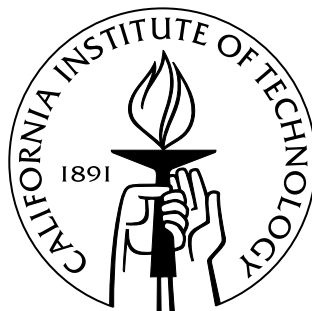


# Chemical-Scale Studies of G Protein-Coupled Receptors and Ligand-Gated Ion Channels

Thesis by

**Ethan Buggie Van Arnam**

In Partial Fulfillment of the  
Requirements for the Degree of  
Doctor of Philosophy



California Institute of Technology

Pasadena, California

2014

(Defended December 12, 2013)

© 2014

Ethan Buggie Van Arnam

All Rights Reserved

*Dedicated to my parents:  
Jean and Peter Van Arnam*

## Acknowledgements

I expected a rich scientific experience at Caltech, but never imagined how personally fulfilling my time here would be. For that I have a great number of people to thank. First and foremost, I want to extend heartfelt thanks to my graduate advisor Dennis Dougherty. I appreciate the extensive freedom he gives his students and yet the compassion and personal dedication he devotes to each one of us. Dennis is a supremely talented communicator, and I especially value the lessons I've learned about speaking, writing, and teaching from him. I also appreciate the support he has given me and the confidence he has instilled in me to tackle hard scientific problems and to explore a new field after Caltech.

Henry Lester, a longtime collaborator of the Dougherty group, is an inexhaustible source of suggestions for new experiments and novel approaches to scientific problems. I value his scientific rigor and his dedication to his students. I also want to thank the members of my thesis committee, Peter Dervan, Doug Rees, and David Tirrell for their support and suggestions, both with regards to my graduate research and my career.

The Dougherty group has been a fantastic place to work (and a fantastic place at times to hang out and not work too). I really look forward to keeping in touch with everyone I overlapped with in the group. When I first arrived in lab, Ariele Hanek, Kiowa Bower, and Kristin Gleitsman had saintly patience while I was learning the ropes, passed on endless sage advice, and made me feel welcome. I'm very glad I got to know Jai Shanata, who always brought a valuable quantitative rigor to scientific discussions and whose worldview is refreshingly optimistic. Sean Kedrowski answered so, so, many of my chemistry questions and put up with all assortment of canned fish and other exciting

lunch items that I brought in. I really enjoyed sitting next to him for three years. Angela Blum and Nyssa Puskar, with whom I overlapped for a good deal of grad school, have great integrity and are supremely capable researchers. Kay Limapichat helped me immensely to get started on fluorescence experiments and was generous with time and with materials she worked long to prepare. Noah Duffy is a hell of a human being. He always has an open mind, really thinks things through, and can fix absolutely anything.

Kristina Daeffler, Ximena da Silva, Maggie Thompson, and I all entered Caltech together and had the wisdom to join the Dougherty group. Maggie has remained a good friend and I deeply admire her bravery to teach middle school science. Ximena (da Silva Tavares Bongoll de Rossi) has the longest name of anyone I know. Kristina and I both cut our teeth on GPCR projects and have commiserated on more than one topic on more than one occasion.

Tim Miles knows how to chart his own path and excel at it. I've appreciated his friendship and look forward to watching what he does in the future. We were ever so fortunate to land Michael Post in the group. He's carrying the nicotinic receptor torch forward and smiles seem to erupt everywhere he goes. Oliver Shafaat and Fan Liu have orders of magnitude more mathematical competence than I do. I really enjoyed my GPCR collaborations with Fan and all of our conversations. The future involves photons for Kayla Busby, Matt (Jefferson) Davis, and Paul (David?) Walton. These folks are breaking new ground, which made my last year in the group a lot more intellectually stimulating. They made it a whole lot more fun too. Betty Wong is bravely taking on some fluorescence work, and is more than up to the task. She hasn't been in the group long, but everybody agrees she's a force for good.

Finally, Matt Rienzo and Clint Regan were my companions in the south bay of desks for the last few years and I'll deeply miss them. Clint is steering the photochemistry ship – making good progress and knowing the right questions to ask. Matt can do very hard chemistry and very hard biology and is a very good person as well.

I had the privilege of working with three truly outstanding undergraduates in lab during my graduate career: Jessica Swallow, Emily Blythe, and Jessica Hsu. All learned the ropes in lab effortlessly and accomplished much, were great to work with, and were often many steps ahead of me.

Friends in the Los Angeles area showed me that this is an awesome place to live and is going to be an especially hard place to leave. Michael Van Vliet has been a fantastic friend and a much needed beacon of normalcy beyond the Caltech community. All of my cycling friends gave me a window into this city I would never have seen otherwise and are all fantastic people. My roommates Alex Goldberg, James McKone, and Judy Lattimer have been great friends and deserve a medal for putting up with my eccentricities.

Finally, I want to thank my family, who I don't thank nearly enough. My parents have always supported me unconditionally and serve as the strongest role models I could ever ask for.

## Abstract

This dissertation describes studies of G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs) using unnatural amino acid mutagenesis to gain high precision insights into the function of these important membrane proteins.

Chapter 2 considers the functional role of highly conserved proline residues within the transmembrane helices of the D2 dopamine GPCR. Through mutagenesis employing unnatural  $\alpha$ -hydroxy acids, proline analogs, and N-methyl amino acids, we find that lack of backbone hydrogen bond donor ability is important to proline function. At one proline site we additionally find that a substituent on the proline backbone N is important to receptor function.

In Chapter 3, side chain conformation is probed by mutagenesis of GPCRs and the muscle-type nAChR. Specific side chain rearrangements of highly conserved residues have been proposed to accompany activation of these receptors. These rearrangements were probed using conformationally-biased  $\beta$ -substituted analogs of Trp and Phe and unnatural stereoisomers of Thr and Ile. We also modeled the conformational bias of the unnatural Trp and Phe analogs employed.

Chapters 4 and 5 examine details of ligand binding to nAChRs. Chapter 4 describes a study investigating the importance of hydrogen bonds between ligands and the complementary face of muscle-type and  $\alpha 4\beta 4$  nAChRs. A hydrogen bond involving the agonist appears to be important for ligand binding in the muscle-type receptor but not the  $\alpha 4\beta 4$  receptor. Chapter 5 describes a study characterizing the binding of varenicline, an actively prescribed smoking cessation therapeutic, to the  $\alpha 7$  nAChR. Additionally,

binding interactions to the complementary face of the  $\alpha 7$  binding site were examined for a small panel of agonists. We identified side chains important for binding large agonists such as varenicline, but dispensable for binding the small agonist ACh.

Chapter 6 describes efforts to image nAChRs site-specifically modified with a fluorophore by unnatural amino acid mutagenesis. While progress was hampered by high levels of fluorescent background, improvements to sample preparation and alternative strategies for fluorophore incorporation are described.

Chapter 7 describes efforts toward a fluorescence assay for G protein association with a GPCR, with the ultimate goal of probing key protein-protein interactions along the G protein/receptor interface. A wide range of fluorescent protein fusions were generated, expressed in *Xenopus* oocytes, and evaluated for their ability to associate with each other.



## Table of Contents

### Chapter 1: Introduction

1.1 Signaling across a membrane: the chemical underpinning of neuroscience.....	1
1.2 Ligand-gated ion channels .....	1
1.3 G protein-coupled receptors.....	5
1.4 Methods for interrogating ion channel and GPCR function .....	7
1.4.1 Electrophysiology as an assay for ion channels and GPCRs .....	7
1.4.2 Unnatural amino acid mutagenesis .....	9
1.4.3 Probing receptor function with fluorescence.....	12
1.5 Summary of dissertation work.....	13
1.6 References.....	14

### Chapter 2: Dissecting the functions of conserved prolines within transmembrane helices of the D2 dopamine receptor

2.1 Abstract.....	16
2.2 Introduction.....	16
2.3 Results.....	20
2.3.1 Experimental approach .....	20
2.3.2 Strategy and general observations .....	20
2.3.3 169 <sup>4.59</sup> , 388 <sup>6.50</sup> , 423 <sup>7.50</sup> : Importance of lacking a backbone hydrogen bond donor .....	24
2.3.4 201 <sup>5.50</sup> : N-substitution as well as lack of a hydrogen bond donor are important .....	25
2.4 Discussion .....	26
2.5 Conclusions.....	29
2.6 Experimental .....	30
2.6.1 Molecular biology .....	30
2.6.2 Oocyte preparation and RNA injection .....	31

2.6.3 <i>Electrophysiology</i> .....	32
2.7 <i>References</i> .....	33

### **Chapter 3: Probing side chain conformations and rearrangements in receptor binding sites**

3.1 Abstract.....	36
3.2 Introduction.....	37
3.2.1 <i>GPCR activation</i> .....	37
3.2.2 <i>nAChR activation</i> .....	40
3.2.3 <i>Probing side chain conformation by mutagenesis</i> .....	42
3.3 Results and Discussion .....	44
3.3.1 <i>Mutagenesis probing conformational rearrangements of C6.47 upon D2 dopamine receptor activation</i> .....	44
3.3.2 <i>Investigating the role of a F6.44/I3.40 switch in activation of the D2 dopamine receptor</i> .....	46
3.3.3 <i>Synthesis and use of 5-aminomethyltryptophan to probe the W6.48 site in GPCRs</i> .....	49
3.3.4 <i>Synthesis, mutagenesis, and modeling of <math>\beta</math>-methyltryptophan and <math>\beta</math>-methylphenylalanine</i> .....	53
3.3.4.1 <i>Synthesis</i> .....	53
3.3.4.2 <i>Mutagenesis</i> .....	54
3.3.4.3 <i>Conformational effects</i> .....	56
3.3.4.4 <i>Double mutant cycle analysis to probe steric effects of <math>\beta</math>-methyl substituents</i> .....	66
3.4 Conclusions.....	72
3.5 Experimental.....	73
3.5.1 <i>Molecular biology</i> .....	73
3.5.2 <i>Microinjection</i> .....	74
3.5.3 <i>Electrophysiology</i> .....	75
3.5.4 <i>Energy calculations</i> .....	76

3.5.5 <i>Synthesis</i> .....	77
3.6 References.....	89

#### **Chapter 4: Binding interactions to the complementary subunit of the $\alpha 4\beta 4$ receptor**

4.1 Abstract.....	92
4.2 Introduction.....	93
4.3 Results.....	96
4.3.1 <i>General strategy</i> .....	96
4.3.2 <i>Mutagenesis studies of the Leu NH</i> .....	100
4.3.3 <i>Mutagenesis studies of the Asn CO</i> .....	101
4.4 Discussion.....	102
4.5 Experimental.....	107
4.5.1 <i>Mutagenesis</i> .....	107
4.5.2 <i>Microinjection</i> .....	108
4.5.3 <i>Electrophysiology</i> .....	109
4.6 References.....	109

#### **Chapter 5: An unusual pattern of ligand-receptor interactions for the $\alpha 7$ nicotinic acetylcholine receptor, with implications for the binding of varenicline**

5.1 Abstract.....	112
5.2 Introduction.....	113
5.3 Results.....	117
5.3.1 <i>Experimental design</i> .....	117
5.3.2 <i>Unnatural amino acid mutagenesis to probe cation-<math>\pi</math> interactions and hydrogen bonds to the protein backbone</i> .....	119
5.3.3 <i>Varenicline interactions with the binding site's principal face</i> .....	121
5.3.4 <i>Probing the canonical hydrogen bond of the nicotinic pharmacophore between agonists and the complementary subunit</i> .....	125

5.3.5	<i>Functional importance of side chains on the complementary face of the binding site</i>	125
5.4	Discussion	126
5.4.1	<i>Cation-<math>\pi</math> interactions to the “aromatic box” residues of the principal face</i>	127
5.4.2	<i>Hydrogen bonding and steric effects on the principal face</i>	128
5.4.3	<i>Hydrogen bonding to the complementary face</i>	129
5.4.4	<i>Interactions with complementary face side chains</i>	132
5.5	Conclusions	133
5.6	Experimental	133
5.6.1	<i>Molecular biology</i>	133
5.6.2	<i>Microinjection</i>	134
5.6.3	<i>Electrophysiology</i>	135
5.7	References	135

## **Chapter 6: Efforts toward single molecule fluorescence imaging of nAChRs**

6.1	Abstract	139
6.2	Introduction	139
6.3	Results and Discussion	142
6.3.1	<i>Background fluorescence from tRNA-BODIPY</i>	142
6.3.2	<i>Optimization of vitelline membrane removal</i>	149
6.3.3	<i>Two-color imaging and single puncta analysis</i>	153
6.3.4	<i>Attempts at fluorophore conjugation by azide-alkyne cycloaddition</i>	159
6.3.5	<i>Design and synthesis of a fluorescence-quenched tRNA</i>	161
6.4	Conclusions	168
6.5	Experimental	169
6.5.1	<i>Molecular biology and in vivo expression</i>	169

6.5.2 <i>Electrophysiology</i> .....	169
6.5.3 <i>Oocyte membrane preparation</i> .....	169
6.5.4 <i>Total internal reflection fluorescence (TIRF) imaging</i> .....	170
6.5.5 <i>Azido dye labeling under copper-catalyzed click conditions</i> .....	171
6.5.6 <i>Two-color TIRF imaging and image analysis</i> .....	171
6.5.7 <i>tRNA synthesis and characterization</i> .....	172
6.6 References.....	174

## **Chapter 7: Attempts to develop a FRET assay for the GPCR-G protein interaction**

7.1 Abstract.....	177
7.2 Introduction.....	177
7.3 Results and Discussion .....	183
7.3.1 <i>Construction of fluorescent protein fusions</i> .....	183
7.3.2 <i>Tests of construct function in vivo</i> .....	185
7.3.3 <i>Formation and fluorescence imaging of plasma membrane sheets</i> .....	188
7.3.4 <i>Attempts to measure FRET between G protein and receptor</i> .....	188
7.4 Conclusions.....	192
7.5 Experimental.....	193
7.5.1 <i>Molecular biology and in vivo expression</i> .....	193
7.5.2 <i>Electrophysiology</i> .....	193
7.5.3 <i>Membrane preparation</i> .....	194
7.5.4 <i>FLIM imaging</i> .....	194
7.6 References.....	194