5-2023

# Planar Lipid Bilayer Analysis of Candidate Rotavirus Antivirals

Emily H. Tamkin
*Bates College*, etamkin@bates.edu

# Planar Lipid Bilayer Analysis of Candidate Rotavirus Antivirals

An Honors Thesis

Presented to

The Faculty of the Department of Biology

Bates College

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

Emily Helen Tamkin

Lewiston, Maine

April 3, 2023

**Acknowledgements**

I'm completely overwhelmed as I consider the number of incredible friends, teachers, mentors, and family members who have continually supported me in my personal and academic endeavors. Firstly, I'd like to thank the Bates College Department of biology for supporting my academic achievements through the course of my college career, for as long as I can remember I knew that I'd find myself studying the living world, and I could not have asked for an establishment more dedicated to facilitating my success. This work was supported by funds from Maine INBRE and institutional funds from the Biology Department Thesis Fund. I am continually grateful for the mentorship and support I've received from my long-time research advisor Dr. Lori Banks. Thank you for giving me the opportunity to answer any of the scientific questions I have – from beer to microbes and beyond.

Anyone looking for me on Bates' campus knows that if I'm not in Bonney Science Center I can be found on the first floor of Ladd Library in the Academic Resource Commons. I want to acknowledge the amazing tutors and professional staff of the learning center. There I've grown as an educator and have attempted to reflect the amazing mentorship that I've received onto my peers. Thank you to Eric Dyer who has always been there to provide guidance, a listening ear, and life-changing, blue, lay-Z-boy couch.

I've been fortunate enough to have been surrounded by exceptional scientific educators who have continuously believed in my abilities and fostered my love of science. From my elementary school science teacher to the faculty of Campolindo High School, thank you for making me first and foremost a lifelong learner.

Most importantly, I want to thank my friends and family who have had my back on and off of the lab bench. To my best friends – Hazel Delehey, Ellie Wolfe, Chloe Arons, Rebecca Feist, Emma Blakeslee, Cici Conroy, and countless others, it's meant the world and more to share my college experience with you. I could spend the rest of my life cackling in our living room together and be the happiest person in the world.

To my amazing parents, who have done everything and more so that I have been able to live the life I've wanted to live. Thank you for always letting me be me, for your unconditional love, and for helping me spread my wings – even if it meant watching my last four years from 3200 miles away.

To everyone who's supported me as a scientist and as a nervous college student – thank you for everything. Without further ado, let's get to it.

## Table of Contents

**Abstract**

Case rates of rotavirus, the leading viral cause of severe acute diarrhea in children under the age of 5, have been steadily declining in countries that have had access to effective mitigation strategies. In children experiencing nutritional insecurity, however, there is a significant decrease in vaccine efficacy. With vaccination and oral rehydration remaining the only rotavirus interventions (preventative and clinical) in place, significant inquiry is required to make antiviral treatment a reality. The NSP4 protein in rotavirus is a viroporin responsible for disturbing the stability of the rough ER membrane of epithelial cells, forming pores that disrupt calcium ion homeostasis. This viral pathway is a prime candidate for target in antiviral drug design, both because of its critical nature towards the function and replication of rotavirus and the myriad of preexisting drugs that block ordinary calcium ion channels. As the Banks lab and its collaborators continue to develop targeted antiviral drugs, an analytical review of existing, FDA-approved ion channel blockers that may cross react with NSP4 is necessary to determine biophysical drug characteristics that are favorable for inhibiting this protein. After performing an extensive literature review, we selected amlodipine, diltiazem, and verapamil as calcium channel blockers to analyze as NSP4 antagonists. We utilized planar lipid bilayer electrophysiology to determine the binding kinetics and efficacy of selected viroporin inhibitors in lipid bilayer. In addition, we will assess the ability of computational modeling systems to confirm these findings.

**List of Abbreviations**

PLB – Planar lipid bilayer

NSP4 – Nonstructural protein 4

ER – Endoplasmic reticulum

ORS – Oral rehydration solution

## Introduction

### Rotavirus Epidemiology:

Rotavirus is an acute gastroenterological disease that predominantly impacts children under the age of 5 in endemic regions. Patients present with general gastrointestinal symptoms including fever, diarrhea, and vomiting. A wide range of severities can present with rotavirus infection. Infants (>3 months of age) have the highest risk for severe diarrhea and gastrointestinal stress. The duration of illness is between 5-7 days, with further symptoms ranging from dehydration and fatigue to abdominal cramping(1).

As the leading cause of diarrheal death in the world, rotavirus leads to an estimated 200,000 deaths annually(2). Of diarrheal deaths in 2019, 19.11% were attributed to rotavirus(3). Disease trends, however, have shifted dramatically following the increased prevalence of vaccine administration. The vaccine is estimated to avert 280,000 clinic and 62,000 emergency department visits annually, in addition to preventing hospitalizations to the tune of 45,000 per year(4).

Transmitted by the fecal-oral route, the viral diarrheal disease is incredibly contagious. Viral particles enter through the mouth and begin replication in the villous epithelium of the small intestine after a 1-3 day incubation period(5). There is significant debate about the impact that sanitation has on disease mitigation. The feces of infected individuals contain trillions of viral particles, and as a highly contagious disease, a very small viral load is required to transmit infection. Infectious particles can remain active in standing water for up to 19 days(6), especially in warmer climates(7). As such, it is hypothesized that countries with inadequate sanitation remain at high risk for contagion and disease outbreak because of this additional viral reservoir. However, another body of research shows that there is no difference in case rate between

countries with much higher water quality versus those lower. Because the rotavirus-specific impact of sanitation on disease spread is inconclusive, public health professionals must look towards other treatment options to mitigate disease outbreak.

Few treatments exist for children infected with rotavirus besides vaccination. Because dehydration is one of the most common symptoms of infection, oral rehydration solutions are the primary treatment option. However, such solutions are only recommended for children experiencing mild to moderate dehydration, severe cases generally require hospitalization and treatment with IV fluids. As the cause of rotavirus fatality, dehydration allowed to persist can become life-threatening. With supply and demand issues and decreased provider outreach in endemic regions, the World Health Organization estimates that two thirds of children with diarrhea in developing countries are unable to receive oral rehydration solutions (ORS)(8). Both for rotaviruses and other gastrointestinal diseases, it is critical that access to ORS be scaled up drastically, yet even with access to these solutions, they are only a temporary treatment for mild-moderate cases. Individuals that lack access to ORS are also assumed to experience barriers toward additional treatment access should their cases become severe. Despite vaccination programs, rotavirus continues to impact endemic communities and further treatment options need to be pursued.

After a fraught history, two rotavirus vaccines remain on the market that have high efficacy rates. The original rotavirus vaccine, Rotashield, released in 1998 was removed from the market after a strong association between the vaccine and intussusception was detected. The remaining vaccines, RV4 (RotaTeq) and RV1 (Rotarix) are both delivered orally and consist of a three-dose and a two-dose series, respectively. Infants recommended for vaccination must begin their dosage before reaching 8 months old. Both vaccines have high effectiveness against

gastroenteritis, with 74% of clinical trial candidates avoiding it altogether(9). The acquired immunity from the vaccine lasts through the first 2-3 years of infant life(9), however there exist inequalities in vaccine uptake and efficacy between various socioeconomic groups. A clinical study conducted in Ethiopia found that families with higher socioeconomic statuses had a much higher concentration of vaccine uptake than those lower(10). Factors such as maternal healthcare access and educational level create such a socioeconomic inequality in childhood rotavirus infection outcomes. Those with education about the severity of gastroenteritis and access to the pursuit of reliable healthcare are more likely to experience minor cases. Areas with high rates of malnutrition also see increases in disease severity, notwithstanding access to vaccines(11).

While vaccination efforts present a foundation for public health response to diarrheal illness, pitfalls exist within access and efficacy of these movements for at-risk children. It is critical that proactive anti-viral development take place to mitigate severe cases in vaccinated individuals, and outbreaks in under-vaccinated populations.

**Rotavirus Virology**

Rotavirus is a double-stranded RNA virus in the family reoviridae. While there are 8 classified strains of the virus, rotavirus A is responsible for around 90% of infections worldwide. The RNA is contained within an icosahedral protein capsid and produces non-enveloped viral particles(12). With ~18,555 nucleotides, the virus codes for 6 structural proteins and 6 nonstructural proteins. Of these nonstructural proteins, our target of interest is nonstructural protein 4 (NSP4). NSP4 is known to contain a viroporin domain - a motif that acts as an ion channel inserted within the lipid membrane of the cell's rough ER(13). When inserted, NSP4 is responsible for increasing intracytoplasmic calcium levels(14). This function is critical for rotavirus viral activity because pools of high $Ca^{2+}$ concentrations are required for viral

maturation and host cell death via oncosis.

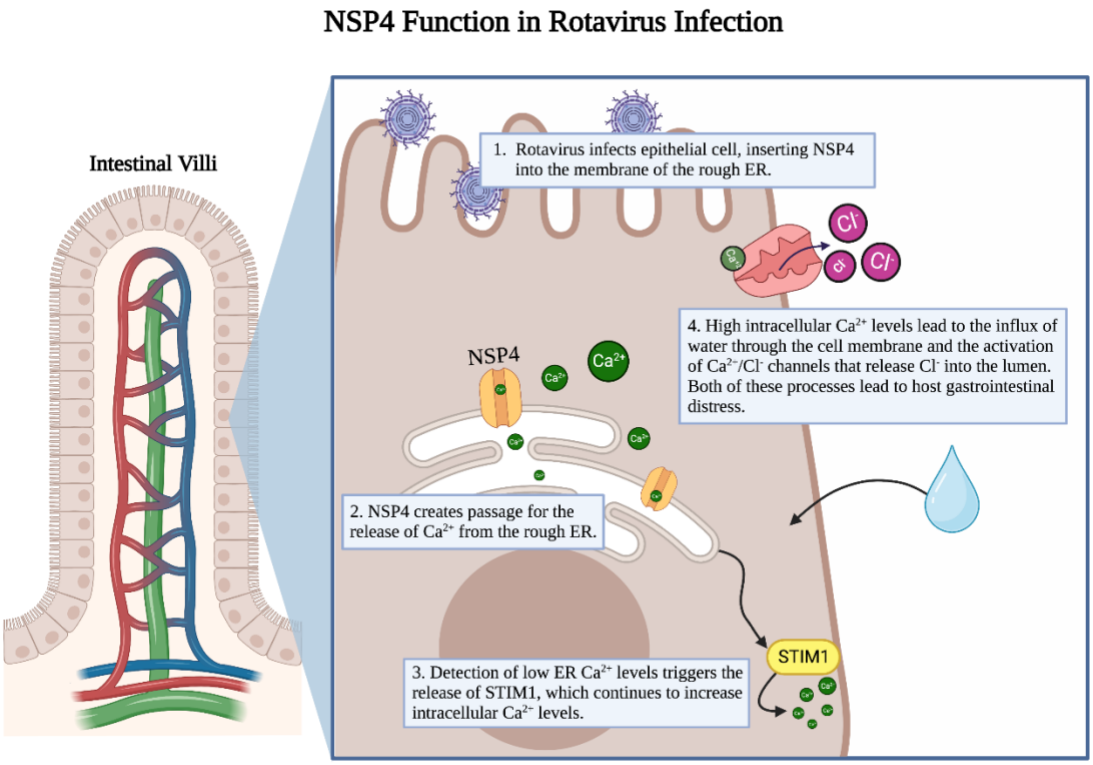


***Figure 1*** *Details the mechanisms by which NSP4 impacts rotavirus symptomatology. Figure created with BioRender.*

The NSP4 protein inserts itself into the membrane of the rough ER of the host cell. There it acts as a calcium ion channel and triggers the release of $Ca^{2+}$ from the ER into the intracellular domain. As the cell senses low $Ca^{2+}$ concentrations in the ER, it triggers the release of STIM1, a transmembrane protein that facilitates the additional release of $Ca^{2+}$ from the plasma membrane. This continues to disrupt ion homeostasis as the intracellular calcium concentrations continue to rise. These high calcium concentrations then activate calcium-dependent chloride channels present in the cell membrane, which release $Cl^-$ into the intestinal lumen. In response to the hypertonic intracellular environment, water rushes in from the lumen through the plasma membrane. The fluid intake causes swelling and cell cytolysis. Increased fluid intake of intestinal villi then disallows water absorption of digestive material, causing diarrhea.

In addition to its symptomatic and cytolytic impact, Pérez et. al. established that the increase in intracellular $Ca^{2+}$ concentration is required to stimulate viral replication(15). Furthermore, rotavirus-associated increases in intracellular calcium result in the disorganization of the actin cytoskeleton of the host cell(16). Actin disorganization is directly related to the abnormal microvilli structure observed in rotavirus cases, which may result in decreased surface area for nutrient absorptivity(17). Determining an NSP4 antagonist would be the first step to antiviral inhibition of rotavirus infection.

Viroporins have become attractive antiviral drug targets because of our growing understanding of their role in the pathology of viral attack. Hepatitis C, HIV-1, Ebola, picornaviruses, and togaviruses all code for viroporins of pathological significance(18-20) and in 2019 it was shown that coronavirus' 3a protein acts as a viroporin responsible for the activation of host inflammasomes in severe acute respiratory syndrome cases(21). The archetypical viroporin in drug targeting is the M2 proton channel of influenza A. M2 is the target of amantadine and rimantadine, two of the three existing antiviral drugs in production for influenza. Viroporins in West Nile and Dengue virus are also being assessed in high throughput screening studies for potential therapeutic blockers(22). As the significance of viroporins in viral replication, maturation, and host cell function continues to be discovered, investigation into their candidacy for antiviral targeting must be prioritized. This avenue has not yet been pursued for rotavirus treatment.

**L-type Calcium Channels**

L-type calcium channels, also known as the dihydropyridine channel, have similar functions to NSP4 in their $Ca^{2+}$ conducting ability. While they are primarily found in smooth muscle tissue, L-type calcium channels are also present in neurons and endocrine cells,

responsible for the activation of various signaling cascades(23). The channels have relatively low rates of activity, once they are activated they tend to stay open generally longer than other channels of the same type(24). This resembles the kinetics of NSP4, whose function is to remain open for the continuous increase in intracytoplasmic calcium concentration.

**L-type Calcium Channel Blockers**

Calcium channel inhibitors function as antiarrhythmics and antihypertensives depending on whether they target vascular or cardiac tissue(23). These inhibitors are generally divided into three subgroups which exhibit different tissue selectivity and allosteric binding mechanisms. There are benzothiazepines, phenylalkylamines, and dihydropyridines. Benzothiazepines and phenylalkylamines are both non-dihydropyridines and are used as antiarrhythmics, whereas dihydropyridines are more common as anti-hypertensives. Despite these subtle differences, many of these drugs are functional in both instances. To analyze a representative sample of calcium channel inhibitors while remaining within the scope of this exploration, we conducted a literature review to select one drug for trial from each subgroup. In the selection of drugs from each class of calcium channel blocker, key factors that influenced these decisions were whether the drugs were commonly prescribed in the rotavirus target patient class - children under 5, drug solubility in water, and efficacy against other viroporin-creating viruses. As the work done by Duffy 2022 found, water insoluble drugs were difficult to apply in wet-lab testing(25).

Antiarrhythmics repress tachycardias, abnormally fast or irregular heart rhythms such as atrial fibrillation, atrial flutter, ventricular tachycardia, supraventricular tachycardia, and ventricular fibrillation. Heart rhythms are controlled by cardiac action potentials, changes in voltage within heart cells which are a result of $Na^+$, $K^+$, and $Ca^+$ ion flux across the membrane(26). Antiarrhythmics are divided by the Vaughan Williams Classification into five

different classes by their pharmacological mechanism. Classes I, III, and IV target the three aforementioned ion channel types respectively. As such, Class IV comprises the non-dihydropyridine calcium channel blockers, the phenylalkylamines and benzothiazepines, which target cardiac tissue more specifically. By interfering with the cardiac action potential via calcium flux, they thereby reduce heart contractility and rate(27).

Phenylalkylamines target the calcium channel from the intracellular side of vascular tissue cell membrane. Of the phenylalkylamines, verapamil is one of the most frequently prescribed and has a water solubility of 83 mg/ml. Furthermore, there is evidence that it blocks both L and T type calcium channels, which theoretically increases the range of viroporin blocking functionality it can be presumed to have. It has also become a frequent candidate for other viroporin blocking studies in influenza, Covid-19 (clinically), various filoviruses, and classical swine fever(28, 29).

The benzothiazepines are commonly considered an intermediate between the properties of the phenylalkylamines and the dihydropyridines. They are prescribed both as an antiarrhythmic and antihypertensive. Diltiazem is the only commonly prescribed benzothiazepine and has a water solubility of 50 mg/ml. It has also been successful in-vitro against viroporin creation in Covid-19(30). *In-vivo* it significantly decreases the viral load of Covid-19 in mouse lung – presumably via the same mechanism(31).

Antihypertensives treat hypertension, high blood pressure, via several different mechanisms. One of the common classes being calcium channel blockers. By preventing calcium movement across the membrane, they cause blood vessel relaxation and therefore increased blood flow through said arteries. This lowers blood pressure and abates hypertension. Dihydropyridines are the choice pharmaceutical for this purpose due to their selective targeting

of vascular tissue for relaxation. They have the fewest side-effects of antihypertensives and are therefore commonly prescribed in children(32).

While the majority of dihydropyridines are not water soluble by nature, amlodipine is considered “slightly soluble”, especially with the addition of an organic solvent such as DMSO. It is the most commonly prescribed antihypertensive for children, making it a prime candidate for a rotavirus antiviral as its primary target is children under 5. While fewer studies exist involving dihydropyridine trials against viroporins, there is evidence of its success against Covid-19 and clinical heart failure induced by encephalomyocarditis virus(33-35).

L-type calcium channel inhibitors are promising candidates for the inhibition of viroporin function, however a significant void exists in the literature regarding the biochemical mechanism of successful calcium channel antivirals. While there is a growing mass of clinical studies exploring them, a lack of in-vitro research leaves a gap in our understanding of their function within the infected host. This complicates the continuation of drug development as researchers do not have an awareness of the vital structures and characteristics creating and increasing antiviral efficacy. By deepening our exploration of these antiviral mechanisms, their principles can be applied to other development efforts and grow a body of research to facilitate and streamline future rational antiviral drug development. This study serves to generate a body of data to understand the characteristics and biomechanics of viroporin blocking as a potential antiviral endeavor.

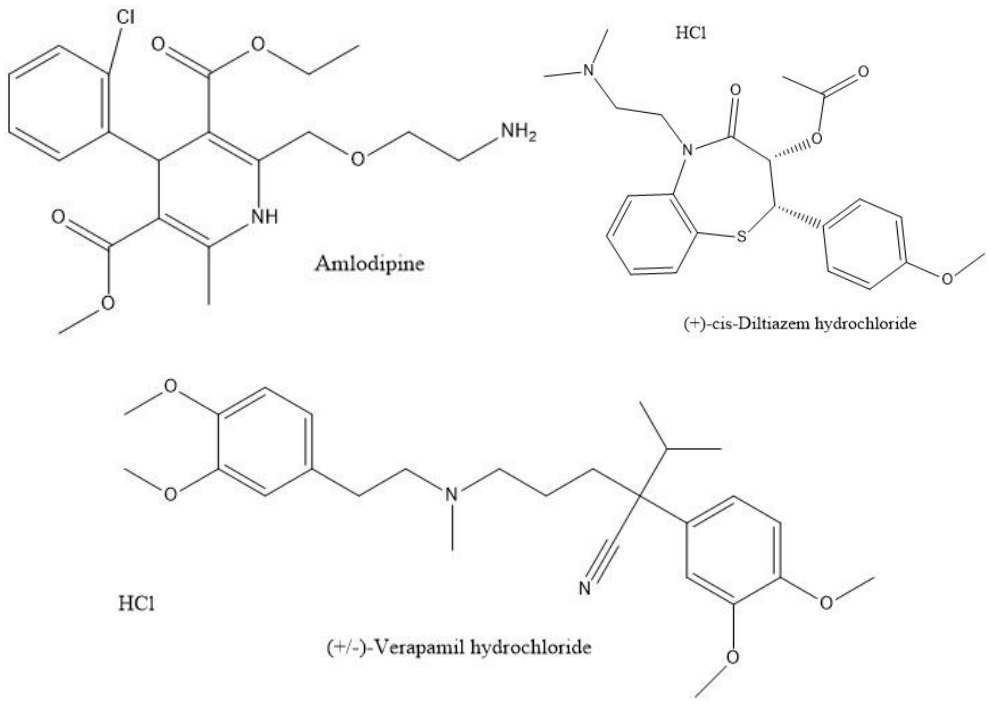


***Figure 2*** *Details the chemical structures of amlodipine, (+)-cis-diltiazem hydrochloride, and (+/-)-verapamil hydrochloride. Figure created with ChemDraw.*

**Planar Lipid Bilayer Electrophysiology**

We utilized planar lipid bilayer (PLB) electrophysiology to assess the impact of the various target drugs on NSP4 membrane protein activity. This technique involves the creation of four concurrent artificial lipid bilayers. After the creation of these artificial membranes, proteins of choice are added into solution and allowed to reconstitute into the lipid bilayer(36). This technique is compelling for the analysis of membrane proteins because researchers are able to track their activity within a true lipid bilayer(37, 38). As the aqueous buffer moves through channel proteins, their activity can be monitored by the change in conductance across the membrane. Closely related to Automated Patch Clamp devices, Planar Lipid Bilayer technology has become an important facet of drug discovery targeting membrane proteins.

Using this technique, we generate planar bilayers with reconstituted NSP4 and are able to track their conductivity traces before and after the addition of our drugs of choice. Characteristic

spikes in conductance represent the opening and closing of proteins in the membrane, therefore allowing aqueous buffer across the barrier and causing the observed conductance spike. In an ideal situation, a drug that results in a decrease of NSP4 activity would show a decrease in the amplitude of these voltage spikes. However, a range of analysis techniques are necessary to explicitly confirm the relationship between observed conductance traces and protein activity.

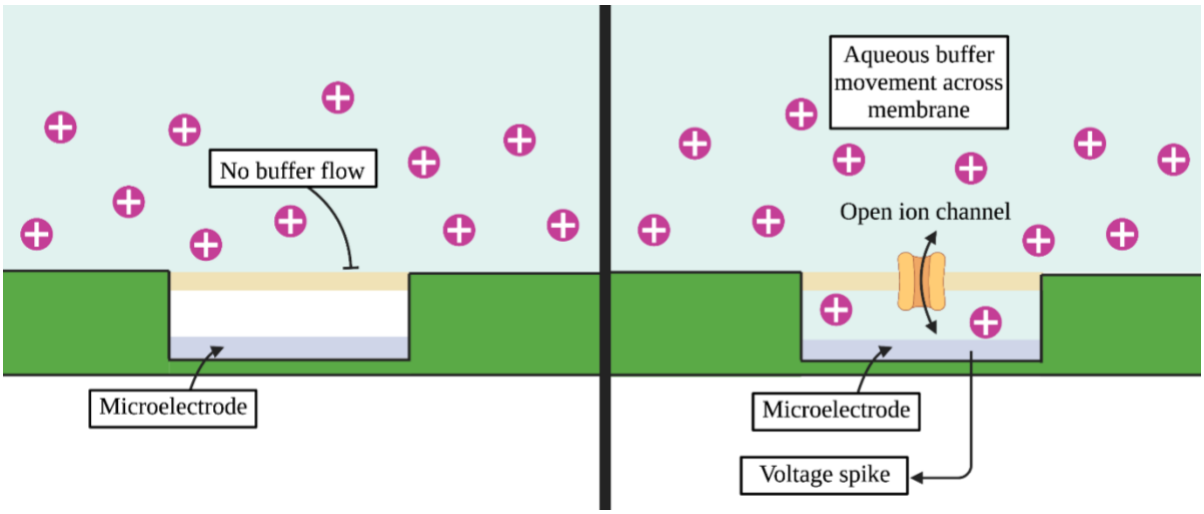


***Figure 3*** *Diagrams the process of planar lipid bilayer electrophysiology. Figure created with BioRender.*

**Process Delay Addendum**

After the literature review process, the Banks Lab experienced a multitude of experimental delays and complications leading to alterations from the originally proposed methods of the work. Initially, experimental methods relied heavily on the usage of thermofluor assays to ascertain binding kinetics between NSP4 and the drugs in question. After the completion of literature review to determine the target drugs for analysis, supply-chain issues with NSP4 production delayed progress extensively. As we were not able to purchase synthesized NSP4 for experimentation at a reasonable cost, it became the work of the lab to isolate NSP4 from E. coli for experimentation. From September to December 2022 researchers in the Banks Lab worked to isolate a significant and pure batch of NSP4 protein. During this

time, methods and processes were determined in order to run thermofluor assays on the drugs in question. These methodological decisions are applicable for future analysis via thermofluor on target drugs for NSP4. Unfortunately, when significant protein stock was developed and ready for analysis after the Winter Recess of 2022, we discovered that the machinery available to the lab would not be able to run the correct melt procedures for the intended kits. As opposed to running the melts with insufficient data collection and temperature range, we made determinations about the change in methods for this project. Despite these changes and limitations, the development and research of thermofluor analysis for future experimentation will be valuable for wet lab binding kinetic study when the lab has the capacity for correct protocol.

On the computational side of the analysis, there was much legwork involved in establishing our capacity to utilize a program that did not end up being the optimal choice for our data. Over the course of Fall 2022, we collaborated extensively with the Bates College IT department to equip computer labs and various lab members with the ability to use the program CHARMM, with which we believed we would perform our computational analysis of the drugs and NSP4. After this extensive collaboration, it became clear that we would be needing a server with a much greater capacity to be able to use the program to its fullest potential. In an attempt to change course to an analytic software that we would have more success with, we pivoted to working with GROMACS, a software with similar abilities. At such time, we made contact with analysts from Clemson University who equipped us with access to their Palmetto Cluster; a supercomputer that we would be able to connect with virtually. While this legwork built the foundation for future computational analytics in the Banks Lab, the timeline of this project did not allow for extensive continued exploration of the Palmetto Cluster for GROMACS applications. Furthermore, our analysis of the preexisting tetramer model for NSP4 concluded

that the known protein structure is of extremely low confidence and is not a strong tool for making conclusions about the binding kinetics of the protein and small drugs.

## Methods and Materials

### Solution Preparation

All electrophysiology took place in PA buffer prepared to the specifications of Pham et. al. (150mM KCL, 5mM Hepes, pH 7.2). The NSP4 solution was grown from E. coli and separated on the STREP-TACTIN column system from IBA Life Sciences to a concentration of 1.77 $\mu$g/ul.

We solubilized amlodipine, (+)-cis-Diltiazem hydrochloride, and (+/-)-verapamil hydrochloride in PA buffer as specified above to create solutions of 10mM concentration. Drug solutions were stored in the -20 degree freezer and thawed for usage.

### Planar Lipid Bilayer Electrophysiology

Planar lipid bilayer (PLB) experiments were performed using the Nanion Technologies Orbit Mini platform and the Elements Data Reader v3 software package. During data collection, samples were taken at a range of 200 pA and a sampling frequency of 1.25kHz, temperature controlled at 27º C. Data were recorded using Ionera 100-$\mu$m MECA recording chips. Chips were double-washed with DI water and ethanol, and reused via manual specifications until nonfunctional. We solubilized 1,2-diphytanoyl-sn-glycero-3-phosphocholine powder from Avanti Polar Lipids, Inc. in reagent-grade octane from Sigma Aldrich to create our 10 mg/ml lipid solution for membrane construction.

To collect data, the Orbit was calibrated to a noise range of -.5-.5 and channels were centered at 0. The chips were coated in 150$\mu$L of PA buffer and lipid membranes were painted onto the channels using a bubble blown by a 10$\mu$L pipette tip dipped in the solubilized lipid. Once the membranes were applied, their resistance and capacitance were tested to ensure resistance readings in the GΩ range and capacitance readings in the 7-10pF range via

manufacturer instructions. If such readings were not obtained, a piece of vinyl was used to re-scrape the channels and their electro physics were assessed until they maintained the correct range.

Once the membranes exhibited appropriate readings, 5$\mu$L of buffer was removed and replaced by 5$\mu$L NSP4 protein solution, pipetted gently next to each aperture. Once the protein was applied, the channels were treated with 2-3 insertion pulses to encourage insertion of the protein into the membrane. Once the membranes showed protein activity via visual analysis, baseline recordings were taken showing the protein activity within the membrane. After this baseline was recorded, equivalent amounts of buffer and drug solution were removed and added, respectively, from the solution above the chip. As with the protein, drugs were gently pipetted near each aperture to evenly distribute the compounds. After gently pipetting up and down over the tops of each channel to simulate drug delivery, we recorded membrane activity with the presence of the drug.

Because the activity of the membrane was able to be measured before and after drug addition, the reading without drug acts as the control for each of the trials. Additionally, to assess whether or not there was a membrane-drug interaction acting as an extraneous variable, negative controls were run at each drug concentration without the presence of protein.

| **Quantity of drug ($\mu$l)** | **Molarity in buffer solution ($\mu$M)** |
|---|---|
| 5 | .333 |
| 10 | .666 |
| 15 | 1.00 |
| 20 | 1.33 |
| 25 | 1.66 |

**Computational Protein Modeling and Analysis**

The amino acid sequence for SA11 rotavirus NSP4 was obtained from National Center for Biotechnology Information (NCBI) (accession number AF087678.1). The sequence was used as input to the Colab notebook version of AlphaFoldv2.1.0 and AlphaFold-Multimer in FASTA format. These restraints included inter-residue distance and oriental distributions, which were predicted by a deep residual neural network. The predicted 3D structures of these algorithms were downloaded as PDB files. In-notebook renderings of models colored by confidence scores (pIDDT) were obtained as screenshots of the notebook page. Refinement of the monomer, dimer, and tetramer models were independently completed using Phenix version 1.20.1-4487 for Alphafold predicted models (www.phenix-pnline.ord), or CCP4Interface (CCP4i) version 8.0.010 (www. https://www.ccp4.ac.uk/). Renderings of each model were created with PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC) for detailed analysis.

**Results**

Figures 4-8 represent the results of planar lipid bilayer experimentation. Each drug type was tested under conditions involving 5 different drug quantities and data are shown in relation to time. The conductance traces are created to generally represent 30s of pre-drug incorporation data on the left and 30s of post-drug incorporation data on the right. Each reading represents the average conductance curves of 4 channel replicates. For each drug type, membrane-drug control results are also represented at each drug quantity. Because the lab does not have the capacity to perform significant statistical analysis on these data, general qualitative assessments can be made about the protein/membrane behavior before and after drug addition, yet substantial conclusions cannot be drawn from these representations alone. During analysis, it also became apparent that many trials were impacted by hardware malfunctions that resulted in data loss or incorrect readings. Such trials are noted in the figures.

Interesting observations that we did note were that there appeared to be no interaction of any of the compounds with our experimental membrane, which suggested that any differences we saw with the addition of the protein were due to protein-drug interactions and not background membrane interactions. As well for our recordings with amlodipine, in the 5, 10, and 15$\mu$l additions, in addition to the channel activity that we observed, there were dips in the baseline conductance. Though the mechanism of this decrease is not determinable in this assay, I hypothesize that there may be some form of NSP4 stabilized that is stuck open, leading to the observation. Similarly, for the 25$\mu$l addition of diltiazem, there was both a drop in the conductance, and a more dentate appearance of the channel activity. I hypothesize that this reading may have been the result of channel deactivation where I was seeing fewer active

channels which allowed us to see more discreet opening and closing events. Here again, the mechanism behind the drop in overall conductance is not clear, however.

Our initial aims were to utilize computational models in assessing the topology of NSP4 in a membrane environment and determine the association of membrane NSP4 with our inhibitor candidates. To achieve these aims, NSP4 sequences were input into AlphaFold2 as either a monomer, dimer, or tetramer to represent multimeric, in solution, forms previously reported for NSP4. Cartoon renderings of each model were colored such that low-confidence regions are shown in red, intermediate regions in green, and high-confidence regions in blue. Prior to any refinement processing, the monomeric structure shows the highest confidence, with the best region being the C-terminus for which there is experimental data in the PDB (Fig10C). Low to moderate confidence was shown for the helices predicted to reside in the membrane. Surprisingly, models for NSP4 multimers do not exhibit the same accuracy confidence, as noted by their mostly red coloring (Fig10A,B). While the algorithm produced 3-4 models that appeared to follow existing conventions of NSP4 self-association from biochemical data, the confidence for each was extremely low. For the tetramer, the structure predicted to be the active form of the multimer, again, we noted intermediate to low confidence in the C-terminal coiled-coil domain but very low confidence in all other regions of the protein (Fig10B). Further assessment of the multimeric models using PyMOL confirmed the presence of steric clashes between predicted membrane helices of adjacent monomers.

In an attempt to resolve the observed steric clashes and increase the confidence of the multimeric structures, we submitted the dimer and tetramer independently to crystallographic refinement software packages Phenix and CCP4i. Both refinement packages have updated interfaces to assist with the processing of computational models lacking the standard files used

to process experimental data, given the recent boom in AI-derived structures. Despite these advances, the refinement of our NSP4 multimers resulted in the removal of most of the residues in our structures due to unfavorable metrics, rather than the adjustment of those residues to accommodate neighboring residues. Due to the lack of a high-confidence NSP4 model, we did not proceed with the molecular dynamics simulations in the membrane environment. Future directions for this portion of the project are to identify additional refinement methods that may increase the usability of the NSP4 models and continue the computational assessment of those models in a membrane environment.

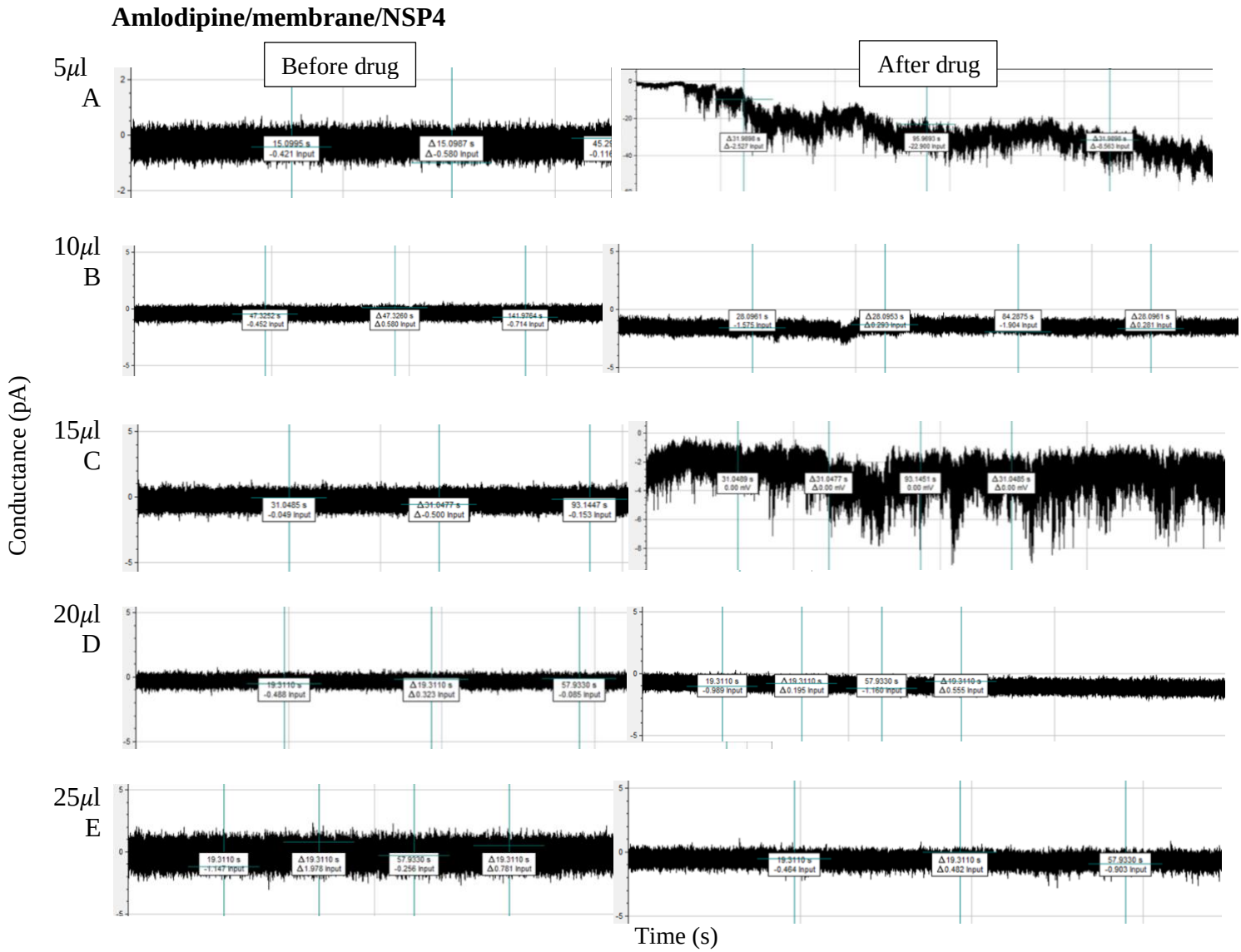


***Figure 4 Membrane electrophysical response to amlodipine drug incorporation***. *Membrane conductance patterns recorded over time of protein-containing planar bilayer trial. Data are separated via volume of solubilized amlodipine added to the environment (n=4). Data on left represent control activity of NSP4 in membrane, while experimental drug conditions are shown after the second axis labelling.*

**Control Data: Membrane/amlodipine**

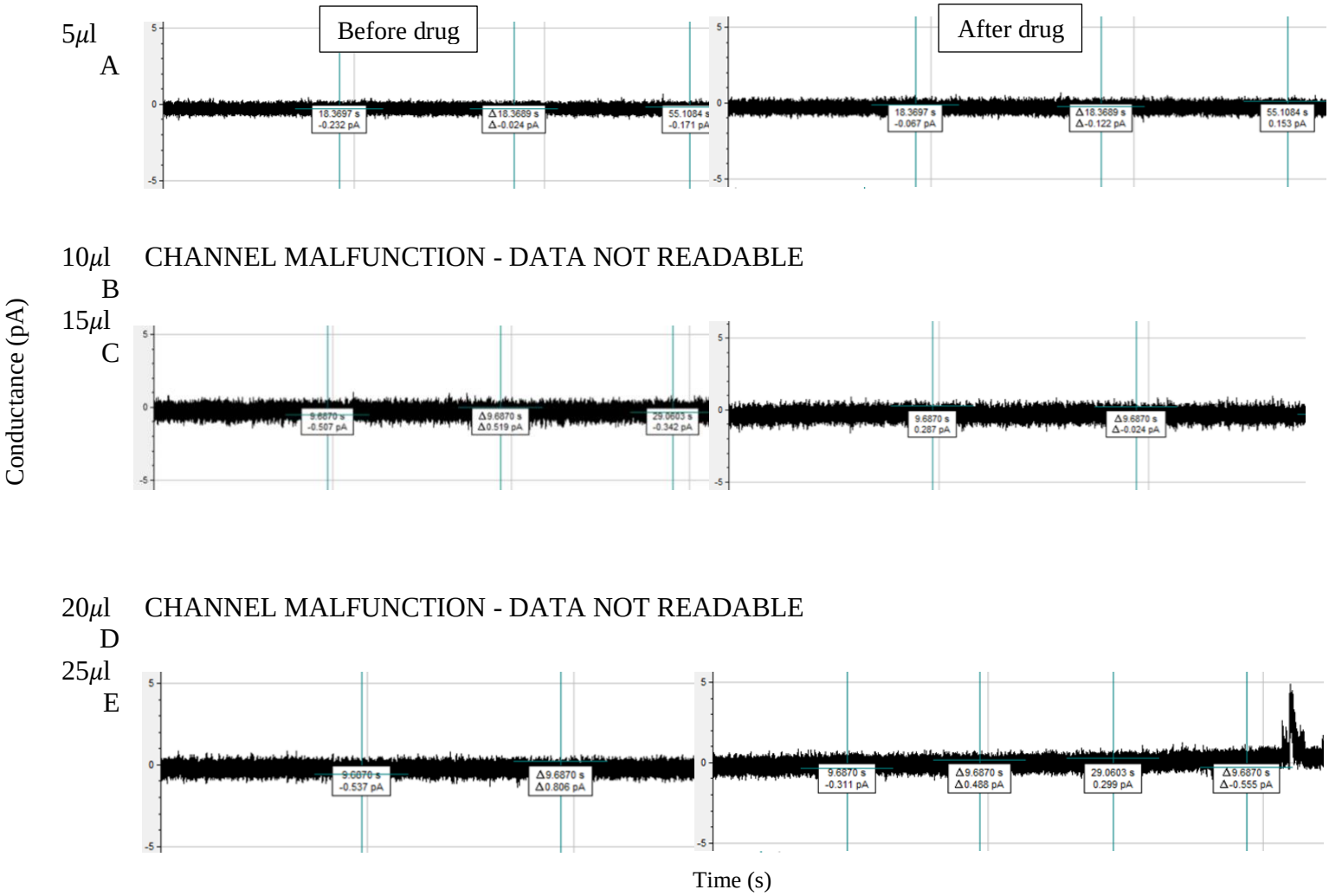


***Figure 5 amlodipine impact on membrane electro physics.*** *Membrane conductance shift in presence of amlodipine. Trials each represent four channel replicates. Data on left represent pure membrane activity while data to right of second axis show the impact of the experimental drug conditions.*

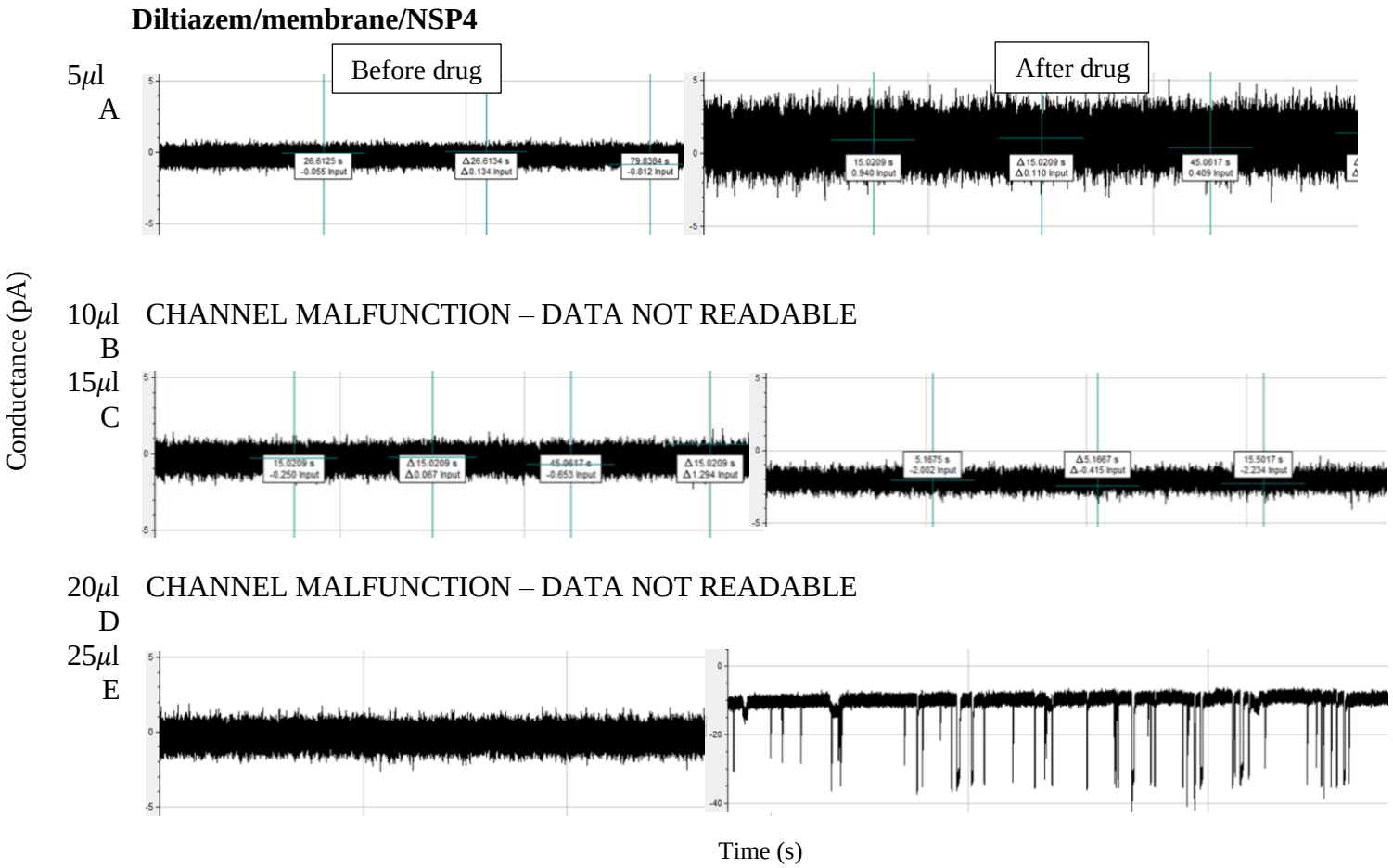


***Figure 6 diltiazem impact on membrane electro physics***. *Membrane conductance shift in presence of diltiazem. Trials each represent four channel replicates. Data on left represent pure membrane activity while data to right of second axis show the impact of the experimental drug conditions.*

**Control Data: Membrane/diltiazem**

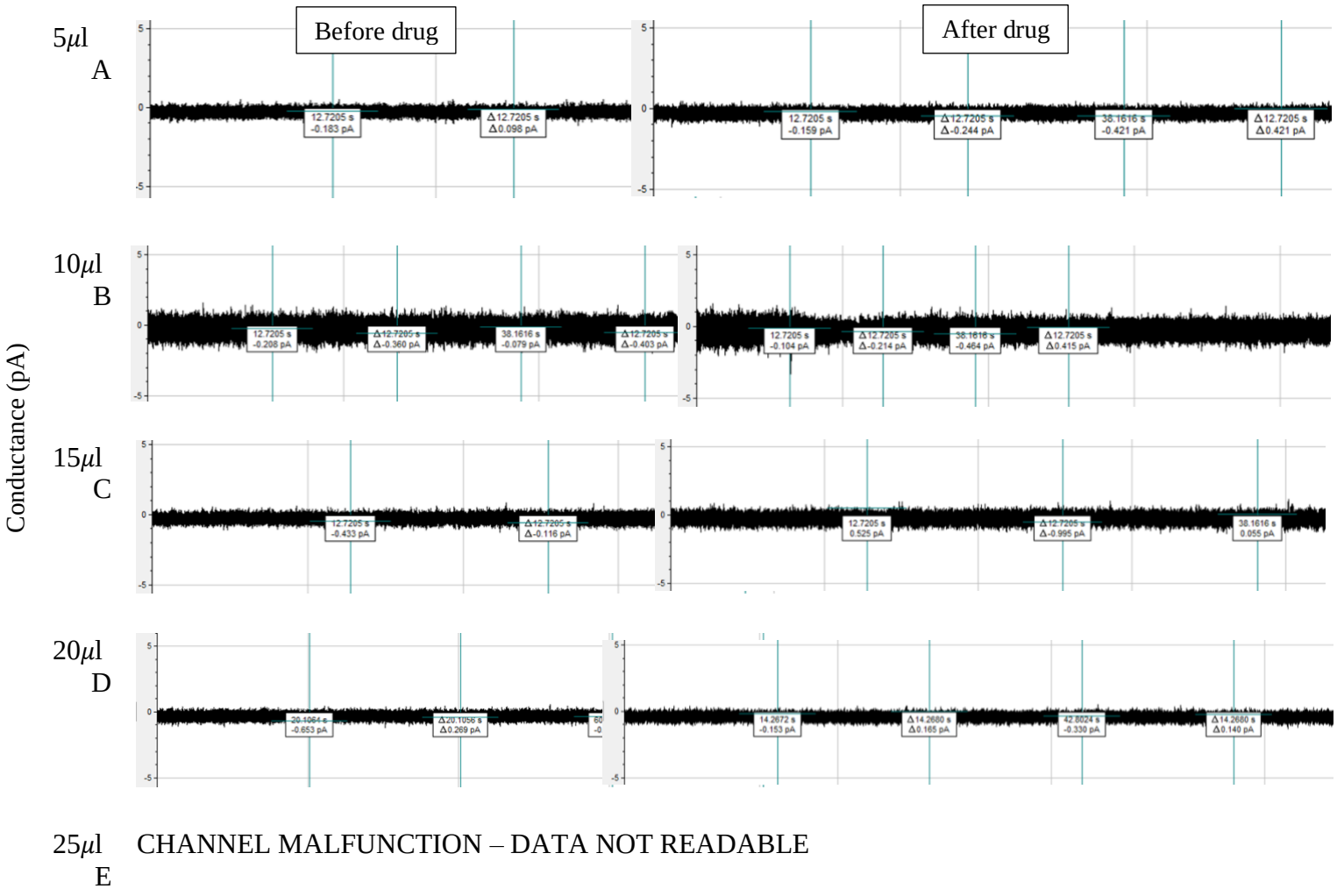


***Figure 7 diltiazem impact on membrane electro physics.*** *Membrane conductance shift in presence of diltiazem. Trials each represent four channel replicates. Data on left represent pure membrane activity while data to right of second axis show the impact of the experimental drug conditions.*

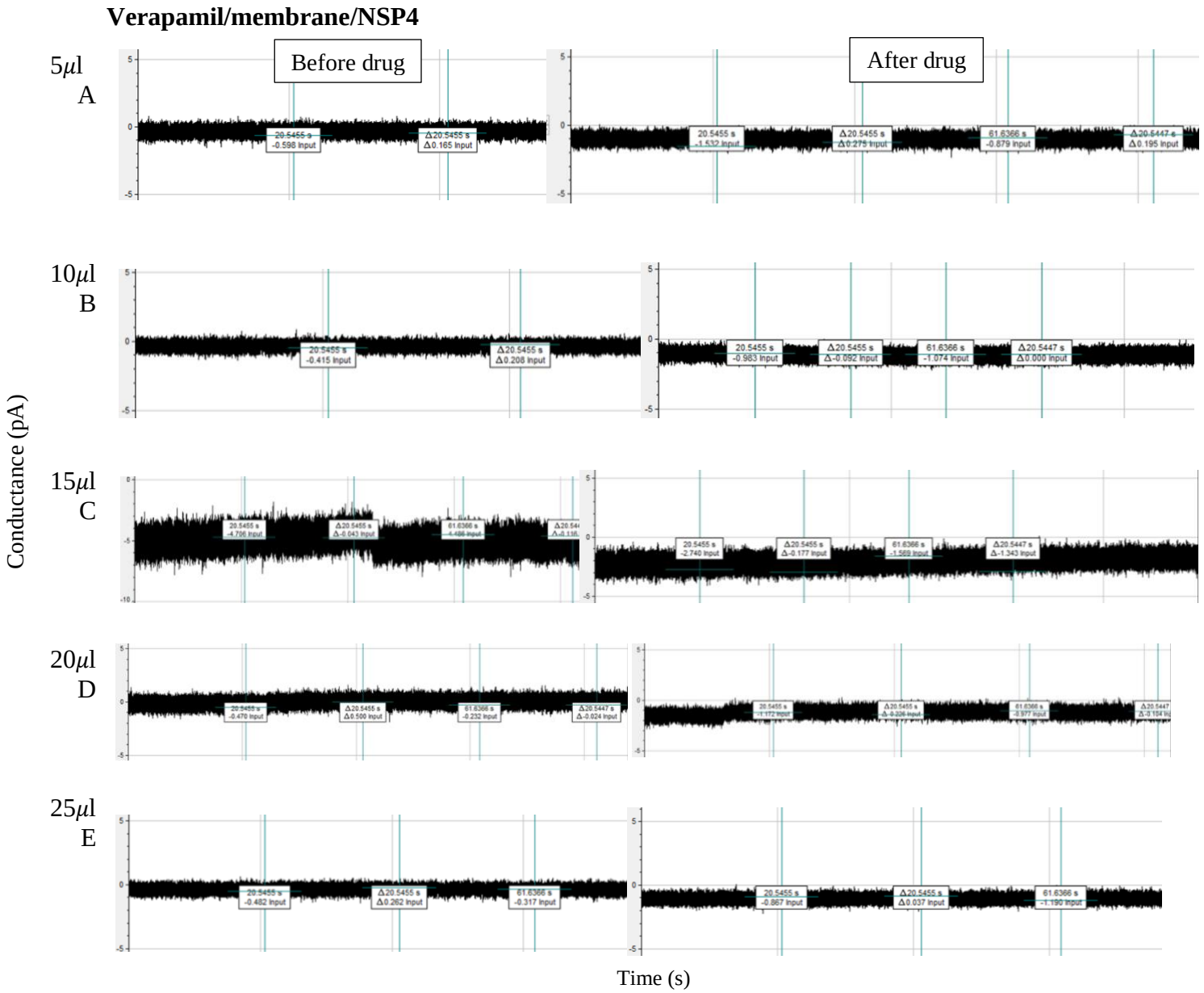


***Figure 8 verapamil impact on membrane electro physics.*** *Membrane conductance shift in presence of verapamil. Trials each represent four channel replicates. Data on left represent pure membrane activity while data to right of second axis show the impact of the experimental drug conditions.*

**Control Data: Membrane/verapamil**

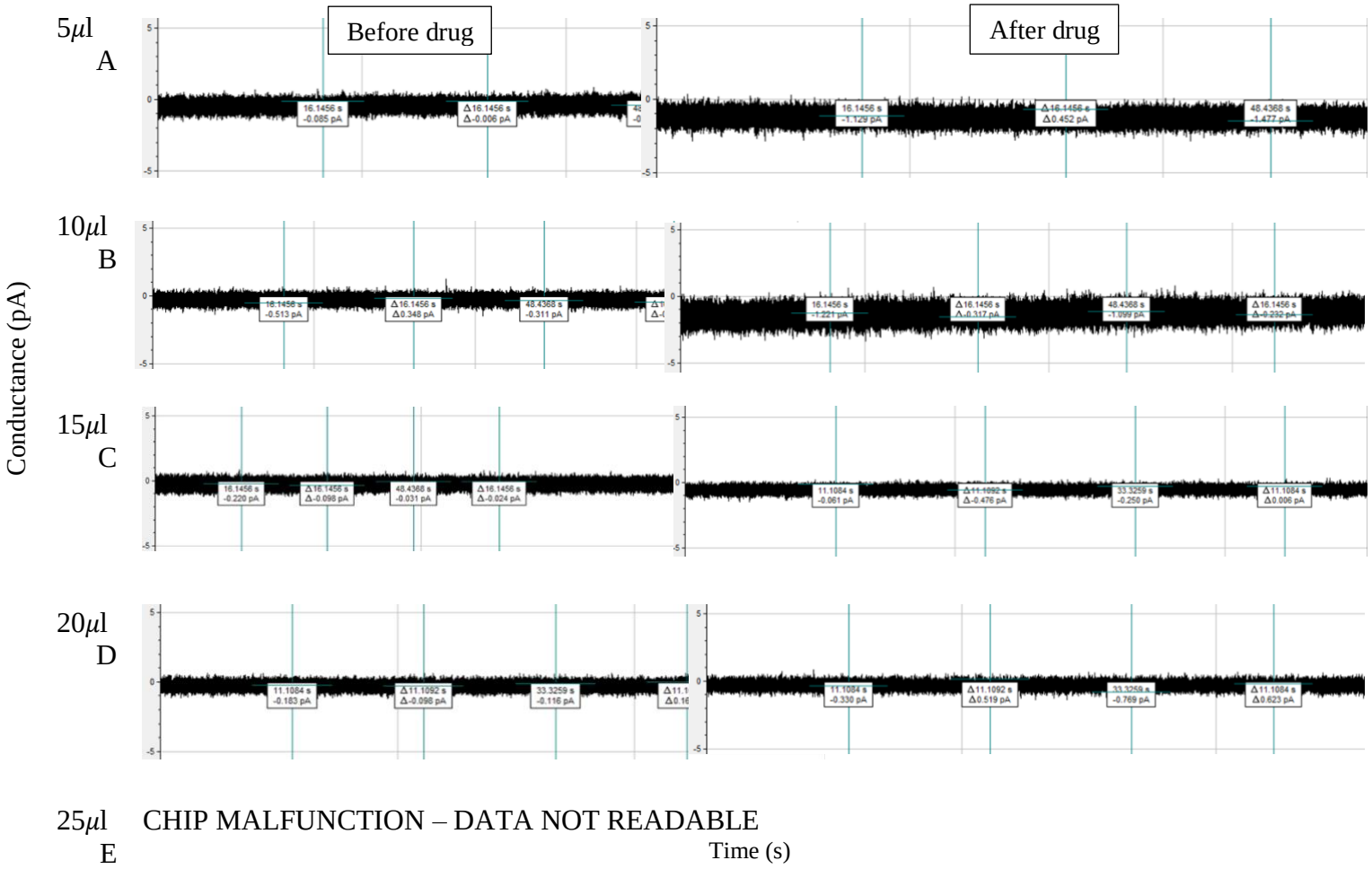


***Figure 9 verapamil impact on membrane electro physics***. *Membrane conductance shift in presence of verapamil. Trials each represent four channel replicates. Data on left represent pure membrane activity while data to right of second axis show the impact of the experimental drug conditions.*

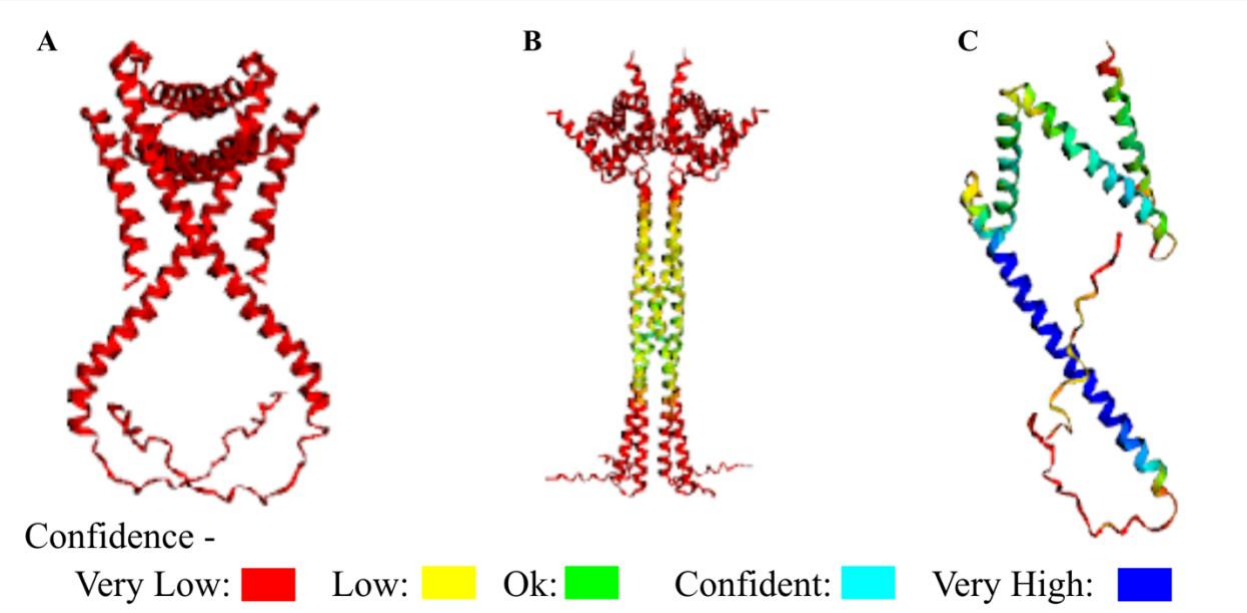


***Figure 10 Alpha fold NSP4 renderings****. Fig10A and B show the computationally generated structural confidence of the NSP4 tetramer. Fig10C shows the structural confidence of the NSP4 monomer.*

## Discussion

The aim of this study was to collect the preliminary electrophysiological data required to inform rational drug design for candidate NSP4 antagonists. Data collected herein provides valuable insight into the potentiality of this category of drugs as promising antiviral candidates. The conductance spikes before drug addition show positive activity of lab-isolated NSP4 reconstituted in lipid membrane, serving as the first trial to document the functionality of this lab-isolate viability and function. In addition, the experimental process itself has continued to formulate the basis for drug testing in the Banks Lab both by developing planar lipid bilayer methods and rationale. The behavior of our replicates are able to point developers towards interactions of interest in future study.

A significant point of interest for our project was that very few interactions were seen in our membrane-drug control trials (Fig5,7,9). The baseline of the membrane conductance curves did not significantly shift after amlodipine or diltiazem addition, however we did see a decrease in those baselines for verapamil. It is difficult to draw concise conclusions from these shifts without additional data analysis. Further, the amplitude of traces during these control assessments did not significantly shift. Showing a lack of interaction between the membrane and this class of drugs is promising because it implies that the drugs of interest have minimal unwanted impact on the membrane structure in this functional assay. While further analysis is necessary to confirm these findings, this also incurs relative confidence in our visually assessed relationships being the result of drug-protein activity as opposed to changes in the membrane environment.

An outstanding observation about the protein-drug interaction is the lowering of the baseline of the conductance curve (Fig4,6,8). As observed in each of the verapamil trials, after

drug addition the baseline of the conductance curves shifts from its calibrated point at 0pA to around -2pA. For verapamil specifically, a slight change in the amplitude of such curves can also be identified for trials with the addition of 15$\mu$l and up of drug addition. In comparison with the control experiments for the same trials, the baseline also shifts down, but much less dramatically with the general downward shift for membrane-drug trials to -.5pA. It is notable that verapamil is the only drug that caused a downshift of baseline in its membrane-drug controls. Diltiazem also caused the downshifting of baseline in its protein interactions, yet because of several channel malfunctions in the diltiazem data collection it is difficult to draw conclusions from its reduced set. With this in mind, we note that, like the verapamil dataset, upon addition of 15$\mu$l of drug and more the baseline of the diltiazem curves begin downward shifts. This trend, however, does not hold upon analysis of amlodipine interactions, which show dramatic changes in membrane conductance, dropping down to baselines around -40pA and more up until the point of 15$\mu$l addition.

Various studies have shown the efficacy of amlodipine as a clinical antiviral, with positive impacts on patient outcome being documented in Covid-19 and encephalomyocarditis viral cases(33-35). The dramatic impact of amlodipine on NSP4 function must be flagged for further exploration because the biochemical basis for its antiviral function is still unknown. It is hypothesized that a greater array of viruses utilizes calcium influx in their pathogenesis than previously known. Thus, channel blockers that target and downregulate these cellular mechanisms must move to the forefront of functional investigation. Additional planar lipid bilayer analysis will be a valuable tool in confirming whether or not these properties are due to L-type calcium channel blockers being effective viroporin antagonists.

It is hard to determine what change in the protein or membrane structure/activity could be causing these downshifts in the conductance baseline. The most effective way for future researchers to parse through this relationship is to perform a $P_o$ (probability open) analysis of the collected data, which would provide statistical probabilities as to whether the reconstituted NSP4 channels in the bilayer are open or closed. A drop in the conductance readings could also imply that contrary to hypothesis, after drug addition more ions are able to freely flow across the membrane. Because we see minimal baseline drop in our control studies besides those for verapamil, this appears to be an isolated protein-drug interaction, possibly leaving NSP4 channels open or in continuous activity(39). This would create a sustained increase in ion movement across membrane channels, leading to continued conductance drop. Harms et. al. is able to investigate the mechanics of gramicidin ion channel activity, using fluorescence microscopy and channel current recordings to determine open-closed transitions. Such analysis would shed light on the more specific single-cell NSP4 mechanism mechanics.

In both amlodipine and diltiazem, the addition of drug resulted in drastic shifts in the signature currents of the membrane conductance environment. Upon addition of small quantities of amlodipine, spikes in membrane conductance became increasingly drastic, as well as data for the 5$\mu$l and 15$\mu$l trials showing a continuous drop in membrane conductance. While it could be assumed that these extreme drops in conductance would result in membrane rupture, they stayed intact for the majority of the drug-protein trials. We therefore conclude that the drug must be impacting the reconstituted viroporins as opposed to these data being a result of a change in the membrane environment(40). Increases in conductance amplitudes suggest greater ion channel activity of NSP4, with pattern spikes representing a burst of openings/closings of the protein. While increases in conductance amplitudes are seen in the low dosage amlodipine trials, those

that received a dosage of 25$\mu$l show a reduction in activity of conductance curves. Desai et. al. use similar amplitude reduction data to confirm the deactivation of malaria-related ion channels(41).

The conductance data for diltiazem show contradictory drug interactions, as low dosages of drug seemed to drastically increase channel activity. Via inspection, a possible explanation for this increase in activity could be that protein activity did not begin in earnest until later in the trial when the diltiazem had been added. Furthermore, diltiazem's 25$\mu$l trial shows very distinct spikes in conductance dropping as low as -40pA. It is possible that this difficult channel data continues to be the result of channel malfunctions as seen in the 10$\mu$l and 20$\mu$l trials. Further testing is required to replicate these findings with more reliable hardware. Benzothiazepines akin to diltiazem are undergoing development as generalized antiviral antidotes. As specific 1,4-Benzothiazepine enantiomers enter clinical testing against respiratory syncytial virus, their biochemical pathways of function are still unknown(42). While clinical pharmacokinetic study is crucial to confirm the true effectiveness of an antiviral against its target, a significant gap in the research exists regarding the mechanistic pathways of these candidate drugs.

Verapamil shows the smallest impact on protein function. As noted above, each trial experiences a downshift in baseline conductance after drug addition, with some similarity between these downshifts and those seen in their membrane-drug control data. Furthermore, their amplitude readings after drug addition are reasonably similar to those before. As such, our body of data conclude that verapamil had the least impact on the functionality of NSP4 in bilayer. However, it remains a candidate for drug design because of its reliability in effect, and the fact that a much greater body of data is required to cement the correlations we've seen in this

preliminary study. Furthermore, it is possible that more acute mathematical analysis is required to understand a closer relationship between verapamil and NSP4.

The modest effect of verapamil on NSP4 activity is surprising given its growing popularity as a viroporin antagonist in a myriad of other viral systems. As an established antagonist, it was vital in the identification of classical swine fever virus (CSFV) nonstructural protein p7 as a viroporin(28). It has been shown further to regulate $Ca^{2+}$ equilibrium in influenza infection, reducing the activity of Raf/MEK/ERK signaling cascades which control production of viral progeny(43, 44). In addition to therapeutic effects specifically related to $Ca^{2+}$ channel blocking, verapamil has been shown to prevent filovirus cell entry and increase the antitumoral efficacy of oncolytic adenoviruses(29, 45). With the development of such promising literature on verapamil's efficacy as an antiviral candidate, an understanding of the biochemical pathway of action in these cases is critical.

These methods are adapted from the patch clamp analysis performed by Hyser et. al. that determined that NSP4 is a calcium conducting viroporin(13). In such work, NSP4 peptides were used at a concentration of 10 ng/ml. In translating that experimentation to PLB, isolated peptides were incorporated at a concentration of 1.77$\mu$g/ul. These results generally follow the conductance trace seen by Hyser's that show defined opening characteristics in bilayer. However, they also resemble those denoting fast kinetics. As such, distinct patterns of open and closed channel pulses are difficult to distinguish as there is significant noise in the data from the large quantity of rapidly pulsing channels. Our fast-kinetic data implies that smaller concentrations of protein are necessary to record explicit channel openings and closings. This is of note for future experimentation but shows promising behavioral patterns for the validation of protein-drug interactions.

Another point of interest in this study and for future research is in relating the drug concentration curves used in these PLB experiments to their effective concentrations. A variety of functional concentrations is present in the literature for all three drugs, and it is difficult to determine the optimum range because there has been very little explicit experimentation regarding these specific drugs and methods. In comparison to other functional drug literature in planar lipid bilayer, this study utilized lower drug concentrations than those expected for experiments performed in cell culture. Literature on diltiazem has shown its efficacy at around 30% in calcium channel blocking in the 20-50$\mu$M range in a perforated patch system(46). amlodipine has shown effective activity at .5$\mu$M in-vitro and 3.5$\mu$M in the reduction of ROS signaling in cell culture(47). On $K^+$ channels in a patch clamp system, verapamil was effective at a concentration of 3$\mu$M(48). A point of consideration in these cases is the level at which these concentrations are representative of the respective concentration expected in the body. As drug discovery trials continue to evolve in the PLB system, further information about effective quantities will inform these findings.

It is apparent that technical issues with MECA chip reuse was having an impact on results. During experimentation, chips were reused for a number of trials via specifications from the manufacturer. While it is noted in Nanion literature that chips are, “usually reusable for several days if not mechanically damaged”, we began seeing issues with chip reliability and functionality much sooner than that in our trials. None of our chips incurred significant mechanical damage and all were used within the correct experimental timeframe. However, many soon lost responsiveness in one or all their channels. Although those that showed unresponsiveness were cycled out of use in these studies, it is impossible for us to know whether there was a spectrum of impact on data reliability for the chips before complete lack of response.

Many also began to continuously read massive, oscillating conductance spikes with or without protein or membrane presence. These issues flag experimental concern about the reliability of our replicates. Because each channel of the four-channel chips is poised to read as a membrane replicate for experiment, there is no concise way for us to remove the chips as a source of error via experimentation. Chip rotational periods were reduced to 1-2 days to continuously cycle fresh hardware into use. Despite their drawbacks, these discoveries inform future experimentation and understanding of the technical issues surrounding the minion system.

Because the planar lipid bilayer is a hyper simplified system modeling NSP4 activity, an important area of study for future drug development will be whether or not the same impacts we see in the bilayer model take place when NSP4 is in its target environment, the rough ER.

While planar lipid bilayer electrophysiology is one of our best available systems to study ion channels, the reproducibility of methods can be quite low due to difficulty establishing uniform membranes. Reconstitution of bilayers is a stop-and-start process, and layers can be exceedingly fragile, requiring the re-establishment during trial(38). Despite these issues, electrophysiology techniques including PLB and the patch clamp remain incredibly important techniques for modern rational drug design systems(49). Ion channels are vital therapeutic targets for drug design, standing as the second largest class of drug targets in FDA approved drugs(50). As such, it is critical that reliable and reproducible methods continue to be established in electrophysiological research such as this.

Regarding clinical drug development relevance, an RNA virus, rotavirus is far more prone to genetic mutation than viruses in the DNA family(51). Because of this rapid evolutionary turnover, rotavirus presents a high quantity of genetically recombinant strains. This in turn makes the development of lasting antiviral therapies tenuous, and drug resistance is common. It

is important to target critical regions of protein and viral function to focus drug development on features that are unlikely to be mutated. The necessity of NSP4 for viral maturation and symptomatic response ensures that antagonists stand the test of viral evolution(52, 53). It is likely that any antivirals developed will be administered as a part of a drug cocktail given to treat severe cases of rotavirus gastroenteritis. A significant body of literature shows that drug cocktails are the most effective way to administer antiviral drugs for the alleviation of symptomology(54). Thus, identifying drug families with unique antagonistic methods against NSP4 for the application of combination therapy is a favorable strategy for continued symptom mitigation. The application of a multitude of antiviral strategies also decreases the likelihood of evolved viral resistance(55, 56). As global attention is turned toward the significance of viral research in the wake of the SARS-CoV-2 pandemic, the importance of proactive antiviral development has never been clearer.

Our computational programs determined that all existing models of the tetramer have exceedingly low confidence in their structural accuracy, as visualized by Figure 10. AI models that generate such structures are intended to work in concert with experimental structure data. As issues then arise with residue alignment, known experimental data can then inform and resolve them. The regions of NSP4 that have been successfully modeled are the result of crystallographic refinement, whereas the insoluble domains remain low confidence. In addition, AI programs are built with training datasets of known protein structures from a wide taxonomic range of organisms. Therefore, as resolves residue locations it is informed by data from protein from related families. Because there does not exist a training dataset for viral proteins, NSP4 lacks yet another cornerstone for the AI validation of protein structure.

**Future Directions**

As drug discovery is an intensive and lengthy process, there are a multitude of avenues for the continuation of NSP4 antagonist research. While electrophysical data provide a reliable functional assay regarding the behavior of the simulated membrane and NSP4, information about the binding kinetics of the drug and protein are essential through the continued drug design process. In future experimentation, the development of further methods to probe the binding affinity of NSP4 and target drugs would be beneficial. There are several widely available experimental techniques available to assess these interactions. Thermofluor assays are widely applicable for the determination of protein-small molecule interaction, and their methods have been reliably validated for the prediction of quantifiable binding affinities(57, 58). Tryptophan fluorescence quenching assays, X-ray crystallography, and solution NMR are also available for these purposes(59-61). Whatever technique is employed, an understanding of ligand-drug binding affinity for developed antivirals is critical for the drug refinement process that takes place between preclinical development and further in-vivo drug testing. Without apt binding kinetics, even the most functional of antivirals will not sustain a response in its target cell.

Dry-lab computation also serves as a valuable tool to understand and confirm drug-protein binding kinetics and functionality. In addition to the general need for a viral training dataset in computational biology, experimental data would greatly improve confidence in viral protein models. This can involve crystallizing NSP4 or employing cryogenic electron microscopy in wet-lab technique, or more complex protein model refinement protocols, specifically molecular dynamics simulations in computation. Furthermore, it is suggested that future AI analysis involve the refinement of individual monomers and the use of docking software to combine and refine them into a higher confidence tetromer.

As noted by Weissmann 2022, reliable proteomic algorithms can create interaction models with nearly wet-lab-generated accuracy(62). These algorithms have the benefit of being able to run interaction simulations in a multitude of membrane/cell environments. With a more reliable structural prediction, the results of these interactions could be documented in a model rough ER membrane – the true target environment of NSP4. Future work into NSP4 drug design in the Banks Lab will capitalize on this approach to continue employing computational methods side-by-side with bench laboratory findings.

No biochemical target will find its perfect match in a pre-existing drug. Dozens of rounds of alteration are required to optimize various characteristics of candidate drugs. Analysis is required to tune binding kinetics, assess functionality while associated with target protein, hone drug delivery methods, and more. In addition to these many avenues of biochemical investigation, further replication of these findings and investigation into the nuances of L-type calcium channel interactions is critical to confirm the findings of this study. As planar lipid bilayer research in the Banks Lab continues, future investigators should make the statistical analysis of such data a priority in order to more effectively quantify the functionality of these drugs while associated with target protein. $P_o$ analysis is a method that while time consuming, provides probability assessments for whether reconstituted ion channels are open or closed, thus establishing quantifiable evidence of drug efficacy.

L-type calcium channel blockers encompass a large family of drugs, and even though we prioritized the testing candidates from all three subdivisions, there are dozens of other calcium channel blocking drugs that could incur greater efficacy in blocking protein activity. A more complete exploration of other candidate channel blockers is required to effectively screen the family for effective antiviral potential. This larger dataset would also give antiviral developers

the opportunity to compare the structure and function of a greater range of molecules and hone the future development process.

The Banks Lab has recently been awarded an R15 grant from the National Institute of Allergy and Infectious Diseases to continue this investigation of NSP4 targeted antivirals. As viroporins continue to be discovered and established as key components in viral pathogenesis, understanding key structural features that may impact the efficacy of channel blockers will be vital to streamline the development of antiviral therapies. In our modern era, the importance of prompt antiviral design and research has become shockingly apparent. As the impact of the covid-19 pandemic continues to shake the world's public health infrastructure, it is imperative that we continue proactively *plugging holes* in our understanding of viroporin targetability.

**Literature Cited**


1. Hall E, Wodi A, Hamborsky J, Morelli V, Schillie S. 2021. Epidemiology and prevention of vaccine-preventable diseases. US Department of Health and Human Services, Centers for Disease Control and ….
2. LeClair CE, McConnell KA. 2022. Rotavirus, StatPearls [Internet]. StatPearls Publishing.
3. Troeger C, Forouzanfar M, Rao PC, Khalil I, Brown A, Reiner RC, Fullman N, Thompson RL, Abajobir A, Ahmed M. 2017. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet infectious diseases 17:909-948.
4. Burnett E, Parashar UD, Tate JE. 2020. Global impact of rotavirus vaccination on diarrhea hospitalizations and deaths among children< 5 years old: 2006–2019. The Journal of infectious diseases 222:1731-1739.
5. Ramig RF. 2004. Pathogenesis of intestinal and systemic rotavirus infection. Journal of virology 78:10213-10220.
6. Rao VC, Seidel KM, Goyal S, Metcalf TG, Melnick JL. 1984. Isolation of enteroviruses from water, suspended solids, and sediments from Galveston Bay: survival of poliovirus and rotavirus adsorbed to sediments. Applied and environmental microbiology 48:404-409.
7. Kraay AN, Brouwer AF, Lin N, Collender PA, Remais JV, Eisenberg JN. 2018. Modeling environmentally mediated rotavirus transmission: The role of temperature and hydrologic factors. Proceedings of the National Academy of Sciences 115:E2782-E2790.
8. Wilson SE, Morris SS, Gilbert SS, Mosites E, Hackleman R, Weum KL, Pintye J, Manhart LE, Hawes SE. 2013. Scaling up access to oral rehydration solution for diarrhea: Learning from historical experience in low–and high–performing countries. Journal of Global Health 3.
9. Carvalho MF, Gill D. 2018. Rotavirus vaccine efficacy: current status and areas for improvement. Human vaccines & immunotherapeutics.
10. Wondimu A, van der Schans J, van Hulst M, Postma MJ. 2020. Inequalities in rotavirus vaccine uptake in Ethiopia: a decomposition analysis. International Journal of Environmental Research and Public Health 17:2696.
11. Markkula J, Hemming-Harlo M, Salminen MT, Savolainen-Kopra C, Pirhonen J, Al-Hello H, Vesikari T. 2017. Rotavirus epidemiology 5–6 years after universal rotavirus vaccination: persistent rotavirus activity in older children and elderly. Infectious Diseases 49:388-395.
12. Beards G. 2017. Rotavirus. WikiJournal of Medicine 4:1-12.
13. Pham T, Perry JL, Dosey TL, Delcour AH, Hyser JM. 2017. The rotavirus NSP4 viroporin domain is a calcium-conducting ion channel. Scientific reports 7:43487.
14. Zambrano JL, Díaz Y, Pena F, Vizzi E, Ruiz M-C, Michelangeli F, Liprandi F, Ludert JE. 2008. Silencing of rotavirus NSP4 or VP7 expression reduces alterations in Ca2+ homeostasis induced by infection of cultured cells. Journal of virology 82:5815-5824.
15. Pérez JF, Chemello ME, Liprandi F, Ruiz M-C, Michelangeli F. 1998. Oncosis in MA104 cells is induced by rotavirus infection through an increase in intracellular Ca2+ concentration. Virology 252:17-27.
16. Brunet J-P, Jourdan N, Cotte-Laffitte J, Linxe C, Géniteau-Legendre M, Servin A, Quéro A-M. 2000. Rotavirus infection induces cytoskeleton disorganization in human intestinal

epithelial cells: implication of an increase in intracellular calcium concentration. Journal of Virology 74:10801-10806.
17. Brunet J-P, Cotte-Laffitte J, Linxe C, Quero A-M, Géniteau-Legendre M, Servin A. 2000. Rotavirus infection induces an increase in intracellular calcium concentration in human intestinal epithelial cells: role in microvillar actin alteration. Journal of virology 74:2323-2332.
18. Fischer WB, Hsu H-J. 2011. Viral channel forming proteins—modeling the target. Biochimica et Biophysica Acta (BBA)-Biomembranes 1808:561-571.
19. Griffin SD. 2009. Plugging the holes in hepatitis C virus antiviral therapy. Proceedings of the National Academy of Sciences 106:12567-12568.
20. Carrasco L. 1995. Modification of membrane permeability by animal viruses. Advances in virus research 45:61-112.
21. Chen I-Y, Moriyama M, Chang M-F, Ichinohe T. 2019. Severe acute respiratory syndrome coronavirus viroporin 3a activates the NLRP3 inflammasome. Frontiers in microbiology 10:50.
22. Lahiri H, Arkin IT. 2022. Searching for blockers of dengue and West Nile virus Viroporins. Viruses 14:1750.
23. Snutch TP, Peloquin J, Mathews E, McRory JE. 2013. Molecular properties of voltage-gated calcium channels, Madame Curie Bioscience Database [Internet]. Landes Bioscience.
24. Lacinová L, HOFMANN F. 2001. Voltage-dependent calcium channels. Heart physiology and pathophysiology 4.
25. Duffy EC, Banks LD. 2022. Preclinical Drug Development of a Novel Antiviral Target in Rotavirus. The FASEB Journal 36.
26. Bkaily G, Jacques D. 2009. L-type calcium channel antagonists and suppression of expression of plasminogen receptors: is the missing link the L-type calcium channel?, vol 105, p 112-113. Am Heart Assoc.
27. Katz AM, Hager WD, Messineo FC, Pappano AJ. 1984. Cellular actions and pharmacology of the calcium channel blocking drugs. The American journal of medicine 77:2-10.
28. Gladue DP, Holinka LG, Largo E, Fernandez Sainz I, Carrillo C, O'Donnell V, Baker-Branstetter R, Lu Z, Ambroggio X, Risatti GR. 2012. Classical swine fever virus p7 protein is a viroporin involved in virulence in swine. Journal of virology 86:6778-6791.
29. Gehring G, Rohrmann K, Atenchong N, Mittler E, Becker S, Dahlmann F, Pöhlmann S, Vondran FW, David S, Manns MP. 2014. The clinically approved drugs amiodarone, dronedarone and verapamil inhibit filovirus cell entry. Journal of Antimicrobial Chemotherapy 69:2123-2131.
30. Jayaseelan VP, Paramasivam A. 2020. Repurposing calcium channel blockers as antiviral drugs. Journal of Cell Communication and Signaling 14:467-468.
31. Wang X, Luo J, Wen Z, Shuai L, Wang C, Zhong G, He X, Cao H, Liu R, Ge J. 2022. Diltiazem inhibits SARS-CoV-2 cell attachment and internalization and decreases the viral infection in mouse lung. PLoS Pathogens 18:e1010343.
32. Sahney S. 2006. A review of calcium channel antagonists in the treatment of pediatric hypertension. Pediatric Drugs 8:357-373.

33. Choksi TT, Zhang H, Chen T, Malhotra N. 2021. Outcomes of hospitalized COVID-19 patients receiving renin angiotensin system blockers and calcium channel blockers. American Journal of Nephrology 52:250-260.
34. Zhang L-K, Sun Y, Zeng H, Wang Q, Jiang X, Shang W-J, Wu Y, Li S, Zhang Y-L, Hao Z-N. 2020. Calcium channel blocker amlodipine besylate therapy is associated with reduced case fatality rate of COVID-19 patients with hypertension. Cell discovery 6:96.
35. Wang WZ, Matsumori A, Yamada T, Shioi T, Okada I, Matsui S, Sato Y, Suzuki H, Shiota K, Sasayama S. 1997. Beneficial effects of amlodipine in a murine model of congestive heart failure induced by viral myocarditis: a possible mechanism through inhibition of nitric oxide production. Circulation 95:245-251.
36. Gutsmann T, Heimburg T, Keyser U, Mahendran KR, Winterhalter M. 2015. Protein reconstitution into freestanding planar lipid membranes for electrophysiological characterization. Nature protocols 10:188-198.
37. Zaitseva E, Obergrussberger A, Weichbrodt C, Boukhet M, Bernhard F, Hein C, Baaken G, Fertig N, Behrends JC. 2021. Electrophysiology on Channel-Forming Proteins in Artificial Lipid Bilayers: Next-Generation Instrumentation for Multiple Recordings in Parallel. Patch Clamp Electrophysiology: Methods and Protocols:67-92.
38. Suzuki H, Tabata KV, Noji H, Takeuchi S. 2006. Highly reproducible method of planar lipid bilayer reconstitution in polymethyl methacrylate microfluidic chip. Langmuir 22:1937-1942.
39. Yelshanskaya MV, Patel DS, Kottke CM, Kurnikova MG, Sobolevsky AI. 2022. Opening of glutamate receptor channel to subconductance levels. Nature 605:172-178.
40. Spencer CI, Uchida W, Turner L, Kozlowski RZ. 2000. Signature currents: a patch-clamp method for determining the selectivity of ion-channel blockers in isolated cardiac myocytes. Journal of Cardiovascular Pharmacology and Therapeutics 5:193-201.
41. Desai SA, Bezrukov SM, Zimmerberg J. 2000. A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. Nature 406:1001-1005.
42. Henderson EA, Alber DG, Baxter RC, Bithell SK, Budworth J, Carter MC, Chubb A, Cockerill GS, Dowdell VC, Fraser IJ. 2007. 1, 4-Benzodiazepines as inhibitors of respiratory syncytial virus. The identification of a clinical candidate. Journal of medicinal chemistry 50:1685-1692.
43. Nugent K, Shanley J. 1984. Verapamil inhibits influenza A virus replication. Archives of virology 81:163-170.
44. Alam M, Mostafa A, Kanrai P, Müller C, Dzieciolowski J. 2016. Verapamil has antiviral activities that target different steps of the influenza virus replication cycle. J Antivir Antiretrovir 8:121-130.
45. Gros A, Puig C, Guedan S, Rojas JJ, Alemany R, Cascallo M. 2010. Verapamil enhances the antitumoral efficacy of oncolytic adenoviruses. Molecular Therapy 18:903-911.
46. Hart J, Wilkinson MF, Kelly ME, Barnes S. 2003. Inhibitory action of diltiazem on voltage-gated calcium channels in cone photoreceptors. Experimental eye research 76:597-604.
47. Saddala MS, Lennikov A, Mukwaya A, Yang Y, Hill MA, Lagali N, Huang H. 2020. Discovery of novel L-type voltage-gated calcium channel blockers and application for the prevention of inflammation and angiogenesis. Journal of neuroinflammation 17:1-23.

48. Ko EA, Park WS, Son YK, Ko J-H, Choi T-H, Jung ID, Park Y-M, Da HH, Kim N, Han J. 2010. Calcium channel inhibitor, verapamil, inhibits the voltage-dependent K+ channels in rabbit coronary smooth muscle cells. Biological and Pharmaceutical Bulletin 33:47-52.
49. Demuro A, Parker I. 2005. "Optical Patch-clamping" single-channel recording by imaging Ca2+ flux through individual muscle acetylcholine receptor channels. The Journal of general physiology 126:179-192.
50. Overington JP, Al-Lazikani B, Hopkins AL. 2006. How many drug targets are there? Nature reviews Drug discovery 5:993-996.
51. Koonin EV. 2017. Evolution of RNA-and DNA-guided antivirus defense systems in prokaryotes and eukaryotes: common ancestry vs convergence. Biology direct 12:1-14.
52. Ng TI, Dorr PK, Krishnan P, Cohen DE, Rhee S, Wang SX, Ruzek MC, Mensa FJ, Kati WM. 2021. Biomarkers for the clinical development of antiviral therapies. Cytometry Part B: Clinical Cytometry 100:19-32.
53. Rhee S-Y, Jordan MR, Raizes E, Chua A, Parkin N, Kantor R, Van Zyl GU, Mukui I, Hosseinipour MC, Frenkel LM. 2015. HIV-1 drug resistance mutations: potential applications for point-of-care genotypic resistance testing. PloS one 10:e0145772.
54. Shyr ZA, Cheng Y-S, Lo DC, Zheng W. 2021. Drug combination therapy for emerging viral diseases. Drug discovery today 26:2367-2376.
55. Baum A, Fulton BO, Wloga E, Copin R, Pascal KE, Russo V, Giordano S, Lanza K, Negron N, Ni M. 2020. Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. Science 369:1014-1018.
56. Sepúlveda-Torres LdC, Rishishwar L, Rogers ML, Ríos-Olivares E, Boukli N, Jordan IK, Cubano LA. 2017. A decade of viral mutations and associated drug resistance in a population of HIV-1+ Puerto Ricans: 2002–2011. Plos one 12:e0177452.
57. Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P. 2001. High-density miniaturized thermal shift assays as a general strategy for drug discovery. Journal of biomolecular screening 6:429-440.
58. Bai N, Roder H, Dickson A, Karanicolas J. 2019. Isothermal analysis of ThermoFluor data can readily provide quantitative binding affinities. Scientific reports 9:1-15.
59. Maveyraud L, Mourey L. 2020. Protein X-ray crystallography and drug discovery. Molecules 25:1030.
60. Yammine A, Gao J, Kwan AH. 2019. Tryptophan fluorescence quenching assays for measuring protein-ligand binding affinities: principles and a practical guide. Bio-protocol 9:e3253-e3253.
61. Li Y, Kang C. 2017. Solution NMR spectroscopy in target-based drug discovery. Molecules 22:1399.
62. Weissman AD. 2022. AI in Proteomics: A Comparison Between Crystallographic and in silico Methods of Protein Modeling.