Appendix A:
Studies of Ionotropic Glutamate Receptors in Mammalian Cells

A.1 Introduction

The use of nonsense suppression to incorporate unnatural amino acids into ion channels has greatly enhanced our ability to probe structure/function relationships in many different ion channels, from Cys-loop ligand-gated ion channels (LGICs) to ionotropic glutamate receptors and GPCRs (*1-5*). One large hurdle for nonsense suppression has been the expansion of this technique into mammalian cells. There are clear benefits to performing structure/function studies in mammalian cells not limited to the opportunity to use a more relevant biological system and the ability to study complex signaling networks. Generally, the *Xenopus laevis* oocyte is the primary heterologous expression system utilized for these studies, primarily due to ease of experimental procedure. Injection of DNA, mRNA, and aminoacyl tRNA into an oocyte (1 mm diameter) is much easier than transferring these material into very small, thin, and transparent mammalian cells. Early studies were able to incorporate small amounts of DNA, mRNA, and aminoacyl tRNA into mammalian cells using an electroporation protocol (*6*).

These protocols were difficult to perform, particularly in a high throughput manner. Additionally, electrophysiological assays performed on a single mammalian cell are not adapted for high-throughput structure/function analysis. Another complication arises due to the availability of the "limiting reagent," aminoacyl tRNA. In order to electroporate mammalian cells, a small dish of adherent cells are used, but within that dish are approximately 5,000 cells. Electroporation results in only several hundred viable cells, an inefficient use of materials. In order to develop the mammalian cell method, we attempted to use new techniques to increase efficiency of transfection and assay analysis. The techniques we utilized in this study were the gene gun (for DNA transfection) and the Flexstation® as a high throughput assay of dose-response relationships.

The gene gun was initially developed as a biolistic particle delivery system for use in transformations performed on plants (*7, 8*). It is a mechanical method for transfecting cells that involves high-speed propulsion of subcellular particles coated with DNA/RNA/tRNA into cells. One feature of biolistic transfection is that the biochemical

nature of the material does not influence the transfection (i.e. being a lipid, etc.), which is a great advantage to the system. Additionally, more than one type of DNA (or additional biological molecule) may be introduced during the transfection. One of the most relevant advantages to this method is its efficiency, which has been demonstrated to be 160-fold more effective than lipofection, 189-fold more than electroporation, and 450-fold more effective than calcium phosphate precipitation, when analyzed by a luciferase activity assay (*9*). Recently, methods using the gene gun have been developed for transfections in mammalian cells and tissue slices, expanding this efficient transfection method to many different cell types (*10-12*). Three basic steps are required to use the gene gun; 1) coating the microcarriers with DNA or other biological material, 2) transferring the microcarriers to a cartridge used to make a "bullet," and 3) firing the microcarriers into a mammalian cell using a pulse of helium gas (*12*).

Using a gene gun requires a hand held gun, such as the Bio-Rad Helios gene gun (used in the studies below). Several metal particles are often used for the microcarriers, such as gold and tungsten. We utilized gold particles due to their uniformity of size and shape and the increased stability of DNA on gold. The microcarriers are turned into a cartridge by attaching them to the inside of a piece of tubing. The cartridges are loaded into the gun and using an inert, diffusible, and low-density gas propellant (typically helium), the microcarriers are stripped from the cartridge and delivered into the cells.

Increasing transfection efficiency is one issue we wanted to address during our experiments with incorporating unnatural amino acids into mammalian cells. The other issue, developing a high-throughput assay, was the second. A new high-throughput system developed for studying mammalian cells was the Flexstation®, produced by Molecular Devices (Sunnyvale, CA). It is a fluorometric plate reader with a fluid transfer system. When used with reagents, such as the Membrane Potential Assay Kit® (Molecular Devices), devised for FLIPR (fluorometric imaging plate reader) and Flexstation®, a change in membrane potential (membrane potential dyes) can be observed and recorded. One advantage to these dyes is their sensitivity and fast response times, enabling accurate detection of changes in membrane potential due to ion channel activation. This membrane potential assay has been utilized in place of traditional patch clamping data (*13-15*).

Project Goals

The structure/function studies we envisioned for our mammalian cell experiments paralleled our previous studies of glutamate receptors in *Xenopus* oocytes (Chapter 2). Our goal was to generate NMDA receptors containing the homotyrosine mutation in the ligand-binding domain, and observe results re-confirming the previously determined relationship between clamshell closure and ligand efficacy. Ultimately, we wanted to use wild type NR1a/NR2B and NR1a/NR2BY705TAG THG73-hTyr NMDA receptors, expressed in human embryonic kidney (HEK293) cells. For our initial experiments, we tested the wild type NR1a/NR2B and NR1a/NR2BY705F mutant NMDA receptors. Although we wanted to perform experiments with the NMDA receptors, we also tested Cys-loop receptors (glycine and 5-HT_3) to gain more experience using the fluorescence assays and the Flexstation®.

A.2 Results and Discussion

Previous studies by K.L. Price and S.C.R. Lummis (University of Cambridge) demonstrated that the Cys-loop LGIC, 5-HT_3, could be studied using both Ca^{2+} and membrane potential-sensitive dyes on the Flexstation® (*16*). We utilized these methods for our examination of the NMDA receptor. The NMDAR experiments did not require the use of aminoacyl tRNA, therefore standard cell electroporation was utilized for transfection of wild type and mutant (NR1a/NR2BY705F) DNA into HEK293 cells. All of the NMDARs were transfected as cDNA and were in the pcDNA3.1 vector optimized for mammalian cell expression. To optimize the transfection efficiency we used the Nucleofector® System from AMAXA Biosystems (Lonza Walkerrsville Inc., Walkerssville, MD). This system allows for direct transfection into the nucleus using electroporation.

Gene Gun Transfection

The gene gun was utilized to co-transfect the LGIC, 5-HT_3AS183TAG cDNA and THG-73-Lah (leucine alpha hydroxy acid) into HEK293 cells. These experiments were performed with John O'Brien and S.C.R. Lummis at the University of Cambridge, Laboratory of Molecular Biology. Using the protocol developed by O'Brien and Lummis (*11, 12*) we generated functional 5-HT_3 mutant receptors that responded to 5-HT (5-hydroxytryptamine) also known as serotonin.

Flexstation Analysis of Ligand Gated Ion Channels

We analyzed both wild type NMDA and 5-HT_3 receptors using the Flexstation. All of the cells were plated in 96-well plates designed for the fluorescence plate reader. Two different assay kits, the Membrane Potential Assay Kit and the Calcium Assay Kit (both from Molecular Devices), were used to analyze the response of the LGICs to varying drug applications. All cells were treated with the Flexstation buffer (see methods) optimized for use on the Flexstation. The buffer is used to wash the cells plated in 96-well plates (3X) prior to incubation with the fluorescent dyes. The dyes are also mixed with the Flexstation buffer (Methods) for dilution. The dyes are diluted as indicated by the protocols provided and incubated for 45 minutes (total) after addition to the cells in 96-well plates. The first 35 minutes the plates are stored in the dark at 37ºC, followed by a 10-minute incubation at room temperature (~25ºC).

The agonist solutions used for NMDA receptor activation were either glutamate and glycine or a NMDA/glycine combination. The agonists were always co-applied at 3X the desired final concentration. For example, to apply a 300 μM glutamate dose, a 900 μM sample was prepared. The concentrations of agonist are added at [3X] and added in 25 μL aliquots to the wells, which already contain 125 μL of solution prior to agonist (drug) application. The same protocol was used for serotonin applications.

Flexstation Acquired Data

Transformation of NMDA receptors expressed in HEK293 cells always resulted in unhealthy cell cultures. The cells would lose adhesion to the plates and become misshapen and often died. When these cells were put in the Flexstation and glutamate

and glycine were applied, often no change in membrane potential was observed. The FLIPR membrane potential assay kit measures a change in the potential across the cell membrane. Hyperpolarization results in less dye inside the cells and leads to a decrease in the signal. Membrane depolarization results in more dye inside the cells and leads to an increase in fluorescence signal. The signal is measured in relative fluorescent units (RFU). Previous experiments with 5-HT_3 receptors produced RFUs of ~5000 to 10,000. The studies with NMDA receptors did not result in consistent results and often the largest concentrations of agonist that were applied (glutamate and glycine) resulted in the largest decrease in fluorescence, indicating cell death (Figure A.1). The responses were inconsistent and further work will be required to increase cell viability after NMDA receptor transfections.

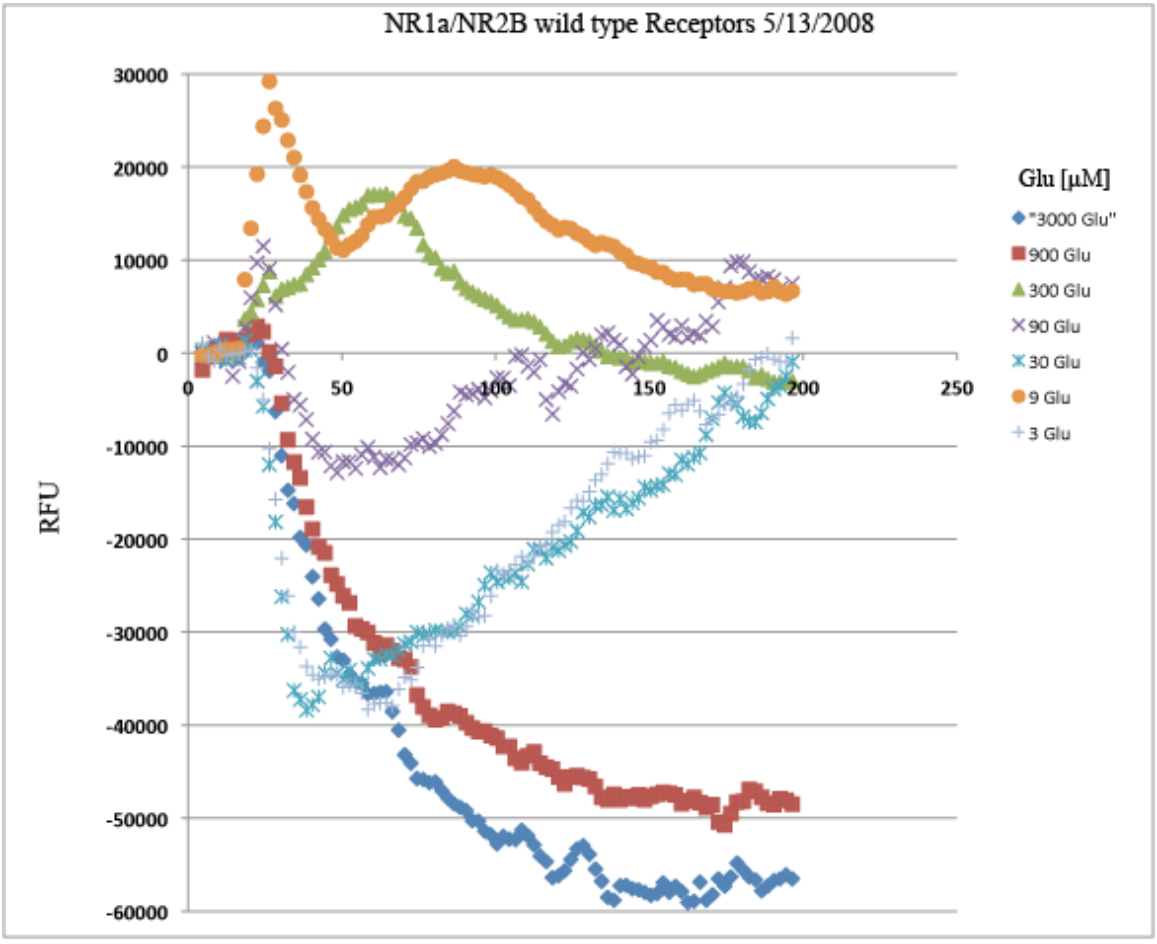

Figure A.1 Wild type NR1a/NR2B receptors response in cells on the Flexstation. The concentrations of glutamate are provided and always given in the presence of 30 µM glycine.

Although we continued to study the NMDA receptors, in order to gain more experience with the fluorescence assays and the Flexstation, we studied wild type 5-HT_3 receptors and the mutation, 5-HT_3A S183TAG Lah-THG73, which were transfected using the gene gun. The wild type 5-HT_3 receptors responded to concentrations of serotonin as expected (Figure A.2). The RFU signal increases as the concentration of applied 5-HT increases.

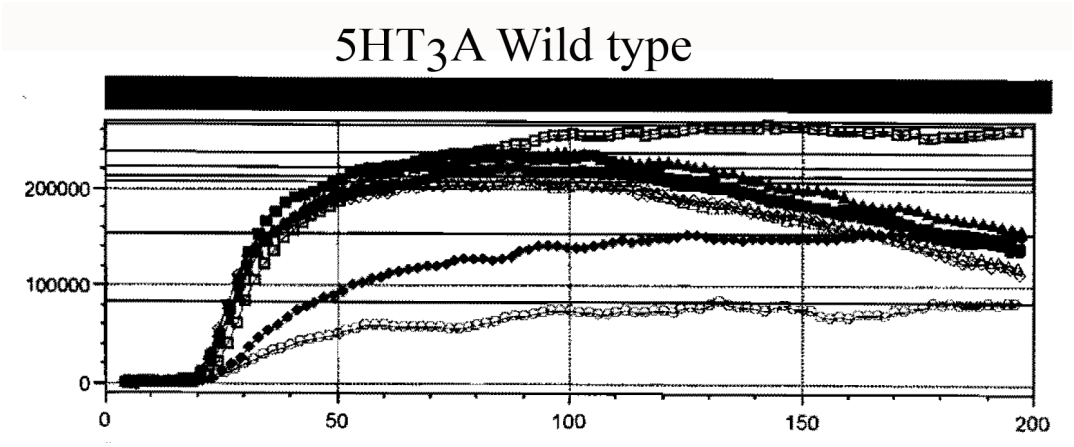

Figure A.2 Wild type 5-HT_3A receptors responding to increasing concentrations of serotonin. All fluorescence measurements are well above 0 RFU (the bottom axis). The largest signals appear over 20,000 RFU, (RFU = y-axis).

Similar results were observed for the 5-HT_3A S183Lah mutation. We only had enough of the aminoacyl Lah-THG73 tRNA to perform one injection with the gene gun, so our goal was to determine if this was a viable method of transfection. We did observe suitable receptor expression. We observed increasing RFU values as the concentration of applied 5-HT increased, similar to wild type 5-HT_3A receptors (Figure A.3).

Control experiments were performed to ensure that untransfected cells (Figure A.4) and cells transfected with only aminoacyl tRNA (Figure A.5) did not produce signals on the Flexstation. These results demonstrated no response in relative fluorescence units in response to varying doses of 5-HT. A very small amount of RFU was detected for one 5-HT application on the tRNA only cells (Figure A.5), which is an anomaly and likely undetectable when compared to cells expressing viable receptors.

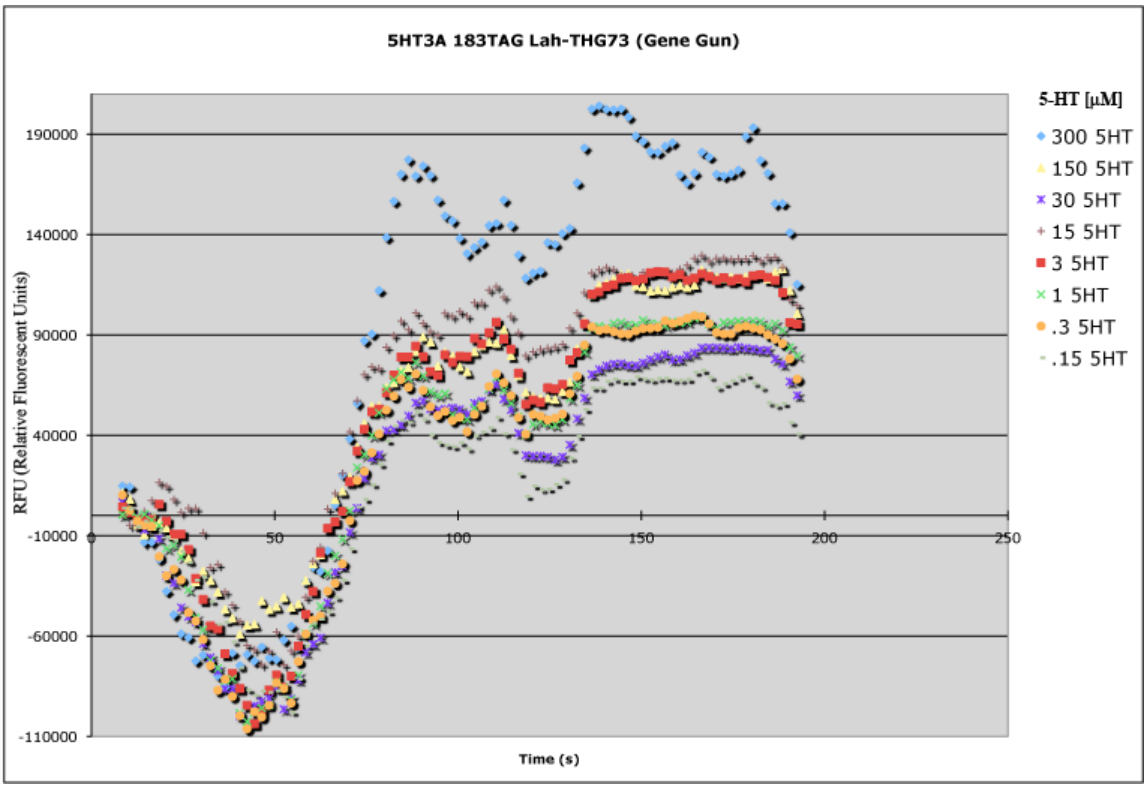

Figure A.3 Fluorescent responses from $5HT_3A$ S183TAG Lah-THG-73 mutant receptors to varying concentrations of serotonin. Both the cDNA and aminoacyl tRNA were transfected using the gene gun.

These results establish that we can generate viable cells via transfection with the gene gun, and that the Flexstation is a useful tool for high-throughput analysis of mammalian cells expressing ion channels via nonsense suppression. These studies should enable future experimentation of effective methods for transfecting aminoacyl tRNA and other biological compounds into mammalian cells. Additionally, our results suggest that using the Flexstation for analysis of ion channels expressed in mammalian cell lines is an effective, high-throughput strategy to parallel our other studies using the OpusXpress and oocytes. Further method development should optimize these procedures for use of mammalian cells to study a variety of LGICs.

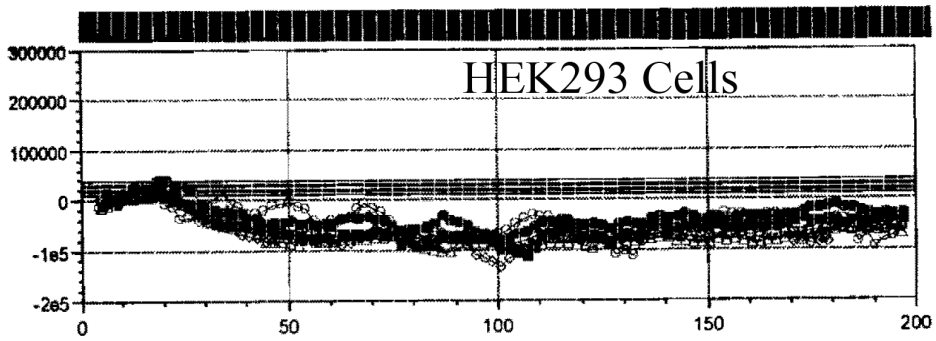

Figure A.4 Flexstation measurements of untransfected HEK293 cells to varying concentrations of 5-HT. No fluorescence was observed, as all above measurements are below zero.

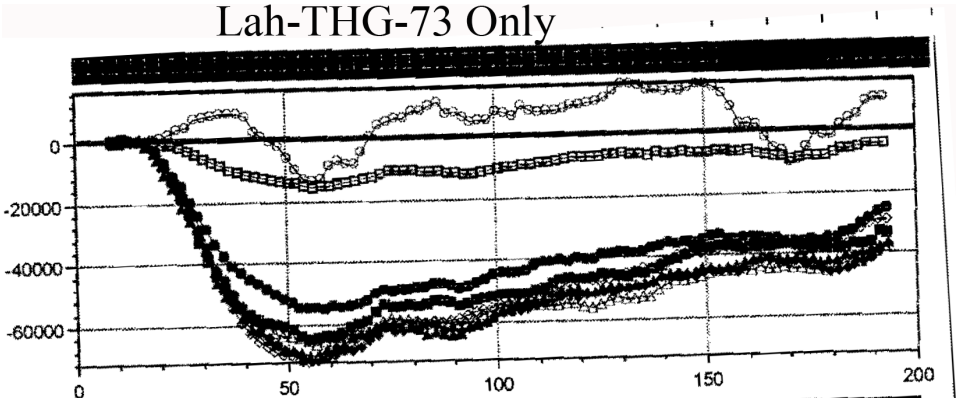

Figure A.5 Flexstation measurements of HEK293 cells transfected with Lah-THG73 tRNA only. No relative fluorescence was observed except for with the first concentration of 5-HT applied.

A.3 Methods

Protocol for NMDAR Transfections:

The following protocol was used:

1) Trypsin Digest 35 mm dish of HEK293 cells
    a) add 1-2 mL EDTA, rinse and remove media
    b) Mix trypsin w/ EDTA and add 1mL/35 mm plate, let sit for 5 minutes
2) Rinse cells with 5 mL media and resuspend 1 mL of cells in 15 mL media in a separate culture tube
3) Spin at 1000 rpm for 5 min.
4) Remove media (pellet should remain at the bottom of the tube)

5)*For use with AMAXA electroporator. Add 100 uL of Solution V to pellet of HEK293 cells and mix

a) Add DNA to solution with cells (1.0 μg/mL and add total of 5 μL cDNA, 2.5 μL of NR1a and 2.5 μL NR2B)

6) Put cells + Solution V + cDNA (~120 μL) into AMAXA cuvette

7) Electroporate with AMAXA

8) Remove cells with 1 mL warm media and resuspend in 15 mL culture tube

9) Plate into 96-well Flexstation® plate. 1.8 mL of cells/media get used per 3 rows of wells. 1 drop/plate well, using 5 mL pipette.

Gene Gun Protocol

The protocols outlined in O'Brien and Lummis (*11, 12*)were adapted for our experiments with aminoacyl tRNA. Microcarriers constructed from gold particles were used. cDNA was plated on a 1.0 μM diameter particle (Bio-Rad) by placing all material in a 1.5 mL microfuge tube and adding 50 μL spermidine with 25 μL cDNA (1 mg/mL) for transfection and 25 μL Lah-THG73 (1 μg/μL). The tube is vortexed and then 50 μL of 1 M $CaCl_2$ is added dropwise. This solution is incubated at room temperature for 5 min. then centrifuged for 3 sec. at 3000 rpm. Bullets were prepared as described in O'Brien and Lummis (*11, 12*).

Flexstation Buffer:

	Concentration (mM)	Volume mL (total of 100 mL)
Na^+	115	11.5
Ca^{2+}	1	1
K^+	1	1
Mg^{2+}	1	1
HEPES	10	10
Glucose	0.9	0.9
H_2O	-	74.6

*All of the cations are in solution with Cl^- counter ions.

Flexstation Data Workup

All data acquired on the Flexstation was analyzed in Excel (Microsoft).

A.4 Cited References

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