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# A Tri-Project Approach: Expanding Three Different Frontiers of Neuroscience

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# A Tri-Project Approach: Expanding Three Different Frontiers of Neuroscience

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**Abstract**

We identify three routes in which we can advance the field of neuroscience's ability to model increasingly complex systems. We identified and reviewed the in vitro neuronal model of HT22 cells, while also offering the field a comprehensive and optimized culturing protocol to produce a matured in vitro model. We also identify a novel optogenetic construct and neural circuitry labeling technique that will base their in vitro modeling within the established HT22 differentiation protocol we present. The constructed optogenetic construct can facilitate dual input recording within the Mesolimbic excitatory neurons, advancing the reach of optogenetic evaluation in the context of the Beaudoin Lab as well as the field at large. The proposed neurocircuitry method also advances the Beaudoin Lab's interest in reward circuitry, as we propose a Notch-Cre and Delta-based mechanism that can ultimately facilitate structural and functional mapping within one experiment without neurotoxicity. Ultimately, we extend the utility of the labeling method to encompass more complex field-wide interests of circuit-specific knock-ins or knock-outs in an immensely efficient and regulated fashion. We detail three novel and distinct models with dual utility for the Beaudoin lab and the field.

**Tri-Project Introduction**

Neuroscience is a burgeoning field defined by the utilization of innovative techniques to model a uniquely complex, unexplored, and integrative discipline. The communication present in neural networks, and the vast array of neurobiological processes that encompass it, are far from a binary or simple connection. However, much of the unforeseen development in tracing these complex and previously inaccessible processes stemmed from model systems as simple as Yeast or incidental novel findings upon observing simple processes (Pfeffer 1994 and Lee et al. 2016). The complex behaviors of a given model can be grouped into distinct categories, and even be studied and taught at the academic course level- but the behaviors themselves are represented by a complex network of activation and inhibition within and between subregions and grand regions at various timescales (Curly et al. 2011, Mogenson 2018, and Krasnegor et al. 1997). Oftentimes, it is essential to backtrace complex processes to their neurobiology of synaptic function at the cellular level, or even further back in the context of neural development and gene expression, to make further advancement at the field level. Since behavior is itself a composite of a vast array of signaling pathways, representing the end output of neural signaling, the study of applicable behavioral neuroscience is limited in the field's knowledge of the foundational. Most research proposals and funding stems from the contention that a given project yields implications that can ultimately be modeled and solved in human populations. In this way, translating behavioral neuroscience from model organisms to humans is the ultimate goal of every long-term project. Yet, these projects cannot be understood, much less achieved,

without prior cell and mechanistic experimentation to build from. By thoroughly modeling and executing projects at the in vitro and ex vivo level, we enhance the depth and breadth of what can be studied and worked towards. More than just advancing the scope of what can be studied, such a neurobiological approach to neuroscience can actually revamp academic pedagogy to enhance how we study and educate (Schwartz et al. 2019). The focus of this Thesis, however, will contextualize how a neurobiological approach to neuroscience can benefit novel research by advancing the foundations of Neuroscience research at large.

Intrinsic in this foundational-based approach to neuroscience are models that allow hyper-focusing on given properties or processes. While foundational research may often involve re-exploring well-defined neural properties such as synaptic transmission or synaptic vesicle formation en route to providing subsequent research with more background, another route is comprehensive technique formation (Krnjevic 1974, Newman 2003, Chanaday et al. 2019, and Brose et al. 2019). Techniques evolve with the field, ultimately allowing the scope of what we may ask to evolve as well. This technique evolution can take the form of a novel groundbreaking method (Notch-Delta Synaptic Labeling) or can take the form of revamping prior constructs and expanding previous methods (ChrimsonR's role in advancing Optogenetics). Sometimes, the evolution is more abstract and can take the form of creating a new model system to facilitate more accessible, accurate, and affordable research (HT22 Cells).

Here, I will explore three different technique models that, at present, have gaps that can be improved upon to advance the field of Neuroscience. Further, I propose three distinct models that I have begun to incorporate en route to offering field-wide solutions to a lack of an

optimized HT22 differentiation and culturing protocol, expression of a functional red-shifted Channelrhodopsin, and an efficient trans-synaptic genetic labeling technique. The projects individually serve as a utility to address a given field need or gap, while also directly interacting with one another. Without an optimized cell line to test construct expression and fidelity of my novel optogenetic and synaptic labeling techniques, the subsequent projects become stalled or even inaccessible. Ultimately, I present the argument that each of the constituent projects in my thesis serves a utility to labs within, and even outside, Neuroscience en route to broadening the scope of future inquiry.

Project 1: Mortalizing the Immortal: Creating an optimized HT22 Differentiation and Culturing Protocol

**Introduction**

Cell culturing is often the initial integral step for most whole-animal or system-based experiments, as many expression-based experiments, genetic experiments, biochemical mechanistic evaluations, and neurobiological research first rely on quantification and qualification in cell-based systems (Mergenthaler et al. 2012 and Bosche et al. 2013). Neurobiological research in cell-based systems is often taxing and expensive due to the various natures of different neural cells of interest in the field requiring complex approaches like stem cell isolation or culturing of primary cells (Uchida et al. 2000, Beaudoin et al. 2012, Herrup and Yang 2007, and Potter and Demarse 2001). This is true in hippocampal systems where neurogenesis is especially regulated, with several key mechanisms that modulate synaptic plasticity (Sorrells et al. 2018 and Feng et al. 2017). It thus becomes difficult to introduce model cultures that may emulate various neuronal subtypes due to the resource and time expenditure in merely procuring a primary or stem cell culture to grow. As a result, subsequent fields in neuroscience become stalled as initial testing of constructs containing channelrhodopsins, proteins of interest to be tested, or drugs of interest must be either upscaled to an in vivo procedure or wait for a successful and suitable primary culture that can withstand such testing. More than halting the progress of already established methods, a lack of an accessible and consistent cell model precludes any evolution of current techniques or innovation of new techniques. Ultimately, the extent of maintenance and effort placed into primary culturing

produces cultures prone to immense experimental error and repeated variation that is often unjustified for simple in vitro experimentation (Xicoy et al. 2017 and Al-ali 2014).

While Neuroscience is famous for its plentiful array of rodent models, it has historically lacked an efficient and universal in vitro system to center around (Ellenbroek and Youn 2016). Recent work to broaden the models utilized has included projects to expand the use of animals like the Zebrafish, the Pig, and the Marmoset for example (Kalueff et al. 2014, Lind et al. 2007, and Kishi et al. 2014). Included in this recent push towards an expanded model system array are in vitro methods to create different types of cell cultures to be utilized in different research projects (Mertens et al. 2016). While extensive work has been done to create neural progenitor cells from somatic cells en route to creating a functional in vitro model, Merterns et al. 2016 describe even more techniques that have arisen to advance neuronal type modeling to match that of excitatory cortical neurons, dopaminergic neurons, motor neurons, interneurons, and glial cells via specific reagent and transcription factor addition (though the conversion to glial cells is distinct from the other categories). Ultimately, the Merterns review showcases a litany of different approaches to create in vitro systems- with Human Pluripotent Stem Cells (hPSC) differentiation being the more expansive and robust approach relative to the induced neuron method that requires specific sets of transcription factors and the induced glial cell method. However, despite the field-wide rally to address a need for in vitro systems, the field had still been left with taxing protocols that require not only several weeks at minimum (and more often months) to produce usable cultures but also several additional surgeries to procure donor tissues, and an array of reagents that vary by safety level clearance. The end product of such

stem cell culturing techniques is often relatively representative of a given project's need for an in vitro model but is often intrinsically distinct from in vivo models.

Alternative in vitro models take the approach of feasible and efficient usage, ultimately aiming at making a usable, but largely unspecified, neuronal model. A common iteration of this approach is to take a given cell line that is neuronal to some degree but also has broad characteristics that make its culturing, subculturing, maintenance, and actual use fairly feasible and cheap. Neuronal models that stem from Neuroblastoma cell lines, such as the SH-SY5Y line which was originally procured from a bone tumor biopsy, often serve this purpose exactly, with frequent division paired with a semi-neuronal cell (Lopes et al. 2010 and Kovalevich and Langford 2013). However, aside from intrinsic issues related to SH-SY5Y's origin from Neuroblastomas, the model has been shown to lack key mature isoforms of tau or broad dopaminergic character (Mandelkow and Mandelkow 2012, Lotta et al. 2010, and Xicoy et al. 2017). This is problematic as its primary usage involves serving as an in vitro model for Alzheimer's and Parkinson's disease, and has been utilized in an unoptimized form for about 82% of the papers that cite using this cell line (Xicoy et al. 2017). The Xicoy review further contextualizes an array of culturing and differentiation protocols, with varying steps, reagents, and results, that are all intentionally used to create a mature SH-SY5Y cell line that can model the same disease (Kovalevich and Langford 2013, Encinas et al. 2002, Sarkanen et al. 2002, Schneider et al. 2002, Teppola et al. 2016, and Yang et al. 2016). The ultimate issue that plagues the SH-SY5Y is much more than its genomic, biochemical, and functional deviations from its in vivo models- but rather its non-uniform methodology and sourcing that precludes any field-wide extrapolation of findings since the field lacks even a consensus on the appropriate

maintenance media which ultimately drastically changes the cells biology (Xicoy et al. 2017, Wu et al. 2009, and Huang et al. 2015). The SH-SY5Y line possesses a litany of intrinsic variance in its tumorigenic origin and different sources to buy from. Further, the line carries extrinsic differences in the vast array of culture methods, its existence as a cell-adhesion or suspension line, the choice to differentiate it, and the various ways in which can be differentiated to different effects. The dual sources of variance weaken the validity and reliability argued for its in vitro modeling capabilities. SH-SY5Y is the most popular in vitro line, though other models such as the PC12 line and H19-7 line operate in the same function as alternatives to primary or stem cell culturing that lack a unified culturing methodology (Wiatrak et al. 2020, Westerwink and Ewing 2007, Hattangady and Rajadhyaskha 2009, Moon et al. 2013, Oh et al. 2008, and Eves et al. 1996).

Here we identify a possible candidate to be introduced as a new model culture for neurons, HT22 cells, that we aim to implement as an in vitro testing apparatus for ranges of experiments such as optogenetics, plasmid expression, and electrophysiology. HT22 cells, which are immortalized hippocampal neurons that are subcloned from HT4 cells, are sometimes used in place of primary cell cultures for hippocampal research and have led to key empirical findings (Maher and Davis 1996, Liuet al. 2009, Stanciu et al. 2000, Suh et al. 2006, and Kenney et al. 2019). The immortalization process stems from transforming the cells with SV40 Large T antigen, which both binds with the Rb-E2F complex to induce profuse proliferation, as well as inhibits p53 to inhibit growth arrest and apoptosis (Anand et al. 2012 and Prasad et al. 1994). HT22 cells' immortalized nature allows for culturing on plates, rapid replication cycles, and easy

maintenance which has provided the field with a faster and more efficient alternative to primary cultures or previously described embryonic stem cell culturing.

HT22 cells are often utilized in glutamate-induced cytotoxicity experiments, as the cell line lacks ionotropic glutamate receptors that normally trigger receptor-induced stress and thus are not susceptible to excitotoxicity (Park et al. 2019, Davis and Maher 1994, and Murphy et al. 1989). HT22 cell's lack of excitotoxicity susceptibility also has led way to immense oxidative stress studies, as He et al. 2013 outlines a field-wide consensus that glutamate inhibits cysteine uptake culminating in cysteine and glutathione depletion en route to oxidative stress (He et al. 2013, Davis and Maher 1994, Sagara et al. 1998, Levinthal and Defranco et al. 2005, Luo and Defranco 2006, Tepkeer et al. 2007, Fukui et al. 2009, Kim et al. 2009, Yoon et al. 2010, and Zhao et al. 2012). Recent literature has highlighted the explosion of HT22 cells in experiments ranging from new evaluations of oxidative death, genomic changes associated with diabetic conditions, genomic changes associated with Alzheimer disease progression, and gene silencing experiments (Park et al. 2020, Wu et al. 2020, Sukprasansap et al. 2020, Liu et al. 2020, Cui et al. 2020, Wang et al. 2019 and Ni et al. 2019). HT22 cells are derived from mouse hippocampal cells which allow for both general modeling of neurons as seen from its utilization in recent research, as well as the potential to offer a representative in vitro model for the complex hippocampal network (Van Strien et al. 2009 and Buzsaki et al. 2003). However, due to their immortalization HT22 cells lose several neuronal properties, namely arrested division as a result of markers keeping neurons in the G0 phase (Yoshikawa 2000 and Buchakjian et al. 2010). HT22 cell division ultimately lends itself to a cascade of mutations and alterations to the cells over time that results in essentially non-neuronal cells that lack the mature neuronal markers

indicative of a mature, differentiated, and functioning neuron (Roskams et al. 1998). The modulation of natural cell maintenance processes culminates into a cascade of downstream effects that, while initially allowing semi-valid in vitro modeling of neurons, produce a cell line with progressive degeneration (Anand et al. 2012). The same issue present in the SH-SY5Y cell is seen within HT22 cells; there is ambiguity on the cell line's origin and even less of established literature and consensus on how to optimize the cell line (He et al. 2013, Liu et al. 2009, Zhao et al. 2012. Zhang et al. 2018, Bonaterra et al, 2018, Dermol-Cerne et al. 2018, Moreau et al. 2017, and Inda et al. 2017).

The Beaudoin lab chose to utilize HT22 cells over the other major hippocampal cell line, H19-7 derived from Rat tissue, due to HT22's mouse origin and relevance to the immediate lab projects (described later) and the field's preferential usage of mouse lines (Brossaud et al. 2013, Eves et al. 1992, Wu et al. 2004, Morrione et al. 2000, and Ellenbroek and Youn 2016). Similarly, PC12 cells, an early in vitro neuronal model established from transplanted rat adrenal pheochromocytoma, originates from rats and are limited in their applicability to mouse model projects in the lab and field (Greene and Tischler 1976). However, both these rat-based models serve immense utility in the path to establishing an optimized HT22 cell line as at least a portion of the core differentiation pathways should be shared, with an immense crossover of some reagent utilization and effects (Greene and Tischler 1976, Aid et al. 2006). Therefore, the creation of a standardized protocol for culturing, transfection, and differentiation will pull from the litany of already present rat in vitro models for reference of some conserved mechanisms. Further, we will build from prior HT22 culturing and differentiation work to guide our study. Limited HT22 differentiation experiments have already shown immense changes in neuronal

character, namely the Zhao et al. 2013 paper which found that with only a one-day differentiation HT22 cells began to express NMDA receptors which operate as ionotropic glutamate receptors (Zhao et al. 2013). This finding that differentiation can induce glutamatergic receptor expression ultimately showcases utility beyond merely its original purpose for glutamate-induced toxicity experiments, and rather can showcase other properties such as excitatory and cholinergic character (He et al. 2013 and Liu et al. 2009).

Here, we identify a protocol of differentiation of HT22 cells that we believe will increase a myriad of parameters that involve mature and differentiated neurons but specifically will increase factors such as transfection efficiency, plasmid expression across a variety of different promoters, induction of mitotic arrest, and expression of mature neuronal markers, promoters, and characteristics. While HT22 cells used in the majority of the literature has been undifferentiated in character, we believe the establishment of an optimized differentiation protocol will increase both internal validity, as they will lose properties of rapid mutation that weakens the power of the study, and external validity of results, as the cells will better mirror neurons if they are differentiated. We hypothesize that by utilizing previous approaches to differentiation of cells seen in the literature and testing different supplements we can optimize a differentiation protocol for HT22 cells to curtail some of the discrepancies that the immortalized line intrinsically comes with. Ultimately, we review the body of work for HT22 cells and test an array of different protocols to provide the field with an optimized and robust method to follow.

We will use the neuron-specific promoter CAMK-II-α as a core primary readout of differentiation since it is a common neuron-specific promoter used for differentiation readouts and is actively expressed in the brain at the synaptic level to facilitate core functions like long-term-potentiation (Ping and Grabowski 2007 and Lisman et al. 2002). HT22 cells in their immortalized state not only lose a lot of the neuronal character but over time can lose the ability to undergo normal metabolic or post-transcriptional modifications (PTM) due to increased genetic mutation. Transfection is empirically difficult with neuronal cultures, with primary culture expression often at around 3%, and added cellular/genetic machinery dysregulation due to immortalization may be expected to decrease this efficiency even lower (Ohki et al. 2001). Thus, we hypothesize that the utilization of Neurobasal media with N2 or B27 supplement, describe in detail below, will both increase the transfection efficiency (the proportion of cells with detectable GFP driven by a CAMK-II-α promoter) and the expression efficiency (the intensity of GFP expression in the cells that are GFP positive) by increasing the differentiation character while also addressing basic cellular dysregulation. Further, we hypothesize that utilizing other known differentiation reagents such as Nerve Growth Factor and Retinoic Acid (described below) will further increase differentiation character and internal regulation such that both expression efficiency and Transfection efficiency will also rise. Since CamK-II-α selectively expresses in glutamatergic neurons and previous literature showcases newfound glutamatergic character in HT22 cells following differentiation, we hypothesize that an interaction effect between differentiation media and supplements will exist to increase both expression and transfection efficiencies of the CamK-II-α driven GFP (Zhao et al. 2013). Finally, we expect that the interaction of multiple supplements together, mimicking in vivo mechanisms

of differentiation, and, to be more significant than individual components in readouts of apoptosis, mitotic arrest, and post-transcriptional regulation.

**Method**

Maintenance of HT22 cultures

*1.1 Cell-culturing in gas regulated chambers*

Cell culturing is often done in gas-tight culture chambers designed for controlled regulation of the internal environment (Morrison et al., 2000). The chamber should continually monitor for conditions of 5% CO2 and 37 degrees Celsius, as well as sterilized before use with 70% ethanol, during culturing procedures with mammalian cells. Proper tubing to the chamber should be installed and monitored to avoid incidental CO2 depletion and death of cells. Additionally, separate cultures of cells should be grown in different chambers to limit cross-contamination and allow ample room for plates.

*1.2 Ensuring Sterile Conditions*

Sterile culturing is done in part by the usage of antibiotics in the media, as well as standard sterile techniques. Usage of a cell culturing hood with positive pressure and laminar flow equipped with filtering systems and UV ray lamps ensures a sterilized cell culturing station, while consistent maintenance and monitoring of the chamber ensure sterile growth. The presence of infected cultures is often immediately visible with several black dots apparent on live in vitro cultures when observed under a phase-contrast inverted scope. Additionally, when staining for imaging with fluorescent dyes like DAPI, microbes that have infected cultures subsequently are stained and apparent when using more advanced microscopes like a confocal scope (Morikawa and Yanagida 1981). Once an infection has been observed, plates are bleached

and the chamber is re-sterilized, as are the hoods and all equipment. Additionally, media is remade entirely with fresh batches of solutions, and new aliquots of trypsin are thawed.

*1.3 Culturing and Subculturing HT22 cells*

Standard tissue culturing plates are used to culture HT22 cells, and the media used to culture HT22 cells consists of Dulbecco's Modified Eagle Medium (Thermofisher 21013024) that is supplemented with 10% Fetal Bovine Serum, L-Glutamine (100 mM), and 1% of penicillin and streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin) (Cesarini et al. 2018). The fetal bovine serum contains all ingredients that are integral for cell growth and is key for use in expression systems involving HT22 cells since previous studies highlight HT22 cell response to serum-deprived conditions (Van der Valk 2005 and Steiger-Barraissoul 2009). Once plates reach 80% to 90% confluence, media is aspirated, and cells are washed with Dulbecco's Phosphate Buffered Saline without Calcium or Magnesium (ThermoFisher A1285601) to remove cell waste and residual FBS that will inhibit trypsinization. HT22 cells are then subcultured for continuation or experiment via a trypsinization reaction for 5-10 minutes at 37 degrees C and transference into new plates at ratios of 1:10 to last for around 2.5 days before the next split (Ricardo 2008). Total resuspension should be observed under a phase-contrast microscope to account for correct splits for continuation and experiment. Additionally, sterility should be ensured under a microscope just before sub-culturing into a new plate and the next day following reattachment.

<u>Expression experiments in HT22 cells</u>

*2.1 Transfection of HT22 cells*

After three passages following thawing, HT22 cells can be split for transfections to induce expression of the encoded protein. Cells should be plated 24 hours before transfection at a cell concentration of 50,000 cells per well for a 24 well. To avoid reattachment to the tissue culturing plates and ensure accurate cell concentrations for transfections, HT22 cells are transferred to a sterile plastic conical tube after trypsinization to be counted for an accurate split using a sterile glass hemacytometer.

Cationic liposome reagent, Lipofectamine 2000, is used to facilitate ectopic gene expression in mammalian cells (Dalby et al. 2004). Lipofectamine is mixed with opti-MEM (Thermo-fisher 51985091) with a ratio of 4ul of lipofectamine in 50µl of opti-MEM per well, while 1ug per well of the plasmid of interest is mixed, separately, in 50 µl of opti-MEM per well. The plasmid is associated with the lipofectamine upon mixing each of the solutions in a 1:1 ratio and letting sit for five minutes. After the association, 100µl of the new solution can be added dropwise to each well before putting the plate back in the chamber.

Transfection efficiency with HT22 cells can be increased by limiting cell plate exposure out of the chamber, as detachment from the coverslips can occur. Additionally, letting the transfection sit for less than 72 hours presents a risk of cell sheeting which results in cells growing off the coverslip. It appears 48 hours works well as a timestamp of both optimized expressions of the ectopic genes and optimized cell concentration.

*2.2 Differentiation of HT22 cells*

Differentiation can be accomplished by replacing the media of the transfected cells after 48 hours with 500µl of Neurobasal media containing either N2 supplement (1:100 dilution from

a 100x stock) or B27 supplement (100mM) with added Nerve Growth Factor (at a 1:1000 ratio from a 100ug/mL stock), and Retinoic Acid (1:1000 ratio from a 25mM stock).

The Neurobasal media works as a serum-free media supplemented with nutrients integral for cell growth, like amino acids, inorganic salts, and vitamins (Brewer et al. 1993). The Neurobasal media contains no ferrous sulfate, reduced osmolarity, and has reduced amino acid content to create a favorable environment for in vitro neural culturing. The use of the serum-free media is designed to support the growth of neural cells by adding cell growth factors in specific concentrations, as well as remove possible toxic elements to neurons present in serum (Barnes & Sato 1980 and Gospodarowicz et al. 1979). The N2 supplement, a media supplement that contains a subset of B27 components, is used to stimulate initial differentiation of HT22 cells into mature neural cells, as well as inhibit differentiation into non-neuronal subsets (Dhara and Stice 2008, Kaech and Banker 2006, Liu 2006, and Price and Brewer 2001). The N2 supplement is used in place of the more commonly used B27 supplement in most neuronal cultures because of its empirically increased efficiency with the denser cell concentrations needed for optimized transfection efficiency (Bottenstein and Sato 1979 and Brewer et al. 1993). However, B27 is also commonly used in neuronal cultures and is a primary reagent for culturing and differentiation of a litany of other cell types (Brewer et al. 1993, Xie et al. 2000, Brewer 1995, Lesuisse and Martin 2002, Vollner et al. 2009, Ying and Smith 2003, and Sato and Clevers 2012). B27 has utility in preventing neuronal death under hypoxic conditions and has been shown to inhibit glycolysis (Sunwoldt et al. 2017).

Nerve growth factor has been shown to increase neuronal survival in vitro, as well as promote and maintain growth of ganglia (Kromer 1987, Levi-Montalcini and Booker 1960, and

Hendry et al. 1974). Nerve growth factor (NGF) receptors are concentrated in the hippocampus and neocortex, and previous literature has established its use in the prevention of cholinergic neuron death after axotomy (Kromer 1987 and Schwab et al. 1979, and Korsching et al 1985). NGF is itself a neurotrophic factor, discovered in her own words "unexpectedly" in 1951 by Dr. Levi-Montalcini, which has been found to be empirically key to morphological differentiation, neuronal gene expression, and maintenance of differentiation character (Aloe et al. 2016, Levi-Montalcini and Hamburger 1951, Cattaneo et al. 2013, and Levi-Montalcini 1987). NGF is thought to be most integral in the initial stages of differentiation, and slowly loses signaling efficacy as the neuron matures- yet still being integral in certain end-stage aspects of some neuronal cell types (Levi-Montalcini 1987 and Lindsay and Harmar 1989). Processes like mitotic arrest, protein methylation, and gene regulation of several hundreds of genes are directly induced by the addition of NGF, highlighting its integral function and immense utility in immature neuronal models (Cragnolini et al. 2012, Cimato et al. 1997, Green et al. 1986, and Angelastro et al. 2000 ).

Retinoic acid (RA) has also been previously employed in HT22 cells, and was found to upregulate endogenous CAMK-II-α expression- boding relevancy as a key element for natural neuronal differentiation as well as utility for selective upregulation of CAMK-II-α driven promoters seen in projects 2 and 3 (Roumes et al. 2016 and Chen and Kelly 1996). RA is often utilized with TPA (12-O-Tetradecanoylphorbol-13-acetate) plays pivotal roles in neuronal differentiation, chiefly via increasing growth associated protein-43 expression, neurite outgrowth, increasing nuclear receptor signaling to modulate gene expression, and even can upregulate expression of K+ and Ca2+ currents (D'Orlando et al. 2007). TPA operates on the

Protein Kinase C (PKC) pathway in a concentration-dependent manner- modulating the pathway en route to establishing differentiated morphological character in Neuroblastoma lines (Perez-Juste and Aranda 1999). Used individually, retinoic acid can still induce differentiated character and gene upregulation at a significant level (D'orlando et al. 2007). In this study, RA and NGF will be supplement additions into one of the three base media, with TPA not utilized as a core element of our protocol due to reagent limitations and crossover functionality with RA (D'Orlando et al. 2007).

2.3 Staining and Imaging Cells

Cells were fixed in 4% Paraformaldehyde for 20 minutes before being washed and were permeabilized with 0.05% Triton in PBS and stained with a 1:10,000 solution of DAPI (5 mg/ml stock in DMSO). DAPI is a DNA intercalating and condensing agent that binds to the minor groove of DNA, localized in the nucleus, that fluoresces blue (Kapuscinski 1995). In this manner, it can be used as a reference point of locations of cell bodies when imaging. Most plasmids used in expression experiments contain GFP, which can be used to detect both levels of gene expression and localization of gene expression (Chalfie et al. 1994).

When imaging with epifluorescent microscopes blue-green co-excitation can result in false positives of expression (Choi et al. 2017). Autofluorescence also presents an issue as weakly expressing ectopic genes can be hard to distinguish from background fluorescence in cell culture. Antibody staining of the GFP can solve both imaging issues by highlighting specifically what is product versus what is background or bleed over (Spitzer et al 2011). Additionally, using a scope with narrower emission filters like a confocal scope or switching the staining agent to a

red-shifted reagent like propidium iodide can eliminate bleed over as well (Jones and Senft 1984). In cases of cell cultures that weakly express a given promoter, a combination of confocal imaging and anti-GFP antibody tagging can greatly amplify GFP fluorescence while simultaneously greatly reducing background fluorescence. The use of antibody staining and confocal imaging greatly reduce issues with autofluorescence and weak expression at the imaging level, while usage of the macro described below can enhance viewer visualization of the constructs of interest.

Antibody staining for GFP, or mature neuronal markers such as Microtubule Associated Protein (1:1000 of mouse antibody) or Beta-III-Tubulin (1:1000 of mouse antibody), can be accomplished with an overnight wash with a primary stain followed by a one-hour wash with a secondary stain (Alexa 488 for Beta-III-tubulin and Alexa 647 for MAP-II-Kinase). Normal Goat Serum was used as a blocking agent Following the secondary stain, the normal protocol of DAPI staining and coverslipping should ensue.

Mature neuronal marker analysis should also be paired Ethidium Bromide and Acridine Orange stain (EB/AO) which can offer both general indications of cell survivability and nature of cell death (Kasibhatla et al. 2006, Ribble et al, 2005, Rackova et al. 2009 Banerjee et al. 2019, and Renvoize et al. 1998). The EB/AO stain plays on the ability of AO to enter all cells and cause green fluorescence, whereas EB can only enter compromised membranes and will dominate over the AO stain to produce red readout (Ribble et al. 2005). To ascertain cell survivability, 100ug/mL of Acridine Orange and 100ug/mL of Ethidium Bromide were mixed in PBS and added at a 1:150 500uL dilution to be added to the cells. Imaging was done on an inverted scope while cells were alive and within a 24 well dish.

Flow cytometry can give a general readout of cell survivability, but this specific staining method is also meant to be visualized as the patterning of the stain can give finely tuned readout; condensed green chromatin would indicate non-necrotic early-stage apoptotic cells whereas condensed red chromatin (presenting as an orange circle) indicates late-stage apoptosis and normal red chromatic expression indicate necrotic death (Renvoize et al. 1998).

## Analysis

### *3.1 Macro details*

A macro was utilized to identify both transfection and expression efficiency of transfections and transductions. The macro identified the DAPI positive nuclei stains of every cell in a given image plane and identified coexpression of GFP in that isolated region. The macro then outputs the total number of cells in a given image, the total number of GFP positive cells in a given image, and the average GFP intensity both within each cell that was GFP positive and the overall GFP for all cells. Limitations of the macro stem from an inability to characterize non colocalized GFP expression, which is common for the membrane-bound GFP tagged to the Channelrhodopsin in the CamK-II-α promoter-driven constructs. Further, this mode of analysis is limited to transfection and expression efficiency read-outs for GFP- and cannot characterize other forms of fluorescence or morphological cues.

### *3.2 Statistical Analysis*

An initial ANOVA will be conducted to determine initial differences in the expression of an array of different promoters.

A two-way between-groups ANOVA test was performed in 3 iterations to determine supplement effects. First, a two-way between-groups ANOVA was done to test the interaction between Media type (N2, B27, or Regular DMEM) and Retinoic Acid. Then, a second two-way ANOVA will be performed to determine the interaction between the same set of Media types and the Nerve Growth Factor. A final two-way ANOVA will be performed to determine the interaction between media and supplement combination (RA + NGF).

To characterize the apoptotic effects induced from differentiation, a final one-way ANOVA will be performed to test the mean incidence of apoptosis and the ratio of apoptosis to necrosis in cells identified as undergoing death from an EB/AO stain. This Data will follow the Thesis submission due to time constraints.

Further, a two-way ANOVA will be utilized to characterize the expression of EF1-α, a constitutive promoter involved in elongation, between differentiated and undifferentiated HT22 cells as a readout of improved post-transcriptional regulation as a proxy readout for internal cellular regulation (Teschendorf et al. 2002). This Data will follow the Thesis submission due to time constraints.

Finally, a three way ANOVA will be performed to ascertain how the supplements interact with N2 and B27 media directy. Thus, offering a lens of comparison across media types.

**Results**

To establish a baseline for plasmid expression in the HT22 cell line, an array of promoters were transfected and analyzed with the aforementioned macro to measure mean GFP and maximal GFP among positive cells. Elongation Factor 1 alpha and Phosphoglycerate Kinase are constitutive promoters involved in translation and glycolysis respectively, while simian virus 40 and cytomegalovirus are viral transient promoters (Cho et al. 1995, Bernstein and Hol 1998, Benoist and Chamben 1981, and Rodova et al. 2013). A hybrid construct containing the Chicken-Beta-Actin promoter with an early CMV enhancer (pCAGGS) is also commonly used as a highly efficient promoter, as it contains constitutive and viral elements to upregulate expression (Alexopoulou et al. 2008). Finally, CAMK-II-α was previously described as a highly active neuron-specific promoter that has been previously used as an in vitro neuronal readout due to its empirical forebrain-specific expression in transgenic mice (Ping and Grabowski 2007 and Tsien et al. 1996 ). A baseline test will be conducted to characterize baseline promoter expression for these 6 promoters in HT22 cells, following previous literature promoter characterization in other cell lines (Qin et al. 2010). The CAMK-II-α plasmid transfected as the modified ChrimsonR construct that was ligated into a halorhodopsin vector and is characterized in project 2. There was a main effect of promoter type on both mean, $F(5,821)= 9.427$, $p= 0.000$, $\eta_p^2= 0.054$, and maximal GFP output, $F(5,821)= 15.707$, $p= 0.000$, $\eta_p^2= 0.084$. An LSD Post-Hoc Test identified that the CAMK-II-α output in regards to Maximal GFP intensity was only significantly different from SV40, P=0.002 (Table 3). In the context of Mean GFP output, it was also found that CAMK-II-α output was only significantly different from SV40, p=0.001 (Table 4). These results and Figures 1 and 2 indicate that undifferentiated HT22 cells selectively express

SV40 promoters, while poorly expressing other promoters such as EF1-alpha and pCAGGS. CMV had detectable expression, though it was still statistically less than SV40. Tables 1 and 2 show the overall trend that only viral promoters have significantly different mean and maximal GFP intensity from baseline, with SV40 having a significantly higher expression than CMV. One notable exception to this is EF1-α, while did not have a significantly different mean GFP expression than baseline but did have a maximal GFP expression than baseline which matches the intense fluorescence seen in Figure 16 when EF1-α becomes operational.

HT22 cells were transfected with either CAMK-II-α driven GFP as shown in figure 15, or SV40 driven GFP as a control, and were either undifferentiated, differentiated with N2 supplement in Neurobasal media, or differentiated with B27 supplement in neurobasal media. NGF and RA were also differentiation supplements that were both withheld and added from every media condition to establish an array of media conditions that may affect GFP expression- with a hypothesis that increased differentiation character would correspond with increased CAMK-II-α expression. To understand the effect the differentiation components may have played on CAMK-II-α readout, an array of 2-way Between Groups ANOVA was performed. Evaluating the B27 condition saw no main effect of NGF supplement on the maximum CAMK-II-α GFP intensity, $F(1,373)=0.899$, $p= 0.344$, $\eta_p^2 =0.002$, observed power= 0.15 There was however a significant effect of RA, $F(1,373)=8.797$, $p= 0.003$, $\eta_p^2 =0.003$, observed power= 0.841. There was also an observed interaction effect between NGF supplement and RA in B27 that suggests each supplement's effect on max intensity changes in the presence of the other, $F(1,373)=0.511$, $p= 0.024$, $\eta_p^2 =0.014$, observed power= 0.616. Figure 3 showcases the negative effect of RA added to B27 media, while also showing a compensatory effect when NGF is

present in the context of the maximal GFP output. Interestingly, Figure 3 shows this trend is reversed for N2 media.

In the context of mean GFP, there was no main effect of NGF, $F(1,373)=0.070$, $p= 0.791$, $\eta_p^2 =0.000$, observed power= 0.058. There was a main effect of RA in B27 media on mean GFP output, $F(1,373)=8.339$, $p= 0.004$, $\eta_p^2 =0.000$, observed power= 0.058. Additionally, there was an observed interaction effect of NGF and RA suggesting that the effect of one supplement on mean CAMK-II-α GFP expression changed in the presence of the other supplement, $F(1,373)=4.807$, $p= 0.029$, $\eta_p^2 =0.013$, observed power= 0.590. Figure 4 maintains the same trends shown in figure 3, with RA having a negative effect in B27 (with NGF compensation) and it having a positive effect in N2 (without apparent NGF compensation) in context of maximal GFP output.

When transfection efficiency was utilized as a readout, there was no main effect of NGF, $F(1,6)=0.252$, $p= 0.633$, $\eta_p^2 =0.040$, observed power= 0.071, or RA, $F(1,6)=2.181$, $p= 0.190$, $\eta_p^2 =0.267$, observed power= 0.239. There was also no interaction effect between the supplements, $F(1,6)=1.460$, $p= 0.272$, $\eta_p^2 =0.196$, observed power= 0.179. Figure 5 shows a contrasting trend from prior figures, as it showcases an increased transfection efficiency in B27 in the presence of RA and decreases efficiency in N2 in the presence of RA. The inverted nature of this data is likely related to background pixel bleed over culminating in false-positive output as these trends seen in Figure 5 do not match the visual output shown in Figures 6 and 7.

These results suggest that neither the addition of NGF nor RA increased the transfection efficiency process, but rather RA by itself decreased the average maximum intensity across all GFP positive cells as well as average fluorescence. Further, there was an observed interaction

effect between RA and NGF within B27 supplement media shown in Table 4 and Figure 3 suggesting the addition of RA without NGF seemingly reduces the mean GFP intensity relative to either RA or NGF supplements alone. These match the phenotype of the transfected cells shown in Figure 6. With the NGF only condition and NGF with RA having readily perceived expressions. Transfection efficiency depicted in figure 5 and the ANOVA readout may be hampered by confounding autofluorescence pixels giving false positive readout.

The same evaluation of supplement effects in N2 media was performed. Similar to the B27 media, there was no main effect of NGF on maximal GFP expression within N2 media, $F(1,263)=2.728$, $p= 0.100$, $\eta_p^2 =0.010$, observed power= 0.377 (Figure 4). Additionally, there was a main effect of RA on maximal GFP intensity, $F(1,263)=7.538$, $p= 0.006$, $\eta_p^2 =0.028$, observed power= 0.781. There was also no observed interaction effect suggesting that NGF and RA did not have different effects on maximal GFP output in the presence of one another, $F(1,263)=0.730$, $p= 0.394$, $\eta_p^2 =0.003$, observed power= 0.730.

The same evaluation was performed for mean GFP intensity, and again finding no main effect of NGF on mean GFP expression in N2 media, $F(1,263)=3.528$, $p= 0.061$, $\eta_p^2 =0.013$, observed power= 0.465 (Figure 3). There was also a main effect RA in N2 media on mean GFP expression, $F(1,263)=4.928$, $p= 0.027$, $\eta_p^2 =0.018$, observed power= 0.599. There was additionally no interaction effect between NGF and RA in N2 media which suggests that neither supplement's effect on mean GFP expression changed in the presence of the other, $F(1,263)=0.589$, $p= 0.444$, $\eta_p^2 =0.002$, observed power= 0.119. When transfection efficiency was utilized as a readout, there was no main effect of NGF, $F(1,6)=1.636$, $p= 0.248$, $\eta_p^2 =0.214$, observed power= 0.191, or RA, $F(1,6)=1.762$, $p= 0.233$, $\eta_p^2 =0.227$, observed power= 0.202.

There was also no interaction effect between the supplements, $F(1,6)=0.042$, $p= 0.845$, $\eta_p^2 =0.007$, observed power= 0.053

These results as they are presented suggest that neither supplement increases the transfection efficiency in N2 media, but rather, RA individually increases both the Mean and Maximal GFP output is driven by a CAMK-II-α promoter. Visual representation in figure 7 showcases this story with an interesting caveat, with the effect of RA supplement alone increasing the brightness and total count of GFP positive cells relative to the other conditions. However figure 8 highlights a unique morphological neurite outgrowth only seen in the dual supplement condition and not characterizable by solely using CAMK-II-α GFP fluorescence intensity as a readout (though the morphological detail highlighted by the fluorescence may be used), Transfection efficiency depicted in figure 7 and the ANOVA readout may be hampered by confounding autofluorescence pixels giving false positive readout and thus poses a need for a more tuned readout.

Finally, a 3-way between-groups ANOVA was performed to elucidate the exact nature of N2 media and B27 media-based differentiation protocols and how they might compare directly to one another. The 3-way ANOVA allows exploration of complex comparisons across both media types, and initially showed no main effect of Media alone, $F(1,636)=0.045$, $p= 0.832$, $\eta_p^2 =0.000$, observed power=0.055. There was an observed interaction effect of RA on mean CAMK-II-α GFP expression such that the addition of RA had significantly different effects on account of what media it was added to, $F(1,636)=12.833$, $p= 0.000$, $\eta_p^2 =0.020$, observed power=0.947. The graphical results are showcased in Figure 10. Indicate a positive effect in N2 media and a negative effect in B27 media. There was also an observed main effect of RA, NGF,

and Media such that the interaction effect was significantly different between the two media types, $F(1,636)=4.035$, $p= 0.045$, $\eta_p^2 =0.006$, observed power=0.520. The graphical trends are showcased in Figure 11, depicts a stronger effect of interaction in the N2 condition relative to the B27 condition, with the all supplement condition culminating in the highest mean for N2 while corresponding to the second-lowest mean for B27. A subsequent 3-way ANOVA was performed to evaluate Maximal GFP expression, and the results were replicated from the mean GFP analysis for the RA-Media interaction effect, $F(1,636)=15.972$, $p= 0.000$, $\eta_p^2 =0.024$, observed power=0.979, and the RA-NGF-Media interaction effect, $F(1,636)=4.619$, $p= 0.032$, $\eta_p^2 =0.007$, observed power=0.574, such that the same trends are shown in Figure 12 and 13.

Figure 9 showcases the Map-II-kinase and Beta-III-Tubulin stains for a mature B27 differentiated neuron. This positive stain provides a distinct mature neuronal readout, though was limited to only one round of staining and only seen within one cell of the B27 differentiated condition and not the N2 condition. The limited number of experiments and cell count makes extrapolation difficult, though this readout does offer preliminary conclusions that staining is possible in this cell line and that these markers can be induced from differentiation.

Further staining with Ethidium Bromide and Acridine Orange was done to ascertain apoptotic effects of media components on HT22 cells. Figure 14 supplements previous findings of RA's negative effects on B27 differentiated HT22 cells, showcasing an increased incidence of necrosis unique to the B27 condition.

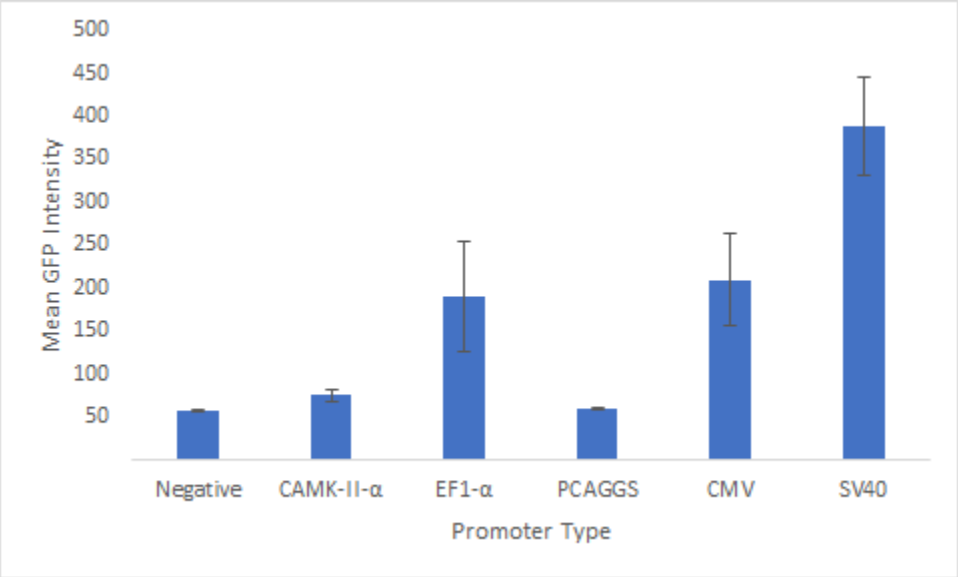


Figure 1. Mean GFP Intensity Across Different Promoter Types within Undifferentiated HT22 Cells. CAMK-II-α corresponds to a ChrimsonR plasmid tagged with GFP to the membrane.

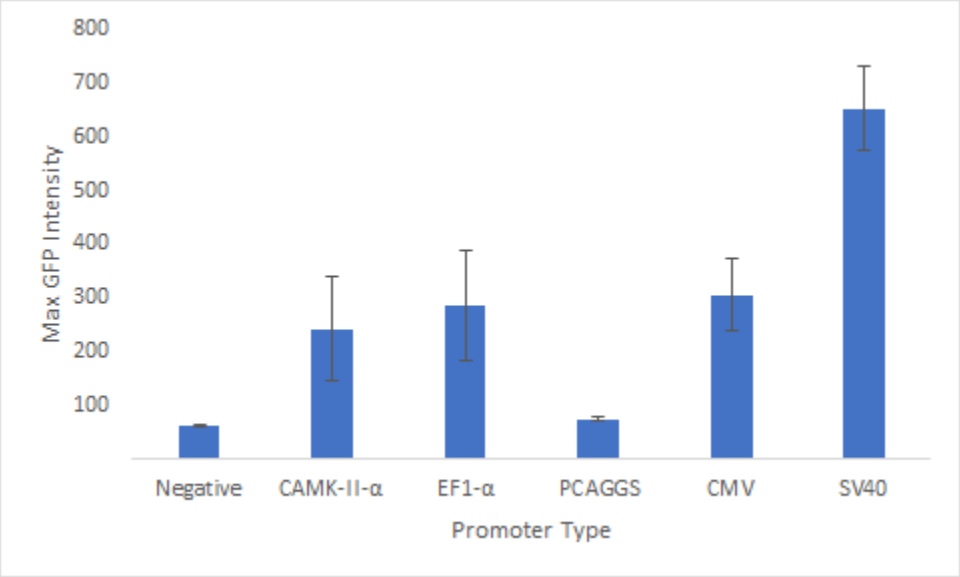


Figure 2. Maximal GFP Intensity Across Different Promoter Types within Undifferentiated HT22 Cells. CAMK-II-α corresponds to a ChrimsonR plasmid tagged with GFP to the membrane.

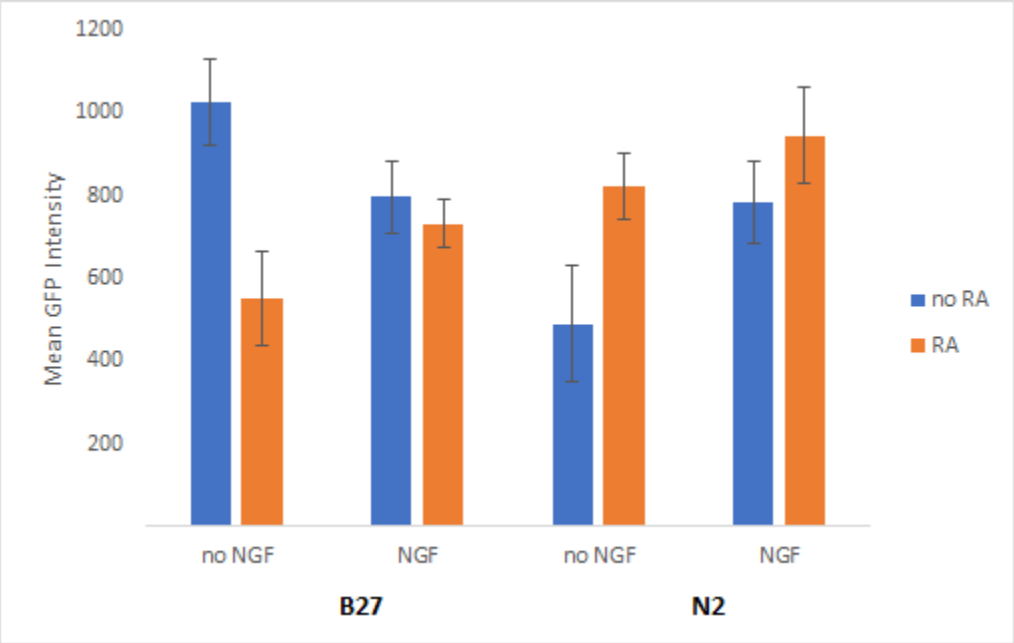


Figure 3. Mean GFP Intensity by Media Condition. Error bars represent Standard Error of the Mean

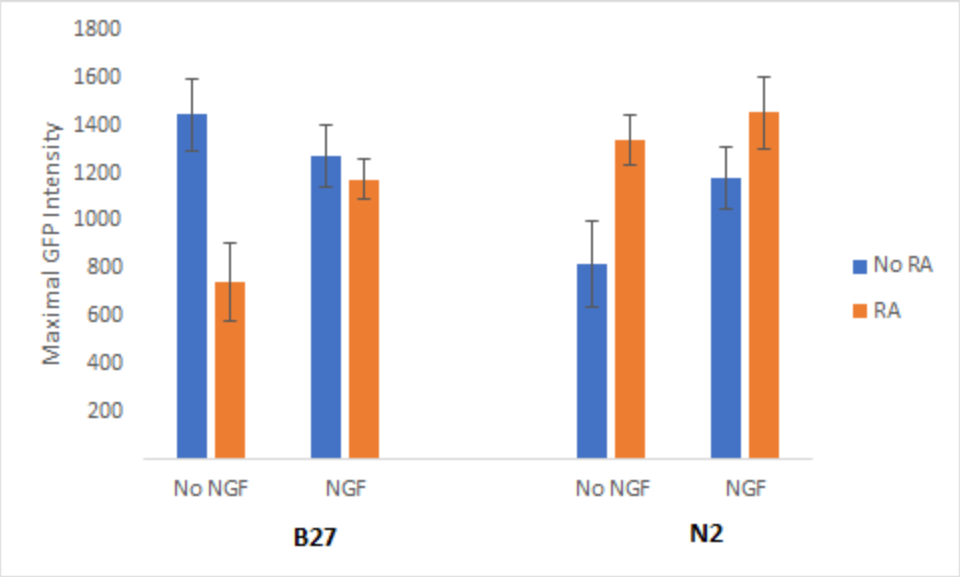


Figure 4. Maximum GFP Intensity by Media Condition. Error bars represent Standard Error of the Mean

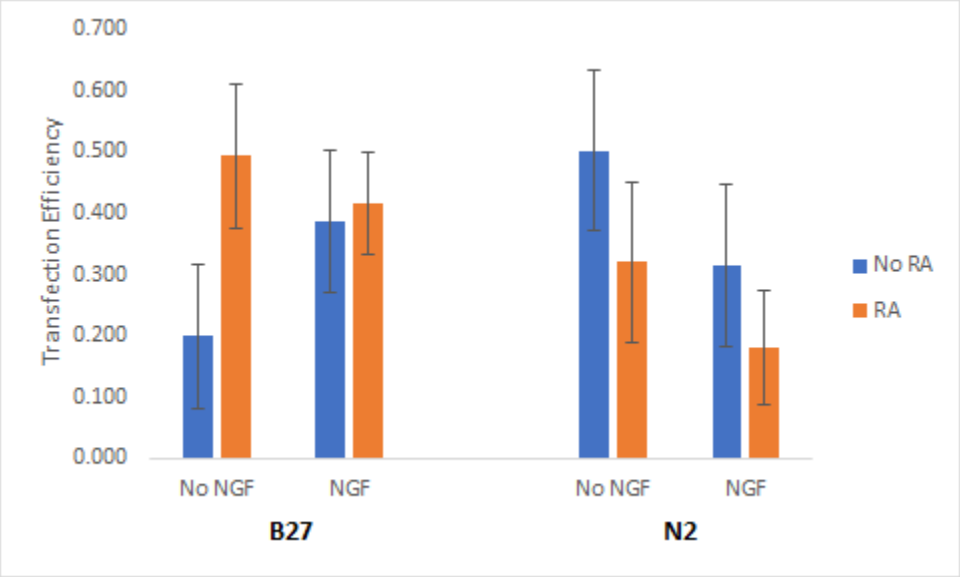


Figure 5. Transfection efficiency by Media Condition. Error bars represent Standard Error of the Mean

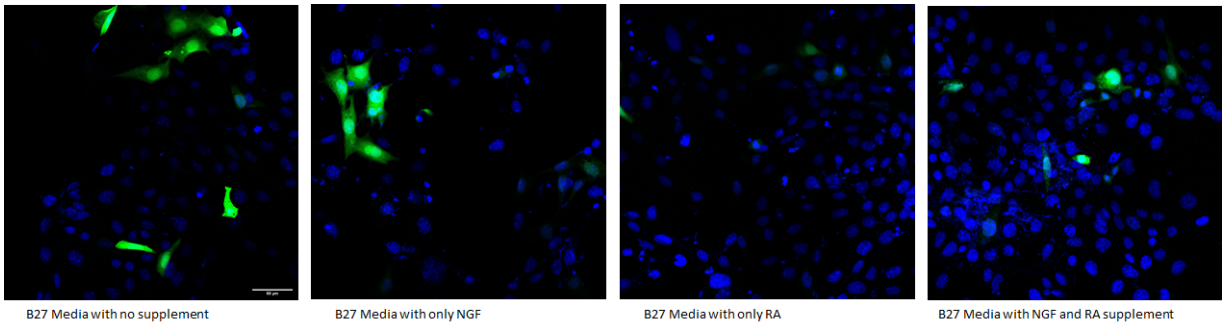


Figure 6. Visual Comparison of CAMK-II-α GFP expression across different B27 media conditions for HT22 cells. Blue stains indicate nuclei while green fluorescence corresponds to GFP. Scale bar is 50µm.

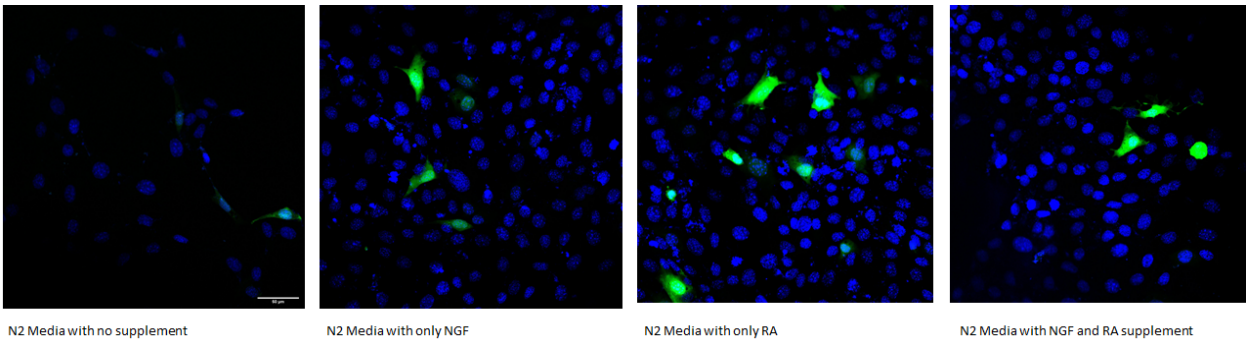


Figure 7. Visual Comparison of CAMK-II-α GFP expression across different N2 media conditions for HT22 cells. Blue stains indicate nuclei while green fluorescence corresponds to GFP. The scale bar is 50µm.

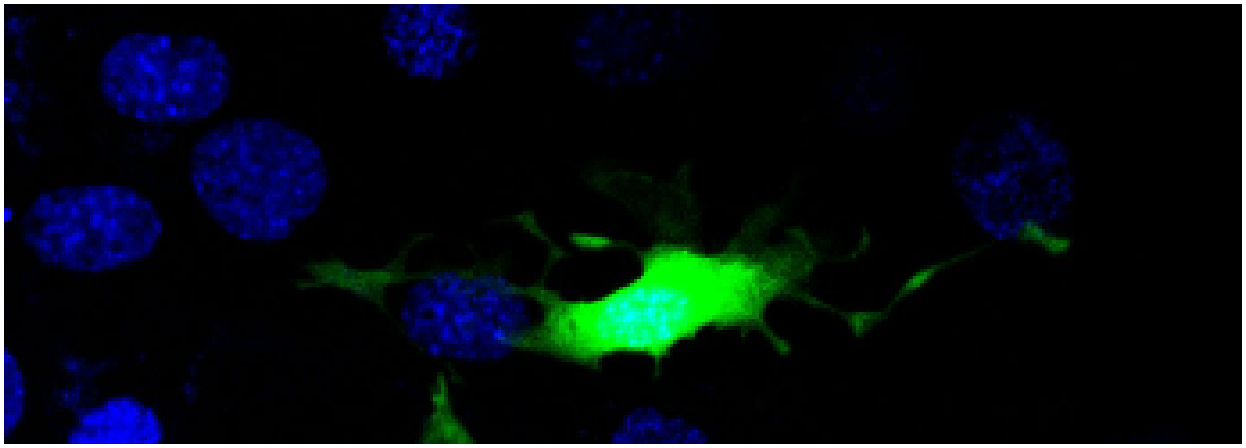

Figure 8. Neurite extension for an N2 differentiated HT22 cell with both NGF and RA. This image is pulled from Figure 7.

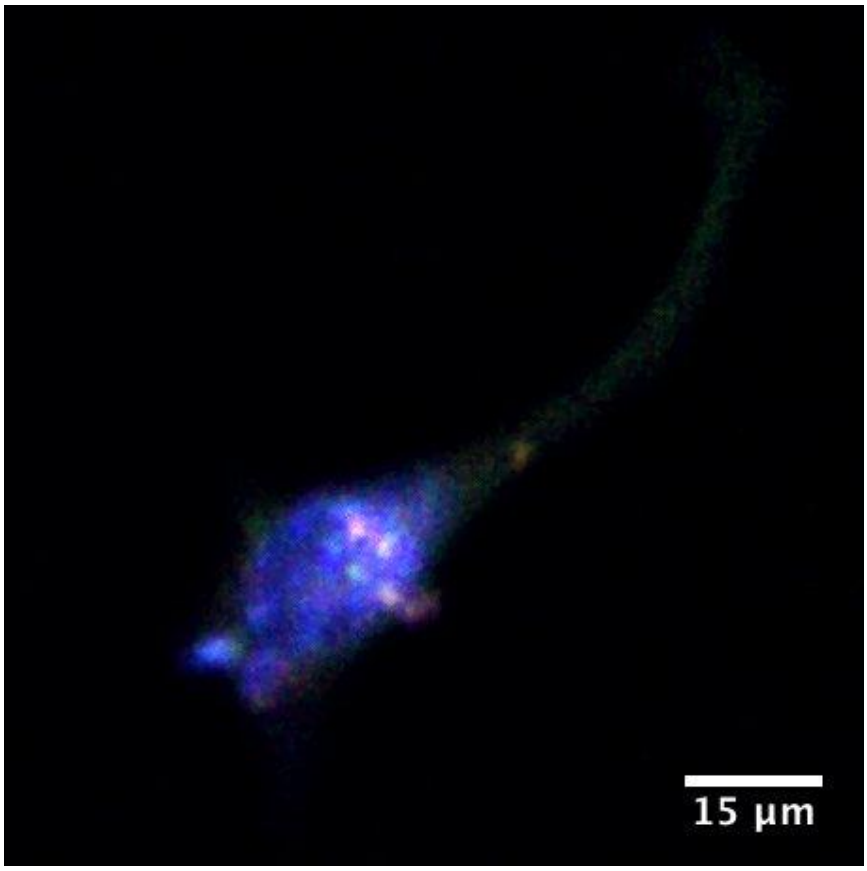


Figure 9. Antibody stain of Beta-III-Tubulin (Green) and MAP-II-Kinase (Red) of a B27 Differentiated Neuron

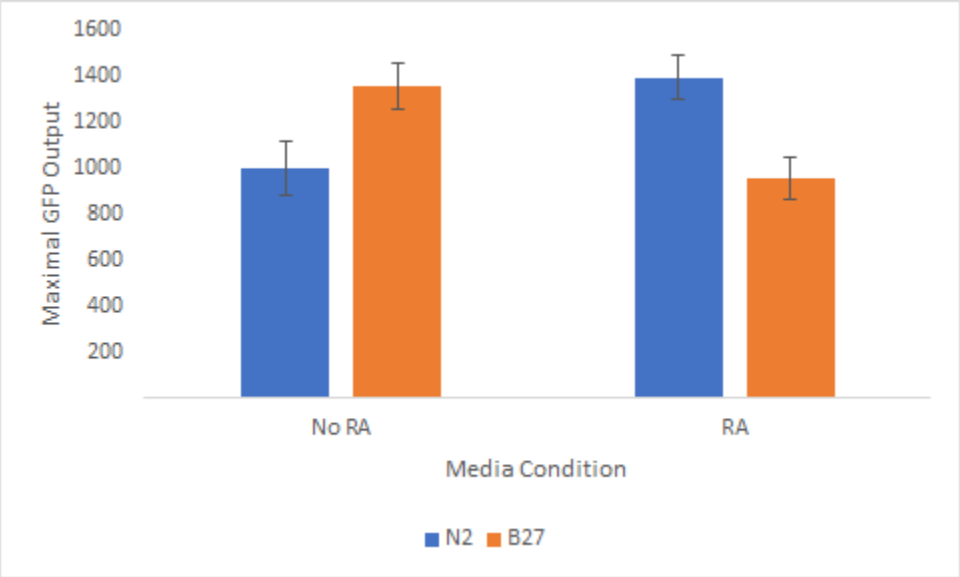


Figure 10. RA's effect on Maximal GFP expression compared directly between Media Types.

Error bars represent the standard error of the mean.

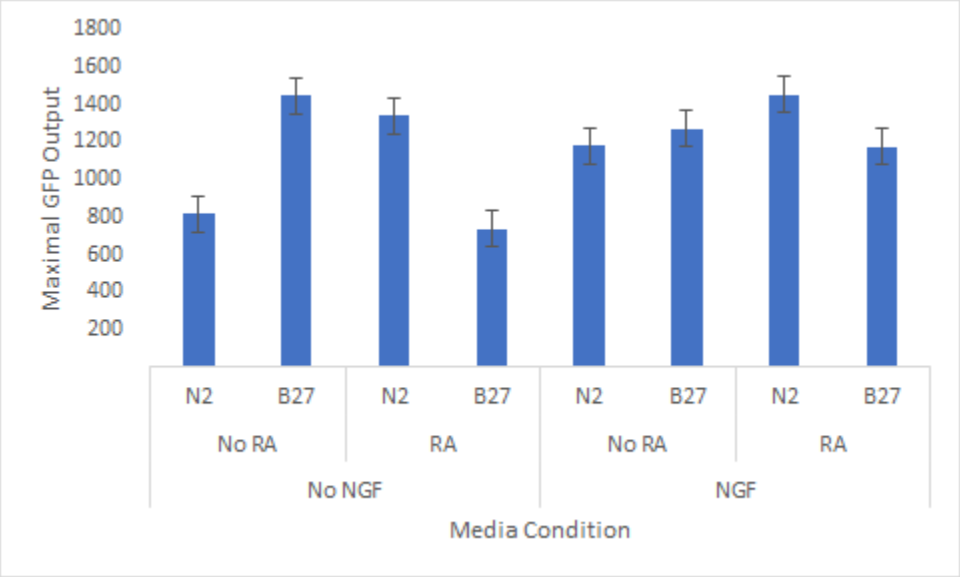

Figure 11. Comparison of the Interaction Effect between N2 and B27 Media on Maximum GFP output

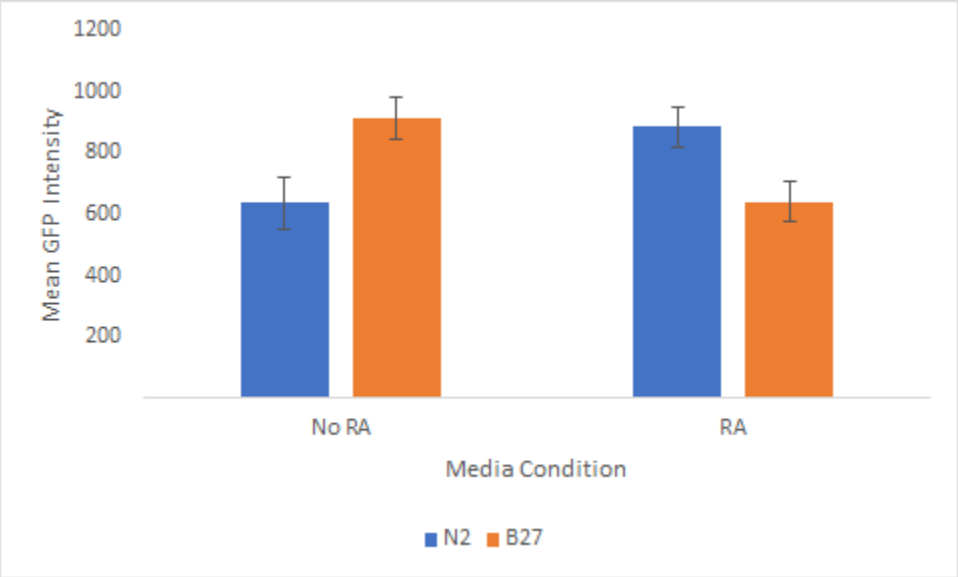


Figure 12. RA's effect on Mean GFP expression compared directly between Media Types. Error bars represent the standard error of the mean.

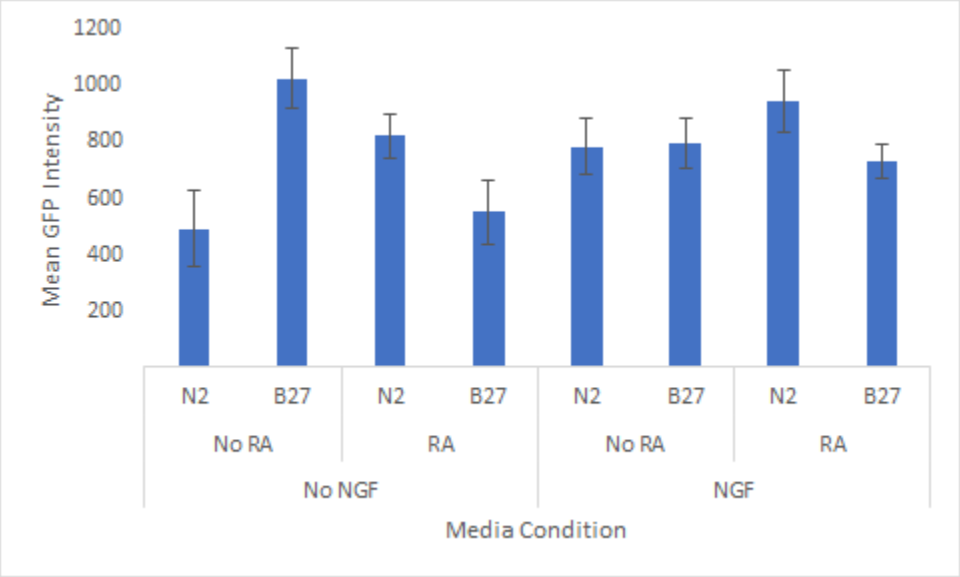

Figure 13. Comparison of the Interaction Effect between N2 and B27 Media on Mean GFP output

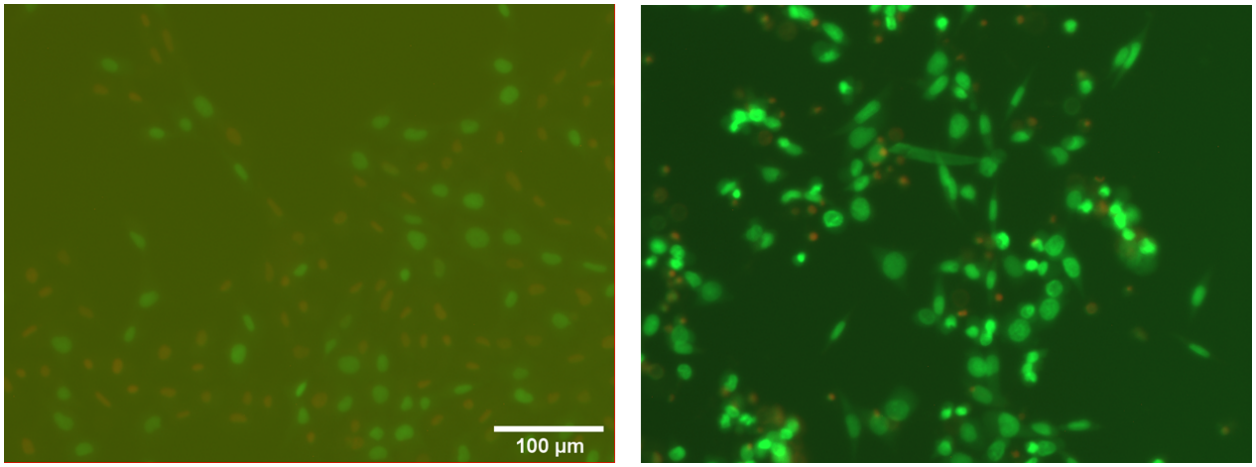


Figure 14. EB/AO Stain of HT22 cells differentiated with B27-RA-NGF (Left) and N2-RA-NGF (Right) for 48 hours. Fragmentation or condensation of the nuclei dictates interpretation, with a full green stain with no red overlay serving as the benchmark for healthy cells. Fragmentation of the green AO serves as the marker for apoptosis, with a paired red stage delineating late stage apoptosis as the membrane has been compromised. Solely red unfragmented ETBR stain indicates necrotic death in the cell population.

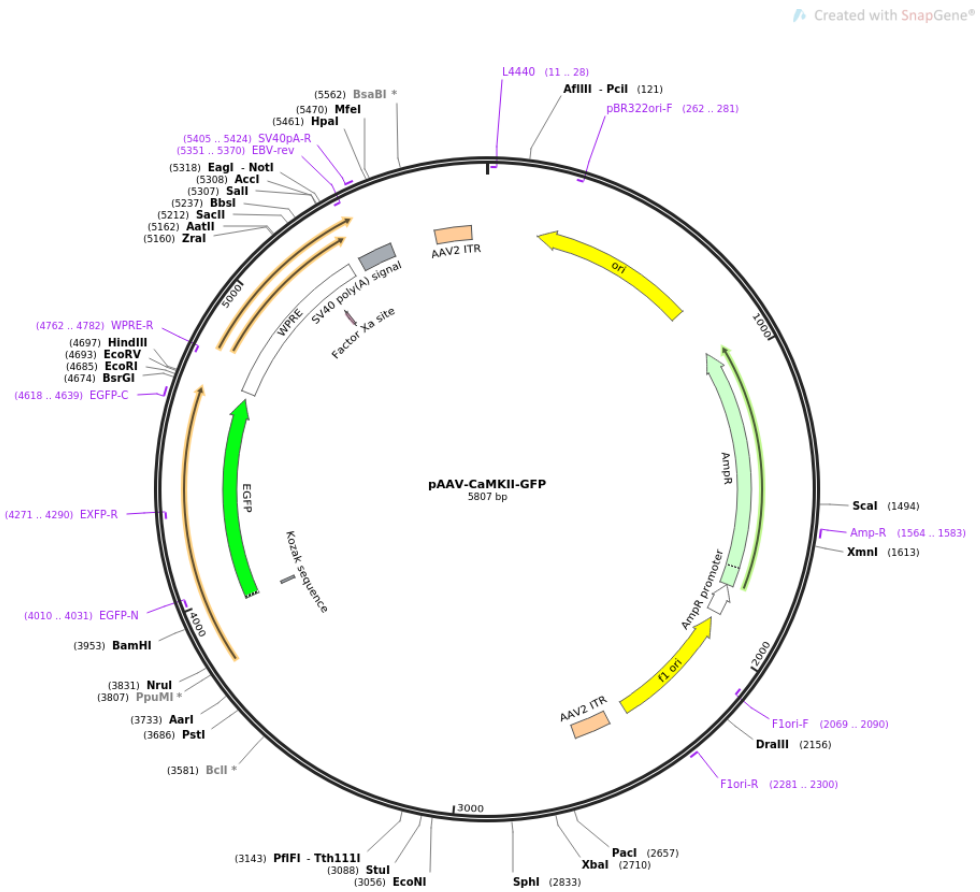


Figure 15. Plasmid Schematic of CAMK-II-α GFP

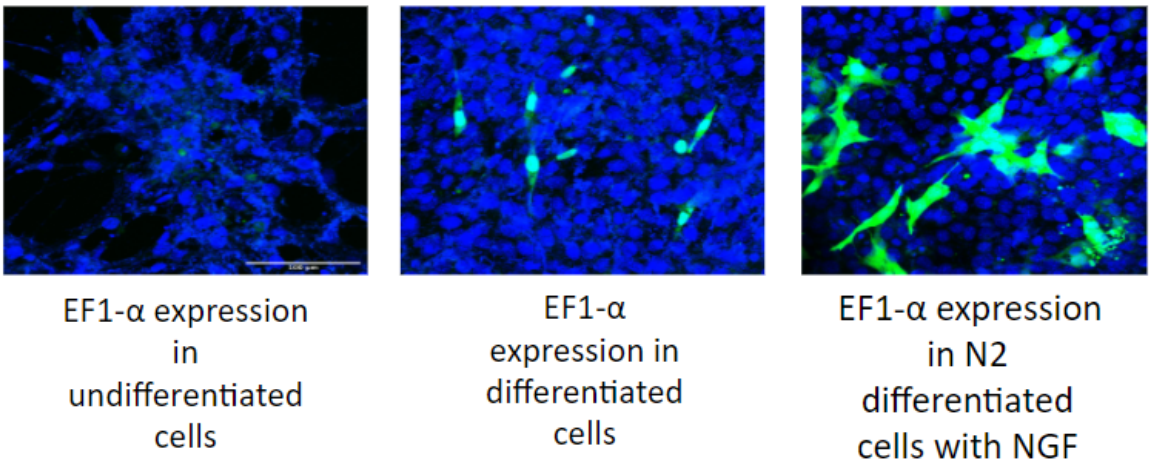


Figure 16. Expression changes of the EF1-α driven GFP across N2 media differentiation conditions

Table 1.

Fisher's Least Significant Difference Post Hoc test for the pairwise evaluation of maximal GFP intensity across promoters

| | Negative | CAMK-II-α | EF1-α | PCAGGS | CMV | SV40 |
|---|---|---|---|---|---|---|
| Negative | 1 | 0.178 | 0.043 | 0.901 | 0.004 | 0.000 |
| CAMK-II-α | | 1 | 0.778 | 0.278 | 0.646 | 0.002 |
| EF1-α | | | 1 | 0.117 | 0.866 | 0.001 |
| PCAGGS | | | | 1 | 0.043 | 0.000 |
| CMV | | | | | 1 | 0.000 |
| SV40 | | | | | | 1 |

Table 2.

Fisher's Least Significant Difference Post Hoc test for the pairwise evaluation of mean GFP intensity across different promoters

| | Negative | CAMK-II-α | EF1-α | PCAGGS | CMV | SV40 |
|---|---|---|---|---|---|---|
| Negative | 1 | 0.854 | 0.099 | 0.978 | 0.013 | 0.000 |
| CAMK-II-α | | 1 | 0.314 | 0.888 | 0.184 | 0.001 |
| EF1-α | | | 1 | 0.182 | 0.813 | 0.013 |
| PCAGGS | | | | 1 | 0.069 | 0.000 |
| CMV | | | | | 1 | 0.003 |
| SV40 | | | | | | 1 |

Table 3.

Effects of Media conditions on Mean GFP fluorescence in N2 Media

| Media Condition | Type II Sum of Squares | df | Mean Square | F | p | $\eta_p^2$ | Observed Power |
|---|---|---|---|---|---|---|---|
| (Intercept) | 131787877.06 | 1 | 131787877.06 | 187.64 | 0.000 | 0.416 | 1 |
| NGF | 2477789.53 | 1 | 2477789.53 | 3.528 | 0.061 | 0.013 | 0.465 |
| RA | 3460904.13 | 1 | 3460904.13 | 4.928 | 0.027 | 0.018 | 0.599 |
| NGF*RA | 413408.957 | 1 | 413408.96 | 0.589 | 0.444 | 0.002 | 0.119 |

| | | | |
|---|---|---|---|
| Error | 184718980.65 | 263 | 702353.54 |

Note. The data was computed using alpha= 0.05

Table 4.

Effects of Media conditions on Mean GFP fluorescence in B27 Media

| Media Condition | Type II Sum of Squares | df | Mean Square | F | p | $\eta_p^2$ | Observed Power |
|---|---|---|---|---|---|---|---|
| (Intercept) | 176482991.04 | 1 | 176482991.04 | 274.777 | 0.000 | 0.424 | 1 |
| NGF | 45049.60 | 1 | 45049.60 | 0.070 | 0.791 | 0.000 | 0.058 |
| RA | 5356162 | 1 | 5356162 | 8.339 | 0.004 | 0.022 | 0.821 |
| NGF*RA | 3087549.05 | 1 | 3087549.05 | 4.807 | 0.029 | 0.013 | 0.590 |
| Error | 239569243.78 | 373 | 642276.79 | | | | |

Note. The data was computed using alpha= 0.05

**Discussion**

Preliminary results have shown never before characterized metrics of HT22 differentiation. While the previously described methods have focused on N2 or NGF effects on HT22 differentiation, we showcase the novel effects of RA and B27 while also offering the field new empirical verification of N2 and NGF. The results seen are supported by the literature, specifically with the novel B27 model of differentiation. Brewer et al. 1993 describe B27 supplements contents' to already contain retinal which is the precursor to retinoic acid (Brewer et al. 1993). The combined effect of retinoic acid's twenty-fold strength over its retinal and prior work that has characterized how increased RA concentration can culminate in abnormal differentiation may explain how the added RA supplement only offers a negative effect (Zasada and Budzisz 2019 and McCaffery et al. 2003). Thus, we contend that an optimized B27 media-based protocol should only incorporate NGF to upregulate neuritic outgrowth and differentiation character while also not reducing CAMK-II-α readout.

N2 supplement, the field staple, offers a different story entirely. In the N2 media condition, there was a positive increase due to RA- which also matches the Brewer et al. 1993 paper's characterization of N2 supplement not containing retinal. NGF did not increase differentiation character via our primary readout of CAMK-II-α, however, we highlight figure 10 as a secondary readout that should be explored in future work: neurite outgrowth. Thus, while NGF was shown to not have a main effect in table 3 and 4 or a strong upwards trend in figure 13 in regards to CAMK-II-α readout, we contend that it ought to be added to differentiation media as it seemingly upregulates other markers of differentiation in the HT22 cell line.

The long branched extensions of the neurites were primarily observed in media conditions that contained NGF- matching the previous characterization of its effect. Recent work has characterized NGF's endogenous presence in the HT22 cell line at a detectable level, and in contrast to RA is shown to activate pathways like Tropomyosin receptor kinase A activation to prevent apoptosis and cell death (Wu et al. 2020, Li et al. 2017, and Oliveira 2015). Thus, the literature posits a very strong neuroprotective role for NGF that exists alongside the already defined neurodevelopmental role it takes. Specific interaction between NGF and RA has also been characterized, showing that RA at high doses can downregulate brain-derived neurotrophic Factor in neurons which normally serve a key neuroprotective and differentiation role (Oliveira et al. 2011 and Oliveira 2015). However, the neurite extension readouts and cell preservation are not readouts directly characterized by the CAMK-II-α GFP outputs and ought to be explored via alternative analysis models. Indeed previous literature aiming to elucidate differentiation character following a variety of distinct differentiation protocols often centers its core readouts around neurite outgrowth assays, and not CAMK-II-α GFP readout (Oh et al. 2008, Das et al. 2004, Rydel and Greene 1987, and Kim et al. 2011). Another common readout for differentiation character is that of antibody staining of mature neuron gene identification, often targeting proteins like Beta-III-Tubulin or MAP-II-Kinase (Roskams and Ronnett 1998, Laferrière and Brown 1996, Cheung et al. 2000, Mak et al. 1999, Bojnordi et al. 2017, and Goto et al. 1994). More than just broad markers of differentiation, these markers can help elucidate more complex natures of our differentiation protocol such as NGF's cellular level mechanism (Pang et al. 1995). Figure 9 identifies the only round of antibody staining the Beaudoin Lab has conducted for this project, ultimately showing advanced readout for HT22 differentiation. The

CAMK-II-α readout actually corroborates the staining readout, as our results emphasize B27's significantly greater baseline effect relative to N2. Figure 14 utilizes the aforementioned EB/AO stain as a means to corroborate our interpretation of the CAMK-II-α GFP readouts, as the stain showcases a higher percentage of apoptotic cells in the B27-NGF-RA media relative to the N2-NGF-RA. Further, Figure 14 identifies a large proportion of late-stage apoptotic cells whereas the apoptotic cells in the N2 media are early stage. These results would support our interpretation of a neurotoxic effect when too much RA is added to HT22 cells, precluding any differentiation mechanism.

Combining readouts of CAMK-II-α-GFP shown here, with future EB/AO, Beta-III-Tubulin/MAP-II-Kinase, and neurite outgrowth assay characterization from already procured data will potentially offer the field a comprehensive defense of our differentiation protocol. Thus far, our results offer an incomplete readout that makes media characterization limited to neuron-specific promoter interaction- which is seemingly confounded with general increases in transfection efficiency on account of internal cellular machinery fixes alluded to in Figure 16. However, early evidence supports the hypothesis of RA's markedly positive effect in N2 media, and NGF's essential role in neurite outgrowth and later stage differentiation. Further, our results also correspond with prior literature in identifying NGF as the sole supplement to be added to B27 media for neuronal differentiation purposes (Brewer et al. 1993, Wu et al. 2020, Li et al. 2017, Oliveira et al. 2011, Zasada and Budzisz 2019, McCaffery et al. 2003 and Oliveira 2015 ). While the initial analysis does not indicate a significant difference on account of media, depending on cell-line purposes our protocol indicates potentially distinct protocols.

Project 2: The Red Pill: Creating, Expressing, and Testing an Optimized Red-Shifted Channelrhodopsin

**Introduction**

Rhodopsins serve as a key light detection method across the metazoa kingdom, with vertebrates and invertebrates alike utilizing the seven-transmembrane protein connection with retinal motif to sense photons (Nagel et al. 2003). Oftentimes the rhodopsin mechanism rests on a conserved g-protein-coupled pathway that opens up channels in a cyclic-nucleotide-dependent way (Kaupp 1995). The first real published finding of the opsins in the microbial kingdom was in 1971, in which a light-gated ion flow process was described in bacteria (Oesterhelt and Stoeckenius 1971). Matsuno-Yagi and Mukohata advanced on this finding a few years later, by identifying Halorhodopsin which operated a chloride transporter-thereby extending the known members of this opsin family and the mechanisms (Matsuno-Yagi and Mukohata 1977 and Deisseroth 2011). Algae, single cell eukaryotic organisms, in specific are known to use rhodopsin for phototaxis, employing light-gated proton channels called channelrhodopsins that generate current (Nagel et al. 2003 and Hegemann et al. 2001). The Nagel et al. 2003 paper isolated a channelrhodopsin receptor of interest (ChR2), and produced a heterologous expression of this protein on mammalian cells (HEK293), and ultimately set the stage for subsequent neuronal work.

The selectivity of channelrhodopsins to certain wavelengths of light and positive ion influx immediately posed great benefit to neurobiological studies interested in controlled neuronal action- better known as optogenetics (Boyden et al. 2005 and Deisseroth 2010). The Deisseroth paper was a groundbreaking one, introducing a novel genetic technique to express

channelrhodopsin within neuronal populations of interest. The thesis was simple: electrophysiological recordings in vivo could not isolate activation in a pathway-specific manner. The immense baseline bleed over from probe insertion innately restricts circuit-specific recording (Deisseroth 2015). However, a technique that would allow selective expression of channelrhodopsin in a given brain subregion would ultimately facilitate recording at never before achievable specificity. Several theoretical methods were introduced to get the region-specific rhodopsin expression involving an array of synthetic genes and supplements, however, it was the groundbreaking paper by Dr. Ed Boyden from the Deiseroth lab in 2005 that showed simple expression of the opsin in neuronal tissue produced light-gated current responses (Zemelman et al. 2002, Banghart et al. 2004, Deisseroth 2010 and Boyden et al 2005). The introduction of this new construct and method, aptly named optogenetics, revolutionized the patch-clamp technique and the depth and breadth of what could be discovered.

The method was conceptually simple; express the channelrhodopsin in the specific regions that one intends to record from, and then shine a light that is between 432 and 490nm wavelength to open the channel. The first barrier is rooted in the ability to utilize genetic techniques to garner expression of the channelrhodopsin in a specific tissue, which was proposed via a lentiviral delivery method shown in hippocampal primary cultures (Boyden et al. 2005). This lentiviral method was utilized in vivo as well, with the Boyden lab showing responses in HEK cells, primary cultures, and rat brain slices (Klapoetke et al. 2014). However, further advances were made in utilizing Adeno-associated Virus, which utilizes a similar triple transfection scheme and dilution series, while also not requiring as intensive of a safety clearance for use (McCarty et al. 2004, Haar et al. 2020, Gradinaru et al. 2010, and Mei and

Zhang 2012 ). The Lentiviral and AAV mechanisms of delivery are essentially the same, except that Lentivirus can package up to a 10kb gene and permanently inserts into the genome, and AAV can package only up to a 5Kb gene and is maintained outside of the genome as an episome (Mei and Zhang 2012). The limitations on package size ultimately restrict the size of the promoter that can be used, and thus the expression efficiency in a given injection site. Since these opsins require large amounts of expression to compensate for low individual conductance and inaccurate surgical injection, weaker promoter expression complicates in vivo work (Zhang et al. 2010 and Yizhar et al. 2011).

The further delineation between AAV and lentiviral delivery is the amount of versatility that AAV vectors can take to overcome packaging constraints. AAV vectors can utilize Cre-recombinase, with promoters driving expression in selected regions based on Cre recombinase expression, such that a floxed constructs' expression occurs only in a given tissue and only after AAV recombination (Mei and Zhang 2012, Sohal et al. 2009, and Tsai et al. 2009 ). Thus, AAV delivery can culminate in strong promoter-driven gene expression of a channelrhodopsin construct in a site-specific manner. Circuit-specific techniques have been employed as well, such as using rabies virus or canine adenovirus, herpes simplex virus 1, or vesicular stomatitis virus to deliver the Cre construct to trigger Cre-dependent channelrhodopsin expression to the neurons projecting to a given injection site (Callaway 2008 and Beier et al. 2011 and Lavoie and Liu 2020). Clever utilization of AAV and other genetic techniques has allowed the already innovative method of Optogenetics to expand even further, and craft an entirely new way to conduct electrophysiological recordings. Even merely at the point of circuit-specific expression of channelrhodopsin, the entire field was advanced- as

neurobiological research could dive even further into synaptic properties and functional connections in the brain while behavioral neurosciences could look to employ this light activated method in the context of eliciting or studying behavior in a wide variety of species (Deng et al. 2017, Zheng et al. 2018, Song et al. 2014, Danksin et al. 2015, Kravitz and Bonci 2013, and Belzung et al. 2014).

However, more than merely introducing a mechanism to depolarize neurons, the field also had a way to hyperpolarize neurons with Halorhodopsin which responds to yellow wavelength light (Zhang et al. 2007). Channelrhodopsin and Halorhodopsin both had different enough spectral responses to allow depolarization and hyperpolarization in either the same neuron (bi-directional control) or in the same circuit to represent complex neural interactions not previously manipulable (Witten et al. 2010 and Mei and Zhang 2012). The inhibitory approach of optogenetics has a litany of different opsins to pull from, such as arcaerhodopsin-3, bacteriorhodopsin, gtARC2 which is a chloride channel, and Guillardia theta rhodopsin-3 (Idnurm and Howlett 2001, Chow et al. 2010, and Park et al. 1989, and Mahn et al. 2018 Gradinaru et al. 2010 ). The excitatory approach chiefly utilizes Channelrhodopsin-2, but Channelrhodopsin-1 procured from Volvox carteri can also be utilized in a similar dual circuit fashion with a response seen around 589nm which is outside of Channelrhodopsin-2's responsive range (Zhang et al. 2008). Both channelrhodopsins have tight millisecond control, but initial work was hampered by wild type Volvox carteri Channelrhodopsin-1's reduced conductance- ultimately driving the Deiseroth lab to create a chimeric construct between two different species channelrhodopsin-1 (Chlamydomonas and Volvox) to equalize conductance for in vivo work, called C1V1 (Yizhar et al. 2011)

The chimeric construct allows some degree of dual synaptic control, but its slow decay rate kinetics precludes efficient spike-train recordings necessary for mimicking or modeling in vivo synaptic transmission (Klapoetke et al. 2014). ReaChr is another red-shifted channelrhodopsin, made via mutations of ChR2, which has been utilized for trans-cranial dual synaptic transmission (Lin et al. 2013).

Chrimson is another red-shifted channelrhodopsin that was seen as a possible solution to the empirical slow kinetics that precludes spike train an ability seen in both ReaChr and the chimeric mutant, C1V1 (Yizhar et al. 2011 and Lin et al. 2013). The Chrimson construct was further optimized with a point mutation at site 176 from Lysine to an Arginine to create ChrimsonR, also being far red-shifted and having even faster control beyond 20Hz (Klapoetke et al. 2014). While previous red-shifted optogenetic methods were employed often, it was only ChrimsonR that was optimized to a point of being able to conduct 50Hz spike trains that could mimic the in vivo neuronal communication that optogenetics was interested in tapping into. The majority of the work with ChrimsonR was done in the context of Retinal Ganglionic Cells (Duaor et al. 2016, Chaffiol et al. 2016, Caplette et al. 2016, Sahel et al. 2016, McGregor et al. 2019, Gauvain et al. 2021 ). However, minor work has been accomplished in cortical neurons utilizing the proposed dual synaptic approach (Schäfer et al. 2021, Francesco et al. 2021, and McGirr et al. 2020). Most of the dual synaptic cortical work utilizes the synapsin promoter, which encodes for critical synaptic vesicle proteins and is highly expressed in some neuronal populations (Thiel et al. 1991 and Jackson et al. 2016). Relative to CAMK-II-α, Synapsin promoters express in more neurons whereas CAMK-II-α has been shown to be specifically and highly active in cortical glutamatergic neurons (Lee et al. 2019).

The Beaudoin Lab empirically showed that an individual input in the addiction reward circuitry could not induce a high firing burst of activity, which may be a crucial detriment in the characterization of neurobiological signaling or the ultimate optogenetically induced behavioral experiments we aim to do. A secondary construct may allow two different opsins to be expressed on a given neuron to induce the high firing burst we are interested in. However, this utility is nested in a dual opsin expression on a given neuron. In line with the field vision of ChrimsonR, we are interested in dual input recordings of a given slice. One iteration of this is testing Long Term Potentiation (LTP) for the Dopamine (DA) neurons we are interested in. Whereas before this type of experiment was inaccessible for DA neurons which characteristically have multiple inputs from different directions, a second optogenetic construct could allow co-activation of two inputs and test how this leads to LTP in the SNc DA neurons the Beaudoin Lab studies (Beier et al. 2015, Watabe-Uchida et al, 2012, Wang et al. 2019, and Beaudoin et al. 2018). Further characterization of the relevant mesolimbic circuitry can be achieved with the same construct, as one input like the subthalamic nucleus (STN) and another input like the Pedunculopontine nucleus (PPN) can each be infected with a distinct opsin and activated individually to ascertain both how an output-defined subpopulations of DA neuron responds to each input (relative to both) or how the STN and PPN respond to each other (Weintraub and Zaghloul 2013, French and Muthusamy 2018, Tykocki et al. 2011, and Morita et al. 2014 ).

In lab in vivo injections have shown poor expression of CAMK-II-α driven ChrimsonR with an SV40 poly-A-tail in the midbrain, suggesting an issue with plasmid expression elements. The limitations induced by poor expression ultimately culminated into months of lost time, and

wasted resources across multiple labs in the field that aimed to utilize the Boyden lab's construct. However, while we propose our new construct should increase in vivo expression to usable levels for the variety of research projects, we specifically contextualize that the Camk-II-α selective expression in glutamatergic neurons has an advantage over the synapsin promoter. Synapsin is neuron-specific, which could culminate in expression across neuronal populations. In contrast, CAMK-II-α promoters facilitate future Beaudoin Lab inquiry into glutamatergic signaling within the mesolimbic reward pathway. Thus, by creating a CAMK-II-α driven construct we can even further selectively express ChrimsonR in glutamatergic neurons within a given subregion- a level of specific expression not currently readily achievable. We propose that switching the vector for the ChrimsonR insert with a known high fidelity plasmid (Halorhodopsin driven by an empirically functional CAMK-II-α promoter and human growth hormone poly-a-tail) should increase the expression efficiencies in vitro and in vivo relative to the old construct. We aim to determine the efficacy of this simple vector switch following the same in vitro CAMK-II-α readouts established in project 1 as well as in vivo expression and red-light response, en route to providing the field with both a novel high fidelity ChrimsonR construct and utilizing this construct to procure more data on the electrophysiological properties of HT22 cells discussed in project 1.

**Method**

*Cloning*

The modified construct was made via a ligation utilizing BamHI and EcoR1 involving two plasmids: the ChrimsonR construct gifted to us by the Boyden Lab and a CAMK-II-α driven

Halorhodopsin (Klapoetke et al. 2014 and Zhao et al. 2016). The ChrimsonR was also driven by a CAMK-II-α promoter, however, analysis of the two promoters yielded several transitions and transversion mutations present in the Boyden construct's promoter. Further, the poly-A tail between the constructs were distinct- with the human growth hormone tail in the Halorhodopsin hypothesized to drive increased transcript stability relative to the sv40 poly-Atail in the old construct.

*Viral production*

The virus was made via AAV packaging with AAV serotype 5 due to its empirically higher transduction efficiency into neuronal populations (Mason et al. 2010). Freestyle 293 cells were maintained in freestyle media Thermo Fisher Scientific: FreeStyleTM 293 Expression Medium., and transfected at a cell concentration of $1x10^6$ cells per mL in 150 mL. A triple transfection of the two AAV helper plasmids, 100.5 µg of pXX6-80 and 75 µg of pXR5-Bam, with 49.5 µg of pAAV-expression plasmid was performed utilizing 562.5uL of transporter5 transfection reagent (Aurnhammer et al. 2012). The protocol described in Aurnhammer et al. 2012 was utilized to grow the AAV virus containing ChrimsonR, but instead utilizing a hand-packed 1 mL Q-Hyper-D column to perform anion exchange chromatography. The column was equilibrated with 10 CV of 0.5 M NaOH followed by 10 CV of 2 M NaCl in 25 mM Tris buffer pH 9.0 (Buffer B) then 20 CV of 25 mM Tris buffer pH 9.0 (Buffer A). Final eluents confirmed to contain virus by qPCR were combined and concentrated in a 30 KDa amicon spin tube and aliquoted to be stored at -80 celsius.

*Proof of Principle Testing*

The construct was tested in vitro and in vivo to determine functional plasmid expression. Differentiated HT22 cells were transfected with the base plasmid to determine in vitro expression, while the purified AAV construct was utilized for viral injections to determine in vivo expression.

The differentiatIon protocol identified in Project 1 was utilized to differentiate the HT22 cells, and both transfection efficiency and expression efficiency was quantified via the macro described in Project 1. All Statistical analysis was performed in the same manner as Project 1, using SPSS and data procured from the macro.

*Animals Protocols*

Mice will be handled in accordance with animal research guidelines of Trinity University's Animal Research Committee.

*Surgery*

BALB/C mice of both sexes, aged a minimum of three months, were used for stereotaxic injections of an AAV-CAMK-II-ChrR2 virus bilaterally into the pedunculopontine tegmental nucleus (PPN). Mice were placed in a stereotaxic frame under 2-3% isoflurane anesthesia with 2 L/min oxygen. A Hamilton syringe needle was used to inject 150 nL of the virus into PPN (ML: ± 1.22 mm, AP: -4.6 mm, DV: -3.7 mm). The injections were conducted at a rate of 50 nL/min at each injection site, and the needle remained in place for 5 minutes post-injection.

Recordings were performed in HT22 cells that were undifferentiated and differentiated to determine both the new construct's optogenetic fidelity, as well as the HT22 cell line's ability to be utilized for electrophysiological recording. The recordings will build upon prior work

characterizing HT22 membrane properties, though with notably distinct methodology using a whole-cell patch configuration instead of the carbon nanopipette (Schrlau et al. 2009).

*Electrophysiology ex vivo*

After a minimum of three weeks post-surgery, mice were anesthetized with 4% isoflurane and decollated. The brain will be dissected rapidly then submerged in an ice-cold artificial CSF (aCSF) cutting solution at 5% $CO_2$ that contains the following (in mM): 2.5 KCl, 1.25 $NaH_2PO_4$, 110 choline chloride, 2.6 $NaCO_3H$, 7 MgCl, 0.5 $CaCl_2$, 10 dextrose, 2.4 sodium pyruvate, 1.3 ascorbic acid. Then, the brain will be sliced horizontally (250 µm) using a Leica vibratome. The slices will recover at 34.5°C for approximately 30 minutes in a recording ACSF solution saturated with 5% $CO_2$ that contained the following (in mM): 125 NaCl, 3.5 KCl, 1.25 $NaH_2PO_4$, 4 $MgCl_2$, 2 $CaCl_2$, 25 NaHCO, 2.4 sodium pyruvate, 1.3 ascorbic acid, and a spatula tip of L-glutathione.

When transferred to a continuously flowing (2.5 ml/min) recording chamber, slices will be placed in a 32.5-35.5°C bath solution of recording aCSF supplemented with 100 µM picrotoxin (TOCRIS) flowing at 1.5-3 ml/min to block $GABA_A$ currents. Glass pipettes (3-7 MΩ resistance) were pulled using the Sutter Instruments Flaming/Brown model P-1000 micropipette puller and filled with the following internal solution (in mM): 10 HEPES, 5 QX-314, 135 CsCl2 , 5 EGTA, 2 $MgCl_2$, 2 ATP, 0.2 (Na2)-GTP, and 0.3% biocytin, titrated to a pH of 7.3 with 1 M and 0.1 M of CsOH.

Whole-cell voltage-clamp recordings will be performed using a Multiclamp 700B amplifier (Molecular Devices), and signals will be digitized with a HEKA instrutech Lih 8+8 data acquisition system. Data will be acquired at a frequency of 50 kHz, which was adjusted to 10 kHz

during analysis with an Axograph X program. While cell-attached, putative dopamine neurons will be identified by a characteristic 1-8 Hz firing rate and >1.5 ms action current half-width. To further confirm the cells are dopamine, a negative voltage of -110 mV will be applied to test for the presence of an $I_h$ current. A 635 nm Opto Engine red laser will be directed towards SNc and fired at a determined optimal intensity (2-5 mV) and duration (0.25-5 ms) of light pulses.

*Staining and Imaging*

Injection site images of both bright-field and yellow fluorescent protein (YFP) were obtained using a 4x objective on an EVOS FL inverted scope. Rig images will also be collected after collecting data from a cell during electrophysiology.

**Results**

The ligation series to insert ChrimsonR into the Halorhodopsin vector was successful, ultimately creating a new construct with altered expression machinery (Figure 1.) We sequenced the two constructs and identified various transitions and transversions in the promoter region between the old construct and the new construct (Figure 2.).

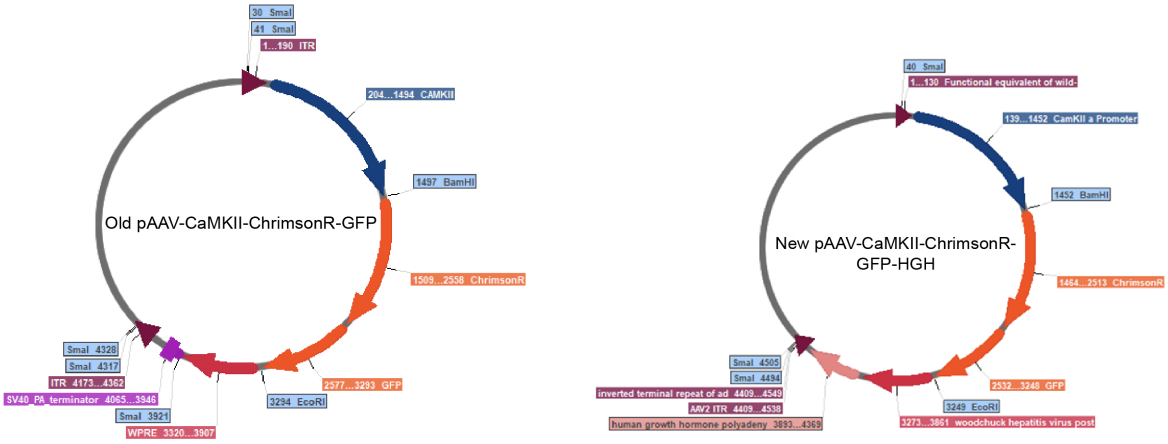


Figure 1. Old ChrimsonR construct (Left) feature comparison to the New ChrimsonR construct (right)

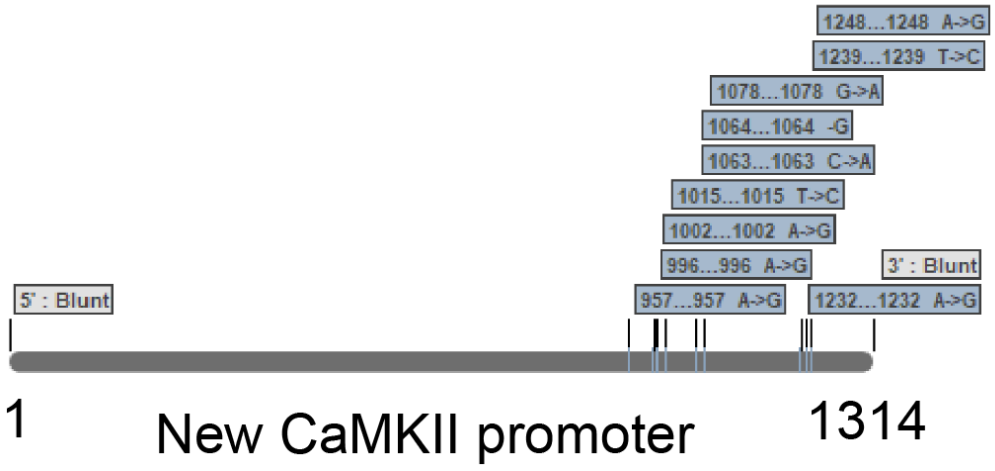


Figure 2. Sequence alignment of the two CAMK-II-α promoters

*Expression Evaluation of the Constructs in vitro*

The effects of Retinoic Acid were characterized between the old and new constructs following the approach of Project One, while also emphasizing the role Retinoic Acid ultimately plays in both the CamK-II-α promoter expression and in the Opsin's function for subsequent electrophysiology tests. A 3-way between groups ANOVA was performed, and there was a main effect of RA, Media, and RA by construct on Mean GFP intensity as showcased in table 1. In the context of the Maximal GFP Intensity readout, there was only a main effect of RA by Construct interaction as showcased in table 2. Figures 3 and 4 showcase the trends of difference, identifying that there was no New construct expression in DMEM media with FBS unless RA was added. In the context of the main effect of media for mean GFP output, it appears that regular media with RA culminated in the highest mean expression. Further, utilizing the maximal GFP output Figure 4, the highest output is also localized in the New construct in regular media. The shared interaction effect of RA by construct between the Mean and Maximal GFP outputs is shown by Figures 3 and 4 to be centered in the increase of New Construct expression with added RA. However, the figures also showcase a weighting of the increase to be in regular media and not differentiation media- with figure 4 actually showcasing a consistent, yet non-significant, decrease of new construct expression in the presence of RA and N2 or B27.

The same analysis was done to elucidate NGF's effect on this CAMK-II-α output, and tables 3 and 4 showcased no significant effect to be found, except in table 3 where there was a main effect of construct on mean GFP expression. Figure 5 showcases a trend of the New

construct generally expressing a slightly larger mean than the old construct across every condition except the DMEM with FBS and NGF condition and the N2 media with NGF condition. Figure 6 showcases a similar trend, though with a much more blunted difference between the old and new construct in the N2 with NGF condition.

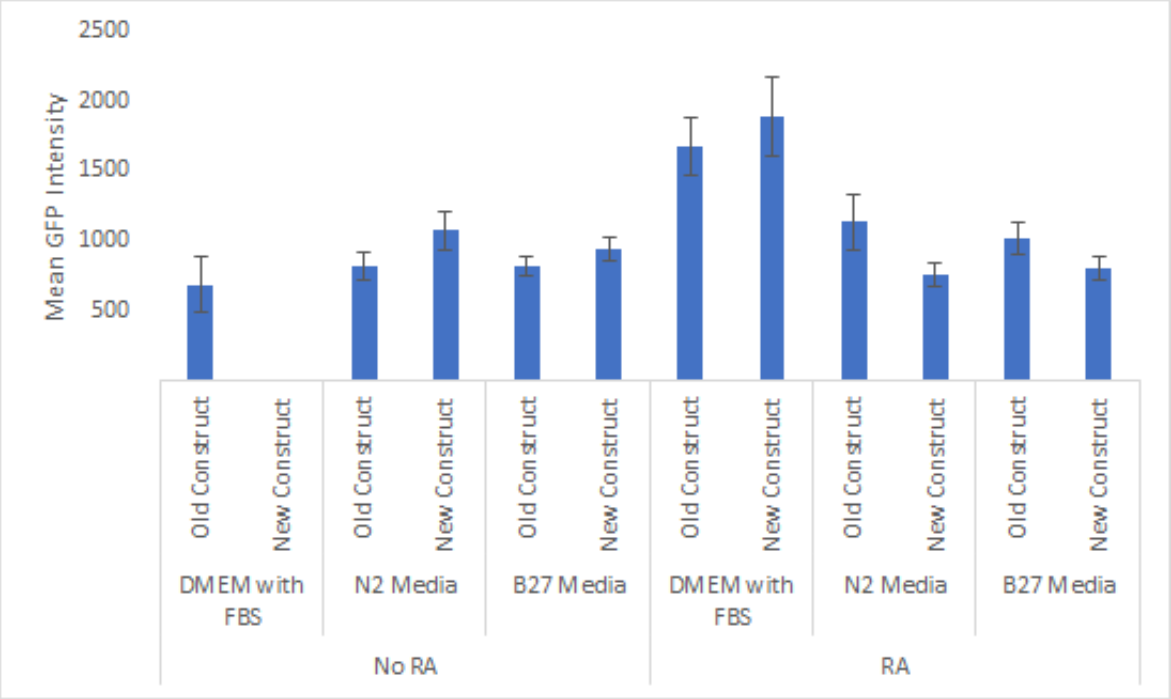


Figure 3. Mean GFP Intensity between the Old and New Construct by Media Condition. Error bars represent the standard error of the mean.

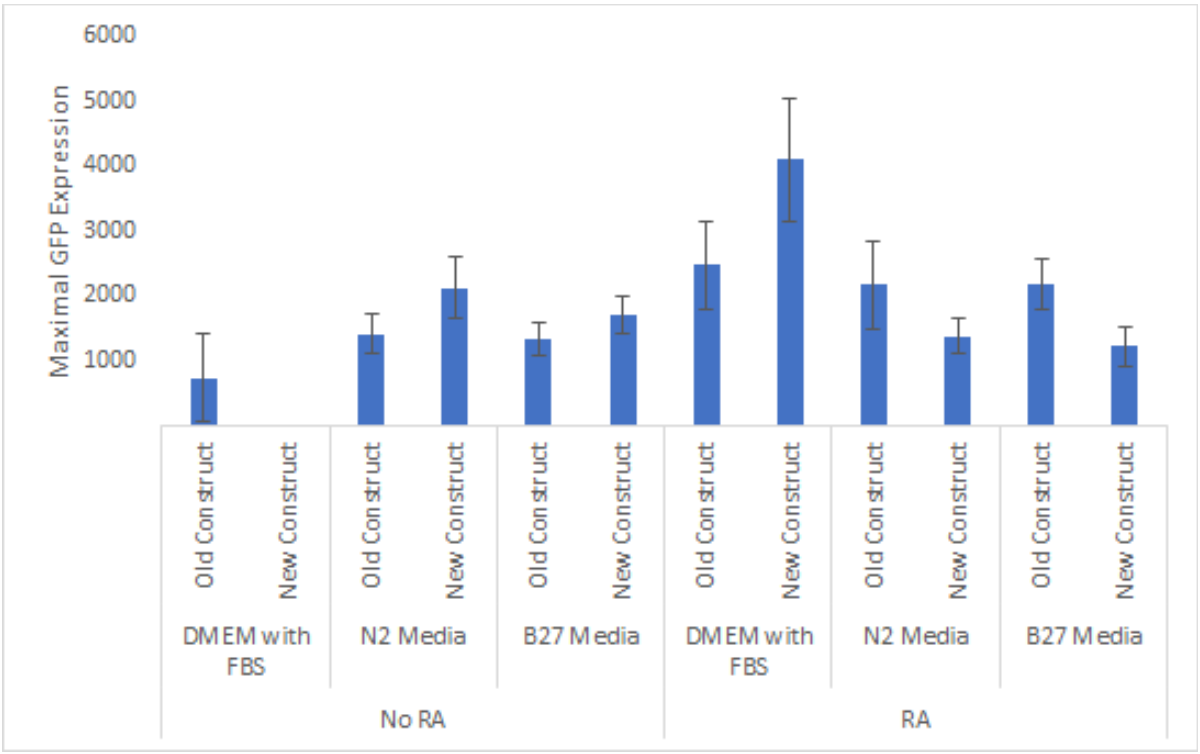


Figure 4. Maximal GFP Intensity between the Old and New Construct by Media Condition. Error bars represent the standard error of the mean.

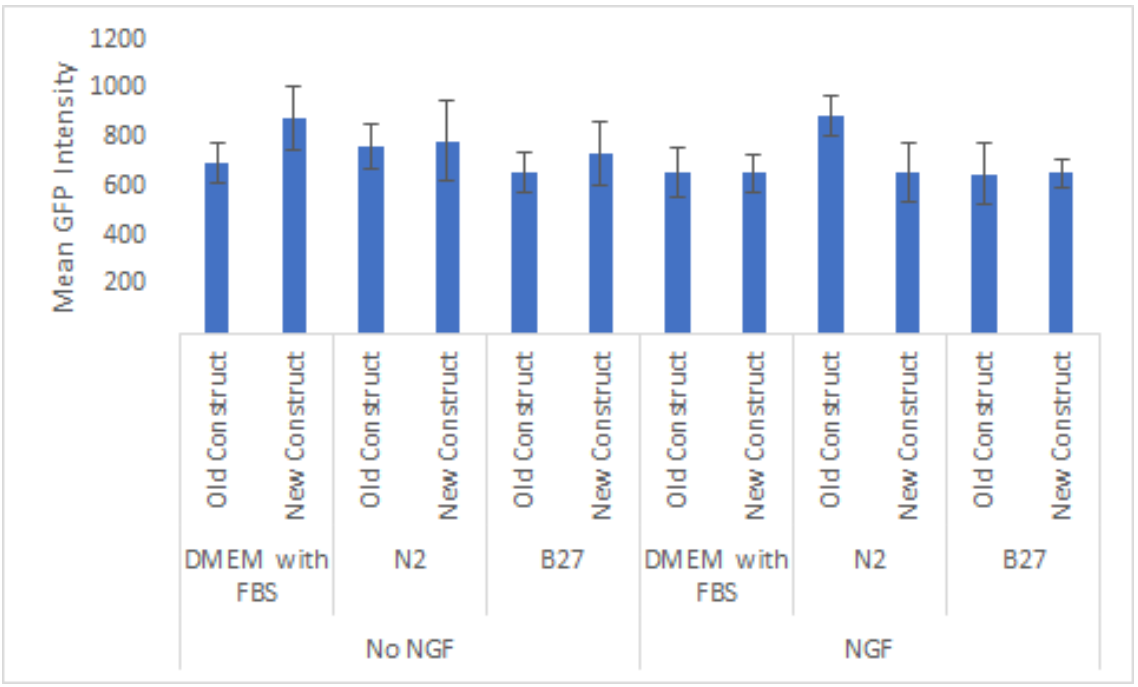

Figure 5. Mean GFP Intensity between the Old and New Construct by Media Condition. Error bars represent standard error of the mean.

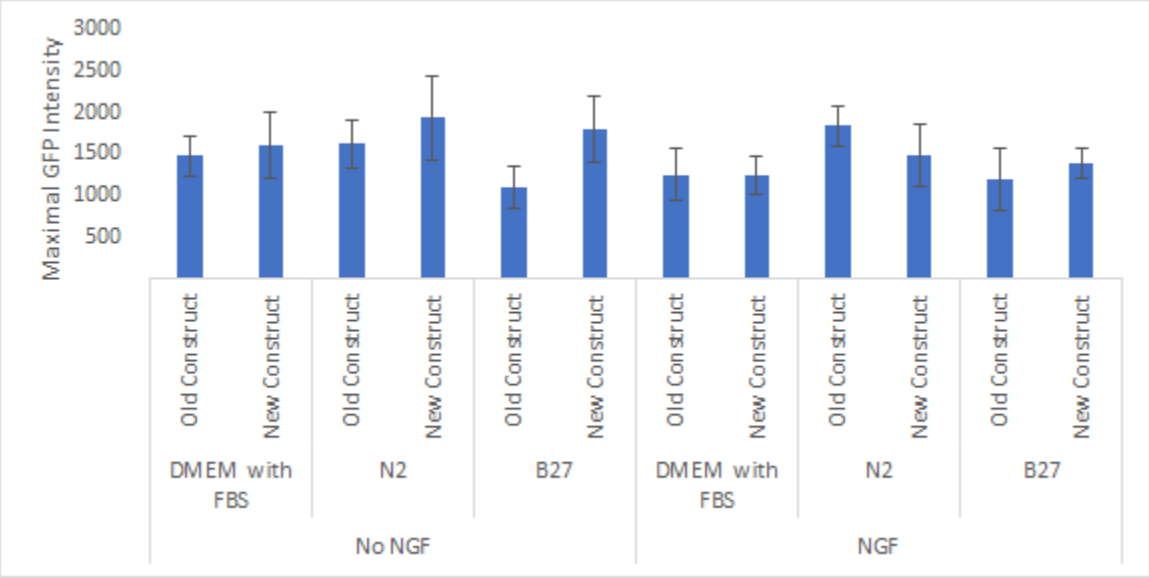


Figure 6. Maximal GFP Intensity between the Old and New Construct by Media Condition. Error bars represent standard error of the mean.

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared | Observed Power |
|---|---|---|---|---|---|---|---|
| Intercept | 30798864.753 | 1 | 30798864.753 | 383.936 | 0.000 | 0.861 | 1.000 |
| RA | 446911.408 | 1 | 446911.408 | 5.571 | 0.021 | 0.082 | 0.642 |
| Media | 980839.378 | 2 | 490419.689 | 6.114 | 0.004 | 0.165 | 0.873 |
| Construct | 23664.977 | 1 | 23664.977 | 0.295 | 0.589 | 0.005 | 0.083 |
| RA * Media | 437083.696 | 2 | 218541.848 | 2.724 | 0.073 | 0.081 | 0.520 |
| RA * Construct | 681686.656 | 1 | 681686.656 | 8.498 | 0.005 | 0.121 | 0.819 |
| Media * Construct | 152947.444 | 2 | 76473.722 | 0.953 | 0.391 | 0.030 | 0.208 |
| RA * Media * Construct | 65398.173 | 1 | 65398.173 | 0.815 | 0.370 | 0.013 | 0.144 |
| Error | 4973559.889 | 62 | 80218.70789 | | | | |
| Total | 66517850.45 | 73 | | | | | |

Table 1. 3-Way ANOVA of Media, RA, and Construct's effect on Mean GFP Intensity

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared | Observed Power |
|---|---|---|---|---|---|---|---|
| Intercept | 102449615.2 | 1 | 102449615.185 | 114.752 | 0.000 | 0.649 | 1.000 |
| RA | 1404145.21 | 1 | 1404145.210 | 1.573 | 0.215 | 0.025 | 0.235 |
| Media | 3469121.553 | 2 | 1734560.776 | 1.943 | 0.152 | 0.059 | 0.388 |
| Construct | 1191086.417 | 1 | 1191086.417 | 1.334 | 0.253 | 0.021 | 0.206 |
| RA * Media | 757873.4202 | 2 | 378936.710 | 0.424 | 0.656 | 0.014 | 0.116 |
| RA * Construct | 5779093.398 | 1 | 5779093.398 | 6.473 | 0.013 | 0.095 | 0.707 |
| Media * Construct | 4033408.043 | 2 | 2016704.021 | 2.259 | 0.113 | 0.068 | 0.443 |
| RA * Media * Construct | 16541.95013 | 1 | 16541.950 | 0.019 | 0.892 | 0.000 | 0.052 |
| Error | 55352896.27 | 62 | 892788.649 | | | | |

Table 2. 3 Way ANOVA of Media, RA, and Construct's effect on Max GFP Intensity

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared | Observed Power |
|---|---|---|---|---|---|---|---|
| Intercept | 73277254.07 | 1 | 73277254.1 | 541.8 | 0.000 | 0.8 | 1 |
| NGF | 125565.7731 | 1 | 125565.773 | 0.928 | 0.337 | 0.005 | 0.160 |
| Media | 204157.4247 | 2 | 102078.712 | 0.755 | 0.472 | 0.008 | 0.177 |
| Construct | 1438.147412 | 1 | 1438.147 | 0.011 | 0.918 | 0.000 | 0.051 |
| NGF * Media | 99792.97626 | 2 | 49896.488 | 0.369 | 0.692 | 0.004 | 0.109 |
| NGF * Construct | 261517.9092 | 1 | 261517.909 | 1.934 | 0.166 | 0.011 | 0.282 |
| Media * Construct | 218777.219 | 2 | 109388.609 | 0.809 | 0.447 | 0.009 | 0.187 |
| NGF * Media * Construct | 44700.25573 | 2 | 22350.128 | 0.165 | 0.848 | 0.002 | 0.075 |
| Error | 24073192.71 | 178 | 135242.656 | | | | |

Table 3. 3-Way ANOVA of Media, NGF, and Construct's effect on Mean GFP Intensity

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared | Observed Power |
|---|---|---|---|---|---|---|---|
| Intercept | 0.040246792 | 1 | 0.040 | 38.537 | 0.000 | 0.734 | 1.000 |
| NGF | 0.000926985 | 1 | 0.001 | 0.888 | 0.362 | 0.060 | 0.142 |
| Media | 0.00019551 | 2 | 0.000 | 0.094 | 0.911 | 0.013 | 0.062 |
| Construct | 0.003292457 | 1 | 0.003 | 3.153 | 0.098 | 0.184 | 0.380 |
| NGF * Media | 0.002935988 | 2 | 0.001 | 1.406 | 0.278 | 0.167 | 0.252 |
| NGF * Construct | 0.001267485 | 1 | 0.001 | 1.214 | 0.289 | 0.080 | 0.177 |
| Media * Construct | 0.003649944 | 2 | 0.002 | 1.747 | 0.210 | 0.200 | 0.305 |
| NGF * Media * Construct | 0.002101189 | 2 | 0.001 | 1.006 | 0.391 | 0.126 | 0.190 |
| Error | 0.014621313 | 14 | 0.00104 4379 | | | | |

Table 4. 3 Way ANOVA of Media, NGF, and Construct's effect on Max GFP Intensity

Electrophysiology

Our findings elucidate the character of both the opsin construct, as well as providing membrane and electrical character of the HT22 cell line that adds to Project 1's evaluation of the model. Figure 7 showcases how basic membrane properties changed within the differentiation conditions such as the resting membrane potential (RMP) and in the input resistance (IR). The B27/NGF/RA condition's RMP hovers near zero, which may correspond to the finding in Project 1 that a large proportion of cells exposed to B27 and RA become necrotic. Interestingly, the B27/NGF/RA condition does have a low IR which would correspond to plasma membrane breakdown in late-stage apoptosis/necrosis- however, it did not have a significantly lower IR than the other media conditions not thought to induce cell death. Figure 8 showcases a dual steady-state unique to N2, RA, and NGF differentiated HT22 cells that is reminiscent of the up and down states of striatal spiny projection neurons and may also be another novel indicator of differentiation character (Plenz and Kitai 1998).

ChrimsonR response in HT22 cells is shown in Figure 9, as a voltage response was procured within the HT22 cell expressing the new ChrimsonR construct. Figure 9 showcases the millisecond response from red-light activation, as well as the characteristic desensitization from a large amount of photon activation. The old and new constructs were compared on an IV plot which identified a similar reversal potential between the constructs, but more current response in the new construct at higher voltage (Figure 10). The new ChrimsonR construct's response to a blue-shifted laser was quantified, with results indicating a similar current response across the blue and red laser 2V pulses. However, figure 11 shows a significant difference in the time to the

peak of the current response in the new construct's response to the two lasers. Ultimately, the data shows both that HT22 cells can express the new ChrimsonR construct- and that the new ChimsonR construct shows some evidence of preferential activity with a red-shifted laser.

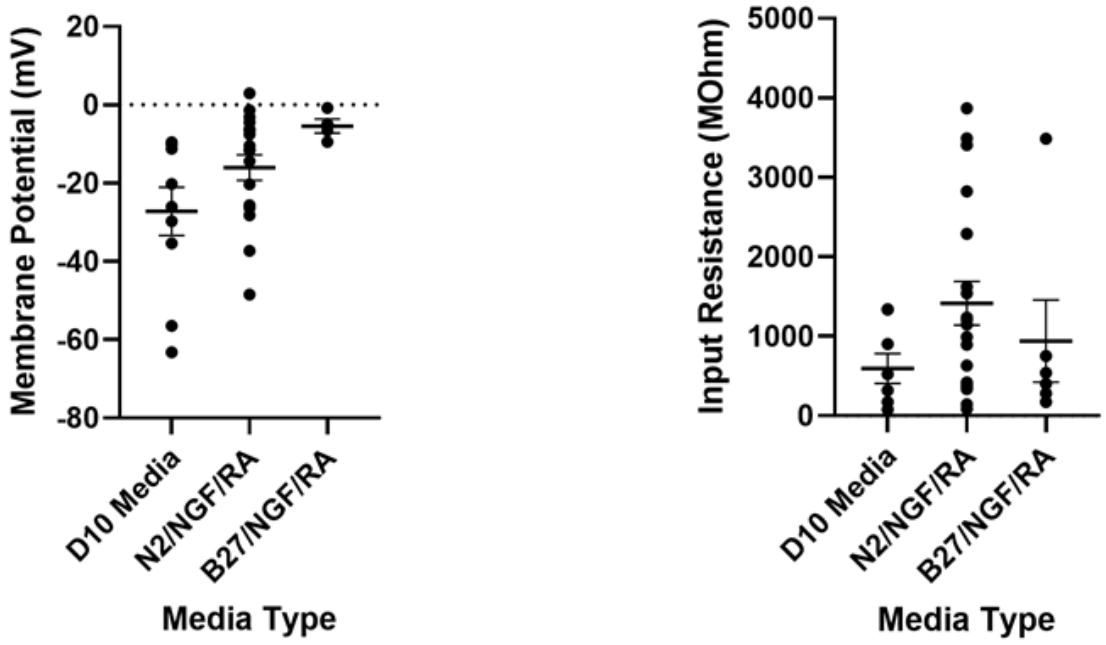


Figure 7. Resting Membrane Potential by Media type (Left) and Input resistance (Right). D10 Media corresponds to undifferentiated media. Error bars correspond to the standard error of the mean.

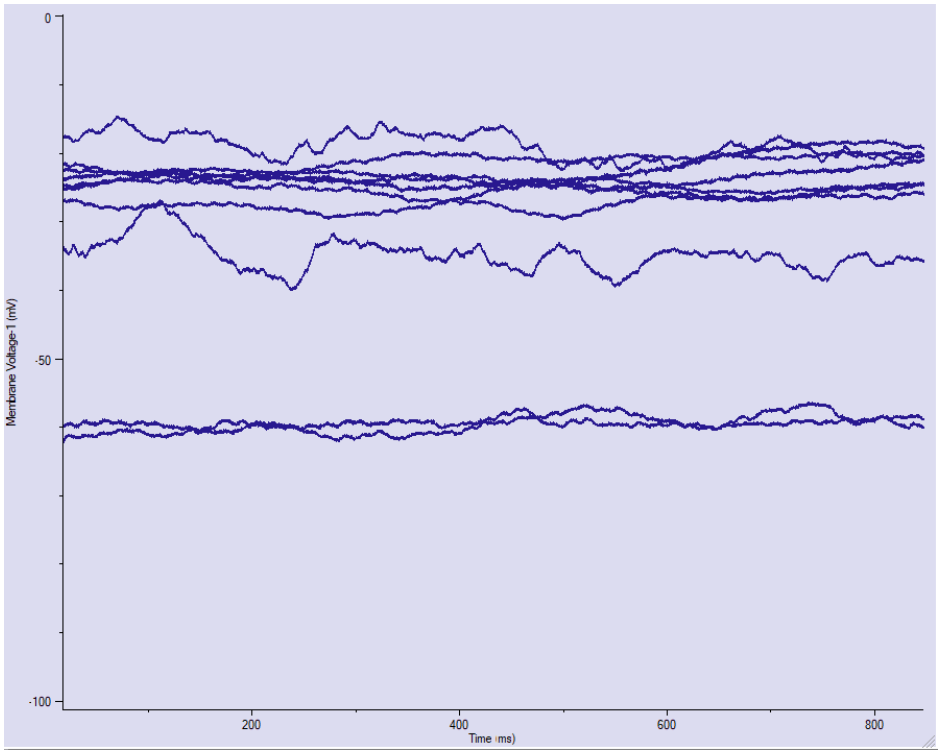


Figure 8. Example of a dual steady state of N2 with NGF and RA differentiated HT22 cell.

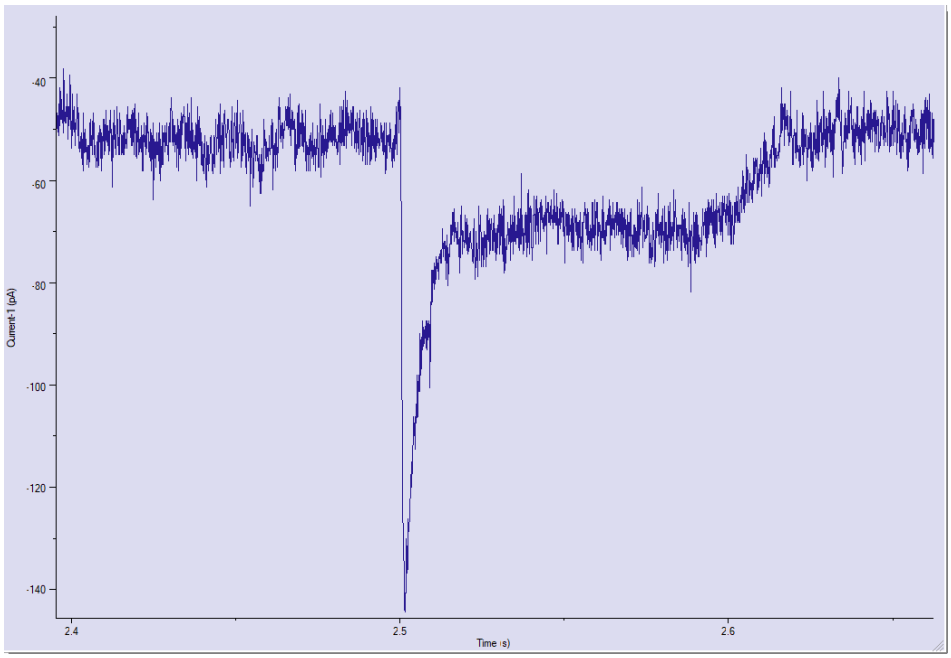


Figure 9. Example of positive current influx following 100ms red light activation of the new ChrimsonR construct at 2.5s

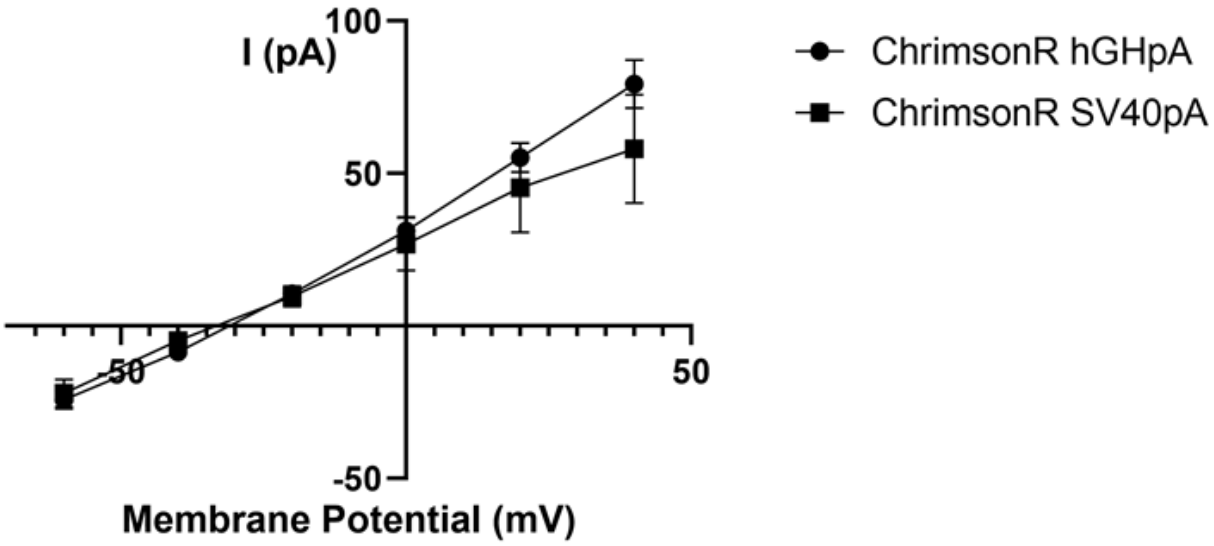


Figure 10. IV plot across HT22 cells differentiated with N2, RA, and NGF and transfected with the other new ChrimsonR construct or the old construct.

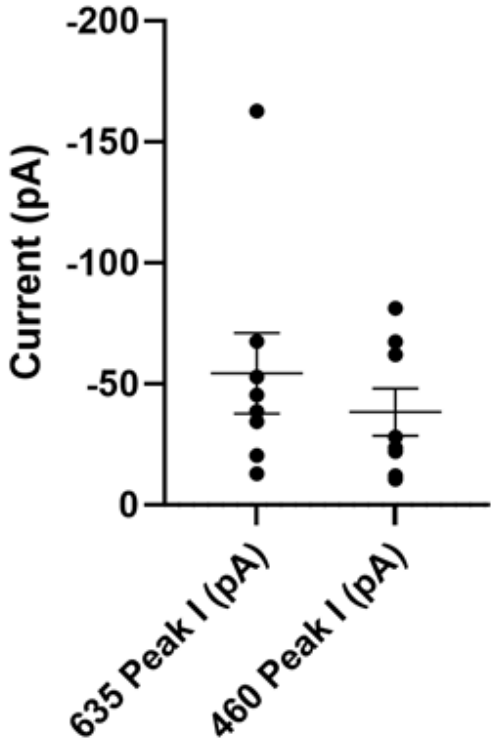

Figure 10. New ChrimsonR construct  current response to 2V activation of the 635 lasers and the 450 laser

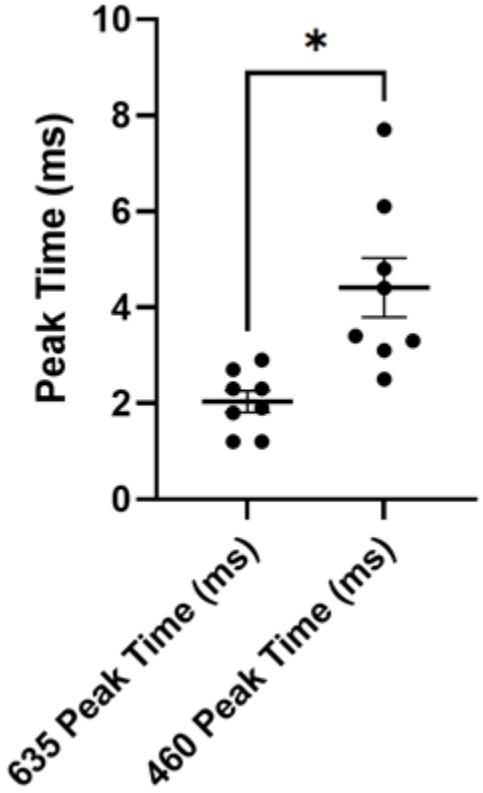


Figure 11. Time to peak response of the new ChrimsonR construct in response to a 2V pulse of the 635nm laser and the 450nm laser

in vivo injection

Viral surgery was performed on purified AAV-ChrimsonR to ascertain if in vivo expression was possible. Preliminary imaging at week 3 was done and is shown below. The low GFP expression is indicative of legitimate in vivo expression that likely needs more expression time to appreciably build up.

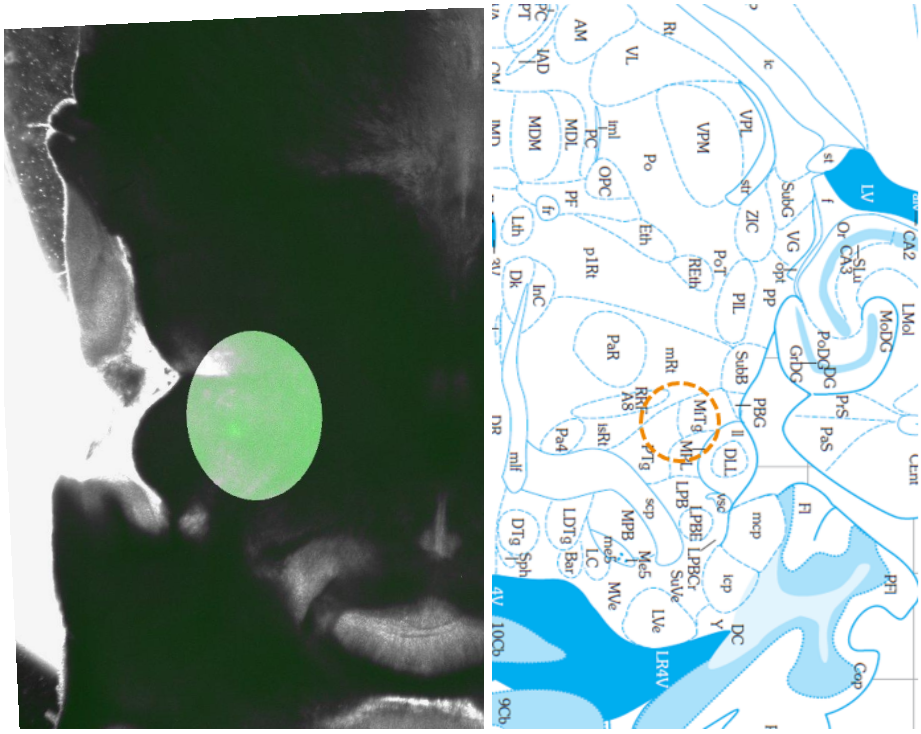


Figure 8. Mouse brain slice injected with PAAV-ChrimsonR in PPN (region highlighted to showcase localized GFP expression) paired with a mouse brain atlas

**Discussion**

Our results build upon project 1's differentiation protocol in the context of unique plasmid machinery such as differing poly-A tails (sv40 and human growth hormone) and point mutations in the neuron-specific promoter. However, one distinction between these CAMK-II-α's readouts between projects 1 and 2 is the localization. While expression occurs throughout the cell in CAMK-II-α GFP, in these ChrimsonR constructs the GFP is tagged to the proteins bound to membranes which can complicate the macro's ability to determine the GFP expression that extends outside of the DAPI stained areas. However, the procured results matched visual trends of the two constructs with notable differences with added RA supplement to induce CAMK-II-α upregulation. These results may suggest one of two outcomes. The first interpretation may be that the new construct's expression efficiency is just lower due to the mutations and/or the poly-A-tail when analyzing in vitro expression. This is not an entirely negative result, as this would solely imply issues with an in vitro expression which could be explained by the human growth hormone poly-A tail needing more complex Post Transcriptional Modification (PTM) that might only occur in vivo. The second interpretation might be centered around the promoter, essentially contending that the basic response elements within the promoter were altered between the original construct's machinery and the halorhodopsin machinery.

While our first interpretation might argue that this would result in decreased expression on account of the old promoter intrinsically operating better in vitro, this second model would contextualize the CAMK-II-α's complex genetic expression. CAMK-II-α is repressed in the early

stages of neuronal development, only expressing several days after birth with regulated expression in the context of Long-term Potentiation and Depression (Mayford et al. 1996). The mutations between the new and old construct promoter may culminate in more repression or inactivation of the new construct that might be less likely in the mutated old construct. Figure 1's characterization of several mutations cannot be understated, as several papers have shown drastic changes in gene expression on account of as few as one point mutation in the promoter in both an upregulated and downregulated fashion (Xu et al. 1999, Fourneir et al. 1999, Kaatz et al. 2005, and). Of course, immense oncological work surrounding p53 expression is centered around how transition point mutations culminate in system-wide dysregulation (Esteller et al. 2001). The array of mutations have not currently been analyzed to ascertain which parts of the mutated promoter region correspond to what function, however, we assume there is an empirical difference to some degree on account of the immense number of base changes. Future work may involve testing the regional importance of the promoter via an array of mutations to fully characterize the CAMK-II-α promoter. Of note, is that the new ChrimsonR construct contains the same promoter, Kozak sequence, GFP sequence, Woodchuck Virus sequence for tertiary RNA stability, and growth hormone tail as the construct utilized in Project 1. However, the visual readouts were differnely not merely in localization of fluorescence but overall intensity and efficiency. This would indicate issues of expression irrespective of the promoter, and possibly due to GFP tagging to the opsin construct and issues associated with folding. Pending data from the PTM readout experiment in project 1 might identify increased efficiency following differentiation, that might ultimately increase ChrimsonR folding efficiency as cellular machinery becomes more regulated. Future work should also be performed to

characterize each of the CAMK-II-α constructs and how fluorescence statistically compares between the two identical machineries.

Ultimately, Figure 3 identifies the end aim of this project: in vivo expression. While the old construct may express better than the new construct in vitro, we have early evidence that in vivo expression of the New construct is visible after only three weeks. Further expression time until the next slice preparation and recording should both increase the GFP output, and thus the ChrimsonR expression in mesolimbic circuitry we are aiming to test. There are a lot of possible implications for the preliminary results, but ultimate analysis and post-hoc testing results will need to be framed with the in vivo data. This would require further work to characterize the old construct's expression in vivo relative to the new, while also performing an array of recordings to identify possible functional results of any differences in expression.

In attempting to characterize optogenetic responses in HT22 cells, we procured data surrounding the membrane properties of HT22 cells that were wholly distinct from prior field findings. The only other paper in the literature with HT22 resting membrane potential data was procured via a novel method that utilizes carbon nanotube pipettes, ultimately establishing a resting membrane potential around -61.5 (+/- 2.97 mV)for the cell line (Schrlau et al. 2009). The Schrlau paper recorded at notably different conditions than us, such as recording at room temperature (we recorded at 34.5 C), using Hank's Balanced Salt Solution (we used Artificial Cerebrospinal Fluid), and not using an internal solution. Alternative recording methods such as perforated patch-clamp recording might be needed as the state of internal ions is not known and the Schlrau paper's high input resistance would indicate that their membranes did not have many ion channels (Linley 2013, Lippiat 2008, and Rae et al. 1991).

Other papers have notated the resting potential of HT22 cells in the context of differentiation, ultimately contending that differentiation actually decreases resting potential (Dermol-Cerne et al. 2018). More work should be done in increasing the number of cells we record from, while also making changes in the recording set up such that we match literature findings. These calibrations would make the interpretation of electrophysiological data more efficient, as the RMP and IR data procured from different cell recordings in different media conditions can actually be statistically analyzed. As of now, we show that differentiated cells have evidence of more mature neuronal properties- at least in the N2 condition with NGF and RA. Further, we establish both that HT22 cells can serve as an in vitro model for electrophysiology and that ChrimsonR can induce a current in response to red light. ChrimsonR did show response to blue light as depicted in Figures 10 and 11, though figure 11 indicates a statistically slower time to peak in response to blue light which is a promising preliminary result. Ultimately, it is the other preliminary trend in Figure 10 that identifies the new construct's higher current response at higher positive voltages that supports our hypothesis that the new construct will function better en route to dual input recordings relative to the old. Of course, Figure 8 identifies the end ex vivo experiment in which we perform these analyses in a more accurate model. However, Project 2 concludes with a justification of Project 1's model, an extension of Project 1's findings, and ultimately positive evidence regarding the new ChrimsonR construct's fidelity.

Project 3: Where There's a Worm, There is a Way: Utilizing Notch-Delta Signaling to Create a Unique Structural and Functional Method to Label Synapses

**Introduction**

*Prior Structural Experimental Techniques (Uni-synaptic)*

The growing nature of the field of structure-function Neurobiology has necessitated an equally innovative imaging toolkit to characterize neurocircuitry. Initial neuroimaging work was chiefly focused on the characterization of the brain on aggregate, utilizing broadscale imaging tools such as Magnetic Resonance Imaging to identify the structure or functional Magnetic Resonance Imaging and Positron Emission Tomography as a means to characterize function (Rosenbloom and Plefferbaum 2008, Glover 2012, and Tai and Piccini 2004). Aside from lacking the level of specificity at individual neural circuits, these techniques also preclude much simultaneous structural and functional work while also restricting the intricacy of much experimental design. Cytoarchitectonics also paved the way for wholescale 3D imaging of the brain, but the same constraints of previous wholescale imaging techniques restricted its utility to broad brain region mapping despite growing interest in circuit analysis. Cytoarchitectonics, however, set the stage for principle neural circuit analysis as its identification of distinct laminar organization and character of cortical neurons was essential in the outlining of the cerebral cortex (Zilles 2018). Even now this technique is being utilized to re-map the cortex, with the aid of modern structural-functional paradigms that this paper emphasizes in circuit analysis (Amunts and Zilles 2015). Cell level staining such as Nissl staining was quintessential for more targeted subpopulation staining of neurons, but such stains are unable to determine fine

circuitry identification nor any functional experimentation (Garcia-Cabezas et al. 2016). The cresyl violet dye used in the Nissl stain operates on the biological principle of ribosomal RNA aggregation in the rough endoplasmic reticulum, ultimately giving a rough overview of brain region characteristics such as the relative concentrations and sub-localization of neurons (Pilati et al. 2008).

Confocal microscopy was a key development and allowed imaging of individual neurons to a novel degree not previously accessible. With the ability to get high-resolution imaging, experiments interested in the structure-function characteristics of individual neurons could be performed (Zheng 2003). The utilization of transgenics and ectopic expression of proteins like GFP allowed proxy measurements of translational efficiency and protein localization at the neuron to neuron level. More intricate and distinct techniques like Golgi staining operate with a similar dynamic of staining individual neurons to identify, at the very least, the morphological character of the stained neurons (Pilati et al. 2008). However, such individual staining techniques often are insufficient in more complex structural characterizations. And when utilized by themselves, these staining techniques offer little to no elucidation of information at the microcircuit level- much less at a macrocircuit level. The focus on the individual neuron is key to understanding the cells that comprise the circuit, but permutations to such methods were required in order to characterize, or even locate, these circuits themselves. Antibody staining may be often paired with other staining techniques for more fine-tuned internal morphology analysis, but circuit level information and neuron-level functional characteristic remains unknown with such staining (Glynn and McAlllister 2006). Further, initial iterations of these staining experiments required the usage of harsh fixatives that not only prevented

functional characterization but also confounded individual neuronal analysis and entirely disrupted an ability to map out what connections existed in a given region (Davis et al. 2014).

As interest in circuitry increased, so did technique development with the aim of proving the existence of the synapse in concept. Electron microscopy often serves as a gold standard for synaptic connection, as clear images of connections and active zones can be visualized broadly as a proof of principle experiment(Gray 1969). However, the immense preparation and expense of EM not only precludes it from ready usage in circuitry analysis- but the information procured, despite being highly valued for evidence of a neuron to neuron connection, is immensely limited in both the scope and detail of information it can provide (Burette et al. 2015). While individual synaptic proteins and connections can be clearly displayed and evaluated, the technique relies on both prior speculations of a synaptic presence in the imaged region and context for evaluation of the given synapse. Void of additional labeling techniques, electron microscopy operates a very expensive magnification method that, at its best, provides immense detail at the cellular level and not a circuitry level. The pairing of ectopic gene expression or antibody labeling facilitates increasing specificity of electron microscopy imaging but is still limited by the staining method's accuracy and information as there are only a few markers that delineate certain types of the synapse (Gray 1969 and Burette et al 2015). Further, staining without broader circuitry knowledge only identifies cells with a given marker and not within a given circuit and myriad of connections. In this way, even neurons with entirely unique staining properties being paired with EM-imaging output would only be able to identify that a synapse can exist for these neurons through a highly detailed image of one cell with an ambiguous connection. Electron microscopy can be taken with transmission electron microscopy using

electrons and thin sheets of a sample or scanning electron microscopy that can scan an entire surface area, ultimately offering wider scale images that are still subject to connection-level ambiguity and functional understanding of the circuit (Wang et al. 2017 and Borges-Merjane et al. 2020).

Even beyond electron microscopy, new techniques such as super-resolution fluorescence microscopy gives us even finer looks at the neuronal structure with resolution far beyond that of conventional microscopy (Guo et al. 2019). Stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy provide further spatial precision via activating photo-switchable fluorophores (PALM) with light at any one time so that each fluorophore can be localized to a precision much finer than the resolution limits of diffraction utilized in conventional microscopy (MacDonald et al. 2015). The STORM and PALM provide data that elucidate information about synaptic distance and growth over time- which is of specific use to projects interested in plasticity, neurogenesis, and synaptic function at large (Dani et al. 2011). Stimulated emission depletion microscopy (STED) also gives super-resolution via depleting fluorescence from surrounding areas but leaving the central focal point of the same activity to emit fluorescence- this method allowed the spatial reconstruction of fly neuromuscular synapses (Badawi and Nishimune 2020 and Badawi and Nishimune 2018). These various techniques offer researchers greater insight into items like synaptic structure, which facilitated synaptic connection analysis to move far beyond mere experimental evidence of synaptic presence.

*Prior Functional Experiments (uni-synaptic)*

Staying at the uni-synaptic level, even the most advanced structural information about the synapse is limited in its implications for more integrated and complex processes such as behavior. Identification of given gene localization, or even protein determinations at the synapses in pre or post-dependent ways, inherently only tells half the story. Even prior works that have determined changing synaptic protein levels following a given stimulus or condition are not sufficient to fully characterize the synapse. Perhaps the most direct method of synaptic functional characterization relies on the utilization of electrophysiology. Electrophysiology allows the selective activation of a presynaptic neuron while recording from a postsynaptic neuron and collecting the resulting data from a myriad of conditions (timing, intensity, treatment, etc.). For synaptic work, electrophysiology is both a key technique to characterize the presence of the synapse experimentally as well as the behavior at the neurobiological level (Moradi and Ascoli 2019, Glasgow et al. 2019, Rohrbough and Broadie 2002, and Li et al. 2019). Void of functional work, the immense structural detail procured would only be correlative in nature (Glasgow et al. 2019).

There are many conventional methods to functionally characterize the synapse, namely targeted excitation of a presumed pre-synapse while recording from the presumed postsynapse. This recording is often ambiguous without the paired structural work, as many different synapses exist in the central nervous system even within a given area (Klenowski et al. 2015). The paired complexity of inhibitory, modulatory and excitatory neurons and other distinct synaptic functional behaviors at the neurobiological level necessitates the projects interested in synaptic research to take a structural and functional approach (Glasgow et al. 2019). And, with synaptic processes like neurogenesis or plasticity- this need for a paired approach is amplified.

However, even intricate structural and functional works that evaluate a marked synapse are limited unless tracing beyond one connection occurs.

*Polysynaptic techniques*

Poly-synaptic tagging mechanisms that can illuminate long circuits of synaptic connection are integral in the creation of a functional neural map. Further, polysynaptic work feeds back into progressing uni-synaptic work, as well as the field at large, as it offers a database that future projects and grants can tap into. Much of the aforementioned ambiguities of early synaptic work stems from the poor characterization of the large scale neural networks

Diffusion Tensor Imaging was key for the characterization of fiber bundles, as it uses the fact that water diffusion in white matter goes along the axon but in the gray matter it is equal. Thus, white matter connections could now be labeled in such a way that the fiber bundles would add in key information detailing the location, density, and path of larger-scale connections. However, the inherent uniformity of gray matter connections limits the utility of this technique- which subsequently, drastically limits the extent of neural mapping that can occur (Wen & Chklovskii, 2005, Gilmore et al. 1996, and Ecker et al. 2013). Further, diffusion tensor imaging only provides detailed information on the scale of millimeters even in the white matter of larger brains such as primate brains.

More apt techniques allowed both white and gray matter labeling, which could ultimately be paired with diffusion tensor imaging, that could label intricate networks in an efficient and easily recordable manner. The first major iteration of path-wide labeling came in the form of tracers, which were divided into anterograde and retrograde labeling pathways. Anterograde tracers can travel down axons to label where the process projects towards

ultimately being absorbed by the corresponding post-synaptic neuron, utilizing a variety of different fluorescent or radioactive imaging methods (Armstrong et al. 1985 and Gerfen et al. 1989). Retrograde tracing occurs in reverse, with axonal uptake of a tracer back to its respective cell body (Wang et al. 2006). Aside from its inherent limitation of only showing projection patterns, these tracers also fail to differentiate specific output patterns as even highly accurate injections bleed over to all cells in a given area. Thus, simple tracers are not efficient neural map creators if prior information and concurrent techniques, such as immunostaining, are not paired to make sense of projection paths. Genetic techniques, such as the utilization of Cre-dependent expression of markers, and viral delivery methods have advanced tracer expression to specific populations of cells that Cre-lines exist for (Lo and Anderson 2011). Additionally, viral and dye polysynaptic labeling is unable to clarify the number of synaptic steps in a given network due to different rates of propagation (Ugolini 1995 and Card et al. 1999).

More integrative techniques are needed to label complex circuitry separated by long distances or multiple connections. The rabies virus has been utilized as a retrograde racer, via modifications to its internal replication and trafficking machinery, to only spread to direct presynaptic cells (Wall et al. 2010). Scaling up one by one, this monosynaptic approach allows a slow iterative process to structurally define immediate circuits. This iterative process allows for some degree of control, however, the monosynaptic labeling approach requires very cytotoxic viruses to be implemented, while simultaneously undersampling presynaptic neurons (Curanovic 2009 and Beier et al. 2011). Complex techniques like GRASP, with functionalized GFP linkages at the synapse as a form of synaptic readout, confound further analysis as novel linkages at the synaptic zone are formed (Wikersham and Feinberg 2012). Thus, even when

evaluating solely structural synaptic labeling techniques- there are severe confounds that limit synaptic readout or even modulate it. This inherently precludes accurate paired functional data that may be procured.

Labeling synaptic connection is accomplished using a myriad of techniques such as dyes, viruses, electron microscopy paired with electrophysiology, and GRASP (Gaffield and Betz 2007, Gillet et al. 1986, Wikersham and Feinberg 2012, Tsetsenis et al. 2014, and Feinberg 2008). These techniques have been integral in the uncovering of specific structural elements of brain regions such as feedback loops integral for learning, Hippocampal Kinetics, and Olfactory input mapping (Cervantes-Sandoval et al. 2017, Klingauf et al. 1998, Miyamichi et al. 2010). Cre labeling of c-fos genes has been utilized as well to create such synaptic maps, but is restricted fundamentally to only activated neurons and not any synaptic connections such that wholescale neurocircuitry labeling is not possible (Liu et al. 2012 and Denardo and Luo 2017). Synaptic labeling historically has opened the field to ask more questions, as previously ambiguity of brain regions and unknown character of regional connections restricted scientific inquiry. The labeling of specific neuronal subpopulation connections allows bodies of literature to guide further structural and functional work in the inquiry of immensely complex processes such as Engram networks or Psychiatric disorders (Choi et al. 2018 and Belmer et al. 2017).

However, overall these techniques are hampered by providing relatively limited functional information that can be procured. Viral labeling techniques can be employed alongside functional techniques such as electrophysiology, but it was impossible to distinguish labeling polysynaptic inputs from monosynaptic inputs until the advent of a modified Rabies

virus method (Braz et al. 2009, Sun et al. 1996, and Wickersham et a. 2012 ). Additionally, viral and dye polysynaptic labeling is unable to clarify the number of synaptic steps in a given network due to different rates of propagation (Ugolini 1995 and Card et al. 1999). Some researchers have switched to a monosynaptic approach, labeling only neurons immediately synaptically connected, to counter the pervasive ambiguity of polysynaptic labeling (Wikersham et al. 2007). However, the monosynaptic labeling approach requires very cytotoxic viruses to be implemented, while simultaneously undersampling presynaptic neurons (Curanovic 2009 and Beier et al. 2011). More intricate techniques like GRASP may actually be confounded in structural analysis, as the GFP links may stabilize or modify transient synapses to depict incorrect networks (Wikersham and Feinberg 2012). While these approaches are common in much of the structural work done with synapses, there are empirical issues that affect both the research process and data procured.

Many of the current structural analysis models are also limited in the amount of functional data that can be drawn from raw data, or procured simultaneously. Viruses and GRASP present real confounds that alter the legitimate neurocircuitry altogether, such that functional representation would be skewed. More appropriate models that elucidate exactly the nature of synaptic connections in basic networks, like that of the Subthalamic Nucleus-Pedunculopontine Nucleus- Substantia Nigra pars compacta (STN-PPN-SNc) dopamine reward pathway, are needed before there can be field-wide progress in studying neurobiological changes surrounding addiction or Parkinson's disease (Beaudoin et al. 2018). Projects that aim to understand even minor neuronal subpopulation changes or interactions are stuck using confounding techniques or intricate combination methods in order to evaluate relevant

structural and functional changes following conditions. The inverse limitation is also present, with many functional techniques like optogenetics or electrophysiology often operating with limited structural roadmapping and identification- severely undermining implications of procured results. Without the presence of a reliable and efficient structural-functional labeling method, further neural mapping will be hindered by this limitation. Thus, we need a trans-synaptic labeling technique that will allow for structural and functional characterization without confounds of inefficient labeling or cytotoxicity.

*Notch-Delta*

The Notch-Delta signaling system is a conserved biological pathway implicated in wholescale development, with relevant gene expression changes in neural development (Guruharsha et al. 2012). While its utilization has been integral for a lot of neuroscience research, particularly projects interested in neurogenesis, it has been studied principally as a process in it of itself (Lasky and Wu 2005, Hämmerle & Tejedor 2007, Granbarbe et al. 2003, Xiao et al. 2009, and Lecourtois & Schweisguth 1997). However, recent work has implemented the conserved Notch-Delta mechanism as a technique to image various tissues, including the brain, in a target-specific mechanism (Vooijs et al., 2007 and Pellegrinet et al., 2011). Upon Notch, a transmembrane protein, interaction with Delta, another membrane protein, a signaling process follows endocytosis of the Receptor ligand pair that is paired with the unfolding of a juxtamembrane negative control region in Notch that is key for the subsequent extracellular cleavage of the Notch protein, by ADAM10, and intracellular cleavage, by gamma-secretase (Kopan 2012, Dunn et al. 2009 and Brunkan and Goate 2005 ). The intracellular domain

functions as a key transcription factor, as its valine and methionine residues at the N-terminus facilitate its bypass of the N-degradation pathway (Tagami et al. 2008). This signaling process plays on a biologically pre-set asymmetry of Notch and Delta protein expression, utilizing a numb signaling pathway to inhibit Notch expression in one cell and ultimately upregulate Notch signaling in another cell (Shao et al. 2017). Ultimately, the signaling pathway only works in a trans-expression dynamic, with internal pathways that determine if the cell will signal or if the cell will receive the signal to prevent accidental cis-type activation (Sprinzak et al. 2010). The compounded trans-specific nature of activation paired with asymmetric expression ensures that this species-wide conservation of signaling only activates under certain paradigms: two adjacent cells expressing Notch and Delta respectively in close proximity.

The homolog of Notch and Delta in C. Elegans is GLP-1 and Lag-2, respectively (Petcherski & Kimble 2000). The pathway is relatively conserved, though C. elegans do show evidence of a soluble delta-like ligand that binds the Notch receptor in an adaptor protein-dependent manner (Komatsu et al. 2008). Overall, there is high conservation of activity of the Notch-Delta signaling pathway such that we contend trans-species activation should be possible.

*Approach*

Here we introduce a labeling method that utilizes the Notch cleavage mechanism in order to label either pre or postsynaptic neurons. Previous work has utilized components of our method to image various tissues of the mouse, including the brain, but have not been implemented in a fashion to label synapses (Vooijs et al., 2007 and Pellegrinet et al., 2011).

Using the Notch approach, unambiguous and controlled structural information may be procured without cytotoxic or synaptic fusing confounds. Additionally, our method would circumvent the permutations required for the prior synaptic labeling techniques to yield functional information. Using a Notch-mediated synaptic readout, functional tests like optogenetics or electrophysiology can be performed without the presence of confounds and with the facilitation of a relatively convenient and targeted readout. Our approach utilizes natural machinery and mechanisms and co-opts them to express reporter genes in either the pre or postsynaptic cell. Projects that utilize the Notch mechanism of synaptic labeling gain an incredible degree of control over the system, as researchers can choose both the neural networks they want to map as well as whether the mapping occurs in a pre or postsynaptic fashion- thereby implicating structural mapping with directional control.

The Notch method also utilizes already established techniques of Cre-recombinase expression and AAV vector injections, thereby allowing controlled area and timing of expression with incredible reliability (Ahmed et al. 2004, Abdallah et al. 2018, Kaspar et al. 2001, French and Annex 2014, and Sengupta et al. 2017). However, while previous approaches utilize viral vectors to label synapses via an incredibly cytotoxic process, our method should not culminate in any noticeable effect in the neurons and label pre-synaptic populations with more efficacy (Grisanti et al. 2013, Curanovic 2009, and Beier et al. 2011). This new method allows a field-wide shift to easily and confidently procure more accurate structural information, while also permitting, in the same research design, a plethora of functional studies to be performed as well. The Notch-mediated synaptic readout promotes a holistic characterization of the synapse that could set a field-wide precedent in how neural networks are ultimately mapped.

Currently, much emphasis is placed on defining merely where connections are with little to no directional characterization and stable multisynaptic labeling options. Our method permits directional characterization in a retro- or anterograde fashion to characterize multiple synapses within one experiment while also inviting paired functional work to be done in order to define implications of newly characterized synapses.

Establishing a dual structural and functional approach in neural mapping encourages the field to build future research questions and projects on a greater foundation. Inquiry into complex neural systems can thus be founded upon unambiguous structural information paired with an in-depth functional understanding, instead of vague networks plagued with confounds (Oishi et al. 2020, Bourdy et al. 2014, and Watabe Uchiha et al. 2012). We posit our Notch-mediated method as a cheaper and more convenient standard in synaptic tracing experiments for both central and peripheral nervous systems. This project builds upon already existing methods that combine Notch and CRE recombinase for tissue labeling and adds in key permutations such that the system can label synapses with the same high fidelity (Grisanti et al. 2013, Pellegrinet et al. 2011, and Liu et al. 2015). Ultimately, our method should offer a universal labeling technique that can be customized within each project to achieve greater structural and functional information.

This technique not only allows easier cross experimentation between structural and functional inquiry but can be directly utilized in novel functional experiments. The sub-region specificity, and freedom to insert any gene in the Cre-dependent plasmid, can permit previously inaccessible experiments that aim to characterize specific neural circuits to be performed via cell-specific optogenetics. Further, the potential for behavioral neuroscience advancement is

evident in the ability to use this method for a Cre-dependent knockout of a gene, such as a receptor or transcription factor, within a specific neuronal subpopulation characterized by their synaptic output in order to determine associated behavioral changes. The potential applications to all fields of neuroscience are diverse, given that we can prove our cross-species approach of utilizing Notch-Delta signaling can occur. We hope this to be a new cornerstone technique in neuroscience, available to be further developed and applied to various sub-disciplines.

The basic mechanism of our method utilizes the conserved Notch and Delta signaling that results in gamma-secretase cleavage in order to activate genetically inserted reporter genes. (Dunn et al. 2009 and Brunkan and Goate 2005 ). This fluorescence is accomplished by altering the portion of the Notch that is released, the intracellular domain, to instead be a Cre-recombinase enzyme that can facilitate the alteration of any loxP flanked gene sequence including activating fluorescence protein expression. By procuring just three constructs, a cre-dependent reporter gene, a C. elegans Notch-Cre construct, and a C. elegans Delta construct, the pieces needed for our synaptic labeling mechanism are complete. Further experiments will involve the construction of an AAV-Notch-Cre plasmid that also contains important structural elements like the woodchuck virus that stabilizes tertiary RNA structure and reporter genes to determine expression. There are currently plasmids available that can be digested and religated in order to easily create our construct of interest. However, limitations on the maximum sizing of our insert in an AAV vector with a promoter and polyA tail is 2.5 kilobases, necessitating the usage of a trans-species construct containing Notch from C. elegans (Gordon et al. 2008). There are key benefits to the usage of a trans-species construct as it

eliminates possibly endogenous Delta ligand binding to the Notch-Cre receptors we express, but it is still essential that we create a set of plasmids that will drive stable expression.

After the C. elegans Notch-Cre construct, the C. elegans Delta construct, and a Cre-dependent GFP plasmid is procured, the next step would be to show proof of concept that our plasmid can express in multiple in vitro systems, and ultimately complete our proposed mechanism. Cre-dependent expression of the GFP plasmid occurs when the Notch and Delta bind, prompting gamma-secretase cleavage of the intracellular domain and trafficking to the nucleus where the cre-dependent GFP plasmid is recombined to activate expression of GFP. The construction of AAV plasmids is not necessary for confirmation of plasmid expression but will be completed in the initial steps of the project for its ultimate use in vivo. At the completion of this specific aim, there should be two sets of our plasmids: One set operating under a viral promoter such that simple expression testing can occur and one set under a neuron-specific promoter for expression and mechanistic testing in vitro, which can also be packed in AAV for in vivo expression

**Method**

*Construct creation*

The GLP-1 construct was synthesized from C. elegans genomic sequence reverse translated using optimized codon usage for mouse cells, enabling expression in mammalian cells (IDT). The sequence containing the GLP-1 sequence was cleaved out utilizing the restriction enzymes Kpn1 and Xba1, and ligated into a pCDNA3 vector with CMV promoter driving GLP-1 and a second SV40 promoter driving expression of GFP. Cre-recombinase was procured via a PCR reaction on a Cre-containing plasmid that was tagged with a NLS sequence (AddGene:49056), and ligated into the GLP-1 pCDNA construct with restriction enzymes PspOM1 and Xba1 (Figure 2). This stage of the ligation will be sufficient for initial proof of principle testing in both the cos7 cells and the HT22 cell cultures and is shown in Figure 1. Subsequent work in more neuronal populations, such as differentiated HT22 cells and primary neuronal cultures, will require a CAMK-II-α driven construct. This will be accomplished using the ChrimsonR plasmid and utilizing restriction enzymes Sal1 and Kpn1 for the GLP1-Cre construct and Xho1 and Kpn1 on the ChrimsonR plasmid which will also contain the optimized poly-A tail described earlier and AAV inverted repeat sites for in vivo work (Figure 4). The success of this final ligation plays on the established Xho1 and Sal1 interaction that results in ligation and loss of the site (Putz et al. 1996).

The lag-2 construct was procured from Dharmacon (OCE1182-202133947 C.elegans CloneId:Y73C8B.4 ORF), and ligated into a pCAGGS-IRES2-tdTomato plasmid utilizing restriction enzymes Xho1 and Btg1 (Figure 3). This construct will be sufficient for in vitro proof of principle

testing in the same HT22 and cos7 cultures, as it is tagged by a red-shifted fluorophore that will not coincide with the GFP fluorescence induced by Notch-Delta binding.

*Proof of Principle Testing*

The method to test cre-dependent expression via our proposed Notch-Delta mechanism will stem from expressing lag2 in one culture and GLP1-Cre in another culture in a 12 well dish. The lag2 transfected cells will then be trypsinized and re-plated onto the GLP1-Cre transfected cells after 24 hours of transfection. The cell combo will then be fixed after 48 hours and imaged for both green and red fluorescence. The initial cell-line utilized will be cos7 cells due to their general model of an adherent eukaryotic cell line, and relatively high transfection efficiency (Cordeiro et al. 2017, Cryan et al. 2004, and Carbajo et al. 2019). The second round of testing will occur in differentiated HT22 cells, in order to model a semi-neuronal culture that can build up to in vivo viral injection. Both the viral-driven and CAMK-II-α driven plasmid sets will be tested in the differentiated cells, ultimately building up to the final round of testing in primary culture.

The primary culturing step will also be co-cultured with glial cells, to induce synaptic formation to test the ability of our method to synaptically label in vitro. After three weeks of culturing, following the method established in Beaudoin et al. 2012, we will observe functional synaptic connections on a 12 well dish and transfect the CAMK-II-α driven GLP1-Cre construct and the CAMK-II-α driven delta into neurons (Beaudoin et al. 2012). The cultures will be carefully observed for labeled neurons and direct synaptic connection will be tested by using electrophysiology, and will subsequently be fixed 2 days after repeating the previously

described transfection scheme. Imaging will follow the outline from Project 1- utilizing a modified macro that identifies both Green and Red readouts. Further analysis will include identifying green readout only in the context of adjacent red fluorescence readout to confirm our mechanism. Ultimately, the green readout will coincide with the in vivo presynaptic cells and the red readout will correspond to the postsynaptic cell during the end stages of the project concerned with structural and functional mapping.

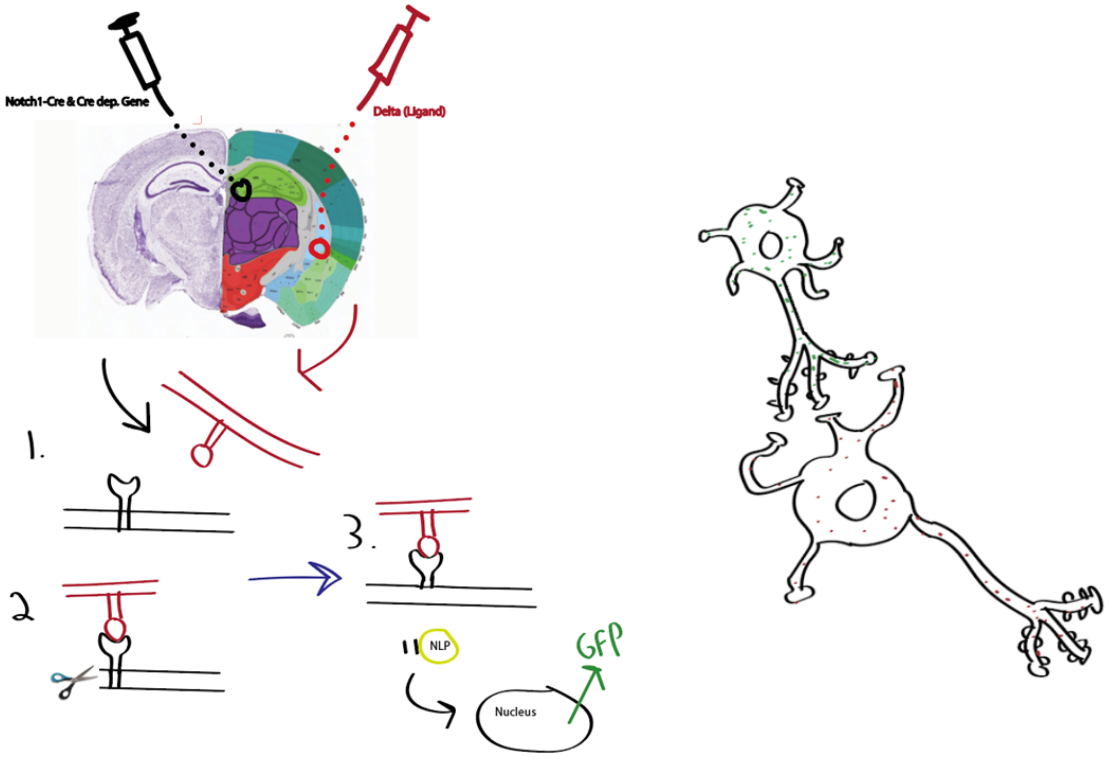


Figure 1. Schematic Representation of the Notch-Cre Delta Mechanism. Green Represents readout in the Presynaptic cell, while Red represents readout in the Postsynaptic cell.

**Results**

Plasmid constructs made for the non-neuron specific proof of principle tests were successfully ligated, screened with Btg1 (GLP1-Cre) and Kpn1 (lag2), and sent for sequencing.

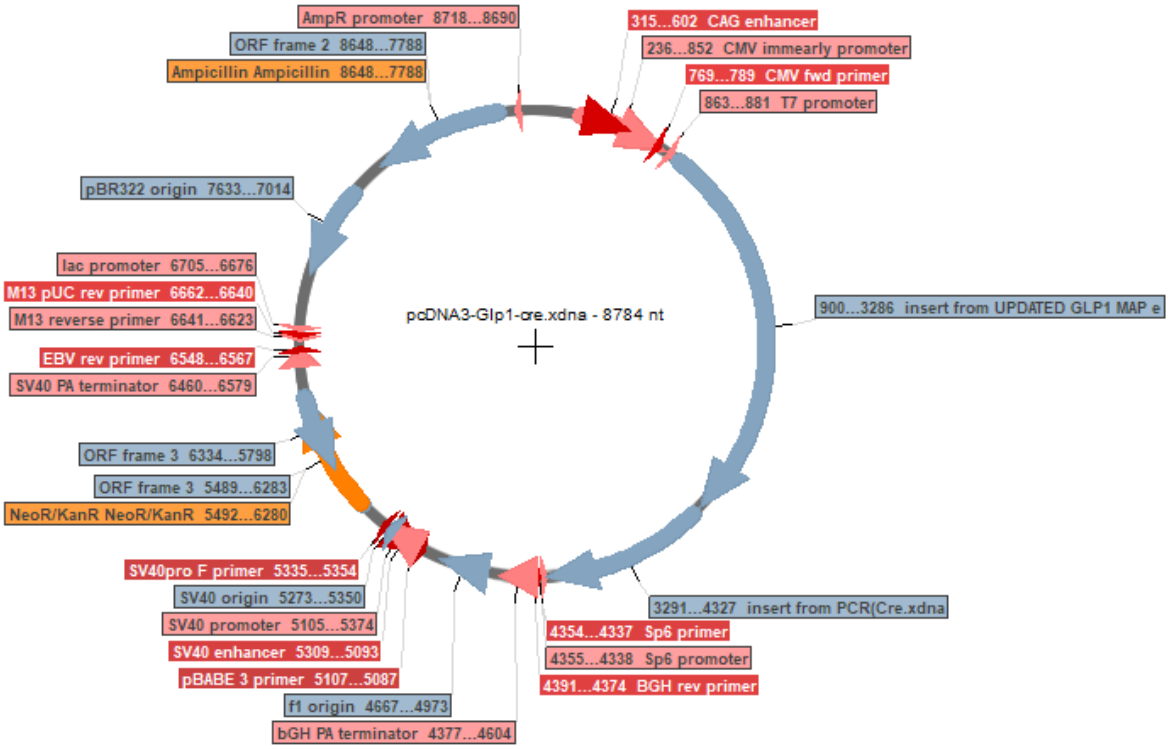


Figure 2. Construct map of GLP-1 Ligated to Cre within a PCDNA vector

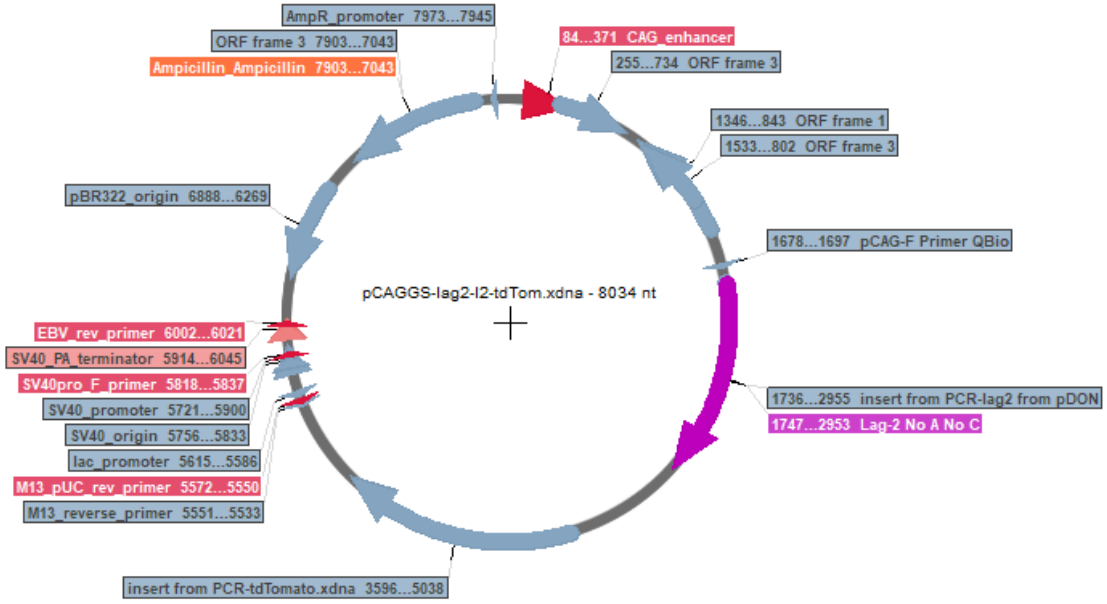


Figure 3. Construct map of lag2 within a PCAGGS vector

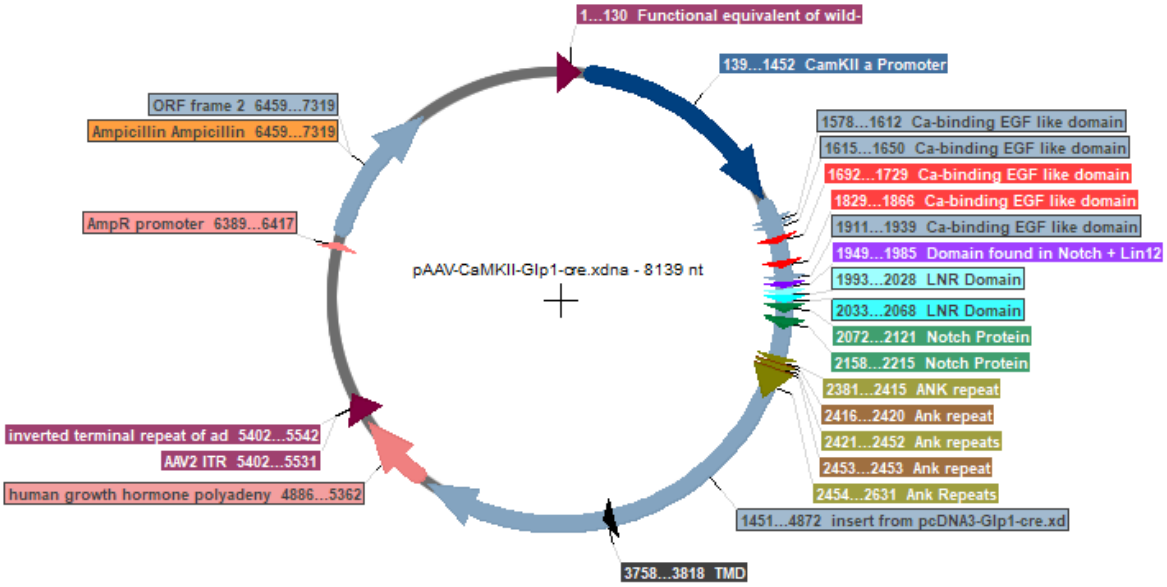


Figure 4. CAMK-II-α driven GLP1-Cre construct with inverted terminal repeats

**Discussion**

Future experiments are still pending until sequencing for the lag-2 construct is finalized. Immediate work following sequencing will be determining the base mechanism and its plausibility outside of C. elegans. The work done in projects one and two were key to set up the cell culturing work in this phase, as we will start with HT22 cells (undifferentiated and differentiated) as our model. The scope of what we feel can be accomplished this semester is simply in vitro proof of principle testing in three different models: undifferentiated HT22 cells, differentiated HT22 cells, and primary hippocampal neurons. The successful output here would include merely GFP fluorescence in the cell with Cre-dependent GFP, as this would indicate the Cre is being trafficked following our proposed mechanism. At the primary cell level, following synaptogenesis, we hope to create functional synapses that would allow proof of principle that our mechanism can be a synaptic specific readout (as opposed to merely being near the lag2 injected cell). Primary cells can also be induced to produce functional synapses, which may facilitate both the structural readout and a functional readout if cre-dependent channelrhodopsin, which was purchased from AddGene, was also transfected.

The biggest forthcoming limitation following successful sequencing is the trans-species nature of the proteins, coming from C. elegans and being brought to an array of murine models. This type of experiment is not unprecedented, as prior researchers have developed methods to transfer yeast DNA for expression in mammalian cells (Davies et al. 1992). We believe that the Notch-Delta pathway is highly conserved, and thus ought to allow this sort of cross-species regulation and coordination. Aside from basic genetic problems, which were largely addressed in utilizing IDT nucleotide correction, there is also concern about intracellular mechanism

coordination. We believe the pathway is conserved enough that downstream recruitment of gamma-secretase can still occur in light of a trans-species construct, however, this has yet to be determined. Injection of C. elegans gamma-secretase is an optional step we may add barring any mechanism issues we encounter, as nuclear transport of Cre-recombinase is certainly conserved. However, in light of any possible issues that may arise we have an alternative approach designed utilizing mutated murine Notch and Delta proteins that are specific to one another and not wild type Notch and Delta. Such an approach is not unprecedented, with Yamamoto et al. 2012 mutating a region of epidermal growth factor repeat-8 to cause selective Delta family affinity (Yamamoto et al. 2012). Another future permutation in the genetics design might involve a cre-dependency of delta, such that cre-mouse lines would selectively express Delta in dopaminergic neurons for example (Zhuang et al. 2005, Vuong et al. 2015, Papathanou et al. 2019, and Pupe and Wallen-Mackenzie 2015).

In accordance with the previous two project's cellular work innovations, we propose an attempt to establish HT22 cells as an in vitro model for forming synapses as several papers have established synaptic character in the cell line (Ding et al. 2018, Kempf et al. 2014, Reddy et al. 2018, Reddy et al. 2021, Shi et al. 2020, and Qu et al. 2021). The field is seemingly moving towards a more comprehensive usage of the HT22 model in the context of synaptic work in 2020 and 2021, although we would contend they are doing this prematurely. However, utilizing the optimized methods in project 1 and the methods described in Beaudoin et al. 2012 and Project 2 we contend that induction of synaptogenesis to test our Notch-Delta mechanism is entirely possible. The majority of this initiative will occur following the completion of this semester, however, we contend that such a method established en-route to proof of principle

testing, similar to project 1's inception, would bolster the Notch-Delta project via offering the field a unique way to structurally/functionally label neurons trans-synaptically while also offering them a system in which they can utilize for novel synaptic evaluation.

Ultimately, the construct will tie into the broader aims of the Beaudoin Lab's interest in reward circuitry, aiming for stereotaxic injection of the AAV plasmids in STN and SNc for the in vivo labeling portion of the project. This stage of the project will have built on the initial cloning, in vitro model creations, and in vitro model testing en route to tying in all 3 projects together to optogenetically evaluate the nuances in the circuit.

**Tri-Project Conclusion**

In summation, we have identified a comprehensive differentiation protocol utilizing a CAMK-II-α based readout as a proxy for differentiation character as CAMK-II-α is a neuron-specific promoter. We argue that N2 with RA and NGF or B27 with just NGF should be used for neuronal differentiation, as both produce CAMK-II-α driven GFP expression while undifferentiated HT22 cells were previously shown to not express CAM-II-α driven GFP above baseline. The addition of NGF despite non-statistically significant results from performed ANOVAS stems from a literature review about the neuroprotective and gene regulation role NGF plays that would not be characterized by mean and maximal CAMk-II-α GFP outputs. Indeed, in our evaluation of mean and maximal GFP values, we noticed a replicated trend of expansive neuritic outgrowth uniquely seen in NGF differentiated conditions that may be further characterized by neurite outgrowth assays. Thus, we established a protocol that we will utilize internally in the Beaudoin lab and continue characterizing en route to creating a comprehensive literature review of prior HT22 differentiation, and supplementing it with our novel findings.

We have also characterized a newly packed ChrimsonR driven by the already thoroughly studied CAMK-II-α promoter. Initial work has been done in characterizing the new ChrimsonR construct relative to the old construct, finding a larger current influx at higher positive voltages for the new construct. Further, the fidelity of the construct was measured via comparing blue and red light responses- ultimately finding that ChrimsonR peak kinetics are faster in the presence of red light relative to blue light. In the pursuit of this quantification, novel electrophysiological properties were established in HT22 cells across various media conditions that furthered Project 1's characterization of supplement effect on differentiation.

Project 1 stemmed from an initial need for a proper in vitro model to test the construct fidelity for Project 2 but ultimately evolved into its own intensive research period to offer a new model to the field. The Notch-Delta project is intertwined with each of the previous two projects, needing both an immortalized neuronal cell line to test proof-of-principle a unique optogenetic construct to ultimately utilize for in vivo expression. Future progress to cross-over these projects would be to create a cre-dependent ChrimsonR channelrhodopsin that could be expressed via our proposed Notch-Delta mechanism, and an optimized in vitro synaptogenesis model to be created to test the mechanisms entirely in cell-culture. We have thus far offered the field with both a thorough and tested culturing protocol for HT22 cells that aims to bolster future results and correct previous studies, as well as offering a novel functional ChrimsonR plasmid vector that expresses in vitro and in vivo.

In the context of the Beaudoin Lab's current focus of excitatory connections within the mesolimbic system that underpin addiction, each of these projects has a key role to play. HT22 cells, after being differentiated and optimized such that they can restore the aforementioned glutamatergic properties, can serve as the in vitro model for any research question inquiring into neuronal behavior and responses to treatment. Our novel CAMK-II-α driven ChrimsonR offers the lab a functional construct that would, unlike current synapsin promoter-driven ChrimsonR, would selectively express in the glutamatergic circuitry the lab is interested in modulating and observing. The dual input approach in the mesolimbic analysis is thus bolstered by further selectivity of the inputs we are interested in and advances the complexity of response measurements we can procure from the PPN and SNc. The innovative Notch-Delta model pushes the envelope the farthest, providing the key map to underscore any circuit-wide

analysis as well as offering even more expression regulation to not only connected neurons but also possibly within Cre-expressing mouse lines to express solely in connected neurons of a given subtype.

The underscoring motivation for each of these projects is method development and fine-tuning in the context of the overall field of Neuroscience. While these projects have immediate utility in the Beaudoin lab's pursuit of increased reward circuitry characterization, we frame them as standalone projects with an innate utility to any project that needs a neuronal in vitro model, a novel ChrimsonR construct for dual input recordings or an innovative and non-toxic method for efficient structural and functional neural mapping. We aim to continue research well beyond the conclusion of this thesis en route to three standalone publications: a paper detailing our work optimizing culturing of HT22 cells, a paper detailing the efficient utilization of our ChrimsonR construct in subcortical neurons, and both a paper detailing the proof of concept of our Notch-Delta method as well as another paper applying the model to ascertain reward circuitry connectivity.

The work accomplished in this thesis also facilitates broader and more ambitious inquiry into the synaptic viability of HT22 cells, or our Notch-Delta model's ability to knock out certain receptor types for behavioral research. We anticipate immense progress in the coming summer research period as these potential exploration projects will no longer be performed blindly. While the initiation of each of the projects described in this Thesis was hindered by the lack of a field-wide standard or approach, or in the context of Project 3 any reference paper for our vision, I have successfully equipped the Beaudion Lab with a proper foundation to reference.

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I also want to dedicate this project's completion to my late grandmother who supported me in this research endeavor early on, with special praise for my "brain work" and my mother who supported me every step of the way as I entered uncharted territory for my family.

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