**Project Number: AJ1 1203**

# Morphological Characterization Cortical Neurons in a mouse model of Rett Syndrome and Design of a Slice Incubation Chamber

*A Major Qualifying Project submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science*


Submitted by WPI Student:

_____

Alexandra Clemente

Conducted in collaboration with:

Jorge Castro, Ph.D

Submitted to:

Project Advisors:

Prof. Anjana Jain, WPI

Prof. Mrignaka Sur, MIT

# Table of Contents

# Table of Figures

# Table of Tables

## Abstract

Rett Syndrome (RTT) is an autism-spectrum disorder caused by a mutation in the gene coding for methyl-CpG-binding protein 2 (MeCP2). In accordance with the theory that RTT arises from a excitatory/inhibitory imbalance caused by dendritic alterations, we hoped to observe reduced arborization in the MeCP2 mutant neurons. The dendritic morphology of pyramidal cells and interneurons proceeding from MeCP2 mutant and non-mutant mice were reconstructed and analyzed. We observed a possible difference in the area covered by the basal dendritic arborization and decreased dendritic complexity reflected in diminished process lengths and number of terminal segments. This could translate to a diminished integrative capacity in MeCP2 KO cells.

.

## Acknowledgements

I would like to thank Prof. Mriganka Sur for giving me the opportunity to work in his lab at the Massachusetts Institute of Technology and for all of the support throughout my project. I would also like to thank Jorge Castro, who was a great help and worked with me on this project. I would like to thank Caroline Runyan for providing the data for the reconstructed Parvalbumin-expressing Interneurons. I would like to thank Prof. Pins and Prof. Ambady for helping me set up this off-campus MQP. I would also like to thank everyone in the Sur lab for making this project a great experience. Last, I would like to thank my major and MQP advisor, Anjana Jain.

# Chapter 1: Introduction

Rett Syndrome (RTT, No.MIM:312750) is a neurological disorder part of the Autism Spectrum and one of the most common forms of cognitive impairment in females. It is characterized a period of normal development for the first 6-18 months, after which there is a period of stagnation and regression. The most common symptoms include decrease in head growth, loss of motor control, irregular breathing, loss of speech, social withdrawal and mental retardation [1, 2]. Patients can to survive into adulthood, but even under extensive treatment, they are unable to recover normal functions. It is known that about 80% of RTT cases are caused by mutations in the X-linked MeCP2 gene (methyl-CpG-binding protein 2) [3-5]

The mechanism of action by which MeCP2 brings about RTT symptoms is still uncertain. It has been proposed that RTT pathology, like other forms of cognitive deficits arises from a prolonged immature state of the nervous system. Anomalies like spine dysgenesis and reduced dendritic arborization are consistent anatomical correlates of neuropsychiatric diseases characterized by cognitive impairment, like Down Syndrome and Fragile X [6, 7]. These changes in dendritic morphology can translate into altered synaptic function, which could account for the distorted cortical circuitry in RTT.

These theories have lead to studies on the morphology of MeCP2 deficient cells. In past studies, it has been proven that MeCP2 mutant pyramidal cells exhibit decreased spine motility, disrupting the normal maturation process [8]. It has also been shown that these MeCP2 mutant cells exhibit decreased spine density and decreased dendritic arborization [9, 10].

Such a detailed analysis has not been conducted in interneurons. The interest in interneurons comes from studies that show how alterations in neuronal GABA signaling causes Rett-like symptoms in mice and that MeCP2 might have a role in controlling GABA synthesis [11]. This and other work suggest that MeCP2 is necessary for proper excitatory/inhibitory balance [12]. Another interesting study indicates that adult GABAergic interneurons exhibit not only functional but also structural plasticity without any gross sensory input alterations [13]. In order to properly centralize and focus our project and also because of the wide variety of interneurons, we will be focusing our project on Fast-Spiking Parvalbumin expressing interneurons. Further clarification regarding the classification of interneurons will be dicussed in Chapter 2.

The disturbance in the excitatory/inhibitory balance could lead to a prolonged immature state of the nervous system, giving rise to the symptoms observed in RTT patients. This imbalance could be

brought about by dendritic alterations in the cortical neurons. Therefore, we hope to observe reduced arborization and decreased process length in the MeCP2 Knock-Out (KO) cortical neurons.

Some of the most important techniques in this study and neuroscience in general is the family of techniques called electrophysiology. Electrophysiology is the study of the electrical properties that exist within biological cells and tissues [14]. By the accurate placement of electrodes along the tissue, one can measure the basal electrical activity of an acute or cultured cell or tissue or their response to an induced electrical current passed through an electrode. It is a technique primarily used in researching the heart, muscles and nervous system.

In neuroscience there are a variety of electrophyisiology techniques that can be used to measure the electrical activity of neurons. For this project, we will be concerned with a technique *whole-cell patch-clamping*. Whole-cell patch-clamping is the gold-standard technique for studying single neuron morphology and firing properties. Whole-cell patch-clamping recording of the electrical activity of neurons *in vivo* and *in vitro* utilizes glass micropipettes to establish an electrical and molecular access to the inside of neurons. The electrical access is substantial enough to record synaptic and ion channel–mediated changes to action potentials and firing properties that enable neurons to compute information and that are affected in brain disorders or by drug treatment. In addition, molecular access to the cell allows the infusion of dyes for morphological visualization and computational reconstruction [15].

For this project we will be conducting whole-cell patch clamp recordings and dye infusions in acute brain slices. The tissue is extracted from mice, sectioned and the slices are incubated in a slice incubation chamber before they are used. A more detailed description of the materials and methods can be seen in Chapter 4. The success of the electrophysiology experiments are dependent on the viability of the brain slices after being incubated [16]. It is therefore extremely important that slices are appropriately preserved in the chamber.

For the design aspect of this major qualifying project, we will be concerned with designing a bioreactor that preserves the slices, allowing for optimal whole-cell patch clamping recording and dye filling. A five-stage prescriptive approach, as described in *Engineering Design: A Project Based Introduction* by Clive I. Dym and Patrick Little was followed. The following is a summary of said project approach and how it pertains to the design of the slice incubation chamber.

The first stage, the problem definition, took the client statement to first clarify the design objectives and identify the constraints. It was also considered pertinent to examine the design stakeholders at this point. The revised client statement, objectives, constraints and stakeholder analysis are described throughout Chapter 5. The design specifications, functions and design alternatives were used to generate four alternative conceptual designs. The means and designs were based on methods and products available on the market. Product and patent searches were also conducted to establish current standards to which compare the final design. The alternative designs were based on functions-means analysis of the desired final product. These four alternative designs were evaluated against the established objectives, constraints and functions to determine a final conceptual design. During the fourth stage, the detailed design, the final design was refined and altered based on testing and the fabrication specifications were determined. The fifth and final stage was the design communication stage in which the design was documented and submitted to the client.

Sections of this study also establish the significance of this work and the impact it may have on aspects such as the environment, economy and current research practices. Finally, conclusions from both the research and design portions of the project were determined in order to establish future recommendations if any of this work were to be carried on in the future.

## 1.1 Key Terms and Abbreviations:

**MeCP2** Methyl-CpG-Binding Protein 2

**RTT** Rett Syndrome

**BDNF** Brain Derived Neurotrophic Factor

**CDKL5** Cyclin-dependent kinase-like 5

**PV** Parvalbumin

**V1** Primary Visual Cortex

# Chapter 2: Literature Review

## 2.1 Rett Syndrome

### 2.1.1 General Description

Rett Syndrome (RTT, No.MIM:312750) is a disorder part of the Autism Spectrum and one of the most common forms of mental retardation in females. It is characterized a period of normal development for the first 6-18 months, after which patients start missing important developmental milestones. This period of developmental stagnation is followed by regression, in which patients lose cognitive and motor abilities. The most common symptoms include decrease in head growth, loss of motor control, irregular breathing, seizures, social withdrawal and cognitive impairment [1, 2]. Patients usually survive until their mid-twenties but can to survive well into their forties. Even under extensive treatment, they are unable to recover normal cognitive and motor functions. There is no specific treatment for RTT and management is mainly symptomatic and individualized. There therapeutics that have been shown to improve multiple symptoms of RTT in animal models [10] currently undergoing clinical trials [17].

It is known that about 90% of RTT cases are caused by loss-of-function mutations in the X-linked *MECP2* gene (methyl-CpG-binding protein 2) [3-5]. This gene codes for MeCP2, a nuclear protein that functions as a transcriptional regulator and epigenetic and is implicated in gene silencing and chromatin remodeling [18]. The MECP2 gene is 112756 bp long and is composed of four exons. The most important functional domains are the methyl-Cp-G-binding domain (MBD), split between exons 3 and 4, and the transcriptional repressor domain (TRD) located in exon 4. The MBD region of the protein binds to methylated CpG sites in DNA while the RD recruits co-repressors like histone deacetylases (HDACs).[19] The MeCP2 protein also contains two nuclear localization signals (NSL) [20]. While MeCp2 expression is ubiquitous throughout the body including lung, liver and spleen, it is abundant in mature neurons in the adult brain [19]. While it is known to be important in the development and maintenance of adult neurons, its particular role or mechanism of action are poorly understood.

To further understand how MECP2 mutations can lead further the understanding of the mechanism of action of MeCP2 several RTT animal models have been generated. Most of these models have been generated by altering the endogenous gene in mice. In some of these models, the MeCP2 protein is undetectable in the brain [21] while in other a truncated non-functional form of the protein is expressed [22] or it is only absent in a specific subset of neurons [11]. For this study, we will be using the

mouse model described by Chen et al. 2001 [22] in which exon 3 of MECP2 is deleted from the neurons of pups using a neuron specific promoter-driven Cre-loxP recombination system.

### 2.1.2 Molecular Characterization of RTT

MeCP2 is a protein that binds to methylated CpGs in DNA regulates transcription by either activating & silencing through histone modification and is mostly found in differentiated mature neurons [4]. MeCP2 controls a variety of genes in the Central Nervous System (CNS), but one of its well known targets is Brain Derived Neurotrphic Factor (BDNF). Membrane depolarization causes the phosphorylation and consequent release of MeCP2 from BDNF promoter III, enabling its transcription[23]. Different studies have shown reduced and aberrant levels of BDNF secretion and expression in MeCP2 mutant brains [24, 25]

BDNF is involved in neuronal survival, axon branching and dendritic arborization and synaptic formation[26, 27]. The way BDNF carries out these diverse functions is through the activation of different signaling pathways through its receptor, TrkB [27]. It is known that BDNF can activate the AKT/mTOR and the MAPK/ERK pathways, both involved in the regulation of protein synthesis and synaptic plasticity and strengthening. The BDNF induced activation of the AKT/mTOR pathway results in the synthesis of proteins that are essential for synaptic organization and plasticity, like PSD-95 [26].

Recently, mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5) have been identified in some RTT patients as well as other forms of mental retardation [28, 29]. CDKL5 mutations in the context of cognitive deficits are characterized by early-onset seizures experienced by these patients. The CDKL5 mutations identified include chromosome translocations, deletions, insertions, nonsense and misense mutations or mutations in the catalytic domain. While CDKL5 is known to be expressed in mature cortical neurons, its kinase activity is somewhat uncharacterized [30]. It has been shown that CDKL5 regulates neuronal morphogenesis *in vivo* [31]. Chen et al. (2010) selectively knocked-down endogenous CDKL5 *in vivo* using shRNA in mice (seen as shRNA#1 in Figure 1). The mice lacking CDKL5 had smaller and less complex neurons throughout the different cortical layers. This morphological deficit was able to be recovered through re-expression of CDKL5. Chen et al.'s work also shows that CDKL5 binds to Rac1, providing evidence that CDKL5 is involved in the BDNF-Rac1 actin-modification pathway.

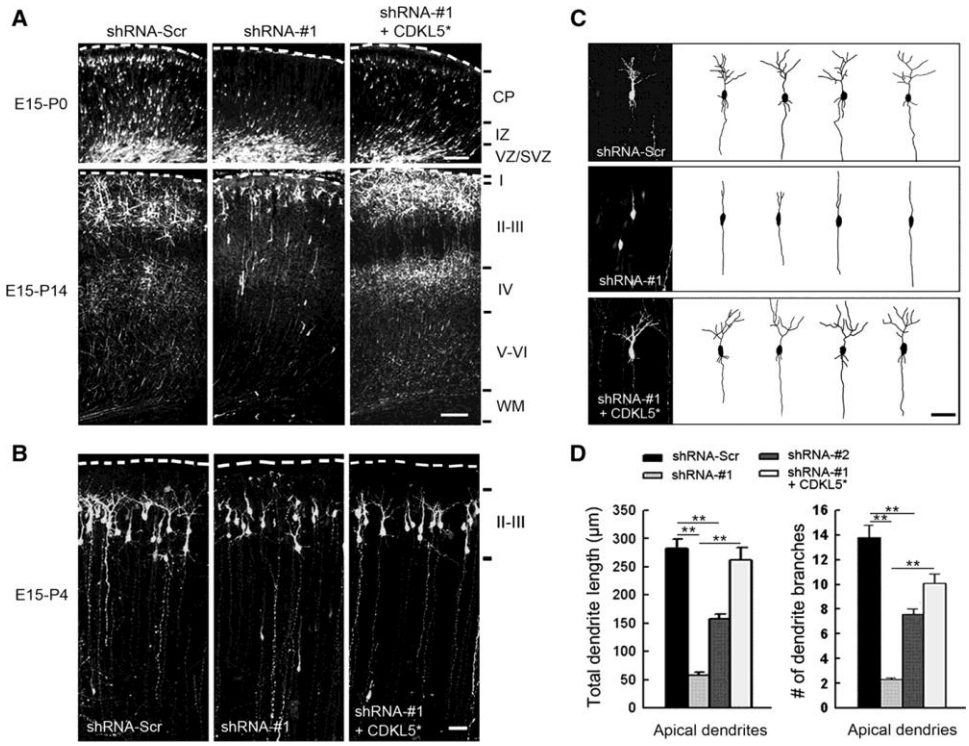



**Figure 1: CDKL5 is required for dendritic arborization in vivo. Taken from Chen et al. (2010)[31]**

## 2.2 Neuron Classification and Morphology

There are a variety of neurons that make up the excitatory and inhibitory units of the cortical circuit. The pyramidal cells are the excitatory unit of the cortex while internuerons are the main inhibitory unit. Both of these types of neurons have distinct morphological, molecular and electrophysiological properties. The appropriate contributions of excitatory and inhibitory synaptic inputs are necessary for proper neuronal functioning. A schematic of how these neurons connect can be seen in Figure 2.

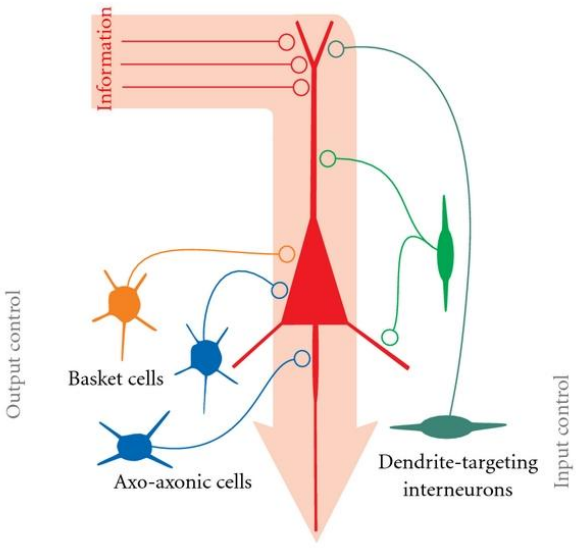



**Figure 2: Oversimplified scheme of the inhibitory control of cortical pyramidal neurons by several general classes of GABAergic interneurons taken from Mendez & Bacci (2011)[32]**

A proper balance between excitation and inhibition is essential for proper neuronal development and functioning. The disturbance in the excitatory/inhibitory balance are thought to lead to a prolonged immature state of the central nervous system, giving rise to the symptoms observed in RTT patients. Therefore it is essential that we appropriately classify and study both populations. In this study, we will be analyzing both types of neuronal populations therefore it is essential that we outline their characteristics features. The following subchapter outlines the characteristics of pyramidal cells and interneurons.

### 2.2.1 Pyramidal Cells

Pyramidal cells are the excitatory unit of the cortex. Pyramidal cells are amongst the largest neurons in the brain and are found in a variety of structures within the cortex. They have a very characteristic dendritic morphology regardless of their location. A simple schematic of pyramidal cell morphology can be seen in Figure 3.

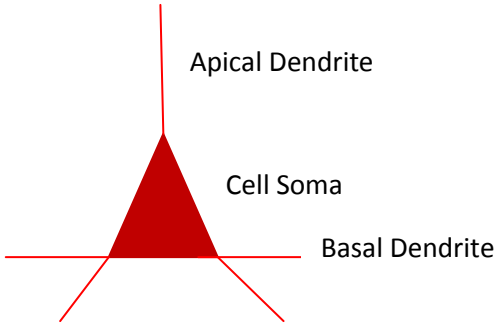



Figure 3: Schematic of morphology of cortical pyramidal cells

Pyramidal cells are characterized by the presence of a single apical dendrite and multiple basal dendrites. The apical dendrite emerges from the apex of the cell. In most cases the primary apical dendrite extends for several hundred microns before branching to form an apical tuft, consisting of dendrites that branch a few times before terminating. Emanating from the primary apical dendrites are several oblique branches, which typically branch once or twice before terminating. Several basal dendrites emerge from the base of the pyramidal soma. Each basal dendrite branches up to several times before terminating. The dendritic arbors are the means by which synaptic inputs are integrated by the cell.

Both the basal and apical dendrites of pyramidal neurons are studded with dendritic spines. These structures serve as the postsynaptic structure at most excitatory synaptic inputs received by the dendritic tree.

### 2.2.2 Interneurons

Interneurons are neuronal cells that target pyramidal cells and modulate their activity. In most cases, interneurons use gamma-aminobutyric acid (GABA) to inhibit the activation of their target cells. A classification of interneurons can be seen in Figure 4.

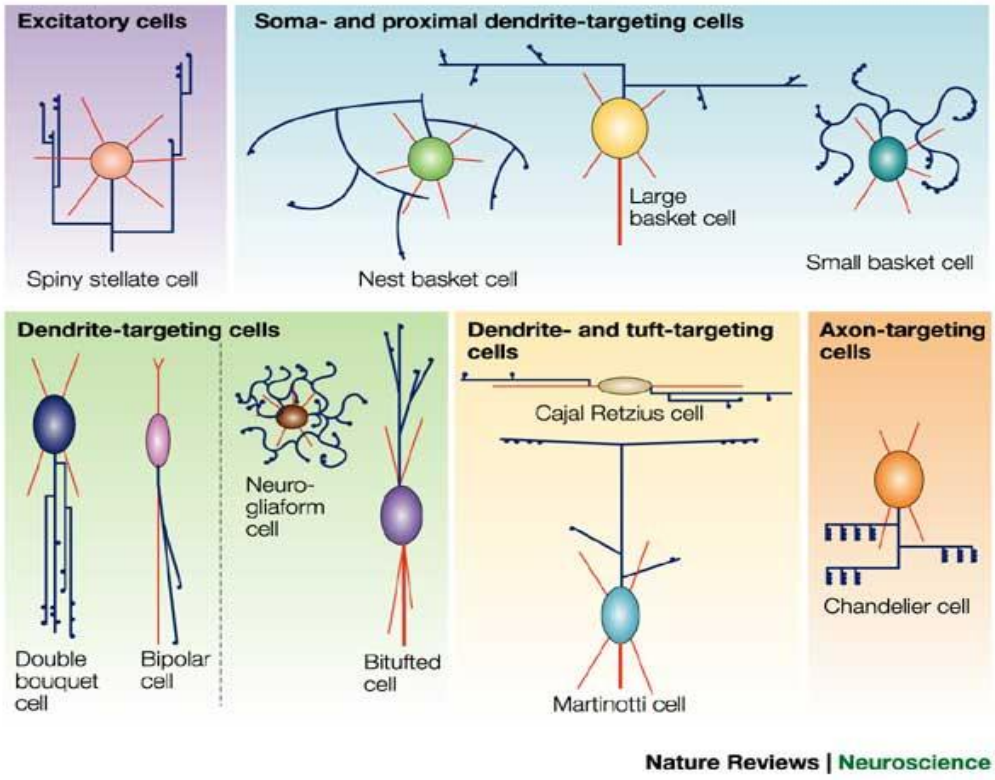



Figure 4: Classification of Interneurons based on target location. Taken from Markram et al. (2004)[33]

Interneurons variety comes not only from can target either the soma, the dendrites or the axon but also based on their molecular expression, location in the brain and cell morphology. For the purpose of our interneuron studies we will only be concerned with cortical interneurons that express parvalbumin.

Parvalbumin is a calcium binding protein that is selectively expressed in a specific and non-overlapping gropup of interneurons in the cortex [34]. Parvalbumin expressing interneurons account for about 40% of the inhibitory neuron population in the cortex and are mostly found in layers II/III. Basket cells that target both the soma and proximal dendrites are the main types of interneurons that express parvalbumin. These cells have also been found to have conserved electrophysiological behavior as they are all classified as Fast-Spiking cells. Fast-spiking cells show increased frequency in firing rate compared to other cells types. A typical firing profile of a Fast-Spiking cell can be seen in Figure 5.

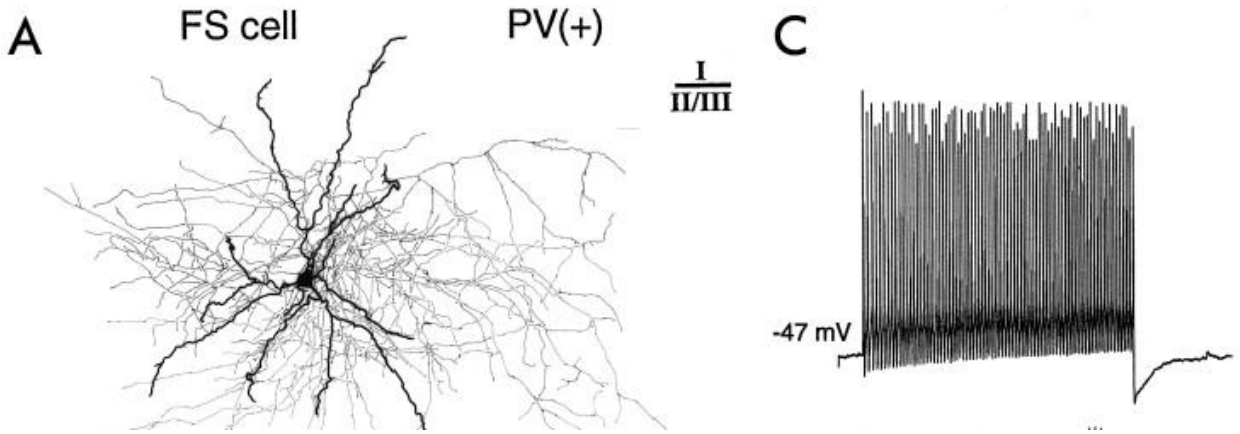



**Figure 5: Reconstruction and firing profile of a Parvalbumin expressing cortical interneuron. Taken from Kawaguchie & Kubota (1998).[35]**

The interest in interneurons comes from studies that show how alterations in neuronal GABA signaling causes Rett-like symptoms in mice and that MeCP2 might have a role in controlling GABA synthesis [11]. This and other work suggest that MeCP2 is necessary for proper excitatory/inhibitory balance [12]. Another interesting study indicates that adult GABAergic interneurons exhibit not only functional but also structural plasticity without any gross sensory input alterations [13]. Because of the wide variety of interneuron morphology, we will be focusing our project on the previously described Fast-Spiking Parvalbumin expressing interneurons. We will not study the alterations in the electrical inhibition within the cortex.

## 2.3 Neuron Morphology in Cognitive Disorders

The mechanism of action by which MeCP2 brings about RTT symptoms is still uncertain. It has been proposed that RTT pathology, like other forms of cognitive deficits arises from a prolonged immature state of the nervous system. Anomalies like spine dysgenesis and reduced dendritic arborization are consistent anatomical correlates of neuropsychiatric diseases characterized by cognitive impairment, like Down Syndrome and Fragile X [6, 7]. These changes in dendritic morphology can translate into altered synaptic function, which could account for the distorted cortical circuitry in RTT.

### 2.3.1 Neuron Morphology in Rett Syndrome

The theory that altered morphology could lead to diminished cognitive functions has lead to the studies of neuronal structure in MeCP2 deficient cells. Past studies have shown that MeCP2 mutant pyramidal cells exhibit decreased dendritic spine motility in early development, disrupting the maturation process of synapses[8, 36]. Insulin-like Growth Factor 1 (IGF-1), a proposed therapeutic for

RTT has been shown to restore spine motility back to WT levels, which correlate to behavioral and physiological improvements in the treated mice [10].

It has also been shown that these MeCP2 mutant cells exhibit decreased spine density and decreased dendritic arborization in their pyramidal cells as can be seen in [9, 10] These structural studies have been correlated to functional studies that show reduction in excitatory synaptic transmission and overall network excitability [36-38].

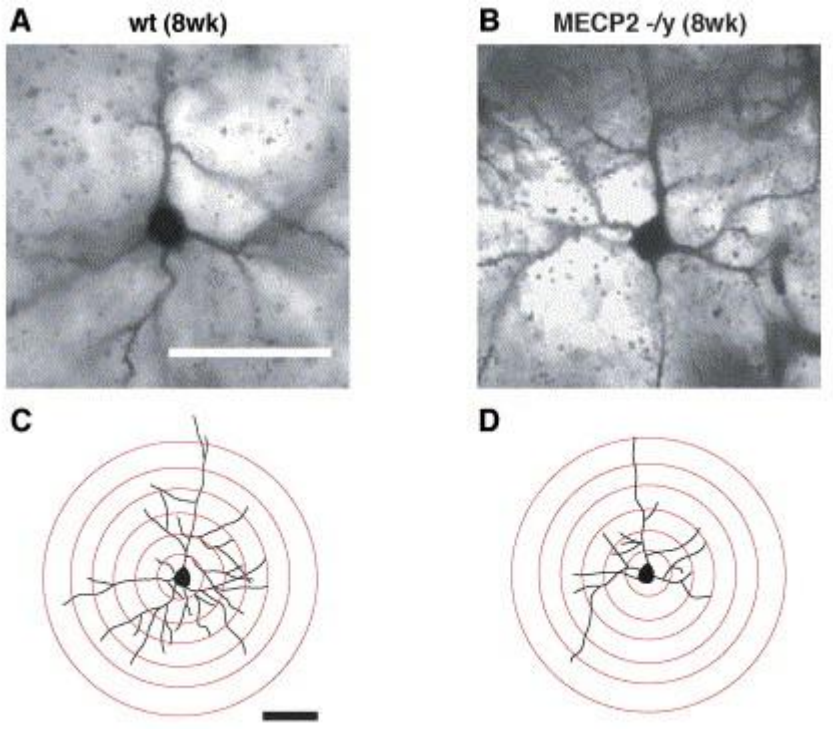



**Figure 6: Pyramidal neurons in MECP2 −/y mice are smaller and less complex than those in wild-type mice. (A and B) Examples of Golgi staining of pyramidal neurons in layer II/III in wild-type (A) and MECP2 −/y mice (B) at 8 weeks of age (C and D). Taken from Kishi & Macklis. [9]**

The precise mechanisms underlying the involvement of MeCP2 in regulating morphological and functional aspects of synaptic signaling remain to be identified. Such a detailed structural and functional analysis has not been conducted in cortical interneurons of MeCP2 mutant mice. The objective of this study will be to characterize the morphology of pyramidal and inhibitory neurons in the cortex of a mouse model for Rett Syndrome.

# Chapter 3: Hypothesis and Project Purpose

The disturbance in the excitatory/inhibitory balance could lead to a prolonged immature state of the nervous system, giving rise to the symptoms observed in RTT patients. This imbalance could be brought about by dendritic alterations on both the excitatory and inhibitory cortical neurons. Therefore, we hope to observe reduced arborization and decreased process length in the MeCP2 Knock-Out (KO) cortical neurons. Our studies will focus both on pyramidal cells and interneurons.

# Chapter 4: Materials and Methods

The methodology used to test the hypothesis included the creation of a transgenic mouse that expressed fluorescent markers in cells of interest, slice physiology experiments and reconstruction analysis using specialized software. A visual schematic of the experimental procedures can be seen in Figure 7. The following chapter explains each step of the procedure, as well as the materials used, in more detail.

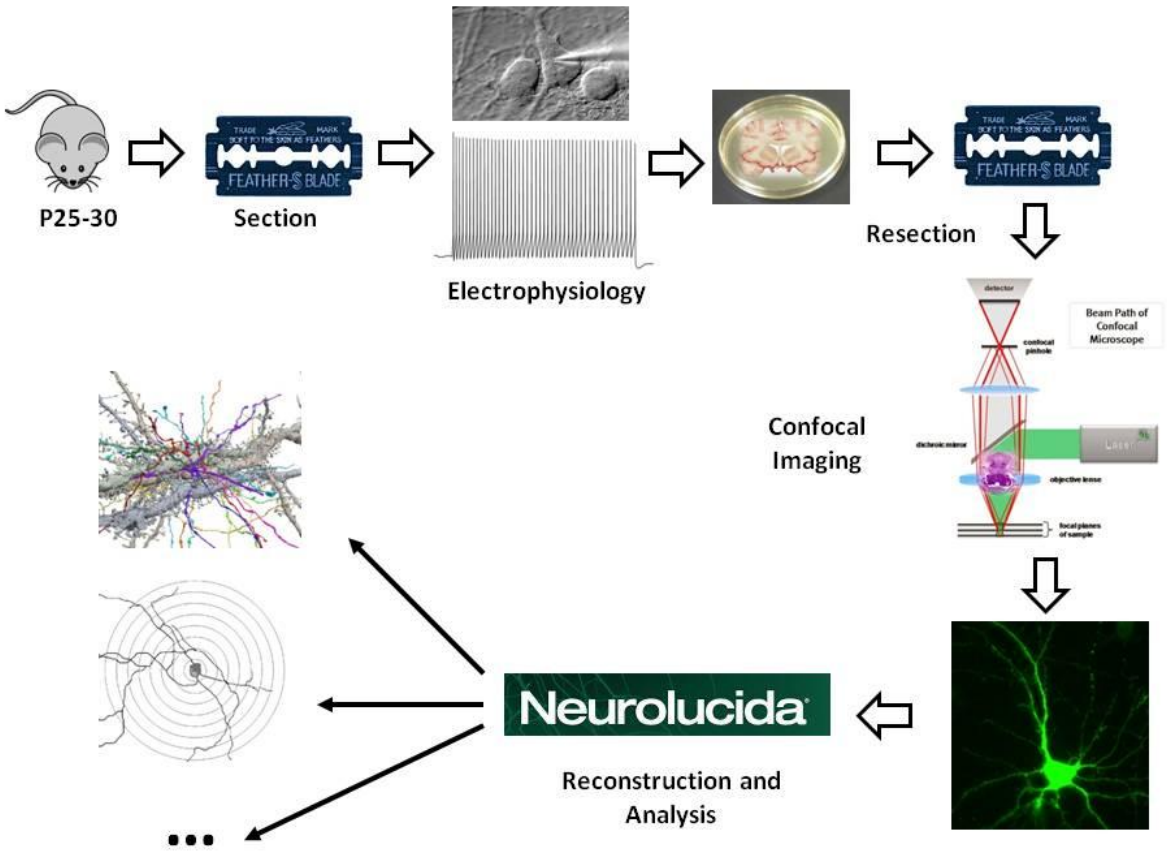
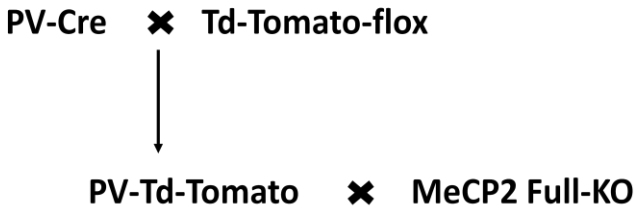



**Figure 7: Illustration of Experimental Procedure**

**Mice:** P28-30 Pv.Td Tomato-MeCP2 hemizygous KO and WT littermates were obtained from breeding heterozygous females [22] with male mice, both on a C57BL/6 background. The breeding scheme to obtain the desired mice is shown below. Mice that are were PV-Cre were crossed with mice which had a floxed Td.Tomato gene to create a PV-Td.Tomato reporter line. These mice express Td.Tomato, a form of Red Flourescent Protein, in their Parvalbumin expressing cells. Male Pv-TdTomato mice were crossed

with heterozygous MeCP2 KO females. To create both MeCP2-KO mice that expressed Td.Tomato in their PV cells and WT littermates that also expressed Td.Tomato in their PV cells.




**Solutions:** Slices were conserved in standard carbogenated (95% O2 and 5% CO2) artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3.5 KCl, 2.5 CaCl2, 1.5 MgSO4, 24 NaHCO3, 1.25 NaH2PO4, and 10 glucose. The recording pipettes was filled with a 5mM or 500µM solution of unconjugated AlexaFlour488 biocytin at 50µM concentration (Invitrogen, Life Technologies) and intracellular solution. The intracellular solution contained (in mM): 100 CsOH, 0.6 EGTA, 5 MgCl2, 8 NaCl, 2 Na-ATP, 0.3 Na-GTP, 40 HEPES, 0.1 Spermine and 1 QX-314.

**Whole-Cell Filling and Recording:** Coronal brain (300 µm) sliced using a Leica VT1200 (Leica Microsystems) were collected from WT and MeCP2 KO mice. Slices were submerged in ACSF at room temperature for at least 1 hr. Experiments were performed in a recording chamber on the stage of an microscope equipped with differential interference contrast (DIC) optics for visualization. Cell response and was recorded from layer IV-V neurons of V1 with an Axon 200B amplifier (Molecular Devices Inc., Sunnyvale, CA, USA) using patch pipettes (3-5MΩ) made from thin-wall (1.5mm outer diameter, 0.86mm inner diameter) glass using a vertical electrode puller. Neurons were voltage-clamped at -60 mV. The recording duration time was 10-20min. Tissue slices containing biocytin-filled cells where fixed by immersion in 4% paraformaldehyde in 0.01mM phosphate buffered saline (PBS) overnight at 4°C.

**Resectioning:** The fixed coronal slices were resectioned by transferring them to a 4% weight-volume low-melting point agar (Sigma-Aldrich) PBS solution. The agar-PBS solution was heated and mixed until uniform. The fixed slice were placed horizontally in the liquid agar-PBS solution and left to solidify for 5-10 minutes. The resulting block is sectioned using a Leica VT1200 (Leica Microsystems) into 80µm slices. The sections were mounted on VWR Superfrost Microslides (VWR Lab, Batavia, IL) using a solution of 0.003 mM phosphate buffer (PB).

**Tyramide Signal Amplification Protocol:** A tyramide signal amplification kit was bought from Invitrogen. The biocytin filled cells on the mounted sections were permeableized with 0.2% Triton-X. The sections were rinsed in PBS and incubated with a 1% blockin solution (Invitrogen) for one hour. The slices underwent 3 PBS washes of 5 minutes each. The slices were incubated labeled with conjugated streptavidin-HRP in a 1:100 solution with the 1% blocking agent for one hour. The sections were again washed with PBS. The sections were incubated in an amplification buffer containing 0.0015% H2O2 and AlexaFLour488-labeled tyramide solution for 10 min at room temperature. The sections were coverslipped with VECTASHIELD Hard-Set Mounting Medium with DAPI (Vector Laboratories) and left to dry overnight.

**Confocal Microscopy and Reconstruction:** Sections were imaged under a LSM 5 Pascal Zeiss Confocal Microscope. Images for reconstruction were obtained using a 63x oil-immersion objective. Reconstruction was done using Neurolucida (MBF Bioscience) and the morphological analysis was done using Neuroexplorer (MBF Bioscience).

# Chapter 5: Projects Strategy

## 5.1 Stakeholders

Before the specifics of the design process are further discussed, it is important to properly identify the stakeholders in this project. The stakeholders were defined as: clients, designers and users. They are show in Figure 8 below:

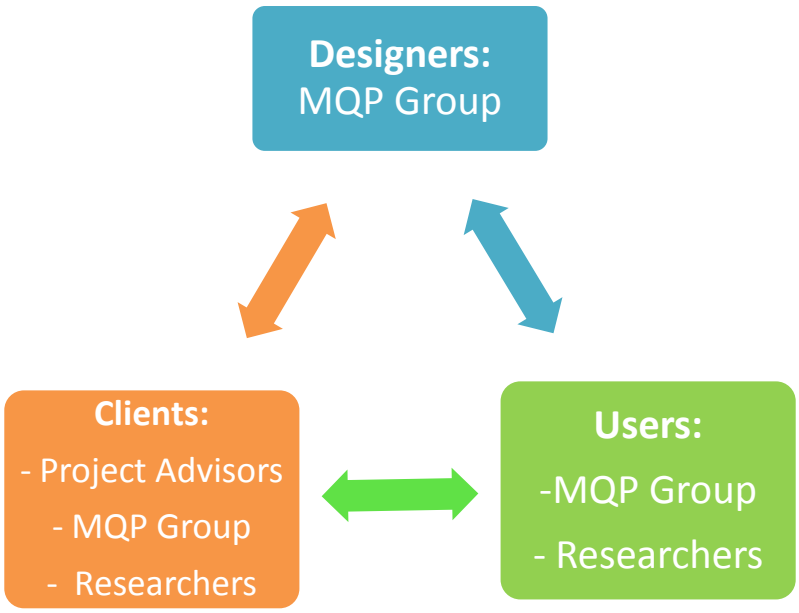



Figure 8: Project Stakeholder Diagram

The main stakeholder in this project is the MQP group. The group will not only design the bioreactor, but also use it in our electrophysiology experiments. Therefore, we are also in charge of determining its objectives and functions.

The clients were considered to be the evaluators of the final design. Therefore, this group includes the users, the MQP group and other researchers in need of a brain slice incubation chamber, who will be determining the efficiency of the design. The Project Advisors were included in the client group as they will be assessing how effectively the design process was carried out and how successfully the objectives and constraints were met.

## 5.2 Initial Client Statement

The clients of this design project are researchers using electrophysiology or a tissue incubation apparatus for their research, as well as the project supervisors. The initial client statement was the result of conversations with other users of slice incubation chambers. The users agreed that the current device was not optimum and that slice viability is indeed hindered, affecting the result of their experiments. The initial, unrefined client statement can be described as follows:

*"Design a bioreactor to: 1) conserve brain slices and optimize their viability for use in electrophysiology experiments, 2) contain the appropriate incubation media and withstand physiological conditions and 3) is inexpensive and/or easy to make"*

This client statement was further refined as the design process progressed. A refined client statement will be presented later on in the chapter

## 5.3 Objectives

In order to develop a more refined client statement, a complete list of objectives was developed. The primary list was pruned into a definite list of objectives. The definite list was divided into primary and secondary objectives using an objectives tree, which can be seen in Figure 9. The primary and secondary objectives were compared using pairwise comparison charts (located in Appendix A: Pairwise Comparison Charts) in order to quantitatively weigh their importance as design considerations. The calculated weight of the primary and secondary objectives can be seen as bolded numbers in Figure 9. The weight of the secondary objectives was calculated based on the weight of the primary objective. The remainder of this section will focus on analyzing and detailing the objectives.

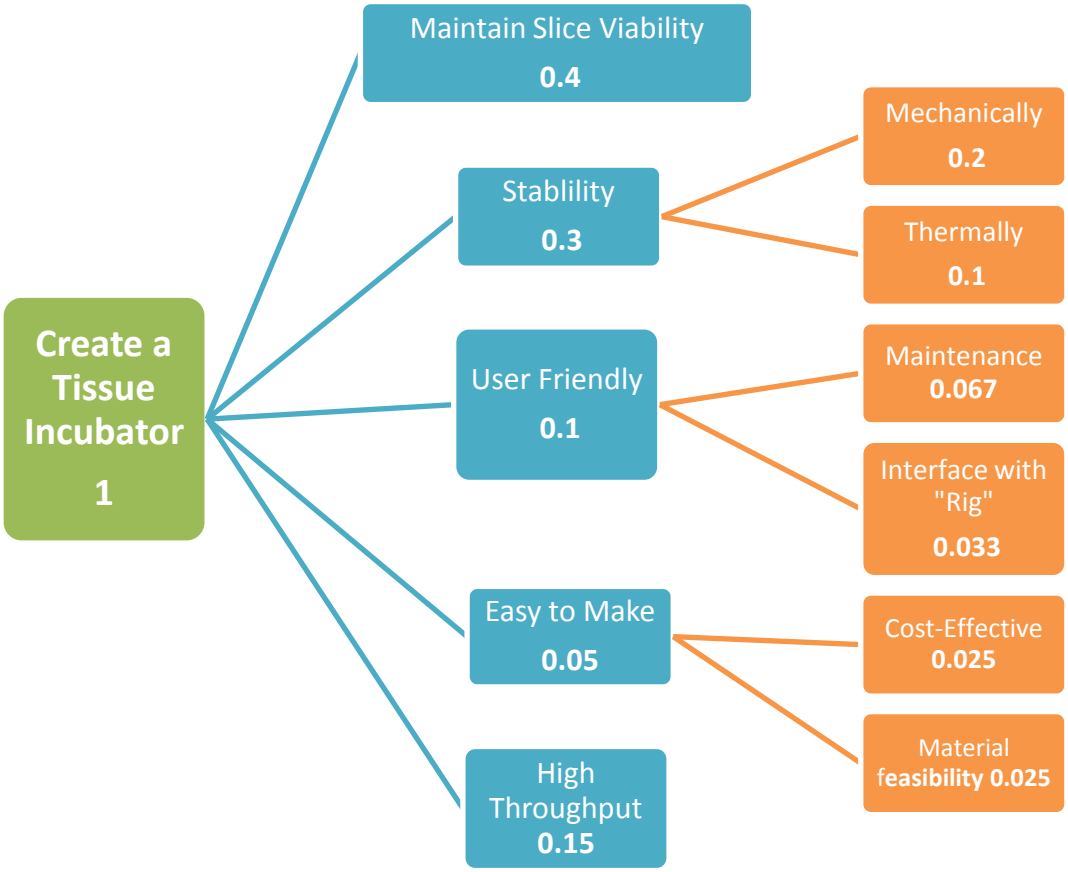



Figure 9: Objectives Tree for Tissue Incubator

### 5.3.1 Maintain Slice Viability

The most important objective, as measured by the Pairwise Comparison Chart in Table 3 in Appendix A: Pairwise Comparison Charts, is that the device be able to maintain the viability of the brain slices. It is the main function this device must be able to carry out. If this objective is not met and the bioreactor actually hinders the brain slice viability, the device will have failed in its purpose and cannot be used. There are many specific functions and specifications which must be met in order for the incubator to maintain slice viability.

One of these functions is that the device must be able to hold oxygenated aCSF at a pH of 7.2-7.5. The aCSF is oxygenated during incubation by the diffusion of carbogen, a gas that

contains 95% $O_2$ and 5% $CO_2$ [16]. Direct contact between the gas bubbles and the tissue is harmful to the tissue's viability and must be avoided [39]. To protect the slices from the bubbling, they are placed on a mesh which disrupts the bubbles before they reach the samples. Another important function is to protect the slices from outside contamination. If the designed device allows uncontrolled exchange with the external environment it could allow the entry of contaminating agents, compromising the slice's viability. These were not separated and evaluated as secondary objectives as they were all initially considered to be equally important.

### 5.3.2 Stability

The purpose of this bioreactor is to incubate the acute brain slices until they reach steady-state conditions [16]. Therefore, this device must be able to maintain a desired condition for an extended period of time. In order to achieve a steady-state condition, the bioreactor must be stable both mechanically and thermally.

Mechanically, the incubator must be sturdy and not agitate or deform the slices. Thermally, the device should be able to keep the slices at a constant temperature and be able withstand temperature around physiological conditions (21-37°C)[40]. If the device fails in any of these cases, the slices will not reach a steady state and their viability will be hindered. Mechanical stability was considered to be more important than thermal stability because more slice deterioration occurs from lack of mechanical stability.

### 5.3.3 User Friendly

As a general objective "user friendly" entails that the device must be simple enough for any researcher to use in a typical experimental setting. In the case of the slice incubator, ease of use entails a variety of secondary objectives including: maintenance and interface with an electrophyisiology recording rig.

The device must be maintainable as it is designed for multiple uses. If the incubator needs to be replaced for every experiment, then it has failed its purpose. Maintainability also entails ease of cleaning and reparability of the device. If the device is difficult to clean, it compromises the integrity of both the slice and incubator and would be considered a failure.

Because the purpose of the device is to conserve slices to be used in electrophysiology, it was established that the new apparatus should be interfaceable with a recording "rig". This would make the experimental preparations more straightforward and diminish the handling of the slices. Maintenance was considered more important than interfaceability because if the final design is difficult to maintain, regardless of it being interfaceable or not, it will be considered a failure.

### 5.3.4 Easy To Make

This objective is very straightforward: if the device is not easily manufacturable, it will be considered a failure. Manufacturability can be subdivided into two main secondary objectives: cost-effectiveness and material feasibility.

The final product should be cost effective in the sense that it should be as inexpensive as possible to make, considering its desired functions. Material feasibility refers to the availability of materials that are qualified to manufacture the device. If the desired functions like little cell interaction, little thermal and electrical conductivity, transparency, specific weight, etc. can only be achieved with a rare or unprocessable material, the desired functions will need to be revisited. Cost-effectiveness and material feasibility were considered to be equally important.

### 5.3.5 High Throughput

For the sake of this project, throughput is defined the ability to handle a large amount of samples at the same time. Many times these experiments require handling slices from different brain samples. It is important that this device be able to provide an appropriate environment to support them. If this device is only able to support slices coming from one brain, then it will be considered a failure. Because this device will handle different samples, it is important that there be separate slice incubation chambers.

## 5.4 Constraints

In order to reduce the design space and produce a design that meets the client's needs, constraints were established. The major constraints are represented in Figure 10. The most important constraints and their implications are further discussed in this chapter. The

constraints were used to evaluate the design alternatives. If a design did not meet an established constraint, it was rejected.

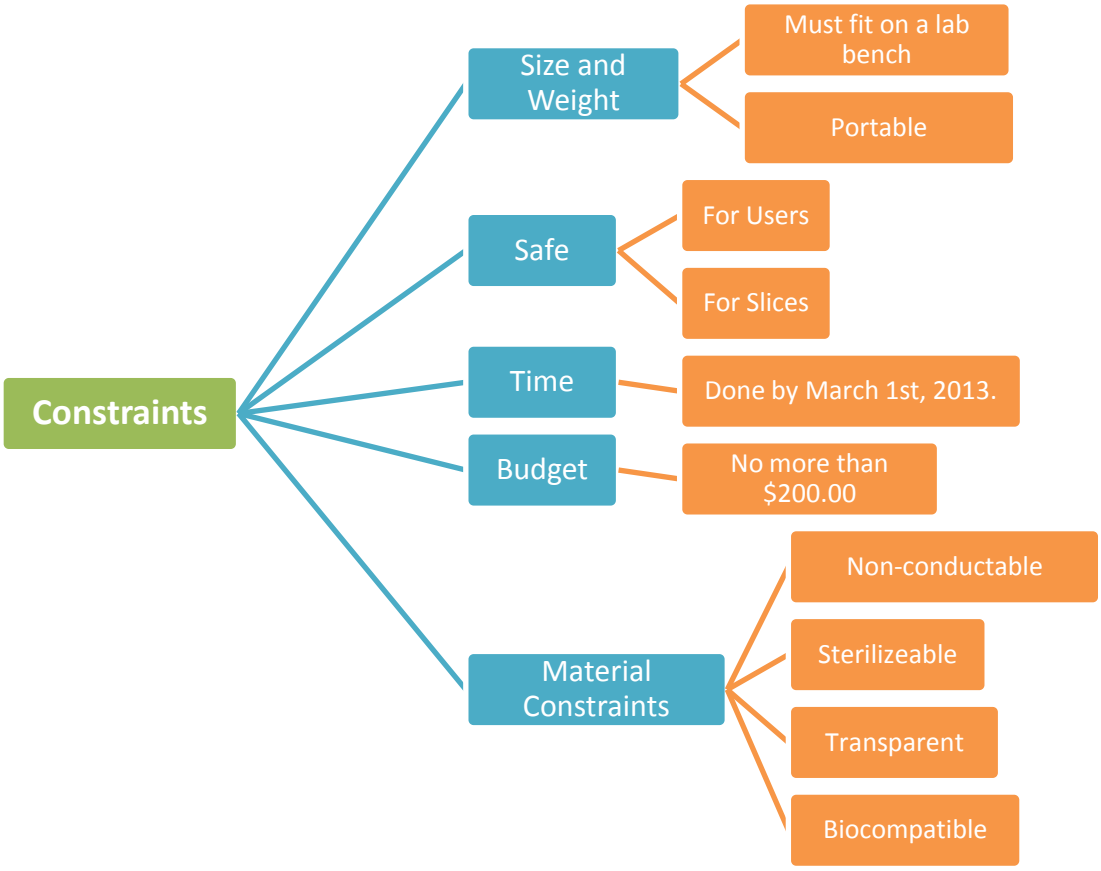



Figure 10: Schematic of main constraints and their implications

### 5.4.1 Size and Weight

The final incubator must be small enough to fit on a crowded lab bench. For successful slice incubation, the slices must be transferred directly from vibratome to the bioreactor. Vibratomes are bulky and occupy an important area of the lab bench. Therefore the final design must be small enough to be able to fit together with the vibratome on a lab bench.

The final incubator also must be portable. If the final design is attachable to the electrophysiology rig, it would be inconvenient to require more than one person to move the device to its required location. This constraint will also be important when choosing a material for the chamber. Size and weight specifications will be further discussed in Chapter 6.

### 5.4.2 Safe

The final design must be safe for both the user and the slices. In regards to user safety, it is important that the incubator does not put the researcher at risk by exposure to biohazardous substances that could be used during specific incubation protocols. It is also important that outer surface of the final design not have sharp edges to avoid glove and skin ruptures that compromise the safety of the researcher. In regards to slice safety, the final design must not compromise the viability of the slice throughout incubation. It must also protect the slice from possible external contaminants.

### 5.4.3 Time

The deadline for this project is currently at the end of C-term, meaning March 1st, 2013.

### 5.4.4 Budget

The final prototype should require no more than $200 to design, test and complete.

### 5.4.5 Material Constraints

It is extremely important to choose the appropriate material out of which to make the incubation chamber. In order to consider a material appropriate it must have the following characteristics: non-conductible, sterilizeable, transparent and biocompatible.

If the material were to conduct heat and electricity from external sources, it would not be able to isolate the slices or keep them at a steady state condition. If the material is not sterilizeable, there are higher chances for contamination of the slices. Transparency of the incubation chamber would allow the researcher to easily locate and monitor the slices. Biocompatibility of the material is essential; the material must not create a cell-toxic environment.

## 5.5 Revised Client Statement

After a detailed analysis of the objectives and constraints and a review of the current technology, the initial project statement was revised and expanded. Aspects that were added to the client statement are underlined in the revised client statement

The revised client statement is as follows:

*"Design a bioreactor which:*

*1) Conserves brain slices and optimize their viability for use in in vitro electrophysiology experiments by providing a sterile and biocompatible environment, protecting the slices from direct contact with gas bubbles, external contaminants and mechanical instability.*

*2) Can contain artificial cerebrospinal fluid (aCSF) oxygenated through carbogen (95% $O_2$ and 5% $CO_2$) bubbling.*

*3) Withstand physiological conditions (pH of 7.2-7.5 and a temperature of 21-37°C.*

*4) Fits on a crowded lab bench and can be handled/moved/carried by one person.*

*3) Meets the specified time (done by March 1sy, 2013) and budget ($200.00 for design and manufacturing) constraints.*

*4) Is made of a non-conductible, sterilizeable, transparent and biocompatible material.*

*5) Is safe for human use in a research setting."*

## 5.6 Project Approach

Once the objectives and constraints were identified, the project approach was developed. But before specific aims could be identified, it is important to understand the slice incubation process. The process was divided into three separate stages, which can be seen in **Error! Reference source not found.**. The device is first filled with aCSF that is oxygenated through bubbling mechanism in the apparatus (1 in Figure 11). A freshly sectioned slice inserted into the device (2 in Figure 11); and after the desired incubation period it is extracted from the bioreactor (3 in Figure 11). These three stages will be discussed in more detail in the project approach chapter.

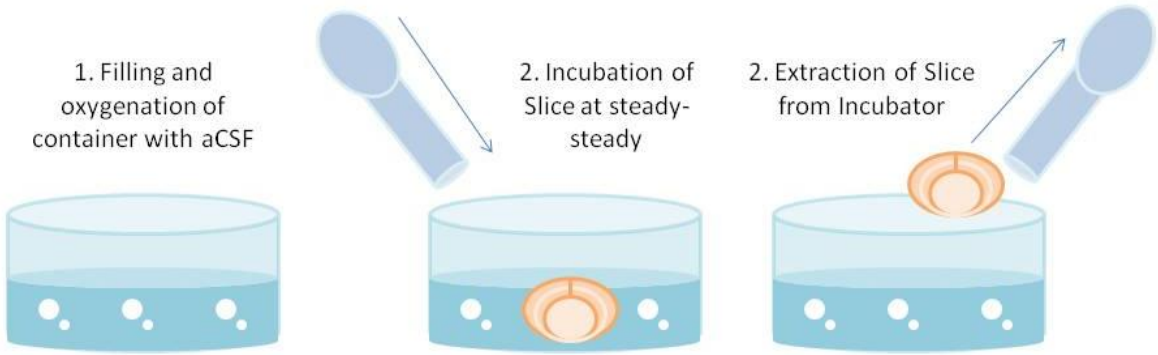



Figure 11: Process of Slice Incubation in Oxygenated artificial CerebroSpinal Fluid (aCSF)

Because of the variety of required functions throughout the process and the anticipated challenges, the project approach was divided into three *Steps.* In order for a design to be a considered a viable solution it will have to successfully pass each step successfully.

***Step 1: Demonstrate that the designed device does not interact with the incubation media and minimizes tissue interaction with external environment.***

In order to pass this "Step", the presented material of which the incubator is made of must not interact with the internal incubating solution. In regards to the internal solution, the composition of aCSF varies depending on the parameters being studied. But chemically aCSF can be classified as a standard $HCO_3$ buffer with the main components being Calcium, Magnesium, Chloride and Sodium. Some formulations use glucose, dextrose or sucrose [40-42]. Some work is also conducted with non-$HCO_3$ buffers, including HEPES and phosphates [40, 43, 44]. All of these materials need to be at physiological pH (7.1-7.5). The most successful incubations occur when concentrations and pH remain unaltered throughout the three steps mentioned above. In order to demonstrate that the designed device does not affect any of these characteristics, the appropriate material must be chosen to make the incubation chambers. This material must be shown to be resistant to the designated pH and not interact with any of the components of aCSF, Hepes and other typical slice incubating solutions.

The motivation behind the isolation of the contents of the incubation chamber is that external conditions are usually beyond a researcher's control and can be very variable. Factors like atmospheric humidity and variable temperature gradients have been shown to hamper brain slice incubation[40]. Therefore the chosen material must have poor conductive properties and the design must physically isolate the slices and incubating solution during stage 2 of the slice incubation process outlined in Figure 11. The design must also allow for minimal interaction with the external environment at stages 1 and 3.

***Step 2: Demonstrate that the designed device provides a stable and sterile environment for brain slices to achieve a steady-state.***

It has been shown that uncontrolled mechanical variations can distress live cells, causing unwanted cell death. Providing a stable environment for the slices during stage 2 of the slice incubation process is essential to maximize the number of live cells that can be used for recording. This objective needs to be met by designing a device that maintains the slices in equilibrium and isolates the internal environment from external vibrations.

In order to provide a sterile environment, the design must be easy to clean and made of material demonstrated to be sterilizeable. The method of sterilization can be varied: through ethanol, autoclaving, etc.

***Step 3: Demonstrate the ability of the design to maintain slice viability by comparing incubated slices with acute non-incubated slices and values found in the literature.***

` The final step for the validation of a design alternative will be to test the viability slice of the incubated slices. The standard method of slice viability testing is to compare a set of parameters in a non-incubated and an incubated slice. Values can also be compared to established *in vivo* values. Slice viability has been characterized through metabolic activity, morphological and electrophysiological characterization [40, 45]. Due to the time and monetary constraints of this project, we can only compare morphological and electrophysiological characteristics. Cell morphology and overall slice appearance of incubated slices can be compared to non-incubated slices through microscopy and electrophyisiological characteristics can be compared to values found in the literature using whole-cell patch-clamping. The reason electrophysiological characteristics cannot be compared to non-incubated slices is because they are not in a steady-state, causing alterations in cell firing, membrane potential and resistivity compared to *in vivo* conditions.

# Chapter 6: Alternative Designs

## 6.1 Needs Analysis

Neurological disorders affect at least 1 billion of the world's population [46]. In many neurological disorders, including Rett Syndrome, the morphology and connectivity of the cells have been to be severely altered. Electrophysiology is the gold standard technique for studying neuron morphology and connectivity. As it was mentioned before, the success of these recordings depend on the viability of the brain sample that is used. Mammalian models (mice and rats usually) are preferred for the study many of these disorders and possible treatments because of the homology with the human central nervous system. The development and maintenance of these animal models can be a very expensive process. Therefore, being able to appropriately conserve these slices to ensure their viability for electrophysiological experiments is crucial. By designing a better slice incubator, the success rate of these experiments can be improved.

## 6.2 Functions, Means and Specifications

In order to aid in the brainstorming process, a functions-means chart was created. The chart can be seen in Table 1. These means not only present a solution to fulfill the necessary functions but are also in line with the objectives and constraints presented in the Chapter 5. The functions-means chart establishes more than one possible mean to accomplish each function. The functions-means chart was used as a tool to develop four design alternatives that will be later discussed in this chapter. This section discusses the functions and the means considered to fulfill each function.

Table 1: Functions-Means Chart for a Slice Incubator

| Functions | Means | | | | |
|---|---|---|---|---|---|
| **Carbogen Exchange** | Attached Exchanger | Separate Exchanger | Separate container for gas exchange | | |
| **Contain Brain Slices** | Included slice holders | Separate slice holders | Slice holding area | | |
| **Contain Media** | Material Properties | Material Coatings | | | |
| **Provide Mechanical Stability** | Mechanical isolation chamber | Material Properties (density) | Design Consideration | Adhesive Bottom | |
| **Provide Thermal Stability** | Thermal Isolation Chamber | Material Properties | | | |
| **Protect Tissue from the Environment** | Isolation Chamber | Attachable Lid | Separate Lid | | |
| **Easy Slice Access** | Attachable Lid | Separate Lid | Window | No Lid | Material Transparency |

### 6.2.1. Carbogen Exchange:

In order to provide the appropriate conditions for cell maintenance, the incubation media (artificial cerebrospinal fluid or aCSF) must be oxygenized with carbogen. Carbogen is a mixture of oxygen and carbon dioxide (usually 95% $O_2$ and 5% $CO_2$) [16]. Carbogen is usually provided through internal piping systems. Three means have been identified to provide the appropriate carbogen exchange with the aCSF, through an attached exchanger, a separate exchanger within the incubator or through a separate gas exchange container.

#### *6.2.1.1 Attached Exchanger:*

Using an attached exchanger, the piping from the carbogen source can be attached directly into the incubator. The attached exchanger would provide a mesh to create small carbogen bubbles that would diffuse through the incubator.

#### *6.2.1.2 Separate Exchanger:*

With the separate exchanger, the piping coming from the carbogen source would need to end in a mesh material that would create bubbles when immersed in the aCSF.

#### *6.2.1.3 Separate Container for Gas Exchange*

Using a separate container for gas exchange would mean that there is no gas diffusion occurring directly in the slice incubator and that the aCSF in the slice incubator would be pre-oxygenated. The diffusion in the separate container could occur with either an attached or separate exchanger.

### 6.2.2 Contain Brain Slices

The whole purpose of designing this device is to be able to incubate brain slices. Therefore it is crucial that the incubator be able to contain them appropriately. To satisfy this function, three means have been identified. The device could have included slice holders, separate slice holders or a slice holding area that is not an individual container.

#### *6.2.2.1 Included Slice Holders*

The included slice holders would be included in the incubator and would not be able to be removed. This provides greater mechanical stability but could also hinder the ease of use and ability to clean the device. If carbogen exchange were to occur in the same container where the slices are incubated, the slice holder bottom would need to be made of a mesh composed of a biocompatible material. The mesh would allow for the carbogen bubbles to not burst on the tissue surface, causing traumatic cell death. These slice holders would be large enough to snuggly fit the length of one brain slice.

#### *6.2.2.2 Separate Slice Holders*

The design considerations of separate slice holders would be the same as the included ones. The only difference would be that these slices would not be attached to the media container of the slice incubator. This would make the device easier to clean but mechanically less stable.

#### *6.2.2.3 Slice Holding Area*

A slice holding area would be included in the incubating device. These areas would be larger than the length of one brain slice and would have divisions to be able to distinguish between different populations of brain slices. This area must also have a mesh bottom of a biocompatible material if the carbogen exchange occurs in the same container as the slice

incubator. This would allow for a higher throughput but would hinder the ease of use and limits its ability to be cleaned and sterilized.

### 6.2.3 Contain Incubation Media

In order to appropriately incubate the slices, the material must be able to contain the appropriate incubation media. The incubation media for brain slice preservation is artificial cerebrospinal fluid (aCSF). There are two ways to achieve this: either by controlling for material properties or by using the proper material coating. In case of material properties, the material used must be hydrophilic in order to properly contain the media and not react with the components of the aCSF. If a desired material would like to be used but does not have one of those both characteristics a material coating could be applied on the surface of the material. The final design should be able to hold 500 mls of aCSF.

### 6.2.4 Provide Mechanical Stability

In order for the slices to reach steady state and minimize cell death, mechanical stability must be maintained throughout the incubation period. A variety of means have been identified that would satisfy this function. These include a separate mechanical isolation chamber, controlling the mechanical and design properties and an adhesive bottom.

#### *6.2.4.1Mechanical Isolation Chamber:*

This chamber could be made of isolation material and would allow the slices to not be affected by the movements of the external environment. The incubator would be placed inside of this device and taken out once the incubation is done.

#### *6.2.4.2 Material Properties:*

By controlling the density and weight of the material one could make a device that would be stable mechanically. The device is specified to be at least 1kg of weight without the aCSF.

#### *6.2.4.3 Design Considerations:*

By taking into account design considerations like a flat bottom, the design would be stable on top of a lab bench.

#### *6.2.4.4 Adhesive Bottom*

An adhesive bottom would allow for added stability during the incubation, it could also support the device through any sudden movements of the external environment.

### 6.2.5 Provide Thermal Stability

The whole purpose of designing this device is to be able to incubate brain slices at a steady state. Therefore it is crucial that there are no sudden or extreme temperature gradients. To satisfy this function, two means have been identified. Much like the mechanical stability function, this device could be contained in a thermal isolation chamber made out of thermally insulating material. Another way to control for this would be by making the incubator out of a thermally unconductive material.

### 6.2.6 Protect the Tissue from the Environment

In order to avoid contamination from the external environment, the device must be able to protect the slices from outside contaminants. The presence from external contaminants could promote cell death. Three means have been established to satisfy this function. A separate isolation chamber could be used during incubation. Another mean would be using an attachable or separate lid. An attachable lid would be included in the design while a separate lid could be something like aluminum foil.

### 6.2.7 Easy Slice Access

To be able to place the slices in the incubator and move the slices from the slice incubator to the electrophysiology rig stage, the slices must be easily accessible. There are a variety of means this could be satisfied. By using an attachable or separate lid or no lid it will allow for easy slice access when needed. Another option is not using a lid but an access window on the material. Another important mean is by using a transparent material that would allow for proper guidance when selecting the slices.

## 6.3 Design Alternatives

Based on the functions and specifications discussed above, four design alternatives were established. These designs are described in detail in this chapter.

### 6.3.1 The "Lobster Pot" Design:

The "Lobster Pot" Design consists of a cylindrical container with an attachable lid to protect the slices from the environment. The device consists of a flat bottom with adhesive material for added mechanical stability. The device would be made of a transparent material. There would attached slice holders and gas exchanger. Sketches of this design can be seen in Figure 12. The functions-means chart for this design can be seen in Table 7.

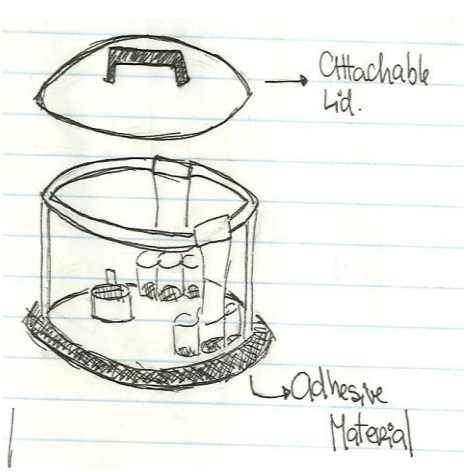
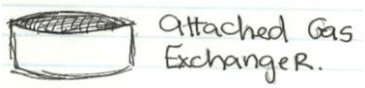




Figure 12: Lobster Pot Design



### 6.3.2 Attachable Slice Holders

These attachable slice holders could be placed on any container. Separate slice holders could be attached in a slice holder holder in order to increase their stability and high throughput of the device. Because this device does not have a media container in itself, it would need a separate gas exchanger. The individual slice holder would be made of a metallic mesh to allow for proper gas diffusion on to the slices. The slices would be protected from the external elements both by the container and a separate lid. Sketches of this design can be seen inFigure 12. The functions-means chart for this design can be seen in Table 8.


Figure 13: Attachable Slice Holder Design

### 6.3.3 Well Slice Holder Design

This design is the most elaborate of all the design alternatives. This device includes a separate gas exchange chamber. Once the aCSF is oxygenated, it flows to the well slice holder through a peristaltic pump or gravity. The slices are incubated in a well slice holder which is constantly infused with the oxygenated aCSF. The aCSF is taken out of the well chamber through another peristaltic pump that deposists the fluid back in the gas exchange container. Sketches of this design can be seen inFigure 12. The functions-means chart for this design can be seen in Table 8.

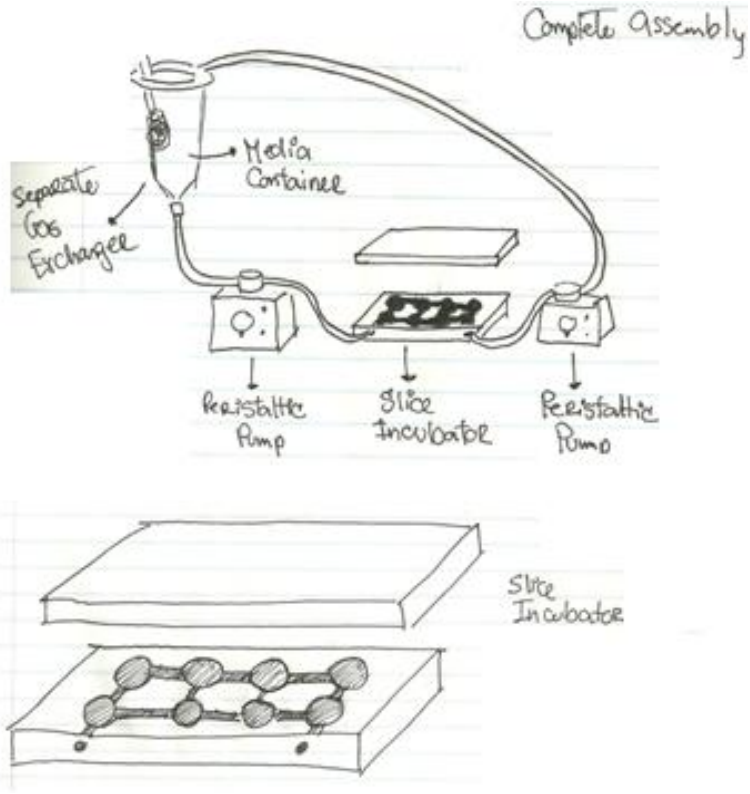



Figure 14: Well Slice Holder

### 6.3.4 "Three Layer" Incubator Design

This design consists of a three layers: a lid, a slice holding area and the media container. The lid would have a movable window to allow for easy slice access. The slice holding area would allow for a higher throughput of slices. This area would interlock with media container to allow for greater mechanical stability of the slices. The third layer would have attached gas exchanger. This was decided based on the tightness of the three layer system that would not allow for tubing to come out of the device. A sketch of the device can be seen in Figure 15 and the functions means chart of the device can be seen in Table 10.

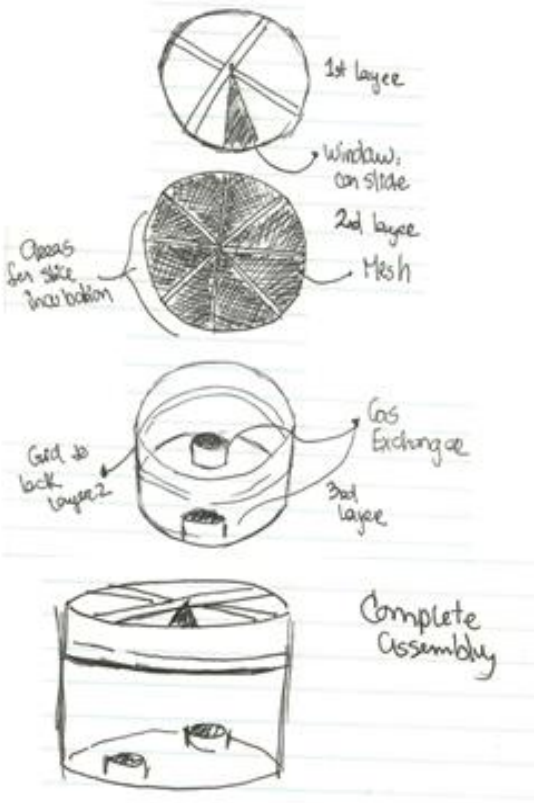



Figure 15: "Three Layer" Incubator

## 6.4 Conceptual Tentative Final Design:

### 6.4.1 Design Selection:

The four alternative designs were evaluated through a design evaluation matrix. The selections matrices for objectives, constraints and functions can be seen in Appendix C: Design Evaluation Matrices. Designs were evaluated on a scale from 0 – 2 depending on how well they met the designated objective, constraint or function. Designs were not disqualified based on not fully meeting a constraint; it was considered that if the design were good enough, those constraints could be worked out. A summary of the design evaluation matrices can be seen in Table 2. The point from the objectives, constraints and functions decision matrices were added up in order to choose a final design. The final conceptual design was the attachable slice holder design.

Table 2: Final Decision Matrix

| | "Lobster Pot" | Attachable Slice Holders | Well Slice Holder | "Three Layer" Incubator |
|---|---|---|---|---|
| Objectives | 6 | 8 | 3 | 8 |
| Constraints | 9 | 9 | 1 | 4 |
| Functions | 13 | 13 | 7 | 12 |
| **TOTAL** | **28** | **29** | **11** | **25** |

### 6.4.2 Attachable Slice Holder Design Details:

The attachable slice holder has many advantages. First of all it is very easy and cheap to manufacture. Second there is no need to design a separate container: a typical beaker or Pyrex ® container provides the material and mechanical properties necessary to contain the media at a stable temperature and not interact with its content. The following section describes in more detail the two main components of the design, the slice holder and the slice holder-holder.

#### *6.4.2.1: Slice Holder:*

As it was explained in the previous chapter, the slice holder would be made of a metallic mesh. It was decided so because of its greater stability and its availability on the market. Further analysis must be conducted on the types of meshes available on the market and their

biocompatibility.The mesh size would be between 0.10 and 0.70mm. The top of the slice holder would be open to allow for sample placement. The internal circumference of the slice holder would be from 3-5cm in diameter. The slice holder would also have an outreaching circumference on the top to allow for the individual slice holder to be mounted on the slice holder holder. This greater circumference would be from 5-7cm. It would not need to be made of mesh material. The most appropriate material could be a simple thermoset polymer, but further investigation must be conducted. For reference, a sketch of the individual slice holder can be seen in Figure 16.

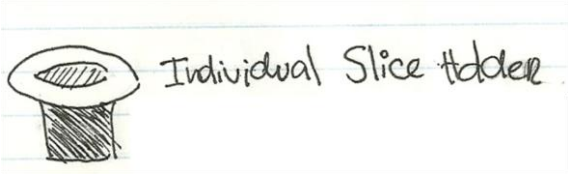



Figure 16: Individual Slice Holder

### *6.4.2.2: Slice Holder Holder:*

The purpose of this device is to be able to increase the stability and sample number that this design can handle. This design must be made of a strong and brittle yet lightweight, therefore we are considering thermoset polymers. This design would attach on to the media container. The current design would allow for three individual slice holders to be contained..For reference, a sketch of a slice holder-holder can be seen in Figure 16.


Figure 17: Slice Holder-Holder

## 6.3 Feasibility Study and Experimental Methodology

### 6.3.1 Feasibility Study:

In order to test the feasibility of manufacture of the design, first a extensive study must be done on what materials and similar devices are currently on the market. Because of the simplistic nature of this design, we do not anticipate a problem with the manufacturability of any of the design parts. If need be, the design should be adapted. In order to test the conceptual design, a prototype can be fashioned with the most readily available material.

Another important part of the feasibility study includes the material selection for the individual parts of the device. The details for the needed qualities of these materials can be seen in Chapter 5 and in earlier sections of this chapter.

### 6.3.2 Experimental Methodology:

In order to test the device, we will Step 3 described in the Project Approach section of Chapter 4. The final step for the validation of a design alternative will be to test the viability slice of the incubated slices. The standard method of slice viability testing is to compare a set of parameters in a non-incubated and an incubated slice. Values can also be compared to established *in vivo* values. Slice viability has been characterized through metabolic activity, morphological and electrophysiological characterization [40, 47]. Due to the time and monetary constraints of this project, we cannot test the efficacy of our chosen design. Studying the changes in cell morphology would be the easiest way of comparing the two designs. Cell morphology and overall slice appearance of incubated slices can be compared to directly harvested non-incubated and slices incubated in distilled water slices through microscopy.

## 6.4 Preliminary Data

As it was explained in the previous chapter, we would need to compare our incubated slices to acute non-incubated slices. In order to establish cell viability, we can look at cell morphology. When neurons undergo apoptosis, their volume increases and they acquire a more rounded shape [16]. The incubated slices should have the least amount of cell volume increase compared to the directly harvested non-incubated slices, which are the positive

control. For the negative control, the slices can be incubated in distilled water. Water lacks the necessary minerals and oxygen required for cell maintenance and therefore will be non-viable slices. Therefore, the cell bodies of the incubated slices must also have less volume than the water incubated slices.

# Chapter7: Results:

## 7.1 Electrophysiological Filling:

Pyramidal cells and interneuron in Primary Visual Cortex (V1) were selectively filled through whole cell patch clamping using biocytin and AlexaFlour 488. Two different mixtures of these component were used. The pyramidal cells were filled using a conjugated form the AlexaFlour 488 and the biocytin. The interneurons were filled with a solution that contained an unconjugated form of the AlexaFlour 488 and the biocytin. By filling the cell with AlexaFlour488, we were able to image the cells as we were recording them to ensure that soma and dendritic arbor were being filled with the biocytin. In Figure 18 we can observe representative images of how the filled cells appear after the recordings are finished.

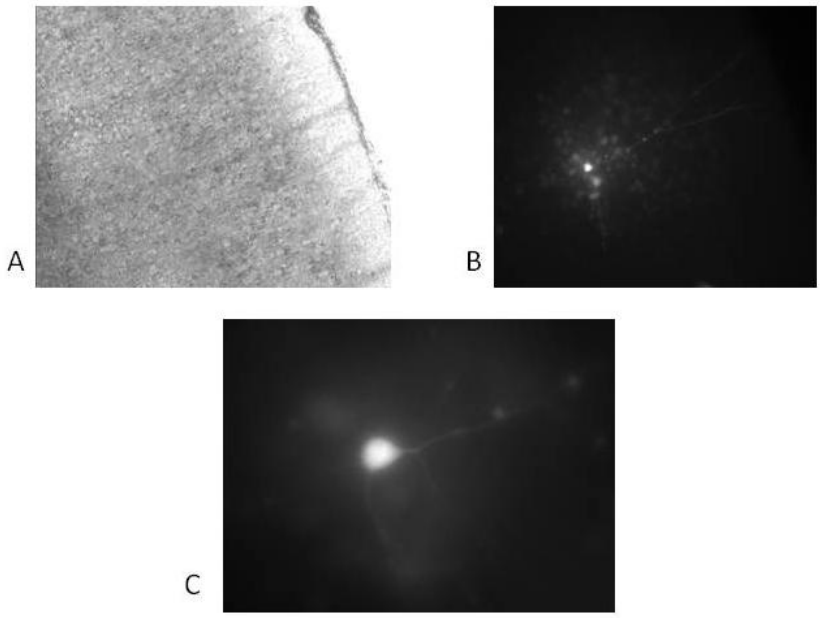



**Figure 18: View of the filled cell in the electrophysiology rig. A. Differential interference contrast (DIC) view of V1 in a brain section at a 10X magnification. B. 10X view of a pyramidal cell filled with the biocytin-AlexaFlour 488 conjugated solution spanning from layer II/III to the plial surface taken using a Green Fluorescent Protein (GFP) filter. C.40X magnification of the cell soma taken using a GFP filter**

For the interneuron experiments, we only used cells that selectively expressed Td.Tomato in their Parvalbumin cells. After locating V1, these cells were identified under a Red Flourescent Protein

(RFP) filter. As the mice were still relatively young (P20-30), Td. Tomato was not ubiquitously expressed in the cell but rather in puncta in the cell soma. This can be more clearly seen in Figure 19.

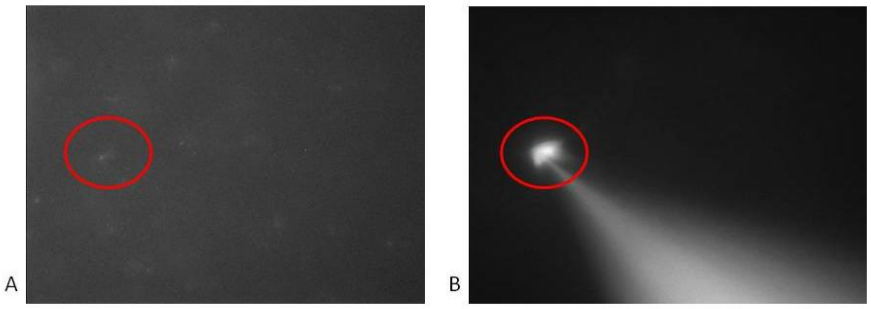



**Figure 19: Selective patching of a PV expressing cell. A. PV expression seen at 40X through a Red Fluorescent Protein (RFP) filter. B. 40X magnification of a cell soma that expresses PV that is being filled with an unconjugated solution of AlexaFlour488 and biocytin.**

## 7.2 Confocal Imaging:

Once the cells were filled and recorded, they were fixed overnight and resectioned using the protocol described in the Materials and Methods section. The pyramidal cells which were filled with the conjugated AlexaFlour488-biocytin mixture were directly placed onto slides and imaged under a confocal microscope. We found that this approach hindered the image quality, as the fluorescence of the cell was significantly reduced. The brain sections containing the interneurons filled with the unconjugated AlexaFlour488 and biocytin solution were subjected to a Tyramide Signal Amplification (TSA) protocol. Tyramide Signal Amplification (TSA™) is an enzyme-mediated detection method that utilizes the catalytic activity of horseradish peroxidase (HRP) to activate an AlexaFlour488 molecule which is bound to streptavidin through a redox reaction. The streptavidin covalently bonds to the biocytin inside the filled cells. This reaction allows for an increased fluorescent signal of the filled cell. All sections were mounted on slides, coverslipped and stained with DAPI.

Using a fluorescent confocal microscope, cells were identified and images were taken on different z-planes. The collections of images obtained at different z-planes are referred to as z-stacks. Cells were identified using 10x and 20x imaging and z-stacks for reconstruction were taken at 63x. Cells were identified using both GFP and DAPI fluorescence filters and images for reconstruction were only taken using the GFP fluorescence filter. An example of images in a z-stack can be seen in Figure 20. These images were used to reconstruct the cell using Neurolucida.

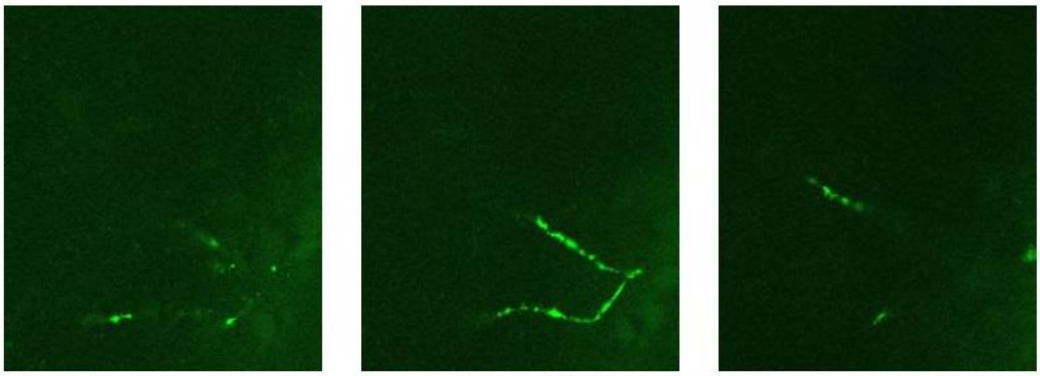


**Figure 20: Three images taken at 63X of the same x and y coordinates with varying z-plane. This cell was subjected to the TSA protocol.**

In some cells it was the case that their dendritic arbor and the cell some were on different brain sections. An example of this case can be seen in Figure 21 . In these cases z-stacks were taken of both sections and reconstructed together using Neurolucida.

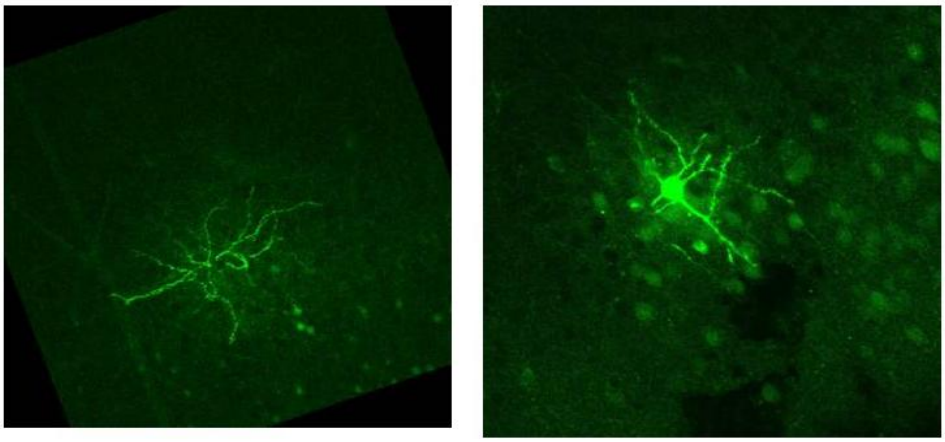


**Figure 21: Dendritic arbor and cell soma taken at 20X from different resectioned brain sections.**

## 7.3 Pyramidal Cells:

A total of six pyramidal neurons, three MeCP2 KOs and three WTs, were filled and reconstructed (Figure 22A and 22B respectively show representative reconstructions of these populations). Pyramidal cells were identified by the presence of an apical dendrite and spines. All four cells were also identified as Regular-Spiking (RS) cells [15]. Three types of analysis were done: branch analysis, Sholl analysis and Convex-Hull Analysis on the basal dendrites of the cells.

1. **Branch Analysis:** Number of branches per branch order, overall process length and average number of terminal ends were measured for each cell (Figure 22C,22D,22E respectively) There was no apparent difference between the MeCP2 KO and WT pyramidal cells.

2. **Scholl Analysis:** Scholl analysis is used to analyze the extent of dendritic branching by counting the number of intersects with concentric circles with increasing radius. While the MeCP2 neurons showed increased number of intersects at a radius larger than 110µm (Figure 22F), the number of samples (n=3) is insufficient to conduct any statistical analysis.

3. **Convex-Hull Analysis:** The Convex-Hull analysis is used to measure the area covered by the basal dendritic arbor. The basal dendritic arbor of the MeCP2 KOs seem to cover less area than the WT pyramidal cells (Figure 22G). These results will have to be further validated by increasing the sample size.

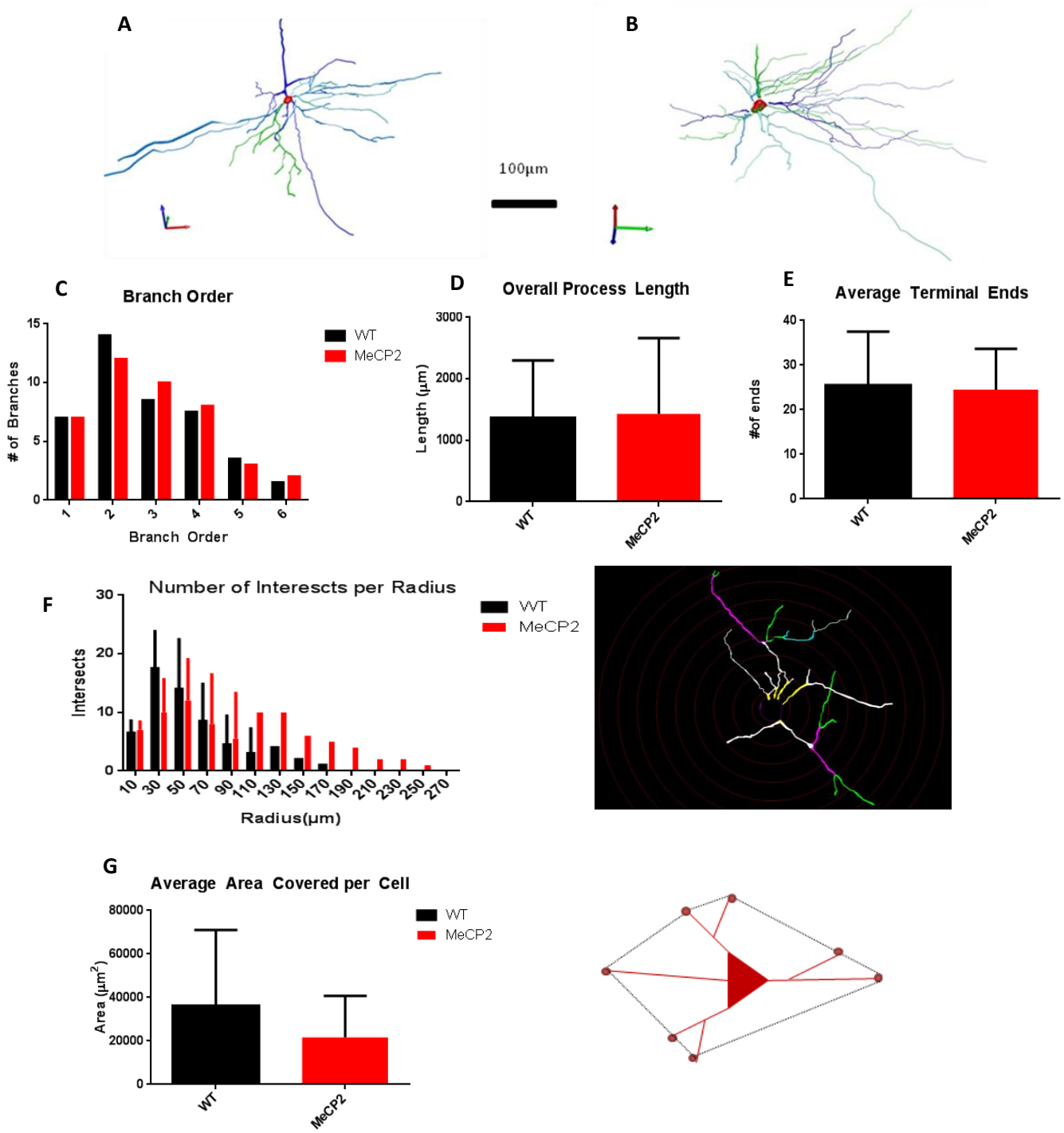



Figure 22: Analysis of pyramidal cells. Reconstruction of MeCP2 KO (n=3) (A) and WT (n=3) (B) cells. Branching analysis including branch order (C), overall process length (D), average number of temrinal ends (E). Number of intersections per Scholl radius (F) with a a representative image. Convex-Hull Analysis, emasuring average area covered per cell (G), with an illustration of how the area was measured.

## 7.4 Interneurons:

A total of three interneurons were filled, one WT neuron and two MeCP2 KO (Figure 22A and 23C respectively). It was realized that these two cells present very different morphology: the cell reconstructed in Figure 23A is clearly a double-bouquet or bitufted cell whereas the cell reconstructed in Figure 23C is clearly a basket cell. It is impossible to compare the morphology between these two cells. Therefore for our analysis it was necessary to use morphological data collected from another member in the lab[48] which can be seen in Figure 23B. Runyan et al. studied the morphology of WT PV-interneurons in V1 using 2-photon microscopy. The same three types of analysis were conducted: branch analysis, Sholl analysis and Convex-Hull Analysis on the dendrites of the cells. While the information of the bitufted cell reconstructed in Figure 23A were included for reference in the figures, they will not form part of our analysis. In Figure 23, the collected from the data from bitufted cell is labeled in black, the data of the WT basket cells from Runyan et al. can be seen in green and the MeCP2 KO basket cells can be seen in red.

1. **Branch Analysis:** Number of branches per branch order, overall process length and average number of terminal ends were measured for each cell (Figure 23D ,23E, 23F respectively) There was an important difference observed between the WT basket cells reconstructed by Runyan et al. and the MeCP2 KO cells. The MeCP2 KO basket cells showed diminished overall process length and number of terminal ends, as can be seen in Figure 23E and 23F. There was not a significant variation within the populations. One can start to infer that these cells seem to be much smaller and possibly cover less area.

2. **Scholl Analysis:** As it can be expected because of its elongated morphology, the WT bitufted cell intersects at circles with a much higher radii than the basquet cells. The WT basket cells of Runyan et al. seem to have both higher number of crosses per radii and longer span than the MeCP2 KO basket cell as it can be seen in Figure 23G. These results align with what was observed in the branching analysis and further suggests that there is a morphological difference between these two populations.

3. **Convex-Hull Analysis:** There was no data for the WT basket cells of Runyan et al. available for the Convex-Hull Analysis. Regardless, the analysis was done with the WT bitufted cell and the MeCP2 KO basket cells. As can be seen in Figure 23H, the dendritic tree of the WT bitufted cell covers more area than the MeCP2 KO basket cell. This could be explained by the innate difference of the morphological characteristics of these cell types or an effect of the

genotype on the average area covered per cell. In order to confirm either of these hypothesis, we must obtain the data both from WT basket cells and MeCP2 KO bitufted cells.

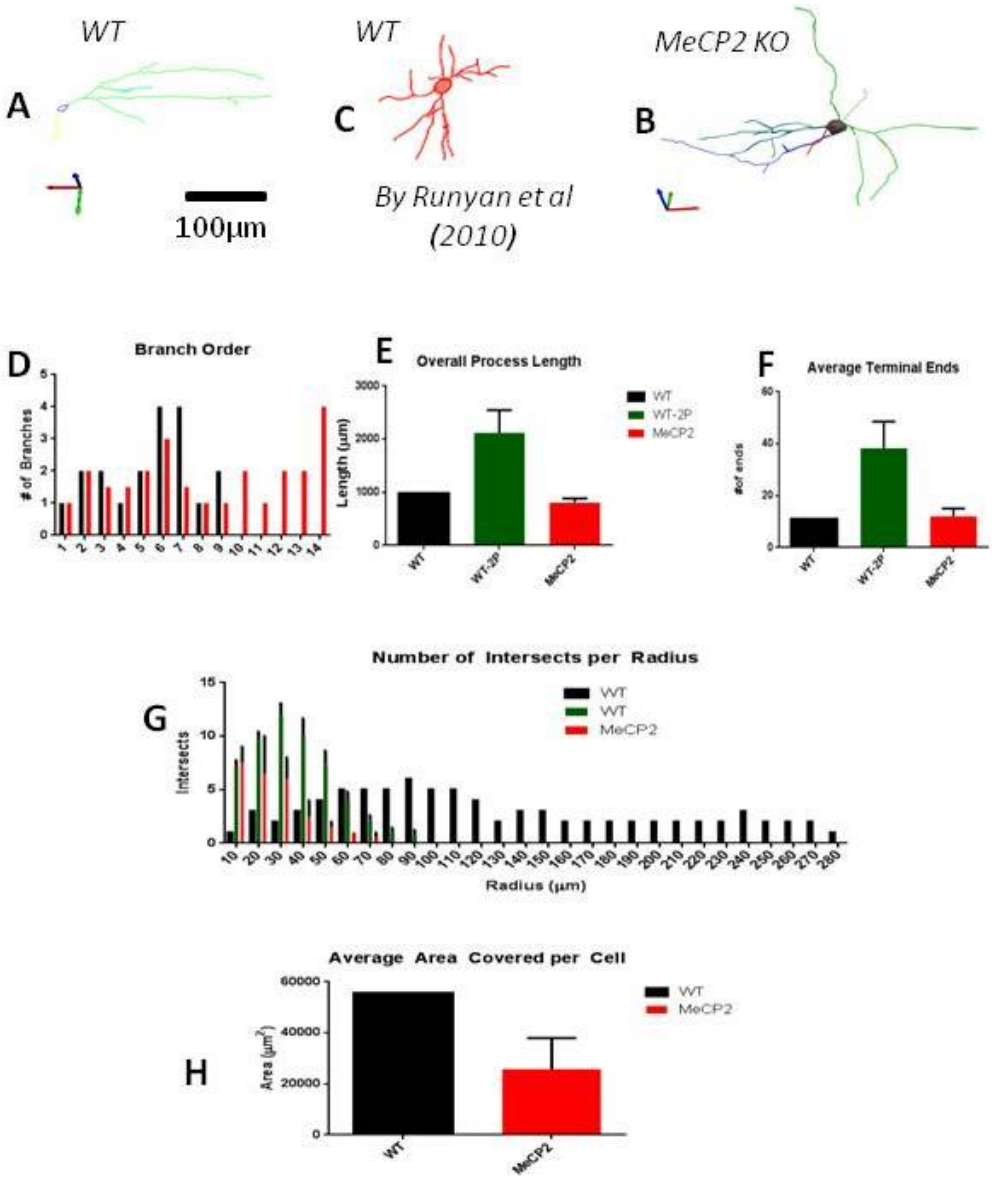



**Figure 23: Analysis of interneurons. Reconstruction of WT bitufted cell (n=1) (A) , WT PV-expressing basket cells from Runyan et al. (n=13) (B) and MeCP2 KO PV-expressing basket cells (C). Branching analysis including branch order (D), overall process length (E), average number of temrinal ends (F). Number of intersections per Scholl radius (G). Convex-Hull Analysis, emasuring average area covered per cell (H).**

# Chapter 8: Conclusions and Future Work

The objective of this study was to do a morphological characterization of cortical neurons in a mouse model for Rett Syndrome. We have studied both pyramidal and interneuron morphology in Primary Visual Cortex (V1). It is important to note that this work is currently ongoing and these results are preliminary.

These initial results hint at possible differences between WT and MeCP2 pyramidal cells in the area covered by the basal dendritic arborization although its overall reach, measured by Sholl analysis and the branching analysis is somewhat similar. In the case of the MeCP2 KO interneurons exhibit decreased area coverage and overall reach per cell as it can be seen in the branching analysis. Sholl analysis demonstrates larger process reach for WT basket cells. Convex Hull analysis demonstrates decrease in the average area covered per cell in the case of the pyramidal cells and possibly the basket cells as well. Both of these results have to be further validated by increasing the sample number of cells reconstructed.

The area coverage and reach decrease for both cell types could lead to a diminished integrative capacity in the MeCP2 knock outs. This is because as the processes are smaller, there is less probability of a synapse forming onto them. This in turn could lead to less synapses forming on the dendritic arbor, causing the cell to sample from a diminished input population and therefore producing an erroneous and non-WT like output. This hypothesis must be further validated with synaptic connectivity analysis using techniques like Immunohistochemistry to locate and count presynaptic and postsynaptic terminals on the dendrites.

Once these observed changes are confirmed through further experimentation, further analysis on the mechanisms of action of MeCP2 will be done. As it was previously stated, MeCP2 could be regulating the actin-modification signaling pathways through the activity of CDK5. CDK5 has been shown to promote neuronal morphogenesis and cortical development *in vivo*[31].

# Appendix

## Appendix A: Pairwise Comparison Charts

Table 3 Pairwise Comparison Charts of Primary Objectives

| | Maintain Slice Viability | Stability | User Friendly | Easy to Make | High Throughput | Total | Weight |
|---|---|---|---|---|---|---|---|
| **Maintain Slice Viability** | | 0.5 | 1 | 1 | 1 | **4** | **0.4** |
| **Stability** | 0.5 | | 1 | 1 | 1 | **3** | **0.3** |
| **User Friendly** | 0 | 0 | | 0.5 | 0.5 | **1** | **0.10** |
| **Easy to Make** | 0 | 0 | 0.5 | | 0 | **0.5** | **0.05** |
| **High Throughput** | 0 | 0 | 0.5 | 1 | | **1.5** | **0.15** |

Table 4 Pairwise Comparison Charts of "Stability" Secondary Objectives

| Stability | Mechanically | Thermally | Total | Weight (out of Weight of Stability) |
|---|---|---|---|---|
| **Mechanically** | | 1 | 1 | **0.20** |
| **Thermally** | 0 | | 0 | **0.33** |

Table 5 Pairwise Comparison Charts of "User Friendly" Secondary Objectives

| User Friendly | Maintenance | Interface with Rig | Total | Weight (out of Weight of User Friendly) |
|---|---|---|---|---|
| **Maintenance** | | 1 | 1 | **0.067** |
| **Interface with Rig** | 0 | | 0 | **0.033** |

Table 6 Pairwise Comparison Charts of "Easy to Make" Secondary Objectives

| Easy to Make | Cost-Effective | Material Feasibility | Total | Weight (out of Weight of Easy to Make) |
|---|---|---|---|---|
| **Cost-Effective** | | 0.5 | 0.5 | **0.025** |
| **Material Feasibility** | 0.5 | | 0.5 | **0.025** |

# Appendix B: Functions-Means Charts for the Alternative Designs

Table 7: Functions Means- Chart for Lobster Pot Design

| Functions | Means | | |
|---|---|---|---|
| **Carbogen Exchange** | Attached Exchanger | | |
| **Contain Brain Slices** | Included slice holders | | |
| **Contain Media** | Material Properties | Material Coatings | |
| **Provide Mechanical Stability** | Material Properties (density) | Design Consideration | Adhesive Bottom |
| **Provide Thermal Stability** | Thermal Isolation Chamber | Material Properties | |
| **Protect Tissue from the Environment** | Separate Lid | | |
| **Easy Slice Access** | Separate Lid | Material Transparency | |

Table 8: Functions-Means Chart for Attachable Slice Holder Design

| Functions | Means | | |
|---|---|---|---|
| **Carbogen Exchange** | Separate Exchanger | | |
| **Contain Brain Slices** | Separate slice holders | | |
| **Contain Media** | Material Properties | | |
| **Provide Mechanical Stability** | Material Properties (density) | Design Consideration | |
| **Provide Thermal Stability** | Material Properties | | |
| **Protect Tissue from the Environment** | Separate Lid | | |
| **Easy Slice Access** | Separate Lid | No Lid | Material Transparency |

Table 9: Functions-Mean Chart for Well Slice Holder Design

| Functions | Means | |
|---|---|---|
| **Carbogen Exchange** | Separate container for gas exchange | |
| **Contain Brain Slices** | Included slice holders | |
| **Contain Media** | Material Properties | |
| **Provide Mechanical Stability** | Material Properties (density) | Design Consideration |
| **Provide Thermal Stability** | Material Properties | |
| **Protect Tissue from the Environment** | Attachable Lid | |
| **Easy Slice Access** | Attachable Lid | Material Transparency |

Table 10: Functions-Mean Chart for "Three Layer" Incubator Design

| Functions | Means | | |
|---|---|---|---|
| **Carbogen Exchange** | Attached Exchanger | | |
| **Contain Brain Slices** | Slice holding area | | |
| **Contain Media** | Material Properties | | |
| **Provide Mechanical Stability** | Material Properties (density) | Design Consideration | |
| **Provide Thermal Stability** | Material Properties | | |
| **Protect Tissue from the Environment** | Attachable Lid | | |
| **Easy Slice Access** | Attachable Lid | Window | Material Transparency |

## Appendix C: Design Evaluation Matrices

Table 11: Objective Evaluation Matrix

| Objectives | "Lobster Pot" | Attachable Slice Holders | Well Slice Holder | "Three Layer" Incubator |
|---|---|---|---|---|
| Maintain Slice Viability | 2 | 2 | 2 | 2 |
| Stability | 2 | 1 | 1 | 2 |
| User Friendly | 1 | 2 | 0 | 1 |
| Easy to Make | 0 | 2 | 0 | 1 |
| High Throughput | 1 | 1 | 0 | 2 |
| **Total** | **6** | **8** | **3** | **8** |

Table 12: Constraint Evaluation Matrix

| Constraints | "Lobster Pot" | Attachable Slice Holders | Well Slice Holder | "Three Layer" Incubator |
|---|---|---|---|---|
| Size and Weight | 2 | 2 | 0 | 0 |
| Safe | 2 | 1 | 1 | 2 |
| Time | 1 | 2 | 0 | 1 |
| Budget | 2 | 2 | 0 | 1 |
| Material Constraints | 2 | 2 | 0 | 2 |
| **TOTAL** | **9** | **9** | **1** | **4** |

Table 13: Function Design Evaluation Matrix

| Objectives | "Lobster Pot" | Attachable Slice Holders | Well Slice Holder | "Three Layer" Incubator |
|---|---|---|---|---|
| Carbogen Exchange | 2 | 2 | 2 | 2 |
| Contain Brain Slices | 2 | 2 | 1 | 2 |
| Contain Media | 2 | 2 | 0 | 1 |
| Provide Mechanical Stability | 2 | 1 | 2 | 2 |
| Provide Thermal Stability | 2 | 2 | 2 | 2 |
| Protect Tissue from the Environment | 2 | 2 | 0 | 1 |
| Easy Slice Access | 1 | 2 | 0 | 2 |
| **Total** | **13** | **13** | **7** | **12** |

# References


1. Chang, Q., et al., *The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression.* Neuron, 2006. **49**(3): p. 341-8.
2. Matijevic, T., et al., *Rett Syndrome: From the Gene to the Disease.* European Neurology, 2009. **61**(1): p. 3-10.
3. Amir, R.E., et al., *Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2.* Nat Genet, 1999. **23**(2): p. 185-8.
4. Chahrour, M. and H.Y. Zoghbi, *The Story of Rett Syndrome: From Clinic to Neurobiology.* Neuron, 2007. **56**(3): p. 422.
5. Caballero, I.M. and B. Hendrich, *MeCP2 in neurons: closing in on the causes of Rett syndrome.* Human Molecular Genetics, 2005. **14**(suppl 1): p. R19-R26.
6. Kaufmann, W.E. and H.W. Moser, *Dendritic Anomalies in Disorders Associated with Mental Retardation.* Cerebral Cortex, 2000. **10**(10): p. 981-991.
7. Dierssen, M. and G.J.A. Ramakers, *Dendritic pathology in mental retardation: from molecular genetics to neurobiology.* Genes, Brain and Behavior, 2006. **5**: p. 48-60.
8. Landi, S., et al., *The short-time structural plasticity of dendritic spines is altered in a model of Rett syndrome.* Sci. Rep., 2011. **1**.
9. Kishi, N. and J.D. Macklis, *MECP2 is progressively expressed in post-migratory neurons and is involved in neuronal maturation rather than cell fate decisions.* Molecular and Cellular Neuroscience, 2004. **27**(3): p. 306-321.
10. Tropea, D., et al., *Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice.* Proc Natl Acad Sci U S A, 2009. **106**(6): p. 2029-34.
11. Chao, H.-T., et al., *Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes.* Nature, 2010. **468**(7321): p. 263-269.
12. Belichenko, N.P., P.V. Belichenko, and W.C. Mobley, *Evidence for both neuronal cell autonomous and nonautonomous effects of methyl-CpG-binding protein 2 in the cerebral cortex of female mice with Mecp2 mutation.* Neurobiology of Disease, 2009. **34**(1): p. 71-77.
13. Lee, W.-C.A., et al., *Dynamic Remodeling of Dendritic Arbors in GABAergic Interneurons of Adult Visual Cortex.* PLoS Biol, 2005. **4**(2): p. e29.
14. Steffenburg, U., G. Hagberg, and B. Hagberg, *Epilepsy in a representative series of Rett syndrome.* Acta Pædiatrica, 2001. **90**(1): p. 34-39.
15. Nelson, E.D., et al., *Selective impact of MeCP2 and associated histone deacetylases on the dynamics of evoked excitatory neurotransmission.* Journal of Neurophysiology, 2011. **106**(1): p. 193-201.
16. Varela, C., D.A. Llano, and B.B. Theyel, *An Introduction to In Vitro Slice Approaches for the Study of Neuronal Circuitry #*, in *T Neuronal Network Analysis*2012. p. 103-125.
17. Pini, G., et al., *IGF1 as a Potential Treatment for Rett Syndrome: Safety Assessment in Six Rett Patients.* Autism Research and Treatment, 2012. **2012**: p. 14.
18. Hendrich, B. and A. Bird, *Identification and Characterization of a Family of Mammalian Methyl-CpG Binding Proteins.* Molecular and Cellular Biology, 1998. **18**(11): p. 6538-6547.
19. Banerjee, A., J. Castro, and M. Sur, *Rett syndrome: genes, synapses, circuits and therapeutics.* Frontiers in Psychiatry, 2012. **3**.
20. Webb, T. and F. Latif, *Rett syndrome and the MECP2 gene.* Journal of Medical Genetics, 2001. **38**(4): p. 217-223.
21. Guy, J., et al., *A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome.* Nat Genet, 2001. **27**(3): p. 322.

22. Chen, R.Z., et al., *Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice.* Nat Genet, 2001. **27**(3): p. 327.
23. Chen, W.G., et al., *Derepression of BDNF Transcription Involves Calcium-Dependent Phosphorylation of MeCP2.* Science, 2003. **302**(5646): p. 885-889.
24. Chang, Q., et al., *The Disease Progression of Mecp2 Mutant Mice Is Affected by the Level of BDNF Expression.* Neuron, 2006. **49**(3): p. 341-348.
25. Wang, H., et al., *Dysregulation of Brain-Derived Neurotrophic Factor Expression and Neurosecretory Function in Mecp2 Null Mice.* The Journal of Neuroscience, 2006. **26**(42): p. 10911-10915.
26. Yoshii, A. and M. Constantine-Paton, *BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation.* Nat Neurosci, 2007. **10**(6): p. 702.
27. Yoshii, A. and M. Constantine-Paton, *Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease.* Dev Neurobiol, 2010. **70**(5): p. 304-22.
28. Mari, F., et al., *CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome.* Human Molecular Genetics, 2005. **14**(14): p. 1935-1946.
29. Scala, E., et al., *CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms.* Journal of Medical Genetics, 2005. **42**(2): p. 103-107.
30. Rusconi, L., et al., *CDKL5 Expression Is Modulated during Neuronal Development and Its Subcellular Distribution Is Tightly Regulated by the C-terminal Tail.* Journal of Biological Chemistry, 2008. **283**(44): p. 30101-30111.
31. Chen, Q., et al., *CDKL5, a Protein Associated with Rett Syndrome, Regulates Neuronal Morphogenesis via Rac1 Signaling.* The Journal of Neuroscience, 2010. **30**(38): p. 12777-12786.
32. #233, P. ndez, and A. Bacci, *Assortment of GABAergic Plasticity in the Cortical Interneuron Melting Pot.* Neural Plasticity, 2011. **2011**.
33. Henry Markram, M.T.-R., Yun Wang, Anirudh Gupta, Gilad Silberberg & Caizhi Wu, *Interneurons of the neocortical inhibitory system.* Nature Reviews, 2004(5): p. 793-807.
34. Rudy, B., et al., *Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons.* Developmental Neurobiology, 2011. **71**(1): p. 45-61.
35. Kawaguchi, Y. and Y. Kubota, *Neurochemical features and synaptic connections of large physiologically-identified GABAergic cells in the rat frontal cortex.* Neuroscience, 1998. **85**(3): p. 677-701.
36. Chao, H.-T., H.Y. Zoghbi, and C. Rosenmund, *MeCP2 Controls Excitatory Synaptic Strength by Regulating Glutamatergic Synapse Number.* Neuron, 2007. **56**(1): p. 58-65.
37. Weng, S.-M., M.E.S. Bailey, and S.R. Cobb, *Rett Syndrome: From Bed to Bench.* Pediatrics & Neonatology, 2011. **52**(6): p. 309-316.
38. Zhang, L., et al., *The MeCP2-null mouse hippocampus displays altered basal inhibitory rhythms and is prone to hyperexcitability.* Hippocampus, 2008. **18**(3): p. 294-309.
39. Adachi, M., et al., *MeCP2-mediated transcription repression in the basolateral amygdala may underlie heightened anxiety in a mouse model of Rett syndrome.* J Neurosci, 2009. **29**(13): p. 4218-27.
40. Lipton, P., et al., *Making the best of brain slices; comparing preparative methods.* Journal of Neuroscience Methods, 1995. **59**(1): p. 151-156.
41. Alvarez-Saavedra, M., et al., *Cell-specific expression of wild-type MeCP2 in mouse models of Rett syndrome yields insight about pathogenesis.* Hum. Mol. Genet., 2007. **16**(19): p. 2315-2325.
42. Nguyen, P.V., *Comparative plasticity of brain synapses in inbred mouse strains.* Journal of Experimental Biology, 2006. **209**(12): p. 2293-2303.

43. Croning, M.D.R. and G.G. Haddad, *Comparison of brain slice chamber designs for investigations of oxygen deprivation in vitro.* Journal of Neuroscience Methods, 1998. **81**(1–2): p. 103-111.
44. Aitken, P.G., et al., *Preparative methods for brain slices: a discussion.* Journal of Neuroscience Methods, 1995. **59**(1): p. 139-149.
45. Amir, R.E., et al., *Influence of mutation type and X chromosome inactivation on Rett syndrome phenotypes.* Ann Neurol, 2000. **47**(5): p. 670-9.
46. Ballas, N., et al., *Non-cell autonomous influence of MeCP2-deficient glia on neuronal dendritic morphology.* Nat Neurosci, 2009. **12**(3): p. 311-317.
47. Voss, L.J., S.A. George, and J.W. Sleigh, *Testing neocortical slice viability in non-perfused no-magnesium artificial cerebrospinal fluid solutions.* Journal of Neuroscience Methods, 2012. **204**(2): p. 273-275.
48. Runyan, C.A., et al., *Response Features of Parvalbumin-Expressing Interneurons Suggest Precise Roles for Subtypes of Inhibition in Visual Cortex.* Neuron, 2010. **67**(5): p. 847-857.