanosine diphosphate (GDP). The presence of an agonist induces conformational changes to the receptor and, in turn, to the G-protein complex. This decreases the G<sub>α</sub> subunits affinity for GDP and increases it for guanosine-5'-triphosphate (GTP). GTP binding induces the release of the G-protein complex from the C-terminus and the disassociation of the G-protein complex into a G<sub>α-GTP</sub> and a G<sub>β</sub>/G<sub>γ</sub> dimer. The extracellular signal is propagated by both the G<sub>α-GTP</sub> and the G<sub>β</sub>/G<sub>γ</sub> dimer. The signal is halted when GTP is hydrolyzed to GDP allowing the reformation of the G-protein complex.

The first step in extracellular signal transduction takes place when the agonist binds to the receptor. It has been shown that transmembrane helices 6 and 7 (TM6 and TM7) are involved in the formation of the agonist binding pocket (Olah et al., 1994). The amino acid residues of transmembrane helix 3 interact with the adenine group of the adenosine molecule (Rivkees et al., 1999). In addition, the residues in transmembrane helix 2 have been shown to be necessary for recognition of the ribose group (Xie et al., 2006).

Several GPCRs have been shown to form functional heteromers with the A<sub>1</sub>AR. Stimulation of the excitatory adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>AR) in the A<sub>2A</sub>AR/A<sub>1</sub>AR heteromer decreases agonist binding and efficacy (Ciruela et al., 2006, Casadó et al., 2010). The A<sub>1</sub>AR also forms heteromers with the purinergic P<sub>2Y</sub><sub>1</sub> receptor (P<sub>2Y</sub><sub>1</sub>R) in several cell types (Yoshioka et al., 2002, Tonazzini et al., 2008, Tonazzini et al., 2007). Activation of the A<sub>1</sub>AR in the P<sub>2Y</sub><sub>1</sub>R / A<sub>1</sub>AR

heteromer led to increased activation of the P<sub>2</sub>Y<sub>1</sub>R while a P<sub>2</sub>Y<sub>1</sub>R agonist decreased activation of the A<sub>1</sub>AR. Stimulation of purinergic P<sub>2</sub>Y<sub>2</sub> receptor (P<sub>2</sub>Y<sub>2</sub>R) in the P<sub>2</sub>Y<sub>2</sub>R/ A<sub>1</sub>AR heteromer had similar effects as the P<sub>1</sub>Y<sub>1</sub>R but the P<sub>2</sub>Y<sub>2</sub>R showed enhanced activity in the absence of A<sub>1</sub>AR activation (Suzuki et al., 2006). The metabotropic glutamate 1 $\alpha$  receptor (mGlu<sub>1 $\alpha$</sub> )/ A<sub>1</sub>AR heteromer resulted in increased efficacy of the mGlu<sub>1 $\alpha$</sub>  with decreased N-methyl-D-aspartate (NMDA) toxicity (Gines et al., 2000).

In addition to other GPCRs, small proteins can bind synergistically with the A<sub>1</sub>AR and induce changes in binding properties. Thus far three such proteins have been identified, adenosine deaminase, Cytoskeletal protein 4.1G and heat shock cognate protein 73 (hsc 73). ADA, an enzyme that metabolizes adenosine to inosine, has been shown to promote the A<sub>1</sub>AR into a high affinity state in several cell types independent of ADA activity (Ciruela et al., 1996, Saura et al., 1996). Cytoskeletal protein 4.1G and heat shock cognate protein 73 (hsc 73) interact with the third intercellular loop of the A<sub>1</sub>AR and inhibited ligand binding (Sarrio et al., 2000, Lu et al., 2004). Hsc 73 binding was decreased in a dose response manner when in the presence of ADA.

Although synergistic effects on A<sub>1</sub>AR are important aspects of how the extracellular signal is translated, internalization is another way external signals can be controlled. Generally GPCRs are internalized through receptor phosphorylation which promotes G-protein complex dissociation and rapid internalization in a vesicle. The A<sub>1</sub>AR has a low basal level of phosphorylation and, in the presence of an agonist, the level of phosphorylation stays static (Gao et al., 1999, Ciruela et al., 1997). Internalization studies indicated that A<sub>1</sub>AR cluster into caveolin like structures after 5-12h of chronic agonist stimulation (Escrache et al., 2003). The A<sub>1</sub>AR is then sorted into vesicles and either degraded or recycled to a non caveolar location at the cell surface. The rate of internalization is affected by allosteric modulation but not by

palmitate attachment (Ferguson et al., 2002). ADA internalizes along with the A<sub>1</sub>AR but is separated and recycled to a different membrane site than the A<sub>1</sub>AR. ADA, Hsc 73 and cytoskeletal protein 4.1G both promote internalization of the A<sub>1</sub>AR.

## Methods and Materials

### *Arctic Ground Squirrels*

AGS tissue was obtained from a tissue bank (courtesy of B. Barnes). All animals were euthanized under protocols approved by the UAF IACUC. AGS were captured near 66°38'N, 149°38'W under permit from the Alaska Department of Fish & Game.

Animals were housed at 22°C on an 18:6 day: night cycle (5/2011-8/2011) and at 2°C on a 4:20 day: night cycle (8/2011-1/2011). Hibernation was monitored using the “shavings added” method where hibernation (torpor) is indicated when shavings placed on the back of the AGS remain undisturbed 24h later (Lyman, 1948; Pengelley and Fisher, 1961). All tissue was harvested from adult male AGS during the summer or winter season. Winter season was defined by evidence of spontaneous hibernation (torpor). Winter AGS were euthanized without being aroused from torpor after demonstrating six to eleven torpor bouts and at least ten but not more than thirteen days in the current torpor bout. Summer season animals were defined as AGS that were captured after the 2010-2011 hibernation season and kept in captivity for two months before being euthanized.

Summer AGS were euthanized by decapitation under a surgical plane of anesthesia. Winter animals were hibernating and did not require anesthesia. Immediately following euthanization the brain was removed and the forebrain, hippocampus, and hypothalamus were isolated and frozen in liquid nitrogen. All tissue was stored at -80°C until use.

### *Isolation of Plasma Membrane for <sup>35</sup>S GTPγS and [<sup>3</sup>H]DPCPX Binding Experiments*

AGS membrane from forebrain, hippocampus, hypothalamus and brainstem were isolated as described previously with modifications (Giuntini et al., 2004). For membrane isolation protocol refer to appendix A. Briefly, tissue was homogenized on ice using an all glass Dounce homogenizer (10-15 strokes) in 20X volume homogenization buffer containing 10 mM HEPES, 2 IU/mL ADA, 640 mM sucrose and protease inhibitor tablets (Roche, Indianapolis, IN) and then further homogenized by polytron for 10-15 sec. The suspension was centrifuged at 1000 x g for 10 min at 4°C. Resultant supernatant was centrifuged at 48000 x g for 15 min at 4°C. Pellets were resuspended in Resuspension buffer containing 10 mM HEPES, 2 IU/mL ADA and protease inhibitor tablets. Suspension was centrifuged at 48000 x g for 15 min at 4°C. Pellets of AGS hippocampus and hypothalamus were suspended in a solution containing 6 mM HEPES, 122 µM GDP and 2.4 IU/mL ADA, the forebrain was suspended in 6mM HEPES, 77 µM GDP, and 0.5 IU/mL ADA and both were incubated at room temperature under gentle rocking for 60 min and then centrifuged at 48000 x g for 30 min. Subsequent pellets were resuspended in Resuspension buffer and stored at -80°C until use.

### *<sup>35</sup>S-GTPγS Binding*

<sup>35</sup>S-GTPγS binding experiments were performed as described previously with modifications (Giuntini et al., 2004). For detailed protocols refer to appendix B. On the day of the experiment, aliquots of summer and winter AGS were thawed on ice. The protein content was then determined by protein analysis (Bio-Rad, Hercules, CA) followed by centrifugation at 48000 x g for 30 min at 4°C. The pellet was then resuspended in previously optimized Assay buffer (refer to appendix C for optimization data) containing 50 mM HEPES, 200 mM NaCl, 10 mM MgCl<sub>2</sub>, 40 µM GDP, 100 µM Saponin, 1 IU/mL ADA and 1 mM DTT, at pH 7.4. 100 µg per ml protein was incubated with 400 pM of <sup>35</sup>S-GTPγS in a total volume of 100 µL for 90 minutes (refer to

appendix D for binding kinetics) under gentle rocking at 27°C. Non-specific activity was determined in the presence of 5  $\mu\text{M}$  GTP $\gamma\text{S}$ . Constitutive activity was defined as binding in the absence of CHA. The reaction was terminated by rapid vacuum filtration and then each well was washed three times with 200  $\mu\text{l}$  of ice cold 50 mM HEPES. The plate was allowed to dry overnight. 40  $\mu\text{L}$  of scintillation cocktail (PerkinElmer, Waltham, MA) was added to each well and  $^{35}\text{S}$  activity was determined in a 1450 Microbeta plus microplate scintillation counter (PerkinElmer, Waltham, MA) utilizing a one minute counting time. The effect of the A<sub>3</sub>AR was determined by preincubating the membrane on ice with an A<sub>3</sub>AR antagonist (MRS 1334, 500 nM) or agonist (Cl-IB-MECA, 132 nM) for at least one hour before conducting the  $^{35}\text{S}$ -GTP $\gamma\text{S}$  binding experiment.

#### *[ $^3\text{H}$ ]DPCPX Binding*

Saturation experiments to determine the K<sub>d</sub> and B<sub>max</sub> of [ $^3\text{H}$ ] DPCPX binding to A<sub>1</sub>AR were conducted on the membrane of the forebrain, hippocampus and hypothalamus of winter and summer AGS following the guidelines of (Hulme and Trevethick 2010) with modification. For binding protocols refer to appendix E. On the day of the experiment, aliquots of summer and winter AGS were thawed on ice. Protein content of each animal was determined by protein analysis (Bio-Rad, Hercules, CA) followed by centrifugation at 48000 x g for 30 min at 4°C. The pellet was then resuspended in a solution containing 50 mM HEPES and 2 IU/ml ADA.

Saturation experiments were performed by incubating 100  $\mu\text{g}/\text{ml}$  protein with nine concentrations of [ $^3\text{H}$ ] DPCPX ranging between 0.4 and 30 nM in the presence of 50 mM HEPES and 2 IU/mL ADA. Non-specific binding was defined in the presence of 7 $\mu\text{M}$  CPT. The solution was allowed to incubate for 90 minutes at room temperature and membrane bound ligand was isolated as described below.

$K_{iLo}$  was determined by displacing 1 nM [ $^3H$ ] DPCPX with nine concentrations of CHA ranging between 100 pM and 10  $\mu$ M in the presence of 100  $\mu$ g/mL protein, 50 mM HEPES, 2 IU/mL ADA and 1 mM GTP.  $K_{iHi}$  was defined by displacing 1 nM [ $^3H$ ] DPCPX (PerkinElmer, Waltham, MA) with CHA in the same manner as the  $K_{iLo}$  experiments but without GTP. The solution was allowed to equilibrate for 90 minutes at room temperature as indicated by kinetic experiments (sup. info). Free and bound [ $^3H$ ] DPCPX was separated through an Inotech glass fiber filter pad (0.35 mM thickness/0.75  $\mu$ M retention) (Inotech Bio. Sys., Derwood, MD) by rapid filtration (0.5 ml per sec per well) with a cell harvester (Tomtec, Hamden, CT). The filter was then allowed to dry overnight. The next morning each well was isolated and placed in a scintillation vial. Scintillation cocktail (PerkinElmer, Waltham, MA) was added and radioactivity was determined (1450 Microbeta Plus, PerkinElmer, Waltham, MA) with a five minute count per well.

#### *Data analysis*

$^{35}S$ -GTP $\gamma$ S specific binding was determined by subtracting non-specific binding from overall binding. Specific binding was converted to percent over constitutive receptor activity.  $pEC_{50}$ , Hill slope and span were determined using the function Log(agonist) vs response – variable slope (four parameters) in Graphpad Prism 5 (v 5.04) (Graphpad Software, La Jolla, CA).

[ $^3H$ ] DPCPX bound was converted from cpm to fmol per mg protein and the specific binding was calculated. A sum of squares F-test was used to determine if a one site or two site model was appropriate.  $K_d$  and  $B_{max}$  were determined with Graphpad Prism (one or two site –total and nonspecific).  $K_{iLo}$  for the displacement of [ $^3H$ ] DPCPX in the presence of GTP was calculated using the average  $K_d$  (one or two site – Fit  $K_i$ ).  $K_{iHi}$  was calculated for the displacement of [ $^3H$ ]

DPCPX without GTP (one or two site – Fit Ki) using the average Kd and  $K_{iLo}$ . Statistical differences were established between summer and winter AGS within the same tissue by way of a Student t-test (Excel 2013) with a threshold of  $p < 0.05$ . Standard error is expressed as standard error of the mean (SEM).



## Results

Summer and Winter AGS were euthanized during the appropriate season and then the binding parameters of the A<sub>1</sub>AR were investigated in three different tissues by probing agonist induced GDP/GTP exchange by way of <sup>35</sup>SGTPγS binding assay, [<sup>3</sup>H]DPCPX saturation experiments and [<sup>3</sup>H]DPCPX displacement by CHA in the presence or absence of GTP.

The effect of CHA on <sup>35</sup>S GTPγS binding in forebrain tissue from AGS collected in the summer season was determined over a range of 100 pm to 100 μM (Fig. 1). The resulting data indicated a Hill slope of less than 0.4. In order to ascertain if the broad range of CHA concentration was evoking binding at more than one site and thus contributing to the low Hill slope, the data was reanalyzed while constricting the highest CHA concentration to 1 μM. The results returned an increase in hillslope to greater than 0.6. In subsequent experiments, CHA concentration was constrained between 100pM to 1 μM which yielded a hillslope of greater than 0.9. Unless stated otherwise, all other <sup>35</sup>S-GTPγS binding experiments were restricted to a CHA concentration range of 100 pm to 1 μM.

A<sub>1</sub>AR agonist induced GDP/GTP exchange was then investigated in the forebrain, hippocampus and hypothalamus of summer and winter AGS (Fig. 2). As expected from the above results, CHA stimulated GDP/GTP exchange in a single site manner. The pEC<sub>50</sub> of CHA in both the hippocampus (7.08 ± 0.050, 7.24 ± 0.030 summer, winter) and hypothalamus (7.07 ± 0.054, 7.27 ± 0.071 summer, winter) was greater in the winter which is indicative of an increase in the potency of CHA. The same effect was not seen in

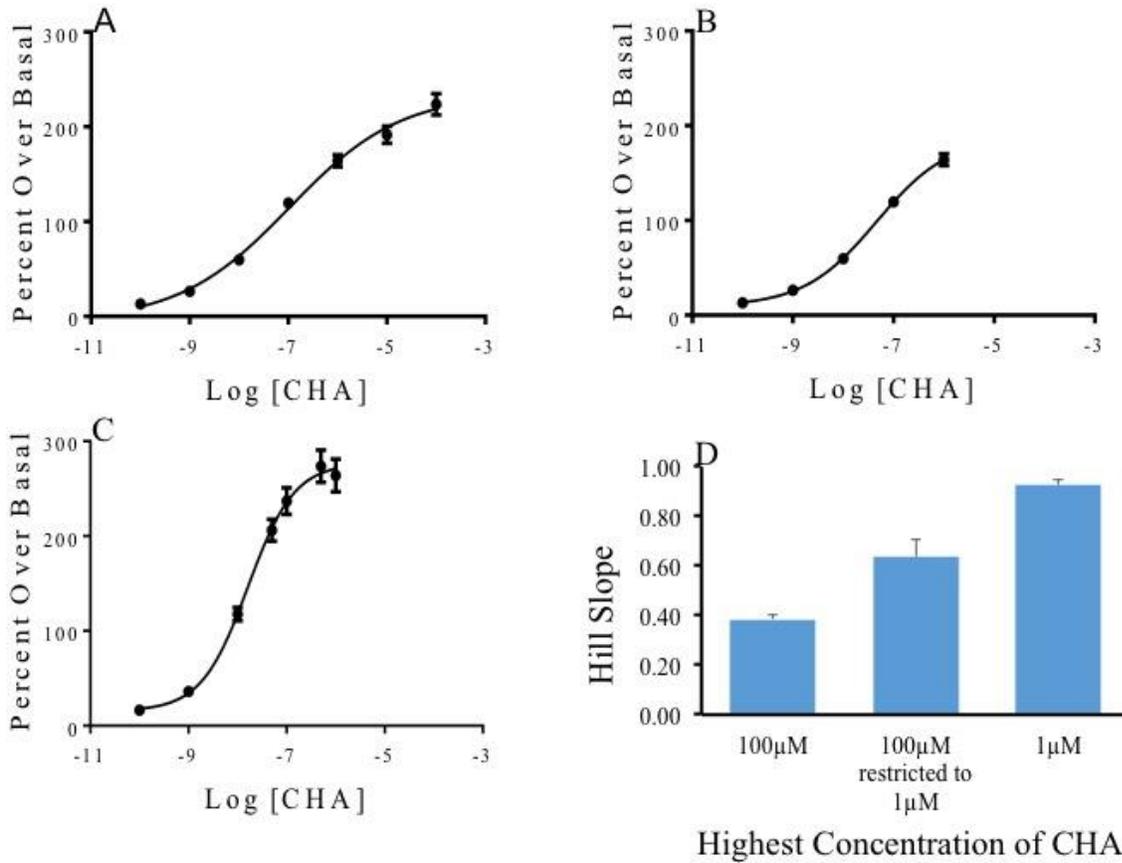


Fig. 1.1 The effect of dose-range on CHA induced GDP/GTP exchange in AGS forebrain. At concentrations greater than 100  $\mu$ M CHA demonstrates a Hill slope of  $0.39 \pm 0.015$  ( $n=5$ ) (A). Subsequent reanalysis with a maximum CHA dose of 1  $\mu$ M returned a Hill slope of  $0.63 \pm 0.070$  ( $n = 5$ ) (B). Repeating the experiment with a maximum dose of 1  $\mu$ M resulted in a Hill slope of  $0.93 \pm 0.018$  ( $n=6$ ) (C). Visual representation of change of Hill slope (D).

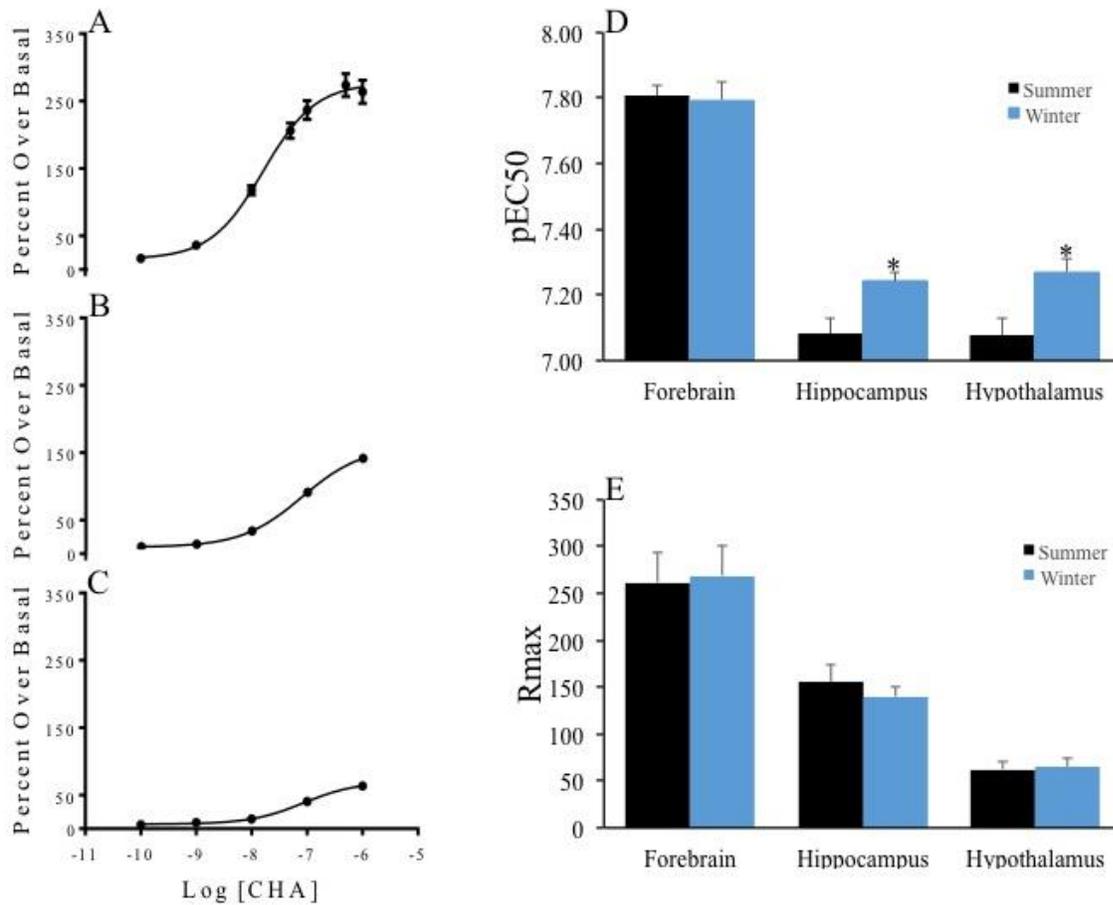


Fig. 1.2 CHA induced GDP/GTP exchange in summer and winter AGS brain tissue. Three brain tissues were analyzed: forebrain (6,5 summer, winter) (A), hippocampus (n= 7,7 summer, winter) (B) and hypothalamus (n=7,6 summer, winter) (C). Analysis of the curves for pEC50 indicated an increase in the potency of CHA in the hippocampus and hypothalamus of winter season AGS (D). There was no difference in Rmax (E). Winter graphs have a similar shape as the summer graphs. \* indicates statistical difference ( $p < 0.05$ ) compared to summer tissue.

the forebrain ( $7.80 \pm 0.035$ ,  $7.79 \pm 0.054$  summer, winter). None of the tissue displayed a difference in the  $R_{max}$  between the winter and summer groups. Subsequent follow up experiments in the forebrain with PIA, an  $A_1AR$  agonist, stimulated GDP/GTP exchange in a single site manner and did demonstrate an effect of season on potency ( $7.46 \pm 0.033$ ,  $7.59 \pm 0.042$  summer, winter) (Fig. 3).

To further characterize the  $A_1AR$  and to determine if a change in affinity or efficacy was driving the seasonal effect on potency, saturation and displacement assays were conducted in the three different brain regions. Saturation experiments using [ $^3H$ ] DPCPX indicated a single site model and yielded  $K_d$  and  $B_{max}$  values that did not differ between groups of animals (Fig. 4, refer to appendix F for more figures). CHA displaced [ $^3H$ ] DPCPX in a two-site model indicating a  $K_{iHi}$  and  $K_{iLo}$  (Fig 5). In the presence of GTP only the  $K_{iLo}$  site was detected.  $K_{iLo}$  values determined by CHA displacement of [ $^3H$ ] DPCPX in the presence GTP and  $K_{iHi}$  values determined in the absence of GTP were also found to be similar between groups (Table 1). Efficacy, measured by the GTP-induced shift in  $K_i$  defined as the ratio of  $K_{iHi}$  to  $K_{iLo}$ , likewise did not differ between groups. One result consistent with increased efficacy of CHA in winter animals was noted by a higher fraction of receptors in the high affinity state in the forebrain from winter animals. We next investigated the low Hill slope seen at concentrations of CHA exceeding  $1\mu M$  in the forebrain of summer and winter AGS. It has been established that CHA has low affinity for the  $A_3AR$  in the rat brain (Mazzoni et al., 1995). We therefore hypothesized that CHA at concentrations above  $1\mu M$  was stimulating the  $A_3AR$ . In order to test this hypothesis we

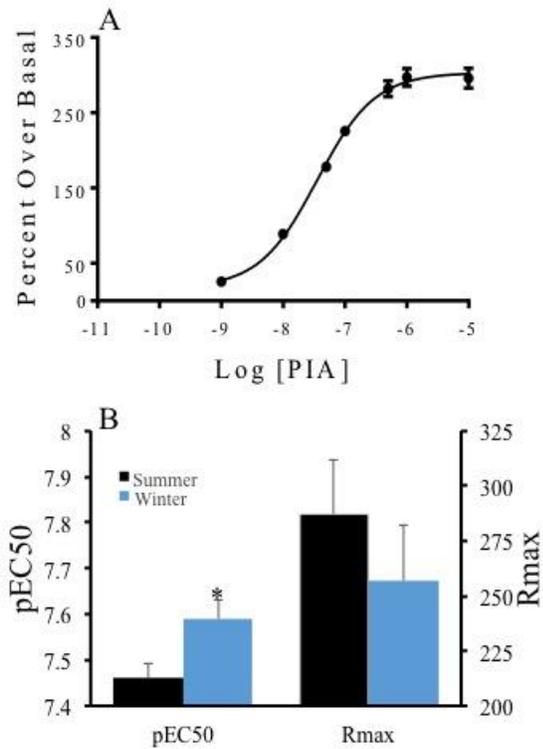


Fig. 1.3 PIA induced GDP/GTP exchange in the forebrain of summer AGS (A). PIA demonstrated an increase in potency in the winter season without effecting the Rmax (n=6,5) (B). \* indicates statistical difference ( $p < 0.05$ ) compared to summer tissue.

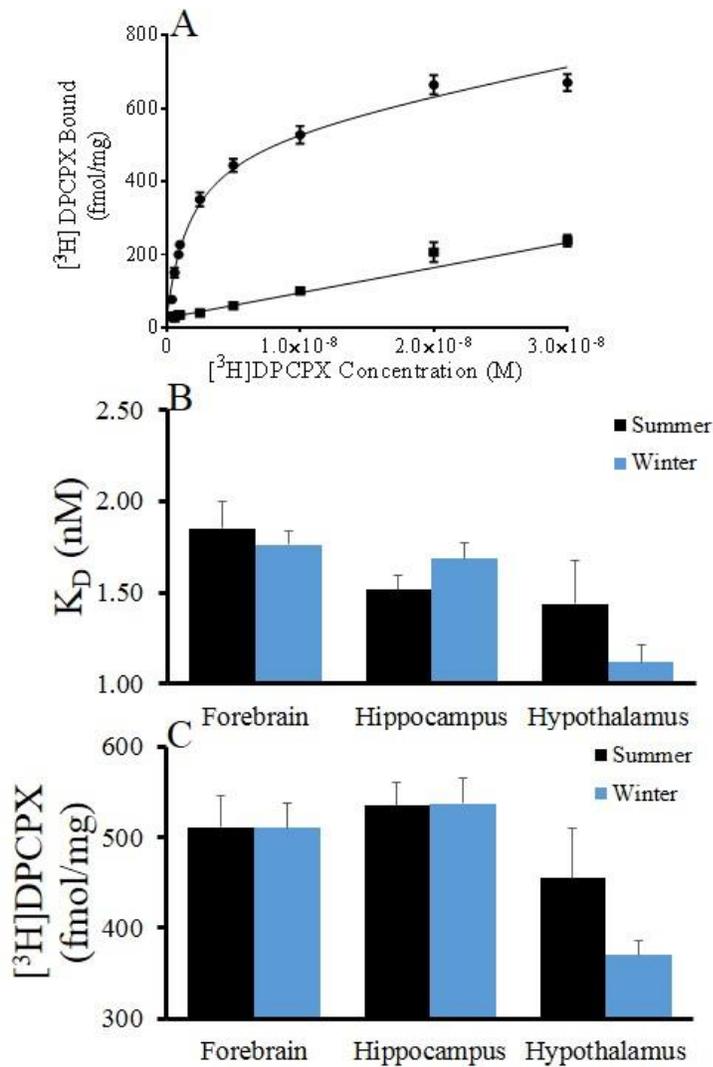


Fig. 1.4 Kinetics of [<sup>3</sup>H] DPCPX binding in summer and winter AGS. The saturation curve of DCPX in the forebrain of summer (A) was similar to that obtained in the winter season (n= 6,5 summer, winter) as well as the hippocampus (n= 8,6 summer, winter) and hypothalamus (n= 5,5 summer, winter) (not shown). [<sup>3</sup>H] DPCPX demonstrated nanomolar affinity for the A<sub>1</sub>AR in the three tissues tested with no effect of season (B). The number of A<sub>1</sub>AR in the forebrain, hippocampus and hypothalamus (C) were similar with no indication of a seasonal effect.

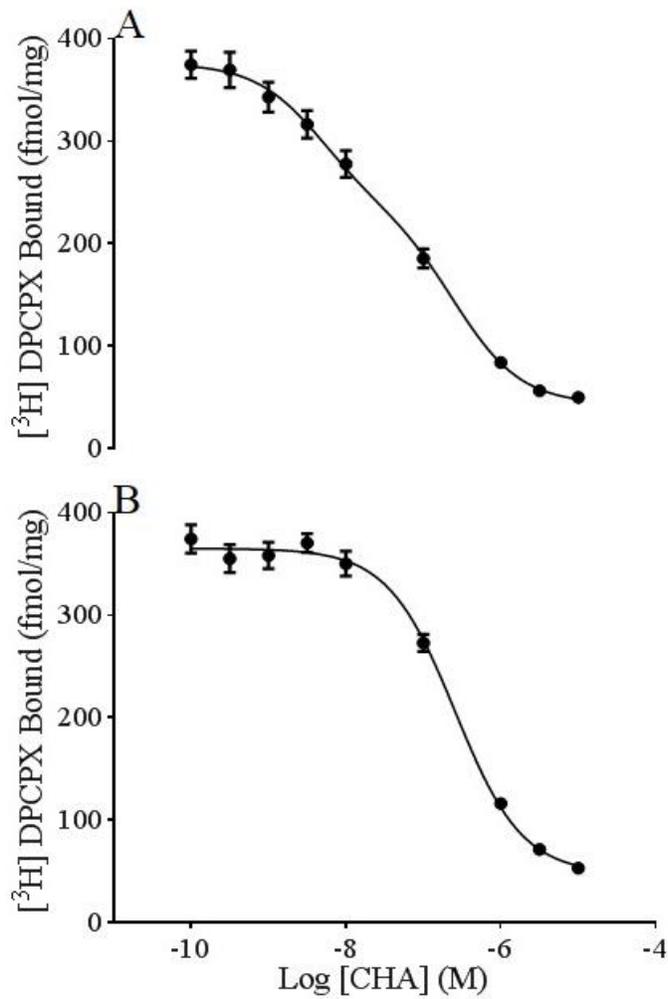


Fig. 1.5 [<sup>3</sup>H] DPCPX displaced with CHA in the presence or absence of GTP. Summer forebrain binding curves were best fit by a two-site model in the absence of 1  $\mu$ M GTP (A) and one-site in the presence of 1  $\mu$ M GTP (B). The general characteristics of these graphs were conserved in the winter season of the forebrain as well as the hippocampus and hypothalamus (not shown/Sup info).

Table 1.1 Parameters of the displacement of [<sup>3</sup>H] DPCPX AGS brain tissue. The ability for CHA to displace [<sup>3</sup>H] DPCPX was similar in the forebrain (n= 6,5 summer, winter), hippocampus (n= 8,6 summer, winter) and hypothalamus (n= 5,5 summer, winter) .  $K_{iHi}$  and  $K_{iLo}$  values were independent of season in all three tissues tested. The fraction of receptors in the high affinity state increased during the winter season in the forebrain but not the hippocampus or hypothalamus. \* indicates statistical difference ( $p < 0.05$ ) compared to summer tissue.

Characterization of CHA binding in summer and winter AGS		
Region and parameter studied	Summer	Winter
<i>Forebrain</i>		
[ <sup>3</sup> H]DPCPX Displacement with CHA + GTP		
$pK_{iLo}$	6.77 ± 0.026	6.76 ± 0.020
[ <sup>3</sup> H]DPCPX Displacement with CHA		
$pK_{iHi}$	8.56 ± 0.054	8.66 ± 0.024
Fraction high	0.41 ± 0.033	0.55* ± 0.024
<i>Hippocampus</i>		
[ <sup>3</sup> H]DPCPX Displacement with CHA + GTP		
$pK_{iLo}$	6.42 ± 0.041	6.49 ± 0.074
[ <sup>3</sup> H]DPCPX Displacement with CHA		
$pK_{iHi}$	8.32 ± 0.065	8.55 ± 0.11
Fraction high	0.51 ± 0.016	0.53 ± 0.020
<i>Hypothalamus</i>		
[ <sup>3</sup> H]DPCPX Displacement with CHA + GTP		
$pK_{iLo}$	6.77 ± 0.030	6.81 ± 0.021
[ <sup>3</sup> H]DPCPX Displacement with CHA		
$pK_{iHi}$	8.39 ± 0.013	8.39 ± 0.017
Fraction high	0.42 ± 0.066	0.55 ± 0.066

blocked the A<sub>3</sub>AR with a single concentration of MRS 1334, an A<sub>3</sub>AR antagonist, before assessing agonist stimulated GDP/GTP exchange by <sup>35</sup>S-GTPγS binding assay with up to 100 μM CHA (Fig. 6). The resulting data indicated that MRS 1334 had no effect on the Hill slope in either the summer (0.345 ± 0.021, 0.391 ± 0.019 DMSO, MRS 1334) or winter (0.430 ± 0.030, 0.482 ± 0.025 DMSO, MRS 1334). MRS 1334 treated tissue did display a decrease in pEC<sub>50</sub> of the same magnitude in both the summer (7.78 ± 0.073, 7.26 ± 0.038 DMSO, MRS 1334) and winter (7.95 ± 0.040, 7.26 ± 0.021 DMSO, MRS 1334). Although the change in potency was similar between the winter and summer seasons, the winter tissue treated with MRS 1334 displayed an increase in R<sub>max</sub> compared to winter DMSO treated tissue.

We next investigated the effect of stimulating A<sub>3</sub>AR with Cl-IB-MECA on agonist stimulated GDP/GTP exchange with up to 1 μM of CHA in the forebrain of summer AGS (Fig. 7). The resulting data indicated no difference in the presence or absence of Cl-IB-MECA in any of the binding parameters.

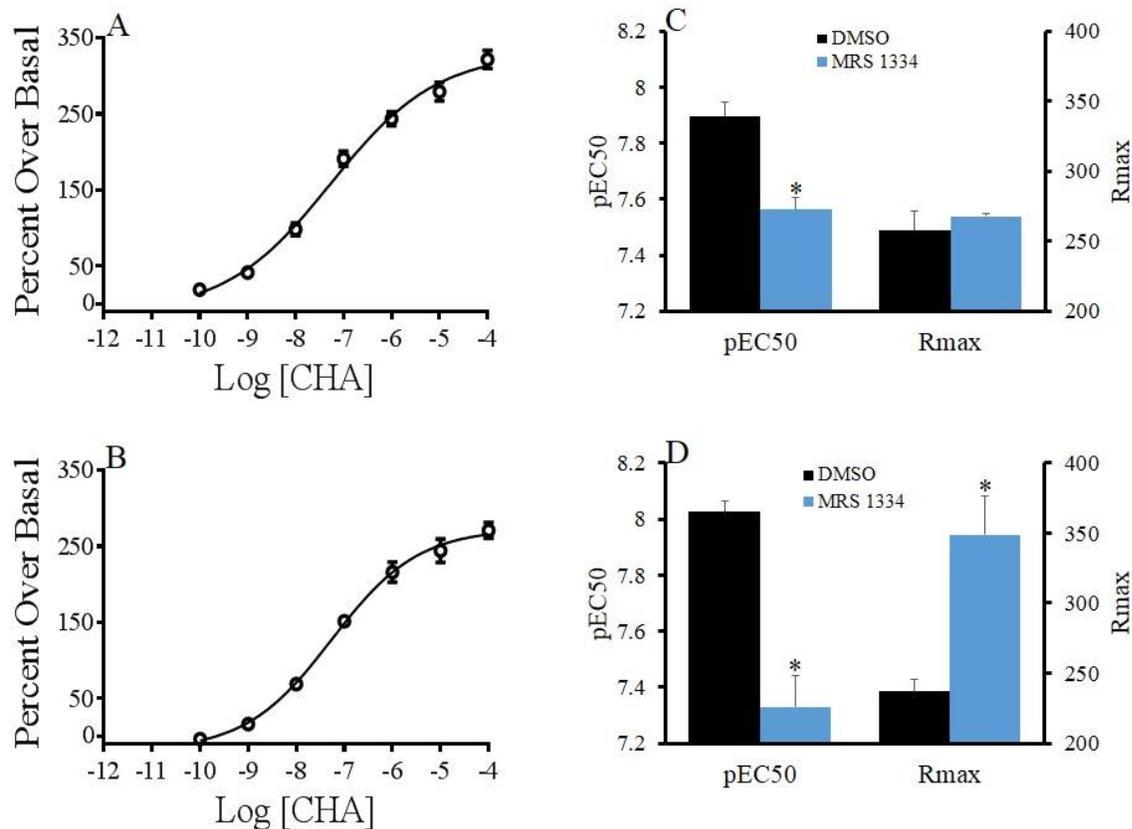


Fig.1.6 CHA induced GDP/GTP exchange in the presence of A<sub>3</sub>AR antagonist. MRS 1334 had no effect on the hill slope of CHA induced GDP/GTP exchange in either the forebrain of summer (n= 3,3 DMSO, MRS 1334) (A) or winter (n= 3,3 DMSO, MRS 1334) (B) season at a range of 100pM to 100μM suggesting the slow hill slope was not due to CHA binding to both the A<sub>1</sub>AR and the A<sub>3</sub>AR. MRS 1334 reduced the potency of CHA by the same magnitude in both the summer (C) and winter (D) season. In the winter MRS 1334 induced an increase in Rmax when compared with DMSO. \* indicates statistical difference (p < 0.05) compared to vehicle treated tissue.

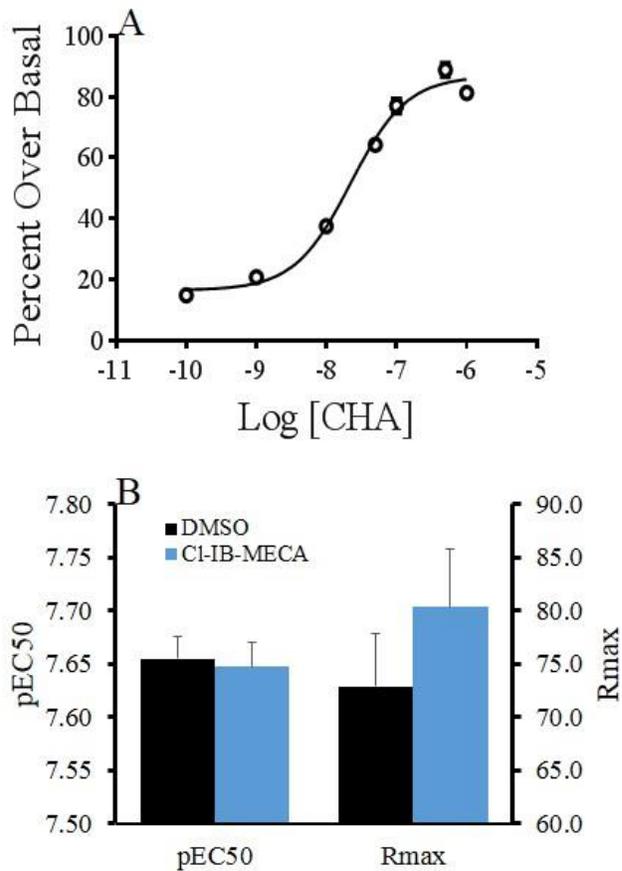


Fig. 1.7 CHA induced GDP/GTP exchange in the presence of A<sub>3</sub>AR agonist. Preincubation with Cl-IB-MECA (n= 4,4 DMSO, Cl-IB-MECA) (A) did not decrease the hill slope of the 100pM-1μM dose range as would be expected if the A<sub>3</sub>AR stimulation was inducing the low hill slope at higher concentrations of CHA. The presence or absence of Cl-IB-MECA had no effect on any pharmacological properties of CHA induced GDP/GTP exchange (B).



## Discussion

Torpor has been a topic of study for several decades. The mechanism that allows animals to enter torpor has yet to be determined. However, the A<sub>1</sub>AR has been shown to be critical for the safe expression of torpor. This study is the first to the author's knowledge to conduct a comprehensive analysis of the binding characteristics of A<sub>1</sub>AR in the brain of summer and winter AGS.

The affinity of adenosine receptor agonists can be species dependent (Klotz et al., 1991). This makes it critical to define the basic binding parameters of agonists such as CHA in each individual species. Direct ligand binding and competition assays have for the first time established K<sub>d</sub>, B<sub>max</sub>, K<sub>iHi</sub>, K<sub>iLo</sub>, the fraction of receptors in the high and low affinity states in AGS brain tissue. As expected [<sup>3</sup>H] DPCPX saturation experiments produced a one-site binding curve at the A<sub>1</sub>AR (Lohse et al., 1987). K<sub>d</sub> for DPCPX was similar to K<sub>d</sub>'s reported in rat brain and smooth muscle preparations as well as sheep pineal membranes (León et al., 2004, Peachey et al., 1994, Falcóna et al., 1997). B<sub>max</sub> for DPCPX binding was surprisingly consistent between brain regions in contrast to differences in R<sub>max</sub> of GTPγS binding. A functional assay such as GTPγS defines efficacy which may reflect the difference in functional surface receptors better than direct ligand binding.

IC<sub>50Lo</sub> and IC<sub>50Hi</sub> for CHA determined by displacing [<sup>3</sup>H] DPCPX was similar for that found in mouse forebrain (Johansson et al., 1997). IC<sub>50</sub> is not a constant and is not directly comparable due to its dependence on experimental conditions. The constant K<sub>i</sub> could not be compared due to a lack of published data. GTP produced a characteristic shift to a single site low affinity state

( $K_{i10}$ ) of the  $A_1AR$  observed in CHA displacement of [ $^3H$ ] DPCPX. In the absence of GTP a 2-site model was consistent with agonist binding to high and low affinity states of the GPCR.

CHA was found to induce GDP/GTP exchange as expected for an  $A_1AR$  agonist as shown in rat brain tissue (Giuntini et al., 2004). Binding characteristics of the hypothalamus and hippocampus indicated an increase in potency of CHA in the winter season. In the current study we report that the potency of CHA for  $A_1AR$  in the winter season increased by 37 percent in the hypothalamus and by 31 percent in the hippocampus. In addition, the potency of PIA in the forebrain increased by 26 percent in the winter, which argues for a global sensitization of the  $A_1AR$  in the AGS brain. In the forebrain, an increase in potency during the winter season is explained by an increase in efficacy indicated by the fraction of receptors in the high affinity state. This interpretation was not consistent with results in the hypothalamus or hippocampus where there was a small but non-significant increase in the fraction of receptors in the high affinity state during the winter season. Interestingly, subsequent power analysis of the data indicated that a sample size of 15 AGS in each group would be sufficient to establish a difference in the fraction of receptors in the high affinity state in the hypothalamus, which would be consistent with the forebrain results. Power analysis also showed that a sample size of 20 AGS in each group would be sufficient to establish a difference in the  $K_{iHi}$  of CHA displacing [ $^3H$ ] DPCPX in both the forebrain and hippocampus. This data indicates that although there is a global change in the potency of CHA in the AGS brain, the means by which the shift in potency is achieved could be tissue specific. These conclusions will need to be further substantiated. The potential dual role of affinity and efficacy to influence the seasonal effect of an agonist could explain why PIA and not CHA showed the seasonal effect on potency. A detailed explanation of

the potential role of affinity and efficacy on individual agonist binding properties is beyond the scope of this paper.

A lower Hill slope noted at higher concentrations of CHA could be due to a second binding site that was found to not be an A<sub>3</sub>AR site. Given that the A<sub>2B</sub>AR is not normally expressed in the brain and CHA has very low affinity for the A<sub>2A</sub>AR the second site is most likely not an adenosine receptor site. The low Hill slope at higher concentrations of CHA could also be due to negative cooperativity that occurs when the A<sub>1</sub>AR form homomers (Gracia et al., 2013).

Interestingly, inhibition of the A<sub>3</sub>AR leads to a decrease in the potency of CHA independent of season. This indicates possible positive cross-talk between the A<sub>1</sub>AR and the A<sub>3</sub>AR. This conclusion was not supported by the stimulation of the A<sub>3</sub>AR which did not increase the potency of CHA. Considering that the A<sub>3</sub>AR is not being stimulated by CHA it is likely that the A<sub>3</sub>AR is already exerting a maximal effect on the A<sub>1</sub>AR. Therefore CHA potency at the A<sub>1</sub>AR would not increase by stimulating the A<sub>3</sub>AR.

Adjustment of GDP/GTP exchange data to reflect the density of the A<sub>1</sub>AR would give an understanding of the functional receptors per total receptor pool. This could give an indication of the number of spare receptors in the system.

In summary, evidence supports increased efficacy of CHA during the winter season due in part to an increase in the proportion of high affinity binding sites. Direct ligand binding and measurements of GDP/GTP exchange in AGS brain tissue yield results consistent with the behavior of the A<sub>1</sub>AR in other species.



## Conclusions

The A<sub>1</sub>AR has been shown to be critical in order for the AGS to enter the hibernation state. [<sup>35</sup>S] GTPγS, [<sup>3</sup>H] DPCPX saturation and displacement assays were performed in order to ascertain the mechanisms that drive A<sub>1</sub>AR involvement in torpor. This work is the first to establish basic pharmacological parameters for A<sub>1</sub>AR CHA induced GDP/GTP exchange as well as K<sub>iHi</sub> and K<sub>iLo</sub>. Basic A<sub>1</sub>AR characterizations of [<sup>3</sup>H] DPCPX binding such as K<sub>d</sub> and B<sub>max</sub> were also established.

The conclusion that an increase in efficacy is responsible for the increase in agonist potency during the winter season is one of several possible explanations. There are several additional explanations that could change agonist potency. One possible explanation is that the A<sub>1</sub>AR undergoes modification by mRNA during the winter season which could change the potency. A second alternative explanation is that a change in the synergistic effects on the A<sub>1</sub>AR by other receptors and proteins are helping drive the seasonal change in potency.

One potential reason why this study did not find an effect of season on the K<sub>d</sub>, B<sub>max</sub>, K<sub>iHi</sub> or K<sub>iLo</sub> is because the AGS were euthanized while hibernating. In Jinka et al., 2011 the effect of CHA was investigated during interbout arousal. If the mechanisms that govern A<sub>1</sub>AR seasonal sensitivity are dynamic between the stages of hibernation then it is possible that an actively hibernating AGS would not demonstrate the seasonal effect. This research cannot address this possibility since tissues from both hibernating and interbout arousal animals was not available. It would be interesting to perform a similar study on animals during interbout arousal as well as during hibernation and the summer season.

A similar effect of CHA induced hibernation should be seen if there is an increase in the concentration of endogenous adenosine. During entrance into hibernation, it is possible that a sudden surge of adenosine acting on A<sub>1</sub>AR drives the entrance into hibernation. For this reason it would be of interest to measure adenosine concentrations as well as binding parameters.

A seasonal increase in potency was established for CHA in the hippocampus and hypothalamus. However, in the forebrain, PIA but not CHA demonstrated increased potency in the winter season. It is also interesting that the forebrain but not the hippocampus or hypothalamus demonstrated an increase in the fraction of receptors in the high affinity state. It has been well established that agonists can interact with a specific signaling path. If this is the case with PIA, this could indicate that discreet but different signaling pathways are being sensitized in order to facilitate the hibernation state.

One requirement of the <sup>35</sup>S-GTPγS binding experiment is the depletion of adenosine from the tissue. Since ADA has been shown to change ligand binding to the A<sub>1</sub>AR it is possible that the addition of ADA results in non-physiological data. It would therefore be of interest to determine binding parameters of CHA in tissue that was depleted of adenosine through a non-ADA method.

In order to determine the possible synergistic influences on the A<sub>1</sub>AR during the summer and winter season a pull down assay could be utilized using the A<sub>1</sub>AR to capture interacting proteins. These proteins would then be analyzed by High-Pressure Liquid Chromatography or Time-of-flight mass spectrometry.

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## Appendix A

### Isolating the Membrane Fraction from Brain Tissue

This protocol details how to isolate the membrane fraction of brain tissue for use in [<sup>35</sup>S] Guanosine 5'-Triphosphate or Direct Ligand binding experiments.

1. Prepare Protease Inhibitor Solution Containing;
  - a. 10mM HEPES
  - b. 2 IU/mL Adenosine Deaminase
  - c. Roche protease inhibitor tablets
2. Prepare Homogenization Buffer Containing;
  - a. Protease Inhibitor Solution
  - b. 636mM Sucrose
3. Prepare Resuspension Buffer Containing;
  - a. Protease Inhibitor solution
4. Prepare GDP Incubation Buffer Containing;
  - a. 50mM HEPES
  - b. 100 $\mu$ M GDP
  - c. 2 IU/mL Adenosine Deaminase
5. Weigh tissue
6. Homogenize in 20x volume of homogenization buffer
  - a. Use an all glass dounce homogenizer
7. Centrifuge at 1,000x gravity at 4°C for 5min.
8. Centrifuge Supernant at 48,000xg for 15min at 4°C.

9. Resuspend pellet in 20x volume of resuspension buffer.
10. Centrifuge at 48,000xg for 15min at 4°C
11. Resuspend pellet in 20x volume of GDP incubation buffer.
12. Incubate solution under gentle rocking at room temperature for 1h.
13. Centrifuge at 48,000xg for 30min at 4°C
14. Resuspend in 10x resuspending buffer.
15. Determine protein concentration.
16. Store at -80°C in single use aliquots.
17. Centrifuge at 48,000xg for 30min at 4°C before use in binding experiments.

## Appendix B

### <sup>35</sup>S-GTP $\gamma$ S: Protocol

Optimal concentration steps modified from PerkinElmer Delfia System

#### A. Optimal Concentration of MgCl<sub>2</sub> and GDP for a 96 Well Plate

Steps:

1. Prepare all solutions on ice.
2. Prepare a Buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
  - c. 200mM NaCl
  - d. 1mM Dithiothreitol
3. All the following solutions should be prepared using the Buffer solution.
4. Prepare four solutions containing between 5 and 75mM MgCl<sub>2</sub>.
  - a. Concentration in well will be between 1 and 15mM MgCl<sub>2</sub>.
  - b. 5, 15, 50 and 75mM MgCl<sub>2</sub> worked well with the A<sub>1</sub>AR.
5. Label the four solutions A, B, C and D
6. Prepare four solutions containing between 0.5 and 200 $\mu$ M GDP
  - a. Concentration in well will be between 0.1 and 40 $\mu$ M GDP.
  - b. 25, 50, 100 and 200 $\mu$ M GDP worked well with the A<sub>1</sub>AR.
7. Label these solutions GTP 1, 2, 3, 4
8. Prepare 0 and 5x agonist solutions.
  - a. Final concentration of 5x in well should be 100\* the drugs K<sub>d</sub>

9. Label these solutions Basal and Agonist.
10. Prepare a membrane solution between 200 to 500 $\mu\text{M}\cdot\text{mL}^{-1}$ .
  - a. Concentration in well will be between 4 and 10 $\mu\text{M}\cdot\text{mL}^{-1}$ .
  - b. Check the plate guidelines for maximal protein allowed.
11. Prepare an 2nM [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  solution.
  - a. Final concentration in well should be 400pM
12. Pipette 20 $\mu\text{L}$  of A into rows A and B.
13. Pipette 20 $\mu\text{L}$  of B into rows C and D.
14. Pipette 20 $\mu\text{L}$  of C into rows E and F.
15. Pipette 20 $\mu\text{L}$  of D into rows G and H.
16. Pipette 20 $\mu\text{L}$  of GTP 1 into wells A (1-6), C (1-6), E (1-6) and G (1-6).
17. Pipette 20 $\mu\text{L}$  of GTP 2 into wells A (7-12), C (7-12), E (7-12) and G (7-12).
18. Pipette 20 $\mu\text{L}$  of GTP 3 into wells B (1-6), D (1-6), F (1-6) and H (1-6).
19. Pipette 20 $\mu\text{L}$  of GTP 4 into wells B (7-12), D (7-12), F (7-12) and H (7-12).
20. Pipette 20 $\mu\text{L}$  of basal in columns 1, 2, 3, 7, 8 and 9.
21. Pipette 20 $\mu\text{L}$  of agonist solution into columns 4, 5, 6, 10, 11, and 12.
22. Pipette 20 $\mu\text{L}$  of 2mM [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  solution into each well.
23. Pipette 20 $\mu\text{L}$  of Membrane into each well.
24. Incubate the plate for 90 minutes at 27 °C under gentle rocking.
25. Terminate the reaction by rapid vacuum filtration.
26. Wash each well three times with 200 $\mu\text{L}$  of ice cold 50mM HEPES.
27. Dry filter plate overnight.
28. Add 40 $\mu\text{L}$  of microsintillation cocktail into each well.

29. Read plate using a 60 second count.
30. Transform data from counts-per-minute into percent over basal.
31. Determine optimal  $\text{MgCl}_2$  and GDP concentration by graphing percent over basal data in a 3-D bar graph.

#### B. Optimal Concentration of NaCl for a 96 Well Plate

This experiment is designed to replicate the experiment on the same plate.

1. Prepare all solutions on ice.
2. Prepare Buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of  $\text{MgCl}_2$
  - e. Optimal concentration of GDP
3. Prepare all the following solutions in buffer
4. Prepare seven different concentrations between 40 to 1000mM of NaCl
  - a. Concentration in well will be 40 to 250mM NaCl
  - b. 40, 80, 200, 400, 600, 800 and 1000mM worked well for the  $\text{A}_1\text{AR}$ .
5. Label these solutions A, B, C, D, E, F, G respectively
6. Label an 8<sup>th</sup> solution H which will contain Buffer but no NaCl.
7. Prepare a 0 and a 4x agonist solutions.
  - a. The final concentration of agonist should be the same concentration as in step A.
8. Label these solutions blank and agonist.

9. Prepare a membrane solution at the same concentration as in step A.
10. Prepare an 1.6nM [<sup>35</sup>S]GTPγS solution.
  - a. Final concentration in well should be 400pM
11. Pipette 25μL of buffer into wells A (1-6) and E (1-6).
12. Pipette 25μL of A into wells A (7-12) and E (7-12).
13. Pipette 25μL of B into wells B (1-6) and F (1-6).
14. Pipette 25μL of C into wells B (7-12) and F (7-12).
15. Pipette 25μL of D into wells C (1-6) and G (1-6)
16. Pipette 25μL of E into wells C (7-12) and G (7-12).
17. Pipette 25μL of F into wells D (1-6) and H (1-6)
18. Pipette 25μL of G into wells D (7-12) and H (7-12).
19. Pipette 25μL of 0 agonist into columns 1-3 and 7-9.
20. Pipette 25μL of 4x agonist into columns 4-6 and 10-12.
21. Pipette 25μL of [<sup>35</sup>S]GTPγS into each well..
22. Pipette 25μL of membrane solution into each well.
23. Incubate the plate for 90 minutes at 27 °C under gentle rocking.
24. Terminate the reaction by rapid vacuum filtration.
25. Wash each well three times with 200μL of ice cold 50mM HEPES.
26. Dry filter plate overnight.
27. Add 40μL of microsintillation cocktail into each well.
28. Read plate using a 60 second count.
29. Transform data from counts-per-minute into percent over basal.

30. Graph Concentration of NaCl vs Percent over basal to determine optimal concentration.

C. Optimal Concentration of Saponin.

This experiment is designed to replicate the experiment on the same plate.

1. Prepare all solutions on ice.
2. Prepare buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of MgCl<sub>2</sub>
  - e. Optimal concentration of GDP
  - f. Optimal concentration of NaCl
3. Prepare all the following solutions in buffer.
4. Prepare seven different solutions between 0.012 and 4mg\*ml<sup>-1</sup> of saponin.
  - a. Concentration in well will be between 3 and 1000μg\*ml<sup>-1</sup>.
  - b. 0.012, 0.04, 0.12, 0.4, 1.2, 2.6 and 4mg\*ml<sup>-1</sup> works well for the A<sub>1</sub>AR.
5. Label these solutions A, B, C, D, E, F and G.
6. Label an 8<sup>th</sup> solution H which contains only buffer.
7. Prepare a 0 and a 4x agonist solutions.
  - a. The final concentration of agonist should be the same concentration as in step A.
8. Label these basal and agonist.
9. Prepare a membrane solution at the same concentration as in step A.

10. Prepare an 1.6nM [<sup>35</sup>S]GTP $\gamma$ S solution.
  - a. Final concentration in well should be 400pM
11. Pipette 25 $\mu$ L of buffer into wells A (1-6) and E (1-6).
12. Pipette 25 $\mu$ L of A into wells A (7-12) and E (7-12).
13. Pipette 25 $\mu$ L of B into wells B (1-6) and F (1-6).
14. Pipette 25 $\mu$ L of C into wells B (7-12) and F (7-12).
15. Pipette 25 $\mu$ L of D into wells C (1-6) and G (1-6)
16. Pipette 25 $\mu$ L of E into wells C (7-12) and G (7-12).
17. Pipette 25 $\mu$ L of F into wells D (1-6) and H (1-6)
18. Pipette 25 $\mu$ L of G into wells D (7-12) and H (7-12).
19. Pipette 25 $\mu$ L of 0 agonist into columns 1-3 and 7-9.
20. Pipette 25 $\mu$ L of 4x agonist into columns 4-6 and 10-12.
21. Pipette 25 $\mu$ L of [<sup>35</sup>S]GTP $\gamma$ S into each well..
22. Pipette 25 $\mu$ L of membrane solution into each well.
23. Incubate the plate for 90 minutes at 27 °C under gentle rocking.
24. Terminate the reaction by rapid vacuum filtration.
25. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
26. Dry filter plate overnight.
27. Add 40 $\mu$ L of microsintillation cocktail into each well.
28. Read plate using a 60 second count.
29. Transform data from counts-per-minute into percent over basal.
30. Graph Concentration of saponin vs Percent over basal to determine optimal concentration.

D. Optimal Concentration of membrane.

This experiment is designed to replicate the experiment on the same plate.

1. Prepare all solutions on ice.
2. Prepare buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of  $MgCl_2$
  - e. Optimal concentration of GDP
  - f. Optimal concentration of NaCl
  - g. Optimal concentration of Saponin
3. Prepare all the following solutions in buffer.
4. Prepare seven different solutions of membrane between 40 and 480  $\mu g \cdot ml^{-1}$ .
  - a. Concentration in well will be between 10 and 120  $\mu g \cdot ml^{-1}$ .
  - b. 10, 20, 40, 60, 80, 100 and 120  $\mu g \cdot ml^{-1}$  works well for the  $A_1AR$ .
5. Label these solutions A, B, C, D, E, F and G.
6. Label an 8<sup>th</sup> solution H which contains only Buffer.
7. Prepare a 0 and a 4x agonist solutions.
  - a. The final concentration of agonist should be the same concentration as in step A.
8. Label these basal and agonist.
9. Prepare a 1.6nM [<sup>35</sup>S]GTP $\gamma$ S solution.
  - a. Final concentration in well should be 400pM

10. Pipette 25 $\mu$ L of Buffer into wells A (1-6) and E (1-6).
11. Pipette 25 $\mu$ L of A into wells A (7-12) and E (7-12).
12. Pipette 25 $\mu$ L of B into wells B (1-6) and F (1-6).
13. Pipette 25 $\mu$ L of C into wells B (7-12) and F (7-12).
14. Pipette 25 $\mu$ L of D into wells C (1-6) and G (1-6)
15. Pipette 25 $\mu$ L of E into wells C (7-12) and G (7-12).
16. Pipette 25 $\mu$ L of F into wells D (1-6) and H (1-6)
17. Pipette 25 $\mu$ L of G into wells D (7-12) and H (7-12).
18. Pipette 25 $\mu$ L of 0 agonist into columns 1-3 and 7-9.
19. Pipette 25 $\mu$ L of 4x agonist into columns 4-6 and 10-12.
20. Pipette 25 $\mu$ L of [<sup>35</sup>S]GTP $\gamma$ S into each well..
21. Pipette 25 $\mu$ L of membrane solution into each well.
22. Incubate the plate for 90 minutes at 27 °C under gentle rocking.
23. Terminate the reaction by rapid vacuum filtration.
24. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
25. Dry filter plate overnight.
26. Add 40 $\mu$ L of microscintillation cocktail into each well.
27. Read plate using a 60 second count.
28. Transform data from counts-per-minute into percent over basal.
29. Graph Concentration of membrane vs Percent over basal to determine optimal membrane concentration.

E. Determination of time to equilibrium.

1. Prepare all solutions on ice.

2. Prepare buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of  $MgCl_2$
  - e. Optimal concentration of GDP
  - f. Optimal concentration of NaCl
  - g. Optimal concentration of Saponin
3. Prepare agonist solution at the same concentration as previous steps
4. Prepare membrane solution at 4x optimal membrane concentration
5. Prepare a 2nM [ $^{35}S$ ]GTP $\gamma$ S solution.
6. Prepare a 20 $\mu$ M GTP $\gamma$ S solution.
7. Choose 16 different time points over a 5h time period.
  - a. For example 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, 180, 210, 240, and 300min.
8. Pipette 25 $\mu$ L of agonist solution into each well.
9. Pipette 25 $\mu$ L of 1.6nM [ $^{35}S$ ]GTP $\gamma$ S solution into each well.
10. Pipette 25 $\mu$ L of buffer into Columns 1-3, 10-12
  - a. Label these columns total binding.
11. Pipette 25 $\mu$ L of GTP $\gamma$ S into Columns 4-6, 7-9.
  - a. Label these columns non-specific binding.
12. Pipette 25 $\mu$ L of membrane solution into wells A (1-6) at time 0 min.
13. Place plate on heater/shaker at 27 °C and shake plate gently.

14. Pipette 25 $\mu$ L of membrane solution into wells B (1-6) at time 60min.
15. Pipette 25 $\mu$ L of membrane solution into wells C (1-6) at time 120 min.
16. Pipette 25 $\mu$ L of membrane solution into wells E (1-6) at time 150 min.
17. Pipette 25 $\mu$ L of membrane solution into wells F (1-6) at time 180 min.
18. Pipette 25 $\mu$ L of membrane solution into wells G (1-6) at time 210 min.
19. Pipette 25 $\mu$ L of membrane solution into wells H (1-6) at time 240 min.
20. Pipette 25 $\mu$ L of membrane solution into wells A (7-12) at time 255 min.
21. Pipette 25 $\mu$ L of membrane solution into wells B (7-12) at time 270 min.
22. Pipette 25 $\mu$ L of membrane solution into wells C (7-12) at time 275 min.
23. Pipette 25 $\mu$ L of membrane solution into wells D (7-12) at time 280 min.
24. Pipette 25 $\mu$ L of membrane solution into wells E (7-12) at time 285 min.
25. Pipette 25 $\mu$ L of membrane solution into wells F (7-12) at time 290 min.
26. Pipette 25 $\mu$ L of membrane solution into wells G (7-12) at time 295 min.
27. Pipette 25 $\mu$ L of membrane solution into wells H (7-12) at time 300 min.
28. Remove plate from heater/shaker and terminate the reaction by rapid vacuum filtration.
29. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
30. Dry filter plate overnight.
31. Add 40 $\mu$ L of microsintillation cocktail into each well.
32. Read plate using a 60 second count.
33. Determine specific binding.
34. Determine time to equilibrium by graphing time vs specific CPM.

F. Determination of Dose-Response Range.

1. Prepare all solutions on ice.
2. Prepare buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of  $MgCl_2$
  - e. Optimal concentration of GDP
  - f. Optimal concentration of NaCl
  - g. Optimal concentration of Saponin
3. Prepare 15 different agonist solutions between 1pM and 1mM.
  - a. For  $N^6$ -Cyclohexyl Adenosine 2, 4, 40, 200, 400pM, 2, 4, 40, 200, 400nM and 2, 4, 40, 200 and 400 $\mu$ M worked well.
  - b. Label these solutions A-O
4. Prepare a solution with only buffer and label it basal.
5. Prepare membrane solution at 4x optimal membrane concentration
6. Prepare a 1.6nM [ $^{35}S$ ]GTP $\gamma$ S solution.
7. Prepare a 20 $\mu$ M GTP $\gamma$ S solution.
8. Pipette 25 $\mu$ L of basal into A (1-6).
9. Pipette 25 $\mu$ L of O into B (1-6).
10. Pipette 25 $\mu$ L of N into C (1-6).
11. Pipette 25 $\mu$ L of M into D (1-6).
12. Pipette 25 $\mu$ L of L into E (1-6).
13. Pipette 25 $\mu$ L of K into F (1-6).

14. Pipette 25 $\mu$ L of J into G (1-6).
15. Pipette 25 $\mu$ L of I into H (1-6).
16. Pipette 25 $\mu$ L of H into A (7-12).
17. Pipette 25 $\mu$ L of G into B (7-12).
18. Pipette 25 $\mu$ L of F into C (7-12).
19. Pipette 25 $\mu$ L of E into D (7-12).
20. Pipette 25 $\mu$ L of D into E (7-12).
21. Pipette 25 $\mu$ L of C into F (7-12).
22. Pipette 25 $\mu$ L of B into G (7-12).
23. Pipette 25 $\mu$ L of A into H (7-12).
24. Pipette 25 $\mu$ L of 1.6nM [<sup>35</sup>S]GTP $\gamma$ S solution.
25. Pipette 25 $\mu$ L of buffer into columns 1-3 and 9-12.
  - a. Label these columns total binding.
26. Pipette 25 $\mu$ L of GTP $\gamma$ S solution into columns 4-6 and 7-9.
  - a. Label these columns non-specific binding.
27. Pipette 25 $\mu$ L of membrane solution into every well.
28. Incubate plate for time determined from step E at 27 °C under gentle rocking.
29. Terminate the reaction by rapid vacuum filtration.
30. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
31. Dry filter plate overnight.
32. Add 40 $\mu$ L of microscintillation cocktail into each well.
33. Read plate using a 60 second count.
34. Determine specific binding and then percent over basal.

35. Graph Percent over basal vs. the log of the concentration.
  36. Determine an eight point dose range from graph.
- G. [<sup>35</sup>S]GTPγS Binding Protocol with modifications from Cooper, T. et al. G Protein-Coupled Receptors in Drug Discovery Vol. 552. Chapter 10. Edited by Leifert, W.R.
- This protocol is designed to repeat the experiment on the same plate.
1. Prepare all solutions on ice.
  2. Prepare buffer solution containing
    - a. 50mM HEPES
    - b. 1 IU/mL Adenosine Deaminase
    - c. 1mM Dithiothreitol
    - d. Optimal concentration of MgCl<sub>2</sub>
    - e. Optimal concentration of GDP
    - f. Optimal concentration of NaCl
    - g. Optimal concentration of Saponin
  3. Prepare each of the following solutions in Buffer.
  4. Prepare 7 different agonist solutions determined from step F.
    - a. Label these A-G
  5. Prepare an aliquot of buffer and label it Basal.
  6. Prepare membrane solution at 4x optimal membrane concentration
  7. Prepare a 1.6nM [<sup>35</sup>S]GTPγS solution.
  8. Prepare a 20μM GTPγS solution.
  9. Pipette 25μL of Basal into A (1-6 and 7-12).
  10. Pipette 25μL of G into B (1-6 and 7-12).

11. Pipette 25 $\mu$ L of F into C (1-6 and 7-12).
12. Pipette 25 $\mu$ L of E into D (1-6 and 7-12).
13. Pipette 25 $\mu$ L of D into E (1-6 and 7-12).
14. Pipette 25 $\mu$ L of C into F (1-6 and 7-12).
15. Pipette 25 $\mu$ L of B into G (1-6 and 7-12).
16. Pipette 25 $\mu$ L of A into H (1-6 and 7-12).
17. Pipette 25 $\mu$ L of Buffer into columns 1-3 and 9-12.
  - a. Label these columns total binding.
18. Pipette 25 $\mu$ L of GTP $\gamma$ S solution into columns 4-6 and 7-9.
  - b. Label these columns non-specific binding.
19. Pipette 25 $\mu$ L of membrane solution into every well.
20. Incubate plate for time determined from step E at 27 °C under gentle rocking.
21. Terminate the reaction by rapid vacuum filtration.
22. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
23. Dry filter plate overnight.
24. Add 40 $\mu$ L of microscintillation cocktail into each well.
25. Read plate using a 60 second count.
26. Determine specific binding and then percent over basal.
27. Graph Percent over basal vs. the log of the concentration.

H. [<sup>35</sup>S]GTP $\gamma$ S Binding Protocol with Second Agonist or Antagonist.

1. Prepare all solutions ice.
2. Repeat step E but this time incubate the membrane for 60min with the 100x the K<sub>d</sub> of the agonist or antagonist.

3. Prepare buffer solution containing
  - a. 50mM HEPES
  - b. 1 IU/mL Adenosine Deaminase
  - c. 1mM Dithiothreitol
  - d. Optimal concentration of  $MgCl_2$
  - e. Optimal concentration of GDP
  - f. Optimal concentration of NaCl
  - g. Optimal concentration of Saponin
4. Prepare each of the following solutions in Buffer
5. Prepare 7 different agonist solutions determined from step F.
  - h. Label these A-G
6. Prepare an aliquot of buffer and label it Basal.
7. Prepare membrane solution at a higher concentration than 4x the optimal membrane concentration
8. Split the membrane into two equal aliquots of known volume.
9. Label these aliquots Membrane A and Membrane B.
10. Add appropriate amount of drug into Membrane A and dilute to proper membrane concentration.
11. Add appropriate amount of buffer to Membrane B to optimal membrane concentration.
12. Incubate the membranes at  $4^{\circ}C$  for 60min.
13. Prepare a 1.6nM [ $^{35}S$ ]GTP $\gamma$ S solution.
14. Prepare a 20 $\mu$ M GTP $\gamma$ S solution.

15. Pipette 25 $\mu$ L of Basal into A (1-6 and 7-12).
16. Pipette 25 $\mu$ L of G into B (1-6 and 7-12).
17. Pipette 25 $\mu$ L of F into C (1-6 and 7-12).
18. Pipette 25 $\mu$ L of E into D (1-6 and 7-12).
19. Pipette 25 $\mu$ L of D into E (1-6 and 7-12).
20. Pipette 25 $\mu$ L of C into F (1-6 and 7-12).
21. Pipette 25 $\mu$ L of B into G (1-6 and 7-12).
22. Pipette 25 $\mu$ L of A into H (1-6 and 7-12).
23. Pipette 25 $\mu$ L of buffer into columns 1-3.
  - a. Label these columns total binding.
24. Pipette 25 $\mu$ L of buffer into columns 10-12.
  - a. Label these columns total binding w/ agonist (or antagonist)
25. Pipette 25 $\mu$ L of GTP $\gamma$ S solution into columns 4-6.
  - a. Label these columns non-specific binding.
26. Pipette 25 $\mu$ L of GTP $\gamma$ S solution into columns 7-9.
  - b. Label these columns non-specific binding w/ agonist (or antagonist).
27. Pipette 25 $\mu$ L of Membrane B solution into columns 1-6.
28. Pipette 25 $\mu$ L of Membrane A solution into columns 7-12.
29. Incubate plate for time determined from step 1 of this experiment at 27 °C under gentle rocking.
30. Terminate the reaction by rapid vacuum filtration.
31. Wash each well three times with 200 $\mu$ L of ice cold 50mM HEPES.
32. Dry filter plate overnight.

33. Add 40 $\mu$ L of microsintillation cocktail into each well.
34. Read plate using a 60 second count.
35. Determine specific binding and then percent over basal.
36. Graph Percent over basal vs. the log of the concentration.
  - a. Graph the w/ agonist or antagonist data using the basal binding from wells A (1-3).
  - b. The w/ agonist or antagonist basal is useful to determine the effect on the system in the absence of the first agonist.
37. Use this data to determine the effect of the secondary agonist/antagonist on the original agonist.



## Appendix C

### Optimization of $^{35}\text{S}$ -GTP $\gamma$ S binding

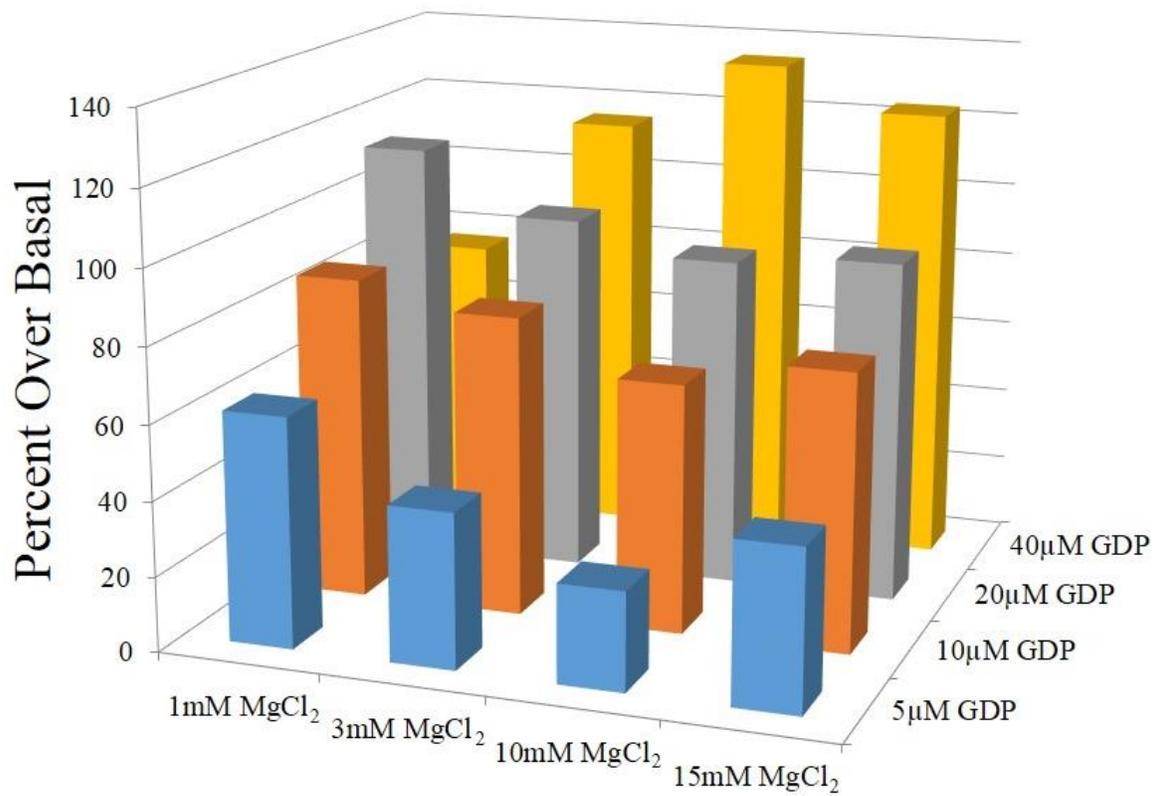


Fig. C.1 The optimization of MgCl<sub>2</sub> and GDP for  $^{35}\text{S}$ -GTP $\gamma$ S binding. Optimal concentrations of MgCl<sub>2</sub> and GDP were determined to be 10mM and 40μM respectively.

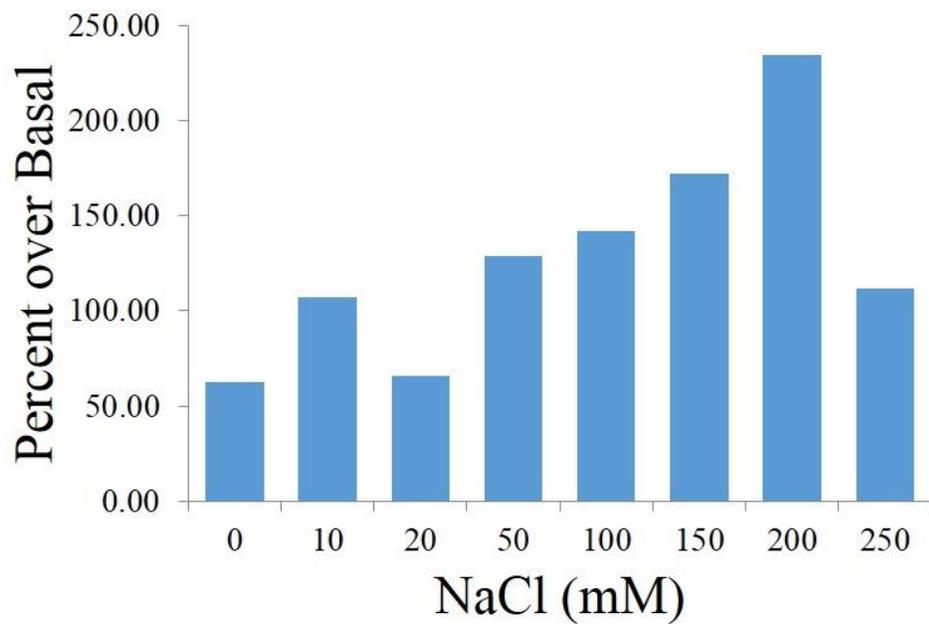


Fig. C.2 The optimization of NaCl for <sup>35</sup>S-GTPγS binding. The optimal concentration of NaCl was determined to be 200mM.

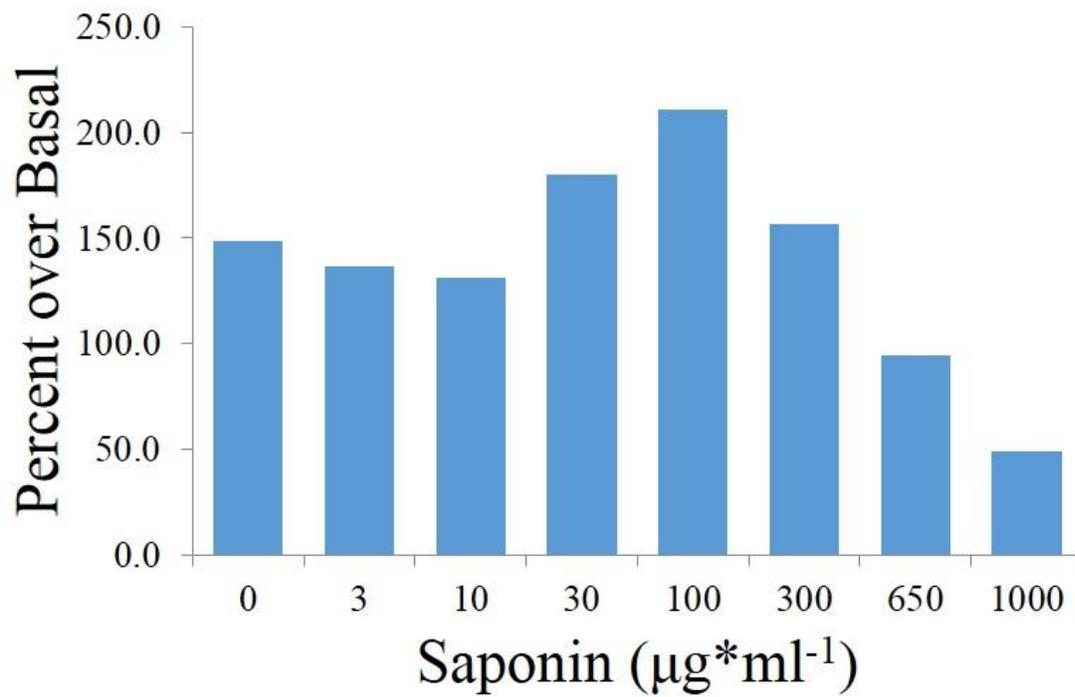


Fig. C.3 The optimization of saponin for <sup>35</sup>S-GTPγS binding. Optimal concentration of saponin was determined to be 100μg\*ml<sup>-1</sup>.

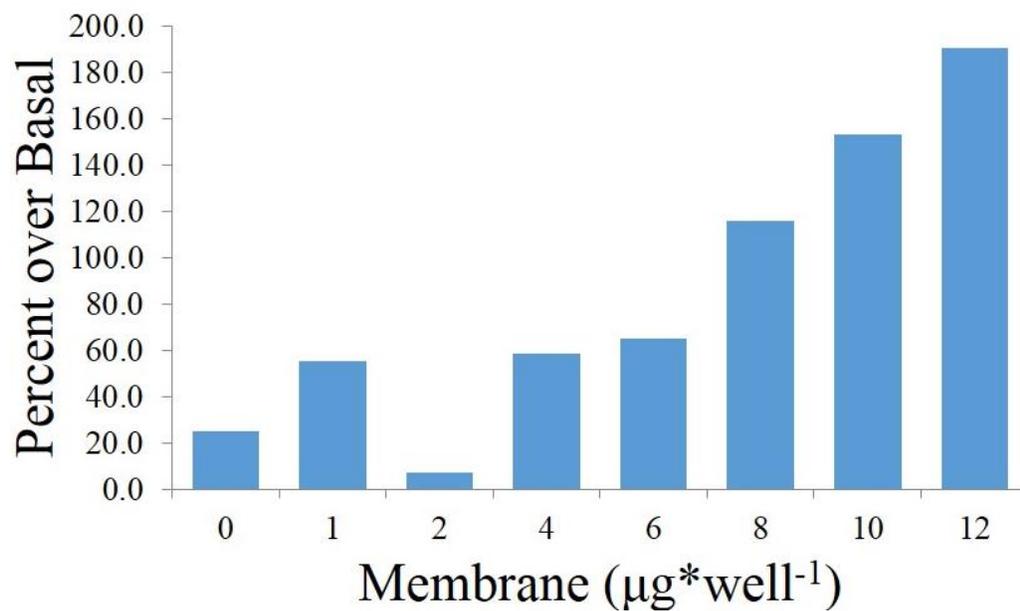


Fig. C.4 The optimization of protein concentration for  $^{35}\text{S}$ -GTP $\gamma$ S binding. Optimal concentration of membrane was determined to be  $10\mu\text{g} \cdot \text{well}^{-1}$ .  $12\mu\text{g} \cdot \text{well}^{-1}$  gave a higher signal but was not used due to the capacity of the filter plate.

Appendix D

Binding kinetics of  $^{35}\text{S}$ -GTP $\gamma$ S and [ $^3\text{H}$ ] DPCPX

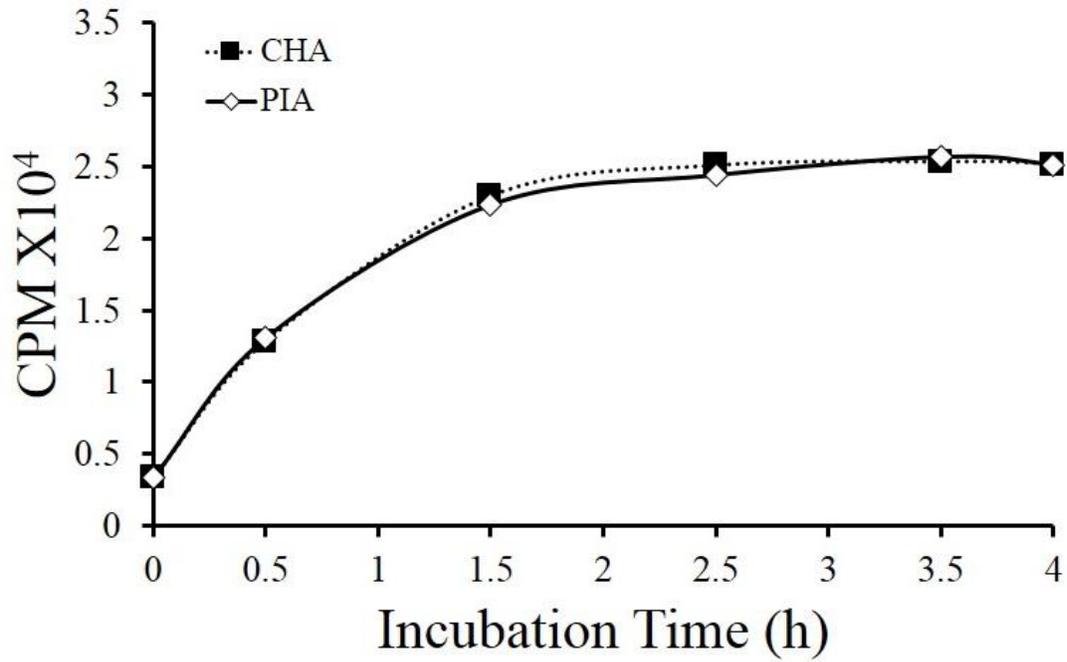


Fig.D.1 Time to equilibrium for CHA and PIA,  $^{35}\text{S}$ -GTP $\gamma$ S binding kinetics. Time to equilibrium for agonist induced GDP/GTP exchange was determined to be 90 minutes in the presence of either CHA (squares) or PIA (diamonds).

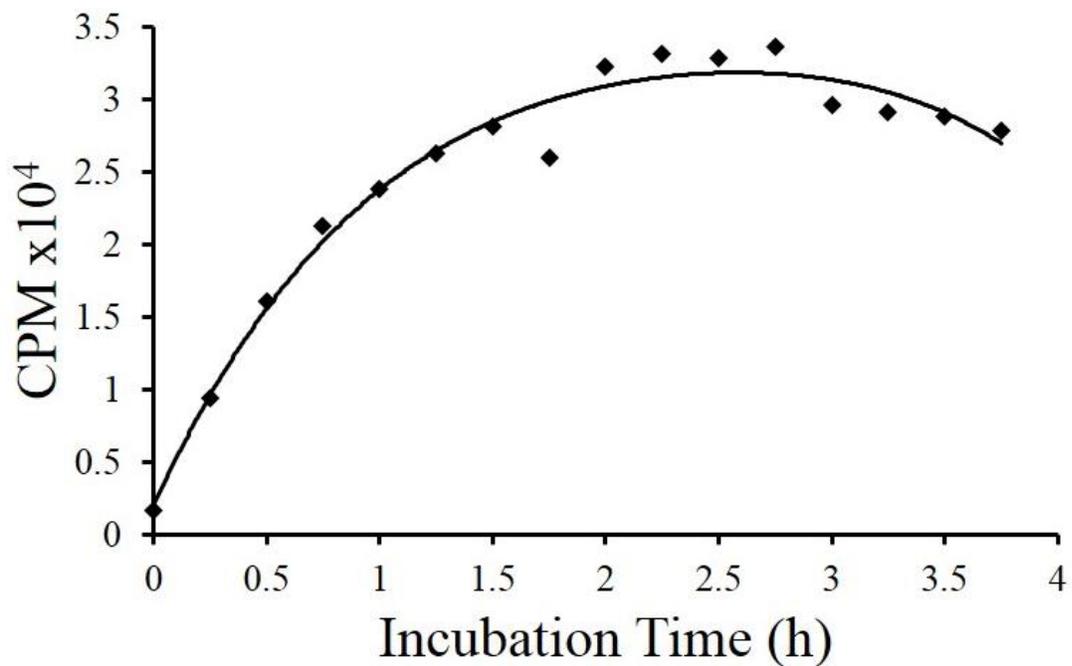


Fig. D.2 CHA equilibrium time with A<sub>3</sub>AR antagonist, <sup>35</sup>S-GTPγS binding kinetics. Time for CHA induced GDP/GTP exchange to reach equilibrium in the presence of MRS-1334 was determined to be 90 minutes.

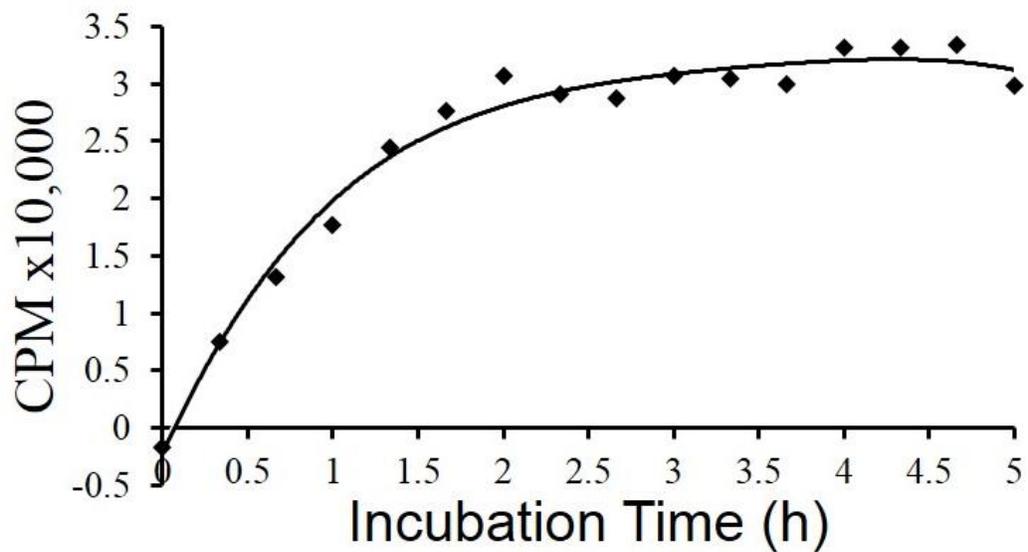


Fig. D.3 CHA equilibrium time in the presence of A<sub>3</sub>AR agonist, <sup>35</sup>S-GTPγS binding kinetics. Time for CHA induced GDP/GTP exchange to reach equilibrium in the presence of Cl-IB-MECA was determined to be 2.5h.

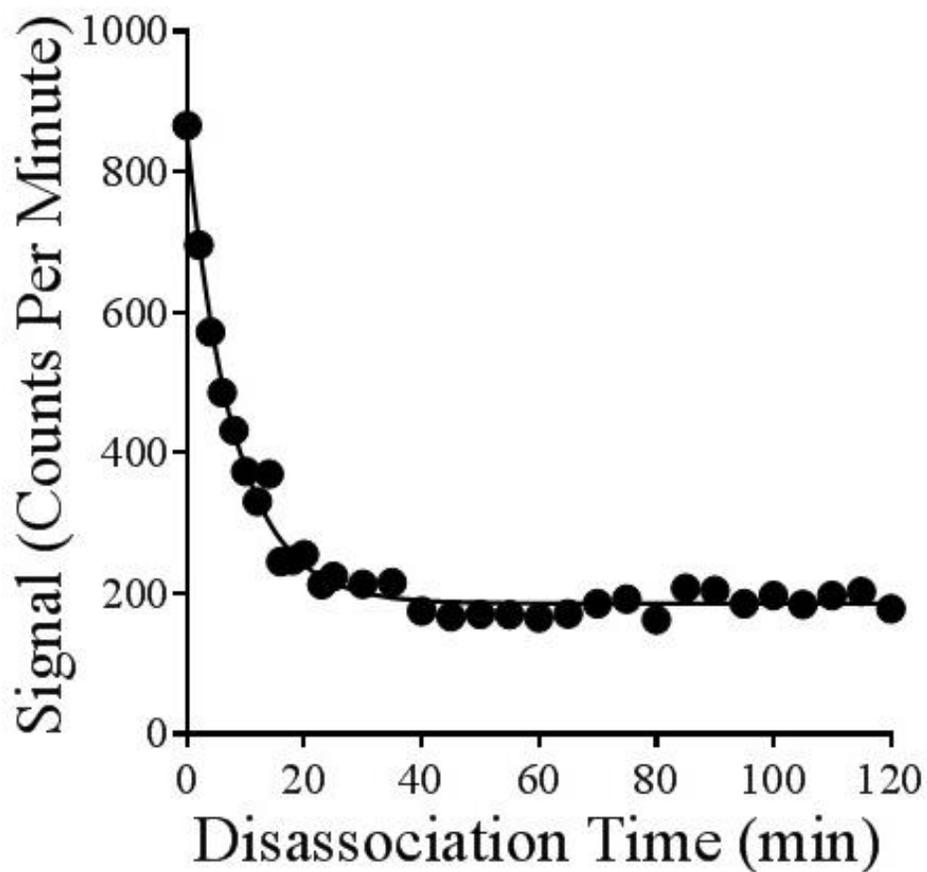


Fig. D.4 Displacement of [<sup>3</sup>H] DPCPX with 1 μM of Cl-IB-MECA. The displacement of [<sup>3</sup>H] DPCPX with 1 μM of IB-MECA. T<sub>1/2</sub> was determined to be 5.6 minutes.

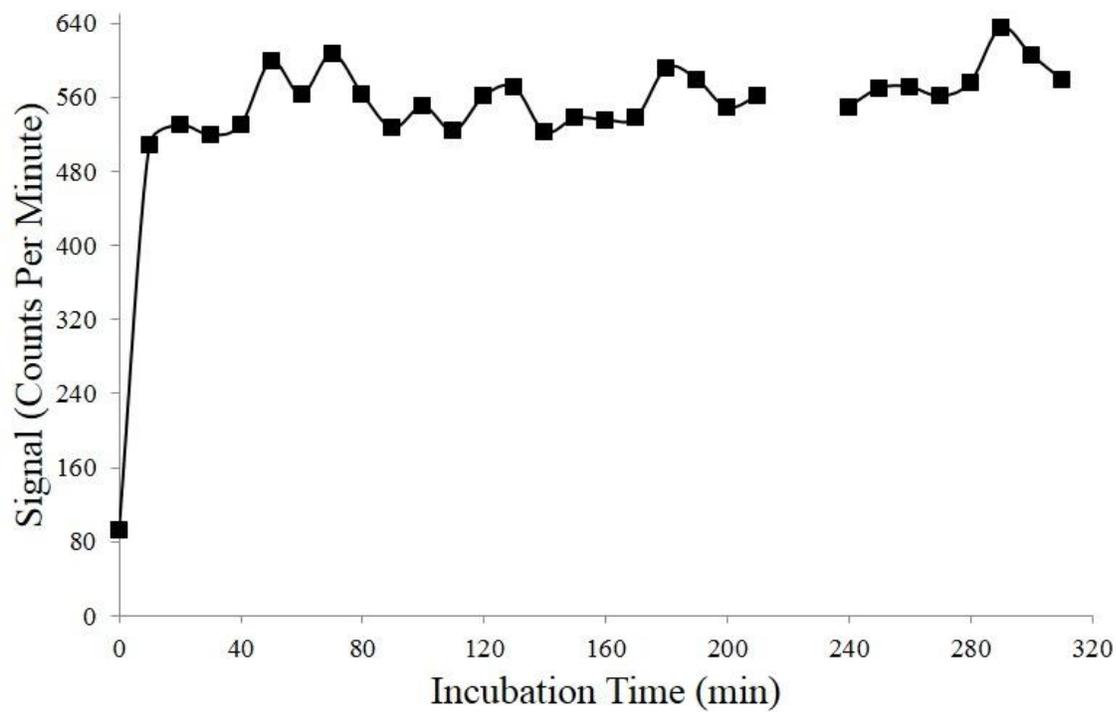


Fig. D.5 Time to equilibrium for the displacement of [ $^3\text{H}$ ] DPCPX by 20pM CHA .The minimum time to equilibrium for the displacement of [ $^3\text{H}$ ] DPCPX by 20pM CHA was determined to be 30 minutes.

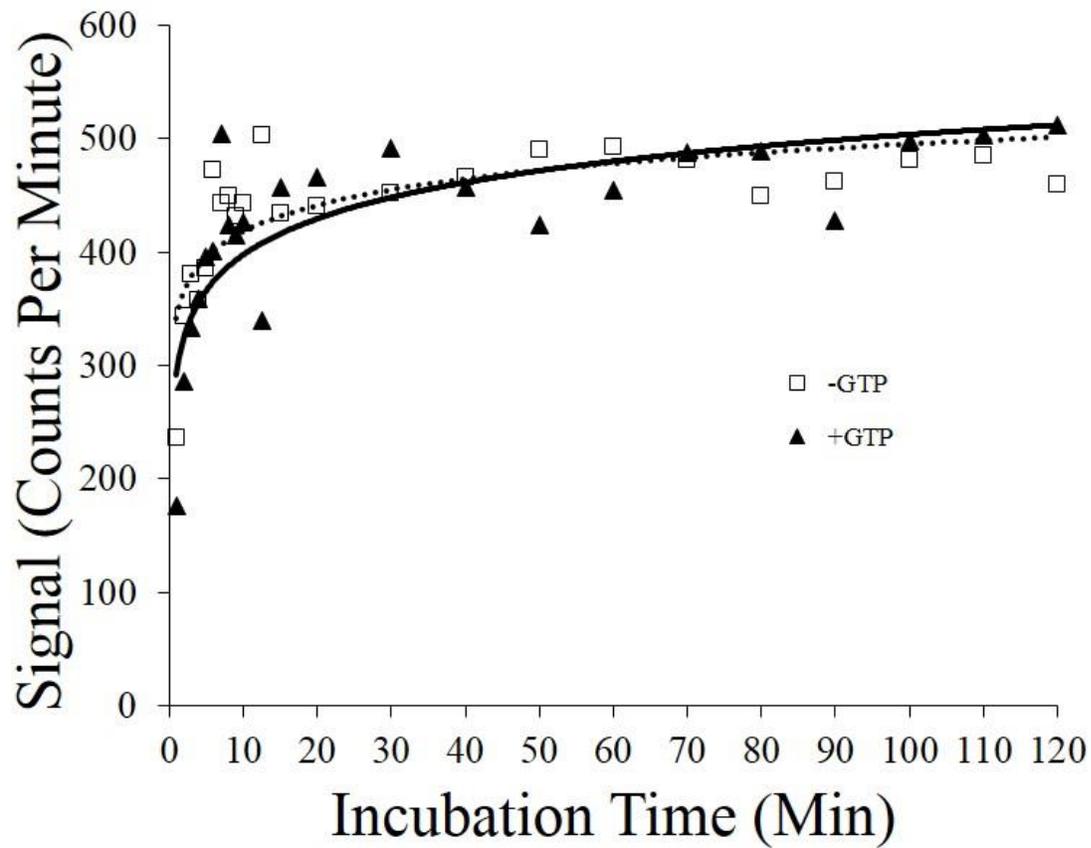


Fig. D.6 Equilibrium time for CHA displacement of [ $^3\text{H}$ ] DPCPX with or without GTP. The time to equilibrium for CHA displacing DPCPX in the presence (triangles, solid line) and absence of 1mM of GTP (squares, dotted line) was determined to be 30 minutes.

## Appendix E

### Direct Ligand Binding: Protocol

#### A. Determination of membrane concentration.

##### Steps:

1. Prepare all solutions on ice.
2. Prepare a Buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
3. Prepare each of the following solutions in buffer.
4. Prepare a solution of 4x radioactive ligand.
  - a. The concentration in the well should be 1-10x the  $K_d$  of the radioactive ligand.
5. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
6. Prepare 15 different concentrations of membrane.
  - a. Remember that each membrane solution should be 4x the desired concentration.
  - b. Label these Membrane A-O.
7. Prepare an aliquot of buffer and label this Membrane P.
8. Pipette 25 $\mu$ L of buffer into each well.
9. Pipette 25 $\mu$ L of radioactive ligand into each well.
10. Pipette 25 $\mu$ L of buffer into columns 1-3 and 10-12.
  - a. Label these columns total binding.

11. Pipette 25 $\mu$ L of non-radioactive ligand into columns 4-9.
  - a. Label these columns non-specific binding.
12. Pipette 25 $\mu$ L of Membrane P into wells A (1-6).
13. Pipette 25 $\mu$ L of Membrane O into wells B (1-6).
14. Pipette 25 $\mu$ L of Membrane N into wells C (1-6).
15. Pipette 25 $\mu$ L of Membrane M into wells D (1-6).
16. Pipette 25 $\mu$ L of Membrane L into wells E (1-6).
17. Pipette 25 $\mu$ L of Membrane K into wells F (1-6).
18. Pipette 25 $\mu$ L of Membrane J into wells G (1-6).
19. Pipette 25 $\mu$ L of Membrane I into wells H (1-6).
20. Pipette 25 $\mu$ L of Membrane H into wells A (7-12).
21. Pipette 25 $\mu$ L of Membrane G into wells B (7-12).
22. Pipette 25 $\mu$ L of Membrane F into wells C (7-12).
23. Pipette 25 $\mu$ L of Membrane E into wells D (7-12).
24. Pipette 25 $\mu$ L of Membrane D into wells E (7-12).
25. Pipette 25 $\mu$ L of Membrane C into wells F (7-12).
26. Pipette 25 $\mu$ L of Membrane B into wells G (7-12).
27. Pipette 25 $\mu$ L of Membrane A into wells H (7-12).
28. Incubate plate at room temperature for 30-90min
  - a. Determine the time based on literature reviews.
29. Filter solution through an all glass filter pad using a cell harvester.
30. Remove the filter and allow to dry overnight.
31. Cut individual wells out of the filter and place them into a scintillation vial.

32. Add scintillation cocktail to each scintillation vial.
33. Read the plate in a scintillation counter using a 5min counting time.
34. Subtract non-specific binding from total binding to determine specific binding.
35. Graph results in specific binding vs. membrane concentration.
36. Choose the membrane concentration that gets the most signal off of the least amount of membrane.

B. Determination of the  $K_{off}$  of the Radioactive Ligand (time to equilibrium for  $K_d$  experiments).

Steps:

1. Prepare all solutions on ice.
2. Prepare a buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
3. Prepare each of the following solutions in Buffer.
4. Prepare a solution of 4x radioactive ligand.
  - a. The concentration in the well should be 1-10x the  $K_d$  of the radioactive ligand.
5. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
6. Prepare a 4x membrane solution based on membrane optimization experiment.
7. Choose 32 time points.
  - a. Look at the literature to determine the appropriate range.
8. Pipette 25 $\mu$ L of Buffer into each well.

9. Pipette 25 $\mu$ L of Radioactive Ligand into each well.
  10. Pipette 25 $\mu$ L of Membrane into each well.
  11. Incubate plate for one to two hours.
  12. Pipette 25 $\mu$ L of non-radioactive ligand into A(1-3).
    - a. The first wells that receive non-radioactive ligand will correspond to the last time point in your experiment.
  13. Continue pipetting 25 $\mu$ L of non-radioactive ligand at each of your chosen time points.
  14. Filter the reaction with cell harvester utilizing an all glass filter.
  15. Remove glass filter from cell harvester and dry overnight.
  16. Cut individual wells out of the glass filter and place into a scintillation vial.
  17. Fill scintillation vials with scintillation cocktail.
  18. Read in a scintillation counter with a 5min counting time.
  19. Insert data into Graphpad Prism.
  20. Calculate the  $K_{off}$  and then the  $t_{1/2}$ .
  21. The incubation time is equal to  $5x t_{1/2}$ .
- C. Determination of the radioactive ligand binding parameters.
- Steps:
1. Prepare all solutions on ice.
  2. Prepare a buffer solution containing
    - a. 50mM HEPES.
    - b. 1 IU/mL Adenosine Deaminase
  3. Make each of the following solutions using the Buffer.
  4. Prepare 15 solutions of radioactive ligand between pM and  $\mu$ M concentrations.

- a. This range should be determined by an extensive literature review.
  - b. Label these RL 1-15.
5. Prepare an aliquot of buffer and label it RL 16
6. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
7. Prepare a 4x membrane solution based on the membrane optimization experiment.
8. Pipette 25 $\mu$ L of buffer into columns 1-3 and 10-12.
  - a. Label these columns total binding.
9. Pipette 25 $\mu$ L of non-radioactive ligand into columns 4-9.
  - b. Label these columns non-specific binding.
10. Pipette 25 $\mu$ L of RL 16 into wells A (1-6).
11. Pipette 25 $\mu$ L of RL 15 into wells B (1-6).
12. Pipette 25 $\mu$ L of RL 14 into wells C (1-6).
13. Pipette 25 $\mu$ L of RL 13 into wells D (1-6).
14. Pipette 25 $\mu$ L of RL 12 into wells E (1-6).
15. Pipette 25 $\mu$ L of RL 11 into wells F (1-6).
16. Pipette 25 $\mu$ L of RL 10 into wells G (1-6).
17. Pipette 25 $\mu$ L of RL 9 into wells H (1-6).
18. Pipette 25 $\mu$ L of RL 8 into wells A (7-12).
19. Pipette 25 $\mu$ L of RL 7 into wells B (7-12).
20. Pipette 25 $\mu$ L of RL 6 into wells C (7-12).
21. Pipette 25 $\mu$ L of RL 5 into wells D (7-12).
22. Pipette 25 $\mu$ L of RL 4 into wells E (7-12).

23. Pipette 25 $\mu$ L of RL 3 into wells F (7-12).
24. Pipette 25 $\mu$ L of RL 2 into wells G (7-12).
25. Pipette 25 $\mu$ L of RL 1 into wells H (7-12).
26. Incubate plate at room temperature for the time determined in the above experiment.
27. Filter solution through an all glass filter pad using a cell harvester.
28. Remove the filter and allow to dry overnight.
29. Cut individual wells out of the filter and place them into a scintillation vial.
30. Add scintillation cocktail to each scintillation vial.
31. Read the plate in a scintillation counter using a 5min counting time.
32. Determine binding parameters of the radioactive ligand using Graphpad Prism.
33. Repeat the experiment with a modified range if the concentrations of radioactive ligand were not correct.

D. Determination of the time to equilibrium of a radioactive ligand in the presence of a competitive ligand.

Steps:

1. Prepare all solutions on ice.
2. Prepare a buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
3. Prepare all the following solutions in Buffer.
4. Prepare a solution of 4x radioactive ligand.
  - a. The concentration in the well should be 1-10x the  $K_d$  of the radioactive ligand.

5. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
6. Prepare a solution of your competitive ligand at the lowest concentration you plan to test.
  - a. The less competitive ligand the longer it takes to equilibrium.
7. Prepare a 4x membrane solution based on membrane optimization experiment.
8. Choose 32 time points.
  - a. Look at the literature to determine an appropriate range.
9. Pipette 25 $\mu$ L of buffer into each well.
10. Pipette 25 $\mu$ L of radioactive ligand into each well.
11. Pipette 25 $\mu$ L of competitive agonist into each well.
12. Pipette 25 $\mu$ L of membrane into well A (1-3).
  - a. These wells will be incubated the longest.
13. Continue pipetting 25 $\mu$ L of membrane at each of your chosen time points.
14. Immediately filter the reaction through an all glass filter using the cell harvester.
15. Remove glass filter from cell harvester and dry overnight.
16. Cut individual wells out of the glass filter and place into a scintillation vial.
17. Fill scintillation vials with scintillation cocktail.
18. Read in a scintillation counter with a 5min counting time.
19. Graph the specific binding vs. time in order to determine the time it takes to reach equilibrium.

E. Competitive Direct Ligand Binding the Determination of the Dose Response Range.

Steps:

1. Prepare all solutions on ice.
2. Prepare a buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
3. Prepare all the following solutions in Buffer.
3. Prepare a solution of 4x radioactive ligand.
  - a. The concentration in the well should be 1-10x the  $K_d$  of the radioactive ligand.
4. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
5. Prepare 15 different solutions of your competitive ligand from pM to  $\mu$ M concentrations.
  - a. This range should be based on a literature review.
  - b. Label these solutions A-O.
6. Prepare an aliquot of buffer and label it P.
7. Pipette 25 $\mu$ L of radioactive ligand into each well.
8. Pipette 25 $\mu$ L of radioactive ligand into three different scintillation vials.
  - a. This will be used to determine the concentration of the radioactive ligand.
  - b. Label these C1, C2, and C3
9. Pipette 25 $\mu$ L of buffer into columns 1-3 and 10-12.
  - a. Label these columns total binding.
10. Pipette 25 $\mu$ L of non-radioactive drug into columns 4-9.
  - a. Label these columns non-specific binding.
11. Pipette 25 $\mu$ L of P into wells A (1-6).

12. Pipette 25 $\mu$ L of O into wells B (1-6).
13. Pipette 25 $\mu$ L of N into wells C (1-6).
14. Pipette 25 $\mu$ L of M into wells D (1-6).
15. Pipette 25 $\mu$ L of L into wells E (1-6).
16. Pipette 25 $\mu$ L of K into wells F (1-6).
17. Pipette 25 $\mu$ L of J into wells G (1-6).
18. Pipette 25 $\mu$ L of I into wells H (1-6).
19. Pipette 25 $\mu$ L of H into wells A (7-12).
20. Pipette 25 $\mu$ L of G into wells B (7-12).
21. Pipette 25 $\mu$ L of F into wells C (7-12).
22. Pipette 25 $\mu$ L of E into wells D (7-12).
23. Pipette 25 $\mu$ L of D into wells E (7-12).
24. Pipette 25 $\mu$ L of C into wells F (7-12).
25. Pipette 25 $\mu$ L of B into wells G (7-12).
26. Pipette 25 $\mu$ L of A into wells H (7-12).
27. Pipette 25 $\mu$ L of membrane solution into each well.
28. Incubate plate for the time you determined in step D.
29. Filter the reaction through an all glass filter using the cell harvester.
30. Remove glass filter from cell harvester and dry overnight.
31. Cut individual wells out of the glass filter and place into a scintillation vial.
32. Fill the scintillation vials along with C1, C2 and C3 with scintillation cocktail.
33. Read in a scintillation counter with a 5min counting time.
34. Subtract non-specific binding from total binding.

35. Enter data into Graphpad Prism to determine competitive binding parameters.

36. You will need the concentration of your radioactive ligand and the  $K_d$  from experiment C to find the  $K_i$ .

F. Direct ligand Binding, Competitive Direct Ligand Binding and GTP Shift on One Plate.

Steps:

1. Prepare all solutions on ice.
2. Prepare a buffer solution containing
  - a. 50mM HEPES.
  - b. 1 IU/mL Adenosine Deaminase
3. Prepare all the following solutions in buffer.
4. Prepare a solution of 4x radioactive ligand.
  - a. The concentration in the well should be 1-10x the  $K_d$  of the radioactive ligand.
5. Prepare a range of 9 other radioactive ligand solutions based on experiment C.
  - a. Label these solutions RL 1-9
6. Prepare an aliquot of buffer and label it RL 10
7. Prepare a solution of 4x non-radioactive ligand to determine non-specific binding.
  - a. The concentration in the well should be 100x the  $K_d$  of the cold ligand.
8. Prepare 9 different solutions of your competitive ligand determined from step E.
  - a. Label these A-I.
9. Prepare an aliquot of buffer label it J.
10. Prepare a 4mM GTP solution.
11. Prepare a 4x membrane solution.
12. Pipette 25 $\mu$ L of radioactive ligand into Columns 1-6 and A-F (7-9).

13. Pipette 25 $\mu$ L of radioactive ligand into three scintillation vials.
  - a. These will be used to determine the concentration of radioactive ligand.
  - b. Label these Z1, Z2, and Z3.
14. Pipette 25 $\mu$ L of J into wells A (1-3) and E (4-6).
15. Pipette 25 $\mu$ L of I into wells B (1-3) and F (4-6).
16. Pipette 25 $\mu$ L of H into wells C (1-3) and G (4-6).
17. Pipette 25 $\mu$ L of G into wells D (1-3) and H (4-6).
18. Pipette 25 $\mu$ L of F into wells E (1-3) and A (7-9).
19. Pipette 25 $\mu$ L of E into wells F (1-3) and B (7-9).
20. Pipette 25 $\mu$ L of D into wells G (1-3) and C (7-9).
21. Pipette 25 $\mu$ L of C into wells H (1-3) and D (7-9).
22. Pipette 25 $\mu$ L of B into wells A (1-3) and E (7-9).
23. Pipette 25 $\mu$ L of A into wells B (1-3) and F (7-9).
24. Pipette 25 $\mu$ L of buffer into columns 1-3 and wells A and B (4-6).
  - a. This is the competitive binding experiment.
25. Pipette 25 $\mu$ L of 4mM GTP solution into wells E-H (4-6) and A-F (7-9).
  - a. This is the GTP shift experiment.
26. Pipette 25 $\mu$ L of buffer into wells C (4-6).
27. Pipette 25 $\mu$ L of 4mM GTP into wells D (4-6).
28. Pipette 25 $\mu$ L of non-radioactive ligand into C, D (4-6).
  - a. These well are the non-specific binding for the displacement experiment with and without GTP.
29. Pipette 25 $\mu$ L of buffer into wells G and H (7-9) and columns 10-12.

30. Pipette 25 $\mu$ L of RL 10 into wells G (7-9).
31. Pipette 25 $\mu$ L of RL 9 into wells H (7-9).
32. Pipette 25 $\mu$ L of RL 8 into wells A (10-12).
33. Pipette 25 $\mu$ L of RL 7 into wells B (10-12).
34. Pipette 25 $\mu$ L of RL 6 into wells C (10-12).
35. Pipette 25 $\mu$ L of RL 5 into wells D (10-12).
36. Pipette 25 $\mu$ L of RL 4 into wells E (10-12).
37. Pipette 25 $\mu$ L of RL 3 into wells F (10-12).
38. Pipette 25 $\mu$ L of RL 2 into wells G (10-12).
39. Pipette 25 $\mu$ L of RL 1 into wells H (10-12).
40. Pipette 25 $\mu$ L of buffer into wells G, H (7, 8) and columns 10 and 11.
  - a. These wells will be your total binding for the direct ligand binding experiment.
41. Pipette 25 $\mu$ L of non-radioactive ligand into G, H (9) and column 12.
  - a. These wells are the non-specific binding for the direct ligand binding experiment.
42. Pipette 25 $\mu$ L of Membrane into each well.
  - a. Use a different syringe for each experiment.
43. Incubate plate at room temperature for length of time indicated in D.
44. Filter the reaction through an all glass filter using the cell harvester.
45. Remove glass filter from cell harvester and dry overnight.
46. Cut individual wells out of the glass filter and place into a scintillation vial.
47. Fill scintillation vials with scintillation cocktail including C1, C2 and C3.
48. Read in a scintillation counter along with C1, C2, and C3 with a 5min counting time.
49. Determine the concentration of your radioactive ligand solution,

50. Determine the  $K_d$  of your radioactive ligand.

51. Use the  $K_d$  and concentration of radioactive ligand to determine the  $K_i$  with and without GTP.



Appendix F

[<sup>3</sup>H] DPCPX saturation and displacement in summer and winter AGS

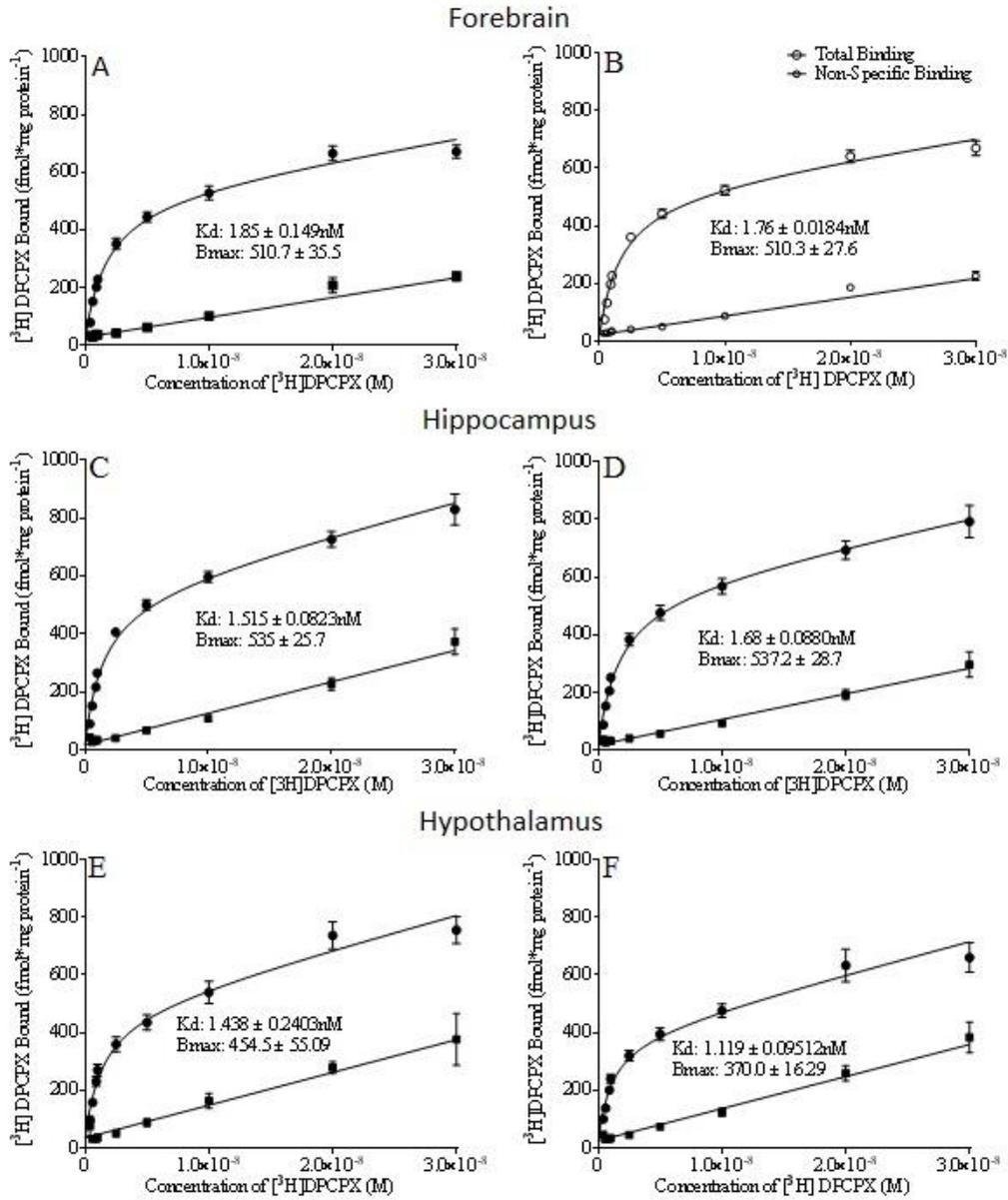


Fig. F.1 [<sup>3</sup>H] DPCPX saturation binding kinetics in AGS brain. [<sup>3</sup>H] DPCPX saturation binding kinetics in AGS forebrain, hippocampus and hypothalamus in the summer (A,C,E) and winter (B,D,F) season. Season had no effect on [<sup>3</sup>H] DPCPX binding.

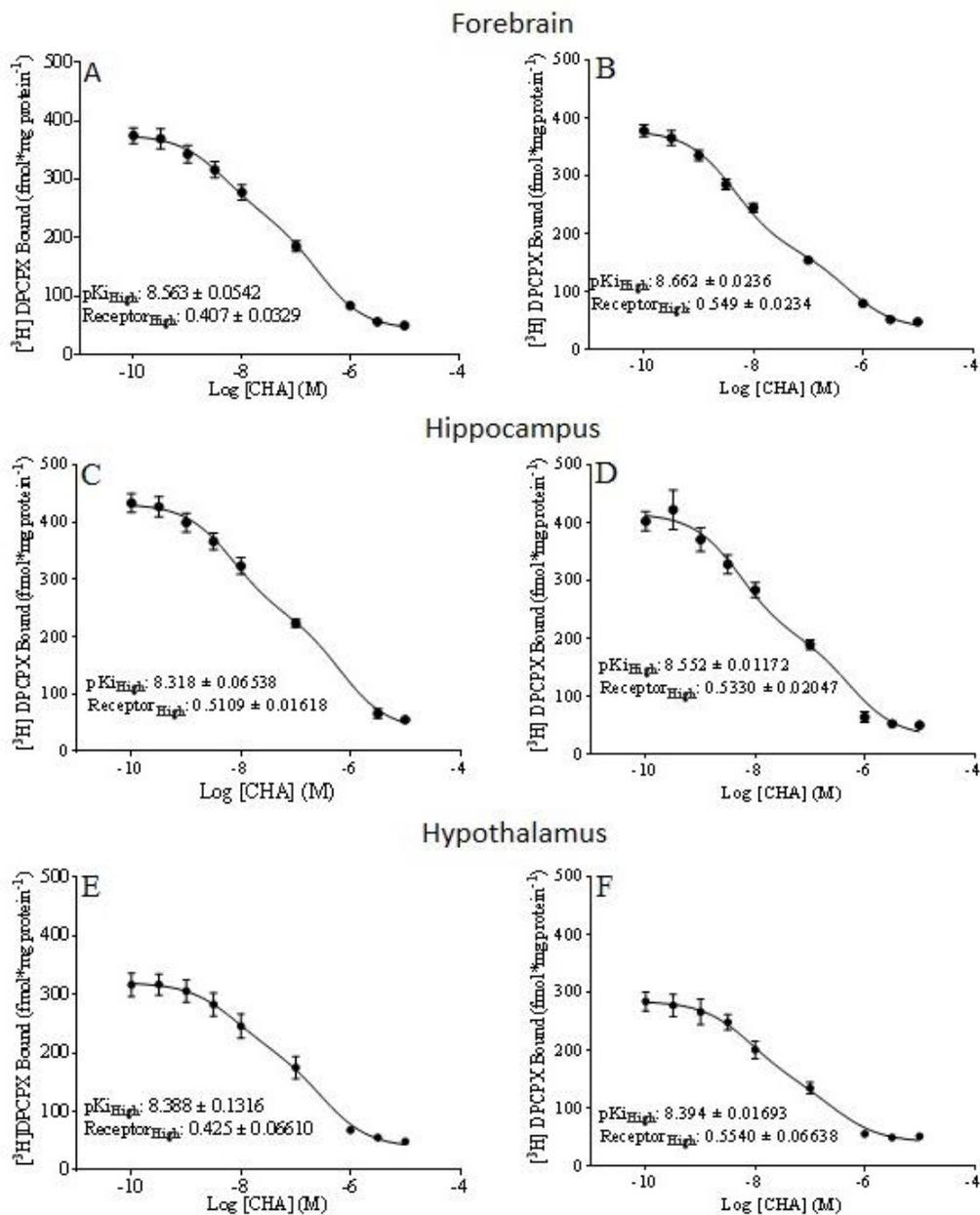


Fig. F.2 Kinetics of [<sup>3</sup>H] DPCPX displacement by CHA in AGS brain. Competition curve showing the displacement of 1nM of [<sup>3</sup>H] DPCPX by various concentrations of CHA in AGS forebrain, hippocampus and hypothalamus in the summer (A,C,E) and winter (B,D,F). Results indicate a statistical difference in the fraction of receptors in the high affinity state ( $Receptor_{High}$ ). \* indicate a statistical difference within the same tissue ( $p < 0.05$ ). Although there were no further differences between the summer and winter season, the winter season tended to have a lower  $K_{iHigh}$  and greater  $Receptor_{High}$ .

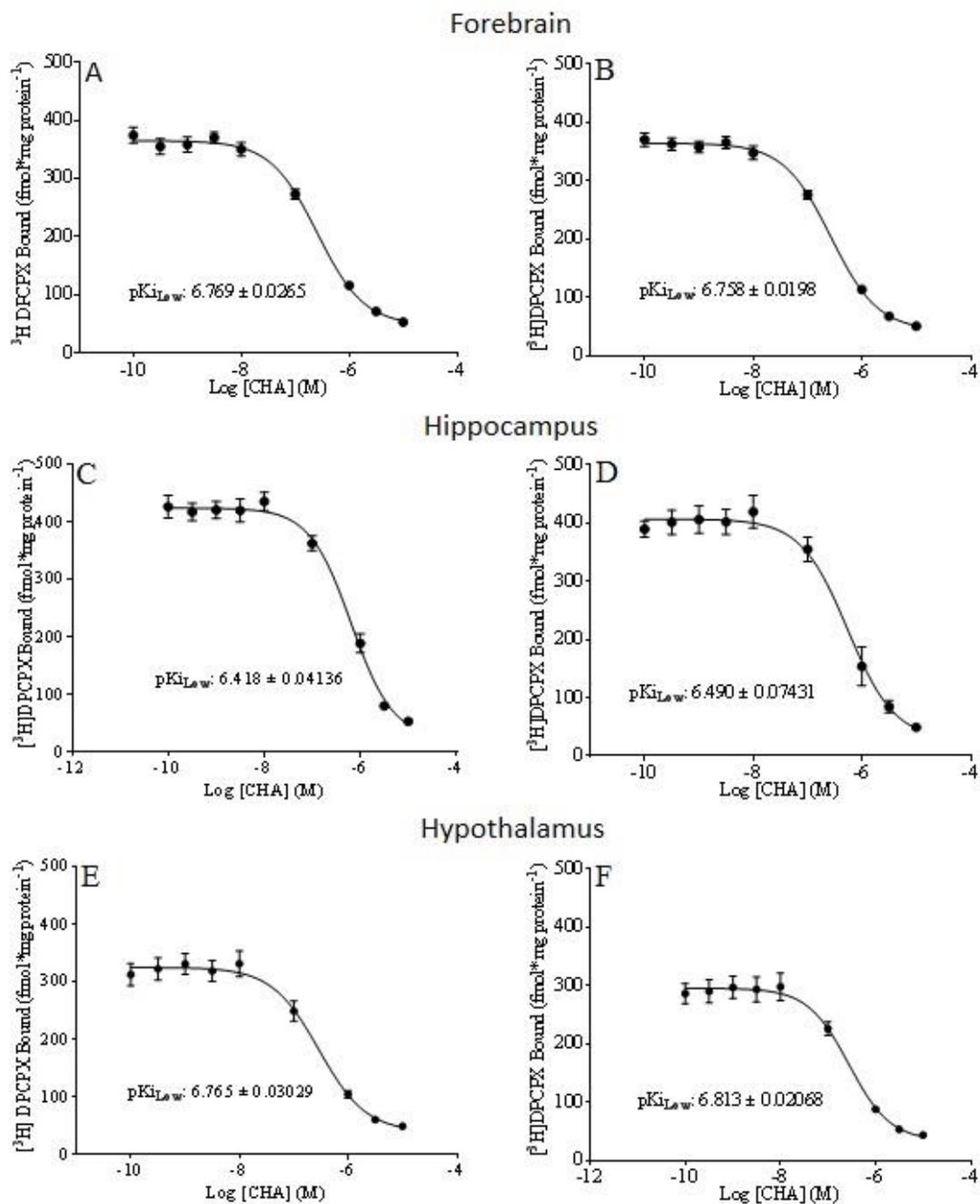


Fig. F.3 Kinetics of  $^3\text{H}$  DPCPX displacement by CHA in AGS brain in the presence of GTP. Competition curve showing the displacement of 1nM of  $^3\text{H}$ DPCPX, in the presence of 1 $\mu\text{M}$  GTP, by various concentrations of CHA in AGS forebrain, hippocampus and hypothalamus in the summer (A,C,E) and winter (B,D,F). The displacement curves depict one site binding isotherms which is indicative of the loss of G-protein coupling.