

***In Vivo* Incorporation of Multiple
Unnatural Amino Acids**

Thesis by:

Erik Ali Rodriguez

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



California Institute of Technology

Pasadena, CA 91125

2009

(Defended December 11, 2008)

©2009

Erik Ali Rodriguez

All Rights Reserved

*Dedicated to my Mother and family,
who have supported me throughout my life.*

Acknowledgements

I have been at Caltech for ten years and the experience has been amazing, starting as an undergraduate and leaving with a doctorate. I must thank Professor Dennis Dougherty, who has been an amazing advisor throughout my undergraduate and graduate research. Not many professors would give an undergraduate their own project that would eventually be published in PNAS. Professor Dougherty has always been helpful in my research, providing insight, new ideas, a different perspective, and being receptive to new ideas that may seem controversial. Throughout my research I have been allowed to develop new methods and molecules that many thought were unnecessary, but Professor Dougherty let me explore as long as the research wasn't too far fetched. Professor Dougherty is an expert of many scientific disciplines, an excellent writer, and an extremely imaginative research advisor and yet he is an amazing person to talk to, an incredible father, and treats everyone humanely and with respect. For all these reasons, it truly has been an honor and privilege to work in the Dougherty lab.

Professor Henry Lester has been like a second advisor with a hands-on attitude towards science. Professor Lester was always interested in discussing research, discussing implications of my work, and fostering new research ideas that combined chemistry and biology. Professor Lester showed me how to use an oscilloscope and calibrate a function generator. The collaboration between the Dougherty and Lester lab has been a great tool to learn different areas of biological research including transgenic mice, atomic force microscopy, fluorescence microscopy, and electrophysiology. I appreciate being able to use the Lester lab equipment and excellent help and suggestions from the Lester lab members. I would also like to thank my thesis committee consisting of Professor David Tirrell, Professor Nate Lewis, and Professor Judith Campbell for all their useful comments on my research and suggestions for my career.

Dr. Sarah May taught me all the basic molecular biology techniques when I began my research as undergraduate, even though my project was different from her research. Sarah also provided extremely large quantities of dCA-W, which was invaluable for all my tRNA screening experiments. I have enjoyed working with Dr. Rigo Pantoja and Dr. Mohammed Dibas on incorporating a fluorescent unnatural amino acid into the nicotinic

acetylcholine receptor. Dr. Joshua Maurer taught me techniques for working with bacterial ion channels, fluorescence microscopy, and electron microscopy. Dr. George Shapovalov performed electrophysiology recordings on MscL incorporated into giant unilamellar vesicles. Sean Gordon taught me how to create giant unilamellar vesicles and took confocal microscopy images of the vesicles. Purnima Deshpande has always been extremely useful for discussions on molecular biology, making biological stocks, and enjoyable conversations at safety meetings. Professor Linda Hsieh-Wilson has allowed me to use the lab's PCR thermal cycler, UV-visible spectrometer, and Li-Cor Odyssey. Heather Murrey has been extremely helpful with training on the Hsieh-Wilson equipment, discussions on molecular biology, and Western blots.

Working in the Dougherty lab as an undergraduate allowed me to interact with many senior graduate students and gain insight into their various personalities. Dr. Sarah May was helpful with molecular biology, very kind, and never gave up on a difficult project. Dr. Gabriel Brandt was knowledgeable about many areas of science. Dr. James Petersson was tremendously hard working, enjoyed science, was willing to lend a hand, and threw a fun party. Dr. Niki Zacharias was persistent with difficult experiments. Dr. David Dahan was helpful with fluorescence microscopy and a great family man. Dr. Don Elmore mainly performed computer simulations, but still performed experimental research and was ready to provide help. Dr. Joshua Maurer was helpful with experiments, very sure of himself, and adventurous in performing research in new areas. Dr. Darren Beene was fun to talk with, a great father, and accepted that I liked molecular biology more than chemistry. Dr. Tingwei Mu was hard working, knowledgeable, and helpful with experiments. Dr. Steve Spronk was always willing to be of assistance and well informed about areas outside of science. From all of them, I would try to assimilate their best attributes into my graduate career.

My graduate research has been enjoyable because of the great people in the Dougherty lab. Dr. Lori Lee was fun to talk to at work or at a party and a great cook. Dr. Amanda Cashin taught me oocyte injection and how to use the OpusXpress. Amanda was also kind and a social butterfly. Dr. Joanne Xiu worked very hard, was fun to work with, and was a great MC for Chinese New Year. Amy Eastwood shared dCA-Ala, is fun to talk to (if I remember or not), and is a great RA of Ricketts House. Michael Torrice is

helpful with experiments and always brings up interesting conversation topics. Katie McMenimen always lent me her laptop because I was too cheap to buy one, was familiar with fun attractions in the area, and good at getting free Bloody Marys. Ariele Hanek is a great opus captain and always gets the job done. Kristin Glietsman is interesting to talk to about her many endeavors including science, collaborations, travel, and dancing. Kiowa Bower is always entertaining and marches to the beat of a different drummer. Jinti Wang was very hard working, good at multi-tasking, amazingly talented at getting experiments done with brute force, and extremely respectful. Kay Limapichat is very talented, able to perform difficult experiments, and a great cameraman. Nyssa Puskar is hard working and was great dancer on Bourbon Street. Jai Shanata seems to always confound me with the single channel equations, but doesn't know what the French flag looks like at night. Angela Blum is a hard worker and hopefully will succeed at her new project. Sean Kedrowski is also hard working and with any luck will get the phosphorylated unnatural amino acid project into ion channels. Shawna Frazier takes on challenging projects, but seems to thrive and is fun to hang out with outside of the lab. The newest members of the lab, Noah Duffy and Darren Nakamura, are entertaining and I am sure they will do well in their research.

In the summer of 2000, I performed research with Professor Jacob Anglister at the Weizmann Institute, Israel. I am extremely grateful for the opportunity and enjoyed working in the Anglister lab. Professor Anglister was extremely kind and very helpful. Dr. Avraham Samson was my graduate mentor and taught me everything about structural biology using NMR. Jane Raymond was influential on my laboratory technique and writing everything in my notebook and I am thankful for the opportunity of being a teaching assistant during my undergraduate studies. Lauren Stolper is an amazing person and I am grateful for being chosen to study abroad at Cambridge University, England. Lauren was also extremely helpful with graduate fellowship applications. I am also thankful to the Minority Student Education office for all their support throughout my undergraduate career, organizing volunteer activities, their mentor program, and many other useful events. Anna Salazar and Patricio Vela were amazing friends that were undergraduates and graduates at Caltech who showed me around the Los Angeles area, took me clubbing, and were great neighbors.

I would also like to thank the Wednesday night crew consisting of Dr. Gavin Murphy, Heather Murrey, Chad Vecitis, David Ebner, Adam Dennis, Cristal Gama, Tammy Campbell, Adam Hartwick, Wally Bugg, Mary Devlin, Adrian Rice, Sarina Mohanty, and Julian Revie. They made graduate school entertaining and definitely more interesting. Gavin, Heather, Chad, Dave, Adam, Cristal, and Tammy were always up for darts, poker, bowling, and hanging out anywhere. We definitely have a lot of crazy stories to tell about graduate school.

Throughout undergraduate and graduate school at Caltech I have received funding from the National Science Foundation Graduate Fellowship, American Chemical Society Division of Biological Chemistry Travel Award, Mellon Minority Undergraduate Fellowship, American Chemical Society's Scholars Program, Benjamin A. Gilman International Scholarship, David and Lucile Packard Foundation Scholarship, Donald R. Beall Scholarship, Target All-Around Scholarship, Ford Salute to Education Scholarship, SEEK, Inc. Scholarship, and El Paso Ronald McDonald Restaurant Owner-Operators Hacer Scholarship. I am very appreciative for the scholarships because they allowed me to attend Caltech and travel abroad.

Finally, I would like to thank my Mom and family for supporting my education throughout all these years. My mom was always there to help me when I needed her, allowing me to dream and apply to the best colleges in the country, supportive of my decisions, and accepting that I wanted to go to school for so long. The rest of the family has always supported my education through gifts of books, donations for my orchestra trips, and applauding my accomplishments. I couldn't have finished without my family.

Abstract

Unnatural amino acid (UAA) incorporation is an invaluable technique that is seeing increased use. THG73 is an amber suppressor tRNA used to incorporate > 100 residues at the UAG, amber stop codon, in *Xenopus* oocytes. We have found that yeast Phe frameshift suppressors (YFFS) can incorporate UAAs at the CGGG and GGGU quadruplet codons *in vitro* and *in vivo*, allowing simultaneous incorporation of three UAAs in the nicotinic acetylcholine receptor (nAChR). The YFFS are more “orthogonal” than the amber suppressor tRNA, THG73, but the frameshift suppressors incorporate UAAs less efficiently than THG73. A library of tRNAs derived from THG73 has produced an amber suppressor that is “orthogonal” and suppresses similarly to THG73. An analogous opal suppressor tRNA allows incorporation of UAAs at the UGA, opal stop codon. The use of the amber, opal, CGGG, and GGGU codons should allow for the simultaneous incorporation of four UAAs *in vivo*. Bioorthogonal labeling of UAAs is useful for the addition of large fluorophores. We incorporated *p*-AcPhe at α 70 of the nAChR and labeled with biotin and Cy5.5 hydrazide. Biotin and Cy5.5 hydrazide consistently labeled three proteins on oocytes not expressing α 70*p*-AcPhe and isn’t useful for site-specific labeling of ketone containing UAAs in oocytes. We explored the known subunit stoichiometry of the nAChR (2 α : β : γ : δ) expressed in oocytes and detected each subunit with the HA tag by Western blot. The α -subunit is present in excess of the other subunits in a ratio of \approx 3:1, which is expected to be 2:1. UAAs are being sold commercially for detection of protein-protein interactions in eukaryotic cells. The UAAs are heterogeneously incorporated and little is known about the effect on protein function and stability. We heterogeneously incorporated UAAs into the nAChR and detected changes in function by shifts in EC₅₀. Many UAAs altered the function of the nAChR. Incorporation of photo-reactive UAAs allowed for detection of cross-linking by Western blot. Heterogeneously incorporated UAAs also altered the functional nAChR expression on the surface of oocytes. Site-specific and heterogeneous incorporation of multiple UAAs are useful techniques for novel experiments to explore protein function, FRET experiments, cross-linking, and protein expression.

Table of Contents

Acknowledgments	iv
Abstract	viii
Table of Contents	ix
List of Figures	xvi
List of Tables	xx
Chapter 1: Vesicle Reconstitution & Characterization of the Synthetic	
Mechanosensitive Channel of Large Conductance	1
1.1 Introduction	2
1.2 Results	5
1.2.1 Vesicle Reconstitution of Synthetic MscL	5
1.2.2 Electron Microscopy of B-Ec-MscL in MLVs	6
1.2.3 Oligomeric State of MscL Analyzed by Cross-Linking in MLVs .	7
1.2.4 Fluorescence Imaging	10
1.2.5 Electrophysiological Characterization of Pt Wire GUVs	15
1.3 Discussion	15
1.4 Experimental Methods	16
1.4.1 Synthetic B-Ec-MscL Samples	16
1.4.2 MLV Preparation and DDM-Mediated Reconstitution of B-Ec-MscL	17
1.4.3 GUV Preparation With Pt Wire Electrodes	17
1.4.4 GUV Preparation With ITO Coverslips	18
1.4.5 Electron Microscopy of B-Ec-MscL in MLVs	19
1.4.6 Cross-Linking of MscL in MLVs	20
1.4.7 Fluorescence Imaging	20
1.4.8 Electrophysiological Characterization of Pt Wire GUVs	21
1.5 References	21
Chapter 2: Evaluating Aminoacylation and Suppression Efficiency of Nonsense	
and Frameshift Suppressor tRNAs <i>In Vitro</i>	27
2.1 Introduction	28

2.2	Results	34
2.2.1	Experimental Design and Western Blot Analysis	34
2.2.2	HSAS Suppression on $\alpha_{\text{NHA}}154\text{UAG}$ and $\alpha_{\text{NHA}}154\text{UAG}374\text{UAG}$	35
2.2.3	HSAS and HSFS _{CCCG} Suppression on $\alpha_{\text{NHA}}154\text{UAG}374\text{CGGG}$	36
2.2.4	HSAS/HSFS and THG73/THG73FS-Ala Suppression on $\alpha_{\text{NHA}}154\text{XXX(X)}$	39
2.2.5	HSAS/HSFS and THG73/THG73FS-Ala Suppression on Single Constructs in Wheat Germ <i>In Vitro</i> Translation	41
2.2.6	HSAS/HSFS and THG73/THG73-W Suppression on the α_{NHA} .	44
2.2.7	Arg Mutations, Changing CGG Triplets to CGC Triplets	50
2.2.8	Optimization of the RRL <i>In Vitro</i> Reactions for Increased Suppression Efficiencies	53
2.2.9	Choosing a New Frameshift Suppressor tRNA, YFFS _{CCCG}	54
2.2.10	HSAS and HSFS Suppression at $\alpha_{\text{NHA}}149\text{XXX(X)}$	57
2.2.11	Suppression Efficiencies of Suppressor tRNAs Using Optimized Conditions	59
2.3	Discussion	60
2.4	Experimental Methods	64
2.4.1	Materials	64
2.4.2	α_{NHA} Mutations and mRNA Preparation	65
2.4.3	Frameshift Suppressor tRNA Gene Construction and tRNA Preparation	66
2.4.4	dCA-aa Synthesis and Ligation to Suppressor tRNAs	68
2.4.5	<i>In Vitro</i> Translation	68
2.4.6	Western Blotting and Densitometric Analysis	69
2.5	References	71
Chapter 3: <i>In Vivo</i> Incorporation of Multiple Unnatural Amino Acids through Nonsense and Frameshift Suppression		
3.1	Introduction	78

3.2	Results	81
3.2.1	Testing Frameshift Suppression Viability <i>In Vivo</i>	81
3.2.2	UAA Incorporation By Frameshift Suppression	84
3.2.3	Masking Effects on Frameshift Suppression	88
3.2.4	Comparison of Frameshift and Nonsense Suppression Efficiencies	89
3.2.5	Comparison of Aminoacylation of Suppressor tRNA and Read-Through of Suppression Sites	91
3.2.6	The Effect of Discriminator and Acceptor Stem Mutations on YFFS _{CCCCG}	92
3.2.7	Incorporation of Two UAAs	93
3.2.8	Incorporation of Three UAAs	95
3.3	Discussion	97
3.4	Experimental Methods	99
3.4.1	Materials	99
3.4.2	Gene Construction and RNA Preparation	101
3.4.3	dCA and dCA-UAA Ligation to Suppressor tRNA	102
3.4.4	<i>In Vivo</i> Suppression Experiments	103
3.4.5	Electrophysiology	104
3.5	References	104
Chapter 4:	Development of Improved Amber and Opal Suppressor tRNAs for Unnatural Amino Acid Incorporation <i>In Vivo</i>. Minimizing Misacylation of Suppressor tRNAs	110
4.1	Introduction	111
4.2	Results	113
4.2.1	Schematic for Site-Specific UAA Incorporation and Aminoacylation	113
4.2.2	Experimental Scheme for Evaluating Aminoacylation and Suppression by Electrophysiology	115
4.2.3	Identifying the Natural aa Placed on THG73 Using Electrophysiology	117

4.2.4	Testing Knob Mutations on THG73	118
4.2.5	Testing Aminoacylation of ENAS and ENAS A71 <i>In Vivo</i>	121
4.2.6	Testing Aminoacylation of THG73 Acceptor Stem Mutations .	122
4.2.7	Aminoacylation Tested at a Highly Promiscuous Site, β A70 ...	127
4.2.8	LysRS Does Not Aminoacylate THG73, TQAS', and TQAS ...	128
4.2.9	Analyzing THG73-W and TQAS-W Interactions with the Translational Machinery <i>In Vivo</i>	129
4.2.10	Comparing Aminoacylation of Amber, Opal, and Frameshift Suppressor tRNAs	132
4.3	Discussion	135
4.4	Experimental Methods	141
4.4.1	Materials	141
4.4.2	tRNA Gene Preparation and tRNA Transcription	141
4.4.3	nAChR Gene Preparation and mRNA Transcription	142
4.4.4	dCA and dCA-W Ligation to Suppressor tRNAs	143
4.4.5	<i>In Vivo</i> Aminoacylation and Suppression Experiments	143
4.4.6	Electrophysiology	144
4.5	References	145
Chapter 5:	Development of Improved Amber and Opal Suppressor tRNAs for Unnatural Amino Acid Incorporation <i>In Vivo</i>. Evaluating Suppression Efficiency of Suppressor tRNAs	152
5.1	Introduction	153
5.2	Results	155
5.2.1	Electrophysiology Assay	155
5.2.2	ENAS and ENAS A71 Suppression Efficiency	156
5.2.3	Testing Suppression Efficiency of THG73 Acceptor Stem Mutations	158
5.2.4	Combining Mutations Causes Averaging of the Suppression Efficiency	160
5.2.5	Suppression Efficiency of the Acceptor Stem Mutations Does Not Correlate With Stability of the Acceptor Stem	161

5.2.6	Testing Amber, Opal, and Frameshift Suppression Efficiency .	162
5.2.7	Natural aa and UAA Incorporation With Selected Suppressor tRNAs	164
5.3	Discussion	167
5.4	Experimental Methods	171
5.4.1	Materials	171
5.4.2	tRNA Gene Preparation and tRNA Transcription	172
5.4.3	nAChR Gene Preparation and mRNA Transcription	173
5.4.4	ΔG Calculations of Each tRNA Acceptor Stem	173
5.4.5	dCA-W and dCA-WF1 Ligation to Suppressor tRNAs	174
5.4.6	<i>In Vivo</i> Suppression Experiments	175
5.4.7	Electrophysiology	175
5.5	References	176
Chapter 6: Bioorthogonal Labeling of <i>p</i> -AcPhe Incorporated in the Nicotinic Acetylcholine Receptor in <i>Xenopus</i> Oocytes & Western Blot Determination of the Subunit Stoichiometry of the Nicotinic Acetylcholine Receptor in <i>Xenopus</i> Oocytes		
6.1	Introduction	182
6.2	Results	185
6.2.1	Screening Sites For Aminoacylation of tRNAs, Read-Through, and UAA Incorporation	185
6.2.2	Suppression of $\alpha 70$ UAG with TQAS-Biocytin	190
6.2.3	Analysis of ^{125}I -Streptavidin and ^{125}I - α -Bungarotoxin Binding to <i>Xenopus</i> Oocytes Expressing Wild-Type nAChR and UAA Incorporation of Biocytin	193
6.2.4	Bioorthogonal Labeling of $\alpha 70$ <i>p</i> -AcPhe With Biotin Hydrazide	195
6.2.5	Attempts to Block Nonspecific Biotin Hydrazide Labeling of Uninjected Oocytes	199
6.2.6	Bioorthogonal Labeling of $\alpha 70$ <i>p</i> -AcPhe with Cy5.5 Hydrazide	200

6.2.7	Analysis of ^{125}I -Streptavidin Binding to <i>Xenopus</i> Oocytes Labeled with Biotin Hydrazide	202
6.2.8	α -, β -, γ -, and δ -Subunit Detection By Western Blot	203
6.2.9	α_{HA} and β_{HA} Detection After Various Incubation Times	205
6.2.10	α_{HA} and β_{HA} Solubilization of Whole Cells or Insoluble Fraction	206
6.2.11	α -, β -, γ -, and δ -Subunit Stoichiometry Determined By Densitometric Analysis	207
6.3	Discussion	208
6.4	Experimental Methods	213
6.4.1	Materials	213
6.4.2	tRNA Transcription and dCA or dCA-UAA Ligation	213
6.4.3	nAChR Gene Preparation and mRNA Transcription	214
6.4.4	<i>In Vivo</i> nAChR Expression Experiments	214
6.4.5	Electrophysiology	215
6.4.6	Oocyte Labeling with Biotin Hydrazide and Cy5.5 Hydrazide ..	215
6.4.7	Oocyte Treatment with Carbohydrazide and NaBH_4	216
6.4.8	Whole Oocyte Homogenization and Membrane Preparation	216
6.4.9	Western Blotting of Proteins Expressed in <i>Xenopus</i> Oocytes ...	217
6.5	References	218
Chapter 7:	Unnatural Amino Acid Replacement Scanning in <i>Xenopus</i> oocytes	228
7.1	Introduction	229
7.2	Results	231
7.2.1	Unnatural Amino Acid Replacement Scanning	231
7.2.2	Met Derivatives	237
7.2.3	Pro Derivatives	238
7.2.4	Val Derivative	239
7.2.5	Leu Derivatives	239
7.2.6	Phe Derivatives	241
7.2.7	Trp Derivatives	243
7.2.8	Cross-Linking the nAChR with PLeu and PMet	245

7.2.9	Unnatural Amino Acid Replacement Scanning Expression	
	Determined by Electrophysiology and Western Blot	251
7.3	Discussion	253
7.4	Experimental Methods	260
7.4.1	Materials	260
7.4.2	nAChR Gene Preparation and mRNA Transcription	261
7.4.3	<i>In Vivo</i> nAChR Expression, UAARS, and Site-Specific UAA Incorporation	261
7.4.4	Electrophysiology	262
7.4.5	UV Irradiation	262
7.4.6	Whole Oocyte Homogenization	262
7.4.7	Western Blotting of Proteins Expressed in <i>Xenopus</i> Oocytes ...	263
7.5	References	263

List of Figures

Figure 1.1	Crystal structure of Tb-MscL	3
Figure 1.2	Diagram of a DOPC lipid and vesicle formation	4
Figure 1.3	Oligomeric analysis of MscL using DSS cross-linking in MLVs	9
Figure 1.4	Fluorescence imaging of B-Ec-MscL in MLVs	12
Figure 1.5	Fluorescence imaging of B-Ec-MscL GUVs prepared with Pt wire electrodes	13
Figure 1.6	GUVs prepared by ITO coverslips and fluorescence imaging	14
Figure 1.7	Diagram of Pt wire electrode chamber for GUV preparation	18
Figure 1.8	Diagram of ITO coverslip chamber for GUV preparation	19
Figure 2.1	UAA incorporation techniques	29
Figure 2.2	Mechanism of nonsense and frameshift suppression	32
Figure 2.3	Nucleotide sequences and cloverleaf structures of suppressor tRNAs tested <i>in vitro</i>	34
Figure 2.4	HSAS suppression on $\alpha_{\text{NHA}}154\text{UAG}$ and $\alpha_{\text{NHA}}154\text{UAG}374\text{UAG}$	36
Figure 2.5	HSAS and HSFS _{CCCG} suppression on $\alpha_{\text{NHA}}154\text{UAG}374\text{CGGG}$	38
Figure 2.6	HSAS/HSFS and THG73/THG73FS-Ala suppression at $\alpha_{\text{NHA}}154\text{XXX(X)}$	40
Figure 2.7	HSAS/HSFS and THG73/THG73FS-Ala suppression at $\alpha_{\text{NHA}}154\text{XXX(X)}$ in wheat germ <i>in vitro</i> translation	43
Figure 2.8	HSAS/HSFS suppression on α_{NHA} is dependent on the amount of tRNA added	46
Figure 2.9	HSAS/HSFS and THG73/THG73FS-W suppression on α_{NHA} or $\alpha_{\text{NHA}}154\text{XXX(X)}$	48
Figure 2.10	Densitometric analysis of HSAS/HSFS suppression on α_{NHA} and $\alpha_{\text{NHA}}154\text{XXX(X)}$	50
Figure 2.11	HSFS _{CCCG} suppression on $\alpha_{\text{NHA}}\text{R182}$ and $\alpha_{\text{NHA}}154\text{CGGGR182}$ mutants	52
Figure 2.12	YFFS _{CCCG-W} and HSFS _{CCCG} suppression at $\alpha_{\text{NHA}}154\text{XXX(X)}$	55

Figure 2.13	YFFS _{CCCG} , YFG73FS _{CCCG} , YFaFS _{CCCG} (-Trp), and HSFS _{CCCG} suppression and reaminoacylation (74 mer) tested at $\alpha_{\text{NHA}}154\text{CGGG}$...	57
Figure 2.14	HSFS _{CCCG} and HSAS suppression at $\alpha_{\text{NHA}}154\text{XXX(X)}$ and $\alpha_{\text{NHA}}149\text{XXX(X)}$	58
Figure 2.15	Average suppression efficiency and reaminoacylation of suppressor tRNAs using optimized Western blot conditions and $\alpha_{\text{NHA}}\text{R182}$	60
Figure 3.1	Multiple UAA incorporation in ion channels expressed in <i>Xenopus</i> oocytes	79
Figure 3.2	tRNAs and UAAs	82
Figure 3.3	nAChR suppression sites used in this research	83
Figure 3.4	THG73FS _{CCCG} anticodon loop mutations	86
Figure 3.5	Simultaneous incorporation of two UAAs, representative traces and fits to the Hill equation	95
Figure 3.6	Simultaneous incorporation of three UAAs	96
Figure 4.1	Site-specific UAA incorporation	115
Figure 4.2	Fits to the hill equation for $\beta 9'$ UAG + THG73 (74mer)/-dCA (76mer) and $\beta 9'$ Q	118
Figure 4.3	THG73 mutations and tRNAs studied	119
Figure 4.4	THG73 Knob mutations	120
Figure 4.5	ENAS and ENAS A71 aminoacylation tested at $\beta 9'$ UAG	122
Figure 4.6	THG73 acceptor stem mutations tested at $\beta 9'$ UAG	124
Figure 4.7	tRNA (74mer/-dCA) aminoacylation tested at a highly promiscuous site, βA70	127
Figure 4.8	tRNA aminoacylation tested at αK145	129
Figure 4.9	Amber, opal, and frameshift suppressor tRNAs tested at $\beta 9'$	135
Figure 5.1	THG73 mutations and tRNAs studied	157
Figure 5.2	ENAS-W and ENAS A71-W suppression at $\alpha 149\text{UAG}$	158
Figure 5.3	THG73 acceptor stem mutations suppressing at $\alpha 149\text{UAG}$	159
Figure 5.4	Amber, opal, and frameshift suppressor tRNAs suppression at $\alpha 149$...	164

Figure 5.5	Fits to the Hill equation for wild-type recovery and UAA incorporation at α 149	166
Figure 6.1	α 70 and aligned positions in the nAChR	186
Figure 6.2	Detection of α 70Biocytin by Western blot	192
Figure 6.3	Analysis of ^{125}I -Streptavidin or ^{125}I - α -Bungarotoxin labeling of <i>Xenopus</i> oocytes expressing wild-type nAChR and UAA incorporation of biocytin	195
Figure 6.4	Bioorthogonal labeling of <i>p</i> -AcPhe with biotin hydrazide	196
Figure 6.5	Detection of α 70 <i>p</i> -AcPhe labeled with biotin hydrazide	197
Figure 6.6	Analysis of <i>p</i> -AcPhe labeled with biotin hydrazide	198
Figure 6.7	Uninjected oocytes labeled with biotin hydrazide after treatment with carbohydrazide or NaBH_4	200
Figure 6.8	Bioorthogonal labeling of α 70 <i>p</i> -AcPhe with Cy5.5 hydrazide	202
Figure 6.9	Analysis of ^{125}I -Streptavidin binding to <i>Xenopus</i> oocytes treated with biotin hydrazide	203
Figure 6.10	Detection of the α_{HA} , β_{HA} , γ_{HA} , and δ_{HA} subunits of the nAChR	205
Figure 6.11	Detection of the α_{HA} and β_{HA} subunits at different incubation times	206
Figure 6.12	Increasing the yield of the α_{HA} and β_{HA} subunits	207
Figure 6.13	Average ratio of the β -, γ -, and δ -subunits relative to the α -subunit	208
Figure 7.1	Unnatural amino acids used in this research	230
Figure 7.2	Unnatural amino acid replacement scanning (UAARS)	234
Figure 7.3	Amino acid composition of the nAChR	236
Figure 7.4	Fits to the Hill equation for UAARS with PLeu, PMet, and F3Leu	240
Figure 7.5	Fits to the Hill equation for UAARS with <i>o</i> FPhe, <i>m</i> FPhe, <i>p</i> FPhe, 2Pyr, 3Pyr, and 4Pyr	242
Figure 7.6	Fits to the Hill equation for UAARS with FTrp and nonsense suppression experiments	244
Figure 7.7	Detecting cross-linking of $\alpha_{\text{HA His}}$ with the HA Ab	247
Figure 7.8	Detecting cross-linking of $\alpha_{\text{HA His}}$ with the His Ab and HA Ab	249

Figure 7.9	Detecting cross-linking of $\alpha_{\text{HA His}}$ with the HA Ab after extended UV irradiation	250
Figure 7.10	UAARS effect on functional nAChR expression detected by electrophysiology	252
Figure 7.11	UAARS effect on nAChR expression detected by Western blot	253

List of Tables

Table 3.1	HSAS and HSFS suppression experiments at the β 9' site	84
Table 3.2	THG73FS _{CCCG} and anticodon loop mutations suppression at α 149CGGG	86
Table 3.3	Wild-type recovery and UAA incorporation by frameshift suppression <i>in vivo</i>	87
Table 3.4	Masking experiments	89
Table 3.5	Comparison of suppression efficiency, aminoacylation, and read-through <i>in vivo</i>	90
Table 3.6	The effect of discriminator base and acceptor stem mutations on YFFS _{CCCG}	93
Table 3.7	Incorporation of two UAAs	95
Table 3.8	Oligos used in this research	100
Table 4.1	THG73 acceptor stem mutations	125
Table 5.1	THG73 acceptor stem mutations	161
Table 5.2	Natural aa and UAA incorporation with THG73, TQAS', TQAS, TQOpS', and TQOpS	166
Table 6.1	Expression tests at the α D70 aligned positions and β A19'	189
Table 7.1	UAARS EC ₅₀ determination	238