Ampa and kainate receptor-potentiating RNA aptamers

Janet L. Lynch
University at Albany, State University of New York, janetlynch40@gmail.com

The University at Albany community has made this article openly available.
Please share how this access benefits you.

Follow this and additional works at: https://scholarsarchive.library.albany.edu/legacy-etd

Part of the Biochemistry Commons, and the Neuroscience and Neurobiology Commons

Recommended Citation
https://scholarsarchive.library.albany.edu/legacy-etd/2737

This Dissertation is brought to you for free and open access by the The Graduate School at Scholars Archive. It has been accepted for inclusion in Legacy Theses & Dissertations (2009 - 2024) by an authorized administrator of Scholars Archive.
Please see Terms of Use. For more information, please contact scholarsarchive@albany.edu.
AMPA and Kainate Receptor-Potentiating RNA Aptamers

by

Janet Lee Phaneuf Lynch

A Dissertation
Submitted to the University at Albany, State University of New York
In Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy

College of Arts and Sciences
Department of Chemistry
May 2021
ABSTRACT

Glutamate receptors act to bring about excitatory transmission in the central nervous system. The receptors are divided into two groups: ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors are ion channels which are activated by an agonist such as glutamate or kainate. The main receptors in the ionotropic glutamate receptor family are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors. In the central nervous system, ionotropic glutamate receptors are found both pre- and postsynaptically. It has been found that most AMPA and NMDA receptors are postsynaptic receptors while the kainate receptors can be pre- or postsynaptic. Underactivity of these receptors has been implicated in many neurological disorders such as depression, learning disabilities, and Alzheimer’s disease (AD). Potentiators of these receptors can be drug candidates for these diseases. For this reason, developing potentiators which specifically target these receptors is important. The work detailed in this thesis has been done to develop an RNA aptamer which potentiates AMPA and kainate receptors.

Currently, small molecule potentiators are being synthesized to potentiate AMPA receptors. Because of low solubility and low selectivity, many of these compounds have failed drug trials and have been deemed to be unsafe to use. In order to find a selective, soluble RNA aptamer which potentiates AMPA and kainate, a systematic evolution of ligands by exponential enrichment (SELEX) study was performed. This is an in-vitro evolutionary method which allows us to isolate RNA sequences (aptamers) which bind specifically to the receptor and may potentiate the receptor.
My use of SELEX against GluA1 will be described in chapter 1, where I detail how I was able to isolate an RNA aptamer, AL3, which potentiates both AMPA and kainate receptors. To find the shortest active sequence for the potentiator, I truncated the AL3 aptamer. The shorter aptamer, named AL3-1, lost activity against AMPA receptors, but specifically and potently potentiates kainate receptors. This was an interesting discovery because research on kainate receptors has been slow, due to the lack of kainate receptor-specific potentiators. The shorter aptamer, AL3-1 has 69 nucleotides, while the longer AL3 has 101 nucleotides. The shorter sequence potentiates GluK1 and GluK2 more potently than the longer sequence does. Both receptors showed no activity on NMDA receptors. Additionally, this work is important because it supports the most recent work of others from this lab showing that aptamers may bind to different receptors with different moieties, and by separating these moieties of the aptamer, a different selectivity profile can be achieved.

Potentiation is an increase in channel conductivity, which can be accomplished in a few different ways. The aptamer most likely potentiates the receptor by opening the channel wider to allow more ions to flow in. Both AL3 and AL3-1 increase the amplitude of channel conductivity while not affecting the steady state, or desensitization of the receptor. The mechanism of most small-molecule potentiators is either slowing desensitization or deactivation of the receptor. Drugs which potentiate in this way are seizurogenic, and many potentiators that work in this way are toxins. However in preliminary tests, small molecule potentiators have been shown to increase memory and learning. The aptamer may be a safer alternative to these small molecule drugs.

In order for RNA aptamers to be functional, chemical modification must be made. I chemically modified a previously found aptamer, FN58, and my own aptamer FL3-1 with 2’-F
nucleotides for A, C, and U. This is necessary because the unmodified RNA is quickly degraded in the presence of ubiquitous RNAse. The 2’ F modification replaces the 2’ OH in all A, C and U nucleotides in the sequence, rendering a more stable aptamer. It has been previously shown in this lab that the 2’-F modified nucleotides have half-lives in the order of days, rather than minutes, for unmodified nucleotides.

In chapter 2, I describe a new method of increasing the transfection yield of green fluorescent protein in HEK-293 cells. In this experiment, I investigated the application of DMSO after transfection. HEK-293 cells are one of the most commonly used cell lines behind CHO cells and HeLa cells. These cells have been particularly useful in electrophysiology and neuropharmacology because they are not typical kidney cells, but contain genetic signatures of adrenal and neurologic cells. Also they are small and faithfully translate membrane proteins, such as glutamate receptors.

Some previous studies have shown that brief applications of DMSO have increased protein yields in different cell lines, but the optimum concentration of DMSO and optimum exposure time seemed to be cell-line dependent. No previous studies have been done on the use of DMSO in HEK-293 cells to improve transfection yield. In my experiment, I found that a brief application of DMSO increases protein yield, and the optimum concentration of DMSO as well as the optimum exposure time for HEK-293 cells were determined.

To quantify transfection efficiency, green fluorescent protein was transfected. The green cells were imaged and classified according to intensity of green, which indicates transfection yield. It was found that a 10% DMSO solution applied for 5 minutes gives approximately 1.7-fold increase in intense green cells. Also, a brief exposure is not noticeably toxic to the cells. To ensure that the DMSO does not affect channel kinetics in glutamate receptors, a whole cell
recording study was performed. This study showed that the channel conductance was not significantly different between cells that were treated with DMSO and hypothermia and those which were not treated. This technique is useful in our lab because much of our research involves transfecting glutamate receptors in HEK-293 cells.
ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Li Niu for his guidance and leadership in the journey of my doctoral study.

I sincerely thank my thesis committee, Dr. Alan Shekhtman, Dr. Alan Chen, Dr. Mehmet Yigit, and Dr. Haijun Chen for their advice and suggestions through the years.

I would like to especially thank Dr. Zhen Huang and Dr. Bill Jaremko for their patience, guidance, and friendship during my time in the lab, and also Wei Wen, Vincen Pierce, Noah Saunders, Samantha Ingenito, Dr. Yumeki Tani, and others who were an important part of my tenure.

Most importantly I would like to thank my husband, Chris Lynch for going through this with me and encouraging me to stand up and fight for it. Also, to my boys, Sam and Jake for being a great encouragement along the way.
PERMISSION TO USE

Figure 1.4 is originally from “Structure and organization of heteromeric AMPA type glutamate receptors” Beatriz Herguedas, Javier Garcia-Nafria, Ondrej Cais, Rafael Fernandez-Leiro, James Krieger, Hinze Ho, Ingo Greger, April 29, 2016 vol 352 no. 6285. Website containing permission statement: http://www.sciencemag.org/help/reprints-and-permissions

Figures 2.1, 2.2, 2.3, 2.5, and 2.6 were originally published in Lynch, J., Chung, J. et al. (2020). “Enhancing transient protein expression in HEK-293 cells by briefly exposing the culture to DMSO” in the Journal of Neuroscience Methods. The images are reproduced here with permission. Some of the wording has been revised for this dissertation. I was the first author for this work, and the material is being included here because it is an important aspect of the research which makes up this dissertation.

This research was originally published in the Journal of Neuroscience Methods. Lynch, J. Chung, J., et al. Enhancing transient protein expression in HEK-293 cells by briefly exposing the culture to DMSO. J Neuro Meth 2020; 350:109058. © Elsevier B.V. Website containing permission statement: https://www.elsevier.com/about/policies/copyright#Author-rights
# TABLE OF CONTENTS

Chapter 1: Development of Aptamers Potentiating AMPA and Kainate Receptors

1.1 Introduction ......................................................................................................................... 1
1.1.1 Glutamate ion channels ................................................................................................. 1
1.1.2 AMPA Receptors subunits and post-translational modifications ................................ 10
1.1.3 Kainate receptor subunits and post-translational modifications .................................. 13
1.1.4 Potentiation of AMPA and Kainate receptors .............................................................. 16
1.1.5 Chemical potentiators of AMPA and Kainate receptors ............................................. 17
1.1.6 Isolation of aptamers using SELEX ............................................................................. 19
1.1.7 RNA aptamers as therapeutics ...................................................................................... 21

1.2 Materials and methods ..................................................................................................... 24
1.2.1 cDNA plasmid preparation ........................................................................................... 24
1.2.2 Cell culturing and cell /receptor expression ................................................................. 24
1.2.3 Preparation of RNA library .......................................................................................... 27
1.2.4 Determining the concentration of receptor in the membrane ....................................... 27
1.2.5 SELEX .......................................................................................................................... 30
1.2.6 Cloning and ligation of the final pool ........................................................................... 35
1.2.7 RNA purification and preparation ................................................................................ 36
1.2.8 Whole cell recording .................................................................................................... 37
1.2.9 Treatment of a putative aptamer for functional assay .................................................. 39
1.2.10 RNA sequence truncation ........................................................................................... 40
1.2.11 Homologous Binding Assay ....................................................................................... 40
1.2.12 Statistical Data Analysis ............................................................................................. 42

1.3 Results ................................................................................................................................ 42
1.3.1 Design of SELEX experiment: target, elution pressure, and negative selection ........ 42
1.3.2 Identifying the enriched RNA sequences and functional assay of the selected aptamers ................................................................................................................. 44
1.3.3 Truncation of AL3 aptamer to generate a minimal length but functional RNA ........... 48
1.3.4 Activity of AL3 and AL3-1 against iGluR subunits ....................................................... 48
1.3.5 Homologous competition assay .................................................................................... 52

1.4 Discussion ........................................................................................................................... 54
Chapter 2: Enhancing transfection efficiency in HEK cells using DMSO

2.1 Introduction ....................................................................................................................... 71
  2.1.1 Membrane proteins in medicine ................................................................................... 72
  2.1.2 Transfecting HEK-293 cells ......................................................................................... 72
  2.1.3 DMSO treatment of cells .............................................................................................. 74
  2.1.4 DMSO increases transfection yield .............................................................................. 74
  2.1.5 My experiment of exploring DMSO treatment in HEK-293 cells ............................... 75

2.2 Materials and Methods ..................................................................................................... 76
  2.2.1 Plasmid DNAs and cell culture .................................................................................... 76
  2.2.2 Transient transfection and DMSO treatment ............................................................... 77
  2.2.3 Cell imaging and quantification ................................................................................... 78
  2.2.4 Cell viability assay ........................................................................................................ 78
  2.2.5 Whole-cell current recording ........................................................................................ 79

2.3 Results ................................................................................................................................ 80
  2.3.1 Effect of DMSO............................................................................................................ 80
  2.3.2 Determining optimum concentration and exposure time ............................................. 82
  2.3.3 Time course of GFP expression post-DMSO exposure ............................................. 84
  2.3.5 Determining the toxicity of a brief DMSO exposure ................................................... 87
  2.3.6 Whole-cell recording assay of ion channel activity for DMSO-treated HEK-293 cells .............................................................................................................................. 90

2.4 Discussion ........................................................................................................................... 93

2.5 Supplementary material ................................................................................................... 96

2.6 References .......................................................................................................................... 97
LIST OF FIGURES

Chapter 1

Fig 1.1 Glutamergic Synaptic Cleft ................................................................................................ 3
Fig 1.2 GluA2 Subunit Topology ................................................................................................... 6
Fig 1.3 iGluR subunits .................................................................................................................... 9
Fig 1.4 Heteromeric and homomeric Glutamate receptors ........................................................... 12
Fig 1.5 GluK2 Subunit Domains .................................................................................................. 15
Fig 1.6 SELEX steps ..................................................................................................................... 20
Fig 1.7 2’-F Modification of RNA Nucleotides ............................................................................ 23
Fig 1.8 Enriched sequences and their functions ......................................................................... 47
Fig 1.9 Truncation of AL3 ............................................................................................................ 50
Fig 1.10 Subunit selectivity of AL3 and AL3-1 ........................................................................... 51
Fig 1.11 Homologous binding study for AL3-1 ......................................................................... 53

Chapter 2

Fig 2.1. Effect of DMSO ............................................................................................................... 81
Fig 2.2 Determining the optimum concentration of DMSO and exposure time ....................... 83
Fig 2.3 Time course of GFP expression post-DMSO exposure ................................................ 86
Fig 2.4 MTT assay of DMSO treated cells ................................................................................... 88
Fig 2.5 Assay of cell viability and growth for DMSO-treated HEK-293 cells using alamarBlue assay .............................................................................................................................................. 89
Fig 2.6 Whole-cell recording assay of AMPA and kainate receptor activities with DMSO- treated and untreated HEK-293 cells ........................................................................................................ 92
LIST OF TABLES

Chapter 1

Table 1.1 Plasmid Amounts for Transfection ................................................................. 26
Table 1.2 Concentrations of Materials for [\( ^3 \text{H} \)] Binding Assay ............................ 29
Table 1.3 Typical SELEX experiment ................................................................................. 34
ACRONYMS AND SYMBOL DEFINITIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazole</td>
</tr>
<tr>
<td>AMPAR</td>
<td>AMPA receptors</td>
</tr>
<tr>
<td>ATD</td>
<td>Amino-terminal domain</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived Neurotrophic Factors</td>
</tr>
<tr>
<td>BPAM344</td>
<td>4-cyclopropyl-3,4-dihydro-7-fluoro-2H-1,2,4-benzothiadiazine 1,1-dioxide</td>
</tr>
<tr>
<td>BPAM591</td>
<td>4-cyclopropyl-7-hydroxy-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTZ</td>
<td>Cyclothiazide</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>Escherichia coli</em> host</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEK-293S</td>
<td>Human embryonic kidney-293S</td>
</tr>
<tr>
<td>IDRA-21</td>
<td>7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide</td>
</tr>
<tr>
<td>iGluRs</td>
<td>Ionotropic ligand gated ion channels</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid or kainate</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>S18986-1</td>
<td>(s)-2,3-dihydro-[3,4] cyclopentano-1,2,4-benzothiazine-1,1-dioxide</td>
</tr>
<tr>
<td>S47445</td>
<td>8-cyclopropyl-3-[2-(3-fluorophenyl) ethyl]-7H-[1,3] oxazino [6,5-g][1,2,3]benzotriazine-4,9-dione</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evolution of Ligands by Exponential Enrichment</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
</tbody>
</table>
Chapter 1: Development of Aptamers Potentiating AMPA and Kainate Receptors

1.1 Introduction

1.1.1 Glutamate ion channels

Glutamate is an essential neurotransmitter which is released from the synapse and activates a group of protein receptors known as glutamate receptors\textsuperscript{1,2}. These receptors are found on both neuronal and non-neuronal cells to control and modulate the central nervous system (CNS) (Fig 1.1)\textsuperscript{2}. Glutamate receptors are subdivided into two classes, which are metabotropic glutamate receptors (mGluR) and ionotropic glutamate receptors (iGluR). These two classifications were identified functionally by observing excitation of neurons\textsuperscript{3}, and observing increasing inositol production caused by the application of glutamate\textsuperscript{4}. MGluRs are G-protein coupled receptors, which activate a second messenger system to alter protein downstream signaling, while iGluRs are ligand-gated ion channels through which ions are permitted to flow into the cells to depolarize the membrane. Both receptor types bind to glutamate; however, it has been found that the mGluR and iGluR interaction is carried out by different molecular mechanisms\textsuperscript{5}. Some iGluRs are thought to act metabotropically as well, where they couple with GTP binding proteins or tyrosine kinase\textsuperscript{6}.

iGluRs are further divided into three subfamilies, i.e., α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors. These separate subtypes of iGluRs were first identified with specific agonists or ligands\textsuperscript{7}. Later, delta receptors were identified. However, the function of delta receptors has not yet been established\textsuperscript{2}. Sequence homology shows that the genes of NMDA, kainate, and AMPA receptors are found on many chromosomes, and the high degree of similarity in sequences shows that the iGluRs share a
similar architecture\(^1\). Unlike other ligand-gated ion channels, iGluRs are transmembrane proteins composed of four subunits assembled into a “dimer of dimers” structure with a channel pore in the middle\(^1\)-\(^2\). AMPA receptors have four subunits, GluA1-4, NMDA receptors have seven subunits, GluN1, GluN2A-GluN2D and GluN3A and GluN3B, whereas kainate receptors have five subunits, GluK1-5\(^1\)-\(^2\). In iGluR topology, each subunit is made up of four domains: a transmembrane domain (TMD), and an intracellular Carboxyl-Terminal domain (CTD) and two extracellular domains, Amino-Terminal domain (ATD), and Ligand Binding domain (LBD), which are connected by peptide linkers\(^1\)-\(^2\).
Fig 1.1 Glutamergic Synaptic Cleft
The depiction of a glutamatergic synaptic cleft containing three subtypes of ionotropic glutamate receptors.
The TMD has four helical segments: M1-M4, of which the M1, M3 and M4 are transmembrane, while the M2 is a re-entrant membrane loop. Specifically, the M2 segment enters the membrane through the cytoplasmic side, forms a narrow area at the channel pore’s base and exits the membrane\(^8\). In AMPA receptors, the GluA subunit is unique in that this segment exists in an edited (R) or unedited (Q) form. Q/R editing changes the calcium permeability of the receptor, and is critical for AMPA receptor activity\(^9-11\). The GluA2R isoform cannot form a functional channel on its own, but can combine with GluA2Q or GluA1\(^12\). Additionally, in GluA1-4, the exon between the M3 and M4 can be R/G edited to form a receptor which has a faster opening rate and a more pronounced desensitization than unedited receptors\(^13\).

The CTD is the intercellular domain, which varies in length among various subtypes and subunits. CTD plays an important role in receptor trafficking. CTD domain is also subject to phosphorylation\(^14\). Phosphorylation of the CTD has been shown to increase channel open probability and enhance channel conductance\(^15-16\). The CTD is also a binding site for intracellular signaling and is subject to posttranslational modifications as well\(^1, 17\). These posttranslational modifications affect the binding of interacting proteins and regulation by phosphorylation in the cytoplasm\(^18\).

Extracellular domains make up most of the mass of a receptor. There are two extracellular domains, the ATD and the LBD. The ATDs of AMPA receptors are made up of approximately 400 amino acids\(^19\). This domain is thought to drive assembly of a receptor and influence the anchoring of the receptor in the synapse\(^20\). The structure of the ATD is composed of two lobes, which is unique for eukaryotic organisms. The two lobes are flexible and may play a role in allosteric regulation due to the flexibility of the lobes. The ATDs of most AMPA and kainate receptors form tight dimers, which restrict mobility, while the NMDA receptor has a loosely
packed ATD\textsuperscript{21}. The LBD is a clamshell shaped structure close to the cell membrane, and acts as an agonist dock in iGluRs. The structure of the LBD has been determined without the ligand (apo), bound to the agonist, and bound to an antagonist\textsuperscript{22}. The domain has two lobes, D1 and D2, which capture the ligand and induce closure of the clamshell shaped structure. This causes a downstream conformational change within the receptor to open the channel gate in the TMD. Quickly following this, the receptor undergoes rapid desensitization or a transient inactivation of the receptor with the ligand remaining bound.
Fig 1.2 GluA2 Subunit Topology
The topology of a GluA2 AMPA receptor subunit shows various domains and post-translational modification areas as well as the Q/R editing site.
There are some functional differences among the three subtypes of iGluRs. NMDA receptors have a high Ca\(^{2+}\) permeability and require both glutamate and glycine as the co-agonist to bind in order to become activated\(^{23}\). Unlike other glutamate receptors, these receptors contain an Mg\(^{2+}\) block, which limits the transmission of fast signals\(^{24}\). Kainate receptors are selectively activated by kainate and are permeable to Na\(^+\) ions, and less permeable to Ca\(^{2+}\) than NMDA receptors\(^{25}\). Some kainate receptors in the presynaptic cleft have been found to have both metabotropic and ionotropic activities. Presynaptic kainate receptors are thought to regulate neurotransmitter release from the presynaptic terminals into the synapse. During long term potentiation, kainate receptors aid in recycling AMPA receptors back to the postsynaptic neuron, an activity requiring protein kinase C\(^{26}\). Both NMDA receptors and kainate receptors are implicated in the induction of long-term potentiation and long-term depression.

AMPA receptors are mostly organized as heteromeric channels in the CNS. Both L-glutamate and kainate activate these receptors, however the binding of kainate to an AMPA receptor does desensitize the receptor\(^{27}\). Upon editing at the Q/R site, the GluA2 AMPA receptor subunit cannot form a functional channel. In contrast, all other AMPA subunits can form functional channels or homomeric channels that are calcium permeable\(^{28-29}\). In early development, calcium permeable GluA2 receptors are exchanged for calcium impermeable AMPA receptors by postnatal week 2\(^{30}\). GluA4 homomers may be present in the synapses at early development and later are exchanged for calcium impermeable GluA2R-containing receptors. However, the population of calcium permeable AMPA receptors spikes again in certain cells during adolescence\(^{31}\). Heteromers that contain GluA2/GluA3 cycle in and out of the synapse while receptors containing GluA1 are moved into the synapse during long term potentiation\(^{32}\). Therefore, the calcium permeable AMPA receptors are tightly regulated in brain
regions and cell types over development and are important in the induction of synaptic plasticity\textsuperscript{33-34}.

Although kainate and AMPA receptors are similar in their overall structures and some post-translational modifications, which I will explain in more detail below, kainate and AMPA receptors have different properties, such as desensitization rates and presynaptic activity. Unlike in AMPA receptors, desensitization of kainate receptors requires sodium ions to be bound to the ligand binding domain of the subunits. Without these ions bound, the receptor does not desensitize\textsuperscript{35}. Additionally, the desensitized state of kainate receptors is more stable than that of AMPA receptors, due to a stabilized D1 interface, strong interactions between D1 and D2, and areas outside of the LBD conferring stability to the desensitized state\textsuperscript{36}. In the hippocampus, kainate receptors are found in both the post-synapse, where they act as ion channel receptors, and in the presynaptic membrane where they act metabotropically, regulating the release of neurotransmitters into the synapse. These presynaptic kainate receptors use calmodulin and other proteins to regulate the release of glutamate and GABA in the synapse and participate in the induction of synaptic plasticity\textsuperscript{37-40}. 
Fig 1.3 iGluR subunits
Ionotropic glutamate receptor subtypes and subunits within each of the subtypes. Not shown here are the delta receptor subunits.
1.1.2 AMPA Receptors subunits and post-translational modifications

The four subunits of AMPA receptors have been classified according to the amino acid sequence. The length of the CTD is also different in different subunits. GluA2, GluA3, and a splice variant of GluA4 have a short CTD, while GluA1, GluA4, and a variant of GluA2 have a longer CTD. The receptors with the short CTD are continually cycled in and out of the synapse, while the receptors with the longer CTD are recruited to the synapse during high neuronal activity.

AMPA receptors are found throughout the brain, inhibitory interneurons, and glia. In the hippocampus, most AMPA receptors are heteromers containing GluA1/GluA2 or GluA2/GluA3, together with a smaller number of homomeric GluA1 receptors. The GluA1 subunits are critical for long term potentiation (LTP) induction, leading to memory storage. Following LTP, GluA1 receptors are exchanged for GluA2/3 receptors in memory consolidation. Phosphorylation of two serine moieties, S831 and S845 on the GluA1 subunit, is associated with LTP. Dysfunction of the GluA1 subunit has been found in conditions such as schizophrenia, learning disabilities, and memory loss. GluA3-containing heteromers are involved in the induction of synaptic potentiation in a process involving cyclic AMP (cAMP).

The AMPA subunits are also diversified by RNA editing, splicing, and posttranslational modification. In the GluA2 subunit, the RNA editing at the Q/R site is controlled by enzyme adenosine deaminase acting on RNA type 2 (ADAR2) and is necessary for normal brain function. The R isoform is unable to form a homomeric functional channel and is not calcium permeable, while all other AMPA subunits are calcium permeable to varying degrees. In the embryonic stage, less than 5% of the receptors are unedited, while in a healthy human adult,
nearly all of the GluA2 receptors are edited. Defects in this editing site are linked to disorders of the CNS such as amyotrophic lateral sclerosis (ALS) and ischemia\textsuperscript{54-55}. Alternative splicing of RNA (R/G editing) is another post-translational modification near the base of the LBD, giving rise to two variants: flip and flop, and all AMPA receptor subunits are subject to this type of RNA editing. In general, flip and flop isoforms exhibit different kinetic properties\textsuperscript{13, 56-57}. The GluA2-4 flop isoforms have a faster channel opening rate and more pronounced desensitization than the unedited subunit. When GluA1/GluA2 heteromeric channels are formed with R/G editing isoform, the flop form of GluA1/GluA2 shows faster channel opening and desensitization rates than the flip counterpart\textsuperscript{13}.

The glutamate receptor structure was first obtained by electron microscopy, and later, crystallographic studies showed a more detailed structure of a rat GluA2 receptor in the membrane\textsuperscript{8}. Other structures of AMPA receptors were more recently identified and a classification of receptors into two structural categories was shown. These structures, namely the Y-shaped, or N-shaped\textsuperscript{21, 58} and O-shaped structures differ in the packing of the ATD where the Y-shaped, or N-shaped receptor is loosely packed and further from the LBD, while the O-shaped receptor structure is tightly packed and more compact due to a cysteine cross-link. The GluA2 receptor was found to have a Y-shape while the GluA2/GluA3 and GluA2/GluA4 heteromers have O-shape\textsuperscript{21, 59-60}. The difference in shape is thought to contribute to differences in kinetics between heteromeric and homomeric channels, as well as affecting the binding of some auxiliary proteins\textsuperscript{61}. 
Fig 1.4 Heteromeric and homomeric Glutamate receptors

NTD layers of a heteromeric GluA2/3 (top left) and a homomeric GluA2 receptor (top right) as viewed from the top [Protein Data Bank Identifier (PDB ID) 3H5V]. Full length cryo-EM structures of heteromeric GluA2/3 (bottom left) and homomeric GluA2 (bottom right) [PDB ID 4UQJ]. From [“Structure and organization of heteromeric AMPA type glutamate receptors” Beatriz Herguedas, Javier Garcia-Nafria, Ondrej Cais, Rafael Fernandez-Leiro, James Krieger, Hinze Ho, Ingo Greger, April 29, 2016 vol 352 no. 6285]. Reprinted with permission from AAAS.
1.1.3 Kainate receptor subunits and post-translational modifications

Kainate receptors have been found to contribute to synaptic plasticity in several ways, including the regulation of neurotransmitter release and cellular excitability in the synapse\textsuperscript{39, 62-63}. Again, there are five kainate receptor subunits, i.e. GluK1 (formerly GluA5), GluK2 (formerly GluA6), GluK3 (formerly KA1) GluK4 (formerly KA2) and GluK5 (formerly KA3). Kainate receptor subunits are classified based on their affinity to the ligand, with low affinity subunits GluK1-3 and high affinity subunits GluK4 and 5. GluK1-3 can assemble into functional homomers, while GluK4 and 5 can only assemble as heteromers with GluK1-3. Most kainate receptors in the CNS exist as heteromers, containing subunits of GluK2 and GluK5\textsuperscript{64}. These heteromeric receptors have been found to control the activity of the AMPA and GABA receptors in the hippocampus and have also been shown to play a role in diseases such as autism in knock-out mice\textsuperscript{65}. Other studies have shown that the GluK2/GluK5 heteromers have been implicated in epilepsy, pain\textsuperscript{66-67}, anxiety\textsuperscript{68}, and memory and learning disabilities such as Alzheimer’s disease\textsuperscript{39, 69}.

The ATD and CTD of kainate receptor subunits also undergo RNA editing and alternative splicing. In GluK1, the alternative splicing in the ATD produces two variants of GluK1\textsuperscript{70}, while the C-terminus have four splicing variants\textsuperscript{71-72}. Some of these C-terminal modifications prevent the subunits from escaping the endoplasmic reticulum, while others affect protein-protein interactions within the cell\textsuperscript{73-74}. GluK1 can be edited in
Q or R form within the pore lining region, affecting biophysical properties such as calcium permeability, and protein-protein interaction. GluK2 can be also edited at its Q/R site, I/V site and Y/C site in the TMD. RNA editing in kainate receptors is developmentally regulated, with 80% of GluK2 and 40% of GluK1 edited after birth. The unedited, or the Q form of GluK2 modulates synaptic plasticity and increases seizure vulnerability, while unedited GluK1 does not show developmental or behavior abnormalities. The I/V and Y/C editing may affect ion permeability, however the activity of kainate receptors with these editing isoforms has not been characterized. Alteration of kainate receptor editing efficiency is decreased in spinal cord injury, epilepsy, mood disorders such as bipolar disorder and depression, and fear conditioning in mice.
Fig 1.5 GluK2 Subunit Domains
Diagram of GluK2 transmembrane domain showing RNA and post-translational editing locations. Red squares indicate locations for splice editing, while blue squares indicate RNA editing areas.
1.1.4 Potentiation of AMPA and Kainate receptors

Potentiation, also known as positive allosteric modulation of an ion channel activity is an enhancement of channel activity. Potentiation can be achieved by slowing the desensitization of the receptor, delaying the deactivation of the receptor, or increasing the opening time of the channel, all of which would result in an increase in channel conductivity. Compounds that bind to and potentiate these channels are called potentiators. The action of potentiators on AMPA receptors has been extensively studied, revealing that the compounds bind in a hydrophobic pocket of the GluA2 AMPA subunit at the interface of the LBD of two adjacent subunits\textsuperscript{86-88}. Mechanistically, these compounds modulate the kinetics of the receptor by affecting both the desensitization and deactivation of the receptor\textsuperscript{89}. Other potentiators, such as Li\textsuperscript{+} have been shown to increase the probability of open GluA3 channels, while not affecting individual channel kinetics. In this case, the potentiation may be due to an increase in trafficking of the receptor to the synapse\textsuperscript{90}. It should be noted, however, potentiatobs which slow desensitization or deactivation may lead to prolonged channel activity, resulting in excitotoxicity\textsuperscript{91}. Thus, enhancing receptor function without potential excitotoxic activity is an important reason to develop potentiators.

In diseases where hypoactivity of glutamate receptors may be a cause, potentiatobs could serve as a treatment. Potentiation of AMPA receptors has been shown to enhance excitatory transmission\textsuperscript{92}, improve episodic and spatial working memory\textsuperscript{93-94}, and rescue long term potentiation and synaptic changes in middle aged and elderly animal models\textsuperscript{69,95}. Learning and memory deficits in diseases such as attention deficit hyperactivity disorder (ADHD), dementia\textsuperscript{96}, Parkinson’s disease\textsuperscript{97}, schizophrenia\textsuperscript{98-100}, and drug
addiction\textsuperscript{101-102} may be contributed by hypoactivity of AMPA receptors. AMPA receptors also stimulate the production of neurotrophins such as brain derived neurotrophic factor (BDNF) which stimulates the growth of new neurons and protects neurons from harm\textsuperscript{103-105}.

Kainate receptors in the hippocampus regulate synaptic transmission and the release of glutamate, and contribute to excitatory transmission, learning and memory\textsuperscript{106}. At certain synapses, kainate receptors can modulate glutamate release bi-directionally depending on the extent these receptors are activated\textsuperscript{107}. An abnormality in the genes of GluK1 have been found in epilepsy patients, while GluK2 gene abnormalities have been found in autism\textsuperscript{65}, and intellectual disability\textsuperscript{108}. Furthermore, GluK2 knockout mice showed signs of mania\textsuperscript{109}. Lower expression of kainate receptor subunits, GluK1-3 have been implicated in Alzheimer’s disease\textsuperscript{110-113}. However, currently the role of kainate receptors in disease is much less well understood than that of AMPA receptors, mainly because of lack of selective, potent, compounds capable of potentiating kainate receptors.

1.1.5 Chemical potentiators of AMPA and Kainate receptors

There are many chemical compounds that can potentiate AMPA receptors. Compounds such as Piracetam, Aniracetam, and Oxiracetam are some of the first AMPA potentiators ever developed. These were studied for their physiological effects on potentiating an AMPA receptor\textsuperscript{114}. Other potentiators such as benzothiadiazines, including Cyclothiazide (CTZ), 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide (IDRA-21), and (s)-2,3-dihydro-[3,4]cyclopentano-1,2,4-
benzothiazine-1,1-dioxide (S18986-1) slow the desensitization, with IDRA-21 enhancing cognitive abilities in animal studies\textsuperscript{115-116}. Compounds classified as benzoxainones and biarylsulfonamides also slow desensitization rate of AMPA receptors\textsuperscript{89,117}. Benzamides (also called ampakines) have been used to determine the effects of AMPA potentiation on memory and learning. These compounds vary in their potentiating action, with some slowing desensitization and some slowing deactivation. One ampakine, 8-cyclopropyl-3-[2-(3-fluorophenyl)ethyl]-7H-[1,3]oxazino[6,5-g][1,2,3]benzotriazine-4,9-dione (S47445) has been studied for its use in a potential treatment in major depressive disorder in Alzheimer’s patients\textsuperscript{93}. This compound showed promise in animal tests but did not, however, show a statistically different result in human trials\textsuperscript{118}. Many of these compounds are not suitable for pharmacological use because of their tendency to promote seizures\textsuperscript{119}.

Developing selective kainate receptor potentiators has been much more challenging. Two benzothiadiazides, 4-cyclopropyl-3,4-dihydro-7-fluoro-2H-1,2,4-benzothiadiazine 1,1-dioxide (BPAM344) and 4-cyclopropyl-7-hydroxy-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide (BPAM591) reversibly slow desensitization of kainate receptors, but it also affects AMPA receptors in the same way\textsuperscript{120}. Some plant lectins, such as Concanavalin-A irreversibly potentiate kainate receptors, acting therefore as toxins. Finding a kainate receptor-specific potentiator is a worthy endeavor because such a potentiator can be used as a probe for better understand the biophysical property of kainate receptors and also as a drug candidate for potential treatment of diseases involving the hypoactivity of kainate receptors.
1.1.6 Isolation of aptamers using SELEX

Aptamers are single strands of DNA or RNA that take on unique tertiary structures and bind to their molecular targets, thereby affecting the function of their targets. Aptamers are selected or isolated from a large library against its target using a process known as systematic evolution of ligands by exponential enrichment (SELEX)\(^{121-122}\). SELEX involves enriching a pool of sequences that bind to a particular biological target of interest through iterative cycles, a process similar to evolution (Fig 1.7). The target can be a soluble protein, a membrane protein, a small molecule, or a cell. Non-binding aptamers are washed away while aptamers which bind to the target are retained and enriched due to reverse transcription (RT)/polymer chain reaction (PCR) (Fig 1.7). Post-SELEX, the enriched nucleic acid candidates are screened against the target for putative biological activity. This approach, discovered in 1990\(^{121-122}\), has been the seminal method of identifying aptamers. Later, different modifications of the original protocol have enabled the discovery of aptamers more quickly and more specifically. For example, the addition of negative SELEX to the protocol, as done in our research group, has improved SELEX against membrane-bound proteins by suppressing evolution of non-specific RNA aptamers\(^{123}\). Furthermore, the introduction of chemical agents which bind to the site of interest in the target increases the chance of obtaining aptamers that may be more specific and selective against the target. One unique feature of applying SELEX to discover aptamers is that such an approach does not depend on the availability of structural information of either the target or specific sites of interaction. This is advantageous for membrane proteins and for a target that does not currently have any high-resolution structures.
Fig 1.6 SELEX steps
The steps in the SELEX protocol include binding, elution, amplification, transcription
Aptamers are capable binding targets specifically and modulating their activities. They are an alternative class of molecular reagents which have many advantages over small molecules. For example, aptamers are inherently water soluble, whereas the majority of the small molecule compounds used as either antagonists or potentiators for both AMPA and kainate receptors have low and poor water solubility. The binding constants of aptamers are generally comparable with antibodies. In addition, aptamers are easier to synthesize in large quantities. Aptamers can be used as potential drug candidates, molecular probes, and diagnostic tools.

RNA aptamers must be chemically modified to resist nuclease-catalyzed degradation when used in vivo. Several different modifications can be applied to nucleotide bases of the RNA either post-SELEX or pre-SELEX\textsuperscript{124}. The more common modifications are those which replace the 2’-OH on one or more of the RNA bases (Fig 1.7). Today, aptamers have been developed as targeted therapeutics and in vitro diagnostics, among other applications\textsuperscript{125-127}. Some RNA aptamers currently approved as drugs include Macugen, which is a drug to inhibit VEGF in patients with age related macular degeneration\textsuperscript{127}, and Mipomersin (Kynamro) which is a modified RNA gapmer, a combination RNA and DNA molecule with DNA in the center and modified RNA on the wings\textsuperscript{128}. Macugen contains both 2’-Fluoro (2’-F) modification and 2’-Methoxy (2’-OMe) modification. For most aptamers, the 2’-F modification seems to be the most popular because the fluorine atom is small enough to render an aptamer that has approximately the same tertiary structure as the original aptamer. Larger modifiers such
as the 2'-OMe may drastically alter the tertiary structure thereby altering the biological activity of the aptamer\textsuperscript{126}.
Fig 1.7 2’-F Modification of RNA Nucleotides
Comparison of a standard RNA nucleotide with a 2’-Fluoro modified nucleotide. Note that the only modification is the replacement of the 2’-OH with a fluorine atom. A 2’-OMe nucleotide (not shown) has Methoxy group in place of the 2’-OH. These modifications result in increased resistance to ribonuclease degradation.
1.2 Materials and methods

1.2.1 cDNA plasmid preparation

Original cDNA plasmids encoding rat AMPA receptor subunits GluA1-3 were provided by the late Steve Heinemann from the Salk institute, and were cloned into a pDNA3.1 vector (Invitrogen). The cDNA plasmid encoding GluA4 (previously called GluRD) was provided by the late Peter Seeburg at the Max Planck Institute for Medical Research. The pDNA3.1 vector contains a simian virus (SV40) replication origin\textsuperscript{129}. Original kainate receptor plasmids GluK5 and GluK1 wild-type and mutant (pcDNA3-myc-GluK1-2b R896A, R897A, R900A, K901A) were provided by Dr. Geoffrey Swanson. Other kainate receptor subunits, i.e. wild-type GluK1 and GluK2 were provided by the late Steven Heinemann. The cDNA plasmids encoding NMDA receptors were generously provided by Dr. John Woodward. All the plasmids were propagated in competent DH5α E.Coli and were purified using a plasmid purification kit (Plasmid Maxi Kit, QIAGEN, Cat No. 12163).

1.2.2 Cell culturing and cell/receptor expression

For SELEX experiments, I used human embryonic kidney 293 (HEK-293) cells to express the receptors through transient transfection. The cells were cultured in 150 mm (17662.5 mm\textsuperscript{2}) tissue culture dishes (Falcon) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin. They were maintained at a 37 °C, 5% CO\textsubscript{2} incubator. For transfection, cells were passaged 24 hrs before and allowed to grow to 50-60% confluence. Calcium
phosphate transfection method was used, and in some cases green fluorescent protein (GFP) was co-transfected in separate dished (35 mm Petri dishes) to monitor transfection in both small and large dishes (Table 1). In other words, cells used for SELEX did not contain GFP. Large T antigen (TAg) was co-transfected to increase the receptor expression in the cells\textsuperscript{130}.

Transfected cells for SELEX were harvested after 48 hrs of growth. First, the cells were washed with cold phosphate buffered solution (PBS). Then ice cold PBS buffer containing 0.5 mM EDTA and 1 mM phenylmethanesulphonyl fluoride (PMSF) was added to the dish and the cells were scraped from the dish and collected. Cell membranes were homogenized using a pestle (Potter-Elveheym) in 50 mM tris acetate buffer (pH 7.4) containing 10 mM EDTA and 1 mM PMSF. The cell membrane was centrifuged at 17,000 g for 25 min to pellet. This same method was used with untransfected cells, to obtain plain cell membranes for negative selection. On average, a 150 mm dish of cells yielded approximately 40 mg of membranes. This method was used for all membrane preparations of the receptors used for SELEX.
<table>
<thead>
<tr>
<th>Subunit 1</th>
<th>Subunit 2</th>
<th>GFP</th>
<th>eGFP</th>
<th>TAg</th>
<th>Mole Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA1: 4 µg</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>N/A</td>
<td>0.4 µg</td>
<td>7:2:1</td>
</tr>
<tr>
<td>GluA2: 4 µg</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>N/A</td>
<td>0.4 µg</td>
<td>7:2:1</td>
</tr>
<tr>
<td>GluA3: 35 µg</td>
<td>N/A</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>0.4 µg</td>
<td>61:3:1</td>
</tr>
<tr>
<td>GluA4: 8 µg</td>
<td>N/A</td>
<td>1.6 µg</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>7:2:1</td>
</tr>
<tr>
<td>GluK1: 8 µg</td>
<td>N/A</td>
<td>1.6 µg</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>7:2:1</td>
</tr>
<tr>
<td>GluK2: 4 µg</td>
<td>N/A</td>
<td>0.8 µg</td>
<td>N/A</td>
<td>0.4 µg</td>
<td>7:2:1</td>
</tr>
<tr>
<td>N1a: 15 µg</td>
<td>N2a: 15 µg</td>
<td>N/A</td>
<td>1.5 µg</td>
<td>N/A</td>
<td>6:6:1</td>
</tr>
<tr>
<td>N1a:15 µg</td>
<td>N2b: 15 µg</td>
<td>N/A</td>
<td>1.5 µg</td>
<td>N/A</td>
<td>6:9:1</td>
</tr>
<tr>
<td>GluAQ</td>
<td>GluAR</td>
<td>N/A</td>
<td>0.4 µg</td>
<td>0.4 µg</td>
<td>7:28:1:1</td>
</tr>
</tbody>
</table>

Table 1.1 Plasmid Amounts for Transfection
Amounts (µg) and mole ratios for transfection of a 35 X 10 mm tissue culture dish of HEK-293 cells.
1.2.3 Preparation of RNA library

The RNA library for the first round of the SELEX experiment contained approximately $10^{14}$ sequences. With this variation in sequences, there were a large variety of structures which may contain unique AMPA and kainate receptor potentiators. The DNA library based on which the RNA library was transcribed was designed and constructed with two 25 nucleotide (nt) conserved regions flanking a random 50 nt region\textsuperscript{131}. The RNA library was prepared by \textit{in vitro} transcription of the DNA library and then purified using a denaturing polyacrylamide gel electrophoresis (PAGE) gel. Specifically, the gel was cast in a column using the prep cell system (Bio-Rad) (Fig. 1.8). The PAGE purified RNA was then precipitated in ethanol. Prior to the SELEX experiment, the RNA was dissolved in external buffer (150 mM NaCl, 3 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES pH 7.4).

1.2.4 Determining the concentration of receptor in the membrane

The quantification of receptors in the membrane or receptor density is important for optimization of SELEX and determination of the binding affinity of the aptamer that targets the receptor. To identify the amount of kainate receptors in the harvested cell membranes, $[^{3}\text{H}]$-kainate was used for quantifying both the AMPA and kainate receptor densities. Here it was assumed that one kainate molecule bound to one receptor subunit, totaling four molecules per receptor, since the receptor is a tetrameric complex.

Binding of $[^{3}\text{H}]$-kainate to the receptor was monitored by titrating various amounts of $[^{3}\text{H}]$-kainate mixed with unlabeled (cold) kainate against fixed amounts of membrane
containing receptors (in mg). To compete the binding of $[^3H]$-kainate to the binding site, cold kainate was used. Table 1.2 lists the amounts of each item used in the experiment.

An experiment was run in 1.5 mL Eppendorf tube (low nucleotide binding) (Eppendorf™, 022431021). The reactions were incubated for 1 hour at 22°C with shaking to bring the binding to equilibrium. Then samples were divided into 3 equal quantities and applied to 3 x 0.45 µm nylon filter tubes (VWR) which were pre-soaked with external buffer. These filter tubes were centrifuged for 2 min at 1,500 RPM on an Eppendorf centrifuge (5417R). The filter was washed 3 x 400 µL of external buffer and centrifuged each time. Finally, the filters were placed in liquid scintillation vials containing 5 mL of liquid scintillation cocktail (Ecoscint A, National Diagnostics) and were rocked at room temperature overnight. The samples were analyzed with a liquid scintillation counter (LS6500, Beckman Coulter). The data was plotted and analyzed using Origin (Origin 7).
<table>
<thead>
<tr>
<th>1 mM [³H]Kainate (µl)</th>
<th>Final Conc. [³H]Kainate (nM)</th>
<th>1×External Buffer (µl)</th>
<th>5 mg/ml Cell Pellet (µl)</th>
<th>10 mM Glutamate (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>300</td>
<td>60</td>
<td></td>
<td>150 (is 0.75 mg)</td>
</tr>
<tr>
<td>60</td>
<td>200</td>
<td>90</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>45</td>
<td>150</td>
<td>105</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>120</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>135</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>7.5</td>
<td>25</td>
<td>142.5</td>
<td></td>
<td>150</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>300</td>
<td>0</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>60</td>
<td>200</td>
<td>30</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>45</td>
<td>150</td>
<td>45</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>60</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>75</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>7.5</td>
<td>25</td>
<td>82.5</td>
<td>150</td>
<td>60</td>
</tr>
</tbody>
</table>

**Table 1.2 Concentrations of Materials for [³H] Binding Assay**
The table above shows the amounts of hot and cold RNA as well as the amounts of membrane containing the GluK2 receptor.
1.2.5 SELEX

The purpose of my SELEX experiment was to find aptamers for AMPA and kainate receptors. I varied the amounts of RNA library, membrane containing the receptor, and volumes of the reactions, using a large amount at the beginning of the experiment to optimize the chances of finding aptamers. For the initial 2-3 rounds, the number of sequences should be maximized. Because of this, I ran the first three rounds against both GluA1 and GluA3 as the targets. After the third round, the RNA pool was split into three portions and the rest of the rounds were run in three different parts: GluA1, GluA3 at the open channel conformation, and GluA3 at the closed channel conformation. The open channel conformation was achieved by adding kainate to the RNA pool, which prevents the receptors from desensitizing. Table 1.3 shows the total amounts of each reagent needed for the entire SELEX experiment against both receptors and the open-channel form of GluA3. A total of 8 SELEX cycles were performed for each receptor.

I planned to run 8 rounds of SELEX. To perform the first round of SELEX, which was divided into 8 experiments, an RNA library was dissolved in 100 µL external buffer to a final concentration of 375 ng/µL. Then it was heated up to 70°C for 10 min and allowed to cool at room temperature for 10 min. The membrane containing the receptor was prepared by suspending 20 mg of membrane in 400 µL of external buffer with SuperRase. In added to the reaction to remove any DNA which might interfere in the binding. The RNA pool was mixed with the membrane and rotated from room temperature to 37°C and back 2 times as follows: 22°C to start, then 37°C for 40 min, then back to 22°C for 10 min, then 37°C for 20 min, then 22°C for 5 min. This
thermocycle allows the RNA library to compete with the receptor at two temperatures, since some of the binding may occur at higher temperatures.

In order to separate bound RNA from unbound, presumably non-specific RNA, the reaction was filtered in a vacuum filtration apparatus. An external buffer-soaked 0.45 µm nitro-cellulose filter paper (Millipore HAWP02500) was used to capture the membrane with bound RNA. Vacuum was applied to pull the unbound RNA through the filter. Then the filter paper containing the membrane and the bound RNA was washed with 16 mL of extracellular buffer under vacuum. The filter paper was removed from the vacuum device and placed in an Eppendorf tube containing elution buffer (8 M urea in nuclease-free deionized water). Starting at round 4, the experiment was divided into three parts, with each part using a different receptor. Also at this point, the elution buffer was supplemented with 10 mM CX546 and 1 mM each of IDRA-21, PEPA, and Aniracetam for eluting potentiating aptamers bound to the same sites or the sites that are mutually exclusive. A filter was incubated at 75°C for 5 min and then vortexed vigorously for 2 min. Then the bound RNAs were extracted from the solution using phenol chloroform treatment and ethanol precipitation with 0.3 M ammonium acetate (at final concentration).

The eluted RNA pool was first reverse transcribed. For the reverse transcription reaction, the eluted RNA pool was dissolved in water and mixed with a 22 nt primer (Primer 22) (5’-ACCGAGTCCAGAAGCTTGTAGT-3’) at a final concentration of 2.5 µM. The solution was incubated at 75°C for 10 min followed by 52°C for 5 min. Then 5X first strand synthesis buffer (final concentration 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂) was added along with 0.01 M dithiothreitol (DTT), 0.625 mM
mixture of all four dNTP’s, 400 units of SuperScript III reverse transcriptase (ThermoFisher, Cat no. 18080093), and 20 units of SuperAse.In (ThermoFisher Cat no. AM2696). The combined solution was incubated in a 52°C water bath for 60 min. Then the solution was removed from the water bath and 5 units of RNase H was added to the reaction, followed by incubation at 37°C for 30 min. The complementary DNA formed from the reverse transcription was amplified in PCR with the following components: PCR buffer (20 mM Tris HCl pH 8.4), 50 mM KCl, 1 mM MgCl₂, 0.75 mM dNTP mixture containing all four dNTP’s, 0.25 µM Primer 40 (5’-GTAATACGACTCACTATAGGGAGAATTCAACTGCCATCTA-3’), 0.125 µM Primer 22, and 20 units of Taq DNA polymerase. After thoroughly mixing, the sample was aliquotted into 100 µL volumes in PCR tubes and run on a thermocycler (Applied Biosystems 2720 Thermal Cycler) with the following settings: 94°C denaturation for one min, 60°C annealing for one min, 72°C for 1.5 min, and 4°C hold. The cycle number was kept as low as possible by running a pilot PCR with a small amount of the mixture to start with. Usually 8 cycles was adequate to produce a sufficiently concentrated DNA pool for transcription. The DNA was visualized in a 10% native polyacrylamide gel using DNA ladder (PCR marker, NEB Cat. # N3234S) to ensure the RNA pool with the correct size was amplified. The DNA pool was purified using phenol/chloroform (pH 7.5) and then washed 3 times and concentrated in water with a spin filter (Amicon 10 kDa MW cutoff).

The corresponding RNA library was used for the next round of SELEX. The library was prepared by transcribing a portion of the DNA amplified above. Transcription was performed by mixing a portion of the DNA pool in HEPES buffer (pH
7.5) (final concentrations 0.25 M HEPES, 0.125 M NaOH, 0.01 M spermidine, 0.2 M DTT, 0.125 M MgCl₂) with 7.5 mM NTP mixture containing all four nucleotides, 40 units of T7 RNA polymerase, 20 units of yeast inorganic pyrophosphatase (YIPP) into a final volume of 20 µL. The reactions were mixed thoroughly and incubated at 37°C for 8 hrs. The resulting RNA pool was purified by first adding Turbo DNase (2 units) incubated at 37°C for 15 min to remove unreacted DNA. Then the RNA was purified using phenol chloroform extraction and precipitated in ethanol. The RNA was dissolved in 100 µL of water, quantified using absorbance at 260 nm, and visualized by running on a 10% urea PAGE gel. The library was then diluted and aliquotted for the next round of SELEX. Quantities used in each round of a typical SELEX experiment are shown in Table 1.3.

A negative selection was run after cycle 3, 4, and 8 to suppress any non-specific RNA. For this process, I layered the pool from a positive SELEX round over the membranes containing all of the other subunits of AMPA and NMDA including GluA2, GluA4, GluK1, GluK2, GluN1/2a and GluN1/2b. The flow-through was collected from the filtration process, and was then reverse transcribed and amplified by PCR before the DNA pool was transcribed into the RNA library for the next SELEX cycle. This negative selection was supposed to filter off any RNA molecules that preferentially bound to any target other than GluA1 and GluA3; GluA1 and GluA3 were the target of my SELEX.
<table>
<thead>
<tr>
<th>Round Number</th>
<th>RNA Pool (µg)</th>
<th>Membrane Lipids (mg)</th>
<th>Binding Reaction Volume (µl)</th>
<th>RT Volume (µl)</th>
<th>PCR Volume (µl)</th>
<th>Transcription Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>600</td>
<td>160</td>
<td>4000</td>
<td>400</td>
<td>10000</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>80</td>
<td>2000</td>
<td>200</td>
<td>5000</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>40</td>
<td>1000</td>
<td>40</td>
<td>2000</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>20</td>
<td>500</td>
<td>40</td>
<td>2000</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>20</td>
<td>500</td>
<td>40</td>
<td>2000</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>20</td>
<td>500</td>
<td>40</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>20</td>
<td>500</td>
<td>40</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>20</td>
<td>500</td>
<td>20</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>20</td>
<td>500</td>
<td>20</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>20</td>
<td>500</td>
<td>20</td>
<td>500</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table 1.3 Typical SELEX experiment**

Example of general amounts of material required in each typical round of a SELEX experiment. The final concentrations of the membrane-bound receptor in the binding mixture for each round is approximately 40 nM as determined by the [3H]-kainate binding. For the negative SELEX rounds, 20 mg HEK-293 cell membrane containing receptors except for the target receptor was used.
1.2.6 Cloning and ligation of the final pool

When SELEX was completed, the sequences of the RNAs which had become abundant in the final pool needed to be known. Although other SELEX experiments in our lab had been analyzed with Next Generation sequencing to achieve a better picture of which sequences were in the final pool, this method of analysis is expensive, and the amount of data is extravagant. The traditional method of Sanger sequencing gives a quicker, more cost effective method of determining the most abundant sequences and was the first choice in analyzing my pools for this SELEX.

For the Sanger sequencing, the PCR product from the round 9 of each SELEX experiment were ligated into the Promega pGEM®-T easy vector system at a 1:1 molar ratio with 2 ng of the DNA pool to 50 ng of the vector. In the reaction, 3 units of T4 DNA Ligase was added with Promega Rapid Ligation Buffer (final concentration 30 mM tris HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP and 5% PEG). The reaction mixture was incubated at 4°C for 8 hrs. This reaction was then transformed into competent DH5α E.Coli.

To transform the bacteria with the ligated DNA pool, the pool was mixed with 10 µL of competent DH5α E.Coli and incubated on ice for 20 min. Then the mixture was heat shocked at 42°C for 20 seconds and returned to ice for 2 min. Following this incubation, super optimal broth with catabolite repression (SOC medium) (0.5% yeast extract, 2% tryptone, 10 M NaCl, 2.5 mM KCl, 10 M MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose) was added to the culture and was incubated at 37°C with shaking for 1 hour. The culture was plated on agar plates containing ampicillin and was incubated at
37°C for 8-10 hrs. Individual colonies were selected, and grown in 3 mL of Luria broth (LB) containing 1 mM ampicillin at 37°C with shaking at 250 RPM for 8-10 hrs. The plasmids were purified from the cultures using Wizard® plus SV mini preps DNA purification kit (Promega).

The Wizard kit is a standard way of isolating plasmids from a bacterial culture using silica columns to bind plasmid DNA. The DNA was eluted from the silica column with water and then a small amount of the sample was cut using a restriction digestion to verify that the plasmid was the expected size. Each purified plasmid contained one sequence from the final pool, which was determined by an outside sequencing facility.

1.2.7 RNA purification and preparation

Selected RNAs must be meticulously purified before they can be tested for biological activity. Purification was needed to separate any other reagents left from the reaction, including nucleotides. I also wanted to make sure that there was only one size of RNA in the sample. In a standard in vitro transcription, some abortive fragments are common. These fragments needed to be removed from the sample of desired size. To remove the DNA template from the transcription product, I used Turbo DNase (Ambion, Cat. No. AM2238, AM2239) and incubated the reaction at 37°C for 15 min. Other reagents were removed through phenol chloroform extraction.

To remove RNA fragments and nucleotides from a transcription reaction and obtain a pure, large quantity RNA sample for functional assay, I used a Bio-Rad Prep cell (Model 491). This was a urea PAGE with continuous flow. This device allowed me to
separate RNA aptamers differing from even few nucleotides in length without staining/de-staining the gel\textsuperscript{133}. The urea polyacrylamide gel mix contained 10% Protogel (National Diagnostics 37:5:1 acrylamide: methylene bisacrylamide solution), 8 M urea, and 1 X TBE. The solution was mixed well and filtered in 0.45 µm filter. To prepare the gel for the Prep Cell, polymerizing agents tetramethylethyldiamine (TEMED) and ammonium persulfate were added and the gel was carefully poured into the column space. Water saturated butanol was applied to the gel surface to remove bubbles and produce a level surface. The gel was allowed to solidify for 12 hrs before use.

The Prep Cell was run at 200 V for 8 hrs at a 1 mL/min flow rate and the RNA elution was monitored using a UV detector set at 254 nm. The pooled fractions were combined and concentrated in an Amicon filtration tube (3 kDa MW cutoff). Then the sample was washed in water 3 times and buffer exchanged to extracellular buffer. The concentration of the RNA aptamers in the purified solution was determined by a Nanodrop 100 spectrophotometer.

1.2.8 Whole cell recording

Putative aptamers selected from the SELEX experiment were tested for their biological activities using whole-cell recording with HEK-293 cells containing the receptors of the target. If an RNA was active against the target of selection, its potency was determined as well using the same technique. HEK-293 cells were transiently transfected with the receptor to be studied, and co-transfected with GFP to identify transfected cells for whole-cell recording. The transfected cells were used for recording 48 hrs after transfection. For the whole-cell assay, glutamate was
used as the agonist with a 3 mM concentration for approximately 96% open channels and 0.1 mM concentration for approximately 96% closed channels for GluA2Q\textsubscript{flip} AMPA receptor channels\textsuperscript{132}. For GluA1 and GluK3, 3 mM concentration and 40-50 µM glutamate concentrations were used for assaying the potency of an aptamer for the open and closed channel forms\textsuperscript{25,134}. Glutamate was applied to the cell with a U-tube flow device having an aperture of 150 µm. The flow of the glutamate and aptamer solution were driven by two peristaltic pumps, while the resulting current with and without the RNA sample was recorded with an Axopatch-200B amplifier. The cutoff frequency was 2-20 kHz with a 4-pole low-pass Bessel filter. A Digidata 1322A digitizer was used to digitize the resting whole cell current at a 5-50 kHz sampling frequency. An intercellular buffer (110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl\textsubscript{2}, 5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.4 with NaOH)) flowed through the pipet. An extracellular solution containing 150 mM NaCl, 3 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES (pH 7.4 adjusted with NaOH) was also used in the cell culture dish. The recording electrodes were fashioned from glass capillaries (World Precision Instruments, Sarasota, FL). These electrodes had ~3 MΩ of resistance. The software used for the data acquisition was Clampfit 10. All cells were voltage-clamped at 60 mV with pH 7.4 at 25°C.

The whole-cell current amplitude in the presence and absence of the aptamer, \(A(P)/A\) was calculated as a function of the concentration of the aptamer. This value was used to show that the aptamer potentiated the receptor. The reciprocal of this value, \(A/A(I)\) was used to denote inhibition of the receptor. This value was used to calculate the apparent potentiation constant \(K_p\) and inhibition constant, \(K_I\). First, the \(A/A(I)\) value was calculated for each aptamer concentration using the following equations:
\[
\frac{A(P)}{A} = \frac{1}{\frac{A}{A(I)}} \quad \text{(Equation 1)}
\]

\[
\frac{A}{A(I)} = 1 + I \frac{(AL_2)}{K_{I,app}}
\]

\[
\left(\bar{AL}_2\right) = \frac{\bar{AL}_2}{A + AL + AL_2 + AL_2} = \frac{L^2}{L^2(1 + \Phi) + 2K_1L\Phi + K_2^2\Phi}
\]

Equation 1 assumes that the inhibitor bound to only one site on the receptor \(^{132,135}\). The open-channel conformation is represented by \((\bar{AL}_2)\) with \(n=2\) (note that the \(n=2\) here was arbitrary and was used to show that the \(K_i\) depends on concentration of the ligand). \(I\) represents to concentration of the inhibiting aptamer while \(L\) represents the ligand concentration and \(\Phi\) represents the equilibrium constant of channel opening. Then the potentiation constant was calculated by the reciprocal of the \(A/A(I)\) value.

1.2.9 Treatment of a putative aptamer for functional assay

An RNA sample chosen for whole-cell recording was subject to a denaturing and refolding cycle to ensure proper and uniform folding of the RNA. For this, the RNA was heated at 95°C for 5 min, then cooled to room temperature. A \(\Psi\)-shaped device (psi tube) was used to flow the aptamer into the Petri dish during the whole-cell recording assay. This was the modified U-tube device. The center tubing of this \(\Psi\)-shaped device was filled with the RNA solution, while the glutamate solution with and without the same RNA was delivered through the U-tube. The use of the \(\Psi\)-tube was to preincubate pure RNA with the receptor prior to the exposure of glutamate to the receptor.
1.2.10 RNA sequence truncation

Once the aptamer activity was determined, I investigated the minimal functional sequence in the RNA molecule. The full length aptamers were approximately 100 nt in length. To find a minimal and functional sequence, a series of truncations and testing was performed. To design shorter aptamers, Mfold, an RNA secondary structure prediction program, was used to identify the most stable RNA secondary structure(s) of the full-length aptamer based on thermodynamic constraints and statistical analysis\textsuperscript{136}. Truncation designing was a trial and error process, so some of the truncations have no significant activity, while others were found to have different activity profiles.

For truncation approximations, stem-loop structures were generally preserved. The preparation of the truncated aptamers was done by first determining the sequence and primers needed to amplify the DNA version. Sequences of templates and primers used to prepare the aptamers detailed in this paper can be found in the supplemental section. These oligoes were purchased from IDT (Integrated Data Technologies, Coralville Iowa). The sequence was amplified in PCR, purified, and then transcribed. The RNA was purified according to the protocol described above. The biological activity of the resulting shorter RNA molecules was assayed using whole-cell recording technique as described earlier.

1.2.11 Homologous Binding Assay

The binding affinity for the target receptor is an important property of an aptamer. To determine the binding affinity, I measured the binding constant of the truncated, potentiating
aptamer against its target, using radioactive aptamer. Specifically, I first prepared radio-labeled aptamer with $^{32}$P. The labelling was performed by a 5’end labelling using T4 polynucleotide kinase (PNK) and calf intestinal phosphatase (CIP) along with dephosphorylation buffer (0.5 M Tris (pH 8.5), 1 mM EDTA (pH 8)) and kinase buffer (0.5 M Tris-HCl (pH 7.5), 0.1 MgCl$_2$, 0.05 M DTT). Following this reaction, the unused nucleotides were washed away using NucAway spin columns (Invitrogen AM10070). The $^{32}$P-labeled aptamer was then mixed with different concentrations of unlabeled or “cold” aptamer of the same sequence to compete for the binding of the hot aptamer, thus allowing me to approximate the binding constant with the decrease in radioactive signal.

Also solutions of external buffer with different concentrations of unlabeled aptamer ranging from 0.1 nM to 5000 nM were prepared, and these were combined with the membrane fragments containing the receptor of interest. The membrane and aptamer binding reactions were incubated at 4°C for approximately 4 hrs to bring the reactions to equilibrium. Then the reactions were each divided into three 100 µL portions and separated by centrifugation (1500 g for 5 min) in a pre-soaked 0.45 µm nylon spin filter (VWR Cat No. 82031-362). The membranes were washed twice with 350 µL of ice cold external buffer and centrifuged at 1500 g for 5 min after each wash. For the last wash, the filters were centrifuged for 5 min so that the filters were completely dry. Then the filters were placed in a liquid scintillation vial containing 5 mL of liquid scintillation cocktail (Ecoscint A, National Diagnostics). The vials were placed on a shaker for 4 hrs. The radioactivity was detected and measured using a scintillation counter (LS6500, Beckman Coulter), with each reading determined over 2 min. The data was analyzed in Excel and plotted in Origin (Origin7) with a non-linear function. I assumed a one-site binding
model, so that the $K_d$ value was determined by the fitting of the points to the following equation:\[ (Equation 2) \]

$$Y = \frac{B_{\text{max}}[\text{hot}]}{[\text{hot}]+[\text{cold}]+K_d} + NSB$$

The [hot] and [cold] values were the concentrations of labeled and unlabeled aptamers; NSB represented any non-specific binding, and $B_{\text{max}}$ was the maximal number of binding sites.

1.2.12 Statistical Data Analysis

The whole-cell recording and radioligand binding experiments were done in triplicate. Every data point was the average of 3 points. All uncertainties shown are standard deviations from the mean. The significance of potentiation was calculated by one-sample two-tailed Student t-test with the assumption that $H_0: \mu = \mu_0 = 1$, with 1 being the value of no potentiation. All graphs, including the non-linear graph to determine the competitive binding constant of the aptamer were done in Origin 7. Also one–way ANOVA and post hoc TUKEY HSD test was performed using software package R-studio (Version 1.0.136) (Team 2013; Team 2015)

1.3 Results

1.3.1 Design of SELEX experiment: target, elution pressure, and negative selection

In order to find RNA aptamers capable of potentiating AMPA and kainate receptors, I used SELEX technique against GluA1Q$_{\text{flip}}$ receptor. Given that the AMPA and Kainate receptors are
similar in sequence and structure\textsuperscript{1}, there was a chance that the aptamer selected would be active against both receptor types. Given the size of a potential RNA aptamer in comparison with those of small molecule potentiators, it may be expected that part of the aptamer may bind to AMPA and part may bind to kainate receptor as well\textsuperscript{138}.

GluA1 homomers have mainly been found in early development, and are thought to be important to the health of developing neurons and the repair of injured neurons\textsuperscript{139}. It has been established that the phosphorylation of GluA1 is important in promoting both long term potentiation and long term depression\textsuperscript{49}. Furthermore, GluA1 can form homomeric channels. GluA1/GluA2 and GluA2/GluA3 heteromeric receptors are the primary types of AMPA receptors in the pyramidal neurons in the hippocampus at any age\textsuperscript{140}. For these reasons, I chose to screen the RNA library against GluA1Q\textsuperscript{flip}.

Because benzamides, benzothiadiazides, biaryl propylsulfonamides, and 3-trifluoromethylpyrazoles are known AMPA receptor potentiators and are available\textsuperscript{141}, I decided to use PEPA, IDRA-21, aniracetam and CX-546 as chemical pressure to elute all the RNA molecules that were bound to their sites or the sites that were mutually exclusive to the binding of these small molecule compounds. Selection of these chemical potentiators include a compound that slows the rate of desensitization and others that are believed to decrease the rate of the channel closing. Both of these mechanisms can lead to prolonging the conductance of the channel or enhancing channel activities.

From an RNA library, SELEX allows selective enrichment of RNA molecules against the target of interest through iterations of operation (Fig. 1.6) or \textit{in vitro} evolution\textsuperscript{121-122}. With the method developed in our lab, I mixed or exposed a pool or library of RNA that contained approximately $10^{14}$ sequences or the equivalent RNA folds to GluA1 receptors as the target of
selection. These receptors were transiently transfected in HEK-293 cells and harvested with the membrane so that the receptor remained intact within the cell membrane and therefore were functional\textsuperscript{132}. In other words, it was the membrane-bound receptors that were chosen for SELEX. It should be noted that due to the presence of membrane, the selection of nonspecific RNAs or those that recognize lipids and other native proteins in HEK-293 cells, but not the target of my interest was more complicated than the use of detergent-solubilized target; however, membrane-bound receptors were thought to be as native as possible to ensure that any selected RNA aptamer could bind to and act on native AMPA and kainate receptors \textit{in vivo}. In order to minimize the evolution or enrichment of non-specific RNAs that could bind to anything other than the target of my interest, I included negative selections (see the Method section) after rounds 3 and 5. In these negative selections, the binding procedure was carried out the same way as before, but HEK-293 cell membrane fragments that expressed GluA2, 3, 4, GluN1 and N2a was used. In other words, a negative SELEX round was expected to suppress the selective enrichment of any other targets, including those close to GluA1, so that the evolution was designed to favor the selective enrichment of GluA1-bound RNA molecules in the positive SELEX cycles. The RNA which eluted from this procedure was collected in one cycle and used for the construction of the biased RNA library for the next cycle.

1.3.2 Identifying the enriched RNA sequences and functional assay of the selected aptamers

After running 9 selection cycles, I terminated the SELEX operation, and executed DNA cloning of the libraries in order to identify those enriched sequences. Specifically, I obtained 72 clones from the RNA library from cycle 9 and sequenced them using traditional Sanger sequencing. The sequences from my SELEX operation were compared with other biologically
active sequences found by our group previously and also from the next generation sequencing data, collected from various SELEX experiments but separate from mine. Two new sequences, termed AL11 and AL3, were found in my sequencing data, as well as two other sequences which were previously found in our lab through other SELEX experiments (Fig 1.9). In the same figure I also listed the copy number for each of the selected DNA sequences. Clearly, a higher copy number of the DNA sequence in the figure reflected a more populous or a more highly enriched RNA sequence in the final selection cycles of the SELEX operation targeting the GluA1 receptor.

In Fig 1.9A, the RNA aptamers with the sequences labeled as M1040 and M1008 were two aptamers previously identified by our lab, and they showed up in my SELEX operation as well. These two RNA molecules have been known as inhibitory RNA aptamers. Therefore, these are excluded from my further pursuit of study, since I was interested in isolating potentiating aptamers. In analyzing the two other remaining RNA sequences, I noticed that the AL3 RNA molecule from my SELEX contains two regions of consensus sequence with CZ RNA aptamer – this is an aptamer that potentiates GluA2 and the RNA has been previously isolated in our lab. I decided to further carry out a functional assay against GluA1 receptor because GluA1 was the target for the SELEX.

Whole-cell recording assay was conducted with HEK-293 cells expressing GluA1 flip homomeric channels. Based on the ratio of the amplitude of the whole-cell current response to glutamate with and without aptamer \( \frac{A(P)}{A} \), I found that the aptamer AL3 potentiated the closed-channel form of the GluA1 channel, but not the open-channel form (Fig 1.9B). As shown in a pair of representative whole-cell traces, it can be seen that the AL3 aptamer increased the
whole-cell current response to glutamate, indicating that the aptamer was indeed a potentiating one. I should note that AL3 did not affect the rate of channel desensitization (Fig. 1.9B and C).

In addition, RNA molecule labeled as AL11 was assayed, but it was found to be an inhibitor (the amplitude in the presence of this aptamer was smaller than the control – see Fig 1.9C). In assaying the selected and enriched RNA molecules, I used an RNA clone which I called AL56 as a non-functional control. The copy number of AL56 was one and it was not listed as “enriched sequences” in Fig. 1.9A. All other single sequence RNA molecules out of these 70 clones were not listed either.
(A) Four RNA sequences enriched in the SELEX against GluA1. The names and frequencies of the sequences are listed below. FN1040 and FN1008 are inhibitors, which have been found previously in the lab. Novel sequences begin with “AL”. AL3 was a single copy but was found to have sequence homology with another potentiator found previously. (B) Whole cell traces of GluA4 with AL3 show that the amplitude increases while the steady state, or recovery of the receptor is not affected. (C) $A_p/A$ plot of an inhibiting sequence (AL11), a non-functional sequence (AL56), and a potentiating sequence (AL3) which is indicated by an increase in current amplitude.
1.3.3 Truncation of AL3 aptamer to generate a minimal length but functional RNA

To understand better the most essential sequence of the AL3 RNA aptamer, I truncated the AL3 aptamer. The idea was to preserve the most essential consensus sequence with CZ aptamer while removing the portion of the RNA that lacked the functionality. Specifically, I removed the lower portion of the aptamer (Fig 1.10). The resulting aptamer, termed AL3-1, was tested against GluA1, but found that it no longer potentiated GluA1. Interestingly, however, functional assay against all other subunits of AMPA and kainate receptors revealed that the truncated aptamer was turned into a kainate receptor-specific potentiating aptamer. Other truncations were attempted based on the shorter AL3-1 sequence, by systematically removing loops and shortening the long stem (Fig 1.10) but those truncations all yielded inactive aptamers (Fig 1.10). Consequently, AL3-1 represented the minimal length, but functional aptamer.

1.3.4 Activity of AL3 and AL3-1 against iGluR subunits

Since the full length aptamer was found to potentiate GluA1Qflip, the aptamer was tested against other subunits of glutamate receptors. By using whole-cell recording, the potentiating ability of the aptamer was found to potentiate the open-channel form of GluA1, 2Q, 3, and 4 homomeric AMPA channels. The most potent target was GluA4. The aptamer also potentiated GluK1 and K2 strongly but only in the closed-channel form (Fig 1.11). No activity was seen with NMDA receptor subunits for this aptamer.

On the other hand, the truncated aptamer, or AL3-1, was tested against GluA1, but it lost biological activity against this subunit. A full activity profile was run on this aptamer which was
found to be selective to kainate receptors only (Fig 1.11). Furthermore, this shortened aptamer potentiated GluK1 and GluK2 stronger than the full-length aptamer, or AL3.
Fig 1.10 Truncation of AL3

(A) The AL3 sequence was truncated to form an aptamer which was kainate receptor specific. This kainate specific aptamer, AL3-1, was further truncated to check for the shortest functional sequence. Colors represent portions of the sequence that were removed for each new aptamer. Aptamers were named according to the number of nucleotides in each. It was determined that AL3-1 is the shortest functional aptamer. (B) The sequences were all tested against GluK2 at 2 µM concentration. Both AL3 and AL3-1 potentiate GluK2 at this concentration. None of the truncated aptamers of AL3-1 potentiate. Two tailed student t-test was done to determine potentiating ability: For AL3 and AL3-1, p>0.05 while for all other cuts: p<0.05.
Fig 1.11 Subunit selectivity of AL3 and AL3-1
Whole cell current recording was used to determine the activity of each of the aptamers. The $A(P)/A$ ratio was measured when 2 µM of the aptamer was applied. Significance of potentiation was determined using a one-way ANOVA with post hoc Tukey HSD test analysis comparing closed-channel AMPA and kainate against $A(P)/A = 1$ indicating no potentiation (See Supplementary Information S 1.3, 1.4). This analysis resulted in $p > 0.5$ for the columns indicated by an asterisk. Black colored columns represent closed channel, while white colored columns represent open channel. All results are based on at least three measurements and the error bars represent standard deviation from the mean of these measurements. (A) Activity profile of AL3 shows potentiation of the closed channel only. (B) The activity profile of AL3-1 shows a stronger potentiation of GluK1 and GluK2 with a loss of activity on AMPA receptors. Neither aptamer potentiated NMDA receptors.
1.3.5 Homologous competition assay

Since the AL3-1 aptamer potentiated GluK2 receptor to the greatest extent, seen in the whole-cell recording assay, it was expected the aptamer would have to bind to the receptor. Therefore, I determined the binding affinity of the AL3-1 aptamer for its target GluK2 in a homologous competition binding experiment. To do this, I first labeled the 5’ end using γ-\(^{32}\)P-ATP and used this hot aptamer, together with the cold aptamer for the assay (see more detail in the Methods section). The decrease in the radioactivity while the cold aptamer concentration increase in this assay showed that the aptamer did bind, and as expected, the binding was dose-dependent, as determined by the radioactivity (Fig 1.12). As a control, I ran a binding the aptamer with a plain-cell membrane or the membrane that contained no GluK2 receptors. As expected, the control showed no detectable radioactivity in this experiment. The binding affinity of the aptamer was further estimated to be 461 ± 4 pM. This value is consistent with the dissociation constants of other aptamers previously found in our lab\textsuperscript{135, 138, 143}.
**Fig 1.12 Homologous binding study for AL3-1**

(A) A homologous binding experiment was run to determine the dissociation constant of AL3-1. Triplicate experiments were run for each point. The original radioactivity was normalized to 100% using the first three data points. The binding constant, $K_d$ was calculated using Equation 2 “Experimental procedures”. The binding constant was calculated to be $461 \pm 4$ pm.
1.4 Discussion

In Chapter 1, I have shown the discovery of two novel RNA aptamers (AL3 and AL3-1) through SELEX, using potentiating compounds for isolating potential RNA aptamers from a library that contains $\sim10^{14}$ RNA molecules. The full-length RNA that was derived from the original library is a 100-nt RNA, and it potentiated not only GluA1, but the rest of the AMPA subunits, namely, GluA2Q, 3 and 4; the potentiation of the GluA3 and 4 are even slightly more potent. Interestingly, the full length aptamer also potentiates GluK1 and GluK2. Yet AL3 does not potentiate NMDA receptors. Surprisingly, the shorter aptamer, AL3-1, which was derived from its parent RNA aptamer AL3, turn out to be a selective potentiator of GluK1 and GluK2. In other words, the truncation of this aptamer leads to one that has lost part of its sequence responsible for potentiating AMPA receptors. Currently, a very limited number of small-molecule compounds have been synthesized to potentiate AMPA receptors, as compared with the small-molecule antagonists for AMPA receptors. For kainate receptors, to our knowledge, a potentiating agent or selective kainate receptor potentiating agent has never been reported. Thus my research has yielded a significant outcome.

From the SELEX experiment, four sequences were identified (Fig 1.9). Among them, AL3 was the only one found to act as a potentiating aptamer. It is interesting to note that the copy number in a limited DNA cloning and sequencing procedure was the lowest (Fig. 1.9). It should be noted, however, the DNA sequence of AL3 has been identified as well with higher appearance rate in the DNA sequencing pools from next generation sequencing. The other three sequences correspond to either RNA aptamers of inhibitory type to AMPA receptors, found earlier by the lab (i.e., M1008 and M1040) or RNA (i.e., AL11) from the current study, but AL11 also turns out to be an inhibitor, rather than a potentiator. In analyzing the sequence of
AL3, I compared with the sequences from a variety of DNA libraries generated from next
generation sequencing. I noticed that the AL3 sequence shares a region of consensus sequence
with another AMPA potentiating aptamer found in our group (CZ aptamer)\textsuperscript{142}. When AL3 was
tested against AMPA receptors, AL3 was indeed shown to be capable of potentiating AMPA
receptors (Fig. 1.11). From these results, it is conceivable that isolating RNA aptamers is more
difficult than isolating inhibitors. This may be attributed to the possibility that site(s) of
potentiation on AMPA receptors is much limited as compared with the number of inhibitory
sites. In fact, the number of small-molecule AMPA receptor inhibitors reported in literature is
far larger than the number of small-molecule potentiators. Obviously, future studies are needed
to better understand the most essential RNA sequence and its possible motif in the AL3 or even
its shorter version, AL3-1, and its relationship with another RNA potentiator aptamer (CZ)\textsuperscript{142}.
CZ aptamer, however, forms a hydrogel, which makes it challenging to quantitatively
characterize its biophysical and pharmacological properties.

There are some interesting findings from my study about the potentiating aptamer. The
potentiating aptamer, AL3 and its shorter aptamer, AL3-1 are shown to potentiate not only
AMPA receptors but also kainate receptors as well (Fig. 1.11); neither, however, potentiate
NMDA receptors. Given the fact that the GluA1 AMPA receptor subunit was used for SELEX
as the target of RNA binding and evolution, and the fact that no kainate receptor has ever been
exposed to the RNA library for binding and selection, my result indicates the site(s) of the AL3
binding to the AMPA receptor is most likely similar to the site(s) of the binding on the kainate
receptors. As such, these potentiating aptamers may also share similar biophysical properties on
both AMPA and kainate receptors. In fact, AL3 and AL3-1 both potentiate AMPA receptors and
kainate receptors by acting only on the closed-channel conformations (Fig. 1.11). Furthermore,
by examining the time-course of the whole-cell current traces in the presence and absence of a potentiating aptamer, I found that an aptamer has not affected the rate of channel desensitization, suggesting an aptamer potentiates both an AMPA and kainate receptor channel not by slowing the channel desensitization rate but by increasing the conductance of the channel. Increasing the conductance of the channel can be achieved effectively by widening the channel conductance, or slowing down the rate of channel closing\textsuperscript{144}, or increasing the channel opening probability. The last scenario is least likely because our lab has previously determined that the channel-opening probability of both AMPA and kainate receptors is close to unity\textsuperscript{25,134}. Furthermore, the fact that AL3 and AL3-1 both only potentiate the closed-channel form, or conformation of any AMPA or kainate receptor, but is ineffective on the open-channel form, is by itself a piece of evidence supporting the mechanism of action by binding to a regulatory site (potentiating) or a noncompetitive site of action. It should be noted as well that by definition, a potentiating agent is mechanistically consistent with being a noncompetitive modulator. Such a modulator cannot be a competitive one. Indeed, compounds with different structures than glutamate or kainate have been found, such as ATPA and 5-iodowillardiine\textsuperscript{106}. However, these compounds are agonists, since they compete with the binding of either kainate or glutamate. Each is able to elicit receptor response. Neither of my aptamers, i.e., AL3 or AL3-1, has shown the ability to be able to evoke receptor response on its own. This absence of the agonist activity has been shown by incubating and applying the aptamer only. No receptor response has ever been detected.

Examination of the sequences of AL3 and its shorter, but functional version, i.e., AL3-1, suggests some interesting features of the RNA structure and function relationship. The shorter aptamer, AL3-1, was created by removing the lower portion of the full-length aptamer. The shorter structure is approximately the top two thirds of the full length aptamer. This portion of
the aptamers shares the sequence with the CZ potentiating aptamer\textsuperscript{142}. When the bottom third of the larger aptamer was removed, it appears that the potentiating activity toward AMPA receptors was lost. This surprising result is, on one hand, good for the discovery of a kainate receptor selective aptamer; on the other hand, my result also led to a hypothesis that an aptamer containing the bottom portion of the aptamer might potenti ate only AMPA receptors. In other words, that the sequence and its predicted structural motif for potenti ating AMPA receptors is separated from another sequence unique for kainate receptors. This phenomena has been previously reported by our lab for the discovery of an AMPA receptor aptamer whose shorter version has turned out to be kainate receptor selective as well, except the fact that that RNA aptamer is an inhibitory aptamer. Based on this hypothesis, I further prepared two aptamers, one with only the bottom third of the full length aptamer, while the other contains the bottom third and the long stem sequences. Both of these aptamers have been prepared for testing. Preliminary assays of the latter sequence shows that it potenti ates GluA1.

To date, no selective kainate receptor potenti ator has been reported in literature. That said, there are compounds that act on both AMPA and kainate receptors non-discriminately. Compounds BPAM 344 and BPAM 591 potentiate both AMPA and kainate receptors\textsuperscript{120}. A large number of AMPA potenti ators have been found, including ampakines, a group of benzothiadiazines which have been used extensively to determine the effect of increasing AMPA receptor activity on brain function. Most potenti ators of AMPA receptors, including ampakines, affect desensitization and deactivation\textsuperscript{141}. Potenti ating AMPA receptors have been shown to be therapeutically beneficial to treat cognitive deficiency in both animal models and human trials\textsuperscript{93, 95-96, 145}. Because of the lack of kainate receptor-selective potenti ators, the potential of targeting kainate receptors and enhancing the activity of kainate receptors selectively has never been
explored in either animal or human diseases\textsuperscript{146}. In this context, the discovery of RNA aptamers, especially a kainate receptor-selective aptamer, is significant. RNA aptamers as potential drug candidates are attractive because they are naturally water soluble, and can be readily modified, such as 2’-OH replacement by 2’-F nucleotides. Chemically modified RNAs are more resistant to ribonucleases so that they are suitable for use \textit{in vivo}. Aptamers can be more potent than small-molecule compounds. An aptamer selective to the target of interest, such as the one I have found, can be isolated successfully without any structural information about its site of binding to the target or without knowing if such a site even exists a priori.
### 1.5 SUPPLEMENTARY INFORMATION

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequences/Oligos needed</th>
</tr>
</thead>
</table>
| AL3-1   | 1) 5’TCCCTTGGCCCTTCCCTCATTTCGGTGGTGTTGCGCGATGGCCGACCTAGATAGTGAGTCTGATTTA3’  
2) 5’TATAACGACTCACTATAGGGGAAATTAAC 3’  
3) 5’TCCCTTGCCCTTCTTCTTTT 3’  
| AL3-1A  | 1) 5’TCCCTTGGCCCTTCCCTCATTTCGGTGGTGTTGCGCGATGGCCGACCTAGATAGTGAGTCTGATTTA3’  
2) 5’TATAACGACTCACTATAGGGGAAATTAAC 3’  
3) 5’TCCCTTGCCCTTCTTCTTTT 3’  
| AL3-1B  | 1) 5’TCCCTTCTTCCCTCCATTTCCGCGATGGCCGCGATGGCCGACCTAGATAGTGAGTCTGATTTA3’  
2) 5’TATAACGACTCACTATAGGGGAAATTAAC 3’  
3) 5’TCCCTTCTTCCCTTCCATT 3’  
| AL3-1C  | 1) 5’GCTTTCCCTTGCCCTTCCCTCCGCGATGGCCGACCTAGATAGTGAGTCTGATTTA3’  
2) 5’TATAACGACTCACTATAGGGGAAATTAAC 3’  
3) 5’GCTTTCCCTTGCCCTTCCATT 3’  
| AL3-1D  | 1) 5’TCCCTTCTTCCCTCCCAATTTCGGTGGTGTTGCGCGATGGCCGACCTAGATAGTGAGTCTGATTTA3’  
2) 5’TATAACGACTCACTATAGGGGAAATTAAC 3’  
3) 5’TCCCTTCTTCCCTTCCATT 3’  

### S 1.1 Sequences and primers needed to create DNA templates

Sequences and primers needed to create DNA templates for the in vitro transcription of each aptamer, AL3-1, AL3-1A, AL3-1B, AL3-1C, and AL3-1D. For each entry, 1) is the initial template, 2) and 3) are the primers. These oligoes were used to create the DNA template through PCR.
<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequences/Oligoes needed</th>
</tr>
</thead>
</table>
| AL3-2A  | 1) 5’ACCGAGTCCAGAAGCTTGTAGTACTTCTTTTCCCTTGCCCTGGCAGTTGAATTCTCCCCTATAGTGAGTCGTATTA 3’  
               2) 5’ TAATACGACTCATATAGGGAGAATTCAACTGCCAGGGC 3’  
               3) 5’ ACCGAGTCCAGAAGCTTGTA 3’ |
| AL3-2B  | 1) 5’ACCGAGTCCAGAAGCTTGTAGTACTTCTTTTCCCTTGCCCTGGCAGTTGAATTCTCCCCTATAGTGAGTCGTATTA 3’  
               2) 5’ TAATACGACTCATATAGGGAGAATTCAACTGCCATCTA 3’  
               3) 5’ ACCGAGTCCAGAAGCTTGTA 3’ |

S 1.2 Sequences and primers needed to create DNA templates

Sequences and primers needed to create DNA templates for the in vitro transcription of each aptamer, AL3-2A and AL3-2B. For each entry, 1) is the initial template, 2) and 3) are the primers. These oligoes were used to create the DNA template through PCR.
<table>
<thead>
<tr>
<th>Subunit 1</th>
<th>Subunit 2</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA2</td>
<td>GluA1</td>
<td>0.999989</td>
</tr>
<tr>
<td>GluA3</td>
<td>GluA1</td>
<td>0.999920</td>
</tr>
<tr>
<td>GluA4</td>
<td>GluA1</td>
<td>0.965182</td>
</tr>
<tr>
<td>GluK1</td>
<td>GluA1</td>
<td>0.000004</td>
</tr>
<tr>
<td>GluK2</td>
<td>GluA1</td>
<td>0.000000</td>
</tr>
<tr>
<td>GluA3</td>
<td>GluA2</td>
<td>0.999035</td>
</tr>
<tr>
<td>GluA4</td>
<td>GluA2</td>
<td>0.985246</td>
</tr>
<tr>
<td>GluK1</td>
<td>GluA2</td>
<td>0.000003</td>
</tr>
<tr>
<td>GluK2</td>
<td>GluA2</td>
<td>0.000000</td>
</tr>
<tr>
<td>GluA4</td>
<td>GluA3</td>
<td>0.910116</td>
</tr>
<tr>
<td>GluK1</td>
<td>GluA3</td>
<td>0.000005</td>
</tr>
<tr>
<td>GluK2</td>
<td>GluA3</td>
<td>0.000000</td>
</tr>
<tr>
<td>GluK1</td>
<td>GluA4</td>
<td>0.000002</td>
</tr>
<tr>
<td>GluK2</td>
<td>GluA4</td>
<td>0.000000</td>
</tr>
<tr>
<td>GluK2</td>
<td>GluK1</td>
<td>0.272998</td>
</tr>
</tbody>
</table>

**S 1.3 Results of Tukey’s HSD analysis of AL03-1 Closed-Channel**

Results of Tukey’s HSD analysis of AL03-1 at 2 µM concentration against closed-channel form. P values ≤ 0.05 are underlined and considered significant.
S 1.4 Results of Tukey’s HSD analysis of AL03-1 Open-channel

Results of Tukey’s HSD analysis of AL03-1 at 2 µM concentration against open-channel form. P values ≤ 0.05 are underlined and considered significant.
1.6 References


alpha-amino-propionic-acid (AMPA)/kainate receptors by antidepressant drugs. *Biol Psychiatry* 2006, 59 (8), 713-20.


90. Gebhardt, C.; Cull-Candy, S. G., Lithium acts as a potentiator of AMPAR currents in hippocampal CA1 cells by selectively increasing channel open probability. *J Physiol* 2010, 588 (Pt 20), 3933-41.


Chapter 2: Enhancing transfection efficiency in HEK cells using DMSO

2.1 Introduction

The study of human genes and the production of recombinant proteins rely on the transient expression of these proteins in mammalian cells. Therefore, it is important to maximize transfection efficiency. One technique that benefits from a higher concentration of proteins on the surface of the cell is the characterization of a protein. Electrophysiology is a technique that measures the changes in potential across cell membranes and would be enhanced by more receptors on the surface of the cell. In this chapter, a method for increasing transfection efficiency in HEK-293 cells using dimethyl sulfoxide (DMSO) is described. A 10% solution of DMSO applied for 5 min at 4 hrs after calcium phosphate transfection, or immediately before media exchange, was found to increase protein expression by approximately 1.7-fold. Cells were transfected with GFP, as a reporter protein, and the relative fluorescence of the cells were compared. Many papers caution against the use of DMSO on cells, however a low dose of DMSO for a brief period was found to be beneficial. The toxicity of the DMSO in this protocol was also monitored with alamarBlue, where it was found that a 10% DMSO solution applied for 5 min did not have appreciable toxicity to the cells. The DMSO was also tested on GluA2Q\textsubscript{flip} and GluK2, membrane proteins which were monitored using whole-cell recording. It was found that the amplitude of the GluA2Q\textsubscript{flip} cells increased by approximately 1.4% in the treated cells compared to the untreated cells, showing that the DMSO can increase the expression of membrane proteins as well as soluble proteins. Furthermore, the desensitization of the membrane proteins GluA2Q\textsubscript{flip} and GluK2 showed no significant difference in the treated cells
compared to the untreated cells. This suggests that the brief treatment of DMSO does not negatively affect the structure of the membrane proteins.

2.1.1 Membrane proteins in medicine

Membrane proteins are the targets of over 60% of drugs\(^1\) and represent nearly one-third of proteins in living organisms. Membrane proteins include ion channels, metabotropic receptors, transport proteins, and ionotropic receptors. Ionotropic glutamate receptors, for example, are membrane proteins in the central nervous system. They can be found on presynaptic or postsynaptic dendrites and bind to glutamate in the synaptic cleft. Ionotropic glutamate receptors have been implicated in disorders such as Parkinson’s disease, learning and memory disorders, ALS, and addiction\(^2\). Recent improvements have been made in the expression of functional membrane proteins\(^3\)-\(^6\), but the expression in sufficient amounts for structural studies remains a challenge. Some reasons for the challenge include the fact that in comparison with soluble proteins, membrane proteins require targeting and translocation, which involves sophisticated recognition and sorting. Eukaryotic expression systems also limit the copy number of membrane proteins because of mechanisms that translocate the protein. A simple transfection modification can be an easy and effective way to increase protein expression.

2.1.2 Transfecting HEK-293 cells

Transient transfection is a method of artificially introducing nucleic acid into cells and allowing the cells to produce proteins in a short period of time. The efficiency of transient
transfection can be improved by effectively inserting the DNA and creating conditions that encourage the production of proteins in the cells. Chemical methods of transient transfection include calcium phosphate, diethyl amino ethyl (DEAE)-dextran, or a cationic lipid-based reagent. Physical methods include electroporation or microinjection. All of these methods aid the cells in taking up the nucleic acid. Calcium phosphate co-precipitation is a transient transfection method introduced in 1973. This method remains popular, mainly because the reagents are easily available and inexpensive. Also, the protocol is simple and works well with different types of cultured cells. With the calcium phosphate method, careful preparation of the calcium phosphate mixture generates a precipitate that is layered onto the cells. This precipitate is then taken up by the cells by endocytosis or phagocytosis.

In transient transfection, the cells are usually harvested or used for cell-based assays 24-72 hrs from transfection for the study of membrane proteins, extraction of proteins, or isolation of nucleic acids. Glutamate receptors are especially challenging to transiently transfect because of the many translational modifications, which are required to produce wild-type like, functional receptors. For this reason, it is important to choose the right cell line. HEK-293 cells are commonly used to express these proteins because of their faithful translational mechanisms and high transfection efficiency, which other cell lines do not have. In particular, HEK-293 cells have the ability to properly fold and assemble proteins. Protein expression is relatively quick to reach its peak level around at 48 hrs, and the small cell size is advantageous for electrophysiological studies of channel proteins. It has been found that HEK-293 cells possess neuronal characteristics, which benefit studies of receptors and proteins found in the central nervous system.
2.1.3 DMSO treatment of cells

The action of DMSO on cell membranes has been studied in simulation models and in cells. The dipolar molecule has long been used as a cryopreservative for cells and tissues. It is capable of penetrating through membranes, leading to intracellular water displacement\textsuperscript{19-24}. DMSO can also modify cell membranes and even enter cells by osmosis\textsuperscript{25} or by dehydrating the phospholipid head groups in the membrane and raising the phase transition temperature\textsuperscript{26}. However, higher concentrations of DMSO and/or longer exposure times have been shown to damage or kill the cells. This may be linked to decreasing membrane integrity since DMSO reduces membrane rigidity and at high concentrations, and even induces pore formation in the membrane\textsuperscript{27}. Within cells, even lower concentrations of DMSO have been cytotoxic through increasing the porosity of mitochondrial membranes\textsuperscript{28}. This can be seen with concentrations of less than 10\% and incubation times of one hour or more. Because of the membrane penetrating and modifying ability of DMSO, brief applications of DMSO during transfection have been found to increase transfection efficiency\textsuperscript{29-30}.

2.1.4 DMSO increases transfection yield

It has been shown that incorporating DMSO with transient transfection protocols increases protein expression in both adherent and non-adherent cell lines. In one study, the expression of a human monoclonal antibody in non-adherent Chinese Hamster Ovary (CHO) cells increased by 2-fold when 0.2\% DMSO was added to the medium\textsuperscript{29}. In another study, 4-minute exposure of DMSO at 30\% v/v concentration to adherent chicken embryo fibroblast cells led to an increase in transfection efficiency\textsuperscript{31}. DMSO was combined with a nanoparticle transfection to increase
transfection yield in cells for tissue regeneration, finding a tunable method for transfection of different stem cells by varying DMSO concentration, incubation time, and nanoparticle concentration\textsuperscript{30}.

Since DMSO is an aprotic solvent, it may interfere with attachment proteins more potently than saline washes. In an experiment using CV-1 cells, the transfection efficiency of SV40 DNA using calcium phosphate was increased with DMSO treatment, but it was found that the application of DMSO increased the detachment of cells from the dish\textsuperscript{32}. This may also cause the lower cell count in the initial assays of the treated samples, which I have found in my experiments.

2.1.5 My experiment of exploring DMSO treatment in HEK-293 cells

In this study, I investigated whether a higher transfection efficiency in HEK-293 cells could be achieved by utilizing DMSO in the transfection protocol. If this was possible, I wanted to determine the optimal concentration of DMSO and the optimal time of DMSO exposure that would achieve the greatest enhancement of transfection yield. It has been shown by many experiments that a higher concentration and/or a longer exposure of DMSO to a living cell can be damaging or even be cytotoxic\textsuperscript{33}, since DMSO reduces cell membrane rigidity and, at high concentrations, it even induces pore formation in the membrane\textsuperscript{27}.

I set out to find whether the exposure of DMSO to HEK-293 cells during transfection would lead to an increase in expression yield. The calcium phosphate protocol for transient transfection of HEK-293 cells\textsuperscript{34} was chosen. The calcium phosphate procedure is a very efficient and simple method for transfecting cells\textsuperscript{8,34-35}. 

75
GFP was transiently transfected as a visual marker or reporter gene\textsuperscript{36-37} allowing me to detect the green fluorescence and determine the transient transfection efficiency by measuring the percentage of cells that had green fluorescence in a population of transfected HEK-293 cells. The percentage of green cells was also monitored over a 6 day period to determine if the DMSO negatively affected the protein expression over time. In order to further test whether the brief application of DMSO was damaging to the cell health, I ran experiments using MTT assay and alamarBlue assay with treated and untreated cells. These reagents assess the cell viability using absorbance and fluorescence. The MTT assay utilizes a tetrazolium dye to assess the mitochondrial activity of live cells\textsuperscript{38}. The alamarBlue is to assay healthy cells\textsuperscript{39-40}. This reagent utilizes resazurin, which fluoresces at 560 ex and 590 em in live cells due to the redox reactions occurring in the cell\textsuperscript{40}. One benefit of using this reagent is that it is non-toxic, allowing me to monitor the cell viability over time.

In a later experiment, I transiently transfected an AMPA receptor, GluA2Q\textsubscript{flip}, and a kainate receptor, GluK2, in HEK-293 cells using DMSO. AMPA and Kainate receptors are subtypes of glutamate ionotropic receptor proteins, which have been found to affect brain development and brain activity, such as memory and learning\textsuperscript{41}. The receptor activity was measured using whole-cell recording in order to determine if DMSO exposure to cells would affect the function of the expressed protein, as shown by the change of whole-cell current amplitude and desensitization\textsuperscript{6}.

2.2 Materials and Methods

2.2.1 Plasmid DNAs and cell culture

All the cDNA plasmids that encode GFP, large T antigen (TAg), and GluA2Q\textsubscript{flip} (i.e., unedited at the Q/R site and flip isoform) were propagated in DH5α E. coli host. They were
purified with a Qiagen kit. HEK-293 (i.e., HEK-293T and HEK-293S) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and penicillin-streptomycin (50 units/ml and 50 µg/ml, respectively, final concentrations). HEK-293 cells were generally cultured at 7% CO2 and 37°C. In some experiments, I moved the culture one day after transfection to a 33°C incubator based on a protocol which was reported to slow the growth of the cells and preserve cell cultures.

2.2.2 Transient transfection and DMSO treatment

For transient transfection, HEK-293 cells were grown to ~70% confluency in 35 mm dishes one day after passage. Cells were transfected using the calcium phosphate co-precipitation method. TAg was co-transfected in all cultures since co-transfection of TAg enhances the yield of the expressed protein. For the imaging study, GFP was transfected as the reporter gene. For the electrophysiological study of the GluA2Q\textsubscript{flip} AMPA receptor and the GluK2 kainate receptor, GFP was co-transfected as a cell marker, and GFP-expressing cells or green cells were used for recording.

Four hrs after transfection, the culture was washed with phosphate-buffered saline (PBS) twice and then exposed to a DMSO solution or PBS alone as control (note that 4 hrs was normally the time when the transfection solution is replaced with the complete DMEM media). I varied the exposure time or the duration of DMSO exposure (Fig 2.2). After I removed the DMSO solution, the cells were washed twice with PBS before I returned the normal media to the dish. The blank control was performed in the same way. In this study, all experiments were performed in triplicate, and I used triplicate data sets for data analysis.
2.2.3 Cell imaging and quantification

Cells were imaged on a Carl Zeiss Axiovert S200 microscope under both bright field and green fluorescence channels. Images were captured using a Sony NEX 3 digital camera with an adapter that has 1.74 X magnification (Model Y1S-EA, McCan Imaging Inc.). In a single dish, a pair of the bright-field and fluorescence images were taken at the same spot, and three sets of images were taken at three randomly selected spots or locations. These images were captured under a 20 X magnification, giving rise to a total magnification of 20 X 1.74 = 34.8 X. By a method previously published, all images were analyzed using Image J (http://rsb.info.nih.gov/ij/) and were used to count the number of green cells and the number of total cells in each view. Based on these numbers, I calculated the percentage of green cells or transfection efficiency, or expression yield. Each green cell was assigned to one of the three fluorescence intensity ranges, and categorized accordingly: an intensity between 29 and 100 was classified as light green, 100-200 was medium green, and over 200 was bright green. A cell with an intensity of <29 was considered non-green, or the cell did not express GFP (an example is displayed in Fig. 2.1).

2.2.4 Cell viability assay

First, MTT assay was done by testing the viability of treated and untreated cells over 2 days. The control cells were transfected normally, while the experimental cells were treated with either 5% or 10% DMSO solutions as described above. On the day of transfection, MTT reagent was added to the dishes 2 hours after treatment. Then 4 hours later, the tetrazolium dye was dissolved in pure DMSO and the absorbance was read at 550 nm. The control absorbance was normalized to 100% and the absorbance of the treated dishes were calculated. Other dishes of
cells were incubated for 2 days, and then analyzed with MTT reagent using the same method. An alamarBlue assay was also done on treated and untreated cells to determine if the DMSO negatively affects the number of viable cells in treated dishes. I completed all analyses in 48 well plates (Corning, Costar flat bottom). Fluorescence was read in a 48 well plate was treated with gelatin and allowed to dry for 1 hour. Then approximately 40,000 cells were passage into 18 wells. After 24 hrs, the cells were transfected with GFP using the same method as the previous experiments. Each well contained 1 mL of transfection mixture and 1 mL of plain DMEM. On the second day after transfection, 20 µL of alamarBlue was added to each well. A blank was also prepared in a well with no cells using DMEM and alamarBlue. I incubated the plate for 2.5 hrs, per the protocol. Then the wells were assayed for fluorescence at 560/590 nM ex/em. On the third day after transfection, the fluorescence was read again.

2.2.5 Whole-cell current recording

The whole-cell recording was used to assess whether DMSO would affect the channel activity of GluA2Q
\text{flip} or GluK2, which individually form a functional, homomeric channel when expressed in HEK-293 cell. The amplitude of the whole-cell current response to glutamate and the desensitization rate constants were measured from receptor-expressing cells treated with DMSO, whereas untreated cells expressing the same proteins were used as control. The procedure for the whole-cell recording was described previously. In brief, recording electrodes were prepared from glass capillaries (World Precision Instruments). Electrode solution contained 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM CaCl2, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted by CsOH). When filled with the electrode solution, the
resistance was approximately 3 MΩ. Extracellular buffer contained 150 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES (pH 7.4 adjusted with HCl). Recordings were made at 22°C with cells clamped at -60 mV using an Axopatch-200B amplifier, with a cutoff frequency of 2 kHz by a built-in 4-pole, low pass Bessel filter. Whole-cell current traces were digitized at a 5 kHz sampling frequency using a Digidata 1322A by Axon Instruments. Patch-clamp data was acquired using pClamp 9 software.

2.3 Results

2.3.1 Effect of DMSO

The effect of DMSO on the transient expression of recombinant proteins in HEK-293 cells was tested by first transfecting GFP. As shown in Fig 1, when I exposed DMSO to the transfected culture 4 hrs after transfection, the number of green cells increased (Fig 2.1B, right panel), when compared to the control (Fig 2.1A, right panel). Fig 2.1C shows that more green cells overall, and more bright-green cells were found from those DMSO-treated dishes (Fig 2.1C). It should be noted that DMSO exposure to cells only occurred 4 hrs after the transfection. Ordinarily, I would layer the transfection solution on top of the cells and leave it there for 4 hrs. After 4 hrs, I would replace the transfection solution with a normal or complete medium. I tried mixing DMSO with the transfection solution and exposed the mixture to cells (this would be equivalent to time zero). Applying the mixture of the transfection solution and DMSO to the cells resulted in much fewer living cells afterward, possibly because incubating DMSO for 4 hrs would be toxic to cells. Also, mixing DMSO with the transfection solution resulted in precipitation in the mixture. Thus, I waited for 4 hrs before the cells were subject to DMSO treatment.
Fig 2.1. Effect of DMSO
(A) Untreated and (B) DMSO treated cells under bright field (left panels) and fluorescence view (right panels). These images were taken 48 h after the transfection. Each view contains approximately 170 cells. (C) A typical HEK-293 cell in 34.8 x magnification contained 3,000 ± 800 pixels. The green color intensity was characterized on a scale of 0 to 255 in a JPEG file, recorded in an 8-bit byte. On this scale, a cell with an average brightness between 29 and 100 was labeled low green intensity, colored as a dark green bar. A cell with an average brightness between 100 and 200 was considered medium intensity, colored as green. Bright green was the color used to label any cell with an average brightness between 200 and 255. On the same scale, a cell with no appreciable fluorescent green color registered an intensity of <29. Thus, black color was assigned to these cells or non-green cells. The percentage of each green group was calculated based on the total cell number, which was determined from the bright-field view of the same viewing area. The white line in each column (C) indicates a break in the y axis from 10% to 30%. Reused with Permission.
Since the brief application of DMSO to cells showed an increase in green fluorescent protein expression, the optimum concentration and exposure time needed to be determined. For this experiment, I transfected 35 mm dishes with GFP plasmid using the calcium phosphate method. All dishes were incubated for 4 hrs at 37°C after which they were removed from the incubator for media replacement. Prior to the media replacement, the cells were washed with PBS and then treated with various concentrations of DMSO ranging from 2% to 20% (v/v) for 5 min at room temperature. The control dishes were incubated with PBS for 5 min. It was found that the 10% DMSO concentration produced a significantly higher percentage of green cells overall as well as a significantly higher percentage of bright green cells (Fig. 2.2A). The 5% DMSO concentration did not give a significantly higher percentage of green cells compared with the control, and concentrations greater than 10% gave a lower percentage of green cells, potentially due to the toxicity of the DMSO at these higher concentrations.

The optimum exposure time was found to be 5 min when I varied the exposure time of the cells from 2-15 min. The cells were incubated with 10% v/v DMSO and incubated at room temperature for these times. It was found that a 5 min exposure yielded the most green cells and most bright green cells. The 2-min exposure was not significantly different from the control, while the 10 and 15 min exposures yielded a reduction in the green cell percentage, potentially due to the toxicity of the DMSO.
Fig 2.2 Determining the optimum concentration of DMSO and exposure time

(A) Different concentrations of DMSO (5%-20%) were used to treat the HEK-293 cells transfected with GFP. Exposure time was kept at 5 min in all experiments. The dishes were then washed with PBS, replenished with complete media, and maintained at 37°C. Images were taken 48 hrs after transfection. For determining the percent of green cells and intensity, three images from three randomly chosen areas were taken under both bright-field and fluorescence view for each dish. (B) Similarly, HEK-293 cells transfected with GFP were exposed to 10% DMSO but with different exposure time, i.e. from 2-15 min. Images were taken 48 hrs after transfection and used for determining the percentage of green cells. The white line shows a break in the y axis from 10% to 30%. Reused with permission.
2.3.3 Time course of GFP expression post-DMSO exposure

Next, the GFP expression was measured over time for dishes treated with DMSO. Specifically, the percentage of green cells was measured over 6-days from DMSO-treated and untreated dishes maintained at 37°C. My data showed that the expression of GFP over this time was consistently higher in the DMSO-treated cells (Fig 2.3A), as compared with untreated ones (Fig 2.3B). Fig 2.3C is the comparison. Both DMSO-treated and untreated cells had the highest level of GFP expression on day 2 (Fig 2.3C). Based on the percentage of the green cells on day 2, I found that on average, there were ~1.7 fold more green cells in a dish treated with a 5 min, 10% DMSO 4 hrs post-transfection.

Starting from day 3, the fluorescence intensity gradually decreased (Fig 2.3C). I asked whether the decline of the number of the green fluorescent cells as in Fig 2.3C was because of the cytotoxicity of DMSO treatment to the transfected cells. Specifically, I monitored the total number of cells between days 2 and 3 using alamarBlue. I also used a lower temperature, precisely 33°C, for cell maintenance after allowing the transfected culture to recover at 37°C overnight. This hypothermic condition, which was reported previously ⁶, allowed me to preserve more cells, as compared with 37°C. As shown in Fig 2.3D, there was approximately a 25% loss in the total number of cell counts between the DMSO-treated and untreated dishes on day 2. This loss was decreased on day 3 to about 12% compared to the untreated cells. The total cell counts on day 3 in each of the experiments are higher on day 3 as compared with day 2, suggesting that the DMSO-treated cells continued to grow at a rate similar to that of the untreated cells. That untreated cells also showed the decline in the number of green cells (Fig
2.3C, lower line) after day 2, which was consistent with the observation I reported earlier for a regular HEK-293 culture (or without DMSO treatment) \(^6\), further suggested that a brief exposure (i.e., 5 min) of DMSO to the transfected HEK-293 cells is unlikely to affect either cell health or cell growth. Taken together, the decline in the percentage of green cells from day 2 was most likely because of intrinsic protein degradation and/or cell division. A previous study reported that the half-life of the attenuation of GFP in mammalian cells is about 26 hrs \(^{45}\).
Fig 2.3 Time course of GFP expression post-DMSO exposure

The percentage of GFP-expressing green cells in dishes treated with 10% DMSO for 5 min (A), 4h after transfection, and untreated dishes (B). The percentage of green cells was determined over 6 days by imaging a dish under bright-field and fluorescence view. Plotted is the average percentage of green cells calculated from three images per dish and three dishes in total. The white line indicates a break in the y axis from 10% to 70%. Unlike in either (A) or (B) where three color intensities are displayed, (C) shows the total percentage of green cells over the 6-day six period for treated cells (solid circle) and untreated cells (solid triangle).  *Reused with permission.*
2.3.5 Determining the toxicity of a brief DMSO exposure

Since DMSO has been shown to be harmful to cells at various concentrations and incubation times depending on the cell line, I wanted to determine whether a brief application of 10% DMSO would be harmful to HEK-293 cells. Initially my experiment showed a reduction in cell activity in both an MTT assay (Fig 2.5) and an alamarBlue assay (Fig 2.6A). To determine whether this reduction was due to cell death or cell loss during washing, each of which would yield lower cell counts, I first collected the washes and counted the cells that were washed away in each well. I found that the treated cells were more prone to be washed away. After transfection and treatment, the remaining cells in the wells were counted and reseeded to the same density (100,000 cells). I incubated them with a 10% solution of alamarBlue in complete DMEM. This reagent is non-toxic to cells and allows the cells to be monitored over a few days. This experiment showed that with the number of cells equalized after transfection, the growth of the cells was not significantly affected by DMSO treatment. Therefore I determined that the brief treatment of DMSO is not cytotoxic, (Fig 2.5 A-D) although DMSO treatment clearly disturbed the cell attachment to the dish such that the treated cells were prone to be washed away during media exchange.
Fig 2.4 MTT assay of DMSO treated cells

The number of healthy cells assayed by MTT in a 5 min and 10 min treatment of DMSO at day 0 and day 2 of incubation at 37°C. The error bar represents error from the mean of three separate dishes at each condition. Control dishes were not treated with DMSO, and the % viable cells of all of the dishes were normalized to 100% of the control. The column with no error bar had error of 0.07%.
Fig 2.5 Assay of cell viability and growth for DMSO-treated HEK-293 cells using alamarBlue assay

After 10% DMSO exposure, cells were recounted and reseeded in 48-well plate with 100,000 cells/well, and all assays were done in triplicate. Immediately following reseeding, alamarBlue was added to the wells at a 10% v/v concentration. The cells were incubated with alamarBlue for 2.5 hrs before fluorescence readout at 560 nm excitation and 590 nm emission on a microplate reader; this fluorescence readout was set as day zero for control (Ctrl), 5-min and 10-min exposure of 10% DMSO to HEK-293 cells transfected with GFP. The fluorescence readout is plotted in relative fluorescence unit (RFU). In (B), the fluorescence intensity of the control well was measured and plotted for days 0, 2, and 3. (C) and (D) are fluorescence intensities from wells treated with 10% DMSO for 5 min and 10 min, respectively. Error bars represent the standard deviation from the mean. Other experimental detail is provided in Materials and Methods. Reused with permission.
2.3.6 Whole-cell recording assay of ion channel activity for DMSO-treated HEK-293 cells

To ensure that the brief exposure of 10% DMSO would not affect the function of a recombinant protein, I designed a functional experiment. Here, an AMPA receptor subunit, i.e., GluA2\text{flip}, was expressed in HEK-293 cells and the activity of the channel was assayed using whole-cell recording. It is known that transient expression of this receptor subunit in HEK-293 cells leads to the formation of a functional, homomeric channel, and the channel activity can be characterized from an electrophysiological study using whole-cell recording (note that the GluA2 channel expressed was the unedited isoform in the Q/R editing site)\textsuperscript{42-43}. As a control, the same receptor was also expressed in HEK-293 cells and tested with cells from untreated with DMSO. Again in both the DMSO-treated and untreated cells, GFP was co-expressed using a separate plasmid as a cell marker for whole-cell recording assay.

Fig 2.6A displays a pair of representative whole-cell current responses to glutamate, the activating ligand. Based on the recording of 15 DMSO-treated cells and also 15 untreated cells, I found that the whole-cell current amplitude increased by 1.6 fold (Fig 2.6B), as compared to the cells from the control (i.e., 7.2 ± 3.7 nA vs. 4.6 ± 2.9 nA). Since the current amplitude was linearly proportional to the number of receptor copies on the surface of a single cell, the increase of the whole-cell current amplitude from those DMSO-treated cells suggested that HEK-293 cells produced on average ~1.6-fold more receptors when the cells were treated with DMSO. It should be noted that the value of 1.6-fold enhancement of receptor expression which I obtained from assaying GluA2Q receptors was in good agreement with the ~1.7-fold increase, which I determined from the analysis of green fluorescence imaging data (Fig 2.3C), where a much
larger population of cells were used for the analysis. Finally, the rate constant of the channel desensitization process ($k_{des}$) was also measured. The channel desensitization is the falling phase of the whole-cell current response (Fig 2.6A), due to the ligand-induced channel closure. The rate of channel desensitization is considered one of the fundamental properties of an ion channel. Specifically, I found that at 1 mM glutamate concentration, the $k_{des}$ for GluA2Qflip measured from the DMSO-treated cells was $125 \pm 13 \text{ s}^{-1}$, whereas $k_{des}$ was $133 \pm 17 \text{ s}^{-1}$ for the same receptor expressed on untreated cells. The first-order rate constant for the channel desensitization was unaffected. Also, the magnitude of $k_{des}$ I observed here is consistent with the value reported earlier from our lab$^{42,47-48}$. These data, therefore, showed that the HEK-293 cells expressed the same receptor with the same channel property when the cells were treated briefly with a 10% DMSO solution, as compared with the control. In short, the brief treatment of transfected HEK-293 cells with DMSO led to the increase or enhancement of a receptor expression but did not affect the property of the receptor.
Fig 2.6 Whole-cell recording assay of AMPA and kainate receptor activities with DMSO-treated and untreated HEK-293 cells

Shown in (A) is a pair of representative whole-cell current response to glutamate mediated through GluK2 kainate receptors expressed in HEK-293 cells. Left trace was from a cell without DMSO treatment (or – DMSO), whereas the trace on the right was from a cell treated with DMSO (or +DMSO). The two traces are scaled to the same amplitude for visual inspection of the channel desensitization rate. The upper bar shows the time scale of a 0.5-mM glutamate application. In (B), the two columns on the left show the channel desensitization rate constant ($k_{des}$) at 3 mM glutamate measured form 15 cells (or N=15) expressing GluA2Qflip AMPA receptors from untreated and treated dishes. Similarly, the two columns on the right represent $k_{des}$, measured at 0.5 mM glutamate concentration for GluK2 kainate receptors expressed in HEK-293 cells. An average $k_{des}$ is shown with an error bar representing the standard deviation from the mean. Also shown on the top for each receptor type is the p-value obtained from a paired two-tailed Student’s t test. Reused with permission.
2.4 Discussion

The productivity of transiently transfected mammalian cultures for the expression of recombinant proteins depends on many factors. These include the host cell, rate of gene transcription and translation, the stability of gene expression, the ability of posttranslational modifications in the cell, and the transfection efficiency. Once a cell source or cell line is chosen, manipulating transfection efficiency is one of the most effective ways to improve the yield of the gene product. Among various attributes that have made HEK-293 cells an attractive choice of transient gene expression system is the fact that these cells can be readily manipulated. Based on this idea, I investigated the effect of briefly exposing DMSO to transfected cells on the productivity of the transient expression yield. I found a 5-min exposure of 10% DMSO to HEK-293 cells, 4 hrs post-transfection, can enhance the expression yield by ~1.7-fold. This value was established from the assay of a reporter gene GFP from a large population of cells by imaging and also the assay of an ion channel receptor at the single-cell level by whole-cell recording.

One important finding from this study is that the exposure of DMSO to cells should occur 4 hrs after exposing the cells to a transfection reagent; in this case, calcium phosphate-DNA coprecipitation. The timing of the medium exchange has been established to minimize cell loss. Mixing DMSO with the transfection solution and layering this mixture on the cells, which would be equivalent to time zero, causes a serious loss of cells. On this note, I also attempted initially the use of the same protocol, i.e., 5 min 10% DMSO treatment, with suspension-adapted HEK-293S cells. I found a similar enhancement in HEK-293S cells has been observed, but the
culture dish must be first coated with collagen; otherwise, a significant amount of the cells would be lost during the removal of DMSO solution and washing of the dish before the regular growth medium returned (data not shown). Therefore, the cell attachment to the dish is an important step to allow us to separate DMSO by pipetting from the cell culture with minimal loss of cell mass. The separation of DMSO solution in the dish by pipetting the solution out is amenable to static culture. It should be noted that the timing of exposing DMSO to the cell culture or 4 hrs after transfection also coincides with the timing of otherwise replacing transfection mix with normal growth medium. Thus, the method I have described here should be simple to implement, especially considering that the calcium phosphate DNA co-precipitation protocol which I used here is a convenient and economical way of introducing foreign genes into mammalian cells. Calcium phosphate transfection has been one of the most popular methods of introducing a gene ever since Graham and Van der Eb reported this method for transfecting adenovirus DNAs and simian virus 40 into mammalian cells in 1973.

The brief treatment of the transfected HEK-293 cells with DMSO, which leads to an increase of transfection efficiency by ~1.7-fold, is most likely attributed to the ability of DMSO molecules to increase membrane permeability by osmosis and DMSO-induced dehydration of phospholipid head groups on the membrane. More cDNA plasmids could enter the cells through endocytosis and finally enter the nucleus. However, a higher DMSO concentration or a longer exposure to DMSO is cytotoxic. Even a lower concentration of DMSO but a prolonged exposure or to the extreme, a chronic incubation of DMSO with the culture medium, can be sufficiently damaging to cells. The magnitude of the reduced cellular viability is thought to correlate with an increase in intracellular water and electrolyte leakage since DMSO is known for its membrane penetrating and intracellular water displacement properties.
Higher concentrations or prolonged interaction of DMSO with lipid molecules could reduce membrane rigidity and even induce pore formation in the membrane\textsuperscript{27}. For these reasons, our method suggests only a 5-min exposure of DMSO solution with HEK-293 cells. Such a procedure has produced no appreciable toxicity to cells, because cell number counts from a population of cells increases over 2 and 3 days similar to untreated cells (Fig 2.3D), the function of the transiently expressed receptors is unaffected (Fig 2.4) and the transfection yield is higher than untreated cells (Fig 2.1-2.3), suggesting that the cells remain healthy.
2.5 Supplementary material

**DMSO increases amplitude in GluA2 whole cell trace**

Testing of the channel activity of AMPA receptors from HEK-293 cells treated with DMSO using whole-cell recording. (A) A pair of whole-cell current response to 3 mM glutamate from HEK-293 cells expressing AMPA receptor GluA2Q\textsubscript{flip}. On the right is a whole-cell trace from an HEK-293 cells treated with 10% DMSO for 5 min, and the trace on the left was the control or an untreated cell. Both cells were chosen for recording 48 hrs after transfection. The fall phase in either trace reflects the channel desensitization rate process. Shown in (B) is the comparison of the average amplitude from 15 cells from the DMSO-treated (right column) and untreated culture (left column). These cells were randomly chosen from dishes for whole-cell recording. The error bar indicates the standard deviation from the mean. A two-tailed Student’s t-test was calculated on this data with a confidence interval of 0.05. *Reused with permission.*
2.6 References


43. Li, G.; Oswald, R. E.; Niu, L., Channel-opening kinetics of GluR6 kainate receptor. *Biochemistry* 2003, 42 (42), 12367-75.


