Phenotype of Neurons in the Nucleus Tractus Solitarius Labeled using an Optogenetic Construct with a CaMKIIa Promoter

Thesis

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Abstract

Optogenetics is a very useful technique used to activate or inhibit specific cells of interest in specific brain regions. The purpose of this study was to determine the phenotype of cells within the nucleus tractus solitarius (NTS) of the rat using an optogenetic construct with a calcium calmodulin type II alpha type (CaMKIIa) promoter. Sixteen adult male rats were microinjected in the NTS with an optogenetic construct that consisted with an adeno-associated viral vector serotype 2 (AAV2) containing a CaMKIIa promoter, a light sensitive channelrhodopsin 2 (ChR2) and an mCherry reporter. Following a 3 to 4 week period after the microinjections, brain tissue was removed and sectioned. Immunohistochemistry was used to identify catecholaminergic neurons and astrocytes. In situ hybridization was used to identify neurons within the NTS that were glutamatergic or GABAergic. The vast majority of neurons that expressed the CaMKIIa promoter were catecholaminergic (87% in 4 rats), and 91% expressed the CaMKIIa enzyme. A subset of neurons expressing the CaMKIIa promoter were glutamatergic (38% in 5 rats). In contrast very few infected neurons were GABAergic (17% in 5 rats). The virus did not appear to infect astrocytes, because no GFAP immunoreactive cells expressed the CaMKIIa promoter. Based on these results, the optogenetic construct appears to be taken up by catecholaminergic neurons in the NTS with minimal or absent infection of GABAergic neurons or astrocytes. A subset of infected neurons were glutamatergic and future studies are needed to determine whether these neurons are also catecholaminergic.

Acknowledgments

I would first like to thank my committee members, Dr. Tom Cunningham, Dr. Ann Schreihofer, Dr. John Planz and my mentor Dr. Steve Mifflin at the University of North Texas Health Science Center for their support and guidance throughout my entire graduate career. I would also like to thank Sharon Ellazar, Joel Little, Parul Chaudhary PhD candidate Dr. Mirza Beig and Dr. Lei Wang for their help in different protocols of the project. And last but not least, I would also like to thank my family for their support and encouragement, especially my wife who has supported me financially and spiritually during my time expanding my horizons. I'm very grateful to have known you all.

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Chapter 1

Introduction:

General Background

Optogenetics is a powerful method used to establish a signaling platform from specific types of cells. Optogenetics uses photostimulation to activate or inhibit cells. Optogenetics uses different types of viral capsules to deliver genetic information to a specific type of cell in a specific area of interest. Once the targeted cell is expressing the gene sequence, it can be activated or inhibited by light depending upon the phenotype as specified by the construct promoter. This is a method of gene delivery that has shown to be useful in analyzing the activity from the direct and indirect pathways of medium spiny neurons that contribute to the motor symptoms Parkinson's disease (Parker et al, 2016).

One of the first steps in administering genetic sequences for optogenetic studies is choosing the type of viral vector to infiltrate the cells of interest for high levels of infection. Types of viral vectors include but are not limited to: Canine-adeno virus type 2 (CAV2), Herpes simplex virus (HSV), Lentivirus (LV) and the adeno-associated virus (AAV) (Zhu and Roth 2015). Noticeably AAV, and LV, have been the preferred vectors for use in optogenetics. Optogenetics allows the control of specific types of cells by photostimulation. This leads to a better understanding of what, when and where cells may have an effect on the physiology of the organism. Once the viral construct enters the neuron, it can integrate its own genetic

sequence within the cell's nucleus, leading to the cell expressing a light sensitive cation channel or Cl^- pump on the membrane that is activated by the photons from a light source. Depending upon the specific protein the construct expresses, this will activate or inhibit the neuron. This enables the investigator to control the cells' activity and observe any changes in the physiology.

Use of Viral Vectors

The ability to selectively express proteins in specific groups or classes of neurons that can then be activated by photo-stimulation or chemical-stimulation has advanced our understanding of neuronal circuitry. Chemogenetics involves the engineering of macromolecules to interact with previously unrecognized small molecules (Urban and Roth, 2015). The AAV, LV, CAV2 and HSV have been involved in chemogenetics for the production of designer receptors exclusively activated by designer drugs (DREADDs) that can control signaling pathways that involve G-protein coupled receptors (GPCRs) (Zhu and Roth, 2015). These viral vectors have had success with high expression levels over long periods of time with little or no reported adverse effects (Zhu and Roth, 2015). Similar to optogenetics, the investigators can identify cells by which the viral vectors are able to infiltrate and begin expressing the DREADDs. This identifies cells that can be manipulated acutely or chronically to observe any changes in behavior when administered a drug that will either act as an agonist or antagonist on the targeted cell (Nation et al, 2016). A previous study concluded that the LV with a human

synapsin promoter predominantly labeled excitatory cortical neurons (Nathanson et al, 2009). Within the same area and using the same promoter, an AAV induced expression in inhibitory cortical neurons (Nathanson et al, 2009). Thus, the selectin of a particular viral vector is essential for targeting specific types of cells of interests.

Viral Vectors

The AAV comes in a variety of serotypes: AAV1-5 and AAV7-9. These are considered true serotypes because AAV6 is almost identical to AAV1 and the serological profiles of AAV10 and AAV11 have not yet been well characterized (Wu et al, 2006). Different serotype(s) have been classified to have successful widespread transduction such as AAV1 and 5 in the central nervous system (CNS), while AAV2 shows transduction throughout the entire midbrain (Wu et al, 2006).

The LV is another viral vector that has had success in optogenetics. This vector is derived from the human immunodeficiency virus (HIV) as well as other immunodeficiency viruses from other species such as the simian, bovine, feline and equine (Wong et al, 2006). However, over time, human cells have evolved mechanisms involving cellular proteins to block key steps of the virus life-cycle suppressing viral replication. These cellular proteins are known as restriction factors that provide an early antiviral defense (Borsotti et al, 2016). Due to its origin, the LV has gone through several improved designs leading to the third generation packaging construct that separates the structural and enzymatic viral proteins, thus

increasing the platform biosafety as well as the transduction efficiency (Borsotti et al, 2016).

Integration of the AAV2 in a host

The AAV2 has grown in popularity in gene therapy due to its lack of pathogenicity, wide range of transduction and the ability to establish long-term transgene expression (Wu et al, 2006). A successful injection of the AAV2 in the area of interest is manifested by a chain reaction which begins with the AAV2 capsule entering the cell. Although the process is unknown, it is suggested that the AAV2 is endocytosed into clathrin-coated vesicles, and with the help of an enzyme called phospholipase A_2, the AAV2 is able to escape the vesicle (Daya and Berns, 2008). Another study concluded that the membrane-associated heparin sulfate proteoglycan serves as the receptor for the AAV2 (Summerford and Samulski, 1998). It is still unclear how the AAV2 enters the nucleus but research has suggested that it is small enough to enter through the nuclear pores (Daya and Berns, 2008). If the host's nuclear DNA contains a sequence that recognizes the promoter associated with the AAV2 construct, the promoter can integrate itself within the genetic sequence of the infected cell. This will begin the process of the integration of the viral sequence in the cell which will result in the cell expressing a light sensitive protein and a reporter used with optogenetics. These light sensitive cation channels integrated on the cell membrane are from 2 different algal species: channelrhodopsin-2 (ChR2) from *Chlamydomonas reinhardtii* and

channelrhodopsin-1 (ChR1) from *Volvox carteri* (Zhang et al, 2010). The cells of interest may also be inhibited, but with the use of another type of light sensitive protein. The NpHR pump is a halorhodopsin which is derived from the archaeon *Natronomonas pharaonic*. Once activated, NpHR transports Cl^- into the cell resulting in hyperpolarization (Guru et al, 2015). ChR2 is the most commonly used light sensitive activation channel in optogenetics. However, ChR2 is also an important factor in ChR2-assisted Circuit Mapping (CRACM), which combines of photostimulation of ChR2-positive neurons and whole-cell recordings of synaptic currents to map the interactions of presynaptic and postsynaptic neurons (Petreanu et al, 2007).

When the cell is expressing one of these proteins, not only can it be activated or inhibited by light, it can also be identified by a reporter with the use of the immunofluorescence microscope. Observation of the reporter indicates that the microinjection of the AAV2, and integration of the viral vectors genetic sequence into the host's cells was successful. Common reporters include but are not limited to Green Fluorescence Protein, mCitrine and mCherry.

Different types of promoters

There are a range of different types of promoters that can be used to induce gene sequencing within cells of interest. (1) A synapsin promoter is typically used when targeting neuronal cells (Kugler et al, 2003), (2) Glial Fibrillary Acidic Protein (GFAP) promoter is used to target astrocytes (Ashpole et al, 2013), (3) Tyrosine

Hydroxylase (TH) promoter to target catecholaminergic neurons (Guo et al, 2014) or (4) Cam Kinase II alpha type (CaMKIIa) to identify cells that express the CaMKIIa enzyme (Yamamoto et al 2014). CaMKIIa promoter has been used to activate and identify specific cell types in distinct brain regions such as glutamatergic and catecholaminergic neurons within the ventral tegmental area (VTA) (Guo et al, 2014), excitatory and inhibitory cortical neurons within the somatosensory cortex (Nathanson et al, 2009) and CaMKIIa-expressing neurons within the hippocampus (Zhang et al, 2008).

The expression of CaMKII is particularly high in neurons comprising up to 2% of total protein in neurons in some brain regions (Griffith, 2004). Knockout of CaMKIIa has revealed the importance of this isoform when it comes to cognition and function of an organism. Mice lacking CaMKIIa have shown numerous deficiencies in learning and neuronal plasticity (Fink and Meyer, 2002). The loss of CaMKIIa has been linked to episodes of epilepsy illustrating the importance of this enzyme, for normal neuronal function (Liu and Murray, 2012).

A previous study from Dr. Mifflin's lab used an AAV2 optogenetic construct with a CaMKIIa promoter within the nucleus tractus solitarius (NTS) (Yamamoto et al, 2014). Photostimulation of infected NTS neurons increased phrenic nerve activity and renal sympathetic nerve activity. The purpose of the present study was to determine the phenotype of NTS neurons infected with the virus, which were detected by the use of in situ hybridization and immunohistochemistry (IHC).

We used an adeno-associated virus serotype 2 (AAV2) viral vector to introduce a genetic sequence into cells within the NTS of the rat hindbrain. AAV2 is typically used in studies concerned with the nervous system due to it being neurotrophic (Yizhar et al, 2011). A typical gene sequence in an AAV2 capsule for this type of experiment encodes a promoter, ChR2 and a reporter (mCherry). Figure 1 is an example of different types of viral vectors and the sequences they may contain.

I will now discuss various NTS neuronal and non-neuronal phenotypes.

Catecholaminergic Neurons

Identification of catecholaminergic neurons is typically done by identifying cells that express TH or Dopamine Beta Hydroxylase. The TH enzyme is the rate limiting step for the synthesis of norepinephrine, dopamine and epinephrine, and the presence of TH defines a catecholaminergic neuron (Daubner et al, 2011). Catecholaminergic neurons in the brain release these principal neurotransmitters which are necessary for central nervous system functions, such as motor control, cognition, emotion, memory processing and endocrine modulation (Kobayashi, 2001).

CaMKIIa-expressing cells

Identification of CaMKIIa-expressing cells determines the specificity of the promoter, and supports the notion that CaMKIIa is found within neuronal tissue. As

stated before, CaMKIIa in neuronal tissue appears to be a factor in neuronal plasticity and is important in learning, memory and general neuronal function.

Astrocytes

Identification of astrocytes in the CNS can be done by identifying cells that express GFAP. GFAP is a protein that is expressed by astrocytes, which are non-neuronal cells that are present in the CNS. These cells play a critical role in modulating neuronal physiology (Ashpole et al, 2013). GFAP is important for astrocyte-neuronal interactions as well as synaptic efficacy in the CNS (Brahmachari et al, 2006).

Glutamatergic neurons

Synaptically released glutamate (Glu) activates three principal types of Glu receptors (GluRs): AMPA receptors (AMPARs), NMDA receptors (NMDARs) and metabotropic Glu receptors (mGluRs) (Gotz et al, 1997). When stimulated, these receptors operate channels that can directly gate $Ca2^{+}$ entry or mediate a Na^{+}-dependent depolarization (Scholz and Palfrey, 1991). With a high abundance of Glu, membrane bound Glu transporter proteins found on the pre and postsynaptic neurons, as well as astrocytes, can remove Glu from the synapse (Attwell and Gibb, 2005). Identifying Glu in a cell is not sufficient enough to refer to a cell as glutamatergic, because neurons contain Glu for other purposes such as cellular respiration. Identifying the mRNA for the protein Vesicular Glutamate Transporter type 2 (VGLUT2), however, does indicate that the neuron produces the protein

VGLUT2 which enables the neuron to release Glu, thus labeling it a glutamatergic neuron. It is reasonable to identify glutamatergic neurons by identifying VGLUT2 region specific mRNA because VGLUT2 is found in the brain stem whereas VGLUT1 and VGLUT3 are localized mainly in the neocortex, hippocampus among others, but not reported to be found in the autonomic regions of the brainstem with VGLUT2 (Liguz-Lecznar and Skangeil-Kramska 2007).

GABAergic neurons

Gamma Aminobutyric Acid (GABA) is an inhibitory neurotransmitter that decreases neuronal excitability (Benson et al, 1992). This is achieved by binding to at least two classes of GABA receptors, $GABA_A$ and $GABA_B$ (Sigel and Steinmann, 2012). Once GABA binds to a $GABA_A$ receptor, the activated receptor channel temporally opens allowing Cl^- to pass into the cell to decrease excitability (Mihic and Harris, 1997). $GABA_B$ receptors are rarely directly activated by synaptically released GABA. However, activation $GABA_B$ receptors can modulate inwardly rectifying K^+ channels (Benke et al, 2012). GABA transporters on the presynaptic cells and glia take GABA away from the synapse to regulate extracellular GABA levels (Nutt, 2006). Although GABA is loaded into a vesicle by the vesicular GABA Transporter (vGAT), in situ hybridization to detect vGAT mRNA has not yielded results as accurate as identifying Glutamate Decarboxylase (GAD) (Jarvie and Hentges, 2012). While the other isoform, GAD65 also identifies GABAergic neurons, it has been reported that the synthesis of GAD65 is frequently lower than that of GAD67

in autonomic related regions of the CNS (Esclapez et al, 1994). The mRNA coding for GAD67 indicates that the neuron produces this enzyme which breaks down glutamate into GABA and CO_2. Though multiple isoforms exist, the GAD67 isoform is found in abundance in autonomic related regions of the brain. Thus, the presence of GAD67 mRNA is an accepted marker for neurons that make GABA to us as a neurotransmitter.

Significance of IHC and in situ hybridization

To identify cells that express TH, CaMKIIa and GFAP, IHC is performed to detect these proteins which are within the soma of the cell. However, for identifying glutamatergic and GABAergic neurons, in situ hybridization has to be undertaken to identify the presence of mRNA that encodes for VGLUT2 and GAD67 proteins. The mRNA is found in the soma of the cell but the proteins are found in the neuronal axon terminals. Therefore, in order to identify the cell bodies, the mRNA must be used as the marker for the proteins.

Our hypothesis states: An AAV2 optogenetic construct with the CaMKIIa promoter microinjected in the NTS will produce mCherry expression in glutamatergic and catecholaminergic neurons. It is also anticipated that GABAergic neurons and glia will not be labeled with the optogenetic construct.

Specific Aims:

All experiments will be performed in rats with injection of an optogenetic contruct (AAV2-CaMKIIa-ChR2-mCherry).

1. To determine if an optogenetic construct with a CamKIIa promoter induces gene expression in catecholaminergic neurons, IHC for TH will be performed.

2. To determine if an optogenetic construct with a CamKIIa promoter induces gene expression in neurons with the enzyme CaMKII, IHC will be performed for CaMKII.

3. To determine if an optogenetic construct with a CamKIIa promoter induces gene expression in glia, IHC for GFAP will be performed.

4. To determine if an optogenetic construct with a CamKIIa promoter induces gene expression in glutamatergic neurons, in situ hybridization for VGLUT2 mRNA will be performed.

5. To determine if an optogenetic construct with a CamKIIa promoter induces gene expression in GABAergic neurons, in situ hybridization for GAD67 mRNA will be performed.

Resource Authentication: Results for this study will provide information for future research as well as fulfilling requirements for NIH grants.

Figure 1:

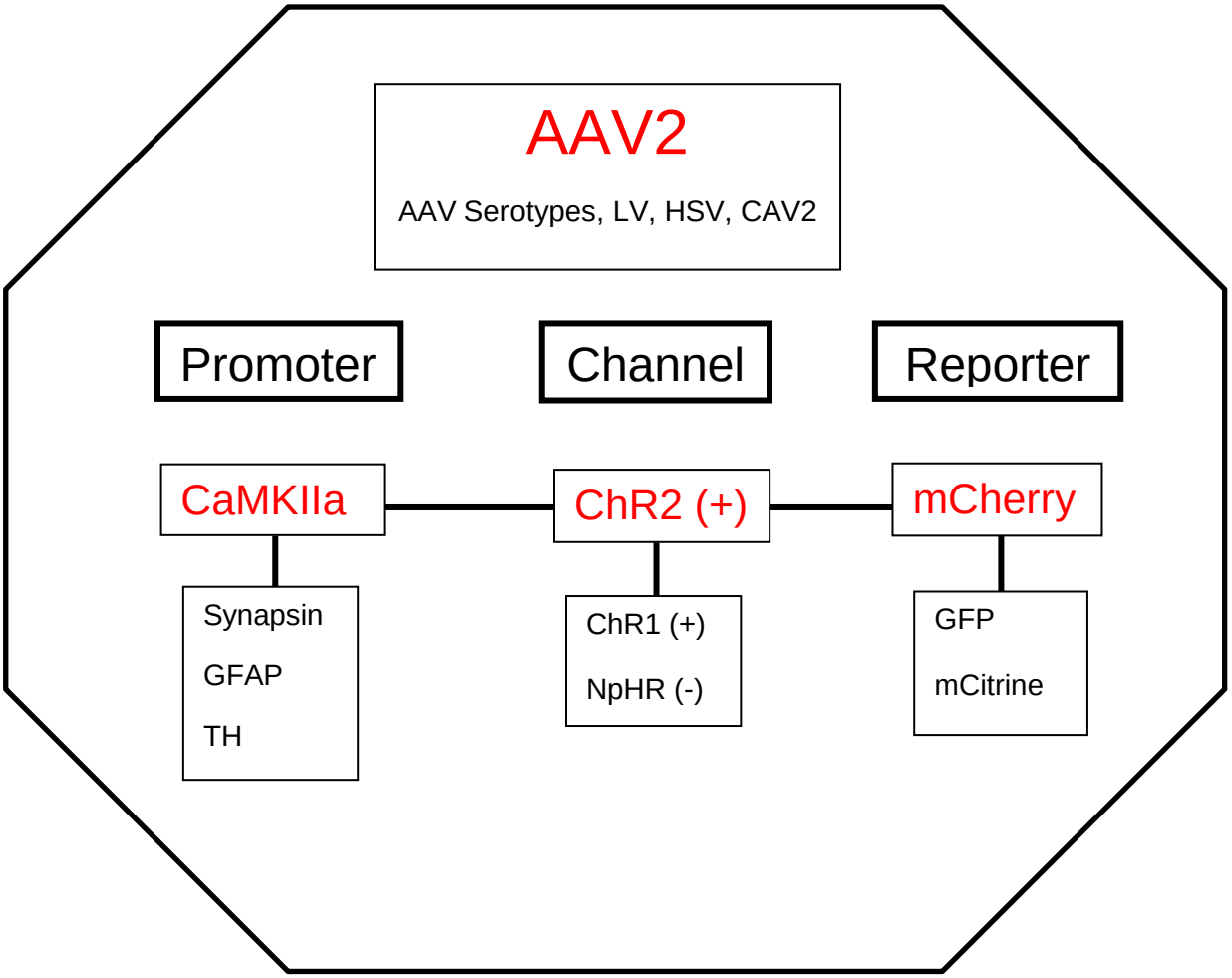

Figure 1: Example of Viral Vectors and what they may contain.

Red letters indicate what was used for this research. AAV: adeno-associated virus, LV: Lentivirus, HSV: Herpes Simplex Virus, CAV2: Canine-adeno virus, TH: Tyrosine Hydroxylase, GFAP:Glial Fibrillary Acidic Protein, Channelrhodopsin1 or 2 (ChR1or2), Halorhodopsin (NpHR), GFP: Green Fluorescence Protein, Activator (+) and inhibitor (-).

Chapter 2

Materials and Methods:

The Institutional Animal Care and Use Committee of the University of North Texas Health Science Center approved all experimental procedures in rats.

Microinjections of AAV2 in the rat NTS

Sixteen young adult male Sprague-Dawley rats (250-330 g) (Charles River Research Facilities, Wilmington, MA) were anesthetized with 4% isofluorane and given 0.2 ml of ketamine ip and 0.14 ml of dexdomitor ip and placed in stereotaxic frame. Adequacy of anesthesia was ensured by the absence of a reflex response after a hind paw pinch. Animals breathed 100% oxygen during the procedure. The dorsal surface of the brain was exposed and the adeno-associated virus AAV2-CamKIIa-hChR2-H134R-mCherry (titer, 10^{12} virus molecules per milliliter, purchased from the University of North Carolina vector core) was injected into the NTS using a glass pipette. One microinjection of the construct was administered at the calamus scriptorius followed by a second microinjection 0.5 mm caudal to the site of the first injection. (See Appendix A for the microinjection protocol). After microinjections, the wounds were sutured. The rats were given a 3-4 week period for observation, recovery and to ensure a high level of gene expression by the optogenetic construct.

Rat Perfusions and Brain Preparation

Four weeks after injections, rats were deeply anesthetized with urethane (1.5 g/5 ml dH_2O, ip) and perfused with 250 ml of saline (pH 7.4) with 0.5 M sodium phosphate buffer followed by 4% paraformaldehyde. For one set, tissues were extracted and post-fixed overnight in the fixative at 4˚C. The next day, tissues were rinsed 3 times 5 minutes each in phosphate buffer, blocked for 30 minutes in 10% normal horse serum (NHS) and the dura mater was removed. Tissue was superglued to a chuck and 30 µm thick slices were cut at room temperature using a Vibratome into 0.1 M of phosphate buffer. Then the sections are mounted on charged slides and allowed to dry overnight at room temperature. Tissues were sliced and sections were grouped to allow the identification of mRNA in multiple brains (See Appendices B and C). A separate set of tissues were removed in the same manner and stored in 30% sucrose solution at 4˚C for later immunohistochemical analysis.

In situ Hybridization

An in situ hybridization protocol provided by Dr. Ruth Stornetta from the University of Virginia (Charlottesville, VA) was followed to detect cells expressing mRNA coded for VGLUT2 and GAD67. RNAscope follows a strategy that is designed to amplify the target-specific signals but not the background noise, resulting in marked improvement in signal-to-noise ratio (Wang et al, 2012). After sections were properly mounted on the charged slides, a 10 ml syringe filled with vacuum

grease was used to make a barrier around the mounted sections. A water bath was heated to 40˚C while a WASH buffer was prepared. A sequence of probes and WASH buffer was applied to the sections to detect VGLUT2 and GAD67 mRNA. Following in situ hybridization, an IHC protocol was performed to identify cells that were positive for mCherry. A rabbit anti-DsRed Express primary antibody at a concentration of 1:20K was administered to the sections followed by a secondary Cyanine (Cy3) donkey anti-rabbit at a concentration of 1:400. Due to the fact that the immuno-fluorescence of mCherry fades over time, this step was necessary to ensure the identification of mCherry labeled cells after the sections had gone through the in situ hybridization protocol. Sections were then cover-slipped with Prolong Diamond to ensure the fluorescence of the labeled cells would remain bright for analysis. See Appendices D Part 1 and Part 2 for the in situ hybridization protocol for mRNA detection.

Immunohistochemistry

Tissues were removed from a 30% sucrose solution and cut into 40 µm thick slices using a cryostat into 3 different groups per hindbrain. Each set of sections were processed through their own respective IHC protocol. One set of sections from all four hindbrains received either: mouse anti-TH, mouse anti-CaMKIIa or mouse anti-GFAP. This allowed the IHC protocol to be applied so 3 different antibodies could be administered to all available hindbrains. A primary mouse anti-TH antibody was applied to one set of sections from 4 different hindbrains at a

concentration of 1:1000 followed by the addition of a secondary antibody, Alexafluor-488 donkey anti-mouse at a concentration of 1:800. See Table 1 for information on antibodies used in this study. This process was repeated with two other primary antibodies; mouse anti-CaMKIIa at a concentration of 1:400 and mouse anti-GFAP at a concentration of 1:400. Both primaries were followed by the secondary Alexafluor-488 donkey anti-mouse at the same concentration of 1:800. See Appendix E for the IHC protocol and Table 1 for information on the antibodies. This protocol allowed us to identify mCherry labeled cells that were also positive for TH, CaMKIIa and GFAP, thus, identifying mCherry labeled cells that were catecholaminergic, cells expressing the CaMKIIa enzyme and astrocytes respectively.

A fluorescent microscope was used to identify immuno-fluorescent cells at the site of the microinjections in the NTS. Sections that contained mCherry labeled cells in the NTS ranged from levels of -13.8 and -14.4 mm respectively bregma to caudal. One wavelength was used to identify cells that were labeled with mCherry and another wavelength was used to identify cells that were immuno-reactive (ir) due to the primary and secondary antibodies as well as cells expressing VGLUT2 mRNA and GAD67 mRNA.

Image J Cellular analysis

Cells were counted after uploading images to the software program Image J. Images of mCherry labeled and antibody labeled cells were merged to identify the

number of mCherry labeled cells that were also phenotypically identified with other markers. From each section there were images that identified cells that were expressing mCherry. Each section also had an image that identified cells that were either: TH-ir, CaMKII-ir, GFAP-ir, VGLUT2^{+} or GAD67^{+}. As an example, an image with mCherry labeled cells is counted, followed by another count of VGLUT2^{+} cells under a different fluorescent filter on the same section. The 2 images of the same section were merged to determine how many of the mCherry labeled cells were also VGLUT2^{+}.

The mCherry labeled cells are visible through the red channel and the VGLUT2 labeled cells (as well as TH-ir, CaMKII-ir, GAD67, and GFAP-ir) are visible through the green channel. Due to the intensity of the mCherry antibody, the merged image had to be manipulated to either increase/decrease the intensity of the mCherry antibody, or by increasing/decreasing the intensity of the VGLUT2 labeled cells. This involved maximizing/minimizing the balance of the red/green channels or increasing and/or decreasing the brightness and contrast of the red and/or green channels.

Data Presentation and Statistical Analysis

All data are presented as means ± SE. Sections were grouped based on the results of the IHC and in situ hybridization. As an example, for the TH group, sections came from 4 different rats. The average number of mCherry labeled cells was calculated for each rat resulting in 4 averages of mCherry labeled cells, TH-ir

cells, mCherry labeled cells that are also TH-ir, and mCherry labeled cells that are not TH-ir. Next, the 4 averages of mCherry labeled cells were averaged for an overall average number of mCherry labeled cells per section. This was repeated for the TH-ir cells, mCherry labeled TH-ir cells and mCherry labeled cells that are not TH-ir. This process described using the TH group was repeated in calculating the average cell counts per section in the other 4 groups of sections. The average mCherry labeled cells phenotypically identified by IHC and in situ hybridization per section was divided by the average number of mCherry labeled cells per section. The product was multiplied by 100 to get the percentage of the mCherry labeled cells that were phenotypically identified by IHC and in situ hybridization.

Statistical analysis was performed using SigmaPlot 12. Differences between labeled cells in a group (mCherry labeled cells, IHC and in situ hybridization labeled cells, and mCherry labeled cells expressing results from the IHC and in situ hybridization) were determined by a One-way ANOVA. A Bonferroni post hoc test was used to identify significant differences among mean values. When the data did not pass the normality test, a non-parametric statistic was used. In this case we used an ANOVA on Ranks test, Kruskal-Wallis, followed by a Tukey post hoc test when a significant difference was observed. (See Appendix F for the non-parametric statistic results). Differences were considered significant when $P<0.05$.

Table 1:

Antibody	Vendor (HQ)	Concentration	Variety	Reactivity
Mouse Anti-TH (1º)	EMD Millipore (Darmstadt, Germany)	1:1000	Monoclonal	Rat Species
Mouse Anti-CaMKIIa (1º)	Thermofisher (Waltham, MA)	1:400	Monoclonal	Rat/Mouse Species
Mouse Anti-GFAP (1º)	Sigma-Aldrich (St. Louis, MO)	1:400	Monoclonal	Rat Species
Rabbit Anti-DsRed Express (1º)	Clontech (Mountain View, CA)	1:20K	Polyclonal	mCherry
Alexafluor-488 Donkey Anti-Mouse (2º)	Jackson ImmunoResearch (West Grove, PA)	1:800	Polyclonal	Mouse Antibody
Cy3 Donkey Anti-Rabbit (2º)	Jackson ImmunoResearch (West Grove, PA)	1:400	Polyclonal	Rabbit Antibody

Table 1: Information about the antibodies that were used for IHC.

Includes the name, vendor and HQ, concentration used, variety and reactivity. TH: Tyrosine Hydroxylase, CaMKIIa: Cam Kinase II alpha type, GFAP: Glial Fibrillary Acidic Protein, DsRed: Discosoma, specifically Red and Cy3: Cyanine specifically orange-red and Alexafluor-488 is green.

Chapter 3

Results:

Catecholaminergic TH-ir cells

Analysis of sections from 4 rats found an average of 15.16±1.79 mCherry labeled cells and an average of 14.52±1.76 TH-ir cells. There was an average of 13.47±1.59 that were mCherry labeled TH-ir cells. 87% of mCherry labeled cells were catecholaminergic. There was a small number of 1.44±0.36 cells per section that were +mCherry labeled but were not positive for TH. Figure 2 illustrates mCherry labeled cells and TH-ir cells that were identified. Figure 3 provides an example of an image where cells were counted at the site of the microinjections in the NTS. Figure 4 provides the group data from sections that were positive for mCherry labeled cells. Due to a failed Shapiro Wilks Normality test, the Kruskal-Walis test was used to determine there were no significant differences among the medians, (P=0.30).

CaMKIIa-ir cells.

Analysis from 4 rats found an average of 15.75±2.46 mCherry labeled cells, 15.45±1.90 CaMKIIa-ir cells, 14.38±2.32 mCherry labeled CaMKIIa-ir cells and 1.38±0.55 mCherry labeled cells that were not CaMKIIa-ir cells per section. 91% of mCherry labeled cells were also CaMKIIa-ir. Figure 5 illustrates single labeled mCherry labeled cells and CaMKIIa-ir cells that were identified. Figure 6 provides

an example of an image where cells were counted at the site of the microinjections in the NTS. Figure 7 provides the group data. The mCherry labeled cells, CaMKIIa-ir cells and the mCherry labeled CaMKIIa-ir cells showed no significant differences among the means, (P=0.90). Statistical analysis was performed using a One-way ANOVA.

Glutamatergic +VGLUT2 cells.

Analysis from 5 rats found an average of 21.59±2.19 mCherry labeled cells, 25.33±2.58 glutamatergic neurons, 8.18±1.14 mCherry labeled glutamatergic neurons and 13.41±2.89 mCherry labeled cells that did not express VGLUT2 mRNA. 38% of mCherry labeled cells were glutamatergic. Figure 8 illustrates mCherry labeled cells and VGLUT2 mRNA expressing cells that were identified. Figure 9 provides an example of an image where cells were counted at the site of the microinjections in the NTS. Figure 10 provides the group data. There were significant differences among the means, ($P<0.05$). This was followed by a Bonferroni post hoc test which determined there was significant differences between the mCherry labeled glutamatergic neurons compared with the glutamatergic neurons, ($P<0.05$) and with the mCherry labeled cells ($P<0.05$).

GABAergic +GAD67 cells

Analysis of 5 rats found an average of 15.75±3.72 mCherry labeled cells, 19.50±3.88 GABAergic neurons, 2.58±0.99 mCherry labeled GABAergic neurons and 15.94±3.17 mCherry labeled cells that did not express GAD67 mRNA. 17% of

mCherry labeled cells were GABAergic. Figure 11 illustrates mCherry labeled cells and GABAergic cells that were identified. Figure 12 provides an example of an image where cells were counted at the site of the microinjections in the NTS. Figure 13 provides the mean data. Due to a failed Shapiro Wilks Normality test, the Kruskal-Walis test was performed and found significant differences among the medians, ($P<0.05$). This was followed by a Tukey post hoc test which determined there was a significant difference between mCherry labeled GABAergic neurons and the GABAergic neurons, ($P<0.05$).

Astrocyte GFAP-ir cells

Analysis of 4 rats found an average of 15.94±3.17 mCherry labeled cells, 45.58±1.45 GFAP-ir cells, no mCherry labeled GFAP-ir cells and 15.94±3.17 mCherry labeled cells that were not GFAP-ir. Based on the information about the AAV2 stated in the introduction about the virus being neurotrophic, these results were expected. Figure 14 illustrates single labeled mCherry labeled cells and GFAP-ir cells that were identified. Figure 15 provides an example of an image where cells were counted within the NTS. Figure 16 provides the group data. Due to a failed Equal Variance test, the Kruskal-Walis test was performed, and there were significant differences among the medians, ($P<0.05$). This was followed by a Tukey post hoc test which determined there was significant difference between GFAP-ir cells and the absence of mCherry labeled GFAP-ir cells, ($P<0.05$).

Figures and Figure Legends

Figure 2:

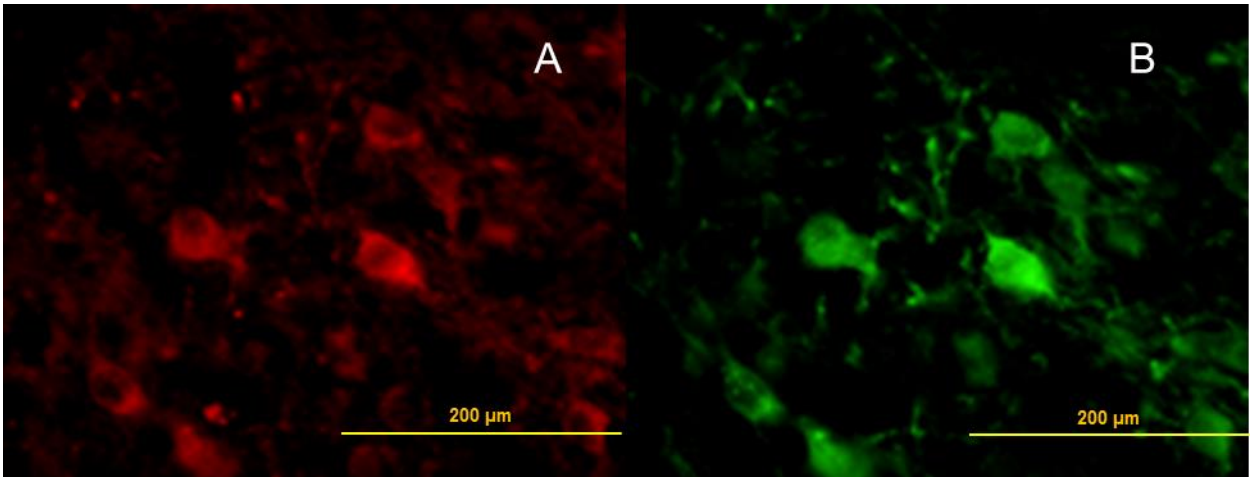

Figure 2: Examples of mCherry labeled cells (A) and TH-ir cells (B).

What is necessary is identifying what cells can be truly identified as mCherry labeled cells and TH-ir cells under different channels of fluorescence. The red channel identified mCherry labeled cells (A) and the green channel identified TH-ir cells (B). This is necessary before these images can be merged to calculate how many mCherry labeled cells were also TH-ir.

Figure 3:

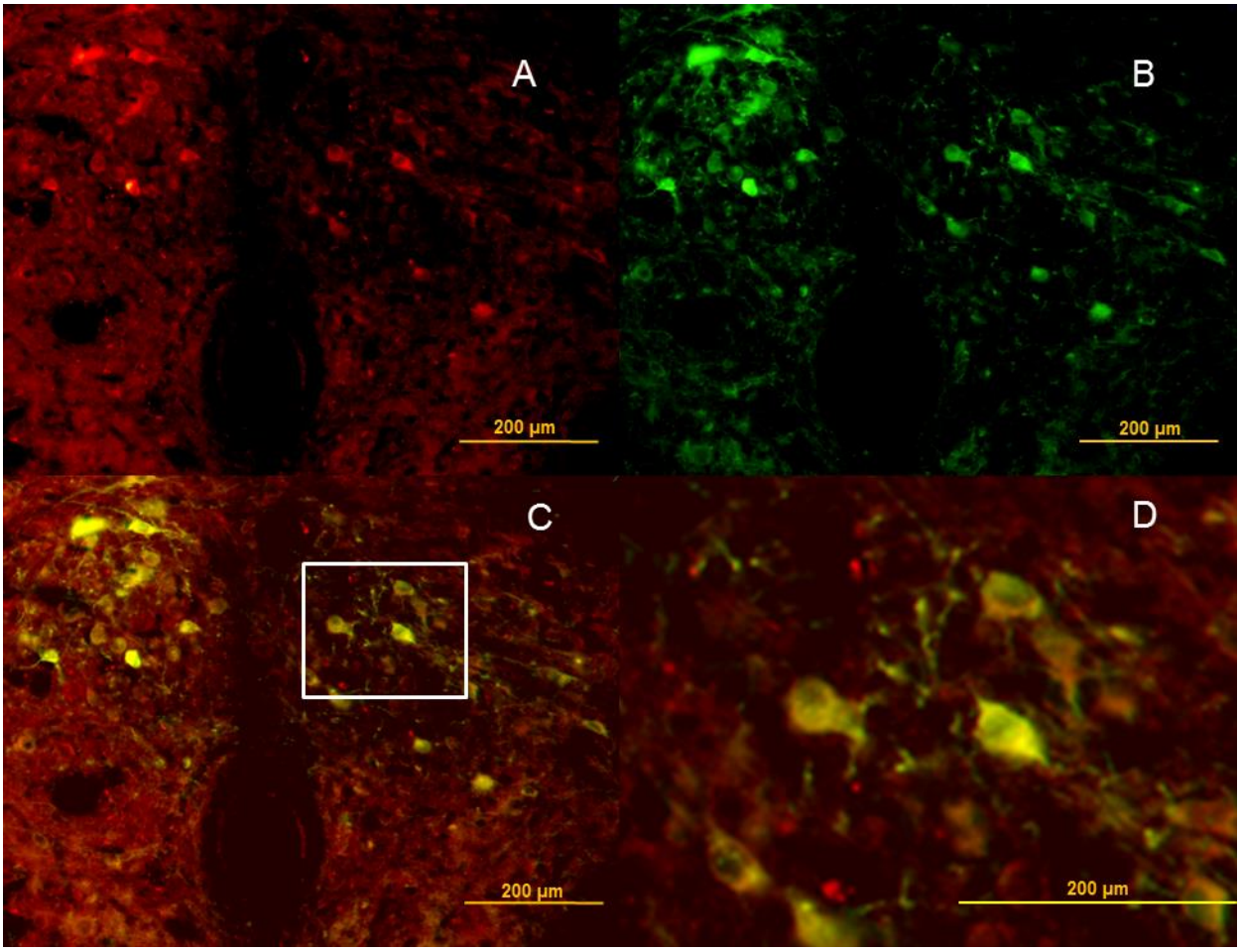

Figure 3: Tyrosine Hydroxylase

mCherry labeled cells (A). TH-ir cells (B). A and B merged showing mCherry labeled TH-ir cells (C). Images A, B and C are at 20X, An area from C at 40X (D).

Figure 4:

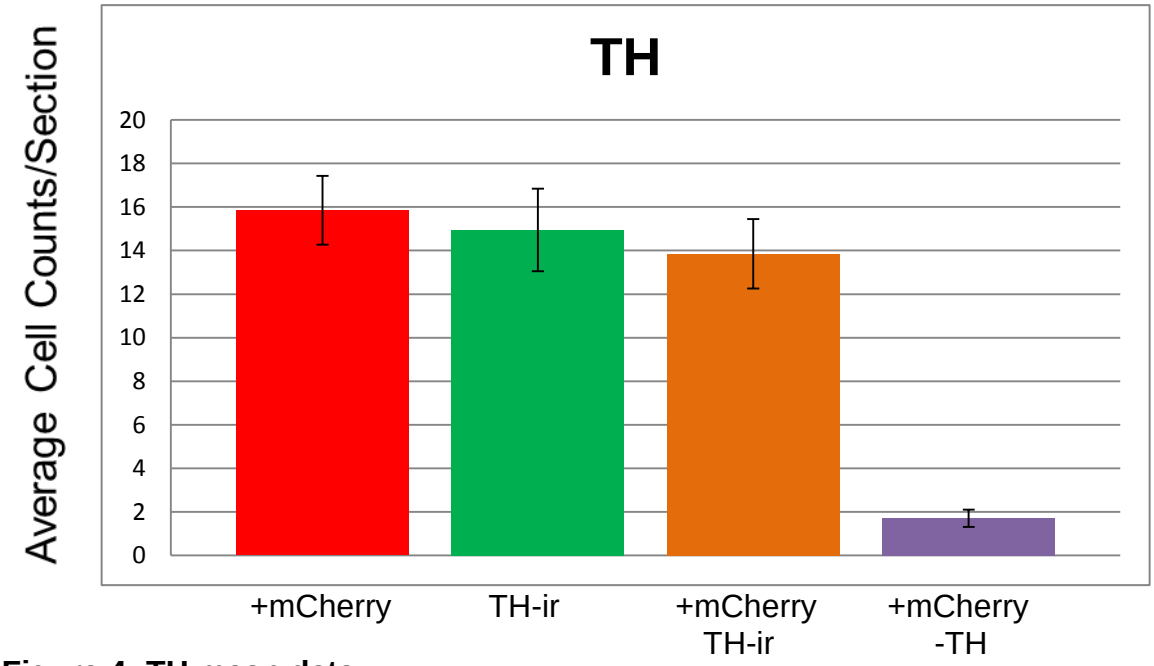

Figure 4: TH mean data

Mean data from 4 rats (Average of 5±1 sections per rat, 20 sections total) showed 15.16±1.79 +mCherry cells, 14.52±1.76 TH-ir cells, 13.45±1.59 +mCherry/TH-ir cells and 1.44±0.36 +mCherry/-TH cells per section per rat. Values for each rat were totaled and divided by the number of sections from the individual rat. 87% of +mCherry cells were also TH-ir. No statistical differences among medians, (P=0.30). Statistical analysis was performed using the Kruskal-Wallis test.

Figure 5:

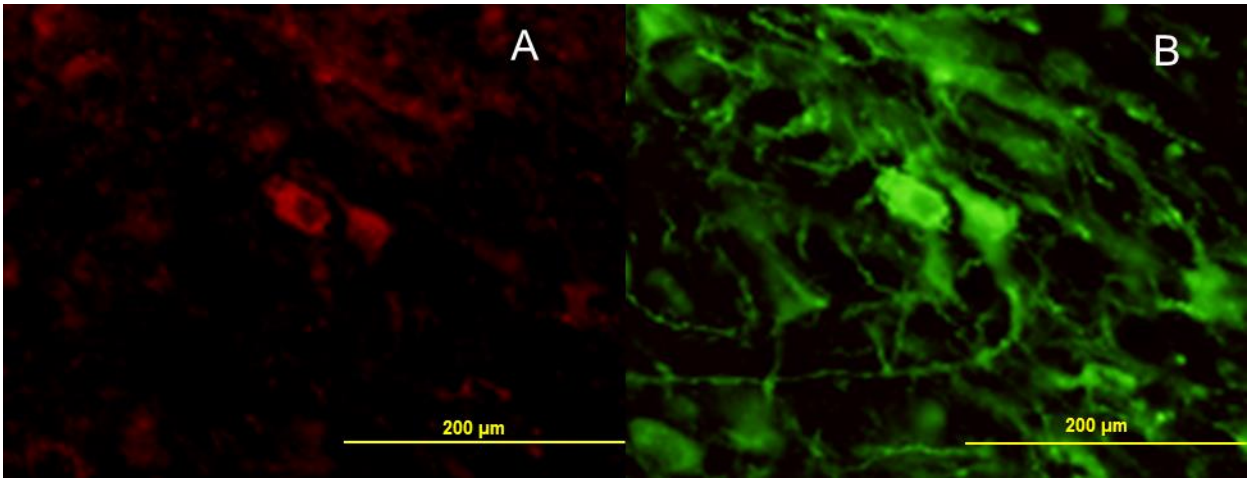

Figure 5. Examples of mCherry labeled cells (A) and CaMKIIa-ir cells (B).

What is necessary is identifying what cells can be truly identified as mCherry labeled cells and CaMKIIa-ir cells under different channels of fluorescence. The red channel identified mCherry labeled cells (A) and the green channel identified CaMKIIa-ir cells (B). This is necessary before these images can be merged to calculate how many mCherry labeled cells were also CaMKIIa-ir.

Figure 6:

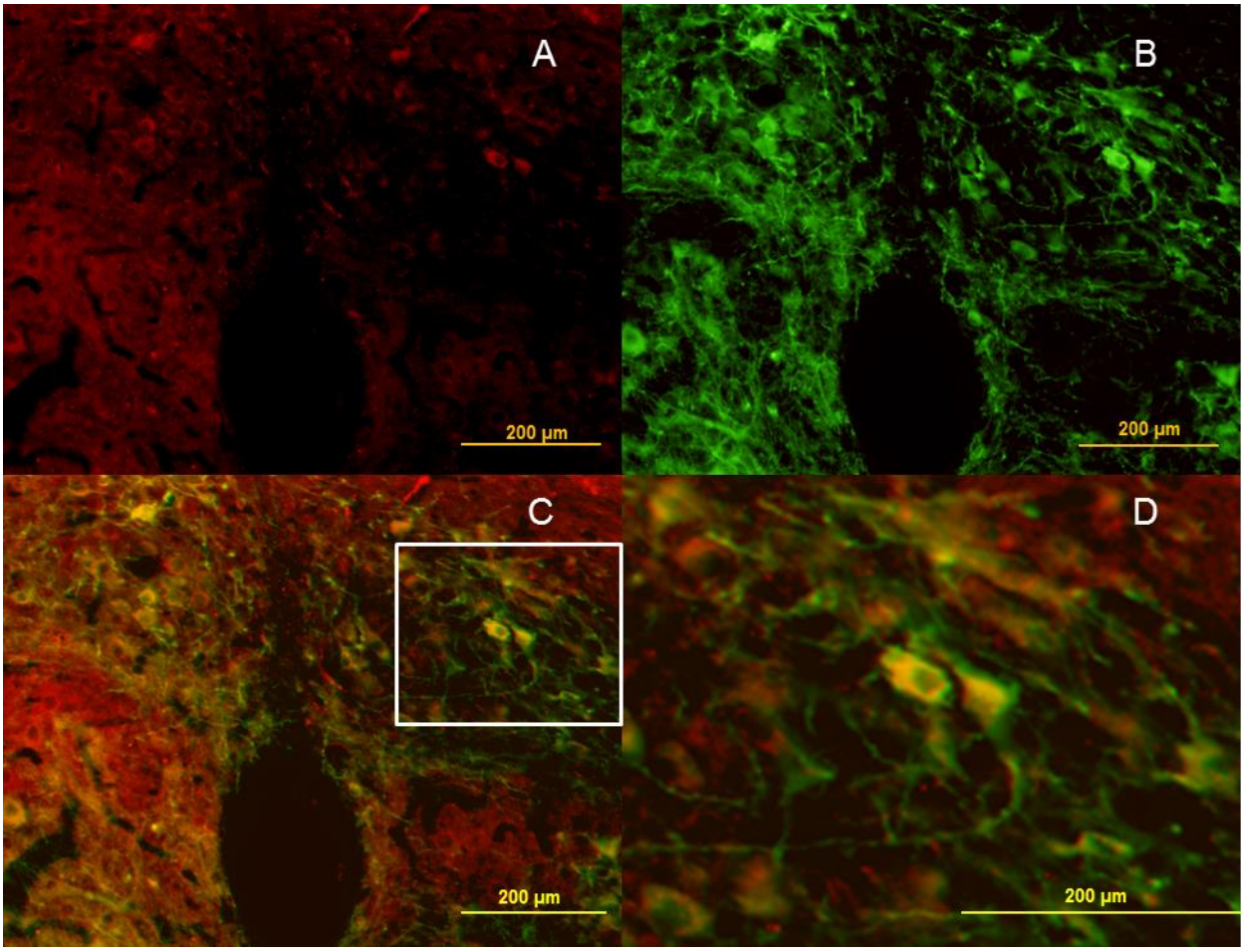

Figure 6: CaMKIIa

mCherry labeled cells (A). CamKIIa-ir cells (B). A and B merged showing mCherry labeled CamKIIa-ir cells (C). Images of A, B and C are at 20X. An area from C at 40X (D).

Figure 7:

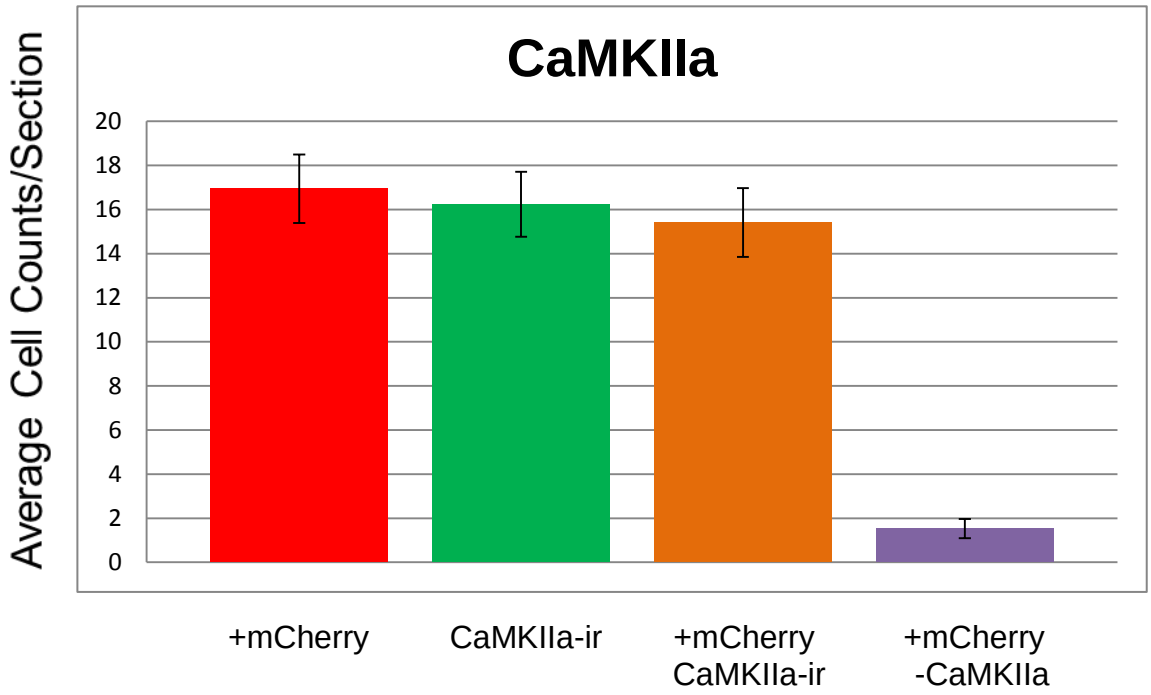

Figure 7: CaMKIIa mean data

Average Mean data from 4 rats (Average of 4±1 sections per rat, 17 sections total) showed 15.75±2.46 +mCherry cells, 15.45±1.90 CaMKIIa-ir cells, 14.38±2.32 +mCherry/CaMKIIa-ir cells and 1.38±0.55 +mCherry/-CaMKIIa cells per section per rat. Values for each rat were totaled and divided by the number of sections from the individual rat. 91% of +mCherry cells were also CaMKIIa-ir. No statistical differences among medians, (P=0.90). Statistical analysis was determined by the One-way ANOVA.

Figure 8:

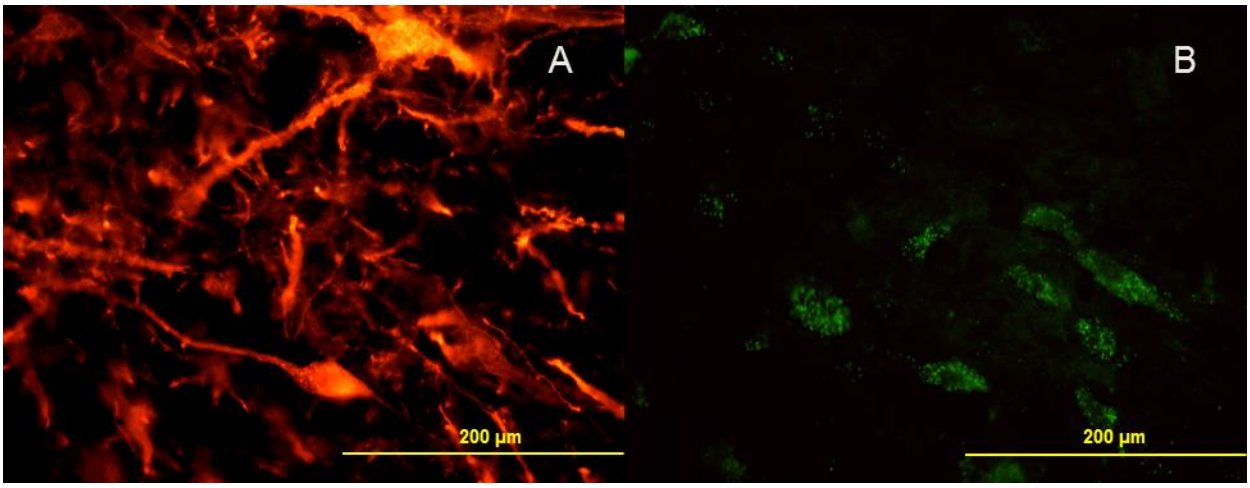

Figure 8: Examples of mCherry labeled cells (A) and VGLUT2$^+$ cells (B).

What is necessary is identifying what cells can be truly identified as mCherry labeled cells and VGLUT2$^+$ cells under different channels of fluorescence. The red channel identified mCherry labeled cells (A) and the green channel identified VGLUT2$^+$ cells (B). This is necessary before these images can be merged to calculate how many mCherry labeled cells were also VGLUT2$^+$.

Figure 9:

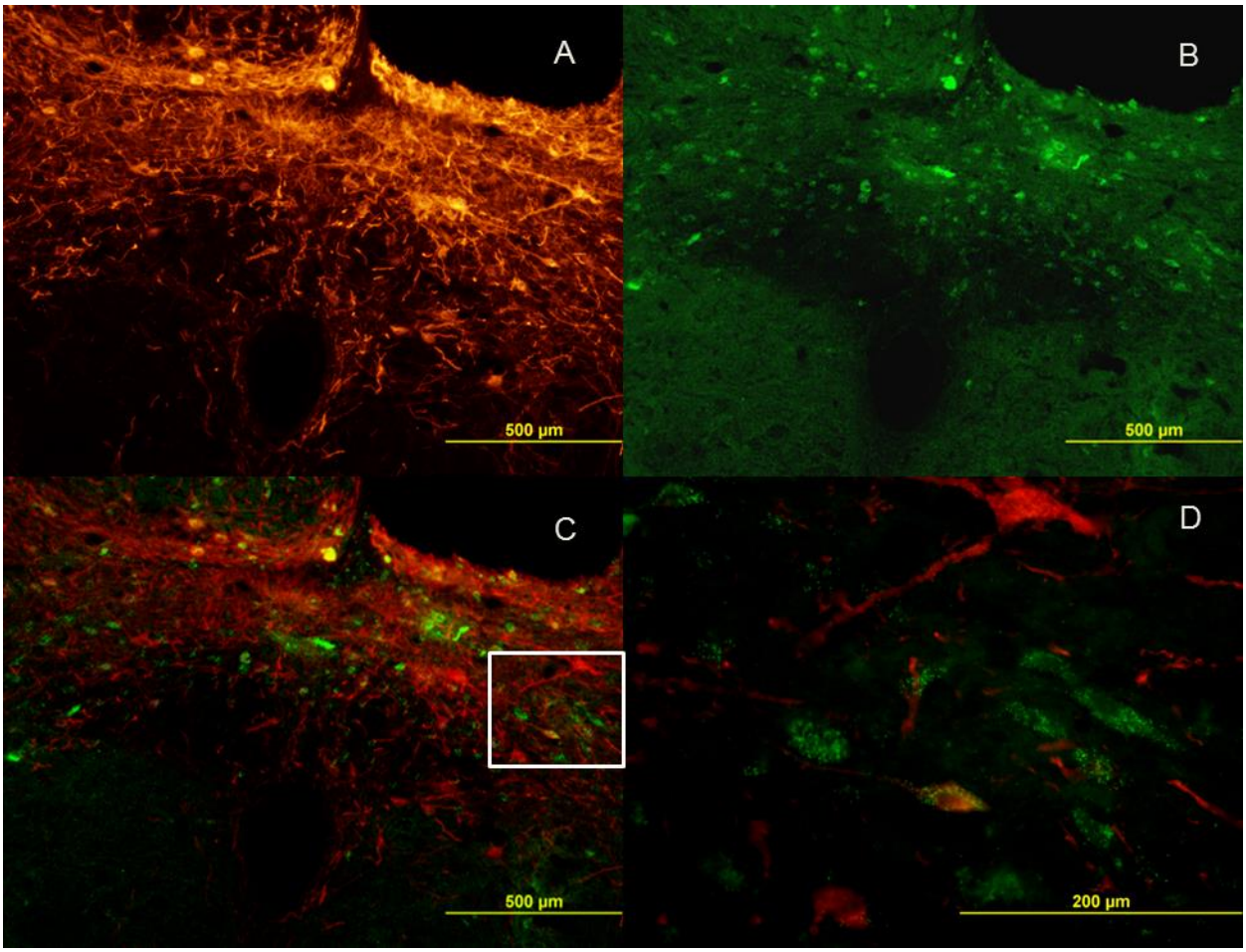

Figure 9: VGLUT2

mCherry labeled cells (A). VGLUT2^{+} cells (B). A and B merged showing mCherry labeled cells and VGLUT2^{+} cells (C). Images of A, B and C are at 10X. An area from C at 40X (D).

Figure 10:

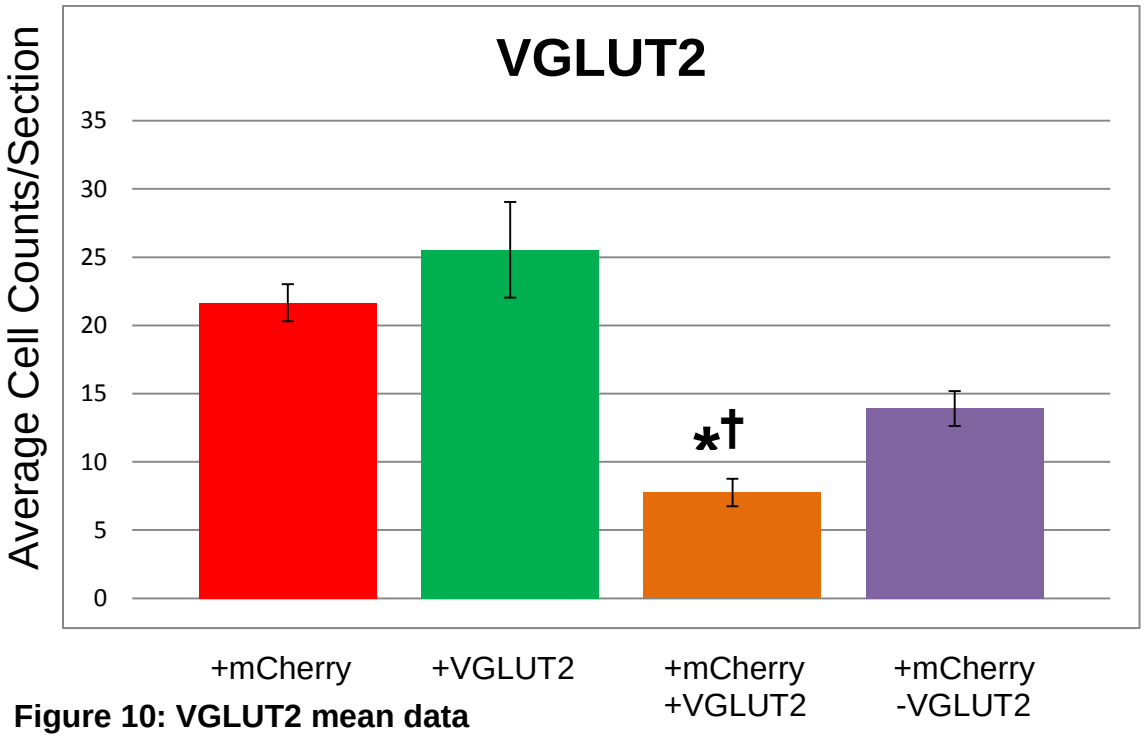

Figure 10: VGLUT2 mean data

Average Mean data from 5 rats (Average of 5±1 sections per rat, 24 sections total) showed 21.59±2.19 +mCherry cells, 25.33±2.58 VGLUT2$^+$ cells, 8.18±1.14 +mCherry/VGLUT2$^+$ cells and 13.41±2.45 +mCherry/-VGLUT2 cells per section per rat. Values for each rat were totaled and divided by the number of sections from the individual rat. 38% of +mCherry cells were also VGLUT2$^+$. Statistical analysis was performed using a One-way ANOVA, ($P<0.05$). The Bonferroni post hoc test was used to determine significant differences between the means. The symbols * and † respectively represent the significant differences of the means between the +mCherry/VGLUT2$^+$ cells with the VGLUT2$^+$ cells, ($P<0.05$), and the +mCherry cells, ($P<0.05$).

Figure 11:

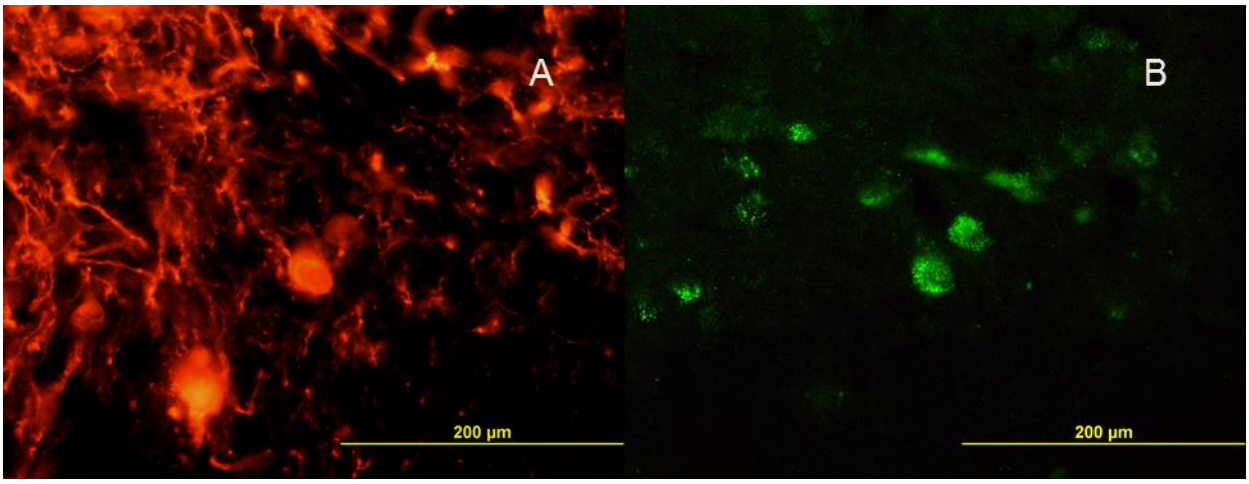

Figure 11: Examples of single labeled mCherry (A) and GAD67$^+$ cells (B).

What is necessary is identifying what cells can be truly identified as mCherry labeled cells and GAD67$^+$ cells under different channels of fluorescence. The red channel identified mCherry labeled cells (A) and the green channel identified GAD67$^+$ cells (B). This is necessary before these images can be merged to calculate how many mCherry labeled cells were also GAD67$^+$.

Figure 12:

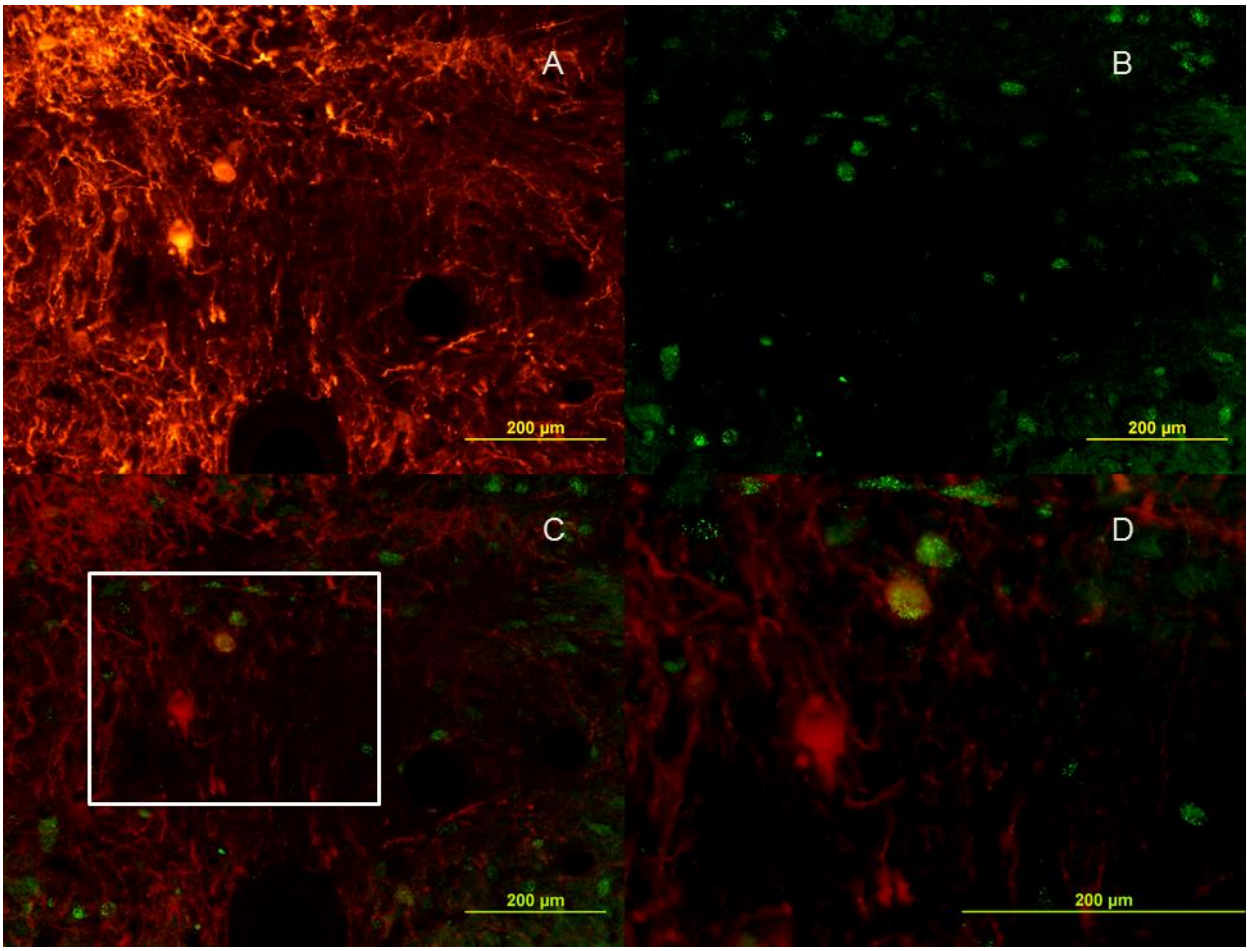

Figure 12: GAD67

mCherry labeled cells (A). +GAD67 cells (B). A and B merged showing +mCherry/+GAD67 cells (C). Images of A, B and C are at 20X. An area from C at 40X (D).

Figure 13:

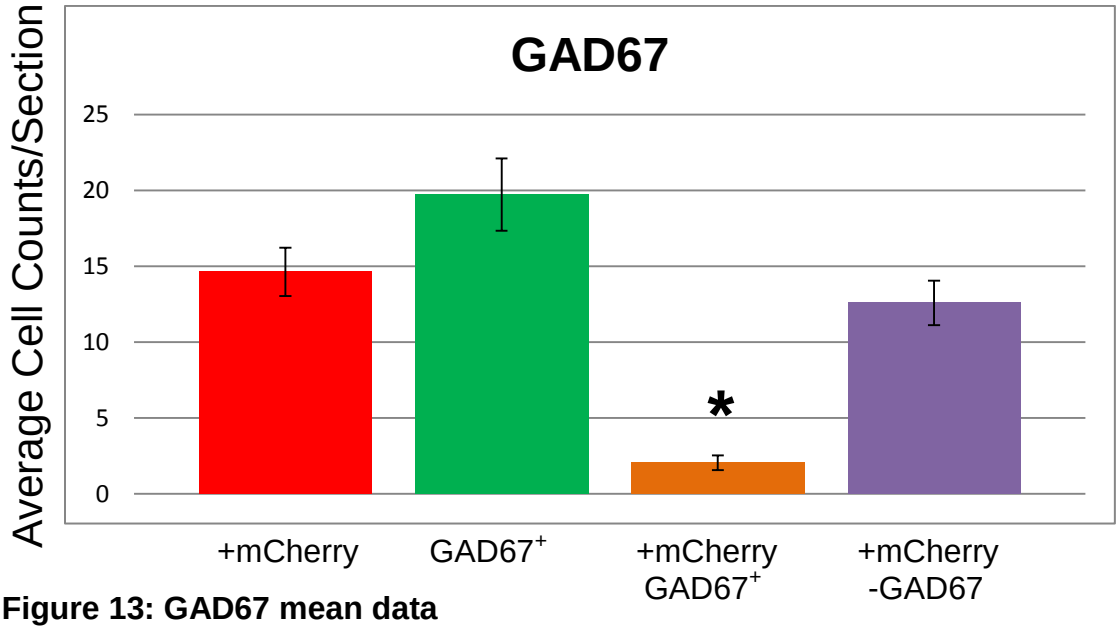

Figure 13: GAD67 mean data

Average Mean data from 5 rats (Average of 4±1 sections per rat, 22 sections total) showed 15.75±3.72 +mCherry cells, 19.50±3.88 $GAD67^+$ cells, 2.58±0.99 +mCherry/$GAD67^+$ cells and 13.17±2.89 +mCherry/-GAD67 cells per section per rat. Values for each rat were totaled and divided by the number of sections from the individual rat. 17% of +mCherry cells were also $GAD67^+$. Statistical analysis was performed using the Krukal-Wallis test, ($P<0.05$). The Tukey post hoc test determined significant differences between the medians. The symbol * represents the significant difference between the +mCherry/$GAD67^+$ cells with the $GAD67^+$ cells, ($P<0.05$).

Figure 14:

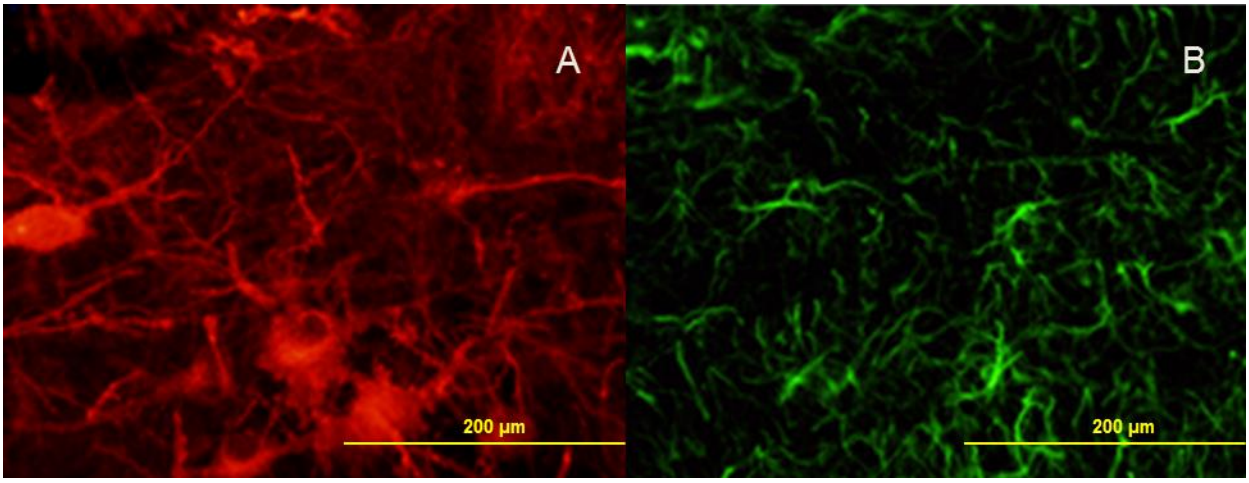

Figure14: Examples of single labeled mCherry (A) and GFAP-ir cells (B).

What is necessary is identifying what cells can be truly identified as mCherry labeled cells and GFAP-ir cells under different channels of fluorescence. The red channel identified mCherry labeled cells (A) and the green channel identified GFAP-ir cells (B). This is necessary before these images can be merged to calculate how many mCherry labeled cells were also GFAP-ir.

Figure 15:

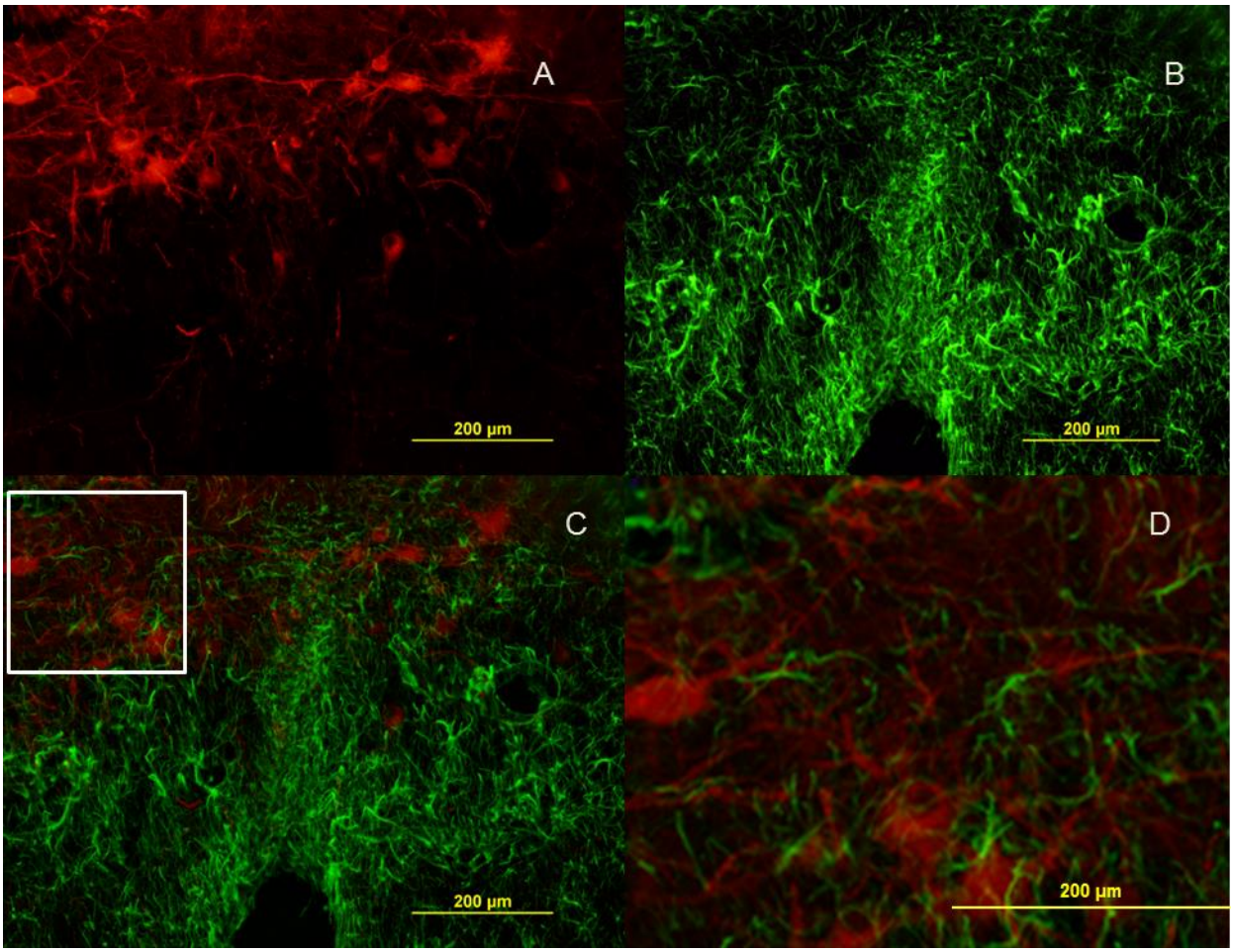

Figure 15: GFAP

mCherry labeled cells (A). GFAP-ir cells (B). +mCherry/+GFAP-ir cells (C). Images of A, B and C are at 20X. An area from C at 40X (D).

Figure 16:

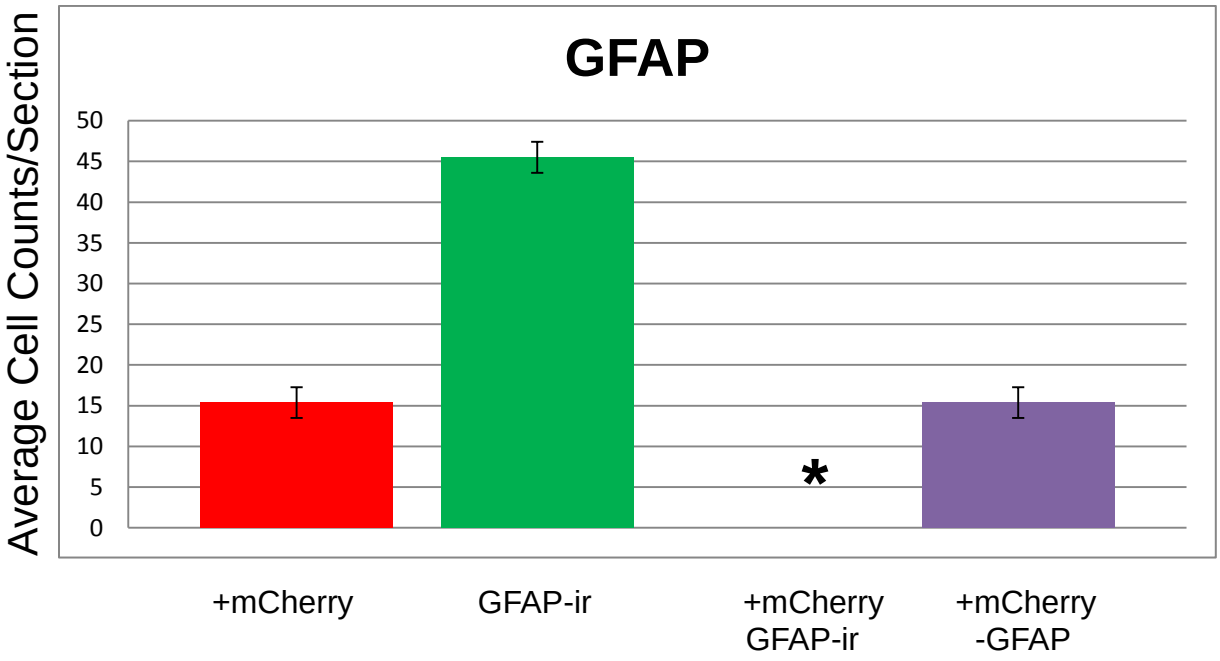

Figure 16: GFAP mean data

Average Mean data from 4 rats (Average of 5±1 sections per rat, 18 sections total) showed 15.94±3.17 +mCherry cells, 45.58±1.45 GFAP-ir cells, 0 +mCherry/GFAP-ir cells and 15.94±3.17 +mCherry/-GFAP cells per section per rat. Values for each rat were totaled and divided by the number of sections from the individual rat. 17% of +mCherry cells were also GAD67^{+}. Statistical analysis was performed using the Krukal-Wallis test, ($P<0.05$). The Tukey post hoc test determined significant differences between the medians. The symbol * represents the significant difference between the +mCherry/GFAP-ir cells with the GFAP-ir cells, ($P<0.05$).

Table 2:

Labels	TH	CaMKIIa	GFAP
β+mCherry	15.16±1.79	15.75±2.46	15.94±3.17
Immunoreactivity	14.52±1.76	15.45±1.90	45.58±1.45
α+mCherry/+Immunoreactivity	13.47±1.59	14.38±2.32	0
+mCherry/-Immunoreactivity	1.44±0.36	1.38±0.55	15.94±3.17
γ%+mCherry/+Immunoreactivity	87%	91%	0%

Table 2: Summary of Immunohistochemistry Results

Displayed are the overall results of the immunohistochemistry experiments. Data presented as means±SE along with the percentage of the +mCherry cells that were immuno-reactive for TH, CaMKIIa and GFAP.

Results presented in row **α** were divided by results from row **β**, in the same column, multiplied by 100 to get the percentage of +mCherry cells that were immuno-reactive, shown in row **γ.**

Table 3:

Labels	VGLUT2	GAD67
β+mCherry	21.59±2.19	15.75±3.72
$mRNA^{+}$	25.33±2.58	19.5±3.88
α+mCherry/$mRNA^{+}$	8.18±1.14	2.58±0.99
+mCherry/-mRNA	13.41±2.45	13.17±2.89
γ%+mCherry/$mRNA^{+}$	38%	17%

Table 3: Summary of In situ Hybridization Results

Displayed are the overall results of the in situ hybridization experiments. Data presented as means±SE along with the percentage of the +mCherry cells that were glutamatergic or GABAergic.

Results presented in row **α** were divided by results from row **β**, in the same column, multiplied by 100 to get the percentage of mCherry labeled cells also phenotypically identified shown in row **γ.**

Chapter 4

Discussion:

Consistent with our hypothesis, there was a high number of mCherry labeled TH-ir cells (87%) as well as mCherry labeled CaMKIIa-ir cells (91%). Based on the results, we conclude that the optogenetic construct with the CaMKIIa promoter is successful in driving gene expression in catecholaminergic neurons as well as neurons expressing the CaMKIIa enzyme (91%) at the site of microinjections in the NTS. Also as expected, there were no GFAP-ir astrocytes that were mCherry labeled. What was unexpected was the low number of mCherry labeled cells that were glutamatergic. We expected to see GABAergic cells that were not mCherry labeled. However, approximately 17% of the mCherry labeled neurons were also GABAergic. Based on the results concerning glutamatergic neurons, CaMKIIa is not a specific marker for glutamatergic neurons as mCherry is also found in most, if not all, TH-ir neurons within the site of injections in the NTS. The TH-ir neurons may be glutamatergic as well.

There have been previous reports of cells labeled using viral constructs with a CaMKIIa promoter in different areas of the brain. TH-ir neurons did not express CaMKIIa and many CaMKIIa positive neurons were not TH-ir (Guo et al, 2014). However, there existed a substantial number of neurons expressing both CaMKIIa and TH in the VTA (Guo et al, 2014). CaMKIIa-ir cells were also found to be glutamatergic and CaMKIIa was not found in GABAergic neurons in the

hippocampus, cerebral cortex and thalamus (Liu and Murray, 2012). Findings concerning GABAergic neurons are variable depending upon the CNS region studied. First, cells within the commissural nucleus of the stria terminalis were positive for CaMKIIa were GABAergic (Liu and Murray, 2012). Second, cerebellar Purkinje GABAergic neurons also expressed CaMKIIa although at lower levels than did forebrain neurons (Liu and Murray, 2012). Another study found that cortical glutamatergic neurons were positive for CaMKIIa, but also found that lemniscal glutamatergic neurons did not express CaMKIIa (Liu and Jones, 1996). It is noted that the data presented by Liu and Murray are based on immunostaining and not optogenetics. The results of these studies suggest the neuronal phenotypes of cells co-localized with CaMKIIa are dependent upon the brain region.

Limitations

There is the possibility that the optogenetic construct may not have entered every single cell that it would be expected to infect. Concerning the immunohistochemistry, a cell may not have contained enough protein to be detected. In addition, the primary antibodies may not have bound to enough protein to be detected. Finally the secondary antibodies may not have bound to enough primary antibodies to be detected. Concerning in situ hybridization, it is possible that the cell was not producing enough mRNA for detection. Based on our results and previous studies, the location within the CNS also appears to be a factor in the

effectiveness of the construct. This leads to the possibility that the injection site variability and the NTS region are other factors to consider.

Significance

The effectiveness of the viral vector cannot be used to extrapolate data from one CNS area to another. As stated before, there are differences involving co-localization and segregation of CaMKIIa with glutamatergic, GABAergic and catecholaminergic neurons. Also, if CaMKIIa is only in a subset of glutamatergic NTS neurons, what is different concerning the implications of CaMKIIa with the functions of the glutamatergic NTS neurons?

Future Directions

Investigation of the subset of the glutamatergic and GABAergic neurons expressing CaMKIIa would be an appropriate next step. Independent of viral infection, the presence of CaMKIIa in glutamatergic and GABAergic NTS neurons could be determined. Future study could examine whether the $VGLUT2^{+}$ neurons that take up the virus are also catecholaminergic. One study reported a range of co-localization of TH and VGLUT2 expressing neurons that ranged from 16-80% indicating differential co-localization depending on regions in the brainstem there was little consistency of the co-localization and segregation of neurons expressing VGLUT2 and TH, as well as suggesting a subset of glutamatergic neurons in the lower brainstem (Stornetta et al, 2002). Due to the co-localization of gene expression due to the CaMKIIa promoter and TH, hypothesizing glutamatergic

CaMKIIa expressing neurons could also be positive for the expression of TH. Finally, there could be a receptor that the majority of x neurons did not express, keeping the AAV2 from entering the cell in the first place. Another study stated that the membrane-associated heparin sulfate proteoglycan is a receptor for the AAV2 and that mutations of the synthesis of these proteoglycans can inhibit AAV2 binding (Summerford and Samulski, 1998). The identification of the possible limiting factors will be a step forward in understanding the effectiveness of this specific construct for future investigations within the NTS.

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Appendix A:

Microinjection Protocol

1. Rats anesthetized with 4% isofluorane and given 0.2 ml of ketamine ip and 0.14 ml of Dormitor ip
2. Placed in stereotaxic frame
3. Hindpaw pinch to test anesthesia
1. 100% oxygen provided during procedure
2. Dorsal surface of the brain is exposed
3. Microinjection of AAV2-CamKIIa-hChR2-H134R-mCherry with a glass pipette (25-30 µm tip diameter) into the NTS
4. 200 nl of virus administered for a period of 5 minutes at the calamus scriptorius 0.5 mm deep
5. Second injection (same amount for same period of time) .5 mm caudal to the calamus scriptorius
6. Suture wounds
7. Application of 0.05 ml antisedan to reverse effects of the anesthesia
8. 3-4 week period for observation recovery and to ensure a high level of cells infected by the AAV2

Appendix B:

Rat Perfusion

1. Make 4% paraformaldehyde (PFA)
2. Fill a beaker to 250 ml saline (.9% normal saline).
3. Fill lines with paraformaldehyde and saline; make sure there are no bubbles.
4. Deeply anesthetize the rat with Nembutal or urethane.
5. Prepare 1 ml syringe with 20G 1.5” catheter/needle filled with Heparin (500 U/1 ml)
    a. Open peritoneal cavity, slit up both sides of lungs, cut through diaphragm.
    b. Insert needle/catheter into the left ventricle and aim toward ascending aorta.
    c. Inject .1 ml heparin (1000U/ml soln)
    d. Remove needle and leave catheter in heart
    e. Attach stopcock/tubing to catheter.
    f. Slit right atria to allow blood to drain
6. Turn on pump, using saline first. The saline should drip out of the body cavity into the bucket at a constant rate. Liver should clear and lungs should remain pink (NOT turn white).
7. When saline solution is almost gone, turn stopcock to deliver the PFA.
8. Hold rat down, as paraformaldehyde will cause muscle constriction for a minute.
9. Decapitate and remove the brain
10. Post-fix 1-2 days in 4% paraformaldehyde in 4ºC. (Weekend storage in fix is ok)
11. Section brain within 1-3 days.

Appendix C:

Brain Tissue Preparation

Day 1:

1. Perfuse rat with buffered saline followed by 4% PFA and remove brain
2. Post-fix brain in same fixative overnight in refrigerator

Day 2:

1. Rinse tissue in phosphate buffer, block tissue with 10% NHS, remove dura, punch in mark if needed, superglue to chuck
2. Cut sections using vibratome at 30 µm thick and store in RNAse-free CP

Note: Storage of tissue before use for in situ hybridization should not be >several days.

3. Mount selected sections onto a charged slide and dry OVERNIGHT at RT.

Day 3:

1. Fill a 10 ml syringe with vacuum grease and pipe it around the mounted sections on the slide to make 1 well.

Appendix D: Part 1

RNAscope protocol (In situ Hybridization)

PREPARE

3 L of 1X WASH BUFFER by adding 2.94 L distilled water and 1 bottle (60 mL) (or 1 ml 50x buffer + 49 ml water) of 50X Wash Buffer to a large carboy. Mix well.

Note: Warm 50X Wash Buffer up to 40°C for 10–20 MIN before making 1X Wash Buffer.

1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month (I have gone for 3-6 months).

PROBES:

1. Warm probes for 10 MIN at 40°C in a water bath or incubator, then cool to ROOM TEMPERATURE (RT).

2. Briefly spin the C2 and C3 probes to collect the liquid at the bottom of the tubes.

3. Mix 1:1:50 ratios of C2, C3, and C1 probes by pipetting1 volume of C2 and 1 volume of C3 probes to 50 volumes of C1 probe into a tube. Invert the tube several times. (per well = 6 ul C2 + 300 ul C1)

Note: Do not mix probes of the same channel.

The mixed Target Probes can be stored at 4°C for up to 6 MONTHS (I have gone up to 12-15 months).

Equilibrate reagents

1. Place AMP1–4FLreagents at RT.
2. Rinse tissue sections in sterile water 2X 2 minutes (NO AGITATION!)
3. Treat with Pretreat 4 for 30 min @ 40ºC (do not pre-heat the Pretreat)
4. Rinse 2X 2 minutes in sterile water
5. Transfer to probes mix for 2 hrs @ 40ºC
6. Wash sections in 1X Wash Buffer for 2X 2 MIN at RT.
7. Transfer sections to AMP1-FL 30 min at 40 C
8. Wash sections in 1X Wash Buffer for 2X 2 MIN at RT.
9. Transfer sections to AMP2-FL for 15 min at 40 C
10. Wash sections in 1X Wash Buffer for 2X 2 MIN at RT.
11. Transfer sections to AMP3-FL 30 min at 40 C
12. Wash sections in 1X Wash Buffer for 2X 2 MIN at RT.
13. Transfer sections to AMP4-FL for 15 min at 40
14. Wash sections in 1X Wash Buffer for 2X 2 MIN at RT.

Appendix D: Part 2

IHC Post-In situ hybridization

1. To combine with immunohistochemistry, after the last wash, add the primary antibody with 10% serum + 0.1% triton on the slide and put in your moist petri dish in the frig overnight.
2. Rinse 2 times for 2 min rinse in TBS or PBS, then put the secondary on the slide (usual concentration) and rinse, mount as described. Cheers, and stay cool!
3. Incubate with primary anti-mCherry antibody (made in rabbit)
4. 10% serum (horse serum), 0.1% triton, and antibody at 1:1000 dilution
5. Overnight in the refrigerator.
6. Rinse in phosphate-buffered saline 2 times (2 minutes each)
7. Incubate with secondary antibody one hour
8. Donkey anti-rabbit - Cy3 (red) at 1:400
    a. % triton and 1% NHS.
9. Rinse 2 times for 2 min each in PBS.
10. Coverslip with Prolong Diamond to preserve the fluorescence of the mCherry antibody and the labeled mRNA.

Appendix E:

Immunohistochemistry Protocol

1. Best to begin the protocol on either a Tuesday or a Friday
2. Take tissue from sucrose solution and cut 40 µm thick slices into 3 different groups per hind brain
3. Place sections in CP for storage in -20˚C freezer
4. Remove sections, when ready, from CP to vial (Tuesday or Friday)
5. PBS rinse 4X, put into fridge and wait 30 min
6. PBS rinse 4X, put into fridge and wait 30 min
7. H_2O_2: 100 µl H_2O_2 + 10 ml of millipore H_2O (mix with disposable pipette)
8. Add 2 ml to each set of sections and leave at RT for 30 minutes
9. PBS rinse 4X, place in fridge for 30 minutes
10. PBS Diluent: 3 ml horse serum (in freezer on 3rd floor) + 250 µl Triton X-100 (Cabinet below hood) + 96.75 ml of PBS. Stir for 10 minutes
11. Add 2 ml of PBS Diluent to sections and leave at RT for 2 hours
12. Remove PBS Diluent from sections but **DO NOT** rinse sections
13. Add 2 ml of Primary to each set of sections: Ab added to PBS Diluent example; 4 µl of TH and 4 ml of PBS Diluent= 1:1000, this would be for only 2 samples)
14. Note: It is recommended that an extra 2 ml of Ab is made to ensure enough is available. (5 samples= 12 ml of Ab in PBS Diluent)
15. Leave at RT for 1-3 hours then put in fridge for 2 days (at least 48 hours)

16. ON THE 3RD DAY
17. PBS rinse 4X, put into fridge and wait 30 min
18. PBS rinse 4X, put into fridge and wait 30 min
19. Add 2 ml of Secondary (In PBS Diluent) to each set of sections
20. Sit at RT for 4 hours in a box, (light sensitive)
21. PBS rinse 4X

After the last rinse, the sections can either be put in 4ºC overnight in a box and then continue the next day mounting the sections on gel coated slides, or, after the last rinse, the sections can directly be mounted on the gel coated slides.

After mounting the sections, place the slides in a slide booklet and put in drawer (in the dark at RT) for 48 hours.

After 48 hours, coverslip with Vectashield Hard-set for 2 hours then take to mircroscope.

CP- Cryoprotectant

RT- Room Temperature

Ab- Antibody

Appendix F:

Statistical Analysis of Non-parametric Data

Tyrosine Hydroxylase

One Way Analysis of Variance Thursday, March 30, 2017, 9:28:27 AM

Data source: Data 1 in Sigma Data 3-29-17

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Thursday, March 30, 2017, 9:28:27 AM

Data source: Data 1 in Sigma Data 3-29-17

Group	N	Missing	Median	25%	75%
Col 1	4	0	16.500	11.375	17.594
Col 2	4	0	16.167	10.938	16.458
Col 3	4	0	14.750	10.188	15.469

H = 2.423 with 2 degrees of freedom. P(est.)= 0.298 P(exact)= 0.327

GAD67

One Way Analysis of Variance Thursday, March 30, 2017, 9:35:03 AM

Data source: Data 1 in Sigma Data 3-29-17

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Thursday, March 30, 2017, 9:35:03 AM

Data source: Data 1 in Sigma Data 3-29-17

Group	N	Missing	Median	25%	75%
Col 17	5	0	14.625	9.200	22.850
Col 18	5	0	21.000	14.850	27.950
Col 19	5	0	1.500	1.350	4.350

H = 10.238 with 2 degrees of freedom. (P = 0.006)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.006)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
Col 18 vs Col 19	44.000	4.400	Yes
Col 18 vs Col 17	13.000	1.300	No
Col 17 vs Col 19	31.000	3.100	No

Glial Fibrillary Acidic Protein

One Way Analysis of Variance Thursday, March 30, 2017, 9:33:03 AM

Data source: Data 1 in Sigma Data 3-29-17

Normality Test (Shapiro-Wilk) Passed (P = 0.160)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Thursday, March 30, 2017, 9:33:03 AM

Data source: Data 1 in Sigma Data 3-29-17

Group	N	Missing	Median	25%	75%
Col 9	4	0	14.250	11.000	22.563
Col 10	4	0	45.417	42.896	48.438
Col 11	4	0	0.000	0.000	0.000

H = 10.240 with 2 degrees of freedom. P(est.)= 0.006 P(exact)= <0.001

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
Col 10 vs Col 11	32.000	4.438	Yes
Col 10 vs Col 9	16.000	2.219	No
Col 9 vs Col 11	16.000	2.219	No