



Mechanisms of axonal pathology in the context of traumatic brain injury

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**MECHANISMS OF AXONAL PATHOLOGY IN THE CONTEXT OF
TRAUMATIC BRAIN INJURY**

A Thesis

Submitted to the Faculty

of

Drexel University

by

Raha Maryam Dastgheyb

in partial fulfillment of the

requirements for the degree

of

Doctor of Philosophy

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Table of Contents

Table of Contents.....	vii
Table of Figures.....	xiii
Abstract.....	xix
Chapter 1: Background	1
1.1 Epidemiology.....	1
1.2 Biomechanics of TBI	2
1.3 Experimental Models of TBI	4
1.3.1 <i>In Vivo</i>	4
1.3.2 <i>In Vitro</i>	5
1.4 Secondary Injuries	6
1.4.1 Beading	7
1.4.2 Membrane Damage	8
1.4.3 Calcium.....	9
1.4.4 Protease Activation	11
1.4.5 Impaired Axonal Transport	12
1.4.6 Mitochondria	13
1.4.7 Oxidative Stress.....	15
1.4.8 Lipid Peroxidation	16
1.5 Other Neurological Disorders.....	17
1.6 Hypothesis and Motivation	18
1.7 Specific Aims.....	19
1.8 Organization of Thesis.....	21
Chapter 2: Materials and Methods.....	22
2.1 Cell Culture	22
2.2 Measurement of Axonal Beading.....	24
2.3 Mitochondrial Morphology	25
2.4 Microtubule Analysis.....	27
2.4.1 Visualization of the Cytoskeleton	27

2.4.2	Quantifying focal disruptions.....	27
2.5	Measurement of Membrane Permeability	28
2.6	Calcium Quantification	29
Chapter 3: The Role of Membrane Damage in Axonal Pathology		31
3.1	Background.....	31
3.1.1	Axonal Pathology	31
3.1.2	Membrane Damage	31
3.1.3	Calcium Influx.....	33
3.1.4	Axonal Beading	35
3.1.5	Hypothesis and summary of experiments	35
3.2	Materials and Methods.....	36
3.2.1	Membrane Permeabilization Methods.....	36
3.2.2	Increasing Axonal Calcium : A23187	37
3.2.3	Chelating Extracellular Calcium: EGTA.....	38
3.3	Results	38
3.3.1	Permeabilizing the Plasma Membrane Results in Pathologies Similar to That of TBI	38
3.3.2	Selectively Increasing Cytosolic Calcium Results in Pathologies Similar to That of TBI.....	47
3.3.3	Blocking Calcium Influx Mitigates Beading after Permeabilizing the Cell Membrane	52
3.4	Discussion.....	54
Chapter 4: The Contribution of Mitochondria to Membrane Damage-Induced Axonal Pathology		57
4.1	Background.....	57
4.1.1	Axonal Mitochondria	57
4.1.2	Causes of Mitochondrial Dysfunction	58
4.1.3	The Mitochondrial Permeability Pore (MPP) and the Mitochondrial Permeability Transition (MPT)	60
4.1.4	Mitochondria and Oxidative Stress.....	61
4.1.5	Hypothesis and Summary of Experiments.....	63

4.2	Materials and Methods	64
4.2.1	Inducing mitochondrial dysfunction with CCCP	64
4.2.2	Inducing Increased Oxidative Stress with TbHp	65
4.2.3	Preventing Increased Oxidative Stress with Antioxidants	65
4.3.4	Chelating Intracellular Calcium with BAPTA-AM	66
4.3.5	Preventing the MPT with Cyclosporin A	67
4.3	Results	68
4.3.1	Disrupting Mitochondria Results in a Pathology seen in TBI.....	68
4.3.2	Increasing Oxidative Stress with TbHp Results in a Pathology seen in TBI	72
4.3.3	Contribution of Oxidative Stress.....	77
3.3.5	Contribution of Intracellular Calcium to Axonal Pathology	86
3.3.5	Calcium Contribution	91
3.3.3	Contribution of the Mitochondrial Permeability Pore	93
4.4	Discussion	98
Chapter 5:	The Role of Secondary Membrane Damage in Axonal Pathology	105
5.1	Background	105
5.1.1	Secondary Membrane Disruption.....	105
5.1.2	Lipid Peroxidation in Traumatic Brain injury	106
5.1.3	Cell Membrane Repair by Poloxamer 188	106
5.1.4	Hypothesis and Summary of Experiments.....	108
5.2	Materials and Methods.....	108
5.2.1	Measurement of Lipid Peroxidation	108
5.2.2	Cell membrane repair with P188	109
5.3	Results	110
5.3.1	Increasing Intracellular Calcium Results in Increased Membrane Permeability	110
5.3.2	Damaging Mitochondria Results in Increased Membrane Permeability..	111
5.3.3	Increasing Oxidative Stress Results in Increased Membrane Permeability	112
5.3.4	P188 Does not reduce beading After Increasing Intracellular Calcium	113

5.3.5	P188 Does Not Reduce Increase in Membrane Permeability Associated with A23187 Treatment	115
5.4.6	P188 Does Not Reduce Cytosolic Calcium Increase Associated with A23187 Treatment	115
5.3.7	P188 Reduces beading after Mitochondrial Dysfunction is Induced with CCCP	116
5.3.8	P188 Reduces Increase in Membrane Permeability Associated with CCCP Treatment	117
5.3.9	Poloxamer Reduces Cytosolic Calcium Increase Associated with CCCP Treatment	118
5.3.10	Poloxamer 188 Reduces beading after Increasing Oxidative Stress.....	119
5.3.11	Poloxamer 188 Reduces Increase in Membrane Permeability Resulting from TbHp Treatment.....	120
5.3.12	Poloxamer 188 Does Not Reduce Increase in Cytosolic Calcium Resulting from TbHp Treatment.....	121
5.3.12	Increasing Intracellular Calcium Results in Lipid Peroxidation	122
5.3.13	Mitochondrial Disruption Results In Lipid Peroxidation	123
5.3.14	Increasing Oxidative Stress Results in Lipid Peroxidation	123
5.4	Discussion.....	124
Chapter 6:	Conclusions and Future Work.....	129
6.1	Summary of Principle Findings.....	129
6.2.	Implications of the current study.....	130
6.4	Future Work	135
Appendices.....		139
Appendix A:	An Automated Interactive Bead Measurement Program	139
	Preprocessing.....	141
	Segmentation.....	142
	Spine.....	143
	Width	144
	Bead Identification.....	144
	Calculations.....	145

Code	148
Appendix B: Quantification of Focal Disruptions in Microtubules	184
Code	187
List of References.....	195

Table of Figures

Figure 1. Hypothesized pathways of cellular death and dysfunction after TBI. Highlighted regions represent components targeted in specific aims	18
Figure 2. Process of Quantifying Mitochondrial Shape..	26
Figure 3. Example of axonal beading in chick forebrain neurons.....	35
Figure 4. Representative image of intracellular Calcein loss before (A) and 20 minutes after (B) the addition of 0.01% Saponin	39
Figure 5. Saponin treatment results in increased membrane permeability	39
Figure 6. Representative image of intracellular Calcein loss before (A) and 30 minutes after (B) the addition of 100nM melittin	39
Figure 7. Melittin treatment results in increased membrane permeability.....	39
Figure 8. Permeabilizing the membrane with saponin results in axonal beading	40
Figure 9. Permeabilizing the membrane with melittin results in axonal beading.....	41
Figure 10. Saponin treatment results in intracellular axonal calcium increase that is prevented when extracellular calcium is chelated with EGTA	42
Figure 11. Representative image of Fluo-3 intensity increase before (A) and 15 minutes after (B) the addition of 0.01% Saponin	42
Figure 12. Melittin treatment results in intracellular axonal calcium increase that is prevented when extracellular calcium is chelated with EGTA.	42
Figure 13. Representative image of Fluo-3 intensity increase before (A) and 25 minutes after (B) the addition of 100nM melittin	42
Figure 14. Cytoskeletal changes after saponin treatment.....	44

Figure 15. Cytoskeletal changes after melittin treatment.....	45
Figure 16. Melittin treatment results in change in mitochondrial morphology	46
Figure 17. Membrane permeabilization with melittin results in a change in mitochondrial morphology.....	46
Figure 18. Membrane permeabilization with saponin results in a change in mitochondrial morphology.....	47
Figure 19. Representative image of Fluo-3 intensity increase before (A) and 30 minutes after (B) the addition 10uM A23187.....	48
Figure 20. Chelating extracellular calcium with EGTA prevents the increase in axonal calcium caused by Calcium Ionophore A23187	48
Figure 21. Increasing intracellular calcium with calcium ionophore A23187 results in a beaded morphology.....	49
Figure 22. Chelating extracellular calcium with EGTA prevents bead formation induced by calcium ionophore	50
Figure 23. Cytoskeletal changes after increasing intracellular calcium with calcium ionophore A23187	51
Figure 24. A23187 treatment results in change in mitochondrial morphology	52
Figure 25. Chelating extracellular calcium reduces axonal beading after permeabilizing the membrane with saponin	53
Figure 26. Chelating extracellular calcium prevents axonal beading after permeabilizing the membrane with melittin.....	54

Figure 27. Disrupting mitochondrial function with CCCP results in a change in mitochondrial morphology	69
Figure 28. Disrupting mitochondria with CCCP results in a beaded morphology	69
Figure 29. Representative diagram of increase in Fluo-3 fluorescence before (A) and 60 minutes after (B) the addition of 100nM CCCP	70
Figure 30. CCCP treatment results in a significant increase in axonal calcium	70
Figure 31. Cytoskeletal changes after CCCP treatment.....	71
Figure 32. Increasing oxidative stress with TbHp results in a beaded morphology.....	73
Figure 33. TbHp treatment results in change in mitochondrial morphology.....	74
Figure 34. Increasing oxidative stress with TbHp results in a change in mitochondrial morphology.....	74
Figure 35. Representative diagram of increase in Fluo-3 fluorescence before (A) and 30 minutes after (B) the addition of 100 mM TbHp	76
Figure 36. TbHp treatment results in a significant increase in axonal calcium.....	76
Figure 37. Cytoskeletal changes after TbHp treatment.....	77
Figure 38. Antioxidant treatment with glutathione (GSH) reduces axonal beading after CCCP treatment.....	79
Figure 39. Antioxidant treatment with catalase reduces beading after CCCP treatment	80
Figure 40. Antioxidant treatment with glutathione (GSH) reduces axonal beading after increasing axonal calcium with calcium ionophore A23187	81
Figure 41. Antioxidant treatment with catalase reduces axonal beading after increasing intracellular calcium with calcium ionophore A23187	82

Figure 42. Antioxidant treatment with glutathione (GSH) reduces axonal beading after increasing membrane permeability with saponin	83
Figure 43. Antioxidant treatment with catalase does not reduce axonal beading after increasing membrane permeability with saponin	84
Figure 44. Antioxidant treatment with glutathione (GSH) reduces beading after increasing membrane permeability with melittin	85
Figure 45. Antioxidant treatment with catalase reduces axonal beading after increasing membrane permeability with melittin	86
Figure 46. Chelating intracellular reduces axonal beading after CCCP treatment	87
Figure 47. Chelating intracellular calcium reduces axonal beading after increasing oxidative stress with TbHp	88
Figure 48. Chelating intracellular calcium does not reduce axonal beading after increasing intracellular calcium with A23187.	89
Figure 49. Chelating intracellular calcium reduces axonal beading after saponin treatment does not reduce axonal beading	90
Figure 50. Chelating intracellular calcium reduces axonal beading after melittin treatment	91
Figure 51. Quantification of cytosolic calcium after CCCP treatment	92
Figure 52. Neurons treated with 100mM TbHp for 30 minutes exhibit a significantly greater change in axonal calcium than control or EGTA-treated cells	93
Figure 53. Preventing the opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after damaging mitochondria with CCCP ..	94

Figure 54. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after increasing oxidative stress with TbHp	95
Figure 55. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after membrane poration with saponin....	96
Figure 56. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after membrane poration with melittin....	97
Figure 57. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) does not reduce axonal beading after increasing intracellular calcium with calcium ionophore A23187	98
Figure 58. Increasing intracellular calcium with calcium ionophore A23187 results in increased membrane permeability.....	111
Figure 59. Disrupting mitochondrial function with CCCP	112
Figure 60. Increasing oxidative stress with TbHp results in increased membrane permeability.....	113
Figure 61. P188 does not reduce axonal beading when cytosolic calcium is increased with A23187	114
Figure 62. A23187 treatment results in increased plasma membrane permeability that is not mitigated when P188 is present.....	115
Figure 63. A23187 treatment results in intracellular axonal calcium increase that is not mitigated when P188 is present	116
Figure 64. P188 reduces axonal beading after CCCP treatment	117

Figure 65. CCCP treatment results in increased plasma membrane permeability that is mitigated when P188 is present	118
Figure 66. CCCP treatment results in increased cytosolic calcium that is mitigated when P188 is present.....	119
Figure 67. P188 reduces axonal beading after TbHp treatment	120
Figure 68. TbHp treatment results in increased plasma membrane permeability that is mitigated when P188 is present	121
Figure 69. TbHp treatment results in increased cytosolic calcium that is not mitigated when P188 is present.....	122
Figure 70. Increasing intracellular calcium with calcium ionophore A23187 results in lipid peroxidation.....	123
Figure 71. Damaging mitochondria with CCCP results in lipid peroxidation.....	123
Figure 72. Increasing oxidative stress with TbHp results in lipid peroxidation	124
Figure 73. User interface for segmenting axons and quantifying axonal beading.	141
Figure 74. Perpendicular lines along the axon in order to calculate the width at every point on the spine	144
Figure 75. Bead identification.....	145
Figure 76. Axon rotation and segmentation in ImageJ before creation of the plot profile.	185
Figure 77. Matlab processing to create plot profile of microtubule intensity	186
Figure 78. Plot profile filtering to identify focal disruptions in microtubules	187

Abstract

Mechanisms of Axonal Pathology in the Context of Traumatic Brain Injury

Raha M. Dastgheyb

Kenneth Barbee, Ph.D

Traumatic Brain Injury (TBI) affects approximately 270,000 people a year and costs the U.S. more than \$48 billion annually. Around 70,000 people die each year from head injuries, but many of those who survive are left with long-term disabilities as a result of their injuries. While there have been many promising therapeutic treatments for TBI, clinical trials have not been successful. *In vitro* studies can provide insight into the cellular mechanisms leading to neuronal death and degeneration after TBI and aid in developing more effective treatments.

The goal of this work was to investigate the mechanisms and pathways by which damage to neurons progresses in the context of Traumatic Brain Injuries by characterizing the behavior and response of cells to individual components of the proposed TBI mechanisms – specifically; membrane damage, increases in intracellular calcium, mitochondrial damage, and oxidative stress and lipid peroxidation. A clear understanding of the injury mechanisms and pathways is a requirement for the development of successful therapeutic interventions after injury.

The mechanisms for cell death and dysfunction after TBI are hypothesized to be initiated by damage to the plasma membrane, which results in a loss of ionic homeostasis.

Calcium flows into the cell and causes an activation of various proteases that disrupt the cytoskeleton and cause deficits in axonal transport. Mitochondria, the primary source of cellular energy, also work in the cell as calcium buffers, but become dysfunctional when they cannot accommodate the excess calcium and result in the release of stored calcium. Damage to mitochondria results in an inability to produce ATP, whereby the cell starves, ATP-driven motors will be arrested, and ATP-driven ion pumps can no longer work to restore ion homeostasis. Damage to the mitochondria can also result in the opening of the mitochondrial permeability pore (MPP) and an increase in oxidative stress. Free radicals can further damage the mitochondria and the plasma membrane, and thus propagate and prolong cellular dysfunction. Damage to the mitochondria and the opening of the MPP also serves to release sequestered calcium from the mitochondria into the cytoplasm. These insults culminate in axonal “beads” and neuronal dysfunction and death.

Chapter 1: Background

1.1 Epidemiology

Traumatic Brain Injury (TBI) is one of the most common causes of disability and death in adults (Langlois, Rutland-Brown et al. 2006). It is predicted that by the year 2020 TBI will be the third leading cause of death and disability in the world (Murray and Lopez 1997). Among patients who die from TBI, about 90% die within 48 hours, usually due to an uncontrolled rise in intracranial pressure (Sahuquillo and Arikan 2006).

Survivors are often left with long term effects including sleep disturbances, persistent headaches, depression, and cognitive defects (Feigin, Barker-Collo et al. 2010). Survivors of mild TBI can suffer from diverse and sometimes disabling symptoms such as chronic headaches, dizziness, and vertigo, difficulty concentrating, problems communicating, depression, irritability, and impulsiveness (DeKosky, Ikonovic et al. 2010). In addition to the social and health related consequences, TBI represents a significant socioeconomic burden (Marshall 2000). The annual costs for TBI-related treatment in the United States was around \$56 billion in 1999 (Maas, Marmarou et al. 2007). Total direct and indirect costs to society have been estimated to exceed \$60 billion annually (Gean and Fischbein 2010). Any successful treatment for TBI would also aid in relieving some of these socioeconomic burdens.

While there have been many promising therapeutic treatments for TBI in the laboratory, clinical trials have not been successful (Schouten 2007, Vink and Nimmo 2009). There are many possible reasons for the failure seen in clinical trials, including drug delivery, timing, dosing issues, and limitations due to side effects. A better understanding of the injury mechanisms and pathways may aid in the development of successful therapeutic interventions.

1.2 Biomechanics of TBI

TBI is initiated by a transfer of kinetic energy to the head (Geddes and Cargill 2001) and can be either focal (from a direct blow or penetrating object) or diffuse (resulting from dramatic accelerations and decelerations of the head) (Waxweiler, Thurman et al. 1995). Mechanisms of brain injury may include direct brain contusion from skull deformation, brain contusion from movements of the brain against the interior of the skull, or subdural hematomas typically from the tearing of the parasagittal bridging veins (Viano, King et al. 1989). Diffuse injuries result from non-contact rotational forces to the brain (Meaney, Smith et al. 1995). Diffuse brain injury can occur without direct impact, but it is dependent on inertial forces such as those from automobile accidents or falls and assaults (Smith, Meaney et al. 2003).

The brain is normally protected from physical injury by the skull (LaPlaca, Prado et al. 2009). Loosely coupled to the skull, the brain has its own inertia, so its movement lags behind that of the skull during incidences of sudden acceleration or deceleration. The

resulting relative displacement causes shear deformations, stretches the blood vessels in the brain, and strains the brain tissue(Viano, King et al. 1989).

Neurons have viscoelastic properties, which may make them more vulnerable to acceleration-dependent injury. When exposed to the rapid deformations associated with brain injury they become brittle(Smith, Meaney et al. 2003) and more prone to injury. Human cadaver studies have shown that the level of injury depends on both the acceleration of the head as well as the duration of acceleration (Gurdjian, Roberts et al. 1966). In addition, the orientation and direction of acceleration are also important (Anderson, Wisner et al. 1997), with angular accelerations being particularly harmful (Ommaya and Gennarelli 1974). Injury can result in the destruction of neural circuits by the primary mechanical forces, or following secondary damage, leading to obvious functional deficits and impairments.

Primary injuries due to the mechanical forces can include immediate cell death, hemorrhaging, and hematomas, as well as diffuse swelling that results in an increase in intracranial pressure (ICP) (Maas, Stocchetti et al. 2008) as well as sub-lethal damage to cells. The possible therapeutic interventions to alleviate these primary effects are limited as the damage occurs over a small time window and there are no known treatments save for surgical interventions for increases in ICP. Brain damage is not limited to the primary insult, as a cascade of events follow, termed “secondary damage”(Gean and Fischbein 2010). Work in animal models has shown that axons are not always directly severed or disconnected unless the level of injury is very severe. Rather, for mild injuries, there are

sub-lethal mechanical injuries that lead axons to undergo a sequence of changes that result in secondary injury.

1.3 Experimental Models of TBI

There are many different models of TBI, both *in vivo* and *in vitro*. There is no single model that perfectly replicates what happens in human TBI, or that allows observations of the entire injury pathway. This highlights the importance of being able to isolate and study pieces of the injury pathway in order to fully understand the mechanisms leading to neurodegeneration and neuronal death.

1.3.1 *In Vivo*

In vivo models of brain injury allow the replication of all cellular mechanisms in a whole, living animal and allow for the observation of overall effects after injury. However, they do not allow for time progression studies or live cellular imaging. The primary *in vivo* models of TBI are the weight drop model, fluid percussion model, and the cortical contusion injury model (Albert-Weissenberger and Siren 2010) often in rodents. Each model has its own strengths and weaknesses which must be considered when choosing or evaluating an injury model.

The weight-drop model (Feeney, Boyeson et al. 1981, Marmarou, Foda et al. 1994, Shohami, Beit-Yannai et al. 1997) is an impact acceleration model that consists of a weight falling freely from a designated height onto an animal's skull. While commonly used, this model has a high mortality rate and is not often reproducible. As with all current *in vivo* models, it does not allow for easy evaluation of the time progression of injury. The cortical

contusion injury (CCI) model (Dixon, Clifton et al. 1991, Sutton, Lescaudron et al. 1993, Huh, Raghupathi et al. 2003) consists of a pneumatic piston which strikes the exposed dura of the rat. This method requires a craniotomy to expose the dura, which may compensate for any intracranial pressure increases that may occur as a result of injury. The fluid percussion model (McIntosh, Noble et al. 1987) may be a more diffuse model of injury than the CCI. It consists of a pendulum that strikes the piston of a reservoir of fluid that generates a pressure pulse to the dura. This method also requires a craniotomy that may compensate for increases in intracranial pressure.

In vivo models are more comprehensive than *in vitro* models of injury in that they can better replicate the anatomy and progression of injury with all cell types and systems in the body. While most *in vivo* injury models are rodent based, which stray from human anatomy, there are non-primate models of rotational acceleration that follow human injury more closely. *In vivo* models represent a more accurate time course of injury, but they are also limited in that they are more complex and have minimal opportunities for observing the progression of injury. When it comes to evaluating therapeutics *in vivo* models provide a better opportunity for understanding drug metabolism and delivery as well as systemic side-effects.

1.3.2 *In Vitro*

The primary *in vitro* models of TBI attempt to replicate the biomechanics of TBI by inducing either shear or stretch forces on cultured neurons. These models often offer several advantages over animal models but are not capable of completely replicating all

the pathological effects of TBI. Importantly, *in vitro* models provide many opportunities for live imaging studies.

The stretch (Ellis, McKinney et al. 1995, Cargill and Thibault 1996, Morrison, Saatman et al. 1998, Smith, Wolf et al. 1999, Geddes and Cargill 2001, Pfister, Weihs et al. 2003, Lusardi, Rangan et al. 2004) model of TBI involves culturing cells on a deformable substrate that can then be stretched either uniaxially or biaxially, thereby transferring strain to the attached cells. The shear (LaPlaca, Lee et al. 1997, Kilinc, Gallo et al. 2007) model of TBI involves applying a fluid shear stress over cultured neurons. Both of these models have been shown to replicate many of the pathologies seen in TBI.

In vitro models may not involve the complexity of *in vivo* models, but in some cases this may be an advantage. *In vitro* models bypass the problem of drug delivery, where in *in vivo* models it may be difficult to determine if the lack of an effect is due to the ineffectiveness of the drug or to failure of delivery to the intended site, especially in studies of the brain where the blood brain barrier poses a formidable obstacle. The primary advantage of *in vitro* models is the opportunity to make real time measurements of cellular responses.

1.4 Secondary Injuries

Secondary injuries are those that occur after the initial injury. They can take several hours to days to develop, which suggests a window of opportunity for therapeutic intervention (Povlishock and Katz 2005) to prevent, attenuate, or delay the resulting neurological deficits (Vink and Nimmo 2009). Secondary damage can include

morphological changes, calcium-mediated damage, mitochondrial dysfunction, and increased oxidative stress (Maas, Stocchetti et al. 2008). Reports for the time span over which this occurs differ based on the injury model and subject. In animals secondary injury can progress over a period of 2-6 hours, though in humans it can take 12 hours or longer (Christman, Grady et al. 1994) providing a greater window of opportunity for intervention.

1.4.1 Beading

Beading is a well-documented phenomenon in neurons that indicates injury severity (Takeuchi, Mizuno et al. 2005). It is the term for focal enlargements in axonal width (Ochs, Pourmand et al. 1996) considered to be the morphological hallmark of DAI. It has gone by other names too, including “axonal varicosities” (Rand and Courville 1946, Coleman 2005), “axonal swellings” or “blebs” (Pullarkat, Dommersnes et al. 2006).

Axonal beading has been identified in human white matter after DAI (Adams 1982, Gentleman, Roberts et al. 1995). The presence of axonal beads is often used to ascertain that an injury has occurred, and to validate experimental injury models. Beading has been shown after stretch injury (Lusardi, Rangan et al. 2004) as well as shear injury *in vitro* (Kilinc, Gallo et al. 2007).

Beading morphology has been associated with disruptions in axonal transport (Smith, Wolf et al. 1999, Coleman 2005) including a misalignment of the cytoskeleton (Povlishock, Becker et al. 1983, Povlishock and Christman 1995, Gaetz 2004). Ultrastructural analysis at beading sites has revealed an aggregation of organelles (Stone, Singleton et al. 2001, Gaetz 2004) along with axolemmal and cytoskeletal defects. In *in*

vitro experiments, beading can be used as a morphological indicator of the extent of neuronal injury.

The mechanisms behind bead formation are not well understood. The beading morphology may be a consequence of osmotic imbalance (Budde and Frank 2010), but this does not explain why the swelling is focal. Cytoskeletal degradation, which is colocalized with beads, has been proposed as a cause of axonal beading (Monnerie, Tang-Schomer et al. 2010) but has not been confirmed. Transport problems have also been hypothesized as an initiator of beading. Pilling et al. have proposed that stationary organelles cause “traffic jams” that grown into axonal swellings through the simple blockage of organelle passage (Pilling, Horiuchi et al. 2006), and have suggested that this may be caused by defects in transport motor function. However, organelles and cytoskeletal alterations may not be required for bead formation as beading can also occur with pure lipid bilayer tubes (Markin, Tanelian et al. 1999).

1.4.2 Membrane Damage

The cell membrane plays a very important role in providing a diffusion barrier against ion transport, to prevent ions from diffusing in the direction of their concentration gradient. Membrane damage is often identified either by the release of intracellular contents or the cellular uptake of membrane impermeable markers such as dextrans. Damage to the plasma membrane has been shown in both *in vivo* and *in vitro* models of TBI (Pettus, Christman et al. 1994, Povlishock and Christman 1995, Pettus and Povlishock

1996, Povlishock and Pettus 1996, LaPlaca, Lee et al. 1997, Okonkwo, Pettus et al. 1998, Borgens 2001, Geddes, Cargill et al. 2003, Geddes, LaPlaca et al. 2003, Stone, Okonkwo et al. 2004, Geddes-Klein, Schiffman et al. 2006, Kilinc, Gallo et al. 2008). This damage can be transient, permanent, or delayed (Farkas, Lifshitz et al. 2006). The plasma membrane may become damaged in a way that does not necessarily kill cells immediately, but results in delayed cell death or dysfunction. It has been hypothesized that mechanical damage to the cell membrane (termed “mechanoporation”) is the originating event that leads to all following secondary damage (Borgens 2001, Kilinc, Gallo et al. 2008, Kilinc, Gallo et al. 2009).

There is also evidence that membrane damage can exist and be exacerbated beyond the original insult (Lafrenaye, McGinn et al. 2012). This suggests the presence of a positive feedback loop that leads to increased membrane permeability and further neuronal degeneration. If true, this could possibly be manipulated as a point of therapeutic intervention.

1.4.3 Calcium

The concentration of free calcium in neurons has been shown to increase after brain injury (Shapira, Yadid et al. 1989, Cargill and Thibault 1996, Zhou, Xiang et al. 2001) and is thought to be linked to many of the neurodegenerative processes that occur following injury. Calcium is a very important ion in cells, and exhibits a large difference in concentration between the outside (1.2mM) and the inside (100nM) of the cell (Gleichmann and Mattson 2011). It is a second messenger that transfers signals locally and globally within the neuron. Calcium has been shown to play an important role in many

aspects of the cell, including neurite outgrowth, synaptogenesis, synaptic transmission, plasticity, and cell survival and degeneration.

Intracellular calcium has been shown to increase within 1 hour following *in vivo* TBI in a variety of experimental models (Shapira, Yadid et al. 1989, Fineman, Hovda et al. 1993, Maxwell, McCreath et al. 1995). Using ion-selective microelectrodes, Nilsson et al. measured interstitial calcium concentrations in the cortex after cerebral contusion injury in rats and found that there was a dramatic decrease in calcium from a baseline of 1.1mM to between 0.1 and 0.3mM implying massive calcium entry into cells in the direction of the concentration gradient (Nilsson, Hillered et al. 1993). In a rat model of DAI the concentration of free calcium in neurons was increased at 30 minutes after injury (Zhou, Xiang et al. 2001). In *in vitro* stretch models, there is an immediate rise in calcium followed by a gradual recovery period, with calcium magnitudes being dependent on the degree of strain (Geddes-Klein, Schiffman et al. 2006).

Elevated intracellular calcium has been linked to a wide array of cellular dysfunction (Trump and Berezsky 1995). An increase in intracellular calcium concentration is well known to initiate degenerative processes in multiple cell types (Stys, Ransom et al. 1990). Persistence of high cytosolic calcium may also be responsible for mitochondrial damage (Pettus, Christman et al. 1994, Lifshitz, Friberg et al. 2003), energy depletion, and signaling for necrotic and apoptotic cell death (Rink, Fung et al. 1995).

While it is agreed that there is an increase in intracellular calcium after injury, there is no clear consensus on the source of the calcium. Calcium may enter the cell from

the extracellular space through nonselective pores or through channels (LoPachin and Lehning 1997, Wolf, Stys et al. 2001). Glutamate receptor activation has also been implicated as a route for calcium entry (Arundine and Tymianski 2004). The N-methyl-D-aspartate (NMDA) receptor is the glutamate receptor most often studied in terms of excitotoxicity after TBI. Substantial sources of intra-axonal calcium might also include the mitochondria (Thayer and Miller 1990, Nicholls, Vesce et al. 2003), which are capable of storing and releasing intracellular calcium (Parekh 2008).

Paradoxically, calcium may also play a protective role by activating vesicle proteins that promote membrane fusion to seal the cell membrane (Fishman, Tewari et al. 1990, Terasaki, Miyake et al. 1997, Fishman and Bittner 2003). By some accounts membrane resealing cannot occur in the absence of extracellular calcium (Steinhardt, Bi et al. 1994). These observations indicate that calcium may play a dual role in TBI.

1.4.4 Protease Activation

Calcium has been implicated in activating certain proteases that contribute to neuronal degeneration, specifically calpains and caspases (Brana, Benham et al. 1999, Buki, Okonkwo et al. 1999, Pike, Zhao et al. 2000, Newcomb-Fernandez, Zhao et al. 2001). Calpains are calcium-activated proteases that can degrade several cytoskeletal proteins including spectrin and tubulin (Johnson, Litsersky et al. 1991, Saatman, Abai et al. 2003, Gaetz 2004). There are two major calpains: μ -calpain and m-calpain, which refer to their micromolar or millimolar requirement for calcium activation.

There have been many studies demonstrating the activation of calpain-mediated degradation of the cytoskeleton with regards to sites of axonal damage (Saatman, Murai et al. 1996, Posmantur, Kampfl et al. 1997, Buki, Okonkwo et al. 1999). In *in vivo* models of TBI, calpains have been shown to be activated as early as 15 minutes after injury (Kampfl, Posmantur et al. 1996). Adult male rats receiving lateral fluid percussion injury showed calpain-mediated spectrin breakdown at 90 minutes in axons (Saatman, Bozyczko-Coyne et al. 1996). Axonal calpain activation has also been observed in repetitive mild brain injury in rats (Huh, Widing et al. 2007). *In vitro* models of TBI have also shown calpain activity that has been linked to beading (Kilinc, Gallo et al. 2009). Caspase activation has also been noted after TBI in rats (Clark, Kochanek et al. 2000), where a caspase inhibitor was able to reduce cellular injury in a cortical contusion model but did not show significant improvements on functional outcome.

Calpain activation has also been noted in other diseases. It has been observed in Parkinson's disease, where experimental models have shown that calpain inhibition is able to attenuate cell death (Chen, Yap et al. 2006). Calpain activation has also been noted in the brain of Alzheimer's patients (Saito, Elce et al. 1993).

1.4.5 *Impaired Axonal Transport*

Deficits in axonal transport have been shown in TBI as well as in many other neurological disorders. To move cargo along the axon there is a requirement for "motors" to move the cargo and "rails" to transport them on (Roy, Zhang et al. 2005). In axons the "rails" consist mainly of microtubules, which run in a longitudinal direction (Hirokawa and

Takemura 2005) . Transport failure can occur when there is a defect in any one of these components.

Microtubules are a major component of the axonal cytoskeleton. Impaired axonal transport, marked by a breakdown of the cytoskeleton, has been shown in a variety of *in vivo* (Pettus, Christman et al. 1994, Maxwell 1996, Maxwell and Graham 1997, Stone, Singleton et al. 2001) and *in vitro* (Kilinc, Gallo et al. 2008, Tang-Schomer, Johnson et al. 2012) models. The loss of microtubules is not limited to the primary injury and can extend over a number of hours (Maxwell 1996, Pettus and Povlishock 1996, Maxwell and Graham 1997). It has been linked to focal disruption of axonal transport along with the accumulation of organelles such as mitochondria (Maxwell 1996, Maxwell and Graham 1997) and has been implicated in beading (Pettus, Christman et al. 1994) .

1.4.6 Mitochondria

The main function of mitochondria is to produce high energy intermediates (NADH and ATP) through the tri-carboxylic (TCA) cycle, also known as the Krebs cycle, and oxidative phosphorylation. Mitochondrial health has crucial links to TBI recovery, and patients with only marginal mitochondrial impairment have been shown to demonstrate a good outcome compared to those with profound mitochondrial impairment, who often have a poor prognosis (Signoretti, Marmarou et al. 2008). Mitochondria are actively transported on the axonal cytoskeleton to areas of high metabolic demand by the motors kinesin and dynein (Ligon and Steward 2000, Hollenbeck and Saxton 2005). This means that defects in mitochondria transport can lead to an altered distribution of mitochondria

along the axon, resulting in an inability to meet local energy demands or participate in calcium buffering.

Mitochondrial dysfunction and accumulation at sites of impaired axonal transport have been noted in *in vivo* (Pettus, Christman et al. 1994) and *in vitro* (Ellis, McKinney et al. 1995) models of TBI. Mitochondria function as calcium buffers and are the main cellular source of reactive oxygen species (ROS)(Rizzuto, Bernardi et al. 2000). *In vitro*, neuronal stretch produces mitochondrial dysfunction and ROS formation (Arundine, Aarts et al. 2004).

Mitochondria also play an important role in calcium regulation. Axonal mitochondria do not normally accumulate calcium(Scarpa, Malmstrom et al. 1976), but when intracellular calcium is increased above 5 μM , the calcium can be sequestered in the mitochondria (Blaustein 1988). However, large increases in intracellular calcium due to extracellular influx have been closely associated with mitochondrial dysfunction (Nasr, Gursahani et al. 2003).

Mitochondria are primarily responsible for the cellular production of reactive oxygen species (ROS). During electron transfer in the inner mitochondrial membrane there is spontaneous leakage of electrons that lead to ROS production. Excessive calcium accumulation can also potentiate mitochondrial ROS production (Dyken 1994, Adam-Vizi 2005) and result in the release of apoptogenic factors such as cytochrome c into the cytosol (Liu, Kim et al. 1996, Fiskum 2000). The release of cytochrome c has been associated with *in vivo* brain injury (Buki, Okonkwo et al. 2000, Fiskum 2000).

An increase in mitochondrial calcium may induce a phenomenon known as the mitochondrial permeability transition (MPT), characterized by a pore in the inner mitochondrial membrane (Gunter and Pfeiffer 1990). The MPT is promoted by excess calcium and inhibited by cyclosporin A (CsA) (Kim, He et al. 2003). The opening of the mitochondrial permeability pore results in the ability of solutes with a molecular mass of up to 1500 Da to nonselectively pass through the inner mitochondrial membrane leading to depolarization of the mitochondria, uncoupling of oxidative phosphorylation, ATP depletion, and mitochondrial swelling (Kim, He et al. 2003). Mitochondrial dysfunction and the MPT have been implicated in brain injury (Okonkwo, Buki et al. 1999, Scheff and Sullivan 1999, Sullivan, Rabchevsky et al. 2000, Alessandri, Rice et al. 2002).

1.4.7 Oxidative Stress

Free radical production has been widely implicated in the pathology of TBI (Hall, Andrus et al. 1996, Shohami, Beit-Yannai et al. 1997, Arundine, Aarts et al. 2004). The primary ROS generated by mitochondria is superoxide (O_2^-), which is a result of leakage from the electron transport chain (Kowaltowski, de Souza-Pinto et al. 2009) where electrons spontaneously leak and react with available O_2 (Won, Kim et al. 2002). Superoxide is transformed into hydrogen peroxide (H_2O_2) through the activity of Mn-SOD as well as Cu-SOD and Zn-SOD. Hydrogen peroxide is diffusible through the membrane and can be removed by an endogenous antioxidant system within the cell. If hydrogen peroxide is not removed, it can generate the hydroxyl radical ($\cdot OH$) through the metal-catalyzed Fenton reaction. It can also react with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$). Excess calcium in the mitochondria can interrupt the electron transport chain

and collapse mitochondrial membrane potential which allows for the accumulation of free electrons which can react with oxygen and produce more superoxide.

1.4.8 Lipid Peroxidation

ROS, while generally damaging to the cell, may also contribute to neuronal degeneration through lipid peroxidation. The neurons are particularly sensitive to this type of damage because of the enrichment of polyunsaturated fatty acids. Lipid peroxidation is the reaction between free radicals and polyunsaturated fatty acids which results in peroxy radical formation and conformational changes in the membrane, leading to a loss of membrane integrity (Girotti 1985, Hall, Braughler et al. 1992).

ROS induction of lipid peroxidation has been implicated in secondary damage after TBI (Braughler and Hall 1992, Hall, Braughler et al. 1992). Increased levels of lipid peroxidation, as measured by quantifying malondialdehyde (Ohkawa, Ohishi et al. 1979), have been shown 1-24 hours after diffuse head injury (Marmarou, Foda et al. 1994, Hsiang, Yeung et al. 1997, Vagnozzi, Marmarou et al. 1999) and 36 hours after weight-drop injury (Inci, Ozcan et al. 1998). Measurements using electron spin resonance have indicated lipid peroxidation 6 hours after TBI (Nishio, Yunoki et al. 1997). Using other methods, lipid peroxidation has been indicated in TBI at 24 hours after weight-drop injury (Promyslov and Demchuk 1995). These studies provide compelling evidence that lipid peroxidation is present after TBI and may be contributing to secondary damage (Lewen, Matz et al. 2000).

1.5 Other Neurological Disorders

Understanding the mechanisms underlying secondary pathologies may be important not only for unlocking management techniques for TBI, but also for other neurological disorders which may not only have similar pathways, but may also be linked. There has been much discussion about a link between TBI and Alzheimer's disease (Heyman, Wilkinson et al. 1984, DeKosky, Ikonomic et al. 2010). Significant amyloid plaques, a hallmark of Alzheimer's, have been reported in TBI patients who died 6-18 days following injury (Roberts, Gentleman et al. 1991). Alzheimer's also exhibits some of the same pathologies seen in TBI, such as axonal swelling and transport deficits (Stokin, Lillo et al. 2005).

A history of repetitive closed head injury has been described to lead to Chronic Traumatic Encephalopathy (CTE), which is a progressive tauopathy with a distinct clinical neuropathological profile that becomes symptomatic many years after an individual has experienced repeated concussive or subconcussive blows to the head. Characteristic features include tau immunoreactive neurofibrillary tangles and astrocytic tangles (Gavett, Stern et al. 2010). Patients with a history of head injury have also been associated with an increased risk for Parkinson's disease (Goldman, Tanner et al. 2006). In addition, a study of Italian soccer players has shown that they have an unusually high risk of Amyotrophic Lateral Sclerosis, possibly linked to repeated head trauma. The correlation between head injury and neurological disorders emphasizes the importance of understanding the mechanism of pathology after head injury.

1.6 Hypothesis and Motivation

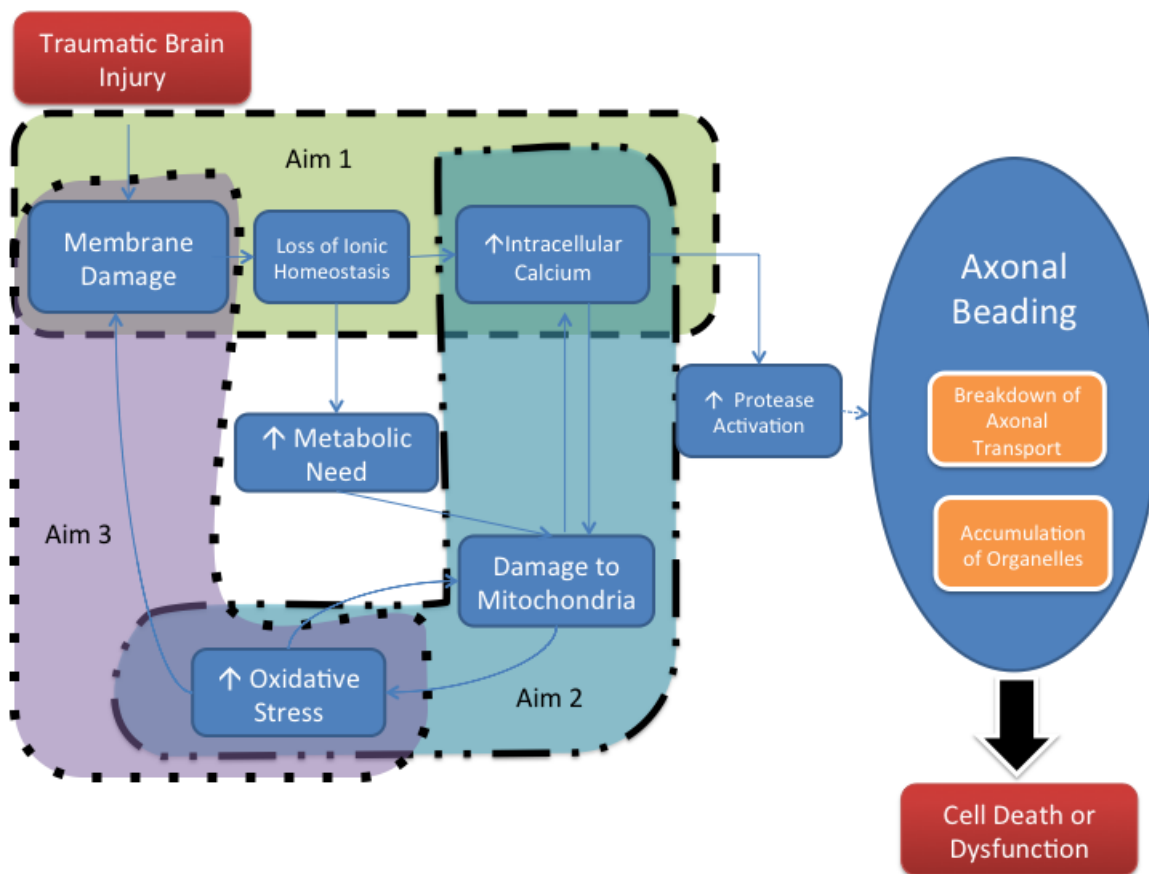


Figure 1. Hypothesized pathways of cellular death and dysfunction after TBI. Highlighted regions represent components targeted in specific aims

The overall hypothesis (Figure 1) is that cell death and dysfunction after TBI are initiated by damage to the plasma membrane. A major consequence of membrane disruption is the loss of ionic homeostasis, resulting in a flux of ions in the direction of their concentration gradient. Thus, cell metabolism and energy demand are increased as ATP-driven ion pumps work to restore the ion gradients. Calcium, which flows into the cell because of the large inward gradient, can cause an activation of various proteases that disrupt the cytoskeleton and cause deficits in axonal transport. Mitochondria, the

primary source of cellular energy, also work in the cell as calcium buffers, but become dysfunctional when they cannot accommodate the excess calcium. Damage to mitochondria results in an inability to produce ATP, whereby the cell starves, ATP-driven motors are arrested, and ATP-driven ion pumps can no longer work to restore the ion gradient. Damage to the mitochondria can also result in the opening of the mitochondrial permeability pore (MPP) and an increase in oxidative stress. Free radicals can further damage the mitochondria and the plasma membrane, and thus propagate and prolong cellular dysfunction. Damage to the mitochondria and the opening of the MPP also serves to release sequestered calcium from the mitochondria into the cytoplasm. These insults culminate in axonal “beads” and neuronal dysfunction and death.

The proposed specific aims are designed to isolate main components in the proposed pathway to validate or revise the injury sequence after TBI. Understanding the relationship between the initial injury and the subsequent pathology is vital to the development of clinically relevant therapeutic interventions.

1.7 Specific Aims

Specific Aim 1: *Determine the role of membrane disruption in axonal pathology.* In this aim, the membrane-permeabilizing agents, saponin and melittin were used to determine the progression of injury in neurons after membrane disruption. EGTA was used to chelate extracellular calcium with models of membrane disruption to investigate the role that calcium influx plays after membrane disruption. Calcium Ionophore A23187 was used to induce increased intracellular calcium without preceding membrane damage.

The hypotheses were that membrane damage and calcium influx would result in a pathology that mimics *in vivo* and *in vitro* models of TBI, including axonal beading, increased intracellular calcium, mitochondrial dysfunction, and microtubule disruption, and that chelating extracellular calcium would prevent axonal beading.

Specific Aim 2: Determine the contribution of mitochondria to membrane damage-induced axonal pathology. In this aim, carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) was used to target mitochondria in order to investigate the isolated component of mitochondrial damage. Intracellular chelation of calcium by BAPTA was used to investigate the interplay between calcium and mitochondria. To investigate the role that oxidative stress plays with disrupted mitochondria, antioxidants glutathione (GSH) and catalase were tested with CCCP, as well as with other isolated perturbations of the proposed pathway. The role of the MPP was explored by preventing its opening with Cyclosporin A (CsA) after various insults. The hypotheses were that isolated mitochondrial injury will result in a pathology that mimic components of the TBI pathway. Increasing oxidative stress would also mimic TBI pathology and antioxidant use would mitigate axonal beading. Chelating intracellular calcium was hypothesized to be neuroprotective and reduce axonal beading, as was the use of CsA to prevent the opening of the MPP.

Specific Aim 3: Determine the role of secondary membrane damage in axonal pathology. In this aim, the development of secondary membrane disruption after isolated insults in the cell injury pathway was investigated by measuring changes in membrane permeability. Poloxamer 188 (P188) was used in conjunction with these isolated insults to test for the therapeutic potential of membrane sealing. Injured cells were tested for

lipid peroxidation. The hypotheses were that secondary steps in the pathway in the absence of preceding membrane damage would lead to secondary membrane damage that can be mitigated by P188. It was also hypothesized that lipid peroxidation measurements will reveal that lipid peroxidation is responsible for secondary membrane damage.

1.8 Organization of Thesis

This thesis is organized in six chapters with a separate chapter for each aim, preceded by a general introduction (present) and a chapter on general methods (Chapter 2) that were used repeatedly. Any reagents or techniques specific to an aim are described in a separate methods section for each aim. Each chapter describing aims (Chapters 3 – 5) includes a background and materials section followed by results and discussion. Chapter 3 discusses the role of membrane disruption in axonal pathology, Chapter 4 investigates the contribution of mitochondria to membrane damage-induced axonal pathology and Chapter 5 explores the role of secondary membrane damage in axonal pathology. Chapter 6 is a final discussion with recommendations for future work. These chapters are followed by appendices with Matlab code and detailed methods for analyzing axonal beading and focal disruptions in microtubules.

Chapter 2: Materials and Methods

2.1 Cell Culture

All of the experiments in this thesis were conducted using chick forebrain neurons. These cells were chosen for culture because of their technical conveniences and because of their similarity to the commonly used rat hippocampal neurons, a common source for neuronal culture. Chick forebrain neurons are able to grow at low density, which allows for the analysis of isolated neurons.

Chick forebrain neurons were dissected using a protocol modified from Heidemann (Heidemann, Reynolds et al. 2003). Fertilized chicken eggs were received from Charles River Laboratories and incubated in humidified chambers to allow for embryo growth. Dissections were performed after 8 days of incubation. The outer surface of the egg was sterilized with 70% ethanol and the top portion of the egg was cracked open and the outer membrane detached so that the embryo could be removed to a petri dish and decapitated quickly. The two forebrain hemispheres were identified and the outer transparent tissue later was removed starting at the central fissure of the forebrain. Traces of blood vessels and meninges were removed, and the hemispheres are mechanically minced. After a brief centrifugation in PBS to clean the tissue, the pieces were incubated for 8 minutes at 37°C in Phosphate-buffered saline without calcium and magnesium (CMF-PBS) to help break up the tissues. This step was followed by more centrifugation to remove the supernatant, followed by enzymatic treatment with trypsin

to digest extracellular components. The cells were incubated for 8 minutes at 37°C in the presence of trypsin, centrifuged, and mechanically triturated again in Hepes-buffered Ham's F12 media (F12HS10) with 10% serum to deactivate the trypsin and further break up the cells. One more centrifugation step removed the trypsin and F12HS10, at which point the cells were counted and plated at a concentration 200-300 cells/mm on flamed glass coverslips that had been coated overnight with 0.01 mg/ml poly-DL-lysine. The plating media was Medium 199 (M199) supplemented with penicillin- streptomycin and 10% fetal bovine serum (FBS). The following day (DIV1) the cells were fed with M199, and the media was partially changed at DIV3. Experiments were conducted after 4-6 days in culture in Ham's F12 Medium supplemented with penicillin-streptomycin and 10mM Hepes (F12H).

For live imaging experiments, cells were plated on video dishes that were created by drilling a 1.5 cm diameter hole in the bottom of a 60mm diameter petri dish and affixing glass coverslips to the bottom of the hole using aquarium sealant and cured at room temperature (Gallo 2004). Dishes were then treated as regular coverslips for standard culture and were coated with 0.1 mg/ml poly-dl-lysine in borate buffer overnight before a suspension of cells was added. The use of a glass coverslip on the bottom of the dish allowed for oil immersion microscopy, and the creation of the well facilitated keeping cells alive in a small volume of media.

2.2 Measurement of Axonal Beading

For beading experiments, images were taken after treatment and fixation. Phase microscopy was used to obtain images of single neurons with discernable axons that did not have connections with other cells. ImageJ was used to measure the length of each axon, and the counting tool was used to manually select the number of beads in each axon. Beading intensity was measured by normalizing the number of beads per axon by the length of the axon in microns.

Axonal Beading was used as a measure of neuronal health in lieu of other methods because it is a morphological measurement based on phase images that potentially allows for the observation of the degradation of a single neuron through time. The use of live/dead assays merely allows for a binary measurement of health: alive or dead. Furthermore, most live/dead assays are based on measurements of membrane permeability. They make the assumption that cells with permeable membranes are no longer alive. Here, we are making the case that increased cell membrane permeability is only the beginning of a cell death pathway and that it can be treated, so these measurements were not sufficient.

For speed and ease of analysis, as well as for comparisons to other labs who use similar methods, the experiments shown here quantify only the number of beads normalized by the length of the axon. However, this may not be the best method of quantification as the size and location of the beads may also be important. To allow for

the quantification of these measurements, an automated and interactive method for the quantification of beading was developed and is presented in Appendix A.

2.3 Mitochondrial Morphology

Mitochondria were visualized using the mitochondrial dye Mitotracker Green, a fluorescent dye that accumulates in the mitochondria due to the membrane potential (Presley, Fuller et al. 2003). Cells were incubated in 50nM Mitotracker Green for 30 minutes and were imaged before and at the end of treatment. Mitochondrial morphology was quantified using ImageJ (Figure 2). Images were background-subtracted and the cell body removed. Mitochondria were then segmented using a fluorescence intensity threshold. If multiple mitochondria appeared to be overlapping they were not included in the segmentation. ImageJ's multi-measure tool was used to select the segments and quantify morphology based on a fit ellipse, measuring the major and minor length. The major axis length was used as a measure of the length of the mitochondria.

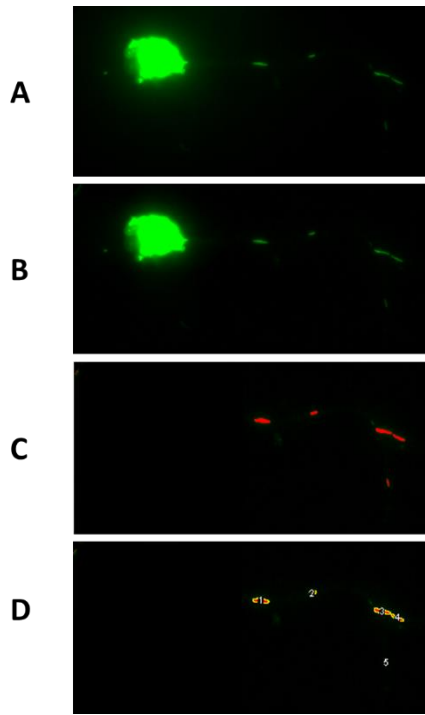


Figure 2. Process of Quantifying Mitochondrial Shape. *A)* Neuron with mitochondria stained using Mitotracker Green. *B)* Background subtraction using a 50 pixel rolling ball radius. *C)* Thresholding using fluorescence intensity. *D)* Mitochondria segmented and ready to be quantified using an ellipsoid fit.

The shape descriptor of roundness was also measured. Roundness was calculated in ImageJ using Equation 1. A value of 1 indicates a perfect circle. Values approaching 0 indicate an increasingly elongated shape.

$$Roundness = 4 \times \frac{Area}{\pi \times (Major Axis)^2}$$

Equation 1. Calculation of Roundness

These measurement were made for each discernable mitochondria in two images for each neuron, before and after treatment. At each time point the measurements for roundness and length were averaged and the percent changes was calculated for the second time point. The average measurements for each axon were used because of the

difficulty in tracking each individual mitochondrion over long periods of time without more frequent images, which would have contributed to mitochondrial dysfunction.

2.4 Microtubule Analysis

Quantifying cytoskeletal disruption involved two general steps: (1) fluorescent staining and imaging of microtubules and (2) quantification of focal disruptions in microtubules.

2.4.1 *Visualization of the Cytoskeleton*

To visualize their breakdown, microtubules must be fixed and stained while removing free tubulin monomers. To do this, a combined fixation and extraction method was used (Gallo and Letourneau 1999). Experimental cultures were fixed in PHEM buffer with 1% Triton X-100 and 0.05% glutaraldehyde for 15 minutes. They were then treated with 2 mg/ml of sodium borohydride to quench autofluorescence and stained with FITC-conjugated DM1A anti-tubulin (1:100) to stain tubulin.

2.4.2 *Quantifying focal disruptions*

An automated program was written and developed in Matlab to allow for the quantification of focal disruptions in microtubules. A detailed explanation of the program and methods is given in Appendix B. Briefly, the images were loaded into Image J and manually rotated and segmented with the user interface already available in Image J. The segmented image was then loaded into Matlab where the plot profile was created by finding the average intensity along the axon. These values were then filtered in a separate Matlab program to identify focal disruptions in microtubule intensity.

2.5 Measurement of Membrane Permeability

Membrane permeability was measured as the loss of an intracellular dye, CellTrace™ Calcein Red-Orange, AM (MW = 789.55, excitation/emission maxima 577/590 nm). Calcein Red-Orange is an acetoxymethyl (AM) ester derivative. Modification of carboxylic acids with AM ester groups results in an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that is well retained by live cells with intact membranes.

Cultured neurons were incubated in a final concentration of 5 μ M of Calcein Red-Orange for 30 minutes at 37°C to load the cell intracellularly with the dye. After incubation cultures were rinsed 3 times with the experimental media, F12H. Imaging was done on a temperature-controlled wide-field fluorescence microscope with an automated motorized stage with a 60x oil immersion objective. Fluorescence images were taken in live cells before and after treatment. ImageJ was used to quantify the total axonal fluorescence for each neuron before and after treatment. These numbers were used to calculate the percent decrease in calcein fluorescence, which is used as a measure of membrane permeability.

Factors other than membrane damage that could result in a change in axonal fluorescence include photobleaching, additional esterase cleavage of the dye, and a change in focus of the microscope. It is assumed that photobleaching and dye conversion

occur at similar rates for both controls and treated cells. Any axons with obvious changes in focus were not included in measurements.

2.6 Calcium Quantification

Calcium measurements were achieved in live cells using the calcium dye Fluo-3 AM. Fluo-3 (769.54) is a fluorescent calcium indicator that exhibits an increase in fluorescence when bound to calcium and can be used to image the spatial and temporal dynamics of intracellular calcium. An increase in Fluo-3 fluorescence is indicative of an increase in cytosolic calcium concentrations. Traditionally, an increase in Fluo-3 fluorescence would be interpreted as a decrease in cytosolic calcium concentrations. However, in these experiments there is a presumed loss of plasma membrane integrity, so a decrease in Fluo-3 fluorescence could also be interpreted as a loss of the intracellular dye. To control for this, the ratio of the cytosolic dye Calcein red/orange was also used with the assumption that the two dyes with similar molecular weights would escape from the cell at the same rate.

Immediately prior to experimentation, cells were incubated in F12H with 5 μ M Fluo-3 AM and 5 μ M of Calcein Red-Orange for 30 minutes at 37°C to load the cell intracellularly with the dyes. After incubation cultures were rinsed 3 times with the experimental media, F12H. Imaging was done on a temperature-controlled wide-field fluorescence microscope with an automated motorized stage with a 60x oil immersion objective. Fluorescence images were taken in live cells before and after treatment.

ImageJ was used to quantify the total axonal fluorescence for each neuron before and after treatment.

Factors other than membrane damage that could result in a change in axonal fluorescence include photobleaching, additional esterase cleavage of the dye, and a change in focus of the microscope. It is assumed that photobleaching and dye conversion occur at similar rates for both controls and treated cells. One major benefit for the ratiometric measurements used here is that any change in focus would affect the view of both dyes, so the ratiometric measurement helps to minimize these artifacts.

Chapter 3: The Role of Membrane Damage in Axonal Pathology

3.1 Background

3.1.1 Axonal Pathology

Collective data has revealed that there are several areas of impairment in TBI. *In vivo* models have demonstrated that after injury there is a loss of integrity in the plasma membrane (Pettus, Christman et al. 1994, Pettus and Povlishock 1996, Okonkwo, Pettus et al. 1998, Stone, Okonkwo et al. 2004), an increase in intracellular calcium (Shapira, Yadid et al. 1989, Fineman, Hovda et al. 1993, Zhou, Xiang et al. 2001), a breakdown of intracellular transport (indicated by focal disruptions in the axonal cytoskeleton and an accumulation of organelles) (Pettus, Christman et al. 1994, Maxwell 1996, Maxwell and Graham 1997, Stone, Okonkwo et al. 2004, Dikranian, Cohen et al. 2008), an increase in oxidative stress (Smith and Hall 1996), mitochondrial damage (Maxwell, McCreath et al. 1995, Dikranian, Cohen et al. 2008), and the formation of axonal beads (Maxwell 1996, Dikranian, Cohen et al. 2008). In many cases the connection and progression between these areas of impairment is unclear. Understanding the pathway that leads to the progression of these axonal pathologies will be an important step to unlocking therapeutic strategies.

3.1.2 Membrane Damage

The cell membrane plays a very important role in providing a diffusion barrier against ion transport, to prevent ions from diffusing in the direction of their concentration

gradient. Membrane damage is often identified either by the release of intracellular contents or the cellular uptake of membrane impermeable markers such as dextrans. Damage to the plasma membrane has been shown in both *in vivo* and *in vitro* models of TBI (Pettus, Christman et al. 1994, Povlishock and Christman 1995, Pettus and Povlishock 1996, LaPlaca, Lee et al. 1997, Okonkwo, Pettus et al. 1998, Borgens 2001, Geddes, Cargill et al. 2003, Geddes, LaPlaca et al. 2003, Stone, Okonkwo et al. 2004, Geddes-Klein, Schiffman et al. 2006, Kilinc, Gallo et al. 2008). The mechanism and structure of membrane damage is not well understood, but has been investigated using various types of markers. Lucifer Yellow (MW 457) has been shown to enter cells in a rat controlled cortical impact model, as has a 3000 MW dextran (Lessing 2008). Dextrans with a molecular weight of around 10,000 have been shown to enter neurons in rat fluid percussion models (Singleton and Povlishock 2004, Kelley, Farkas et al. 2006) as well as rat impact acceleration models (Stone, Okonkwo et al. 2004, Farkas, Lifshitz et al. 2006). Dextrans as large as 40,000 Da have been shown to enter cells in rat fluid percussion models (Pettus and Povlishock 1996, Singleton and Povlishock 2004) and impact acceleration models (Stone, Okonkwo et al. 2004). Horseradish Peroxidase, also with a molecular weight of around 40,000, has been shown to enter cells in a cat fluid percussion model (Pettus, Christman et al. 1994) and a rat fluid percussion model (Singleton and Povlishock 2004) as well as an impact acceleration model in rats (Koob, Duerstock et al. 2005). All of these studies indicate damage to the plasma membrane that can vary based on the model and location of injury. This damage can be transient, permanent, or delayed (Farkas, Lifshitz et al. 2006).

The plasma membrane may become damaged in a way that does not necessarily kill cells immediately, but results in delayed cell death or dysfunction. It has been hypothesized that mechanical damage to the cell membrane (termed “mechanoporation”) is the originating event that leads to all following secondary damage (Borgens 2001, Kilinc, Gallo et al. 2008, Kilinc, Gallo et al. 2009). There is also evidence that membrane damage can exist and be exacerbated beyond the original insult (Lafrenaye, McGinn et al. 2012), suggesting the presence of a feedback loop that leads to increased membrane permeability. Damage to the plasma membrane results in a loss of ionic homeostasis as ions move in the direction of their concentration gradient. In neurons, this involves an efflux of potassium and an influx of sodium and calcium ions.

3.1.3 Calcium Influx

Loss of plasma membrane integrity will cause calcium to diffuse down the concentration gradient and into the cell. Many studies have shown increased cytosolic calcium in TBI. A weight drop model of rat brain injury showed increased calcium content one hour after injury (Shapira, Yadid et al. 1989). In a rat fluid percussion injury model, calcium accumulation was demonstrated immediately after injury (Fineman, Hovda et al. 1993). Measurements of extracellular calcium after injury showed a sharp drop two hours after injury, indicating influx into neurons (Nilsson, Laursen et al. 1996). Stretch injury models of rat hippocampal neurons result in a sharp rise in intracellular calcium (Lusardi, Wolf et al. 2004). The concentration of free calcium in neurons has been shown to increase after brain injury (Shapira, Yadid et al. 1989, Fineman, Hovda et al. 1993, Cargill

and Thibault 1996, Zhou, Xiang et al. 2001) and is thought to be linked to many of the neurodegenerative processes that occur following injury. The source of this increased calcium is not completely clear, but it is likely to be an influx from the extracellular space or released intracellularly from mitochondria or endoplasmic reticulum.

Calcium is an essential ion in cells, and exhibits a large change in concentration between the outside (1.2mM) and the inside (100nM) of the cell (Gleichmann and Mattson 2011). It is a second messenger that transfers signals locally and globally within the neuron. Elevated intracellular calcium has been linked to a wide array of cellular dysfunction (Trump and Berezsky 1995). An increase in intracellular calcium concentration is well known to initiate degenerative processes in multiple cell types (Stys, Ransom et al. 1990). Oxidative stress, mitochondrial dysfunction, and protease activation have been proposed to lead to neuronal degeneration through calcium-dependent pathways in neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease (Nilsson, Hillered et al. 1993). Loss of calcium homeostasis is thought to be the final common pathway of neurodegeneration. Paradoxically, calcium may also play a protective role by activating vesicle proteins that promote membrane fusion to seal the cell membrane (Fishman, Tewari et al. 1990, Terasaki, Miyake et al. 1997, Fishman and Bittner 2003). These observations indicate that calcium may play a dual role in TBI, both in propagating neurodegeneration and as a signal to promote membrane repair.

While it is agreed that there is an increase in intracellular calcium after injury, there is no clear consensus on the source of the calcium. Calcium may enter the cell from the extracellular space through nonselective pores or through channels in the plasma

membrane (Blaustein 1988, LoPachin and Lehning 1997, Wolf, Stys et al. 2001). Substantial sources of intra-axonal calcium might also include the mitochondria (Thayer and Miller 1990, Nicholls, Vesce et al. 2003), which are capable of storing and releasing intracellular calcium (Parekh 2008).

3.1.4 Axonal Beading

Beading (Figure 3) is a well-documented phenomenon in neurons that indicates injury severity (Takeuchi, Mizuno et al. 2005). It is a

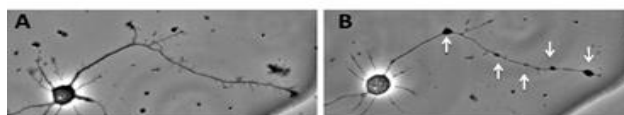


Figure 3. Example of axonal beading in chick forebrain neurons. A) Neuron before injury B) The same neuron 30 minutes after fluid shear stress injury. Beads are denoted by white arrows

term that describes focal bead-like swellings that have been shown in diffuse axonal injury after brain injury. In models of TBI, beads are often co-localized with areas of cytoskeletal damage and an accumulation of organelles (Povlishock, Becker et al. 1983, Povlishock and Christman 1995, Smith, Meaney et al. 2003, Takeuchi, Mizuno et al. 2005, Greenwood, Mizielinska et al. 2007, Kilinc, Gallo et al. 2009). In *in vitro* experiments, beading can be used as a morphological indicator of the extent of neuronal injury.

3.1.5 Hypothesis and summary of experiments

Damage to the plasma membrane due to the application of mechanical forces, termed “mechanoporation,” has been hypothesized to be the initiating factor that precedes the sequence of secondary injury mechanisms after TBI. If this is true, then membrane damage in the absence of mechanical loading would be expected to result in

the same sequence of events including mitochondrial dysfunction, increased intracellular calcium, cytoskeletal disruption, and mitochondrial damage. To simulate membrane damage, membrane permeabilizing agents were used to simulate membrane damage and allow non-selective transport across the membrane in the absence of mechanical injury to see if the same pathologies in TBI would occur. A major consequence of membrane disruption is the influx of calcium, so a calcium ionophore was used in the absence of preceding membrane disruption to investigate the role of calcium in TBI. To further investigate the role that calcium influx plays in membrane disruption, EGTA was used to chelate extracellular calcium after membrane disruption.

3.2 Materials and Methods

3.2.1 *Membrane Permeabilization Methods*

Two separate methods were used to increase membrane permeability; the detergent saponin and the pore-forming peptide melittin. The use of different agents with different mechanisms of membrane permeabilization ensured that different possible effects of membrane permeabilization would be represented.

Saponin

Saponin is a detergent commonly used in fixed cells for immunohistochemistry and at low concentrations it can be used in live cells to disrupt the cell membrane (Gogelein and Huby 1984). It is reported to interact with membrane cholesterol, selectively removing it and leaving pores in the membrane (Jamur and Oliver 2010). The

cholesterol-selectivity of saponin makes it more likely that it will permeabilize the cell membrane of the neuron, which is rich in cholesterol, and avoid permeabilizing organelles such as mitochondria which are almost cholesterol free(Addabbo, Montagnani et al. 2009).The size of pores created by saponin differs depending on the substrate, but in membranes it is reported to result in 3-5 nm holes(Seeman 1967). Saponin treatment is likely to allow for the exchange of ions as well as proteins.

Melittin

Melittin, the major toxin in bee venom, is an amphiphilic pore-forming peptide with antimicrobial properties. At low concentrations it can induce transient pores, and produces more stable pores at higher concentrations. It has been proposed that melittin integrates into the bilayer creating a torroidal (or “wormhole”) pore (Yang, Harroun et al. 2001, Lee, Sun et al. 2013). Reports of the size of melittin-induced pores range from a diameter of 1-4nm (Ladokhin, Selsted et al. 1997, Rex and Schwarz 1998, Yang, Harroun et al. 2001) . Melittin is likely to allow for the exchange of ions, but less likely than saponin to allow for the efflux of intracellular proteins.

3.2.2 Increasing Axonal Calcium: A23187

Increased intracellular calcium without preceding membrane poration can be achieved by using the calcium ionophore A23187(Reed and Lardy 1972). It is commonly used for research purposes to increase intracellular calcium in living cells because it shuttles free calcium in the direction of the concentration gradient.

3.2.3 *Chelating Extracellular Calcium: EGTA*

Ethylene glycol tetraacetic acid (EGTA) chelates calcium. It can be added to the extracellular medium to prevent calcium influx from the extracellular medium.

3.3 Results

3.3.1 *Permeabilizing the Plasma Membrane Results in Pathologies Similar to That of TBI Beading*

Beading is the hallmark morphological change of TBI, and if increased membrane permeability is leading to downstream secondary effects, increasing membrane permeability in the absence of mechanical trauma should also lead to beading. To test this hypothesis, saponin and melittin were used to chemically increase plasma membrane permeability. First, it was established that these agents were able to increase membrane permeability by loading cells with calcein red/orange and imaging before and after the addition of saponin or melittin (Figure 4, Figure 6). The percent loss of calcein fluorescence intensity in the axons was used as a measure of membrane permeability.

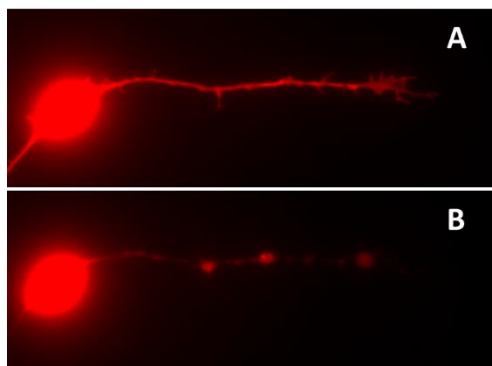


Figure 4. Representative image of intracellular Calcein loss before (A) and 20 minutes after (B) the addition of 0.01% Saponin

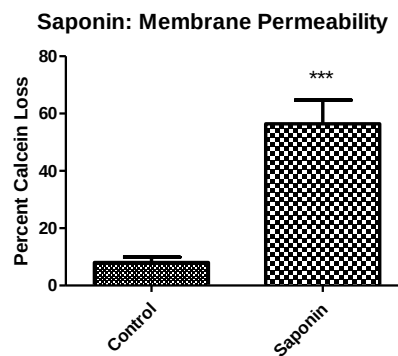


Figure 5. Saponin treatment results in increased membrane permeability. Neurons were loaded with Calcein Red/Orange and imaged before and 20 minutes after the addition of 0.01% Saponin. (One-tailed t-test, $p < 0.0001$. $n > 15$ cells)

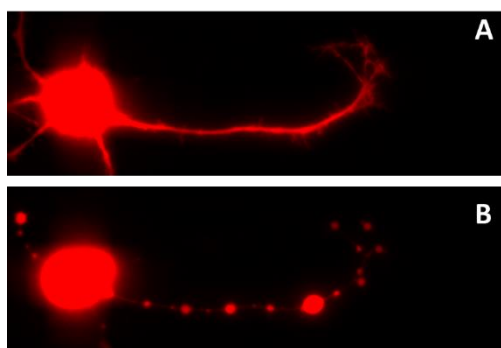


Figure 6. Representative image of intracellular Calcein loss before (A) and 30 minutes after (B) the addition of 100nM melittin

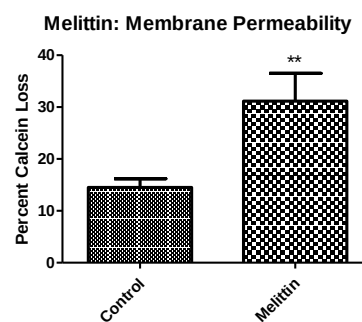


Figure 7. Melittin treatment results in increased membrane permeability. Neurons were loaded with Calcein Red/Orange and imaged before and 30 minutes after the addition of 100nM melittin (One-tailed t-test, $p < 0.01$. $n > 15$ cells for each condition). All values are presented as means $\pm$ SEM.

Both saponin and melittin treatment resulted in a significant increase in membrane permeability as compared to controls. Saponin treated cells lost more than 50% of their initial fluorescence after 20 minutes of incubation and melittin treated cells

lost more than 35%, while control cells lost less than 10% of their initial calcein fluorescence on average.

These findings were consistent with previous reports that saponin and melittin increase membrane permeability and validate their use as a model for membrane damage. Specifically, a study by Pratt in 2005 used a similar technique of observing calcein efflux to establish that melittin permeabilized the membrane of sheep lymphocytes (Pratt, Ravnic et al. 2005) and in 1997 Matsuzaki also used Calcein efflux to measure melittin pore formation in phospholipid vesicles (Matsuzaki, Yoneyama et al. 1997).

Phase imaging before and after the addition of saponin (Figure 8) and melittin (Figure 9) showed that both membrane-permeabilizing agents resulted in beading. These observations establish that membrane permeabilization even in the absence of mechanical injury is sufficient to induce beading.

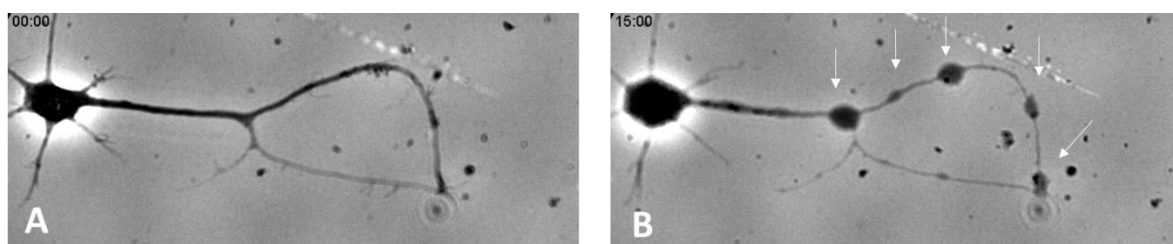


Figure 8. Permeabilizing the membrane with saponin results in axonal beading. *A*, Phase image of chick forebrain neuron before the addition of saponin. *B*, the same neuron 10 minutes after the addition of 0.01% saponin. Beads indicated by white arrows.

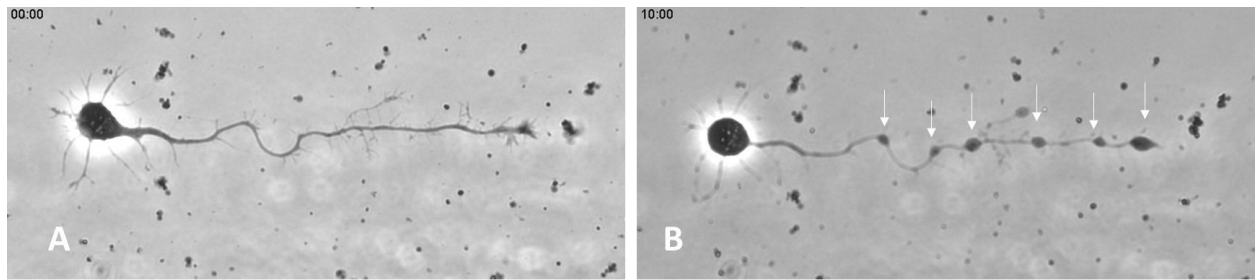


Figure 9. Permeabilizing the membrane with melittin results in axonal beading. *A*, Phase image of chick forebrain neuron before the addition of melittin. *B*, the same neuron 10 minutes after the addition of 100nM melittin. Beads indicated by white arrows.

Increased Axonal Calcium

Intracellular calcium is increased after TBI, and this increase is hypothesized to be mainly the result of calcium influx from the extracellular space after membrane poration. To test this, the calcium indicator dye Fluo-3 was used to measure relative changes in axonal calcium before and after increasing membrane permeability with saponin (Figure 10) and melittin (Figure 12). Changes in axonal Fluo-3 intensity were normalized by changes in Calcein Red/Orange intensity to account for dye loss due to membrane damage. Axonal calcium changes were quantified and compared to control cells where no membrane-permeabilizing agents were added. To establish that increased calcium was a result of extracellular influx, EGTA was used to chelate extracellular calcium. Saponin treatment resulted in an almost two-fold increase axonal calcium that was prevented with the addition of EGTA (Figure 11). Similar results were seen with melittin (Figure 13), confirming that increases in cytosolic calcium are due to influx from an extracellular source.

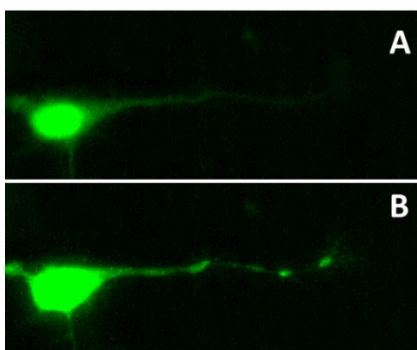


Figure 10. Representative image of Fluo-3 intensity increase before (A) and 15 minutes after (B) the addition of 0.01% Saponin

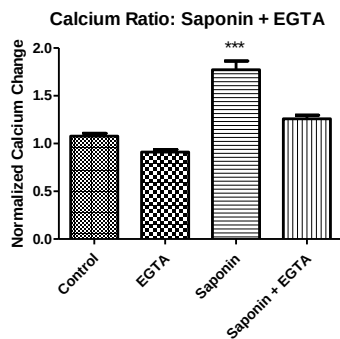


Figure 11. Saponin treatment results in intracellular axonal calcium increase that is prevented when extracellular calcium is chelated with EGTA. Cells were treated with 0.01% Saponin for 15 minutes and extracellular calcium was chelated with 1mM EGTA, which reduced the calcium increase back to baseline levels. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 8$ cells for each condition). All values are presented as means $\pm$ SEM.

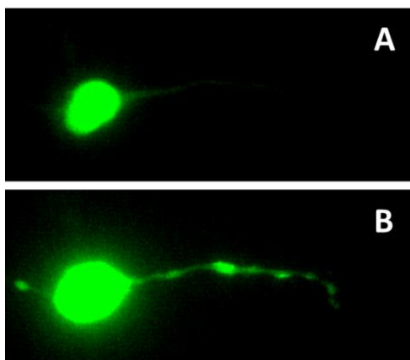


Figure 12. Representative image of Fluo-3 intensity increase before (A) and 25 minutes after (B) the addition of 100nM melittin

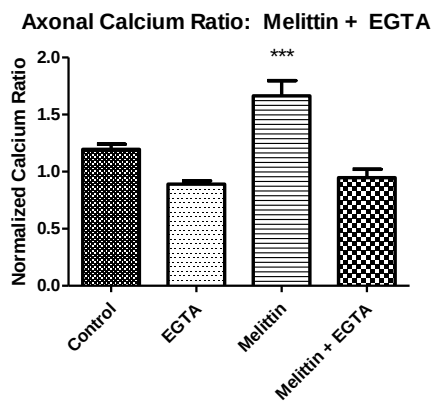


Figure 13. Melittin treatment results in intracellular axonal calcium increase that is prevented when extracellular calcium is chelated with EGTA. Cells were treated with 100nM melittin for 25 minutes and extracellular calcium was chelated with 1mM EGTA, which reduced the calcium increase back to baseline levels. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 8$ cells for each condition). All values are presented as means $\pm$ SEM.

Cytoskeletal Disruption

Cytoskeletal disruption is a pathology seen in TBI. In the hypothesized pathway (Figure 1), mechanoporation leads to cytoskeletal disruption and beading, presumably as a result of calcium-activated protease cascades that degrade the cytoskeleton. If this is the case, then increasing membrane permeability without mechanical damage should also lead to similar effects. Neuronal membranes were permeabilized using saponin and melittin and then underwent a simultaneous fixation and extraction procedure to remove free tubulin before staining for microtubules. Axons were imaged and focal disruptions in microtubules were quantified as a measure of cytoskeletal disruption. Both saponin (Figure 14) and melittin (Figure 15) treatment resulted in a statistically significant increase in focal disruptions.

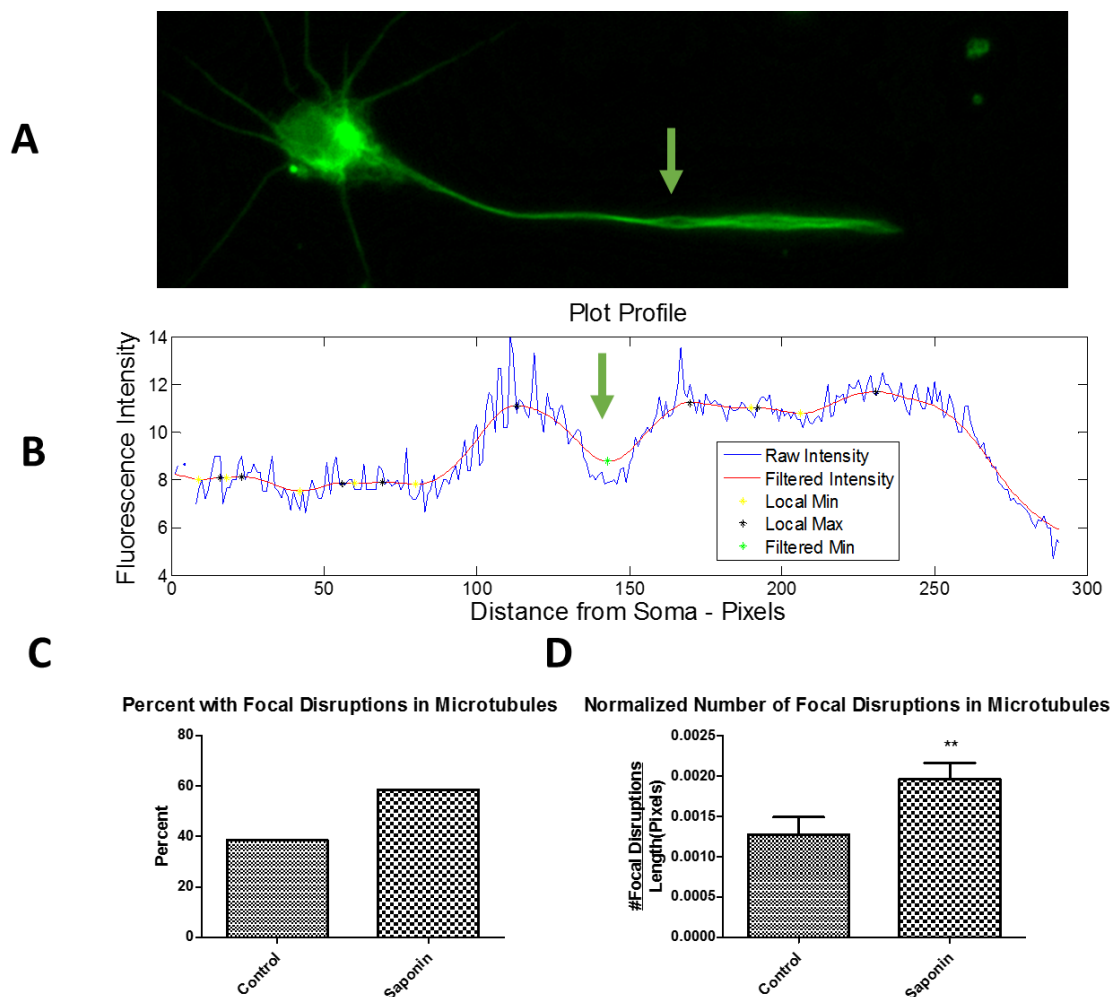


Figure 14. Cytoskeletal changes after saponin treatment. Cells were treated with 0.01% saponin for 30 minutes, after which they underwent a simultaneous fixation and extraction of free tubulin before being stained for tubulin. **A**, Representative image of saponin-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. **B**, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. **C**, Saponin treated cells have a higher percentage of cells with focal disruption in microtubules. **D**, Saponin treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p < 0.005$. $N > 80$ cells from three coverslips for each condition. Error bars represent SEM.

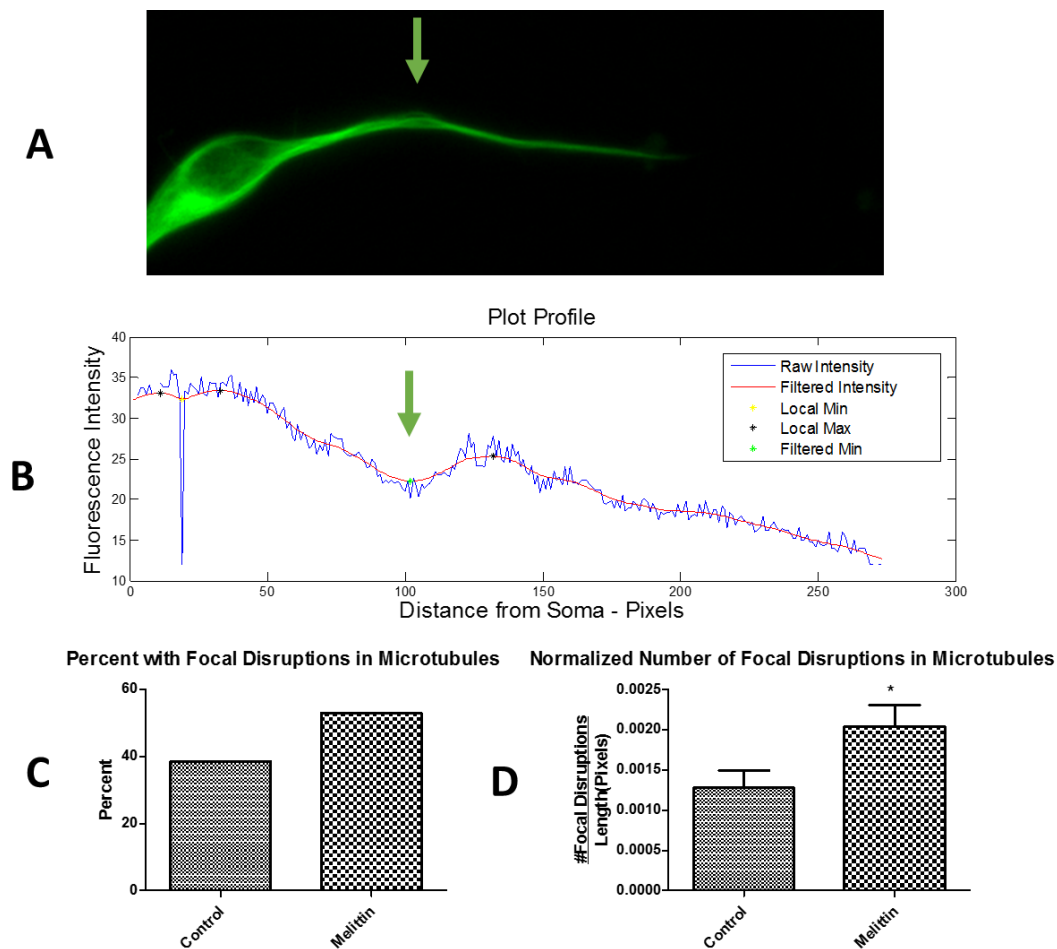


Figure 15. Cytoskeletal changes after melittin treatment. Cells were treated with 100nM melittin for 30 minutes, after which they underwent a simultaneous fixation and extraction of free tubulin before being stained for tubulin. **A**, Representative image of melittin-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. **B**, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. **C**, Melittin treated cells have a higher percentage of cells with focal disruption in microtubules. **D**, Saponin treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p < 0.05$. $n > 65$ cells from three coverslips for each condition. Error bars represent SEM.

Mitochondrial Dysfunction

Mitochondrial dysfunction is seen after TBI (Lifshitz, Sullivan et al. 2004). In the hypothesized pathway (Figure 1), this occurs as a result of excess calcium sequestration and increased energy need in order to restore ionic homeostasis. Since mitochondrial dysfunction results in changes in mitochondrial morphology, mitochondria were stained

with Mitotracker Green so that mitochondrial changes could be observed. Treating cells with melittin resulted in mitochondria that were shorter and rounder, indicating mitochondrial dysfunction (Figure 16).

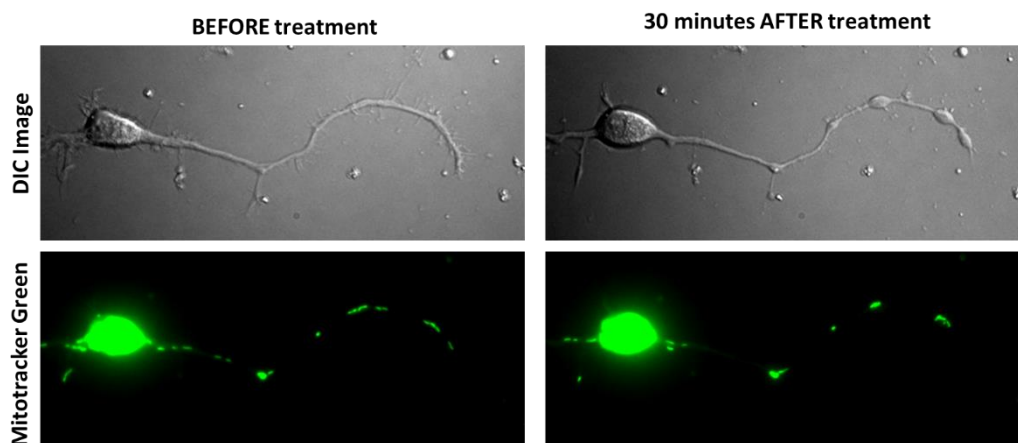


Figure 16. Melittin treatment results in change in mitochondrial morphology. Representative image of a neuron before and 30 minutes after the addition of 100nm melittin. The change in axonal mitochondria, stained with Mitotracker Green, can be seen.

Changes in mitochondrial morphology were quantified and melittin treatment was shown to result in mitochondria that were rounder and less elongated (Figure 17).

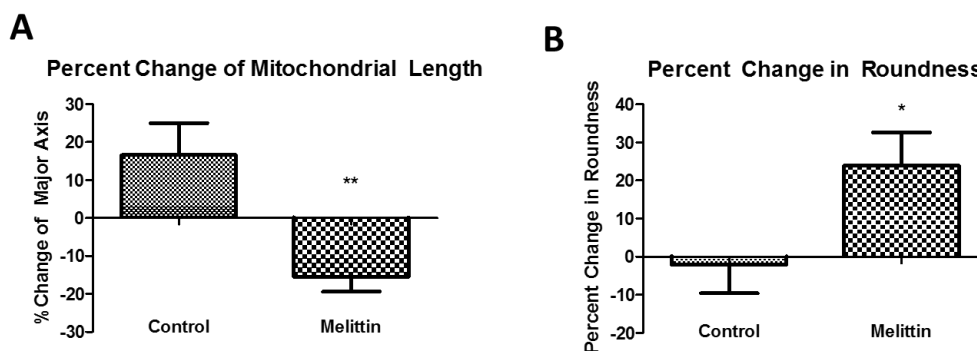


Figure 17. Membrane permeabilization with melittin results in a change in mitochondrial morphology. *A*, 30 minutes of 100nm melittin treatment results in a decrease in mitochondrial length ($p < 0.01$) and *B*, an increase in mitochondrial roundness ($p < 0.05$). (One-tailed t-test. $n > 100$ mitochondria from at least 15 cells for each condition). All values are presented as means $\pm$ SEM.

The mitochondria in cells treated with saponin followed a similar trend. While control cells became slightly longer and more elongated, saponin-treated cells became significantly shorter and rounder, indicating mitochondrial dysfunction (Figure 18).

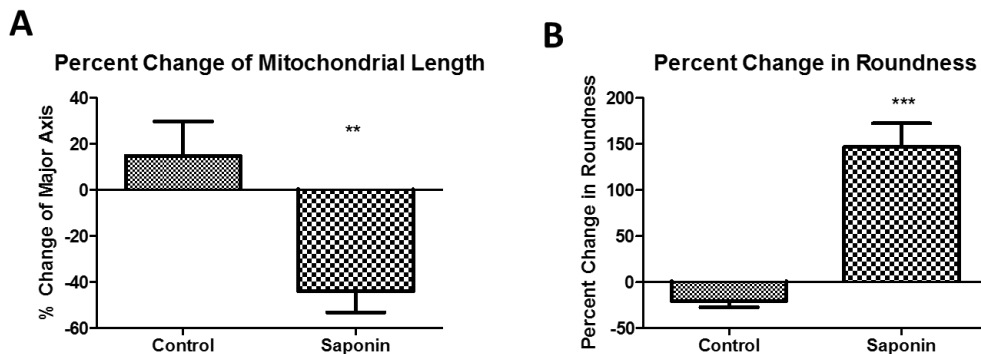


Figure 18. Membrane permeabilization with saponin results in a change in mitochondrial morphology. **A**, 30 minutes of 0.01% saponin treatment results in a decrease in mitochondrial length ($p < 0.01$) and **B**, an increase in mitochondrial roundness ($p < 0.001$). (One-tailed t-test. $n > 30$ mitochondria from at least 8 cells for each condition). All values are presented as means $\pm$ SEM.

3.3.2 Selectively Increasing Cytosolic Calcium Results in Pathologies Similar to TBI

Beading

Permeabilizing the membrane using saponin and melittin was shown here to result in pathologies seen in TBI. Membrane permeabilization also induces the influx of calcium into the cell. To examine the contribution of calcium to axonal pathology, calcium ionophore was used to allow for the influx of extracellular calcium without preceding membrane damage. First, it was established that A23187 was increasing intracellular

calcium by using Fluo-3 AM to make measurements of relative calcium changes (Figure 19).

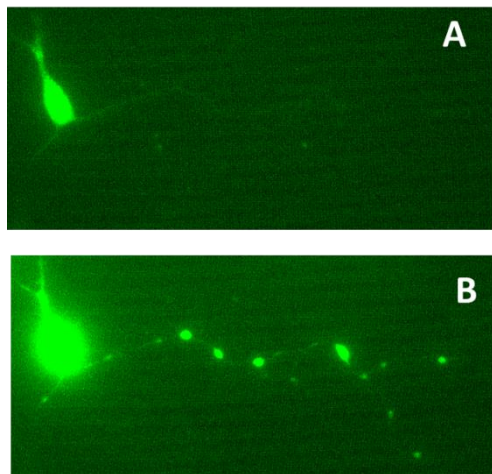


Figure 19. Representative image of Fluo-3 intensity increase before (A) and 30 minutes after (B) the addition 10 μ M A23187.

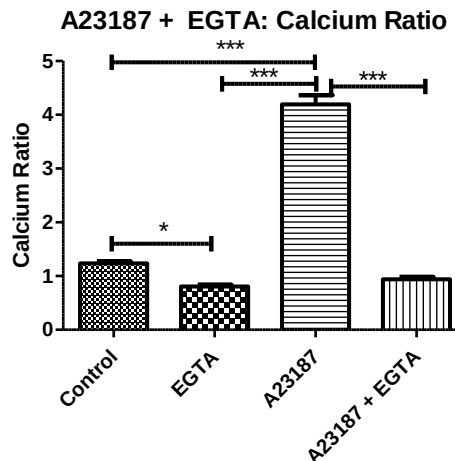


Figure 20. Chelating extracellular calcium with EGTA prevents the increase in axonal calcium caused by Calcium Ionophore A23187. Neurons were treated with 10 μ M A23187 for 30 minutes. The addition of 1mM EGTA reduced axonal calcium changes back to baseline levels (One-way ANOVA followed by Tukey's Multiple Comparison test, $n \geq 20$ cells for each condition). All values are presented as means $\pm$ SEM.

The addition of calcium ionophore A23187 to the extracellular media resulted in more than a four-fold increase in axonal calcium, indicating that A23187 is successful in increasing axonal calcium. This increase was completely prevented when EGTA was added to the extracellular media to chelate extracellular calcium, indicating that the intracellular calcium increase seen with A23187 treatment is purely a result of calcium influx from the

extracellular media (Figure 20). Increasing intracellular calcium without preceding membrane damage resulted in the formation of axonal beads (Figure 21).

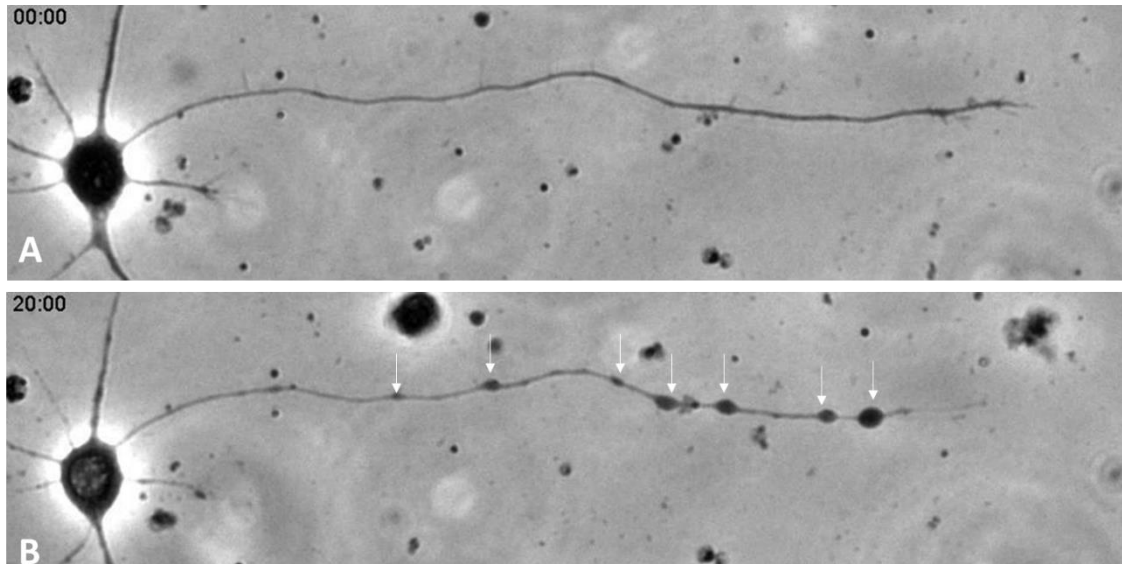


Figure 21. Increasing intracellular calcium with calcium ionophore A23187 results in a beaded morphology. *A*, Phase image of chick forebrain neuron before the addition of Saponin. *B*, the same neuron 20 minutes after the addition of 10uM A23187. Beads indicated by white arrows.

To confirm that beading was a result of calcium influx with A23187, EGTA was used to chelate extracellular calcium and completely prevented the formation of axonal beads (Figure 22).

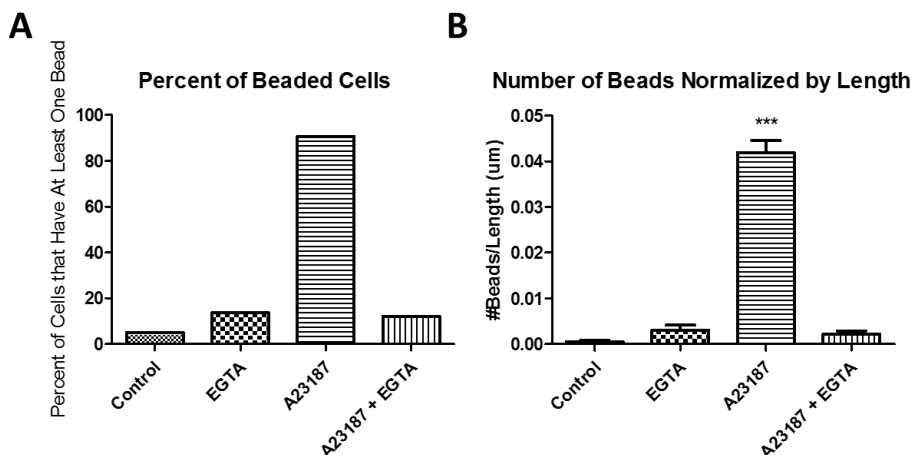


Figure 22. Chelating extracellular calcium with EGTA prevents bead formation induced by calcium ionophore. Cells were incubated in 10μM A23187 and 1mM EGTA for 20 minutes. **A**, EGTA added with A23187 reduces the percent of beaded cells compared to A23187 treatment alone. **B**, A23187 treated cells show significantly more beads than the control or EGTA sham cells. Adding EGTA to the A23187 treated cells brings beading back to baseline levels (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 75$ cells from three experiments). All values are presented as means $\pm$ SEM.

Inducing calcium entry with calcium ionophore A23187 resulted in over 80% of cells presenting with beads. Preventing calcium entry by chelating extracellular calcium with EGTA completely prevented A23187-induced bead formation.

Cytoskeletal Disruption

Increased cytosolic calcium, a result of increased membrane permeability, is thought to lead directly to cytoplasmic disruption through an activation of proteases and calpains. Therefore, increasing cytosolic calcium even in the absence of mechanical damage or membrane disruption should lead to cytoskeletal disruption. To test this, neurons were incubated in 10μM A23187 to increase intracellular calcium and then underwent a fixation and extraction procedure to stain microtubules. The plot profile for each axon was created and quantified in ImageJ, yielding the normalized number of

microtubule disruptions in each axon. When compared to controls, A23187 treated cells had more than twice the number of focal disruptions (Figure 23).

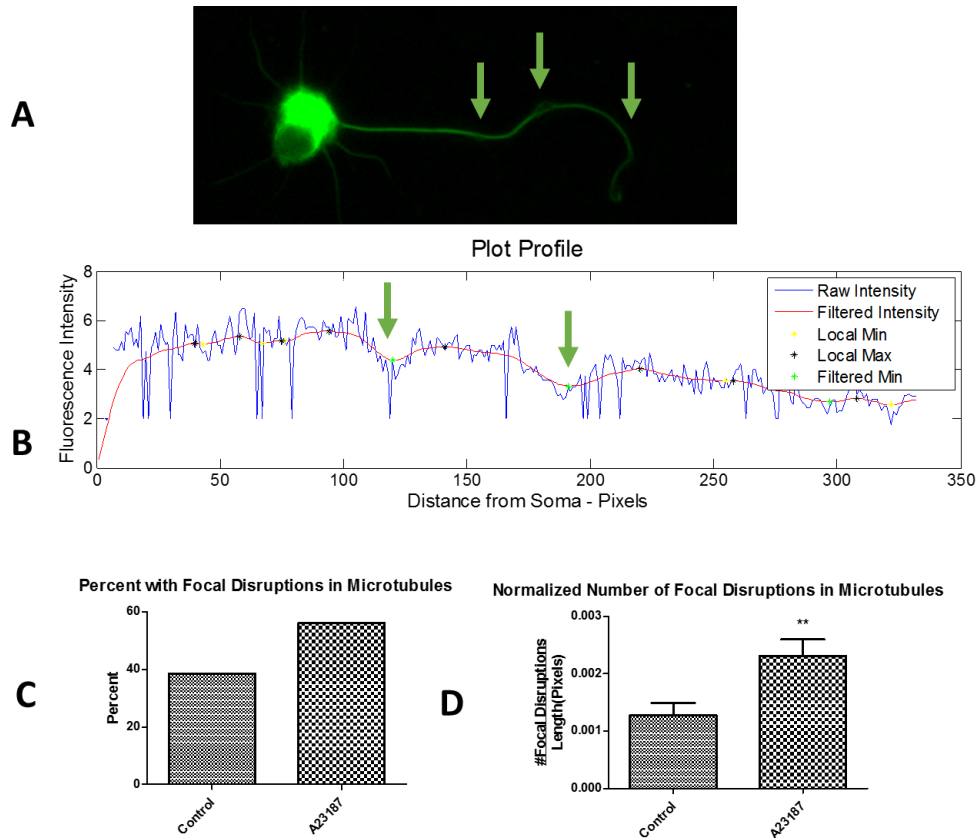


Figure 23. Cytoskeletal changes after increasing intracellular calcium with calcium ionophore A23187. Cells were treated with 10 μ M A23187 for 30 minutes, after which they underwent a simultaneous fixation and extraction of free tubulin before being stained for tubulin. **A**, Representative image of A23187-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. **B**, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. **C**, A23187 treated cells have a higher percentage of cells with focal disruption in microtubules. **D**, A23187 treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p < 0.005$. $N > 80$ cells from three coverslips for each condition. Error bars represent SEM.

Mitochondrial Dysfunction

Excess intracellular calcium is sequestered by the mitochondria, which become overloaded and damaged as they are overtaxed. Damaged mitochondria exhibit a change

in shape, from elongated to more circular. To establish that increasing intracellular calcium with calcium ionophore A23187 also resulted in mitochondrial dysfunction, mitochondria were stained with Mitotracker Green so that mitochondrial changes could be observed. Treating cells with A23187 resulted in mitochondria that were shorter and rounder, indicating mitochondrial dysfunction (Figure 24).

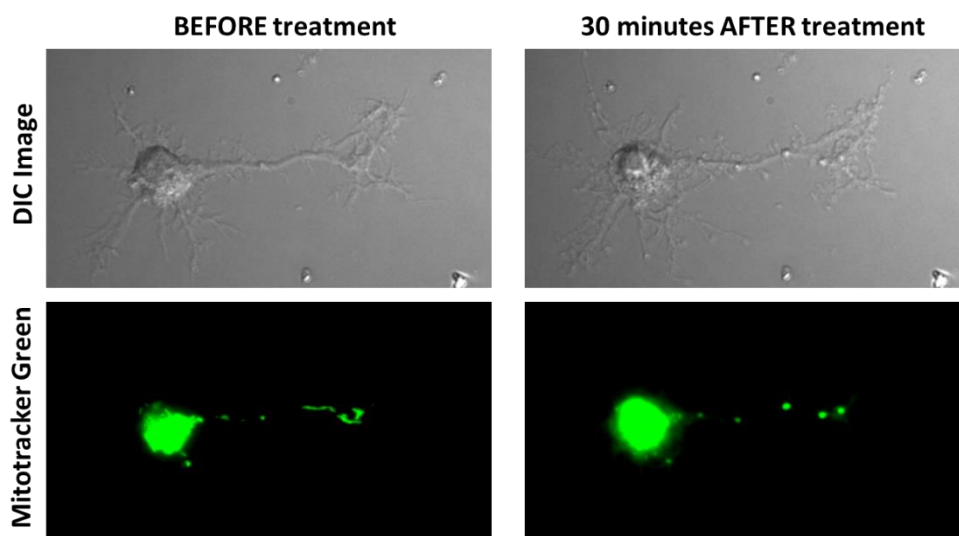


Figure 24. A23187 treatment results in change in mitochondrial morphology. Representative image of a neuron before and 30 minutes after the addition of 10 μ M A23187. The change in axonal mitochondria, stained with Mitotracker green, can be seen.

3.3.3 Blocking Calcium Influx Mitigates Beading after Permeabilizing the Cell Membrane

Permeabilizing the cell membrane is hypothesized to lead to axonal beading through a variety of mechanisms. Membrane permeabilization results in a loss of ionic homeostasis and an increase in metabolic need as mitochondria work to produce the ATP required to restore ionic homeostasis. One major contributor to the loss of ionic homeostasis is the influx of calcium, which has been shown to lead to beading without general membrane permeabilization. It is clear that calcium contributes to axonal

beading, but not that it is the only contributor. To evaluate the role of calcium in axonal beading after membrane permeabilization, EGTA was used to chelate extracellular calcium and prevent calcium influx after membrane permeabilization. Permeabilizing the cell membrane with saponin resulted in axonal beading that was reduced by more than 50%, but not abolished, when extracellular calcium was chelated with EGTA (Figure 25).

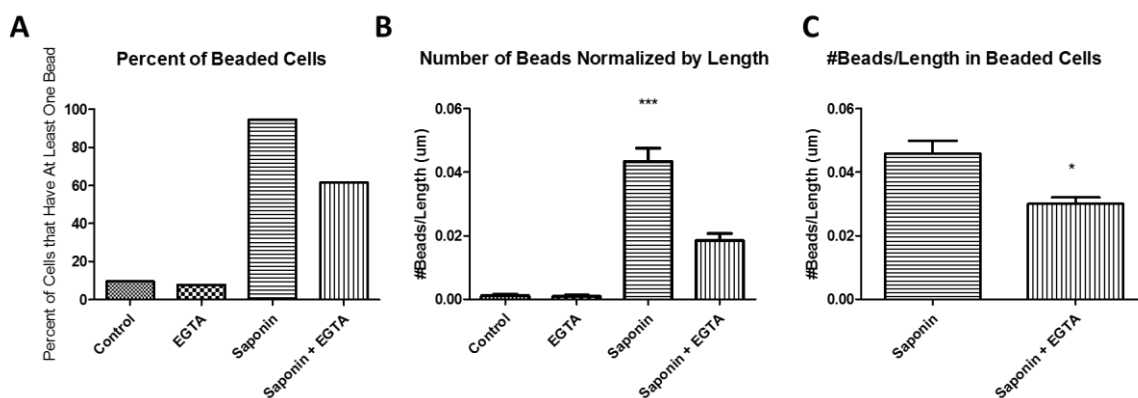


Figure 25. Chelating extracellular calcium reduces axonal beading after permeabilizing the membrane with saponin. Cells were incubated in 0.01% saponin and 1mM EGTA for 20 minutes. **A**, EGTA added with saponin reduces the percent of beaded cells compared to saponin treatment alone. **B**, Saponin treated cells show significantly more beads than the control or EGTA sham cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.01$, $n > 35$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with saponin and EGTA have significantly less beading than cells treated with saponin alone (Mann Whitney test, $p < 0.05$, $n \geq 35$ cells from three experiments). All values are presented as means $\pm$ SEM.

The contribution of extracellular calcium to axonal beading after membrane damage was also investigated using melittin to permeabilize the cell membrane. Like saponin, melittin permeabilizes the cell membrane and allows for ion exchange, but is less likely than saponin to allow for protein exchange. Chelating extracellular calcium with EGTA when permeabilizing the membrane with melittin produced similar results as when saponin was used to permeabilize the membrane, beading was mitigated but not abolished (Figure 26).

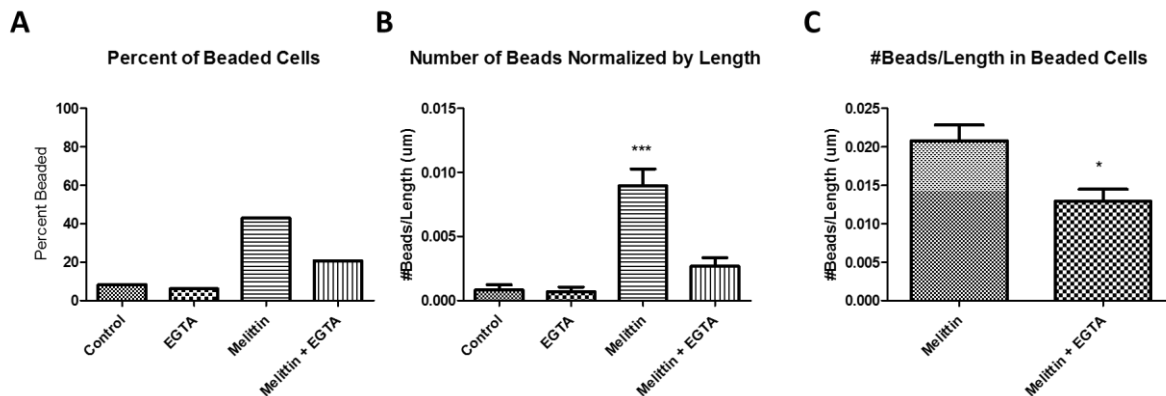


Figure 26. Chelating extracellular calcium prevents axonal beading after permeabilizing the membrane with melittin. Cells were incubated in 100nM melittin and 1mM EGTA for 20 minutes. A, EGTA added with melittin reduces the percent of beaded cells compared to melittin treatment alone. B, Melittin treated cells show significantly more beads than the control or EGTA sham cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 80$ cells from three experiments). C, In cells that present at least one bead, cells treated with melittin and EGTA have significantly less beading than cells treated with melittin alone (Mann Whitney test, $p < 0.05$, $n \geq 15$ cells from three experiments). All values are presented as means $\pm$ SEM.

3.4 Discussion

Membrane damage is hypothesized to be the initiating factor in all subsequent secondary damage after mechanical injury in TBI. Secondary damage includes increased intracellular calcium, cytoskeletal disruption, mitochondrial damage, and axonal beading. To evaluate the role of membrane damage in axonal pathology, neuronal membranes were permeabilized using both saponin and melittin and secondary pathologies were observed. Both treatments were successful in permeabilizing the membrane, increasing intracellular calcium, disrupting microtubules, and producing axonal beads.

To further investigate the mechanism of axonal pathology after membrane damage, the calcium portion of the pathway was isolated and calcium ionophore A23187 was used to increase intracellular calcium without preceding membrane damage. Increasing intracellular calcium was also able to initiate common pathologies of TBI,

including cytoskeletal disruption, mitochondrial damage, and axonal beading. This confirmed that calcium influx plays a major role in axonal pathology after injury. However, it is not the only important factor. When extracellular calcium was chelated using EGTA and the cell membrane was permeabilized, axonal beading was reduced but not completely prevented, implying that there are more factors at play than just calcium.

Work from the Povlishock lab has shown that mechanoporation is occurring after TBI and that a subset of mechanoporated neurons progress to cellular degeneration and subsequent death (Singleton and Povlishock 2004, Farkas, Lifshitz et al. 2006). Similar results have been shown *in vitro*, reaffirming the importance of membrane poration (Geddes, Cargill et al. 2003, Kilinc, Gallo et al. 2007, Kilinc 2008, Kilinc, Gallo et al. 2008, LaPlaca, Prado et al. 2009). Here, we show that membrane poration in the absence of mechanical damage is sufficient to induce the secondary pathologies seen in TBI.

George et al. showed that axotomy-induced neuronal death is dependent on extracellular calcium, indicating that calcium influx is a mechanism for neuronal degeneration and death (George, Glass et al. 1995). Gwag et al have shown that calcium ionophore can lead to neuronal degeneration via two mechanisms; low concentrations lead to apoptosis whereas high concentrations result in necrosis (Gwag, Canzoniero et al. 1999). Bellomo et al have shown that calcium ionophore A23187 can induce blebbing that is prevented when extracellular calcium is not present, and that ionophore use also results in cytoskeletal alterations (Bellomo and Mirabelli 1992). Emery et al. have also shown that A23187 leads to mitochondrial abnormalities and cytoskeletal disruption in neurons (Emery and Lucas 1995).

Here, we show that increasing axonal calcium is sufficient to induce the secondary damage seen in TBI and that preventing calcium influx after membrane permeabilization is able to reduce, but not completely prevent, axonal beading. Extracellular calcium plays an important role in axonal pathology, but is not the only factor at play after membrane damage. Even if calcium is not present there is still a loss of ionic homeostasis involving the influx of sodium and an efflux of potassium as well as other ions. The restoration of ionic homeostasis taxes the mitochondria. If the mitochondria become dysfunctional, the cell will be unable to restore homeostasis. Disrupted mitochondrial function also results in an increase in oxidative stress and a release of sequestered mitochondrial calcium, which may also contribute to beading.

The results presented here show that membrane permeabilization with saponin and melittin result in an influx of extracellular calcium because saponin and melittin treatment results in an increase in intracellular calcium that is prevented when EGTA is used to chelate extracellular calcium. However, these studies do not provide evidence for intracellular calcium release. It is likely that permeabilizing the membrane also allows for the influx of EGTA into the cell, where it can chelate intracellular calcium as well.

Chapter 4: The Contribution of Mitochondria to Membrane

Damage-Induced Axonal Pathology

4.1 Background

Mitochondrial impairment has been reported after TBI (Maxwell, McCreath et al. 1995, Xiong, Gu et al. 1997, Dikranian, Cohen et al. 2008). Mitochondrial health has been associated with TBI recovery, and patients with marginal mitochondrial impairment demonstrate a better outcome than those with profound mitochondrial impairment, who have a poor prognosis (Signoretti, Marmarou et al. 2008). *In vitro* experiments have suggested that beads may form where mitochondria are present, suggesting that they play a significant role in axonal pathology (Greenwood, Mizielinska et al. 2007, Kilinc, Gallo et al. 2008). Mitochondria are vital organelles that provide energy and act as calcium buffers. Mitochondria are hypothesized to play multiple important roles in axonal pathology: as an energy source, ROS source, and calcium source. TBI has been shown to result in a rapid loss of ATP (Sullivan, Keller et al. 1998), which can lead to necrosis due to a lack of available ATP to fuel membrane pumps, preventing the restoration of homeostasis and leading to neuronal death (Santos, Moreno et al. 1996).

4.1.1 Axonal Mitochondria

Mitochondria perform multiple vital roles inside the cell. They are the main source of cellular energy, and also serve to store and release intracellular calcium (Rizzuto, Bernardi et al. 2000, Parekh 2008). Mitochondria are dynamic organelles which localize

to regions of high energy use along the axon (Bereiter-Hahn 1990, Hollenbeck and Saxton 2005). They travel along microtubules using kinesin and dynein in a combination of saltatory and bidirectional movements, as well as stationary docking (Ligon and Steward 2000, Hollenbeck and Saxton 2005, Pathak, Sepp et al. 2010). Importantly, mitochondria have been shown to accumulate in beads (Greenwood, Mizielska et al. 2007).

The main function of mitochondria is to produce high energy intermediates (NADH and ATP) through the tri-carboxylic acid (TCA) cycle and oxidative phosphorylation (Gleichmann and Mattson 2011). The TCA cycle feeds the electron transport chain which converts oxygen to water and pumps protons from the mitochondrial matrix to generate a proton gradient, which in turn is used by an ATPase to generate ATP from ADP. The movement of protons creates a negative membrane potential in the mitochondria, so cations such as calcium will tend to accumulate in the mitochondrial matrix (Rizzuto, Bernardi et al. 2000).

4.1.2 Causes of Mitochondrial Dysfunction

Calcium

Mitochondria play an important role not only for energy production but also for calcium signaling. Mitochondria serve as calcium buffers, helping to regulate the cytosolic concentration of calcium by sequestering it inside the inner mitochondrial matrix if it gets too high. Axonal mitochondria do not normally accumulate calcium (Scarpa, Malmstrom et al. 1976), but when intracellular calcium is increased above 5 μM , the calcium can be

sequestered in the mitochondria (Blaustein 1988). However, large increases in intracellular calcium due to extracellular influx have been closely associated with mitochondrial dysfunction (Nasr, Gursahani et al. 2003).

The transport of calcium into the inner mitochondrial matrix depolarizes the mitochondria and inhibits oxidative phosphorylation and ATP synthesis (Won, Kim et al. 2002). Calcium accumulation in mitochondria can also result in a loss of matrix components, impairment of mitochondrial function, and swelling of the organelle leading to outer membrane rupture (Lemasters, Nieminen et al. 1998). Accumulated intra-mitochondrial calcium can also lead to the opening of what has been termed the mitochondrial permeability pore (MPP). This is defined as a non-selective increase in the permeability of the inner membrane to solutes of molecular weight less than approximately 1,500 Da (Fiskum 2000) and has been associated with excessive calcium accumulation (Fiskum 2000, Hansson, Mansson et al. 2004).

Oxidative stress

Under conditions of oxidative stress, mitochondria experience a change in permeability of the inner mitochondrial membrane that results in a complete collapse of the mitochondrial membrane potential. This loss of membrane potential is accompanied by mitochondrial swelling and the release of cytochrome c into the cytoplasm, where it activates certain caspases and induces apoptotic death (Simpkins, Yi et al. 2010). The loss of ATP can also cause ATP-dependent membrane transporters to fail which can lead to necrosis from increased osmotic stress.

4.1.3 The Mitochondrial Permeability Pore (MPP) and the Mitochondrial Permeability Transition (MPT)

Under certain stress conditions (including high calcium concentrations and oxidative stress) mitochondria undergo what is referred to as the Mitochondrial Permeability Transition (MPT) which involves an opening of the Mitochondrial Permeability Pore (MPP). The MPP refers to a channel of the inner mitochondrial membrane thought to be ~3nm (Bernardi, Krauskopf et al. 2006) that allows for the free diffusion of solutes less than 1500 Da (Halestrap 2009). While the details of the MPP are not well understood, the dominant hypothesis is that the pore spans the mitochondrial inner and outer membranes and is composed of proteins from both membranes and from the mitochondrial matrix (Kim, He et al. 2003). The MPT causes mitochondrial swelling and uncoupling of oxidative phosphorylation (Gunter and Pfeiffer 1990). This prevents mitochondria from producing ATP and leads to ATP depletion and bioenergetic failure (Halestrap 2009).

The MPT can also occur in an unregulated fashion. This mechanism of pore opening does not require calcium for conductance and is not blocked by calcium chelators or drugs known to prevent the opening of the MPP (He and Lemasters 2002). It has been reported that unregulated MPP opening occurs at high concentrations of various chemical inducers and that the size of regulated and unregulated pores is similar (Kim, He et al. 2003).

4.1.4 Mitochondria and Oxidative Stress

Mitochondrial dysfunction can result in a decrease in essential ATP and an increase in the production of reactive oxygen species (ROS) (Lemasters, Nieminen et al. 1998, Simpkins, Yi et al. 2010). The brain may be particularly vulnerable to oxidative damage because of its high rate of metabolic activity, the non-replicating nature of neurons, and the high ratio of membrane to cytoplasm (Evans 1993, Shohami, Beit-Yannai et al. 1997). Mitochondria are one of the main sources ROS (Lemasters, Nieminen et al. 1998), where free oxygen radicals are generated as byproducts from the electron transport chain (Adam-Vizi 2005, Cozzolino and Carri 2012). *In vivo*, neuronal injury has been shown to result in mitochondrial dysfunction and ROS formation (Arundine, Aarts et al. 2004). The formation of ROS has also been documented in multiple models of TBI (Kontos and Povlishock 1986, Ikeda and Long 1990, Povlishock, Erb et al. 1992, Muir, Lyeth et al. 1995, Hall, Andrus et al. 1996, Shohami, Beit-Yannai et al. 1997, Arundine, Aarts et al. 2004).

Oxidative stress is caused by an imbalance between ROS generation and oxidative defense systems (Ebadi, Srinivasan et al. 1996, Jenner 1996, Simonian and Coyle 1996). An increase in oxidative stress can be caused by either an increase in ROS, a decrease in antioxidants, or both. ROS, or free radicals, are highly reactive molecules that contain an unpaired electron in the outermost orbit, increasing their potential for chemical reactivity(Ikeda and Long 1990). They are normal byproducts of oxidative metabolism that are constantly produced and are also tightly controlled by endogenous antioxidant mechanisms. Sources of ROS in the brain include mitochondria, nitric oxide synthase

(NOS), and arachidonic acid metabolism (Hall, Yonkers et al. 1992, Schulz, Lindenau et al. 2000). Mitochondria produce ROS when electrons spontaneously leak from the electron transport chain and react with available O_2 to produce superoxide ($O_2^{\cdot -}$) [Equation 2]. The superoxide radical is the most commonly occurring cellular free radical and is produced when an oxygen molecule gains an electron (Gilgun-Sherki, Rosenbaum et al. 2002). Superoxide can be converted to H_2O by superoxide dismutases (SODs) and glutathione peroxidase (Won, Kim et al. 2002). If not converted, the superoxide can be processed to produce the hydroxyl radical ($\cdot OH$) via the Fenton reaction [Equation 6] or to produce peroxynitrite ($ONNO$) by reacting with nitric oxide [Equation 7]. These ROS also inhibit the electron transport chain in the mitochondria and can amplify the generation of free radicals (Choi, Klintworth et al. 2011).

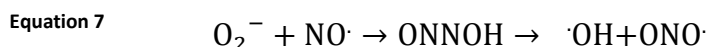
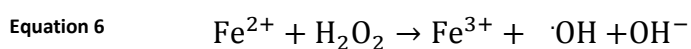
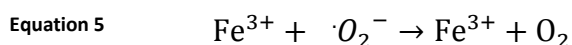
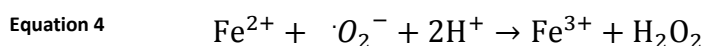
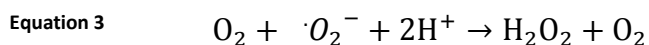
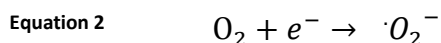


Table 1. Equations for the main reactions responsible for cellular oxidative stress

Cells normally have a number of mechanisms to defend against increased oxidative stress (Evans 1993, Simonian and Coyle 1996). Natural antioxidants fall into two categories: enzymes and low molecular weight antioxidants (LMWAs). Enzymes include

SOD and catalase (Gilgun-Sherki, Rosenbaum et al. 2002). The LMWA group contains tocopherols (Vitamin E), glutathione, and ascorbic acid (Vitamin C). These serve to protect the cell from increased oxidative stress under normal conditions of ROS production.

4.1.5 Hypothesis and Summary of Experiments

The goal of this chapter is to investigate the role that mitochondria play in axonal pathology, including common consequences of mitochondrial dysfunction: increased oxidative stress and increased cytosolic calcium released from intracellular sources. First, mitochondrial function was disrupted using Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and downstream pathologies were observed, hypothesizing that mitochondrial dysfunction would induce axonal beading, increased cytosolic calcium, and cytoskeletal disruption. Since increased oxidative stress is a consequence of mitochondrial damage, the component of oxidative stress without preceding mitochondrial or membrane damage was isolated using the hydroperoxide donor tert-butyl hydroperoxide (TbHp), hypothesizing that this too would lead to axonal beading, mitochondrial dysfunction, increased cytosolic calcium, and focal disruption of microtubules. The component of oxidative stress was then further investigated using the antioxidant glutathione as a general scavenger and catalase as a hydrogen-peroxide-specific antioxidant to mitigate beading after isolated perturbations to mitochondria, cytosolic calcium, and membrane permeability. It was assumed that if oxidative stress was playing a role in downstream pathology after these insults then antioxidant use would decrease axonal beading.

Mitochondrial damage can lead to the release of mitochondrially sequestered calcium, thereby increasing cytosolic calcium. To investigate the role of intracellularly-released calcium, the intracellular calcium chelator BAPTA-AM was used to chelate intracellular calcium with the assumption that if increased cytosolic calcium was contributing to neuronal degeneration then chelating intracellular calcium would prevent beading after isolated perturbations to the damage pathway. To elucidate the source of increased cytosolic calcium, relative calcium measurements were used while chelating extracellular calcium with EGTA after disrupting mitochondrial function with CCCP and increasing oxidative stress with TbHp, hypothesizing that if intracellular calcium release was contributing to higher axonal calcium levels then EGTA would not be able to abolish the calcium increase. Finally, the role of the mitochondrial permeability transition (MPT) was investigated using Cyclosporin A (CsA) to prevent the regulated opening of the MPP and damaging the neuron using CCCP to target mitochondria, TbHp to induce oxidative stress, saponin and melittin to increase membrane permeability, and A23187 to increase cytosolic calcium. It was hypothesized that if the MPT was playing a role in downstream pathology after these perturbations then CsA would be able to reduce beading.

4.2 Materials and Methods

4.2.1 *Inducing mitochondrial dysfunction with CCCP*

Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is a proton ionophore used extensively to study energy coupling in mitochondria (Benz and McLaughlin 1983). It

transports hydrogen ions across membranes and acts as an uncoupler of oxidative phosphorylation (Vaur, Sartor et al. 2000). This is because the electron transport chain pumps protons out of the mitochondrial matrix in order to create an electrochemical gradient (Werth and Thayer 1994). Therefore CCCP uncouples electron transfer and inhibits ATP synthesis in the mitochondria, collapsing the proton gradient and membrane potential across the inner mitochondrial membrane (Suzuki, Higuchi et al. 1999).

4.2.2 Inducing Increased Oxidative Stress with TbHp

Tert-butyl hydroperoxide (TbHp) was used to induce oxidative stress in cells. It is a membrane-permeable hydrogen peroxide donor widely used to induce oxidative stress in a variety of cells (Altman, Zastawny et al. 1994, Nardini, Pisu et al. 1998, Sestili, Guidarelli et al. 1998, Kanupriya, Prasad et al. 2007).

4.2.3 Preventing Increased Oxidative Stress with Antioxidants

Two different antioxidants were used to investigate the contribution of oxidative stress to axonal pathology: Glutathione (GSH) and catalase. GSH was used as a general scavenger for all ROS and catalase was used specifically for its interaction with hydrogen peroxide.

Glutathione

Oxidative stress was battled using Glutathione (GSH), which acts as an antioxidant and is the most abundant non-protein thiol found in the brain (Shohami, Beit-Yannai et al.

1997). It is a tri-peptide consisting of glycine, cysteine, and glutamic acid. GSH reacts directly with radicals in nonenzymatic reactions (Dringen, Gutterer et al. 2000). Glutathione has a molecular weight of 307.32 and is not cell membrane permeable. For these experiments, GSH was used as a general scavenger to decrease oxidative stress from all sources. Cells were pre-incubated in GSH before the addition of any damaging insults in order to increase the probability of GSH entering the cell.

Catalase

Catalase (MW 250 kDa) was used to specifically target hydrogen peroxide by catalyzing its conversion into water and oxygen. Hydrogen peroxide is freely diffusible through the cell membrane, so extracellular catalase would still be able to reduce hydrogen peroxide concentrations.

4.3.4 Chelating Intracellular Calcium with BAPTA-AM

1,2-bis-(2-aminophenoxy)ethane-4,4',5,5'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) is a cell membrane permeable analog of the calcium chelator BAPTA. It cannot chelate calcium until the acetoxymethyl group is removed by cytoplasmic esterases and it is converted to BAPTA (Tymianski and Tator 1996) Therefore BAPTA-AM is a useful tool to chelate intracellular calcium while not affecting extracellular calcium.

4.3.5 *Preventing the MPT with Cyclosporin A*

Cyclosporin A (CsA) inhibits the MPT (Fournier, Ducet et al. 1987, Crompton, Ellinger et al. 1988, Broekemeier and Pfeiffer 1989, Pastorino, Snyder et al. 1993, Nieminen, Saylor et al. 1995, Crompton, Virji et al. 1998, Bernardi, Krauskopf et al. 2006). In addition to preventing the regulated opening of the MPP, CsA also inhibits the calcium-dependent phosphatase calcineurin (Ho, Clipstone et al. 1996) but its ability to block the MPT is unrelated to its immunosuppressive activity (Kim, He et al. 2003). Cyclosporin A does not block an unregulated opening of the MPP (He and Lemasters 2002, Kim, He et al. 2003). He et al. have proposed two functional modes for the MPP: one activated by calcium and inhibited by CsA, and a second unregulated mode that is calcium independent and insensitive to CsA. In some cases the inhibition of the MPT by CsA can be transitory or ineffective (Kristal and Brown 1999).

In one theorized model of pore formation and the MPT, pores are formed by the aggregation of misfolded and damaged integral membranes in the mitochondria. Chaperone-like proteins block conductance through the misfolded protein clusters but increased calcium opens these pores in a manner which can be blocked by CsA – referred to as “regulated” pore opening. However when the protein clusters exceed the chaperone proteins available to block conductance CsA is no longer able to block the pore opening and this is referred to as “unregulated” pore opening. It is thought that CsA inhibits the MPT by interfering with the binding of cyclophilin D to the adenine nucleotide translocator within the mitochondria (Connern and Halestrap 1994, Connern and Halestrap 1996, Nicolli, Basso et al. 1996, Sullivan, Rabchevsky et al. 2005). When CsA

binds to cyclophilin D in the MPP complex, cyclophilin D remains associated with the complex but the complex is no longer able to form a pore (Connern and Halestrap 1994).

4.3 Results

4.3.1 *Disrupting Mitochondria Results in a Pathology seen in TBI*

In the hypothesized pathway (Figure 1), damaged mitochondria prevent cell recovery and contribute to axonal pathology by increasing oxidative stress and releasing sequestered calcium which can lead to microtubule disruption and beading. Therefore isolated mitochondrial impairment in the absence of mechanical and chemical membrane disruption should lead to the cellular pathologies seen in TBI. To test this, CCCP was used to disrupt mitochondrial function to see if mitochondrial disruption led beading and increased cytosolic calcium

Mitochondrial Dysfunction

First, it was established that CCCP was capable of disrupting mitochondrial function by quantifying changes in mitochondrial morphology (Figure 27) after CCCP treatment.

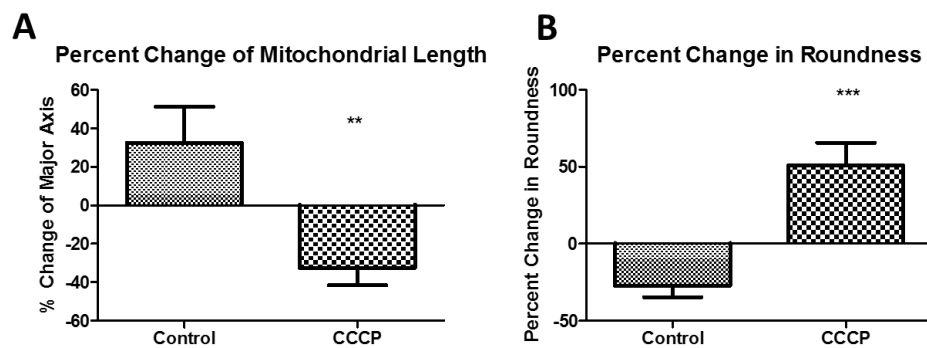


Figure 27. Disrupting mitochondrial function with CCCP results in a change in mitochondrial morphology. 60 minutes of 100 μ M CCCP treatment results in *A*, a decrease in mitochondrial length ($p < 0.01$) and *B*, an increase in mitochondrial roundness ($p < 0.0001$). (One-tailed t-test. $n > 100$ mitochondria from at least 8 cells for each condition). All values are presented as means $\pm$ SEM.

Beading

When chick forebrain neurons were treated with CCCP for 60 minutes, the result was a beaded morphology similar to that obtained by mechanical injury models (Figure 28).

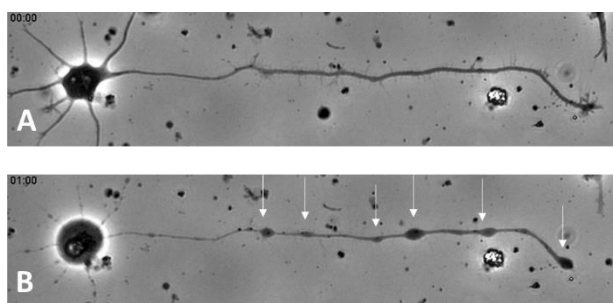


Figure 28. Disrupting mitochondria with CCCP results in a beaded morphology. *A*, Phase image of chick forebrain neuron before the addition of CCCP. *B*, the same neuron 60 minutes after the addition of 100 μ M CCCP. Beads indicated by white arrows.

Calcium Increase

It is hypothesized that one of the ways in which mitochondria contribute to the propagation of neuronal degeneration is through the release sequestered calcium. To determine if intracellular calcium is increased as a result of mitochondrial dysfunction,

CCCP was used to disrupt mitochondrial function without preceding membrane damage. Fluo-3 was used as a calcium indicator (Figure 29). Ratiometric calcium measurements were made using Calcein Red/Orange to correct for possible loss of dye, comparing fluorescence ratio before treatment and 60 minutes after the addition of 100nM CCCP. It was shown that CCCP results in a significant increase of axonal calcium (Figure 30).

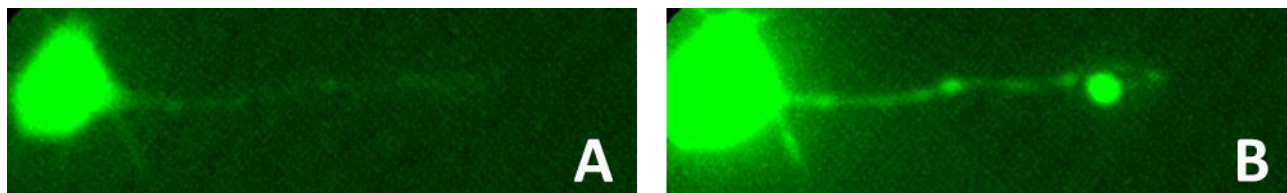


Figure 29. Representative diagram of increase in Fluo-3 fluorescence before (A) and 60 minutes after (B) the addition of 100nM CCCP

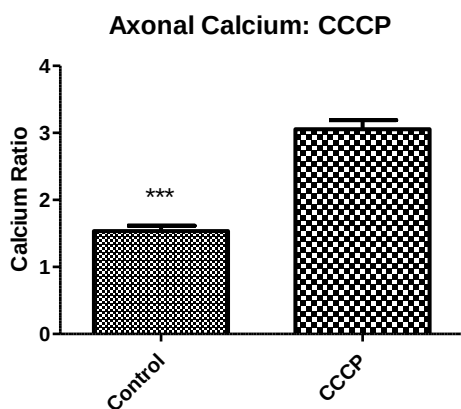


Figure 30. CCCP treatment results in a significant increase in axonal calcium. Calcium ratio was calculated as the normalized ratio of axonal Fluo-3 to Calcein Red/Orange fluorescence before and 60 minutes after the addition of 100nM CCCP (One-tailed t-test, $p < 0.0001$, $n > 20$ cells for each condition). All values are presented as means $\pm$ SEM.

Cytoskeletal Disruption

Inducing mitochondrial dysfunction is thought to result in an increase in cytosolic calcium through the release of mitochondrially sequestered calcium. High intracellular calcium concentrations have been correlated with microtubule disruption through an activation of proteases. To see if disrupting mitochondrial function leads to focal disruptions of microtubules, neuronal cultures were treated with 100 nm CCCP for 60

minutes before undergoing a fixation-extraction procedure and being stained for tubulin. Custom Matlab code was used to create and filter plot profiles of fluorescence intensity along the axon to identify focal disruption in microtubules (Figure 31). CCCP treatment resulted in an increase in percentage of cells that presented with focal disruptions – from 43% to 72%. There was also a statistically significant increase in the normalized number of focal disruptions.

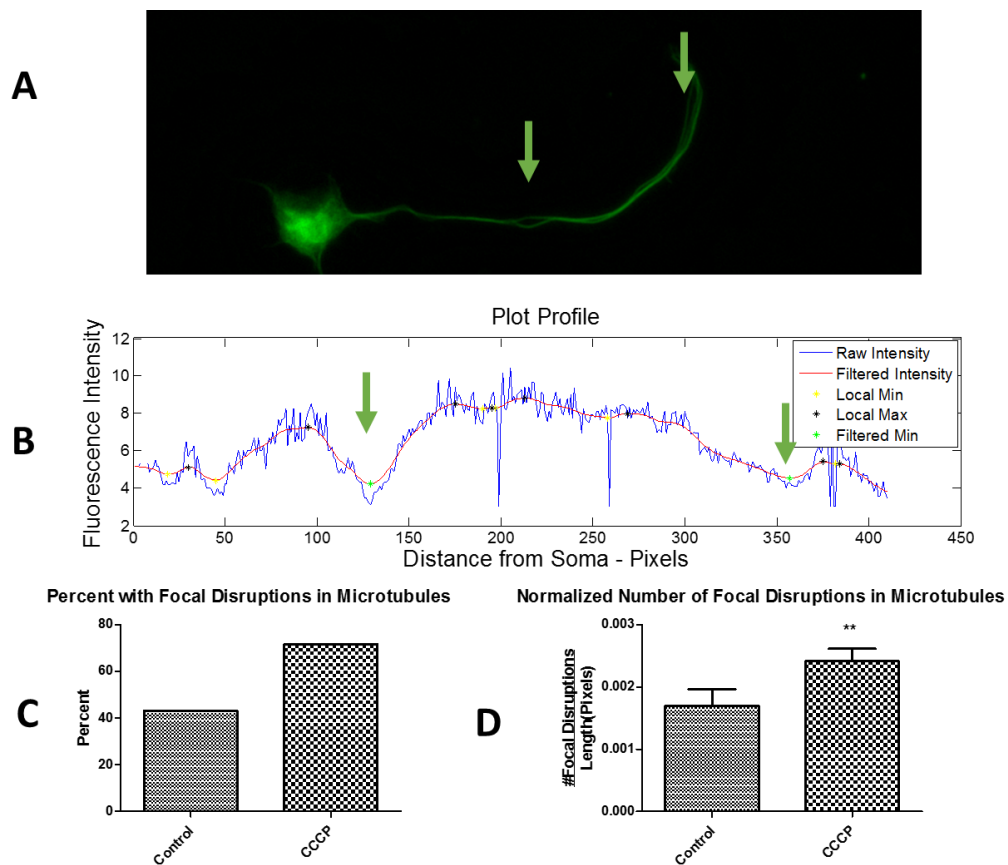


Figure 31. Cytoskeletal changes after CCCP treatment. Cells were treated with 100nM CCCP for 60 minutes, after which they underwent a simultaneous fixation and extraction of free tubulin before being stained for tubulin. **A**, Representative image of CCCP-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. **B**, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. **C**, CCCP treated cells have a higher percentage of cells with focal disruption in microtubules. **D**, CCCP treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p < 0.01$. $N > 75$ cells from three coverslips for each condition. Error bars represent SEM.

4.3.2 *Increasing Oxidative Stress with TbHp Results in a Pathology seen in TBI*

In the hypothesized TBI pathway, oxidative stress is increased as a result of mitochondrial dysfunction and can contribute to further impairment of the mitochondrial function. Therefore increased oxidative stress damages the mitochondria preventing the ATP-dependent restoration or maintenance of ionic homeostasis and the release of mitochondrially-sequestered calcium. Calcium can propagate the injury pathway through caspase and calpain-dependent pathways. The increased oxidative stress is also assumed to lead to lipid peroxidation and increased membrane permeability, thereby resulting in a loss of ionic homeostasis and an influx of extracellular calcium as well as furthering the bioenergetics crisis and need for mitochondrial ATP production.

If the hypothesized pathway (Figure 1) is correct, increasing oxidative in the absence of preceding membrane disruption and mitochondrial damage should lead to the cellular pathologies seen in TBI. To test this, the hydroperoxide donor TbHp was used to see if increasing oxidative stress led to beading, increased axonal calcium, mitochondrial disruption, and cytoskeletal alterations.

Beading

Increased oxidative stress is hypothesized to lead to beading through two main calcium-dependent pathways. The first is through damaging mitochondria and increasing cytosolic calcium through the release of mitochondrially sequestered calcium. The second is by causing secondary membrane damage through lipid peroxidation leading to an influx of extracellular calcium. High intracellular calcium levels then lead to beading through protease activation and cytoskeletal disruptions. If these hypotheses are correct then

increasing oxidative stress should lead to beading. To test this neurons were treated with TbHp to see if axonal beads would form, and it was confirmed that increasing oxidative stress with TbHp results in the formation of axonal beads (Figure 32).

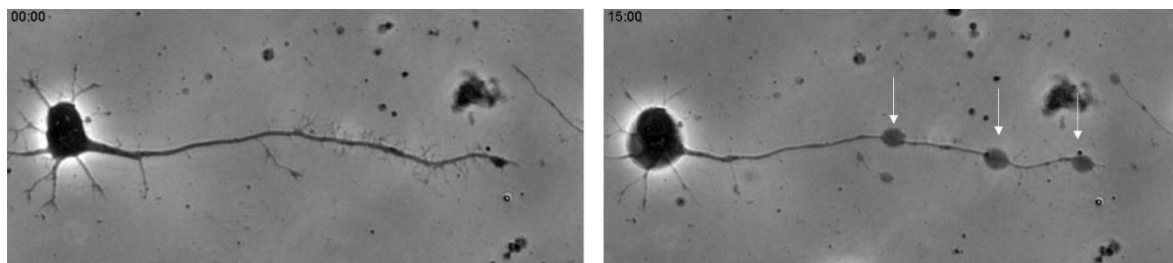


Figure 32. Increasing oxidative stress with TbHp results in a beaded morphology. *A*, Phase image of chick forebrain neuron before the addition of TbHp. *B*, the same neuron 15 minutes after the addition of 200mM TbHp. Beads indicated by white arrows.

Mitochondrial Dysfunction

Increased oxidative stress disrupts mitochondrial function. To see if increasing oxidative stress with TbHp results in mitochondrial dysfunction in cultured neurons, mitochondria were stained with Mitotracker Green and treated with 100mM TbHp for 30 minutes (Figure 33). Cells were imaged before and after treatment and changes in mitochondrial morphology were quantified.

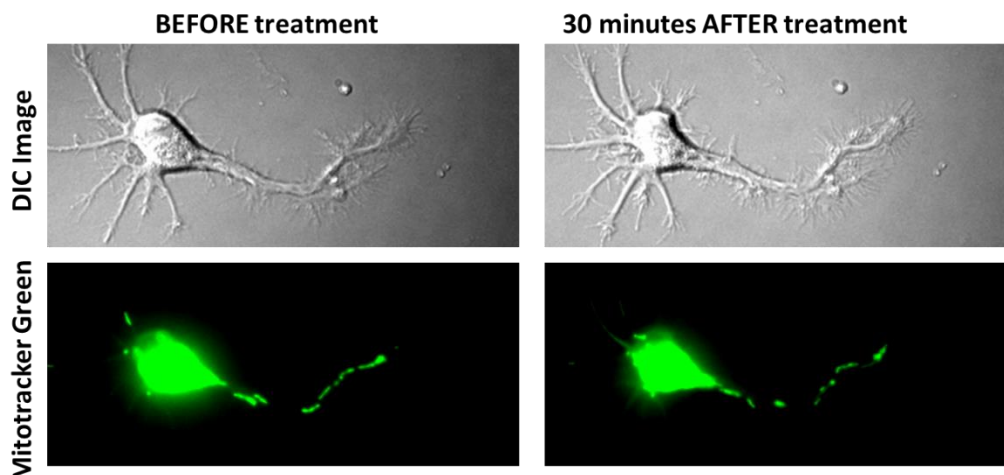


Figure 33. TbHp treatment results in change in mitochondrial morphology. Representative image of a neuron before and 30 minutes after the addition of 100mM TbHp. The change in axonal mitochondria, stained with Mitotracker Green, can be seen.

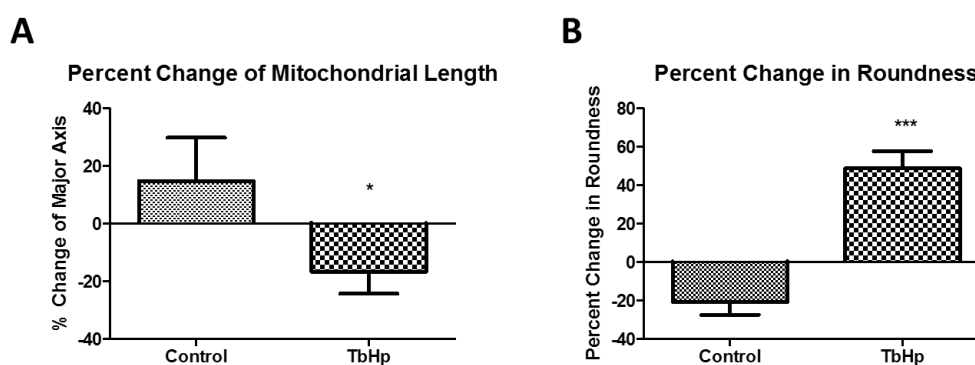


Figure 34. Increasing oxidative stress with TbHp results in a change in mitochondrial morphology. 30 minutes of 100mM TbHp treatment results in **A**, a decrease in mitochondrial length ($p < 0.05$) and **B**, an increase in mitochondrial roundness ($p < 0.0001$). (One-tailed t-test. $n > 100$ mitochondria from at least 7 cells for each condition). All values are presented as means $\pm$ SEM.

TbHp treatment resulted in shorter and rounder mitochondria (Figure 34), indicative of mitochondrial dysfunction.

Calcium Increase

Increasing oxidative stress is assumed to disrupt mitochondria and result in the release of sequestered mitochondrial calcium. Oxidative stress is also thought to increase membrane permeability through the oxidation of membrane lipids, thereby leading to the

influx of extracellular calcium. To determine if intracellular calcium is increased as a result of increased oxidative stress, TbHp was used to increase oxidative stress without preceding mitochondria or membrane disruption. Fluo-3 was used as a fluorescent calcium indicator (Figure 35). Ratiometric calcium measurements were made using Calcein Red/Orange to correct for possible loss of dye. The fluorescence ratios before treatment and 60 minutes after the addition of 100mM TbHp were compared. It was shown that increasing oxidative stress with TbHp results in a significant increase of axonal calcium (Figure 36).

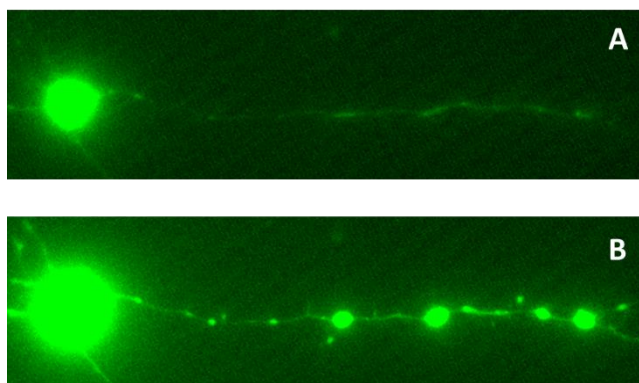


Figure 35. Representative diagram of increase in Fluo-3 fluorescence before (A) and 30 minutes after (B) the addition of 100 mM TbHp

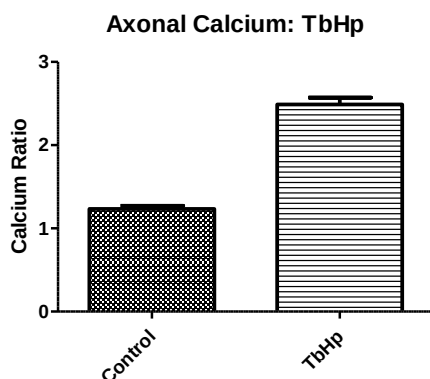


Figure 36. TbHp treatment results in a significant increase in axonal calcium. Calcium ratio was calculated as the normalized ratio of axonal Fluo-3 to Calcein Red/Orange fluorescence before and 30 minutes after the addition of 100 mM TbHp (One-tailed t-test, $p < 0.0001$, $n > 25$ cells for each condition). All values are presented as means $\pm$ SEM.

TbHp – Microtubule disruption

Increased oxidative stress is hypothesized to lead to microtubule disruption through multiple pathways. Oxidative stress disrupts mitochondrial function and results in a release of mitochondrially sequestered calcium, which can activate proteases that disrupt the cytoskeleton. Increased oxidative stress is also hypothesized to induce lipid peroxidation and secondary membrane damage, leading to an influx of extracellular calcium which can also activate proteases and lead to microtubule disruption. To see if increased oxidative stress leads to cytoskeletal disruption, neurons were treated with 100mM TbHp for 30 minutes before undergoing a simultaneous fixation extraction procedure and being stained for tubulin (Figure 37). The results showed an increase in the

percentage of cells presenting any focal disruptions—from 39% to 57% -- as well as a statistically significant increase in the normalized number of focal disruptions.

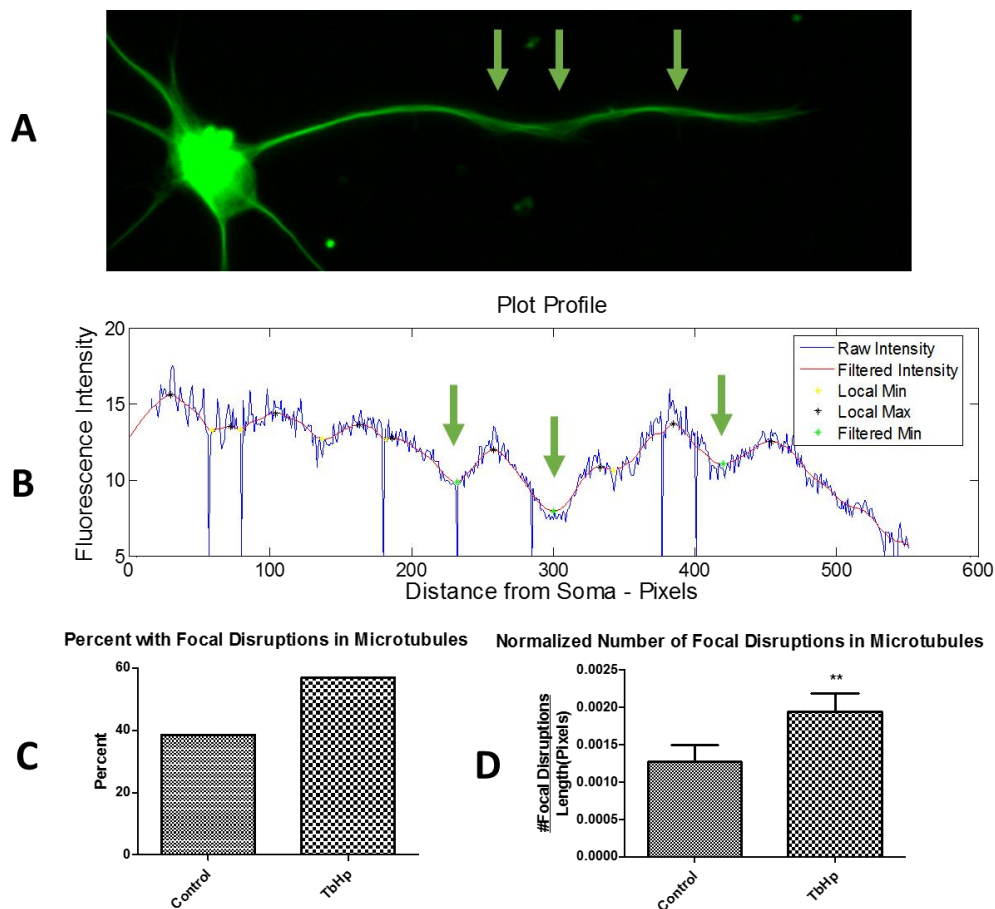


Figure 37. Cytoskeletal changes after TbHp treatment. Cells were treated with 100mM TbHp for 30 minutes, after which they underwent a simultaneous fixation and extraction of free tubulin before being stained for tubulin. **A**, Representative image of TbHp-treated neuron stained for tubulin. Arrow denotes focal disruption of microtubules. **B**, Plot Profile of intensity of representative neuron, arrow corresponds with area identified as having disrupted microtubules. **C**, TbHp treated cells have a higher percentage of cells with focal disruption in microtubules. **D**, TbHp treated cells have more focal disruptions in Microtubules. One-tailed mann-whitney test, $p < 0.01$. $n > 70$ cells from three coverslips for each condition. Error bars represent SEM.

4.3.3 Contribution of Oxidative Stress

Oxidative stress is thought to lead to axonal beading by damaging mitochondria, thereby resulting in the release of mitochondrially sequestered calcium and even more

oxidative stress as a result of mitochondrial ROS production, as well as through leading to an influx of extracellular calcium after increasing plasma membrane permeability through lipid peroxidation. In the hypothesized damage pathway, oxidative stress is thought to occur downstream of mitochondrial disruption, which occurs as a result of increased axonal calcium following increased membrane permeability. Therefore, reducing oxidative stress after any of these steps should serve to reduce beading. To test this, the antioxidants glutathione and catalase were combined with isolated perturbations to the damage pathway and axonal beading was quantified.

CCCP + Glutathione

Mitochondria play a crucial role in propagating the injury pathway and also in promoting recovery after injury. After damage to the plasma membrane, recovery of ionic homeostasis is an energy intensive process requiring the production of ATP by mitochondria. Damage to the mitochondria results in an increased production of ROS, which in turn serve to further damage mitochondria participating in a positive feedback loop. These are all hypothesized to promote axonal beading by impairing the extrusion of calcium by plasma membrane pumps, the release of calcium from mitochondria, and the increase in membrane permeability. To investigate the contribution of oxidative stress to this pathway, the antioxidant glutathione was used in conjunction with CCCP to damage mitochondria (Figure 38). Cells were pre-incubated in 1mM GSH for 60 minutes before being treated with 100 μ M CCCP for 50 minutes in the presence of GSH. When cells were pre-treated with GSH before CCCP treatment beading was reduced to 13 percent as opposed to the 43 percent of cells that presented with beads when CCCP was used alone.

When beading was quantified, pre-treatment with GSH reduced beading back to baseline levels (Figure 38).

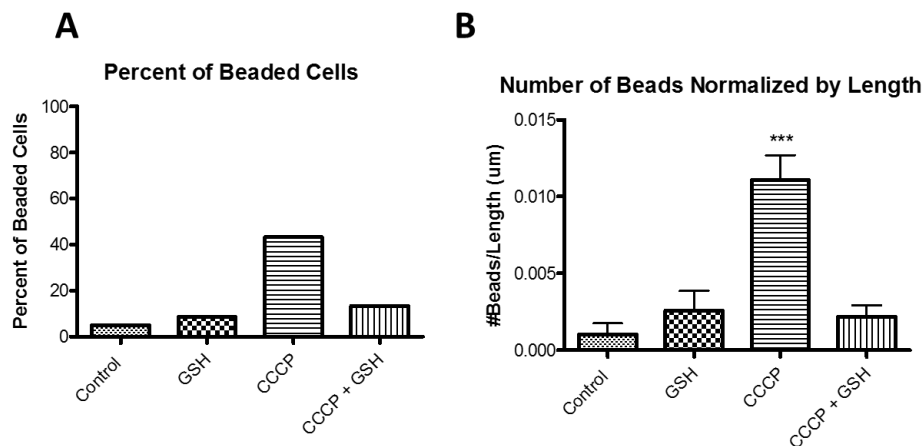


Figure 38. Antioxidant treatment with glutathione (GSH) reduces axonal beading after CCCP treatment. Cells were incubated in 100μM CCCP for 60 minutes after pretreatment with 1mM glutathione for 60 minutes. **A**, Glutathione reduces the percentage of beaded cells compared to CCCP treatment alone. **B**, CCCP treated cells show significantly more beads than the control or glutathione treated cells. Pre-incubating cells with glutathione before CCCP treatment results in significantly less beading than CCCP treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 80$ cells from three experiments). All values are presented as means $\pm$ SEM.

CCCP + Catalase

The contribution of hydrogen peroxide to beading after CCCP treatment was investigated using catalase to catalyze the decomposition of hydrogen peroxide to oxygen and water. Cells were pretreated with 100μg/ml of catalase before the addition of 100μM CCCP. While catalase reduced the percentage of cells with beads (from 47% to 23%), in the fraction of cells that did have beads catalase did not reduce the number of beads per length (Figure 39).

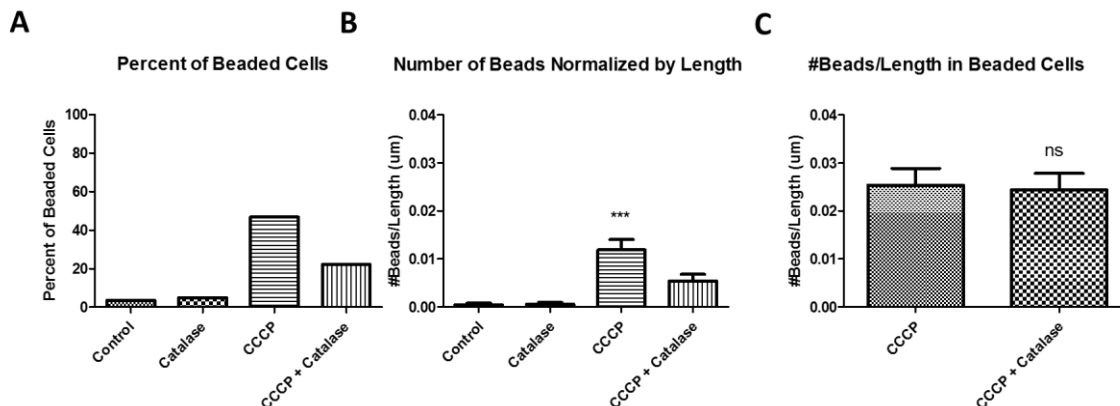


Figure 39. Antioxidant treatment with catalase reduces beading after CCCP treatment. Cells were incubated in 100 μ M CCCP after pretreatment with 100 μ g/ml catalase for 60 minutes **A**, Catalase reduces the percent of beaded cells compared to CCCP treatment alone. **B**, CCCP treated cells show significantly more beads than the control or catalase treated cells. Pre-incubating cells with catalase before CCCP treatment results in significantly less beading than CCCP treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 75$ cells from three experiments). **C**, In cells that present at least one bead, cells pre-treated with catalase do not have significantly less beading after treatment with CCCP than that were not pretreated (Mann Whitney test, $p > 0.05$, $n > 15$ cells from three experiments). All values are presented as means $\pm$ SEM.

A23187 + Glutathione

One of the ways in which increasing axonal calcium is hypothesized to promote beading is through the disruption of mitochondrial function. Influx of calcium leads to structural alterations of the inner mitochondrial membrane, which may increase ROS formation due to disorganization of the electron transport chain (Kowaltowski, Castilho et al. 1995). To investigate the role of increased oxidative stress after increased axonal calcium, calcium ionophore A23187 was used to isolate the effect of calcium overload without membrane disruption. If calcium overload leads to beading via ROS production, GSH ought to inhibit it. Cells were pretreated with 1mM GSH before the addition of 10 μ M A23187. The presence of GSH slightly reduced the percentage of beaded cells from 77% to 60% and had a statistically significant decrease in the number of beads per length as

well as a reduction in beading in the fraction of cells that presented with beads (Figure 40).

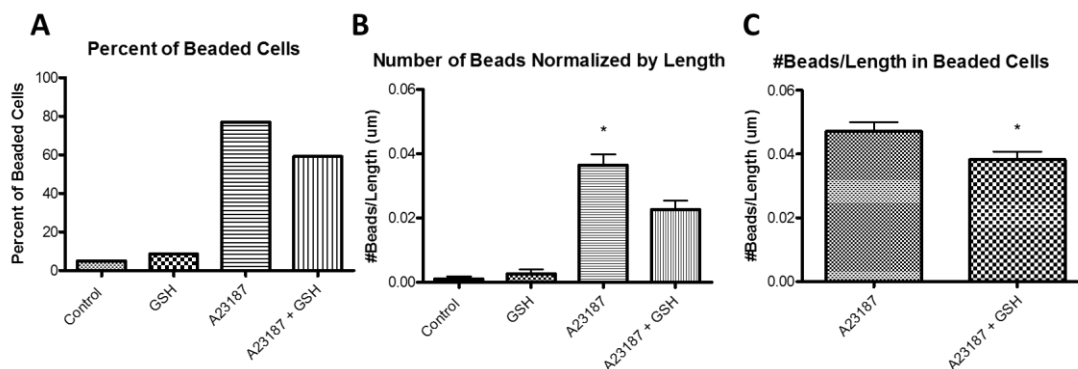


Figure 40. Antioxidant treatment with glutathione (GSH) reduces axonal beading after increasing axonal calcium with calcium ionophore A23187. Cells were incubated in 10μM A23187 after pre-incubation with 1mM GSH for 60 minutes. **A**, GSH added with A23187 reduces the percent of beaded cells compared to A23187 treatment alone. **B**, A23187 treated cells show significantly more beading than the control or GSH sham cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.05$, $n > 60$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with A23187 and GSH have significantly less beading than cells treated with A23187 alone (Mann-Whitney test, $p < 0.05$, $n > 40$ cells from three experiments). All values are presented as means $\pm$ SEM.

A23187 + Catalase

Having established that oxidative stress is playing a role in calcium-induced axonal beading, the contribution of hydrogen peroxide to A23187-induced beading was investigated using catalase. Catalase reduced the percentage of beaded cells from 73% to 33% and brought the number of beads per length back to baseline levels as well as showing a statistically significant reduction in the number of beads per length in the cells that presented with beads (Figure 41).

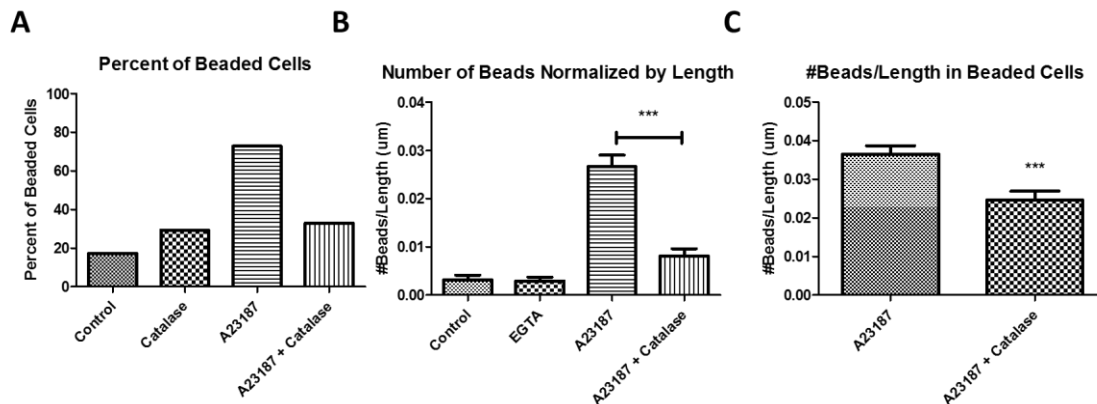


Figure 41. Antioxidant treatment with catalase reduces axonal beading after increasing intracellular calcium with calcium ionophore A23187. Cells were incubated in 100μM catalase for 20 minutes after pre-incubation with 100μg/ml catalase for 60 minutes. **A**, Catalase added with A23187 reduces the percentage of beaded cells compared to A23187 treatment alone. **B**, A23187 treated cells show significantly more beading than the control or catalase treated cells, and significantly more beads than cells treated with A23187 and catalase. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n > 80$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with A23187 and catalase have significantly less beading than cells treated with melittin alone (Mann Whitney test, $p < 0.001$, $n > 25$ cells from three experiments). All values are presented as means $\pm$ SEM.

Saponin + Glutathione

Damage to the plasma membrane leads to a loss of ionic homeostasis, which must be recovered in an energy-dependent process involving the mitochondria. The production of ATP through the electron transport chain results in the formation of ROS. Under injury conditions, this can result in an increase of oxidative stress which propagates the injury pathway by further damaging the mitochondria and preventing neuronal recovery. To investigate the contribution of oxidative stress to the injury pathway after membrane injury, plasma membrane permeability was increased using saponin, and the antioxidant GSH was used to reduce any increase in oxidative stress. Cells were pre-treated in 1mM GSH for 60 minutes before the addition of 0.01% Saponin for 15 minutes. The presence of glutathione reduced the percentage of beaded cells from 70% to 40% as well as resulting in a 50% reduction in the number of beads per length and a statistically

significant decrease in normalized beading in the fraction of cells that presented with beads (Figure 42).

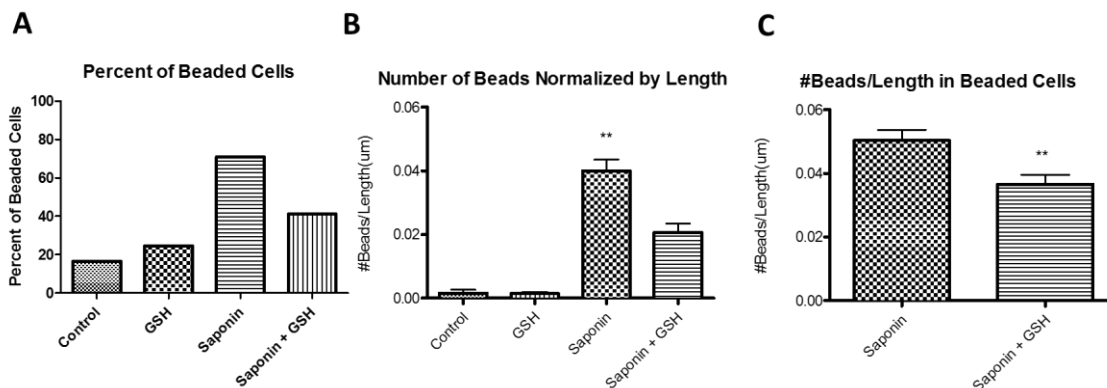


Figure 42. Antioxidant treatment with glutathione (GSH) reduces axonal beading after increasing membrane permeability with saponin. Cells were incubated in 0.01% saponin and 1mM GSH for 15 minutes after being pre-treated with 1mM GSH for 60 minutes. **A**, GSH added with saponin reduces the percent of beaded cells compared to saponin treatment alone. **B**, Saponin treated cells show significantly more beads than the control or GSH sham cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.01$, $n > 65$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with saponin and GSH have significantly less beading than cells treated with saponin alone (Mann Whitney test, $p < 0.01$, $n \geq 35$ cells from three experiments). All values are presented as means $\pm$ SEM.

Saponin + Catalase

Having established that oxidative stress is playing a role in saponin-induced axonal beading, the contribution of hydrogen peroxide after saponin treatment was investigated using catalase. When catalase was added to saponin-treated cells, it was not found to be neuroprotective. Pre-treatment with catalase before the addition of saponin resulted in an increased percentage of beaded cells as well as increased beading in the overall cell population, but did not change beading in cells that presented with beads (Figure 43).

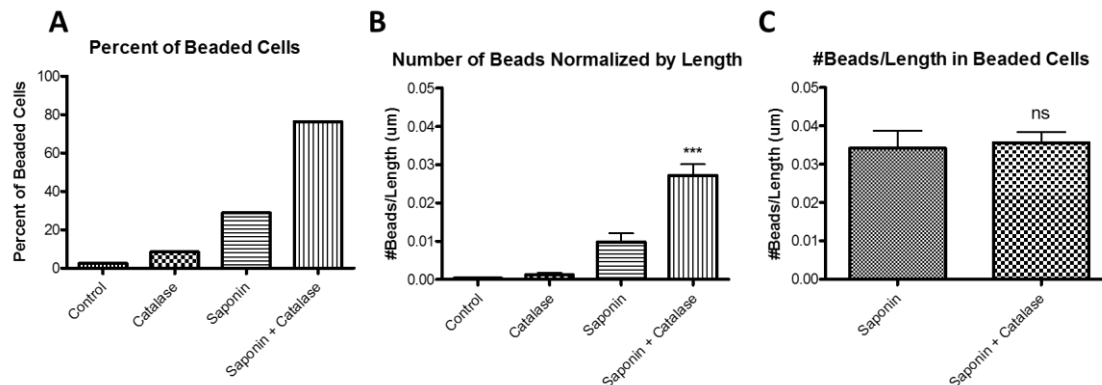


Figure 43. Antioxidant treatment with catalase does not reduce axonal beading after increasing membrane permeability with saponin. Cells were incubated in 0.01% saponin after pre-incubation with 100 $\mu\text{g}/\text{ml}$ catalase for 60 minutes. **A**, Catalase added with saponin increases the percentage of beaded cells compared to saponin treatment alone. **B**, Cells treated with saponin and catalase show significantly more beading than the control or catalase treated cells, as well as cells treated with saponin alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n > 55$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with saponin and catalase do not have less beading than cells treated with saponin alone (Mann Whitney test, $p > 0.05$, $n > 20$ cells from three experiments). All values are presented as means $\pm$ SEM.

Melittin + Glutathione

The contribution of oxidative stress to beading after membrane damage was also investigated using melittin to permeabilize the membrane, which may result in a different type of membrane permeability than saponin. Melittin is thought to result in smaller protein-lined pores that can allow for ion flux but not protein exchange. Neurons were pre-incubated in 1mM GSH for 60 minutes before the addition of 50nM melittin for 20 minutes. The presence of GSH halved the percentage of beaded cells (from 56% to 28%) and the number of beads per length compared to melittin treatment alone by more than half. Then number of beads per length in the fraction of cells that presented with beads was reduced from an average of 0.035 beads/ μm to 0.024 beads/ μm (Figure 44).

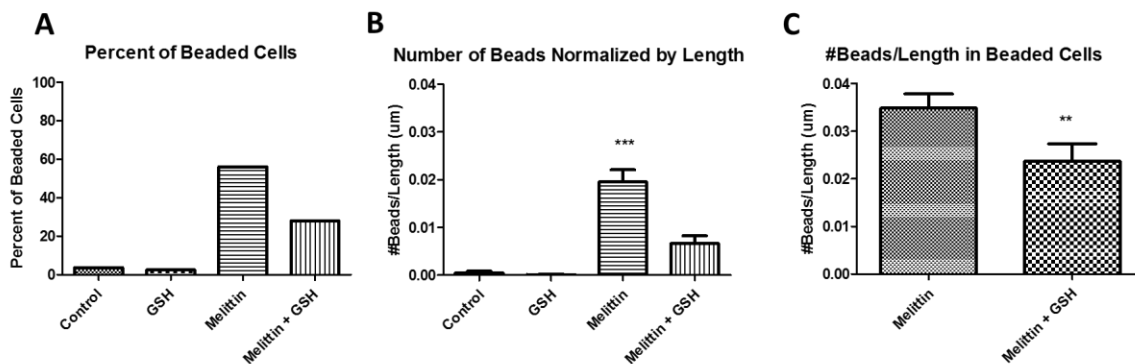


Figure 44. Antioxidant treatment with glutathione (GSH) reduces beading after increasing membrane permeability with melittin. Cells were incubated in 50nM melittin and 1mM GSH for 20 minutes. *A*, GSH added with melittin reduces the percent of beaded cells compared to melittin treatment alone. *B*, Melittin treated cells show significantly more beading than the control or GSH sham cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n > 65$ cells from three experiments). *C*, In cells that present at least one bead, cells treated with melittin and GSH have significantly less beading than cells treated with melittin alone (Mann Whitney test, $p < 0.01$, $n > 15$ cells from three experiments). All values are presented as means $\pm$ SEM.

Melittin + Catalase

Having established that oxidative stress is playing a role in melittin-induced beading, contribution of hydrogen peroxide specifically was investigated using catalase. Neurons were pre-treated with 100 μ g/ml catalase for 60 minutes before the addition of 100nM melittin for 15 minutes. The presence of catalase reduced the percentage of beaded cells from 34% to 20% and brought beading in the overall population back to baseline levels as well as showing reducing the number of beads per length in the fraction of beaded cells by over 50% (Figure 45).

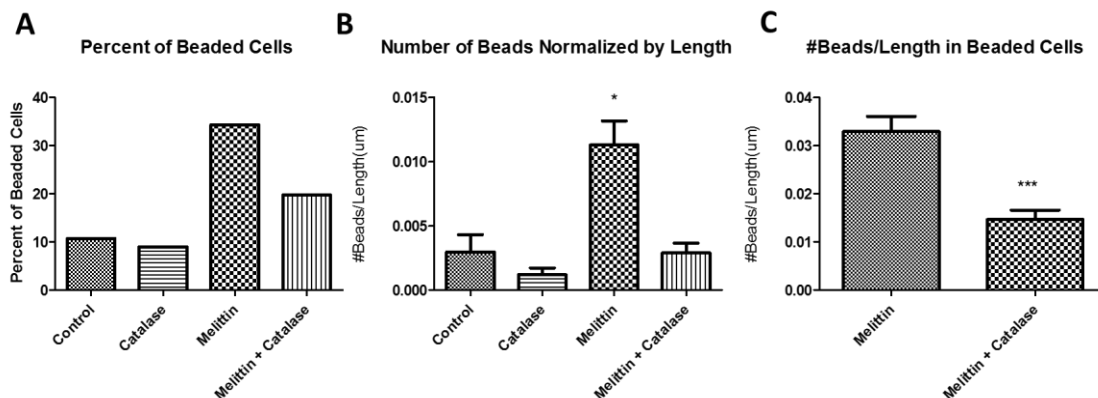


Figure 45. Antioxidant treatment with catalase reduces axonal beading after increasing membrane permeability with melittin. Cells were incubated in 100nM melittin for 15 minutes after pre-incubation with 100 μg/ml catalase for 60 minutes. **A**, Catalase added with melittin reduces the percentage of beaded cells compared to melittin treatment alone. **B**, Melittin treated cells show significantly more beading than the control or catalase treated cells. Adding catalase brings beading back to baseline levels. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.05$, $n > 75$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with melittin and catalase do not have significantly less beading than cells treated with melittin alone (Mann Whitney test, $p < 0.0001$, $n \geq 15$ cells from three experiments). All values are presented as means $\pm$ SEM.

3.3.5 Contribution of Intracellular Calcium to Axonal Pathology

An increase in cytosolic calcium is seen after neuronal injury. This calcium can come from two sources; the influx of extracellular calcium or the release of intracellularly sequestered calcium. The intracellular calcium is found mostly sequestered in the mitochondria and endoplasmic reticulum. In the hypothesized pathway (Figure 1) calcium release from both sources plays a role in axonal pathology. Here, we investigate the role of intracellular calcium by creating isolated perturbations in the cell and chelating intracellular calcium using BAPTA-AM.

CCCP + BAPTA

Mitochondria represent a significant source of intracellular calcium. They buffer excess calcium, but when damaged they release the calcium that they have sequestered. CCCP treatment was shown to result in an increase in cytosolic calcium (Figure 30) and it

is hypothesized that this increased calcium leads to CCCP-induced beading (Figure 27). Therefore, chelating intracellular calcium should be neuroprotective. To investigate the contribution of intracellular calcium to axonal beading after mitochondrial injury, BAPTA-AM was used to chelate intracellular calcium when damaging mitochondria with CCCP. Chelating intracellular calcium with BAPTA reduced the percentage of beaded cells from 92% to 54% and brought the number of beads per length back to baseline levels as well as reducing beading in the fraction of cells that presented with beads (Figure 46).

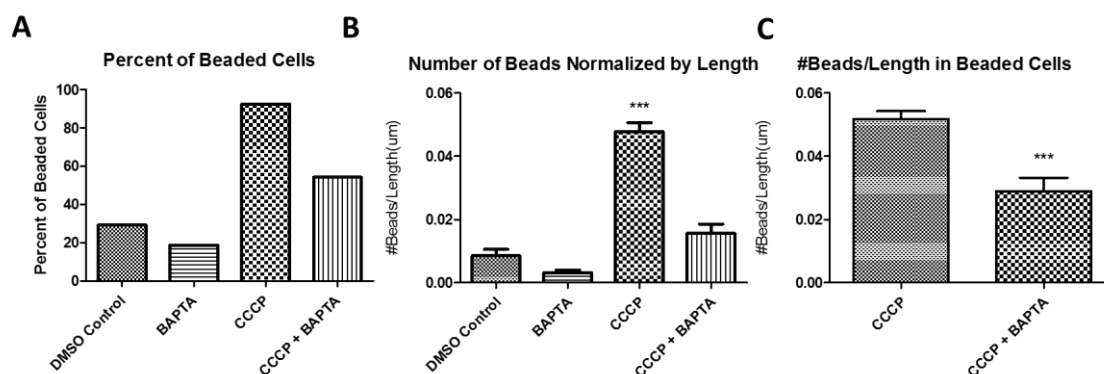


Figure 46. Chelating intracellular reduces axonal beading after CCCP treatment. Cells were incubated in 100μM CCCP after pretreatment with 100μM BAPTA-AM for 10 minutes **A**, BAPTA-AM reduces the percent of beaded cells compared to CCCP treatment alone. **B**, CCCP treated cells show significantly more beads than the control or BAPTA-AM treated cells. Pre-incubating cells with BAPTA-AM before CCCP treatment results in significantly less beading than CCCP treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n > 70$ cells from three experiments). **C**, In cells that present at least one bead, cells pre-treated with BAPTA-AM have significantly less beading after treatment with CCCP than that were not pretreated (Mann Whitney test, $p < 0.0001$, $n > 35$ cells from three experiments). All values are presented as means $\pm$ SEM.

TbHp + BAPTA

Increased oxidative stress plays into a positive feedback loop with mitochondria, damaging the mitochondria and resulting in even more oxidative stress. Damaged mitochondria are hypothesized to contribute to axonal pathology through a multitude of pathways, including the release of sequestered calcium. Therefore, one of the ways in

which increased oxidative stress is thought to lead to axonal beading is through the release of sequestered intracellular calcium. To test the contribution of oxidative-stress induced intracellular calcium release to axonal pathology, BAPTA-AM was used to chelate intracellular calcium after inducing oxidative stress with TbHp. Intracellular calcium was chelated by pre-incubating neuronal cultures in 10 μ M BAPTA-AM for 20 minutes before the addition of 200mM TbHp for 40 minutes. The chelation of intracellular calcium resulted in a 50% reduction in the percentage of beaded cells and brought the number of beads per length back to baseline levels, as well as reducing the number of beads per length in the fraction of beaded cells by over 50% (Figure 47).

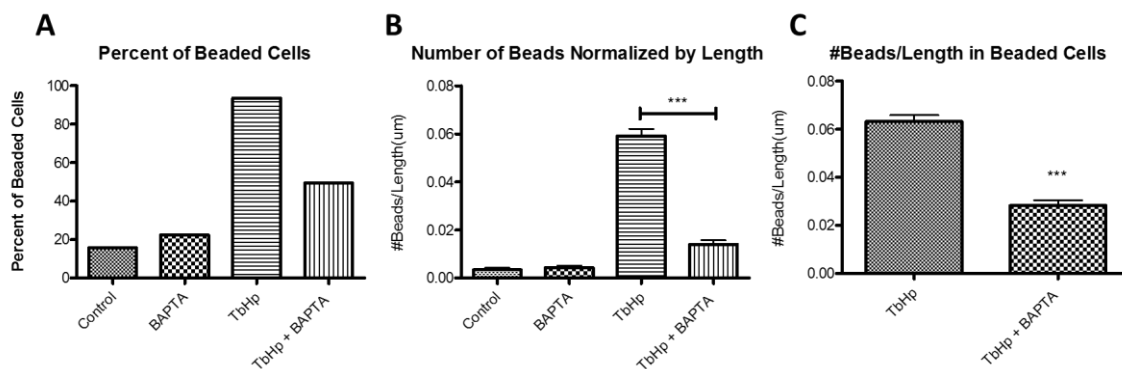


Figure 47. Chelating intracellular calcium reduces axonal beading after increasing oxidative stress with TbHp. Cells were incubated in 200mM TbHp for 40 minutes after being pre-incubated in 10 μ M BAPTA-AM for 20 minutes. **A**, BAPTA-AM reduces the percent of beaded cells compared to TbHp treatment alone. **B**, TbHp treated cells show significantly more beads than the control or BAPTA-AM treated cells. Pre-incubating cells with BAPTA-AM before TbHp treatment results in significantly less beading than TbHp treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.0001$, $n \geq 85$ cells from three experiments). **C**, In cells that present at least one bead, cells pre-treated with BAPTA-AM have significantly less beading after treatment with TbHp than that were not pretreated (Mann Whitney test, $p < 0.0001$, $n \geq 45$ cells from three experiments.) All values are presented as means $\pm$ SEM.

A23187 + BAPTA

Permeabilizing the cell membrane results in an influx of extracellular calcium that damages the mitochondria and results in the release of sequestered mitochondrial

calcium. To investigate the role that intracellular calcium release plays when extracellular calcium is introduced inside the cell in the absence of membrane permeabilization BAPTA-AM was used to chelate intracellular calcium while inducing calcium influx with calcium ionophore A23187. BAPTA-AM did not reduce beading levels in ionophore treated cells (Figure 48).

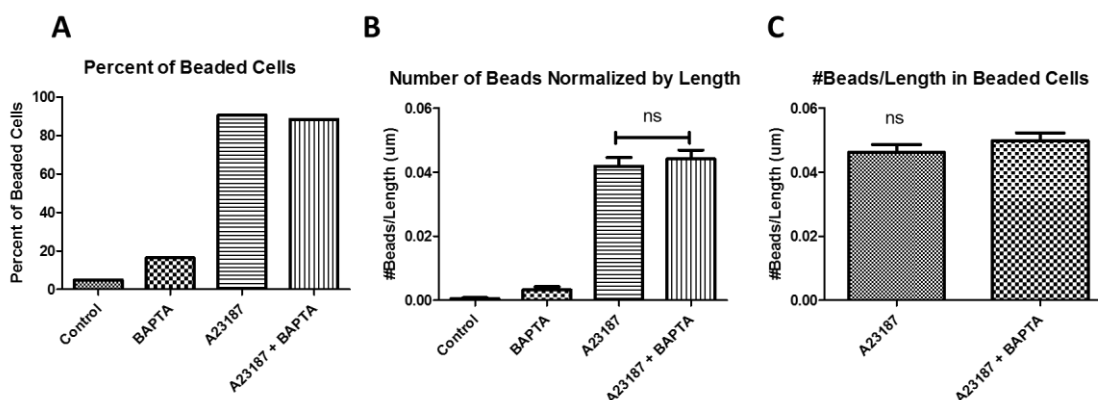


Figure 48. Chelating intracellular calcium does not reduce axonal beading after increasing intracellular calcium with A23187. Cells were incubated in 10 μ M A23187 after being pre-incubated in 10 μ M BAPTA-AM for 20 minutes. **A**, BAPTA-AM does not reduce the percent of beaded cells compared to A23187 treatment alone. **B**, A23187 treated cells show significantly more beads than the control or BAPTA-AM treated cells. Pre-incubating cells with BAPTA-AM before A23187 treatment does not result in less beading than A23187 treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p > 0.05$, $n \geq 75$ cells from three experiments). **C**, In cells that present at least one bead, cells pre-treated with BAPTA-AM do not have less beading after treatment with A23187 than those that were not pretreated (Mann Whitney test, $p > 0.05$, $n > 65$ cells from three experiments). All values are presented as means $\pm$ SEM.

Saponin + BAPTA

Permeabilizing the membrane results in an influx of extracellular calcium. Mitochondria attempt to sequester this calcium, but once damaged release the calcium they have sequestered. To investigate the contribution of the intracellular calcium release to axonal pathology, BAPTA-AM was used to chelate intracellular calcium while

permeabilizing the cell membrane with saponin (Figure 49). Chelating intracellular calcium with BAPTA-AM did not mitigate axonal beading

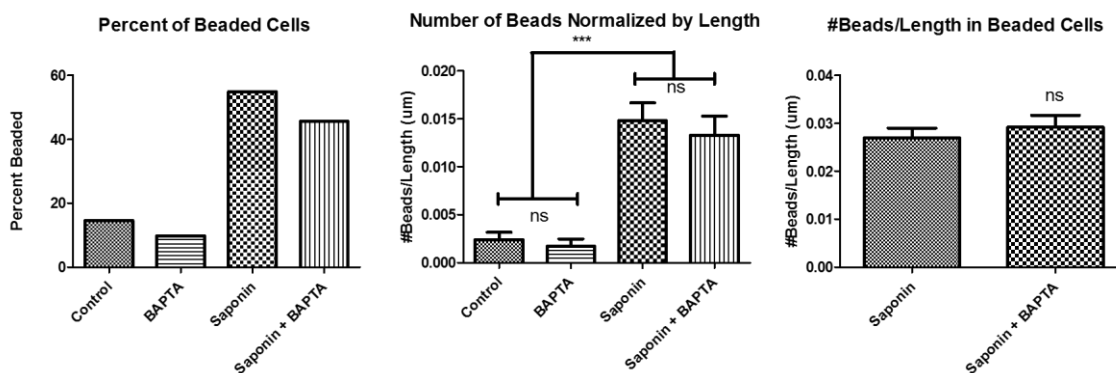


Figure 49. Chelating intracellular calcium reduces axonal beading after saponin treatment does not reduce axonal beading. Cells were incubated in 0.01% Saponin after being pre-incubated in 10 μ M BAPTA-AM for 20 minutes. **A**, BAPTA-AM slightly reduces the percent of beaded cells compared to saponin treatment alone. **B**, Saponin treated cells show significantly more beads than the control or BAPTA-AM treated cells. Pre-incubating cells with BAPTA-AM before saponin treatment results in significantly less beading than saponin treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p > 0.05$, $n \geq 75$ cells from three experiments). **C**, In cells that present at least one bead, cells pre-treated with BAPTA-AM do not have less beading after treatment with saponin than that were not pretreated (Mann Whitney test, $p > 0.05$, $n > 35$ cells from three experiments). All values are presented as means $\pm$ SEM.

Melittin + BAPTA

The role that intracellular calcium release plays after membrane damage was also investigated using melittin to increase membrane permeability. Cells were pre-incubated in 10 μ M BAPTA-AM for 20 minutes before the addition of 50nM melittin for 20 minutes. The presence of BAPTA reduced the percentage of beaded cells by more than 50%, as well as reducing beading in the overall population by over 50% and reducing beading in the fraction of cells that present with beads (Figure 50).

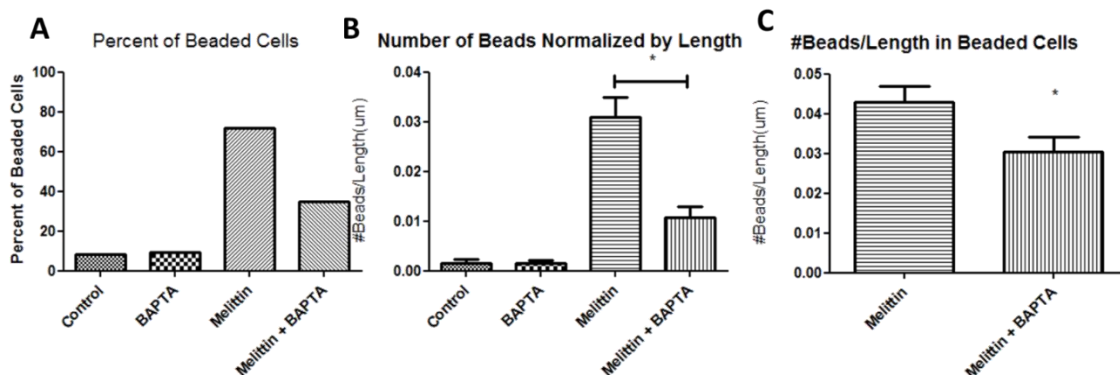


Figure 50. Chelating intracellular calcium reduces axonal beading after melittin treatment. Cells were incubated in 50nM melittin after being pre-incubated in 10 μ M BAPTA-AM for 20 minutes. A, BAPTA-AM reduces the percent of beaded cells compared to melittin treatment alone. B, Melittin treated cells show significantly more beads than the control or BAPTA-AM treated cells. Pre-incubating cells with BAPTA-AM before melittin treatment results in significantly less beading than melittin treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.05$, $n \geq 50$ cells from three experiments). C, In cells that present at least one bead, cells pre-treated with BAPTA-AM have significantly less beading after treatment with melittin than that were not pretreated (Mann Whitney test, $p < 0.05$, $n > 20$ cells from three experiments.) All values are presented as means $\pm$ SEM.

3.3.5 Calcium Contribution

CCCP + EGTA

As part of the hypothesized pathway (Figure 1), damaged mitochondria release sequestered calcium. CCCP treatment to disrupt mitochondrial results in an increase in axonal calcium (Figure 30). To investigate the source of the calcium, EGTA was used to chelate extracellular calcium and prevent extracellular calcium influx. When EGTA was used in conjunction with CCCP, the calcium increase was prevented (Figure 51).

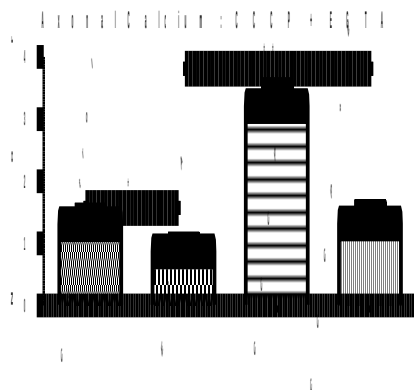


Figure 51. Quantification of cytosolic calcium after CCCP treatment Neurons treated with 100 μ M CCCP for 60 minutes exhibit a significantly greater change in axonal calcium than control or EGTA-treated cells ($p < 0.001$). When 1mM EGTA is added in addition to CCCP treatment the axonal calcium ratio is significantly greater than the EGTA controls ($p < 0.01$). (Kruskal-Wallis followed by Dunn's multiple comparison test, $n > 20$ cells for each condition). All values are presented as means $\pm$ SEM.

TbHp + EGTA

Increased oxidative stress is thought to contribute to increased cytosolic calcium through multiple mechanisms: first, the release of mitochondrially sequestered calcium and second, the influx of extracellular calcium after lipid-peroxidation induced secondary membrane damage. To test this hypothesis, TbHp was used to increase oxidative stress and EGTA was used to chelate extracellular calcium. The results showed that TbHp treatment resulted in increased axonal calcium compared to control cells, and that adding EGTA to TbHp treated cells reduced the increase in calcium but that it was still statistically higher than controls (Figure 52).

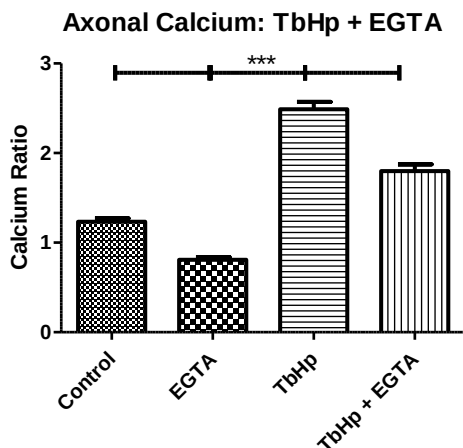


Figure 52. Neurons treated with 100mM TbHp for 30 minutes exhibit a significantly greater change in axonal calcium than control or EGTA-treated cells. When 1mM EGTA is added in addition to TbHp treatment the axonal calcium ratio is reduced but still remains higher than control values. (One-way ANOVA followed by Tukey's multiple comparison test. $p < 0.0001$, $n > 25$ cells for each condition). All values are presented as means $\pm$ SEM.

3.3.3 Contribution of the Mitochondrial Permeability Pore

Excess intracellular calcium is buffered by mitochondria which can sequester it up to a certain point. When mitochondria are overloaded with calcium, they release the sequestered calcium through an opening of the MPP, which can open in either a regulated or unregulated fashion. Cyclosporin A has been shown to prevent the regulated opening of the MPP. To investigate the contribution of regulated MPP opening, Cyclosporin A was used to prevent the regulated MPT while adding a chemical insult to neurons. Axonal beading was quantified as a measure of the extent of neuronal damage.

CCCP + CsA

Damage to the mitochondria is hypothesized to result in an opening of the MPP, which contributes to beading through a release of sequestered calcium and a continuation of the hypothesized injury pathway. In order to test for the contribution of the MPT pore to beading after mitochondrial injury, CsA was added with CCCP to disturb

mitochondrial function and prevent the opening of the MPP (Figure 53). CsA treatment was shown to reduce beading in CCCP treated cells.

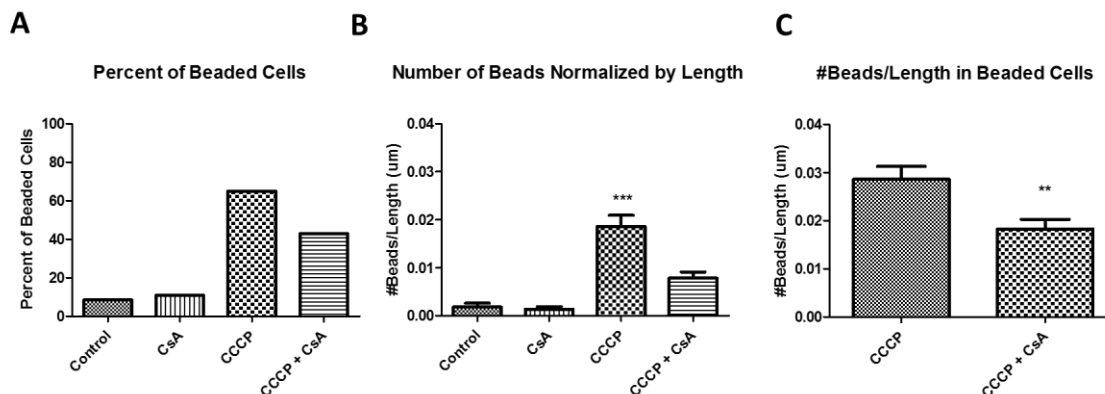


Figure 53. Preventing the opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after damaging mitochondria with CCCP. Cells were incubated in 100 μ M CCCP and 1 μ M CsA for 60 minutes. **A**, CsA reduces the percent of beaded cells compared to CCCP treatment alone. **B**, CCCP treated cells show significantly more beads than the control or CsA treated cells. Adding CsA to CCCP treated cells results in significantly less beading than CCCP treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n > 80$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with CsA and CCCP do not have significantly less beading after treatment with saponin alone (Mann Whitney test, $p < 0.01$, $n > 35$ cells from three experiments). All values are presented as means $\pm$ SEM.

TbHp + CsA

Increased oxidative stress is hypothesized to lead to beading through a variety of pathways. One is through inducing lipid peroxidation in the plasma membrane which in turn results in increased membrane permeability and a loss of ionic homeostasis, including an influx of calcium which can overload and destabilize mitochondria as well as activate proteases that lead to beading. Another posited theory for the contribution of oxidative stress to beading is direct damage to the mitochondria and induction of the MPT, which results in a release of sequestered calcium. To test for the contribution of the MPT to beading, oxidative stress was increased using TbHp and the regulated opening of the MPT was prevented using CsA. CsA reduced the percentage of beaded cells from 66%

to 49% which accounts for a statistically significant reduction in beads per length in the entire population, but no change in beading when looking at the fraction beaded (Figure 54).

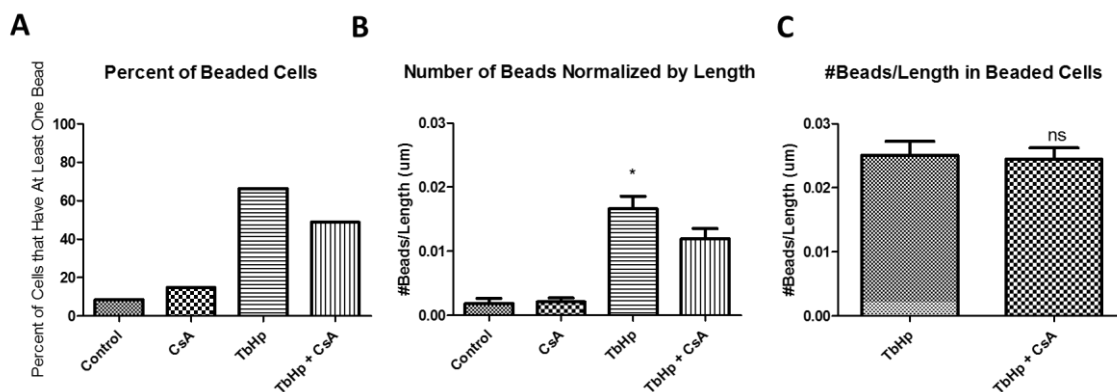


Figure 54. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after increasing oxidative stress with TbHp. Cells were incubated in 100mM TbHp and 1μM CsA for 30 minutes *A*, CsA reduces the percent of beaded cells compared to TbHp treatment alone. *B*, TbHp treated cells show significantly more beads than the control or CsA treated cells. Adding CsA to TbHp treated cells results in significantly less beading than TbHp treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.05$, $n \geq 80$ cells from three experiments). *C*, In cells that present at least one bead, cells treated with CsA and TbHp do not have significantly less beading after treatment with TbHp alone (Mann Whitney test, $p < 0.01$, $n > 40$ cells from three experiments). All values are presented as means $\pm$ SEM.

Saponin + CsA

An increase in membrane permeability results in a loss of ionic homeostasis which the cell has to work to restore in an energy-dependent manner, taxing the mitochondria which produce that energy. The loss of ionic homeostasis also results in an influx of calcium which can overload the mitochondria and lead to an opening of the mitochondrial permeability pore. This sort of mitochondrial disruption results in a bioenergetics crisis, releases sequestered calcium, and increases oxidative stress. To investigate the role that the MPT plays after membrane injury, Cyclosporin A was used to prevent regulated MPP

opening after membrane permeabilization with saponin. CsA reduced the percentage of beaded cells and brought the number of beads per length back to baseline levels.

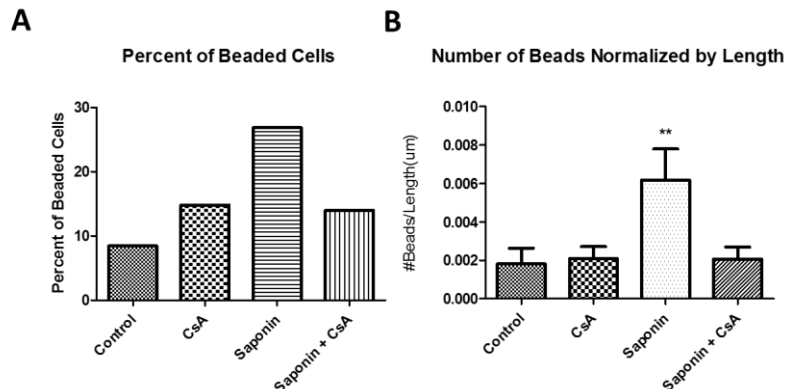


Figure 55. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after membrane poration with saponin. Cells were incubated in 0.01% saponin and 1μM CsA for 20 minutes *A*, CsA reduces the percent of beaded cells compared to saponin treatment alone. *B*, Saponin treated cells show significantly more beads than the control or CsA treated cells. Adding CsA to saponin treated cells results in significantly less beading than saponin treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n > 80$ cells from three experiments). All values are presented as means $\pm$ SEM.

Melittin + CsA

The contribution of the MPT to axonal beading after membrane damage was also investigated using melittin to permeabilize the membrane and CsA to prevent the regulated opening of the MPP. Pre-treatment with CsA reduced the percentage of beaded cells by 50% and brought the number of beads per length back to baseline levels as well as resulting in a statistically significant decrease in beading in the fraction of beaded cells (Figure 56).

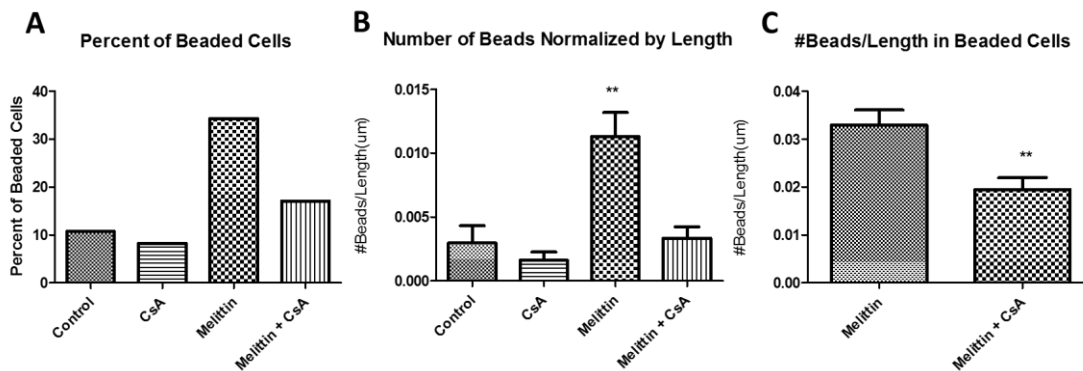


Figure 56. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduces axonal beading after membrane poration with melittin. Cells were incubated in 100nM melittin and 1 μ M CsA for 15 minutes. **A**, CsA reduces the percent of beaded cells compared to melittin treatment alone. **B**, Melittin treated cells show significantly more beads than the control or CsA treated cells. Adding CsA to melittin treated cells results in significantly less beading than melittin treatment alone and shows no difference from control cells. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.01$, $n > 80$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with CsA and melittin have significantly less beading than treatment with melittin alone (Mann Whitney test, $p < 0.01$, $n \geq 14$ cells from three experiments). All values are presented as means $\pm$ SEM.

A23187 + CsA

If a regulated opening of the MPP is contributing to beading when intracellular calcium is increased, then preventing that opening should reduce downstream effects including axonal beading. In order to explore the contribution of the MPP, intracellular calcium was increased with calcium ionophore A23187 and the regulated opening of the MPP was prevented using CsA. When CsA was used in conjunction with A23187 there was no statistically significant change in beading in the overall cell population and the number of beads in beaded cells was actually increased (Figure 57).

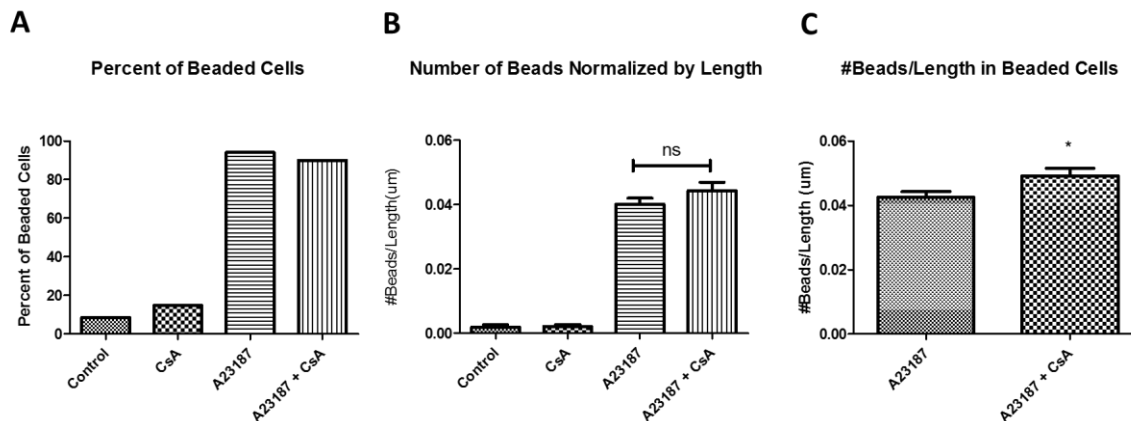


Figure 57. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) does not reduce axonal beading after increasing intracellular calcium with calcium ionophore A23187. Cells were incubated in 10 μ M and 1 μ M CsA for 20 minutes. **A**, CsA does not reduce the percent of beaded cells compared to A23187 treatment alone. **B**, A23187 treated cells show significantly more beads than the control or CsA treated cells. Adding CsA to A23187 treated cells does not result in less beading than A23187 treatment alone. (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p > 0.05$, $n \geq 80$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with CsA and A23187 have significantly more beading after treatment with A23187 alone (Mann Whitney test, $p < 0.05$, $n > 70$ cells from three experiments). All values are presented as means $\pm$ SEM.

4.4 Discussion

It is hypothesized that mitochondrial dysfunction, secondary to mechanical trauma, plays an important role in axonal pathology in TBI. Consistent with that hypothesis, directly impairing mitochondrial function with CCCP without preceding mechanical damage CCCP resulted in axonal beading (Figure 28), focal disruptions in microtubules (Figure 31) and increased intracellular calcium (Figure 29, Figure 30), consistent with *in vivo* axonal pathologies seen in TBI. They are consistent with experiments by Werth and Vaur which showed increased intracellular calcium after CCCP treatment (Werth and Thayer 1994, Vaur, Sartor et al. 2000). The expectation here was that disrupting mitochondrial function with CCCP would cause calcium release from

mitochondria that could lead to plasma damage and the influx of extracellular calcium. To inspect the source of CCCP-induced axonal calcium, relative axonal calcium levels were quantified using EGTA to chelate extracellular calcium and showed that when EGTA was present in the extracellular media intracellular calcium levels were decreased but were still significantly higher than EGTA controls (Figure 50), implying a dual source of cytosolic calcium increase. These findings are consistent with those of Vaur who concluded that CCCP activated calcium influx from the extracellular medium (Vaur, Sartor et al. 2000) as well those of Werth which indicated that CCCP treatment resulted in a release of calcium from the mitochondria (Werth and Thayer 1994).

In the hypothesized pathway (Figure 1), increased oxidative stress damages the mitochondria and increases membrane permeability via lipid peroxidation. This results in an increase of axonal calcium via both the release of mitochondrially sequestered calcium and an influx of calcium from the extracellular media. Increasing the oxidative stress of the cells using the hydrogen peroxide donor TbHp resulted in beading (Figure 32), focal disruption of microtubules (Figure 37) and increased axonal calcium (Figure 35, Figure 36) consistent with this hypothesis. Chelation of extracellular calcium with EGTA partially inhibited the cytosolic calcium increase in response to TbHp (Figure 48). When compared to A23187 treatment where the only calcium source was extracellular (Figure 21) this provides support for the conclusion that TbHp causes both intracellular release of calcium as well as influx from the extracellular space. These findings are consistent with those of others that showed that treatment with hydrogen peroxide treatment resulted in beading (Lim, Lee et al. 2002, Roediger and Armati 2003) and microtubule disruption and

that increasing oxidative stress via TbHp treatment results in increased intracellular calcium (Kanupriya, Prasad et al. 2007).

The antioxidant glutathione was used to investigate the role of oxidative stress in axonal pathology after various isolated injuries. It is hypothesized that membrane injury, increased intracellular calcium, and mitochondrial damage each lead to an increase in oxidative stress. Therefore, reducing the increase in oxidative stress with an antioxidant should help to prevent some of the downstream effects. This proved to be the case as glutathione prevented beading when mitochondria were targeted with CCCP (Figure 38), intracellular calcium was increased with calcium ionophore A23187 (Figure 40), and membrane permeability was increased using saponin(Figure 42) and melittin(Figure 44). The success of glutathione in preventing beading after these treatments provides evidence that oxidative stress is playing a role in the downstream pathology. Others have also shown that oxidative stress is increased under these isolated perturbations of the damage pathway. Bonfoco et al showed that calcium overload of mitochondria leads to the generation of superoxide anion $O_2^{\cdot -}$ (Bonfoco, Krainc et al. 1995).

To further probe the type of oxidative stress, the antioxidant catalase was also used in conjunction with these chemical insults. Since catalase is an antioxidant specific to hydrogen peroxide, the effectiveness of catalase in preventing beading would show that hydrogen peroxide is contributing significantly to downstream pathology. Catalase was indeed effective in preventing beading when mitochondria were targeted with CCCP (Figure 39), intracellular calcium was increased with calcium ionophore A23187 (Figure 41), and membrane permeability was increased using melittin (Figure 45). Surprisingly,

catalase was not neuroprotective when membrane permeability was increased using saponin (Figure 43) and in fact resulting in a statistically significant increase in beading. This is in stark contradiction to melittin, the other method used to permeabilize the membrane. Perhaps the difference between the two can be explained by the type of membrane permeabilization and possible size of pores. Melittin creates smaller, possibly transient pores that likely allow for ion exchange but not the flow of larger molecules. Catalase is not permeable to intact membranes (Schimmel and Bauer 2002) and has a stoke's radius of 5.12 nm. It is possible that larger and more prolonged membrane poration with saponin could allow for the influx of catalase into the cell. Here, instead of reducing extracellular hydrogen peroxide catalase may be interacting directly with mitochondria.

In neurons, the primary source of ROS is from mitochondria, which are also one of the primary sources of intracellular calcium. Increased oxidative stress damages the mitochondria and also results in the release of sequestered calcium. To investigate the role that intracellular calcium plays in axonal pathology, intracellular calcium was chelated using BAPTA-AM. BAPTA-AM reduced beading caused by damaging mitochondria with CCCP (Figure 46), increasing oxidative stress with TbHp (Figure 47), and increasing membrane permeability with melittin (Figure 50), indicating that intracellular calcium plays a significant role in downstream pathology after these treatments. Chelating intracellular calcium was not neuroprotective when intracellular calcium was increased using calcium ionophore A23187 to allow for the entry of extracellular calcium (Figure 48). This was the expected result because intracellular calcium release is small

compared to the amount of calcium that is constantly introduced into the cell due to ionophore treatment.

Cytosolic calcium has been shown to be increased after TBI. Understanding the source of this calcium is important to understanding the pathways and mechanisms of axonal pathology after brain injury. Cytosolic calcium can be increased by either entering the cell from the extracellular media or being released from intracellular sources such as the mitochondria or endoplasmic reticulum. To investigate the source of the increased cytosolic calcium, extracellular calcium was chelated using EGTA and live calcium imaging was performed to quantify relative changes in calcium before and after treatment.

The release of sequestered mitochondrial calcium is said to occur due to a change in mitochondrial permeability due to the opening of the MPP. To test the contribution of the MPT, the regulated opening of the MPP was prevented using Cyclosporin A. Neuroprotective effects of CsA were interpreted as evidence that the MPT was occurring. CsA treatment reduced beading caused by disrupting mitochondria with CCCP, indicating that the MPT is playing a role in beading with CCCP treatment. Niemenin et al. have tried observing calcein entry into mitochondria as an indicator of the MPT but concluded that CCCP depolarized mitochondria but did not induce a permeability transition on its own (Niemenin, Saylor et al. 1995). These conflicting results may be due to differences in concentrations and timing of reagents used, or differences in mitochondria due to cell types, as mitochondria from different cells may behave differently (Andreyev and Fiskum 1999). Niemenin et al used cultured hepatocytes and used 10 μ M CCCP for less than 20 minutes, compared to the 100 μ M treatment for 60 minutes that is used here.

CsA treatment was neuroprotective when oxidative stress was increased with TbHp (Figure 54), indicating that the MPT is occurring when oxidative stress is increased. This has also been shown by Nieminen et al. who observed that after the addition of TbHp mitochondria quickly filled with Calcein, indicating the onset of the MPT (Nieminen, Saylor et al. 1995), even at a lower concentration of 100 μ M. Imberti et al. also showed that TbHp treatment uncoupled mitochondria, an event they associated with an MPT and showed that CsA protected against mitochondrial depolarization and cell death (Imberti, Nieminen et al. 1993).

CsA treatment was neuroprotective when increasing membrane permeability with saponin and melittin. This indicates that regulated opening of the MPP is happening downstream of increased membrane permeability and that is contributing significantly to axonal beading.

CsA treatment was not neuroprotective when increasing intracellular calcium with calcium ionophore A23187. This indicates that A23187 is not resulting in MPT, or that the MPT is occurring in a fashion that is not mitigated by CsA. However, many others have shown that calcium ionophore A23187 specifically is capable of inducing the MPT (Petersen, Castilho et al. 2000). If the MPT is occurring here, it is possible that it involves an unregulated pore opening that does not require calcium for conductance and is not blocked by CsA (Kim, He et al. 2003). This is the most likely scenario as it is well established that increased calcium results in an MPT, and specifically that it can occur following exposure to calcium ionophore. The contradictions between these results may be explained by differences in concentrations of ionophore used. For example, Peterson et

al showed MPT induction that was mitigated with CsA while using concentrations of A1287 that ranged from 0.1 – 4 μ M, which is much less than the 10 μ M used in these experiments.

Further evidence of MPP involvement in TBI may be found in *in vivo* models using CsA that have shown it to be neuroprotective. *In vivo* CsA preserved mitochondrial function (Okonkwo, Buki et al. 1999) and reduced cortical cell loss. Importantly, CsA has been shown to promote functional recovery, including motor and cognitive function (Alessandri, Rice et al. 2002). *In vitro*, CsA has been shown to be neuroprotective by delaying axotomy after axonal stretch injury (Staal, Dickson et al. 2007). Evidence for CsA neuroprotection is so strong that it has even been suggested to be responsible for the relatively mild neurological impairment suffered by a TBI patient who had been undergoing treatment with CsA for its immunosuppressive properties at the time of his injury (Gogarten, Van Aken et al. 1998).

Brain injury is not the only neurological condition involving mitochondrial dysfunction or oxidative stress. Other diseases that involve energy deficits and increased oxidative stress as a result of mitochondrial damage include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and Freidrich's ataxia (Simpkins, Yi et al. 2010). Thus, understanding the role that mitochondria and oxidative stress can play in TBI pathology may also lead to advancements in treating these other neurological disorders.

Chapter 5: The Role of Secondary Membrane Damage in Axonal Pathology

5.1 Background

5.1.1 *Secondary Membrane Disruption*

Immediately following a traumatic insult to the brain, the plasma membrane of neurons is disrupted and potentiates detrimental processes which lead to cell death and dysfunction (LaPlaca, Prado et al. 2009). Membrane disruption has been identified as one of the earliest events after experimental traumatic brain injury (Pettus, Christman et al. 1994, Povlishock, Marmarou et al. 1997, Shi and Borgens 2000, Borgens 2001). This membrane disruption can be transient, permanent, or delayed (Farkas, Lifshitz et al. 2006). As membrane disruption is hypothesized to be a precursor to all events in the injury pathway, it is an important therapeutic target to prevent downstream pathologies. However, if membrane damage only occurs as a result of mechanical trauma at the time of injury the therapeutic time window for membrane protection may be unfeasibly small. However, if secondary membrane damage is also occurring after the initial mechanoporation, there may be hope for the therapeutic potential of plasma membrane repair at later time points.

There is some evidence *in vivo* for the occurrence of secondary membrane damage. In a rat model of head injury, it was noted that persistently elevated intracranial pressure post-injury was associated with chronic membrane poration (Lafrenaye, McGinn et al. 2012). *In vivo* experiments using permeability markers delivered after injury have

also noted that increased membrane permeability may not be limited to the moment of injury. These experiments include controlled cortical impact models in mice (Whalen, Dalkara et al. 2008) and impact acceleration models in rats (Koob, Duerstock et al. 2005, Farkas, Lifshitz et al. 2006).

5.1.2 Lipid Peroxidation in Traumatic Brain injury

Oxygen radical-mediated lipid peroxidation has been suggested to be an important factor in posttraumatic neuronal degeneration (Braugher and Hall 1992, Hall, Yonkers et al. 1992). TBI has been shown to result in a rapid, and sometimes biphasic, increase in lipid peroxidation (Marmarou, Foda et al. 1994, Promyslov and Demchuk 1995, Hsiang, Yeung et al. 1997, Sullivan, Keller et al. 1998, Vagnozzi, Marmarou et al. 1999). Lipid peroxidation leads to perturbations of the plasma membrane that increase membrane permeability (Girotti 1985).

5.1.3 Cell Membrane Repair by Poloxamer 188

Poloxamer 188 (P188) is a compound that has membrane-protecting properties. It is a tri-block copolymer that consists of two hydrophilic polyethylene oxide chains flanking a central hydrophobic polyoxypropylene molecule (Moghimi and Hunter 2000). It has been shown to rescue cells from many membrane-damaging insults such as ionizing radiation (Hannig, Zhang et al. 2000), thermal damage (Padanilam, Bischof et al. 1994), electroporation (Lee, River et al. 1992, Sharma, Stebe et al. 1996), and shear stress injury (Kilinc, Gallo et al. 2007) by interacting with the cell membrane (Marks, Pan et al. 2001, Maskarinec and Lee 2003). P188 prevents axonal beading and reduces membrane permeability in a mechanical shear stress model of axonal injury in chick forebrain

neurons(Kilinc, Gallo et al. 2007) . P188 has also been shown to successfully rescue neurons from NMDA injury (Marks, Pan et al. 2001). Because of its ability to seal damaged membranes P188 is a potential therapeutic as well as a tool to investigate the contribution of membrane damage to cellular degeneration after injury. The role of P188 as a therapeutic agent will be dependent on the time window during which membrane damage is occurring and can be reversed.

Poloxamers are triblock copolymers made of poly(ethylene glycol)-poly(propylene glycol)-poly(ethyleneglycol). Polaxamers are synthesized by the sequential addition first of propylene oxide and then ethylene oxide to a low molecular weight water-soluble organic compound, propylene glycol. The hydrophobic portion is the inner polyoxypropylene glycol which changes from a water soluble to a water-insoluble polymer as the molecular weight goes above 750(Schmolka 1977). Poloxamers are non-ionic surfactants that currently have many approved medical applications, including uses as food additives, stool softeners, wound cleansers, emulsifying agents in intravenous fat emulsions, organ perfusates, and enhancing drug delivery. They have also been shown to be therapeutic in situations where the cell membrane may be disrupted or permeabilized such as radiation injury, thermal burns, frostbite, reperfusion injury, spinal cord injury and electrical shock (Schmolka 1977, Maskarinec and Lee 2003, Borgens, Bohnert et al. 2004, Curry, Wright et al. 2004). Poloxamers with different hydrophilic: hydrophobic ratios have different properties and poloxamers with low hydrophilic: hydrophobic ratios have been shown to destabilize membranes(Moghimi and Hunter 2000).

5.1.4 Hypothesis and Summary of Experiments

In the hypothesized injury pathway, secondary membrane damage can occur after the initial mechanoporation of the membrane as a consequence of lipid peroxidation due to increased oxidative stress. This increase in oxidative stress is presumed to come about as a result of mitochondrial dysfunction, which occurs when mitochondria are overloaded with sequestered calcium.

To test whether secondary membrane damage can occur downstream of the injury pathway, axonal membrane permeability measurements were made after increasing axonal calcium, damaging mitochondria, and increasing oxidative stress. To see if this membrane damage could be treated by P188, P188 was added to the treatment solutions and axonal beading was quantified. Lastly, lipid peroxidation measurements were made to investigate the mechanism of secondary membrane damage, lipid peroxidation measurements were made.

5.2 Materials and Methods

5.2.1 Measurement of Lipid Peroxidation

Lipid Peroxidation measurements were made using Molecular Probes® Image-iT® Lipid Peroxidation Kit for live cell analysis. The reagent localizes to the cell membrane and displays a shift in peak fluorescence emission from ~590 nm (Red) to ~510 nm (Green) when it is oxidized by lipid hydroperoxides. This oxidation-dependent emission shift enables ratiometric fluorescence imaging of lipid hydroperoxides in live cells.

The Molecular Probes® protocol was adapted to measure lipid peroxidation in axons. Neurons in video dishes were incubated with 1mM of the Lipid Peroxidation Sensor for 30 minutes at 37°C. Cells were rinsed three times and baseline fluorescence images were taken using an excitation/emission of 581/591 nm (Texas Red® filter set) for the reduced dye, and the other at excitation/emission of 488/510 nm (traditional FITC filter set) for the oxidized dye. Treatment was then added and a second fluorescence image was taken after the allotted treatment time.

To quantify changes in lipid peroxidation, ImageJ was used to measure the total integrated axonal fluorescence intensity at each wavelength for each image. The ratio of Green: Red fluorescence was calculated for each image, and the fold change for each axon was calculated by normalizing by the baseline fluorescence ratio.

$$\text{Fold Change} = \frac{G_2/R_2 - G_1/R_1}{G_1/R_1}$$

Equation 8. Calculation for Fold Change measurement for Lipid Peroxidation. G_1 = Total integrated Green Fluorescence before treatment, R_1 = Total integrated Red Fluorescence before treatment, G_2 = Total integrated Green Fluorescence after treatment, R_2 = Total integrated Red Fluorescence after treatment,

5.2.2 Cell membrane repair with P188

P188 is used here both a therapeutic and investigative tool. The goals were to determine its capability to protect membrane integrity and to reduce axonal beading. This provided evidence that secondary membrane damage was occurring after isolated perturbations to the damage pathway and that this damage could be treated with P188.

P188 has been demonstrated to successfully seal permeabilized membrane bilayers (Maskarinec and Lee 2003). While the mechanism by which it seals damaged membranes is not completely understood, there is some evidence of insertion into the

membrane (Marks, Pan et al. 2001). Experiments using Langmuir monolayers (Wu, Majewski et al. 2004) and bilayers (Wu, Majewski et al. 2005) have provided evidence that poloxamers can selectively insert into low lipid-density regions of membranes. Here, poloxamer is used as a tool to protect membrane integrity.

5.3 Results

5.3.1 Increasing Intracellular Calcium Results in Increased Membrane Permeability

To determine if membrane damage occurs downstream of increased axonal calcium, calcium ionophore A23187 was used to increase axonal calcium without preceding membrane damage. The loss of the intracellular dye Calcein Red/Orange was used to indicate axonal permeability. In the hypothesized pathway (Figure 1) axonal calcium is increased as a result of calcium influx due to membrane permeability and overloads the mitochondria resulting in increased oxidative stress and membrane damage through lipid peroxidation. Control cells lost less than 10% of their baseline calcein fluorescence after 30 minutes while cells treated with 10 μ M A23187 lost more than 40% (Figure 58), indicating that increasing axonal calcium with A23187 results in increased membrane permeability.

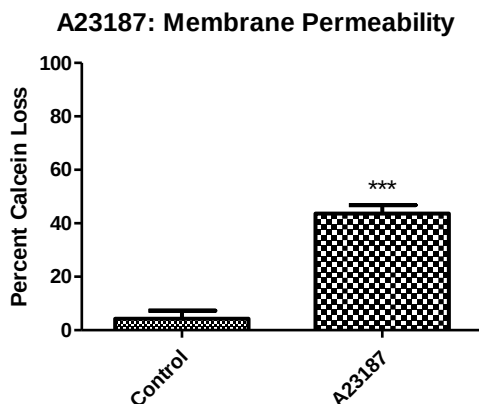


Figure 58. Increasing intracellular calcium with calcium ionophore A23187 results in increased membrane permeability. The Percent Calcein Loss was calculated as percentage decrease in axonal fluorescence intensity 20 minutes after the addition of 10 μ M A23187. (One-tailed t-test, $p < 0.0001$, $n > 20$). All values are presented as means $\pm$ SEM.

5.3.2 Damaging Mitochondria Results in Increased Membrane Permeability

To determine if membrane damage occurs downstream of mitochondrial damage, CCCP was used to damage mitochondria without primary membrane damage or increased axonal calcium. The loss of the intracellular dye Calcein Red/Orange was used to indicate axonal permeability. In the hypothesized pathway (Figure 1), damaged mitochondria increase the oxidative stress of the cell, leading to increased membrane permeability as a result of lipid peroxidation. Control cells lost less than 20% of their baseline calcein fluorescence after 60 minutes while cells treated with 100 μ M CCCP lost almost 40% (Figure 59), indicating that damaging mitochondria with CCCP results in increased membrane permeability.

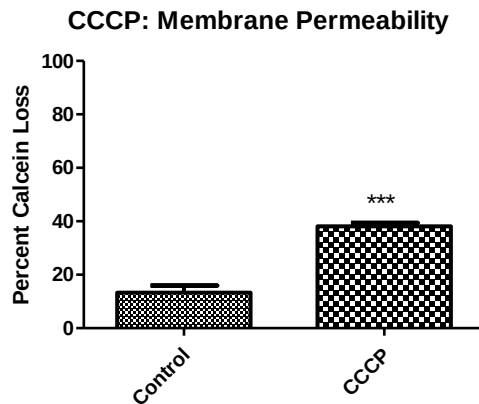


Figure 59. Disrupting mitochondrial function with CCCP results in increased membrane permeability. Percent Calcein Loss was calculated as percentage decrease in axonal fluorescence intensity 60 minutes after the addition of 100 μ M CCCP. (One-tailed t-test, $p < 0.0001$, $n \geq 35$). All values are presented as means $\pm$ SEM.

5.3.3 Increasing Oxidative Stress Results in Increased Membrane Permeability

To determine if membrane damage occurs downstream of increased oxidative stress, TbHp was used to increase the oxidative stress of the cell without preceding mechanoporation, increased axonal calcium, or mitochondrial injury. In the hypothesized pathway (Figure 1), oxidative stress is increased as a result of mitochondrial damage and leads to increased membrane permeability through lipid peroxidation. The loss of the intracellular dye Calcein Red/Orange was used to indicate axonal permeability. In the hypothesized pathway, damaged mitochondria increase the oxidative stress of the cell, leading to increased membrane permeability as a result of lipid peroxidation. Control cells lost less than 10% of their baseline Calcein fluorescence after 60 minutes while cells treated with 100mM TbHp lost almost 30% (Figure 60), indicating that damaging mitochondria with TbHp results in increased membrane permeability.

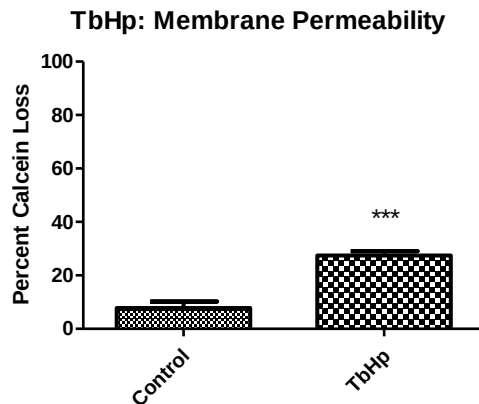


Figure 60. Increasing oxidative stress with TbHp results in increased membrane permeability. Percent Calcein Loss was calculated as percentage decrease in axonal fluorescence intensity 20 minutes after the addition of 100 μ M TbHp. (One-tailed t-test, $p < 0.0001$, $n \geq 15$). All values are presented as means $\pm$ SEM.

5.3.4 P188 Does not reduce beading After Increasing Intracellular Calcium

In the hypothesized pathway (Figure 1), axonal calcium is increased due to mechanoporation after injury and leads to beading through a pathway that includes protease and calpain activation. Increased axonal calcium is also hypothesized to lead to beading through a second, less direct pathway, through lipid peroxidation that occurs as a result of increased oxidative stress from mitochondria that are damaged when overloaded with calcium. Since beading is occurring directly downstream of increased axonal calcium, protecting the membrane with P188 should not eliminate beading, but it may slightly decrease beading due to secondary membrane damage downstream of

increased axonal calcium. When Poloxamer 188 was added in conjunction with calcium ionophore A23187, it did not decrease beading (Figure 61).

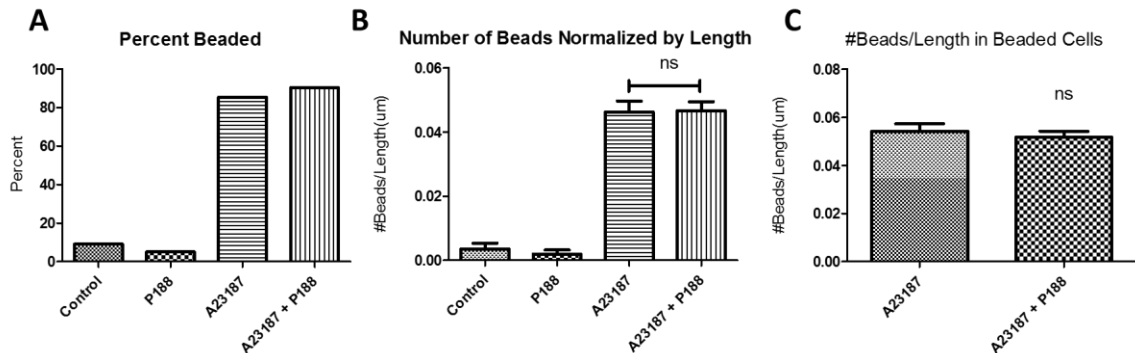


Figure 61. P188 does not reduce axonal beading when cytosolic calcium is increased with A23187. Cells were incubated in 10μM A23187 and 100μM P188. **A**, P188 did not reduce the percent of beaded cells. **B**, A23187 treated cells show significantly more beading than controls, but P188 does not cause any change in beading levels (Kruskal-Wallis test followed by Dunn's Multiple Comparison test, $p > 0.05$, $n > 65$ cells from three experiments). **C**, In cells that present at least on bead, cells that have been treated with P188 in addition to A23187 show no difference in beading as compared to those treated with A23187 alone (Mann Whitney test, $p > 0.05$, $n \geq 70$ cells from three experiments). All values are presented as means $\pm$ SEM.

P188 is known to help preserve membrane integrity and calcium ionophore treatment has been shown to disrupt membrane integrity (Figure 58), but P188 did not reduce axonal beading when added with calcium ionophore A23187. This could be because membrane permeability per se plays a relative small contribution in beading as compared to increased axonal calcium, which is a major consequence of membrane permeability. It is also possible that the type of membrane damage induced by ionophore treatment cannot be prevented with P188.

5.3.5 P188 Does Not Reduce Increase in Membrane Permeability Associated with A23187 Treatment

The potential for P188 neuroprotection is premised on its ability to protect plasma membrane integrity. To see if P188 was able to protect membrane integrity after cytosolic calcium increase with A23187, P188 was added at the same time as A23187 and changes in membrane permeability were measured by quantifying loss of the intracellularly loaded dye Calcein. After 30 minutes of incubation control cells lost less than ten percent of their initial fluorescence while cells treated with 10 μ M A23187 lost around 40% (Figure 62). There was no change in permeability when P188 was present.

Membrane Permeability: A23187 + P188

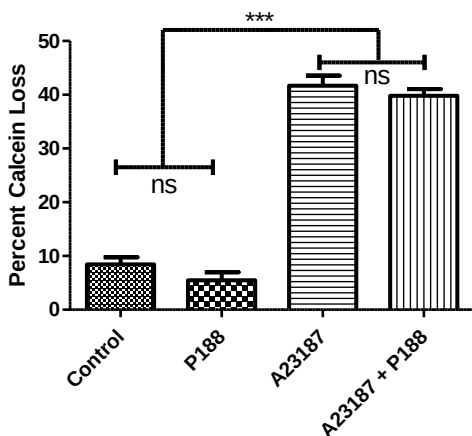


Figure 62. A23187 treatment results in increased plasma membrane permeability that is not mitigated when P188 is present. Cells were treated with 10 μ M A23187 and 100 μ M P188 for 30 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p > 0.05$, $n > 25$ cells for each condition). All values are presented as means $\pm$ SEM.

5.4.6 P188 Does Not Reduce Cytosolic Calcium Increase Associated with A23187 Treatment

As P188 does not protect the plasma membrane from increased leakiness due to A23187 treatment, it would also not be expected to prevent the increase in cytosolic

calcium. To ensure that this was the case, P188 was added at the same time as A23187 and relative changes in cytosolic intracellular calcium were measured. Neurons were imaged before treatment and again after 30 minutes of treatment with μM A23187 and 100 μM P188. A23187 treatment resulted in a four-fold increase in cytosolic calcium and there was no significant change when P188 was on board (Figure 63).

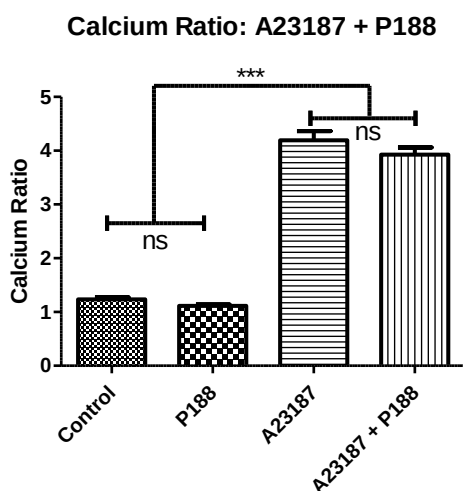


Figure 63. A23187 treatment results in intracellular axonal calcium increase that is not mitigated when P188 is present. Cells were treated with 10 μM A23187 and 100 μM P188 for 30 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p > 0.05$, $n > 25$ cells for each condition). All values are presented as means $\pm$ SEM.

5.3.7 P188 Reduces beading after Mitochondrial Dysfunction is induced with CCCP

In the hypothesized pathway (Figure 1), mitochondrial injury leads to increased oxidative stress and a subsequent increase in membrane permeability due to lipid peroxidation. If beading is a result of the effects of the increased membrane permeability, then protecting the membrane using Poloxamer 188 should reduce beading. When CCCP was used to disrupt mitochondrial function, P188 was effective in reducing axonal beading (Figure 64).

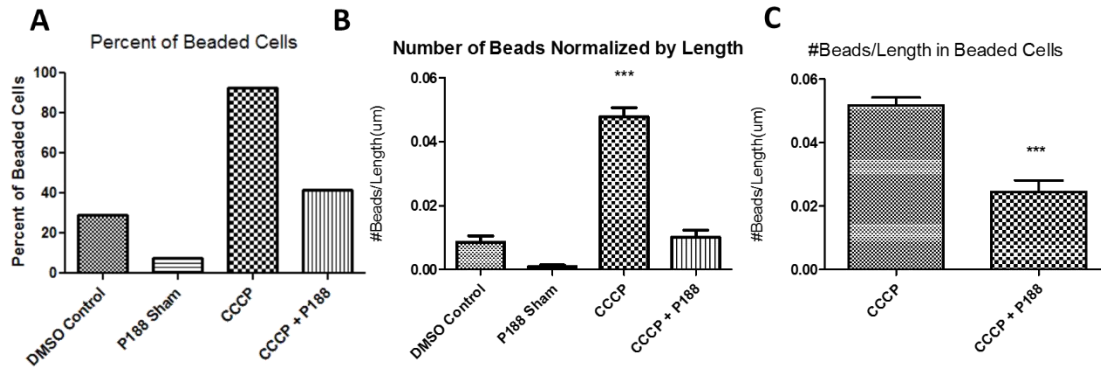


Figure 64. P188 reduces axonal beading after CCCP treatment. Cells were incubated in 100 μ M CCCP and 100 μ M P188. **A**, P188 added with CCCP reduces the percent of beaded cells compared to CCCP treatment alone. **B**, CCCP treated cells show significantly more beads than the DMSO Control or P188 Sham. Adding P188 to the CCCP treated cells brings beading back to baseline levels (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.001$, $n \geq 65$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with CCCP and P188 have significantly less beading than cells treated with CCCP alone (Mann Whitney test, $p < 0.001$, $n > 25$) cells from three experiments. All values are presented as means $\pm$ SEM.

Disrupting mitochondria with CCCP has been shown to result in increased plasma membrane permeability (Figure 59) and treatment with P188 which is thought to protect membrane integrity is able to prevent beading. These results support the hypothesis that secondary membrane damage can occur downstream of mitochondrial disruption and that the degenerative effects of this membrane damage can be mitigated with P188.

5.3.8 P188 Reduces Increase in Membrane Permeability Associated with CCCP Treatment

The effectiveness of P188 in mitigating axonal beading after CCCP treatment is presumed to be a result of preventing secondary membrane damage induced by CCCP. To ensure that this is the case, membrane permeability changes were measured with CCCP and P188. Neurons were imaged before and after 60 minutes of treatment and changes in axonal fluorescence for the intracellular dye calcein were quantified. Control

cells lost around 15 percent of their initial fluorescence while CCCP-treated cells lost more than 30 percent. When P188 was added at the same time as CCCP, the percent of calcein loss was less than 25%. There was a statistically significant decrease from cells with CCCP alone, and no statistical significance from control cells (Figure 65).

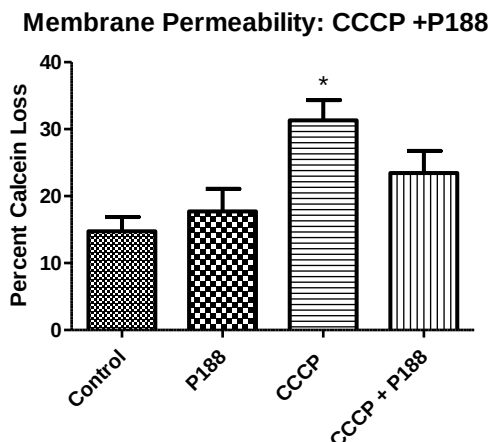


Figure 65. CCCP treatment results in increased plasma membrane permeability that is mitigated when P188 is present. Cells were treated with 100 μ M CCCP and 100 μ M P188 for 60 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p < 0.05$, $n > 15$ cells for each condition). All values are presented as means $\pm$ SEM.

5.3.9 Poloxamer Reduces Cytosolic Calcium Increase Associated with CCCP Treatment

CCCP treatment has been shown to result in secondary membrane damage which is mitigated when P188 is present. This is presumed to be a result of P188's ability to preserve membrane integrity. One effect of secondary membrane damage after CCCP treatment should be a loss of ionic homeostasis and a flow of ions in the direction of their concentration gradient. Cytosolic calcium is increased after CCCP treatment (Figure 30) and if the influx of extracellular calcium through a leaky membrane is contributing to that increased calcium then protecting the membrane with P188 should reduce that increase. Relative changes in cytosolic calcium were measured after CCCP treatment. The increase after 60 minutes of 100 μ M CCCP treatment was twice the increase shown in control cells.

When 100 μ M P188 was present in addition to CCCP the increase in calcium was statistically less than CCCP alone but still more than controls (Figure 66).

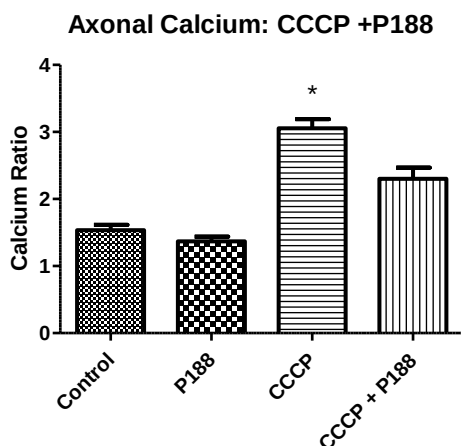


Figure 66. CCCP treatment results in increased cytosolic calcium that is mitigated when P188 is present. Cells were treated with 100 μ M CCCP and 100 μ M P188 for 60 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p < 0.05$, $n > 15$ cells for each condition). All values are presented as means $\pm$ SEM.

5.3.10 Poloxamer 188 Reduces beading after Increasing Oxidative Stress

In the hypothesized pathway (Figure 1), increased oxidative stress as a result of mitochondrial damage results in further mitochondrial disruption and membrane damage through lipid peroxidation. The damage to the membrane results in a loss of ionic homeostasis which ultimately leads to beading. If P188 can protect the membrane, it should also be able to reduce beading. When 100 μ M P188 was added in with 200mM TbHp to increase oxidative stress, the percentage of cells which presented with beads was not reduced but the intensity of beading was decreased (Figure 67).

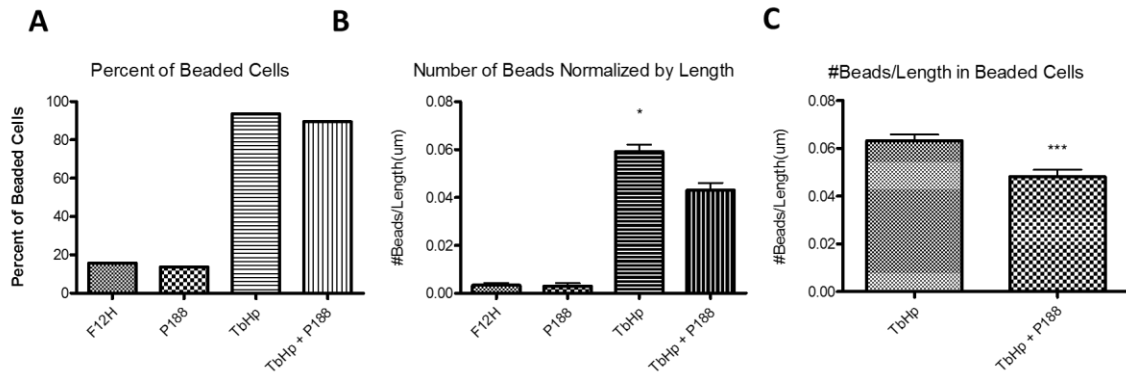


Figure 67. P188 reduces axonal beading after TbHp treatment. Cells were incubated in 200mM TbHp and 100 μ M P188. **A**, P188 added with TbHp does not reduce the percentage of cells showing at least one bead. **B**, Adding P188 to TbHp treated cells significantly reduces beading (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test, $p < 0.05$, $n \geq 85$ cells from three experiments). **C**, In cells that present at least one bead, cells treated with P188 and TbHp have significantly less beading than cells treated with TbHp (Mann Whitney test, $p < 0.001$, $n \geq 85$ cells from three experiments). All values are presented as means $\pm$ SEM.

It is worth noting that in this experiment almost every single TbHp treated cell presented with beads. It is possible that using a lower concentration of TbHp where not every cell was beaded would also result in P188 reducing the percentage of beaded cells. Taken with the results showing that TbHp treatment increases membrane permeability (Figure 60) this provides supporting evidence that increased oxidative stress disrupts the plasma membrane in a manner that is mitigated by P188.

5.3.11 Poloxamer 188 Reduces Increase in Membrane Permeability Resulting from TbHp Treatment

P188 was successful in reducing axonal beading due to increased oxidative stress. This is presumed to be a result of P188's ability to protect the integrity of the membrane. In order to make this interpretation it must first be shown that P188 is in fact protecting the plasma membrane from increased membrane permeability. To test this, neurons

were imaged before and 30 minutes after treatment and changes in axonal fluorescence for the intracellular dye calcein were quantified. Control cells lost less than ten percent of their initial fluorescence while TbHp-treated cells lost almost thirty percent. The addition of 100 μ M P188 with 100mM TbHp reduced the loss of fluorescence to an average of less than 15 percent, which was not a statistically significant change from controls (Figure 68).

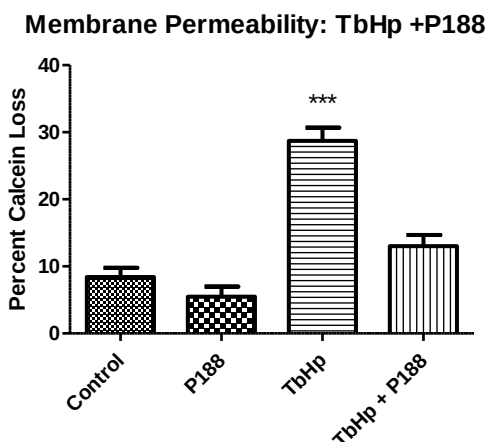


Figure 68. TbHp treatment results in increased plasma membrane permeability that is mitigated when P188 is present. Cells were treated with 100 mM TbHp and 100 μ M P188 for 360 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p < 0.001$, $n > 20$ cells for each condition). All values are presented as means $\pm$ SEM.

5.3.12 Poloxamer 188 Does Not Reduce Increase in Cytosolic Calcium Resulting from TbHp Treatment

TbHp treatment results in an increase in cytosolic calcium (Figure 36), a major contributor to which comes from intracellularly sequestered calcium release (Figure 52) as well as some from extracellular calcium influx, presumably as a result of secondary membrane damage due to TbHp treatment. When P188 is present at the time of TbHp treatment there is no significant increase in membrane permeability, meaning that there is limited potential for the influx of extracellular calcium. To see how P188 protection of the membrane affected cytosolic calcium changes, relative changes in intracellular calcium were measured with 100mM TbHp and 100 μ M P188. TbHp treatment resulted

in a relative change in intracellular calcium that was more than two-fold that of controls, with no discernable change when P188 was present (Figure 69).

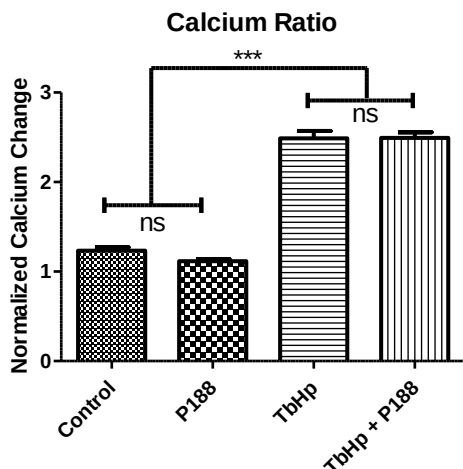


Figure 69. TbHp treatment results in increased cytosolic calcium that is not mitigated when P188 is present. Cells were treated with 100mM TbHp and 100 μ M P188 for 360 minutes (one-way analysis of variance followed by Tukey's multiple comparison, $p < 0.001$, $n > 20$ cells for each condition). All values are presented as means $\pm$ SEM.

5.3.12 Increasing Intracellular Calcium Results in Lipid Peroxidation

In the hypothesized pathway (Figure 1), calcium overload causes mitochondrial dysfunction and an increase in oxidative stress which results in lipid peroxidation and increased membrane permeability. To see if increasing intracellular calcium resulted in lipid peroxidation, calcium ionophore A23187 was used to induce an influx of calcium and changes in lipid peroxidation were measured. Cells treated with 10 μ M A23187 for 30 minutes had higher levels of lipid peroxidation than controls (Figure 70).

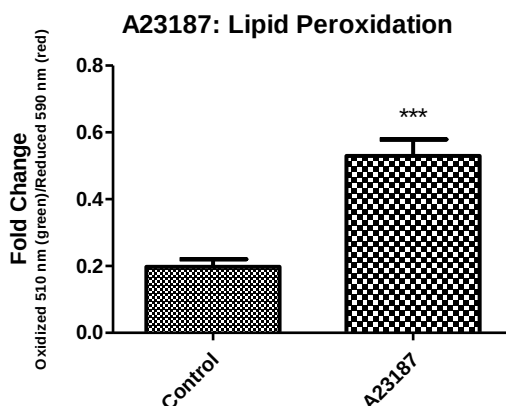


Figure 70. Increasing intracellular calcium with calcium ionophore A23187 results in lipid peroxidation. Cells were incubated 10 μ M A23187 for 20 minutes after a 30 minute incubation with 1 mM Image-iT[®] Lipid Peroxidation Sensor at 37°C. , The fold change was calculated as the ratiometric measurement of axonal fluorescence of 610nm/590nm normalized to the baseline fluorescence before treatment. (One-tailed t-test, $p < 0.001$, $n > 25$). All values are presented as means $\pm$ SEM.

5.3.13 Mitochondrial Disruption Results In Lipid Peroxidation

Mitochondrial disruption should lead to an increase in oxidative stress, resulting in lipid peroxidation of the plasma membrane. To test for this, CCCP was used to disrupt mitochondria and lipid peroxidation was measured, revealing greater levels of lipid peroxidation in CCCP-treated cells than control cells (Figure 71).

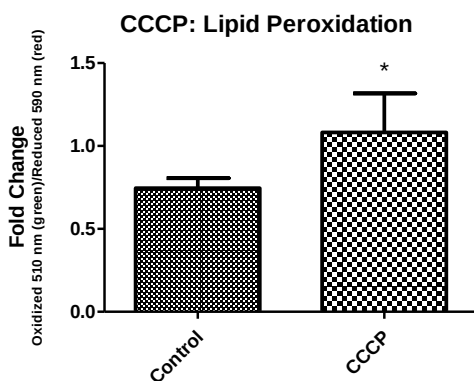


Figure 71. Damaging mitochondria with CCCP results in lipid peroxidation. Cells were incubated 100 μ M CCCP for 60 minutes after a 30 minute incubation with 1 mM Image-iT[®] Lipid Peroxidation Sensor at 37°C. , The fold change was calculated as the ratiometric measurement of axonal fluorescence of 610nm/590nm normalized to the baseline fluorescence before treatment. (One-tailed t-test, $p < 0.05$, $n > 15$). All values are presented as means $\pm$ SEM.

5.3.14 Increasing Oxidative Stress Results in Lipid Peroxidation

Lipid peroxidation should occur under conditions of increased oxidative stress, therefore increasing oxidative stress with TbHp should result in increased levels of lipid

peroxidation. To test for this, TbHp was used to increase the oxidative stress of the cell and changes in axonal lipid peroxidation were measured, revealing increased levels of lipid peroxidation in TbHp-treated cells as compared to controls (Figure 72).

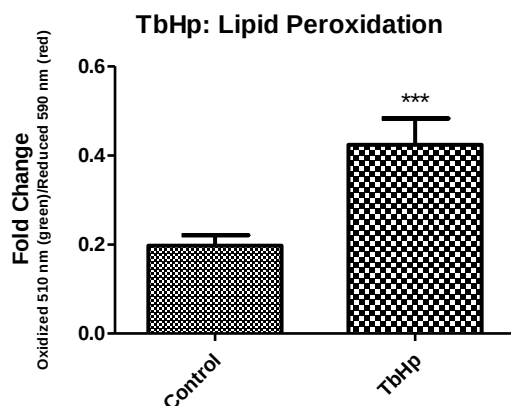


Figure 72. Increasing oxidative stress with TbHp results in lipid peroxidation. Cells were incubated in 100 mM TbHp for 20 minutes after a 30 minute incubation with 1 mM Image-iT® Lipid Peroxidation Sensor at 37°C. The fold change was calculated as the ratiometric measurement of axonal fluorescence of 610nm/590nm normalized to the baseline fluorescence before treatment. (One-tailed t-test, $p < 0.001$, $n > 30$). All values are presented as means $\pm$ SEM.

5.4 Discussion

The results in this chapter provide evidence that secondary membrane damage is occurring after targeted insults to the hypothesized TBI pathway. These insults included increasing cytosolic calcium, disrupting mitochondrial function, and increasing oxidative stress, and that lipid peroxidation is playing a part in these effects. It also provides evidence that P188 is able to reduce membrane permeabilization and axonal beading after disrupting mitochondrial function and increasing oxidative stress, but not after increasing cytosolic calcium with A23187.

In order for plasma membrane protection to be a valid therapeutic target for TBI, there must be a reasonable therapeutic time window for membrane damage. If membrane damage is occurring only at the moment of mechanical injury, the time

window of application for membrane-protective treatments such as P188 would transient and brief, limiting the potential for membrane protection. However, if membrane damage is ongoing or recurring then targeting the membrane has greater therapeutic potential.

Post-injury treatment of P188 has been shown to be neuroprotective in neurons undergoing shear injury when it was added after the mechanical insult (Kilinc, Gallo et al. 2007). This indicates that there is a window of opportunity for a therapeutic application of P188 after mechanical injury. The results here show that increased membrane permeability, as well as lipid peroxidation, are occurring after increased cytosolic calcium, mitochondrial damage, and increased oxidative stress.

Primary membrane damage at the time of injury is hypothesized to lead to a loss of ionic homeostasis and an influx of calcium, leading to beading through protease activation. A secondary pathway is hypothesized to lead in a less direct manner to neuronal degeneration by damaging mitochondria and increasing oxidative stress, leading to secondary membrane permeability through lipid peroxidation. Therefore inducing an influx of calcium in the absence of preceding membrane damage should also lead to increased membrane permeability and lipid peroxidation. Indeed, A23187 application did result in increased membrane permeability (Figure 62) as well as lipid peroxidation (Figure 70). P188 was not able to reduce A23187-induced beading or calcium influx. This is to be expected as A23187-induced calcium influx is likely to be much greater than that caused by secondary membrane damage and leads more directly to beading. However, P188 was also unsuccessful in reducing the changes in membrane permeability. P188 was able to

reduce the changes in membrane permeability after CCCP and TbHp application, all of which are hypothesized to be caused by the same mechanism. The fact that P188 was unable to protect the cell membrane from damage after increasing cytosolic calcium with A23187 implies a different mechanism of increasing membrane permeability or a different form of damage that is not treatable with P188. It is also possible that the kinetics of A23187-induced damage are so extreme compared to the other types of damage that P188 is unable to mitigate it.

Mitochondrial damage, a consequence of TBI, is hypothesized to lead to increased oxidative stress and increased membrane permeability via lipid peroxidation, as well as a release of mitochondrially sequestered calcium which can lead to beading. Secondary membrane damage can also lead to beading by allowing an influx of extracellular calcium. To investigate these ideas, CCCP was used to induce mitochondrial dysfunction without preceding membrane damage or calcium influx and was shown to result in increased membrane permeability (Figure 65) and lipid peroxidation (Figure 71), providing evidence for secondary membrane permeability. Indirect evidence for secondary membrane permeability was also given by the fact that P188 was able to bring axonal beading back to baseline levels (Figure 64), and calcein imaging to measure changes in membrane permeability confirmed that P188 was bringing increases in membrane permeability back to baseline levels (Figure 65). Calcium imaging with P188 also showed that changes in cytosolic calcium were reduced but still higher than controls. Similar to the experiment where EGTA used to chelate extracellular calcium reduced cytosolic calcium levels after CCCP treatment to a level that was still higher than EGTA controls (Figure 66), this

provides supporting evidence for a dual source of calcium from extracellular influx as well as intracellular release.

Increased oxidative stress in TBI is hypothesized to lead to secondary membrane damage through lipid peroxidation, thereby leading to an influx of extracellular calcium which can lead to beading. Increased oxidative stress can also induce mitochondrial dysfunction, inducing the release of mitochondrially sequestered calcium and leading to axonal beading through that pathway. Increasing oxidative stress with TbHp was shown to result in increased membrane permeability (Figure 68) and lipid peroxidation (Figure 72). Calcein imaging to measure changes in membrane permeability with P188 shows that the presence of P188 brought membrane permeability back to baseline levels (Figure 68) and P188 reduced axonal beading (Figure 67). P188 did not, however, reduce axonal calcium (Figure 69), providing even more evidence for the release of calcium from intracellular stores.

The most therapeutically significant finding in this chapter is the evidence of secondary membrane permeability through lipid peroxidation. This means that increased membrane permeability in TBI is not limited to the initial mechanoporation and there may be an extended time window for therapeutic intervention through membrane protection. P188 was used here as one option to protect membrane integrity, but the opportunity for other membrane-protection methods exists.

These findings, in the absence of initial mechanoporation, are not specific to TBI and can possibly be applied to other neurological conditions involving mitochondrial

dysfunction and increased oxidative stress. Oxidative stress has been implicated in the pathogenesis of Alzheimer's Disease (Volicer and Crino 1990), Parkinson's disease (Adams, Graham et al. 1991, Schulz, Lindenau et al. 2000) , and ALS (Barber, Mead et al. 2006). Mitochondrial dysfunction has also been implicated in neurodegenerative disease such as Alzheimer's disease, Parkinson's disease, ALS, and Huntington's disease. If mitochondrial dysfunction and increased oxidative stress lead to increased membrane permeability in other disease conditions, this opens the door for more therapeutic interventions.

Chapter 6: Conclusions and Future Work

6.1 Summary of Principle Findings

The work presented here contributes to the understanding of injury mechanisms and the role that the disruption of plasma membrane integrity plays in the initiation and progression of axonal pathology in the context of Traumatic Brain Injury. It shows that increasing membrane permeability, even in the absence of mechanical injury, is sufficient to induce many of the consequences of TBI including axonal beading, focal disruption of microtubules, increased cytosolic calcium, and mitochondrial dysfunction. Increasing cytosolic calcium, even without preceding membrane damage, was also sufficient to cause these pathologies, and chelating extracellular calcium reduced axonal beading, providing further evidence of the deleterious effects of calcium influx. These findings are consistent with previous work regarding mechanoporation and secondary damage due to calcium influx.

Mitochondrial dysfunction, in the absence of preceding membrane damage, also led to axonal beading, focal disruption of microtubules, and increased cytosolic calcium. Oxidative stress, a major consequence of mitochondrial damage, resulted in similar effects including further disruption of mitochondrial function. Reducing oxidative stress with antioxidants after isolated perturbations to membrane integrity, mitochondrial function, and cytosolic calcium, prevented axonal beading which supports the idea that increased oxidative stress is occurring after these insults and that it plays a role in beading. Evidence for dual sources of calcium were provided by calcium measurements made after

increasing oxidative stress or disrupting mitochondrial function while preventing the influx of calcium from the extracellular space, revealing that cytosolic calcium increases may result influx from the extracellular space as well as intracellular release, most likely from mitochondria. Chelating intracellular calcium reduced axonal beading after disruption of mitochondrial function, increased oxidative stress and membrane disruption with melittin, but not after membrane disruption with saponin or increasing intracellular calcium with calcium ionophore A23187. Preventing the regulated opening of the mitochondrial permeability pore with Cyclosporin A (CsA) reduced axonal beading after disrupting mitochondrial function, increasing oxidative stress, and permeabilizing the membrane but not after increasing cytosolic calcium with A23187.

Finally, the possibility of secondary membrane damage and therapeutic interventions was investigated by measuring changes in membrane permeability and protecting membrane integrity with P188. It was demonstrated that increased membrane permeability and lipid peroxidation are induced under conditions of oxidative stress, mitochondrial dysfunction, and increased axonal calcium. P188 was able to prevent axonal beading after increasing oxidative stress or inducing mitochondrial dysfunction, but not after increasing cytosolic calcium with calcium ionophore A23187.

6.2. Implications of the current study

The experiments here support the hypothesized injury pathway and allow for the exploration of different therapeutic targets. Understanding the link between different

parts of the TBI pathway allows for drug development that can be targeted to appropriate pieces of the pathway. The potential targets shown here include the plasma membrane, intracellular calcium, mitochondria, and oxidative stress.

Membrane damage, at the beginning of the TBI pathway, is first initiated through mechanical forces (“mechanoporation”). Loss of plasma membrane integrity leads to many of the secondary injuries seen in TBI, including increased cytosolic calcium, mitochondrial dysfunction, cytoskeletal disruption, and axonal beading. Membrane damage on its own does not have to be a death sentence. The cell has mechanisms for repairing damaged membranes and restoring cellular integrity in ATP-dependent processes. However, depending on the extent of the damage, this is not always possible. Since membrane damage initiates the proceeding damage sequences, it is likely the best target for saving the cell. However, if membrane damage is limited to the moment of injury, the time window for membrane-protecting interventions may not be therapeutically viable. Here, we provide evidence that membrane damage can be ongoing due to secondary damage that results in lipid peroxidation and further increases in membrane permeability. We also provide evidence that this increase in membrane permeability can be mitigated using P188, which has already been shown to be neuroprotective in mechanical models of neuronal injury. Since P188 can be protective of the membrane even without direct insults to membrane integrity, there seems to be great therapeutic potential that warrants further study of membrane-targeted neuroprotection.

Calcium, both from extracellular and intracellular sources, was shown to play a significant role in axonal pathology. Increasing cytosolic calcium, even in the absence of mechanical trauma or primary membrane damage, results in a similar pathology to that of TBI. Preventing the influx of extracellular calcium is able to prevent beading after targeting isolated components of the TBI pathway, as is chelating intracellular calcium. All of these point towards calcium as being an important contributor to neuronal degeneration and death after injury, meaning that calcium may be an important target for neuroprotection. However, chelating calcium is not a valid therapeutic target because of the many important roles that calcium plays in neurons – most notably as part of the production of an action potential. Removing calcium may prove to be more detrimental. However, there may be other, more therapeutically valid, options for mitigating the uncontrolled increase in cytosolic calcium. Influx of calcium through a leaky membrane cannot be alleviated unless the membrane damage is repaired, but calcium through other sources may be a potential target. In mild-stretch injuries where mechanoporation may be temporary or more limited, calcium may still enter the cell through other channels, and may be functionally coupled to sodium entry (Wolf, Stys et al. 2001). Therefore, adjusting calcium entry through membrane channels may be a valid target. For example, NMDA receptor activation has been shown to result in calcium influx and in some cases NMDA-receptor agonists have been neuroprotective. However, in all cases they have failed in clinical trials (Ikonomidou and Turski 2002). Cytosolic calcium can also be increased as a result of release from intracellular stores, and is increased after disruption

of mitochondrial function and increased oxidative stress. These routes of release may also be valid therapeutic targets.

Mitochondria were shown to be dysfunctional (through morphological changes) after damage to the plasma membrane, increased intracellular calcium, and increased oxidative stress. They contribute to the progression of axonal injury by releasing sequestered calcium and increasing oxidative stress. Although not investigated here, mitochondrial dysfunction may also prevent neuronal recovery when there is not enough ATP to fuel the energy-dependent processes of repairing the plasma membrane and restoring ion homeostasis. If these dysfunctions are a result of the MPT, then that is a potential therapeutic target as well.

Oxidative stress is another major aspect of the TBI Pathway. It was shown here that increasing oxidative stress, even in the absence of preceding membrane damage or mitochondrial dysfunction, is sufficient to mimic the pathology seen in TBI. Increased oxidative stress causes mitochondrial dysfunction, results in the release of calcium from intracellular stores, results in focal cytoskeletal disruption, and is able to induce secondary membrane permeability and lipid peroxidation. In addition, antioxidant use was successful in mitigating axonal beading after increased membrane permeability, increased cytosolic calcium, and disruption of mitochondrial function. These results indicate that targeting the oxidative stress part of the damage pathway is a valid therapeutic strategy.

Many attempts have already been made to target these parts of the pathway, specifically with cyclosporin A and antioxidant use. No drugs have passed phase III clinical trials (Schouten 2007, Jain 2008). The reason for this is often due to adverse side effects or drug delivery issues. The timing of drug therapy may also be very important, depending on the temporal progression of injury. Drug delivery to the brain is incredibly difficult due to the defense of the blood brain barrier (BBB). Brain injury can result in a breakdown of the BBB (Zhou, Xiang et al. 2001). This has been suggested as a way to target drug delivery to impaired areas of the brain. Assuming that compromised areas of the BBB are co-localized with mechanoporated neurons, this could be a great advantage that should be taken advantage of. In this possible scenario, an understanding of the injury pathway and progression of neurodegeneration would guide treatment strategies. Targeting the plasma membrane with membrane-protecting drugs could have great potential, but may also seal the BBB and prevent successful administration of other drugs to combat oxidative stress or mitochondrial dysfunction.

There are several limitations to the work shown here, some of which can be addressed with simple experiments referenced in the future work section. While these experiments provide insight into the pathway of neuronal degeneration after TBI, they do not provide information about the time course of injury progression which is also important for deciding on therapeutic strategies. They can, however, provide insight into the sequence of events even if the time course is contracted compared to human TBI. These experiments include only non-myelinated neurons in isolation, ignoring the contribution of other cell types. Injury in DAI is primarily seen in the white matter of the

brain, which consists mainly of axons. In these experiments measurements are only made on axons, but insults are given to the entire cell. Time course measurements were not made, primarily due to the damage inflicted by fluorescence imaging which limited the number of measurements that could be made.

In spite of the limitations to this work, great progress has been made in understanding the pathway and mechanisms of axonal pathology related to TBI. The experiments shown here support the hypothesis that membrane damage leads to calcium influx and subsequent mitochondrial disruption. Damaged mitochondria undergo the MPT through an opening of the MPP and release sequestered calcium and increase the production of ROS, increasing the oxidative stress of the cell. This results in lipid peroxidation and further damage to the plasma membrane, propagating and potentiating the cycle of calcium influx ultimately leading to axonal beading. Most importantly this work provides evidence of an injury cycle instead of a linear progression, opening up more opportunities for therapeutic intervention.

6.4 Future Work

There are a number of obvious extensions of the work presented here. The ability to isolate and perturb aspects of the TBI pathway opens many doors to understanding the many pieces of the injury puzzle. The ultimate goal is to understand the mechanisms of injury in order to develop therapies to restore functional recovery.

The work presented here shows measurements of mitochondrial morphology and cytoskeletal disruption as evidence of axonal injury that matches TBI pathology. However,

in addition to being evidence of injury these can also be used as metrics for neuronal health. Here, neuronal health was measured morphologically by quantifying axonal beading, but future work can also look at cytoskeletal and mitochondrial alterations to quantify the protective effects of EGTA, BAPTA-AM, antioxidants, CsA, or P188 as well as other agents that provide clues as to the mechanism of neuronal degeneration. Additionally, making these measurements may help progress the understanding of the mechanisms behind axonal beading.

Along the same lines, a study of axonal transport may also be important. Transport deficits have been shown with TBI, as well as many other neurological disorders. The dynamics of vesicular transport with and without injury can be examined through live imaging of mitochondria. These membrane-bound organelles are good models for transport because they are relatively easily to visualize and travel both anterogradely and retrogradely on the axonal cytoskeleton.

The importance of mitochondrial health and the contribution of the MPT was indirectly observed here using CsA. While the neuroprotective effects of CsA have often been used as evidence of the MPT, this is not a direct measurement. Direct measurements of the MPT and the opening of the MPP can be made to give a better understanding of when this transition is occurring relative to other changes in the cell.

Mitochondrial health should also be investigated with regards to cell death, as well as promoting neuronal regeneration. For functional recovery the ultimate strategy may not be to keep neurons alive, but to promote plasticity or to induce the controlled death

of neurons not functioning properly. Neurons undergoing necrotic death may induce excitotoxic injury on nearby, initially healthy, cells and promote the secondary injury sequence that way. Apoptosis, or controlled cell death, is an energy-dependent programmed form of cell death (Choi, Klintworth et al. 2011). Mitochondrial dysfunction results in ATP-depletion which may promote necrotic death (Cheng, Kong et al. 2012).

The contribution of cytoskeletal integrity to neuronal health has been investigated before, and a link has been made between calpain activation and axonal beading after increased intracellular calcium due to mechanoporation. The work presented here does not address calpain activation specifically. However, to further the understanding of the mechanism behind bead formation one could inhibit calpains while inducing isolated perturbations to the damage pathway.

In vivo there may be other cell types contributing to both the degeneration and restoration of neuronal health. Most notably, astrocytes appear to have higher GSH levels than neurons, both in vivo and in culture (Cooper and Kristal 1997, Dringen, Gutterer et al. 2000). Simplified experiments with isolated neurons can help to provide a better understanding of the progression of axonal pathology, but the presence of other cell types can help to add another layer to this story.

Diffuse axonal injuries are known for occurring in the white matter of the brain, which consists primarily of axons. The work presented in this dissertation includes the entire cell, including the soma, which is undoubtedly affected by the insults being given. To better replicate what is happening in TBI it would be a good idea to isolate the

application of the insult to the axon only, using microfluidics devices or campenot chambers.

The most important aspect of recovery after TBI is not whether or not a neuron is alive and looks healthy, but if it is signaling properly. This is a requirement for functional recovery, which is what really matters. The assumption is being made here that neurons with a normal morphology will also have normal signaling patterns, but it will be important to investigate that directly.

Appendices

Appendix A: An Automated Interactive Bead Measurement Program

Beading is a well-documented phenomenon in neurons that parallels neuronal damage. It is a term that describes focal bead-like swellings that have been shown in diffuse axonal injury after brain trauma and other neurological conditions such as epilepsy, amyotrophic lateral sclerosis, and multiple sclerosis (Takeuchi, Mizuno et al. 2005). It is often colocalized with areas of microtubule disruption and an accumulation of organelles (Povlishock, Erb et al. 1992, Smith, Meaney et al. 2003, Takeuchi, Mizuno et al. 2005, Greenwood, Mizielinska et al. 2007, Kilinc, Gallo et al. 2008). In *in vitro* experiments, beading can be used as a morphological indicator of the extent of neuronal injury. However, difficulty in quantifying the degree of beading has made this problematic.

Previous attempts to quantify beading have been modest at best. The most primitive method involved simply stating the presence of beads. Some researchers compare the ratio of beaded or unbeaded axons (Swann, Al-Noori et al. 2000, Roediger and Armati 2003, Takeuchi, Mizuno et al. 2005). One of the most advanced methods of quantifying beading has simply involved normalizing the number of beads by the length of the axon (Kilinc, Gallo et al. 2008). Beads are often associated with areas of disrupted transport and organelle accumulation, which results in a tendency of the human eye towards dark swellings which may not actually have a significantly larger diameter than

the rest of the axon. Some scientists attempt to limit the subjectivity of deciding what is and is not a bead by setting the minimum size of a bead to be at least two times the width of the axon(Beirowski, Nogradi et al. 2010). However, these methods leave much to be desired. Which section of the axon represents the true width? Normalizing by the length of the axon does not take the size of the bead into account – what if a big bead is a larger sign of injury than a small bead? The most advanced method of analyzing axonal beading to date (Kilinc, Gallo et al. 2009) attempts to address this subjectivity but is unable to achieve satisfactory agreement between what is identified as a bead and what a human participant would choose. To address these concerns the Automated Interactive Bead Measurement program was developed with a complete user interface to allow users to quantify beading in single cultured axons.

While there have not been many successful attempts to quantify being in neurons, there have been many attempts to segment axons and quantify different aspects of neuronal morphology. These attempts have met with many of the same challenges as this beading program. The work down towards these efforts shows a lot of insight that and advice that can be incorporated into the program. For example, Neurphology J (Ho, Chao et al. 2011) acknowledges some problems with skeletonization and undesired branches. Eric Meijering has provided a nice review of various neuronal quantification methods(Meijering 2010).

To develop the Automated Interactive Bead Measurement Program, all functions were written separately in Matlab script and tested on images to ensure that all of the pieces were working properly, and were then compiled into a user interface to allow the

user to easily and efficiently quantify beading in their images (Figure 73). The interface allows the user to process images one by one, or all at once. Since many of the functions are time intensive, automating many images at once saves the user time by allowing them to accomplish other tasks while the program runs, and then returning for validation.

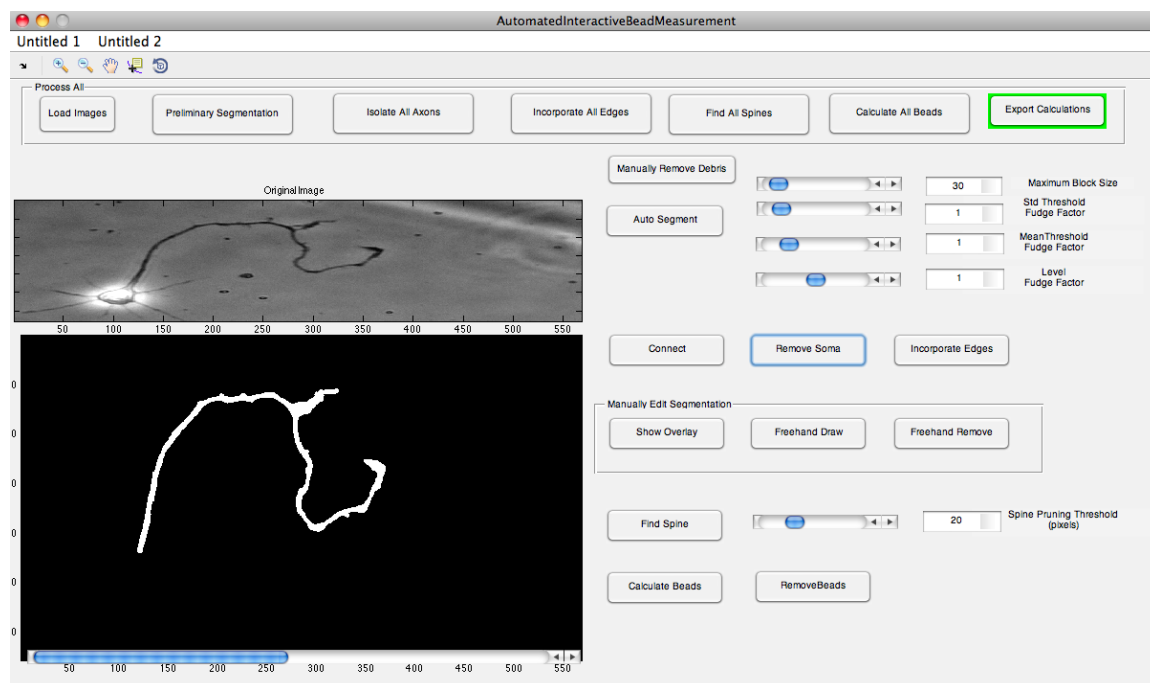


Figure 73. User interface for segmenting axons and quantifying axonal beading.

The input to the program is a list of phase microscopy images of single cultured neurons. The important functions of the program are preprocessing, axon segmentation, determination of the axon spine, axon width measurement, bead identification, and cell morphology calculations.

Preprocessing

The first step to the image processing is to run it through median filters. This is especially important for the edge finding that is incorporated into the segmentation. The

median filter smooths the image without losing important details and prevents the edge functions from finding too many unnecessary edges. Images of cultured neurons often contain debris that can affect the segmentation if it is too close to the cell. To remedy this, the user interface includes a “remove debris” button that allows the user to interactively select regions to be removed and uses Matlab’s “roifill()” function to smoothly interpolate that region

Segmentation

The axon segmentation part of the program is a combination of global and local thresholding combined with edge detection and user validation. The axon segmentation section of the program is one of the most vital. Small deviations in the axon are incredibly important for bead identification, which is why this section allows for user validation and edits in each step of the process. The first step is a global otsu filter on the image, which is used to identify the halo of the cell which helps to identify the cell body. This is necessary because identifying the cell body is important both for removing it from the axon and for orienting the axon for future calculations. The second step of the segmentation is to locally threshold the axon based on the local standard deviation. This was done by breaking the image up into multiple blocks and calculating the standard deviation for each block. Low standard deviations are assumed to be the background which should be mostly uniform. High standard deviations are assumed to be areas that contain edges, and are then locally thresholded using Matlab’s built-in Otsu filter. The user interface allows the user to select “fudge factors” for the various thresholds used (maximum block size, standard deviation, and level). The maximum block size is important

because if a block falls entirely within an axon, it may also have a small standard deviation and be mistaken for the background. This can be remedied by including a mean intensity threshold into the background identification.

Sometimes this segmentation may result in an axon that is not fully connected. Since the segmentation algorithm includes a step to identify the axon by selecting the largest region, any breaks in the segmentation may result in a significantly shorter axon. To remedy this, the user interface includes a “connect” button which allows the user to draw a line connecting the pieces of axon if they have been separated and prevent pieces from being lost.

Edge detection is used to further improve the segmentation. Edges are found in the original image using a canny filter. Small segments are removed. The edges are first subtracted from the segmented axon, which helps to remove any extra bits in the segmentation. The edges are then added back to the axon, dilated, and holes are filled. Any extraneous edge pieces not attached to the axon are then removed. This serves to add in important curves that the segmentation may have missed.

Spine

The identification of the spine of the axon is an important step for quantifying the morphology of the axon. This is achieved using Matlab’s `bwmorph` ‘thin’ function instead of ‘skel’. This is because ‘thin’ does a better job of finding the centerline of the axon without including as many unnecessary branches. However, there is no convenient way to find the main spine of the axon. The spine was pruned by identifying each branch of

the skeleton and then removing the ones shorter than the threshold set by the user through the user interface.

Width

Accurate width identification is important since the bead identification is based on the width. Finding the width of the axon at each point on the skeleton is difficult because the axon is

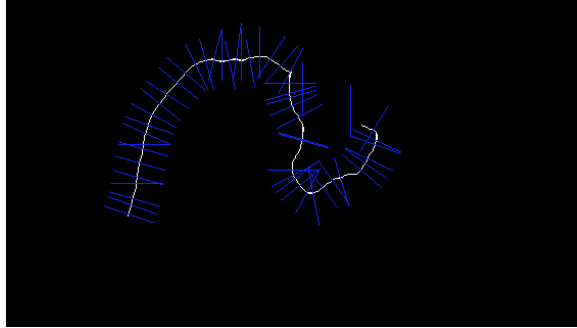


Figure 74. Perpendicular lines along the axon in order to calculate the width at every point on the spine

curved and it is hard to distinguish the two points to measure between. Early attempts to measure the width involved creating a circle at every points on the skeleton, and choosing the diameter of the circle that had the best overlap. This was very time intensive, so a new method was used based on methods for finding the width of blood vessels. The slope of the spine was found for every points, so that a line perpendicular to each point could be plotted (Figure 74). The length of the area of the line that overlapped with the axon was used as the measurement of axonal width at that point.

Bead Identification

The method used to identify what was a bead was any region that was significantly larger than the average region. To do this, all the regional maxima were identified as possible beads. While they required additional filtering to decide what was a bead, it was also useful in identifying all the areas that were definitely not beads. The width of all the points that were not possible beads were averaged to find the average width of the axon, and the standard deviation was also found because it would help to determine what was

significantly larger than the average axon. The possible beads were then filtered using the average width and standard deviation to find all points on the spine that had significantly larger widths. The area of these beads was also estimated by assuming they were circular. Since this method could result in overlapping beads, and beads that were within a certain threshold of each other ($2 \times \text{width}$) were combined into one. Because not all focal enlargements are not signs of injury and should not be considered beads (i.e. growth cones), the user interface includes a “remove beads” button which allows the user to click on misidentified beads to remove them.

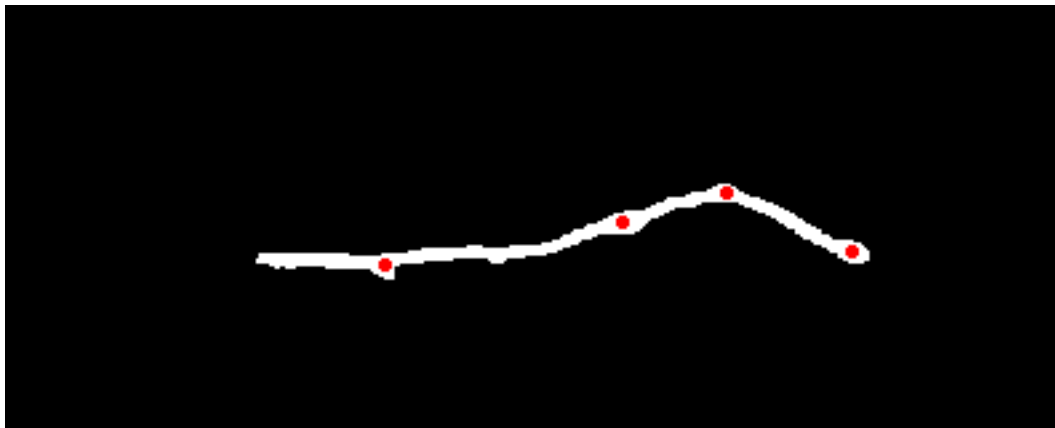


Figure 75. Bead identification

Calculations

The average width of the axon having already been identified, the remaining calculations remaining were the length of the axon, the number of beads, the ‘Beading Index’, and the tortuosity. The method used to identify the length of the axon was to count the number of pixels in the spine. In order to quantify beading, a unitless number was devised to describe the area of the cell that was beading:

$$BeadingIndex = \frac{\sum_{i=1}^n Area_i}{widthAxon * LengthAxon}$$

The beading index for each axon sums the area of the beads and divides it by the length*width of the axon, which means that the axon is approximated as being a rectangle.

Tortuosity was defined as the linear distance between the two endpoints of the axon divided by the length of the axon. This means that a tortuosity value of 1 is a completely linear axon. In order to find the beginning and end points of the axon, the previously identified soma was used to find the beginning points of the axon and then traced until the end. Once all the calculations are complete, the program allows the user to export important calculations to the Matlab workspace for the user to save, further analyze, or export into Excel or statistical software.

The **Automated Interactive Bead Measurement Program** provides a tool to objectively and quantitatively examine axonal beading in in vitro cultures. When used in conjunction with a brain injury model it can be used to better evaluate the effectiveness of therapeutic agents so that those that go on to clinical trials have a greater chance of being effective. In future studies, it should be used to assess many different therapeutic agents alone and in combination.

Future work to improve the program involves making the program more efficient so that it takes less time to analyze pictures. There are also some bugs that should be fixed, including checking for the existence of a value before accessing it. This will protect the user from running into an error and using all data. The initial segmentation may also

be improved by incorporating a sliding window threshold instead of a local block threshold. More complicated skeleton pruning would also be helpful and would make the length measurement more accurate.

Code

```

% Author      : Raha Dastgheyb
% File and function name : AutomatedInteractiveBeadMeasurement.m
% Version     : 1.0
% Date of completion    : 20 March 2013
% Inputs      : An array of phase (and possibly fluorescent) images
% of single cultured neurons. Make sure that there are at least 2 images
% selected when you load the images
%
% Outputs     : Number of beads, beading index, average width,
% stdwidth, length, tortuosity, and an assortment of images
%
% Description :
%             Interactively quantifies beading in single cultured neurons

function varargout = AutomatedInteractiveBeadMeasurement(varargin)
% AUTOMATEDINTERACTIVEBEADMEASUREMENT MATLAB code for
AutomatedInteractiveBeadMeasurement.fig
% AUTOMATEDINTERACTIVEBEADMEASUREMENT, by itself, creates a new
AUTOMATEDINTERACTIVEBEADMEASUREMENT or raises the existing
% singleton*.
%
% H = AUTOMATEDINTERACTIVEBEADMEASUREMENT returns the handle to a
new AUTOMATEDINTERACTIVEBEADMEASUREMENT or the handle to
% the existing singleton*.
%
% AUTOMATEDINTERACTIVEBEADMEASUREMENT('CALLBACK', hObject,eventData,handle
s,...) calls the local
% function named CALLBACK in AUTOMATEDINTERACTIVEBEADMEASUREMENT.M
with the given input arguments.
%
% AUTOMATEDINTERACTIVEBEADMEASUREMENT('Property','Value',...)
creates a new AUTOMATEDINTERACTIVEBEADMEASUREMENT or raises the
% existing singleton*. Starting from the left, property value pairs
are
% applied to the GUI before
AutomatedInteractiveBeadMeasurement_OpeningFcn gets called. An
% unrecognized property name or invalid value makes property
application
% stop. All inputs are passed to
AutomatedInteractiveBeadMeasurement_OpeningFcn via varargin.
%
% *See GUI Options on GUIDE's Tools menu. Choose "GUI allows only
one
% instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help
AutomatedInteractiveBeadMeasurement

```

```

% Last Modified by GUIDE v2.5 20-Mar-2013 00:25:06

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                  'gui_Singleton',   gui_Singleton, ...
                  'gui_OpeningFcn',  @AutomatedInteractiveBeadMeasurement_OpeningFcn, ...
                  'gui_OutputFcn',   @AutomatedInteractiveBeadMeasurement_OutputFcn, ...
                  'gui_LayoutFcn',   [], ...
                  'gui_Callback',    []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargin
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT

% --- Executes just before AutomatedInteractiveBeadMeasurement is made
% visible.
function AutomatedInteractiveBeadMeasurement_OpeningFcn(hObject,
eventdata, handles, varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles     structure with handles and user data (see GUIDATA)
% varargin   command line arguments to
AutomatedInteractiveBeadMeasurement (see VARARGIN)

% Choose default command line output for
AutomatedInteractiveBeadMeasurement
handles.output = hObject;

% Update handles structure
handles.blocksegment = {};
handles.axon = {};
handles.skeleton = {};
handles.finalAxon = {};
handles.beadImage = {};
handles.tortuosity = [];
handles.originalImages = {};
handles.currentIndex = 1;
handles.img = {};
handles.cF = 0;

guidata(hObject, handles);

% UIWAIT makes AutomatedInteractiveBeadMeasurement wait for user response
(see UIRESUME)

```

```

% uiwait(handles.figure1);

% --- Outputs from this function are returned to the command line.
function varargout = AutomatedInteractiveBeadMeasurement_OutputFcn(hObject, eventdata, handles)
% varargout cell array for returning output args (see VARARGOUT);
% hObject handle to figure
% eventdata reserved - to be defined in a future version of MATLAB
% handles structure with handles and user data (see GUIDATA)

% Get default command line output from handles structure
varargout{1} = handles.output;

% --- Executes on button press in LoadImage_button.
function LoadImage_button_Callback(hObject, eventdata, handles)
% hObject handle to LoadImage_button (see GCBO)
% eventdata reserved - to be defined in a future version of MATLAB
% handles structure with handles and user data (see GUIDATA)

[FileName, PathName] = uigetfile({'*.jpg'}, 'Load Image Files', 'MultiSelect', 'on');

handles.FileNames = FileName;
handles.numImages = length(FileName);
for i = 1:handles.numImages
    F = fullfile([PathName, char(FileName(i))]);
    o = imread(F);
    o = o(:, :, 1);
    o = mat2gray(o);
    o = im2uint8(o); % change scale to be from 0 to 255, not an intensity
    image
    handles.originalImages{i} = o;

    im = o;
    im = Filter(im);
    im = Filter(im);
    im = Filter(im);
    handles.img{i} = im;

    handles.blocksegment{i} = zeros(size(o));
    handles.axon{i} = zeros(size(o));
    handles.skeleton{i} = zeros(size(o));
    handles.finalAxon{i} = zeros(size(o));
    handles.beadImage{i} = zeros(size(o));
    handles.Names{i} = FileName(i);
end

sliderStep = [1, 1]/(handles.numImages-1);

set(handles.scroll_slider, 'SliderStep', sliderStep);

```

```

set(handles.scroll_slider, 'Max', handles.numImages);
axes(handles.OriginalImage);
imagesc(handles.originalImages{1});
colormap(gray);
title('Original Image')
guidata(hObject, handles);

function scroll_slider_CreateFcn(hObject, eventdata, handles)

% --- Executes during object creation, after setting all properties.

Max = 2;
if exist('handles.numImages','var')
    Max = handles.numImages;
end
set(hObject, 'Value', 1);
if(Max>1)
    set(hObject, 'Max', Max);
end

sliderStep = [1, 1]/(Max-1);

set(hObject, 'SliderStep', sliderStep);

guidata(hObject, handles);

% --- Executes on button press in AutoSegment_Button.
function AutoSegment_Button_Callback(hObject, eventdata, handles)
% hObject      handle to AutoSegment_Button (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
%set(hObject,'string','running','enable','off');
handles.cF = 1;
cI = handles.currentIndex;
img = handles.img{cI};
axes(handles.EditedImage);
colormap(gray);
blocksize = get(handles.MaxBlockSize_Slider, 'Value');
stdFF= get(handles.StdThresh_Slider, 'Value');
meanFF= get(handles.MeanFF_slider, 'Value');
threshFF= get(handles.threshFF_slider, 'Value');
%img = adapthisteq(img);
%img = imagesc(img);
img = AdaptOtsu(img, blocksize, stdFF, meanFF, threshFF);
img = Cleanup(img);
imshow(img);
handles.blocksegment{cI} = img;
handles.finalAxon{cI} = img;
%set(hObject,'string','Auto Segment','enable','on');

```

```

guidata(hObject, handles);
% -----
function Untitled_1_Callback(hObject, eventdata, handles)
% hObject    handle to Untitled_1 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% -----
function Untitled_2_Callback(hObject, eventdata, handles)
% hObject    handle to Untitled_2 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% -----
function Untitled_3_Callback(hObject, eventdata, handles)
% hObject    handle to Untitled_3 (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

function [filteredimage] = Filter( inputImage )
%UNTITLED2 Summary of this function goes here
% Detailed explanation goes here
filteredimage = inputImage;

filteredimage = medfilt2(filteredimage);

function [segmentedImage] = AdaptOtsu( img, maxBlock, stdFF, meanFF,
threshFF)%create an anonymous function handle to calculate the indices
based on
%blocksize
ind = @(x, rc) 1+((x-1)*rc);

%first, cut it up into blocks and make a matrix of standard deviations
im = imcomplement(img);
[r c] = size(img);
[blkr blkcr] = bestblk(size(img), maxBlock);

stdMatrix = zeros(size(img));

for i = 1:r/blkr-1
    for j = 1:c/blkcr-1
        window = im(ind(i, blkr):ind(i+1, blkr),ind(j, blkcr):(ind(j+1,
blkcr)));
        stdevIntensity = std2(window);
        stdMatrix(ind(i, blkr):ind(i+1, blkr),ind(j, blkcr):(ind(j+1, blkcr)))
= stdevIntensity;
    end
end

%also make a matrix of the mean values, which will help with the
%thresholding later

meanMatrix = zeros(size(img));

for i = 1:r/blkr-1

```

```

    for j = 1:c/blkc-1
        window = im(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkc):(ind(j+1,
blkc)));
        meanIntensity = mean2(window);
        meanMatrix(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkc):(ind(j+1,
blkc))) = meanIntensity;
    end
end

```

%make a final matrix of the max of the nearest neighbors of the stdMatrix

%middle points

```

finalStd = zeros(size(img));
    for i = 2:r/blkcr-2
        for j = 2:c/blkc-2
            s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkc):ind(j, blkc)));
            s2= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkc):ind(j+2, blkc)));
            s3= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkc):ind(j+1, blkc)));
            s4= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkc):ind(j+1, blkc)));
            s = [s1 s2 s3 s4];
            finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkc):(ind(j+1,
blkc))) = max(s);
        end
    end

```

%corner values

%top left corner

```

    for i = 1
        for j=1
            s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkc):ind(j+2, blkc)));
            s2= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkc):ind(j+1, blkc)));
            s3= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j+1,
blkc):ind(j+2, blkc)));
            s = [s1 s2 s3];
            finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkc):(ind(j+1,
blkc))) = max(s);
        end
    end

```

%top right corner

```

    for i = 1
        for j= round(c/blkc)-1
            s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkc):ind(j, blkc)));

```

```

        s2= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
        s3= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
        s = [s1 s2 s3];
        finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);

    end
end

%bottom left corner

for i = round(r/blkcr)-1
    for j= 1
        s1= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
        s2= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j+1,
blkcr):ind(j+1, blkcr)));
        s3= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkcr):ind(j+2, blkcr)));
        s = [s1 s2 s3];
        finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);
    end

end

%bottom right corner

for i = round(r/blkcr)-1
    for j= round(c/blkcr)-1
        s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
        s2= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
        s3= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
        s = [s1 s2 s3];
        finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);

    end
end

%top row
for i = 1
    for j= 2:round(c/blkcr)-2
        s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
        s2= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkcr):ind(j+2, blkcr)));
        s3= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
        s = [s1 s2 s3];

```

```

        finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);
    end
end
%bottom row

    for i = round(r/blkcr)-1
        for j= 2:round(c/blkcr)-2
            s1= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
            s2= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkcr):ind(j+2, blkcr)));
            s3= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
            s = [s1 s2 s3];
            finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);
        end
    end
%left column
    for i = 2:round(r/blkcr)-2
        for j= 1
            s1= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
            s2= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j+1,
blkcr):ind(j+2, blkcr)));
            s3= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
            s = [s1 s2 s3];
            finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);
        end
    end
%right column
    for i = 2:round(r/blkcr)-2
        for j= round(c/blkcr)-1
            s1= mean2(stdMatrix(ind(i-1, blkcr):ind(i,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
            s2= mean2(stdMatrix(ind(i, blkcr):ind(i+1,blkcr), ind(j-1,
blkcr):ind(j, blkcr)));
            s3= mean2(stdMatrix(ind(i+1, blkcr):ind(i+2,blkcr), ind(j,
blkcr):ind(j+1, blkcr)));
            s = [s1 s2 s3];
            finalStd(ind(i, blkcr):ind(i+1, blkcr),ind(j, blkcr):(ind(j+1,
blkcr))) = max(s);
        end
    end

%calculate the mean of the ones less than the std, and the mean of the
ones
%greater than the std
n = find(stdMatrix>0);
meanStd = mean(stdMatrix(n));
stdThresh = meanStd*stdFF;

```

```

meanLow = [];
meanHigh = [];
levelLow = [];
levelHigh = [];

for i = 1:r/blkcr-1
    for j = 1:c/blkcr-1
        window = meanMatrix(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr)));
        meanIntensity = mean2(window);
        std = mean2(stdMatrix(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))));
        level = graythresh(im(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))));
        if (std < stdThresh)
            meanLow = [meanLow meanIntensity];
            levelLow = [levelLow level];
        else
            meanHigh = [meanHigh meanIntensity];
            levelHigh = [levelHigh level];
        end
    end
end

meanThresh = meanFF* mean(meanLow);

thresholded = zeros(size(img));
%decide how to threshold

im = imcomplement(img);

for i = 1:r/blkcr-1
    for j = 1:c/blkcr-1
        window = im(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,      blkcr):(ind(j+1,
blkcr)));
        meanIntensity = mean2(meanMatrix(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))));
        std = mean2(finalStd(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))));
        if (std < stdThresh && meanIntensity < meanThresh) %defenitely
background
            thresholded(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))) = 0;
        elseif (std < stdThresh && meanIntensity > meanThresh) % probably
foreground
            thresholded(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))) = 0; %im2bw(window, mean(levelLow)*threshFF);
        elseif (std > stdThresh && meanIntensity < meanThresh) %maybe a
noisy background, or a small corner of edge
            thresholded(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))) = im2bw(window, mean(levelLow)*threshFF);
        elseif (std > stdThresh && meanIntensity > meanThresh) %definitely
foreground
            thresholded(ind(i,      blkcr):ind(i+1,      blkcr),ind(j,
blkcr):(ind(j+1, blkcr))) = im2bw(window, threshFF*graythresh(window));
    end
end

```

```

        end

    end
end

blocksegment = imclearborder(thresholded);
blocksegment = im2bw(blocksegment);
segmentedImage = blocksegment;

% --- Executes on slider movement.
function MaxBlockSize_Slider_Callback(hObject, eventdata, handles)
% hObject      handle to MaxBlockSize_Slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'Value') returns position of slider
%         get(hObject,'Min') and get(hObject,'Max') to determine range of
slider
sliderValue = get(hObject,'Value');
set(handles.MaxBlockSize_String,'String', sliderValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function MaxBlockSize_Slider_CreateFcn(hObject, eventdata, handles)
% hObject      handle to MaxBlockSize_Slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% Hint: slider controls usually have a light gray background.
if isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUiControlBackgroundColor'))
    set(hObject,'BackgroundColor',[.9 .9 .9]);
end

% --- Executes on slider movement.
function StdThresh_Slider_Callback(hObject, eventdata, handles)
% hObject      handle to StdThresh_Slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'Value') returns position of slider
%         get(hObject,'Min') and get(hObject,'Max') to determine range of
slider
sliderValue = get(hObject,'Value');
set(handles.StdThresh_String,'String', sliderValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);

% --- Executes during object creation, after setting all properties.
function StdThresh_Slider_CreateFcn(hObject, eventdata, handles)
% hObject      handle to StdThresh_Slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

```

```

% Hint: slider controls usually have a light gray background.
if isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor',[.9 .9 .9]);
end

function MaxBlockSize_String_Callback(hObject, eventdata, handles)
% hObject    handle to MaxBlockSize_String (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of MaxBlockSize_String as
text
%          str2double(get(hObject,'String')) returns contents of
MaxBlockSize_String as a double
textValue = str2num( get(hObject,'String') );
set(handles.MaxBlockSize_Slider,'Value',textValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function MaxBlockSize_String_CreateFcn(hObject, eventdata, handles)
% hObject    handle to MaxBlockSize_String (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called

% Hint: edit controls usually have a white background on Windows.
%         See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

function StdThresh_String_Callback(hObject, eventdata, handles)
% hObject    handle to StdThresh_String (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of StdThresh_String as
text
%          str2double(get(hObject,'String')) returns contents of
StdThresh_String as a double

textValue = str2num( get(hObject,'String') );
set(handles.StdThresh_Slider,'Value',textValue)
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function StdThresh_String_CreateFcn(hObject, eventdata, handles)
% hObject    handle to StdThresh_String (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called

```

```

% Hint: edit controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if      ispc      &&      isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% --- Executes on key press with focus on MaxBlockSize_Slider and none of
its controls.
function MaxBlockSize_Slider_KeyPressFcn(hObject, eventdata, handles)
% hObject      handle to MaxBlockSize_Slider (see GCBO)
% eventdata    structure with the following fields (see UICONTROL)
%   Key: name of the key that was pressed, in lower case
%   Character: character interpretation of the key(s) that was pressed
%   Modifier: name(s) of the modifier key(s) (i.e., control, shift)
pressed
% handles      structure with handles and user data (see GUIDATA)

function img = Cleanup(img)
%fill holes
img = imfill(img, 'holes');

%remove small bits
img = bwareaopen(img, 700);

function [axon, soma] = RemoveSoma(img, blocksegment)

%calculate halo to add to image later
Halobw = im2bw(img, graythresh(img));
%add halo to image so that it can be eroded properly
im2 = imadd(Halobw, blocksegment);
im2 = bwmorph(im2, 'close');

%fill holes
im2 = imfill(im2, 'holes');
holesfilled = imfill(im2, 'holes');
s = holesfilled;
l = bwlabel(s);
while (max(max(l)) >1)
    s = bwmorph(s, 'erode');
    l = bwlabel(s);

    if (max(max(l))==1)
        break;
    end

end

%get it to 50 pixels, and then find the centroid

stats = regionprops(s, 'all');
centroid = cat(1, stats.Centroid);

```

```

c = 0;
soma = holesfilled;
while (length(find(soma>0))> 0)
    soma = bwmorph(soma, 'erode');
    c = c+1;
end

c = c-1;
centers = round(centroid);
soma(round(centroid(2)), round(centroid(1))) = 255;

xc=centers(2);
yc=centers(1);

r=(c+20)^2;
[x y]= find(soma<275);
d=find(((x-xc).^2+(y-yc).^2) <= r);

for i=1:length(d)

    soma(x(d(i)),y(d(i)),:)=255;

end
%subtract soma from rest of image

axon = imsubtract(blocksegment, soma);
axon = im2bw(axon);

%now find the longest region. This should be the axon

labeled = bwlabel(axon);
stats = regionprops(axon, 'all');
lengths = cat(1, stats.MajorAxisLength);
id = find(lengths == max(lengths));
axon = ismember(labeled, id);

% --- Executes on button press in roifill_push.
function roifill_push_Callback(hObject, eventdata, handles)
% hObject      handle to roifill_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
%img = handles.img;
cI = handles.currentIndex;
axes(handles.OriginalImage);
img = roifill(handles.img{cI});
handles.img = img;
axes(handles.OriginalImage);
imagesc(img);

colormap(gray);
guidata(hObject, handles)

```

```

% --- Executes on slider movement.
function MeanFF_slider_Callback(hObject, eventdata, handles)
% hObject      handle to MeanFF_slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'Value') returns position of slider
%         get(hObject,'Min') and get(hObject,'Max') to determine range of
slider
sliderValue = get(hObject,'Value');
set(handles.MeanFF_string,'String', sliderValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function MeanFF_slider_CreateFcn(hObject, eventdata, handles)
% hObject      handle to MeanFF_slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% Hint: slider controls usually have a light gray background.
if isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor',[.9 .9 .9]);
end

function MeanFF_string_Callback(hObject, eventdata, handles)
% hObject      handle to MeanFF_string (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of MeanFF_string as text
%         str2double(get(hObject,'String')) returns contents of
MeanFF_string as a double
textValue = str2num( get(hObject,'String') );
set(handles.MeanFF_slider,'Value',textValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function MeanFF_string_CreateFcn(hObject, eventdata, handles)
% hObject      handle to MeanFF_string (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% Hint: edit controls usually have a white background on Windows.
%         See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

```

```

% --- Executes on slider movement.
function threshFF_slider_Callback(hObject, eventdata, handles)
% hObject    handle to threshFF_slider (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'Value') returns position of slider
%         get(hObject,'Min') and get(hObject,'Max') to determine range of
slider
sliderValue = get(hObject,'Value');
set(handles.threshFF_string,'String', sliderValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function threshFF_slider_CreateFcn(hObject, eventdata, handles)
% hObject    handle to threshFF_slider (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called

% Hint: slider controls usually have a light gray background.
if isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUiControlBackgroundColor'))
    set(hObject,'BackgroundColor',[.9 .9 .9]);
end

function threshFF_string_Callback(hObject, eventdata, handles)
% hObject    handle to threshFF_string (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of threshFF_string as
text
%         str2double(get(hObject,'String')) returns contents of
threshFF_string as a double
textValue = str2num( get(hObject,'String') );
set(handles.threshFF_slider,'Value',textValue);
AutoSegment_Button_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function threshFF_string_CreateFcn(hObject, eventdata, handles)
% hObject    handle to threshFF_string (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns called

% Hint: edit controls usually have a white background on Windows.
%         See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUiControlBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% --- Executes on button press in connect_push.
function connect_push_Callback(hObject, eventdata, handles)

```

```

% hObject    handle to connect_push (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;
handles.cF = 1;
%user uses lines to connect segments
axes(handles.EditedImage);
img = handles.finalAxon{cI};
h_i = imshow(img);
h = imline();
b = createMask(h, h_i);
b = bwmorph(b, 'dilate');
img = img + b;
img = im2bw(img);
imshow(img)
handles.blocksegment{cI} = img;
handles.finalAxon{cI} = img;
guidata(hObject, handles)

```

```

% --- Executes on button press in RemoveSoma_push.
function RemoveSoma_push_Callback(hObject, eventdata, handles)
% hObject    handle to RemoveSoma_push (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
set(hObject, 'string', 'running', 'enable', 'off');
cI = handles.currentIndex;
handles.cF = 1;
axes(handles.EditedImage);
img = handles.img{cI};
block = handles.blocksegment{cI};
[axon, soma] = RemoveSoma(img, block);
handles.finalAxon{cI} = axon;
imagesc(axon)
handles.Soma{cI} = soma;
set(hObject, 'string', 'Remove Soma', 'enable', 'on');
guidata(hObject, handles)

```

```

% --- Executes on button press in edges_push.
function edges_push_Callback(hObject, eventdata, handles)
% hObject    handle to edges_push (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;
set(hObject, 'string', 'running', 'enable', 'off');
handles.cF = 1;
axes(handles.EditedImage);
img = handles.img{cI};
axon = handles.finalAxon{cI};
edges = Edges(img, axon);
axonEdges = subtractEdges(axon, edges);
imagesc(axonEdges)
hold on;
[xc xr] = find(edges);
handles.Edges{cI} = edges;
handles.finalAxon{cI} = axonEdges;

```

```

set(hObject,'string','Incorporate Edges','enable','on');
guidata(hObject, handles)

function edges = Edges(img, axon)

%segment using edges

BWcanny = edge(img, 'canny');
BWcanny = im2uint8(BWcanny);
BWcanny = im2bw(BWcanny);
BWcanny = imclearborder(BWcanny);
%remove short edge lines
lengthThresh = 60;
labeledEdge = bwlabel(BWcanny);
Estats = regionprops(BWcanny, 'all');
Elengths = cat(1, Estats.MajorAxisLength);
ide = find(Elengths>lengthThresh);
edges = ismember(labeledEdge, ide);

function axon = subtractEdges(axon, edges)
%Combine edges and segmented axon
%subtract edges from axon to see what was extra

subEdges = imsubtract(axon ,bwmorph(edges, 'dilate'));
subEdges = im2bw(subEdges);
subEdges = bwmorph(subEdges, 'clean');
subEdges = bwmorph(subEdges, 'spur');
subEdges = bwmorph(subEdges, 'clean');
subEdges = bwmorph(subEdges, 'spur');
subEdges = bwareaopen(subEdges, 100,8);
subEdges = bwmorph(subEdges, 'clean');
subEdges = bwmorph(subEdges, 'spur');
subEdges = bwmorph(subEdges, 'clean');
subEdges = bwmorph(subEdges, 'spur');

%add edges, fill holes, then remove thin lines
added = imadd(subEdges, edges);
added = bwmorph(added, 'close');
added = imfill(added, 'holes');
%remove all thin unconnected lines

% Morphologically open image
se = strel('square',2);
added2 = imopen(added,se);

%the largest objects are the axon
l = bwlabel(added2);
Estats = regionprops(added2, 'area');
area = cat(1, Estats.Area);
iarea = find(area >100);
axon = ismember(l, iarea);

%fill any holes, and make sure there is only one object there
axon = bwmorph(axon, 'close');
l = bwlabel(added2);

```

```

Estats = regionprops(added2, 'area');
area = cat(1, Estats.Area);
iaarea = find(area == max(area));
axon = ismember(1, iaarea);

% --- Executes on button press in beads_push.
function beads_push_Callback(hObject, eventdata, handles)
% hObject      handle to beads_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
handles.cF = 2;
set(hObject, 'string', 'running', 'enable', 'off');
cI = handles.currentIndex;
skel = handles.Skeleton{cI};
axon = handles.finalAxon{cI};
%find slope
slope = slopeArray(skel);
slopeImg = slopeimg(axon, skel);
%find width
widthAxon = widthaxon(axon, skel, slope);

%find possible beads
widthI = widthImg(axon, skel, slope);

%find nonbead properties
[meanAxonWidth, stdAxonWidth] = nonBeadProps(widthI);
%filter beads
beadImg = filterbeads(widthI, 1);
nb1 = 0;
%find close beads and combine them. Repeat until there are no more changes
distthresh= meanAxonWidth*2;
beads = beadImg;
c=0;
while(nb1 ~= length(find(beads)))
nb1 = length(find(beads));

beads = combinedBeads(beads, distthresh);

end

imshow(axon)
[beadx beady] = find(beads);
hold on;
plot(beady, beadx, 'r.')
b = traceSpine(skel, handles.Soma{cI});
tort = calcTort(skel, b);
handles.Tortuosity{cI} = tort;
len = length(find(skel));
handles.AxonLength{cI} = len;
handles.AxonWidth{cI} = meanAxonWidth;
beadIdx = find(beads);

sumB = 0;
for i = 1:length(beadIdx)

```

```

        sumB = sumB + beads(beadIdx(i));
    end

    beadingIndex = sumB/(len*handles.AxonWidth{cI});

    handles.stdAxonWidth{cI} = stdAxonWidth;
    handles.widthImg{cI} = widthI;
    handles.BeadIndex{cI} = beadingIndex;
    handles.numBeads{cI} = length(find(beads));

    handles.Length{cI} = len;

    handles.beadImage{cI} = beads;
    set(hObject, 'string', 'Calculate Beads', 'enable', 'on');
    guidata(hObject, handles);

% --- Executes on button press in Export_Calculations.
function Export_Calculations_Callback(hObject, eventdata, handles)
% hObject      handle to Export_Calculations (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

assignin('base', 'axon', handles.finalAxon);
assignin('base', 'beads', handles.beadImage);
assignin('base', 'numBeads', handles.numBeads);
assignin('base', 'skel', handles.Skeleton);
%all done finding beads! now do the calculations
assignin('base', 'Len', handles.Length);
%numberofbeads
%use improfile to find beads in each third of the axon
%tortuosity
assignin('base', 'meanAxonWidth', handles.AxonWidth);
assignin('base', 'Tortuosity', handles.Tortuosity);
assignin('base', 'stdAxonWidth', handles.stdAxonWidth);
assignin('base', 'widthImg', handles.widthImg);
assignin('base', 'beadingIndex', handles.BeadIndex);
assignin('base', 'OriginalImage', handles.originalImages);
assignin('base', 'Names', handles.Names);

guidata(hObject, handles);

function skel = skeleton(axon)
axon = imfill(axon, 'holes');
skel = bwmorph(axon, 'thin', Inf);
skel = bwmorph(skel, 'spur');

function skel = prune(skel, lengthresh)
branchpoints = bwmorph(skel, 'branchpoints');
b = skel-branchpoints;
l = bwlable(b);

```

```

%regionprops was not finding all labels, so wor with the number of pixels
in bw label
f = [];

for i = 1:max(max(l))
    if length(find(l==i))<lengthresh
        f = [f i];
    end

end

remove = ismember(l,f);
skel = skel-remove;
%now connect close segments
skel = bwmorph(skel, 'dilate');
skel = bwmorph(skel, 'dilate');
skel = bwmorph(skel, 'dilate');
skel = bwmorph(skel, 'erode');
skel = bwmorph(skel, 'erode');
skel = bwmorph(skel, 'erode');

%remove anything that is not the longest segment

l = bwlabel(skel);
for i = 1:max(max(l))
    ll(i) = length(find(l==i));
end
skel = ismember(l, find(ll==max(ll)));

function [S1, S2] = findSpine(skel)
[S1,S2]= find(skel==1);

function slope = slopeArray(skel)
[S1, S2] = findSpine(skel);
r = 3;
for k = 1:length(S1)
    %draw a box around that point
    sx = S1(k); %x and y coordinates
    sy = S2(k);
    disk = skel(sx-r:sx+r, sy-r:sy+r);
    %find all the x and y points in the box
    [y x] = find(disk);
    %calculate the slope
    %slope(k) = diff(y')/diff(x');
    coeff = polyfit(x, y,1);
    sk = coeff(1);
    nnn = isnan(sk);
    if(nnn== 1)
        slope(k) = 1000;
    else
        slope(k) = sk;
    end
end

function plotSlope = plotSlope(axon, skel, slopeArray)

```

```

[S1, S2] = findSpine(skel);
imshow(axon)
hold on;
for i = 1:5:length(S1)

    xp = [1 2 3 4 5];
    yp = xp*slopeArray(i);

    %center the third point of the x axis at S2(i)
    xp = S2(i)-2:S2(i) +2;
    %raise the third point of the y-axis to match the center point of S1(i)
    d = S1(i) - yp(3);
    yp = yp+d;
    plot(xp, yp);
end

function slope = slopeimg(axon,skel)
[S1, S2] = findSpine(skel);
slope = slopeArray(skel);
for i = 1:length(S1)
    slope(S1(i),S2(i)) = slope(i);
end
handles.slopeImg = slope;

function widthA = widthaxon(axon,skel, slope)

[S1, S2] = findSpine(skel);
perp = -1./slope;
l = 50; % length of tangent line
for i = 1:length(S1)

    %use the pythagorean theorem to find the range of x values
    u = perp(i);
    lengthx = round(sqrt(((l/2)^2)/(1+u^2))); %length on each side of
    centerpoint
        if lengthx == 0 %in the case of a flat line
            lengthx = round(l/2);
        end

    xp = 1:2*lengthx+1;
    yp = xp*u;

    %in case of a vertical line
    %center the middle point of the x axis at S2(i)
    xd = S2(i) - xp(lengthx);
    xp = xp+ xd;

    %raise the middle point of the y-axis to match the center point of S1(i)

    d = S1(i) - yp(lengthx);
    yp = yp+d;

    if abs(u) >50
        yp = 1:2*lengthx+1;
        yp = yp+round(S1(i)/2);
    end
end

```

```

    %and there will only be one x-value
    for j = 1:length(yp)
        xp(j) = S2(i);
    end

end

%also check the minimum and maximum ranges of yp
if(min(yp)<1 || max(yp) > length(axon))
    for j = 1:length(xp)
        yp(j) = d;
    end
end
yp = round(yp);

C = improfile (axon, xp, yp);

%the number of 1s in C will be the width at that point (in pixels);
%it is possible that the line will cross the axon at multiple locations,
%so look for the region that contains the center point

C = bwlabel(C);
if max(max(C)) > 1
    n = find(xp == S2(i));
    C = ismember(C, C(n));

end
w = length(find(C));
widthA(i) = w;

end

function plotAxon = plotAxon(axon,skel, slope)
imshow(axon)
hold on
%use the pythagorean theorem to find the range of x values
u = perp(i);
lengthx = round(sqrt(((1/2)^2)/(1+u^2))); %length on each side of
centerpoint
    if lengthx == 0; %in the case of a flat line
        lengthx = round(1/2);
    end

xp = 1:2*lengthx+1;
yp = xp*u;

%in case of a vertical line
%center the middle point of the x axis at S2(i)
xd = S2(i) - xp(lengthx);
xp = xp+ xd;

%raise the middle point of the y-axis to match the center point of S1(i)

```

```

d = S1(i) - yp(lengthx);
yp = yp+d;

if abs(u) >50
    yp = 1:2*lengthx+1;
    yp = yp+round(S1(i)/2);
    %and there will only be one x-value
    for j = 1:length(yp)
        xp(j) = S2(i);
    end
end

%also check the minimum and maximum ranges of yp
if(min(yp)<1 || max(yp) > length(axon))
    for j = 1:length(xp)
        yp(j) = d;
    end
end
yp = round(yp);
plot(xp, yp);
C = improfile (axon, xp, yp);

%the number of 1s in C will be the width at that point (in pixels);
%it is possible that the line will cross the axon at multilpe locations,
so look for the region that contains the center point
C = bwlabel(C);
if max(max(C)) > 1
    n = find(xp == S2(i));
    C = ismember(C, C(n));

end
w = length(find(C));

function widthimg = widthImg(axon, skel, slope)
width = widthaxon(axon, skel, slope);
[S1, S2] = findSpine(skel);
widthimg = zeros(size(axon));
for i = 1:length(S1)
    widthimg(S1(i),S2(i)) = width(i);
end

function localMaximg = localMax(widthI)
localMaximg = imregionalmax(widthI);
handles.localMaxImg = localMaximg;

function [meanAxonWidth, stdAxonWidth] = nonBeadProps(widthI)
localMaximg = localMax(widthI);
nonBeads = widthI;
nonBeads(find(localMaximg))= 0;
indnb = find(nonBeads);% List of all radii of non beads
for i = 1:length(indnb)
    nonBeadR = nonBeads(indnb); %list of all the radii of non beads
end

```

```

meanAxonWidth = mean(nonBeadR);
handles.meanAxonWidth = meanAxonWidth;
stdAxonWidth = std(nonBeadR);
handles.stdAxonWidth = stdAxonWidth;

function beadImg = filterbeads(widthI, stdThresh)
possibleBeads = localMax(widthI);

[meanAxonWidth, stdAxonWidth] = nonBeadProps(widthI);

beadIdx = find (possibleBeads); % location of the current maxes
for i = 1:length(beadIdx)
    if (widthI (beadIdx(i)) < (meanAxonWidth + stdThresh*stdAxonWidth))
        possibleBeads (beadIdx(i)) = 0;
    end
end

%the value of each bead is the area of the bead. Assume that it is a
%circle, so use r = width/2 to calculate it
beads = im2double(possibleBeads);
b = find(beads);
for i = 1:length(b)
    r = widthI(b(i))/2;
    beads(b(i)) = pi*r^2;
end

beadImg = beads;

function beads = combinedBeads(beadImg, distthresh)
%
beadimg2 = beadImg;

%if some beads are really close, combine them into one
[x y] = find(beadImg);
%find points of the beads

[X, Y] = meshgrid (x, y);

Dx = X-X';
Dy = Y-Y';

distances = hypot(Dx, Dy);
%get rid of 0s

distances = distances + diag(nan(1, numel(x)));

[xi yi] = find(distances < distthresh);

%there will be double entries for every two points, so remove the
%duplicates

for i = 1:length(xi)
    if i <= length(xi)

```

```

        yvalue = yi(i);
        n = find (xi == yvalue);
        xi(n) = [];
        yi(n) = [];
    end
end

for i = 1:length(xi)
    %each row contains the index of two points that need to be combined.
    y1 = y(xi(i));
    x1 = x(xi(i));
    y2 = y(yi(i));
    x2 = x(yi(i));

    %set the coordinates of the combined points to be the midpoint of the two
    points

    newx = round((x1 + x2)/2);
    newy = round((y1+y2)/2);
    beading2(x1,y1) =0;
    beading2(x2, y2) =0;

    %calculate the overlap of the two points to see what the new area value
    %is. Do this by plotting both circles in a new image. Then count the
    %total number of pixels
    temp1 = zeros(size(beadImg));
    temp2 = zeros(size(beadImg));
    tempimg = zeros(size(beadImg));
    r1 = sqrt(beadImg(x1, y1)/pi);
    r2 = sqrt(beadImg(x2, y2)/pi);

    temp1(x1, y1) = 1;
    temp1 = bwdist(temp1);
    n = find(temp1<=r1);
    tempimg(n) = 1;
    temp2(x2, y2) = 1;
    temp2 = bwdist(temp2);
    n = find(temp2<=r1);
    tempimg(n) = 1;

    sizebead = length(find(tempimg));
    beading2(newx, newy) = sizebead;

end
beads = beading2;

function plotBeads = plotBeads(axon, beadImg)
[beadx beady] = find(beadImg);
imshow(axon)
hold on;
plot(beady, beadx, 'r.')

```

```

% --- Executes on button press in FindSpine_push.
function FindSpine_push_Callback(hObject, eventdata, handles)
% hObject      handle to FindSpine_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;
set(hObject, 'string', 'running', 'enable', 'off');
handles.cF = 3;
axes(handles.EditedImage);
%find skeleton
axon = handles.finalAxon{cI};
skel = skeleton(axon);
spineThresh= get(handles.SpineThresh_slider, 'Value');
%prune skeleton
skel = prune(skel, spineThresh);
handles.Skeleton{cI} = skel;
imshow(axon)
[skel1 skel2] = find(skel);
hold on;
plot(skel2, skel1, '.');
set(hObject, 'string', 'Find Spine', 'enable', 'on');
guidata(hObject, handles);

% --- Executes on slider movement.
function SpineThresh_slider_Callback(hObject, eventdata, handles)
% hObject      handle to SpineThresh_slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject, 'Value') returns position of slider
%         get(hObject, 'Min') and get(hObject, 'Max') to determine range of
slider
sliderValue = get(hObject, 'Value');
set(handles.SpineThresh_string, 'String', sliderValue);
FindSpine_push_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function SpineThresh_slider_CreateFcn(hObject, eventdata, handles)
% hObject      handle to SpineThresh_slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% Hint: slider controls usually have a light gray background.
if ~isequal(get(hObject, 'BackgroundColor'),
get(0, 'defaultUicontrolBackgroundColor'))
    set(hObject, 'BackgroundColor', [.9 .9 .9]);
end
guidata(hObject, handles);

function SpineThresh_string_Callback(hObject, eventdata, handles)

```

```

% hObject      handle to SpineThresh_string (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'String') returns contents of SpineThresh_string as
text
%             str2double(get(hObject,'String')) returns contents of
SpineThresh_string as a double
textValue = str2num( get(hObject,'String') );
set(handles.SpineThresh_slider,'Value',textValue);
FindSpine_push_Callback(hObject, eventdata, handles);
guidata(hObject, handles)

% --- Executes during object creation, after setting all properties.
function SpineThresh_string_CreateFcn(hObject, eventdata, handles)
% hObject      handle to SpineThresh_string (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% Hint: edit controls usually have a white background on Windows.
%         See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% --- Executes on button press in RemoveBeads_push.
function RemoveBeads_push_Callback(hObject, eventdata, handles)
% hObject      handle to RemoveBeads_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
handles.cF = 2;
cI = handles.currentIndex;
axes(handles.EditedImage);
axon = handles.finalAxon{cI};
beads = handles.beadImage{cI};
imshow(axon)
hold on;
[xp yp] = find(beads);
plot(yp, xp, 'o', 'MarkerFaceColor','g')

[yd,xd] = ginput(1); %lets user click on points that are not beads
%find the point that it is closest to.  Make sure that the distance is
not
%too far
d = [];

for i = 1:length(xp)
    d(i) = sqrt((xd-xp(i))^2 + (yd - yp(i))^2) ;
end

minI = find(d == min(d)); %return index of the smallest distance, so the
point corresponding to the clicked point

```

```

%now remove that point

beads(xp(minI), yp(minI)) = 0;

[xp yp] = find(beads);
imshow(axon)
hold on;
plot(yp, xp, 'o', 'MarkerFaceColor', 'g')

beadIdx = find(beads);
sumB = 0;
for i = 1:length(beadIdx)
    sumB = sumB + beads(beadIdx(i));
end
beadingIndex = sumB/(handles.Length{cI}*handles.AxonWidth{cI});
handles.BeadIndex{cI} = beadingIndex;
handles.numBeads{cI} = length(find(beads));

handles.beadImage{cI} = beads;

guidata(hObject, handles);

function traced = traceSpine(skel, soma)
stats= regionprops(soma, 'centroid');
centr = stats.Centroid;
[xp yp] = find(skel);
%find the point on the skeleton that is closest to the centroid
yd= centr(1);
xd = centr(2);
d = [];

for i = 1:length(xp)
    d(i) = sqrt((xd-xp(i))^2 + (yd - yp(i))^2) ;
end

minI = find(d == min(d));

P = [xp(minI) yp(minI)];
%b = bwtraceboundary(skel, P, 'E');
b = bwtraceboundary(skel,P,'E',8,length(find(skel)), 'clockwise');
traced = b;
handles.boundary = traced;
%plot(b(:,2),b(:,1), 'g', 'LineWidth',2);
%plot(P(2),P(1), 'gx', 'LineWidth',2);
%plot 25th point from the beginning:
%plot(b(25,2),b(25,1), '.');

function tort = calcTort(skel, b)
x1=b(1,1);
y1 = b(1,2);
x2 = b(length(b), 1);
y2 = b(length(b),2);

```

```

enddist = sqrt((x2-x1)^2 + (y2-y1)^2);
skelLength = length(find(skel));

%tortuosity is the length between endpoints divided by the total length.
A
%tortuosity of 1 implies a perfectly straight line
tort = enddist/skelLength;

% --- Executes on button press in FreehandDraw_push.
function FreehandDraw_push_Callback(hObject, eventdata, handles)
% hObject    handle to FreehandDraw_push (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;
handles.cF = 4;
axes(handles.EditedImage);
%plot the original image, with the points on the axon over it
orig = handles.originalImages{cI};
h = imshow(orig);
hold on;

%get all the points on the axon, and plot on top.

axon = handles.finalAxon{cI};
[x y] = find(axon);

plot(y, x, '.');

imf = imfreehand(gca);
M = imf.getPosition;
addX = M(:, 1);
addY = M(:, 2);
plot(addX, addY, '.');
mask = createMask(imf,h);
axon = axon + mask;

imshow(orig);
[x y] = find(axon);
hold on;
plot(y, x, 'y.');

handles.finalAxon{cI} = axon;
% Update handles structure
guidata(hObject, handles);

% --- Executes on button press in FreehandRemove_push.
function FreehandRemove_push_Callback(hObject, eventdata, handles)
% hObject    handle to FreehandRemove_push (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;

```

```

handles.cF = 4;
axes(handles.EditedImage);
%plot the original image, with the points on the axon over it
orig = handles.originalImages{cI};
h = imshow(orig);
hold on;

%get all the points on the axon, and plot on top.

axon = handles.finalAxon{cI};
[x y] = find(axon);

plot(y, x, 'r.');

imf = imfreehand(gca);
M = imf.getPosition;
addX = M(:, 1);
addY = M(:, 2);
mask = createMask(imf,h);
mIdx = find(mask);
axon(mIdx) = 0;

imshow(orig);
[x y] = find(axon);
hold on;
plot(y, x, 'y.');

handles.finalAxon{cI} = axon;
% Update handles structure
guidata(hObject, handles);

% --- Executes on button press in Overlay_push.
function Overlay_push_Callback(hObject, eventdata, handles)
% hObject      handle to Overlay_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
cI = handles.currentIndex;
handles.cF = 4;
axes(handles.EditedImage);
%plot the original image, with the points on the axon over it
orig = handles.originalImages{cI};
h = imshow(orig);
hold on;

%get all the points on the axon, and plot on top.

axon = handles.finalAxon{cI};
[x y] = find(axon);
plot(y, x, 'y.');
guidata(hObject, handles);

% --- Executes on slider movement.

```

```

function scroll_slider_Callback(hObject, eventdata, handles)
% hObject      handle to scroll_slider (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

% Hints: get(hObject,'Value') returns position of slider
%          get(hObject,'Min') and get(hObject,'Max') to determine range of
slider

%change the original Image axes to be the current image. Also change the
title

sliderValue = round(get(hObject,'Value'));
handles.currentIndex = sliderValue;
cI = sliderValue;

axes(handles.OriginalImage);
imshow(handles.originalImages{cI});

%now change the second image depending on what you had been looking at

axes(handles.EditedImage);
cla;
cF = handles.cF;

if(cF == 1)
    imshow(handles.finalAxon{cI}) % show segmented axon
end

if(cF == 2)
    imshow(handles.finalAxon{cI}) % show segmented axon with bead
    overlay
    hold on
    beads = handles.beadImage{cI};
    [xp yp] = find(beads);
    plot(yp, xp, 'o', 'MarkerFaceColor', 'b')
end

if(cF == 3)
    imshow(handles.finalAxon{cI}) % show segmented axon with spine
    overlay
    hold on
    spine = handles.Skeleton{cI};
    [xp yp] = find(spine);
    plot(yp, xp, '.', 'r')
end

if(cF == 4)
    imshow(handles.img{cI}) % show original axon with segmented overlay
    hold on
    overlay = handles.finalAxon{cI};

```

```

    [xp yp] = find(overlay);
    plot(yp, xp, 'y.')
```

end

```

guidata(hObject, handles)
% --- Executes on button press in SegmentAll_push.
function SegmentAll_push_Callback(hObject, eventdata, handles)
% hObject      handle to SegmentAll_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)v
set(hObject, 'string', 'running', 'enable', 'off');
handles.cF = 1;

for i = 1:handles.numImages
    cI = i;
    handles.currentIndex = i;
    set(handles.scroll_slider, 'Value', i);
    scroll_slider_Callback(hObject, eventdata, handles)
    img = handles.img{cI};
    blocksize = get(handles.MaxBlockSize_Slider, 'Value');
    stdFF= get(handles.StdThresh_Slider, 'Value');
    meanFF= get(handles.MeanFF_slider, 'Value');
    threshFF= get(handles.threshFF_slider, 'Value');
    img = AdaptOtsu(img, blocksize, stdFF, meanFF, threshFF);
    img = Cleanup(img);
    handles.blocksegment{cI} = img;
    handles.finalAxon{cI} = img;
end

cI = 1;
set(handles.scroll_slider, 'Value', i);
axes(handles.OriginalImage);
imshow(handles.originalImages{cI});
axes(handles.EditedImage)
imshow(handles.finalAxon{cI}) % show segmented axon

set(hObject, 'string', 'Preliminary Segmentation', 'enable', 'on');
disp('Done Segmenting');
guidata(hObject, handles);
% --- Executes during object creation, after setting all properties.
function LoadImage_button_CreateFcn(hObject, eventdata, handles)
% hObject      handle to LoadImage_button (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      empty - handles not created until after all CreateFcns called

% --- Executes on button press in IsolateAll_push.
function IsolateAll_push_Callback(hObject, eventdata, handles)
% hObject      handle to IsolateAll_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
set(hObject, 'string', 'running', 'enable', 'off');
handles.cF = 1;
for i = 1:handles.numImages
    handles.currentIndex = i;
    cI = i;
```

```

        set(handles.scroll_slider, 'Value', i);
        img = handles.img{cI};
        block = handles.blocksegment{cI};
        [axon, soma] = RemoveSoma(img, block);
        handles.finalAxon{cI} = axon;
        handles.Soma{cI} = soma;
    end
    cI = 1;
    set(handles.scroll_slider, 'Value', i);
    axes(handles.OriginalImage);
    imshow(handles.originalImages{cI});
    axes(handles.EditedImage)
    imshow(handles.finalAxon{cI})    % show segmented axon

    set(hObject, 'string', 'Isolate All Axons', 'enable', 'on');
    disp('Done Isolating Axons');
    guidata(hObject, handles);

% --- Executes on button press in AllEdges_push.
function AllEdges_push_Callback(hObject, eventdata, handles)
% hObject      handle to AllEdges_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
set(hObject, 'string', 'running', 'enable', 'off');
handles.cF = 1;
for i = 1:handles.numImages
    handles.currentIndex = i;
    cI = i;
    set(handles.scroll_slider, 'Value', i);
    scroll_slider_Callback(hObject, eventdata, handles)
    img = handles.img{cI};
    axon = handles.finalAxon{cI};
    edges = Edges(img, axon);
    axonEdges = subtractEdges(axon, edges);
    [xc xr] = find(edges);
    handles.Edges{cI} = edges;
    handles.finalAxon{cI} = axonEdges;

end

cI = 1;
set(handles.scroll_slider, 'Value', i);
axes(handles.OriginalImage);
imshow(handles.originalImages{cI});
axes(handles.EditedImage)
imshow(handles.finalAxon{cI})    % show segmented axon

disp('Done Incorporating Edges');
set(hObject, 'string', 'Incorporate All Edges', 'enable', 'on');
guidata(hObject, handles);

% --- Executes on button press in SpineAll_push.
function SpineAll_push_Callback(hObject, eventdata, handles)
% hObject      handle to SpineAll_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)

```

```

handles.cF = 3;
set(hObject,'string','running','enable','off');
for i = 1:handles.numImages
    handles.currentIndex = i;
    cI = i;
    set(handles.scroll_slider,'Value', i);
    %find skeleton
    axon = handles.finalAxon{cI};
    skel = skeleton(axon);
    spineThresh= get(handles.SpineThresh_slider, 'Value');
    %prune skeleton
    skel = prune(skel, spineThresh);
    handles.Skeleton{cI} = skel;
    [skel1 skel2] = find(skel);
end

cI = 1;
set(handles.scroll_slider,'Value', i);
axes(handles.OriginalImage);
imshow(handles.originalImages{cI});
axes(handles.EditedImage)
imshow(handles.finalAxon{cI}) % show segmented axon
hold on;
spine = handles.Skeleton{cI};
[xp yp] = find(spine);
plot(yp, xp, '.')

set(hObject,'string','Find All Spines','enable','on');
disp('Done Finding Spines');
guidata(hObject, handles);

% --- Executes on button press in CalcAllBeads_push.
function CalcAllBeads_push_Callback(hObject, eventdata, handles)
% hObject      handle to CalcAllBeads_push (see GCBO)
% eventdata    reserved - to be defined in a future version of MATLAB
% handles      structure with handles and user data (see GUIDATA)
handles.cF = 2;
set(hObject,'string','running','enable','off');
for i = 1:handles.numImages
    handles.currentIndex = i;
    cI = i;
    set(handles.scroll_slider,'Value', i);
    skel = handles.Skeleton{cI};
    axon = handles.finalAxon{cI};
    %find slope
    slope = slopeArray(skel);
    slopeImg = slopeimg(axon,skel);
    %find width
    widthAxon = widthaxon(axon,skel, slope);
    %find possible beads
    widthI = widthImg(axon, skel, slope);
    %find nonbead properties
    [meanAxonWidth, stdAxonWidth] = nonBeadProps(widthI);
    %filter beads
    beadImg = filterbeads(widthI, 1);
    nb1 = 0;

```

```

    %find close beads and combine them. Repeat until there are no more
changes
    distthresh= meanAxonWidth*2;
    beads = beadImg;
    c=0;
    while(nb1 ~= length(find(beads)))
        nb1 = length(find(beads));
        beads = combinedBeads(beads, distthresh);
    end

    [beadx beady] = find(beads);
    b = traceSpine(skel, handles.Soma{cI});
    %will update all of the following in another function that can also
be run after removing beads
    tort = calcTort(skel, b);
    handles.Tortuosity{cI} = tort;
    len = length(find(skel));
    handles.AxonLength{cI} = len;
    handles.AxonWidth{cI} = meanAxonWidth;
    beadIdx = find(beads);
    sumB = 0;
    for j = 1:length(beadIdx)
        sumB = sumB + beads(beadIdx(j));
    end

    beadingIndex = sumB/(len*handles.AxonWidth{cI});
    handles.stdAxonWidth{cI} = stdAxonWidth;
    handles.widthImg{cI} = widthI;
    handles.BeadIndex{cI} = beadingIndex;
    handles.numBeads{cI} = length(find(beads));
    handles.Length{cI} = len;
    handles.beadImage{cI} = beads;
end

cI = 1;
set(handles.scroll_slider, 'Value', i);
axes(handles.OriginalImage);
imshow(handles.OriginalImages{cI});
axes(handles.EditedImage)
imshow(handles.finalAxon{cI}) % show segmented axon
beads = handles.beadImage{cI};
[xp yp] = find(beads);
plot(yp, xp, 'o', 'MarkerFaceColor', 'b')

set(hObject, 'string', 'Calculate Beads', 'enable', 'on');
disp('Done Calculating Beads');
guidata(hObject, handles);

```


Appendix B: Quantification of Focal Disruptions in Microtubules

The quantification of focal disruptions in microtubules involved manual segmentation in ImageJ, the automatic creation of a profile plot in Matlab, and further filtering analysis and thresholding in Matlab to identify focal disruptions in microtubule intensity.

ImageJ was used for the segmentation part of the analysis due to the ease and speed of the user interface, which could then be integrated into the Matlab code (Figure 76). The image was imported into ImageJ and rotated so that the soma would be oriented towards the left of the screen. This was important because the Matlab code used later would identify the left-most point of the axon in order to find the anterograde-retrograde direction. The image was then cropped so that only the axon was included, background subtracted using a 50-pixel rolling ball radius, and converted to an 8-bit image using a custom macro with build-in ImageJ functions. The image was then thresholded so that the axon could be segmented out of the original image. This axon was then imported into Matlab for further processing

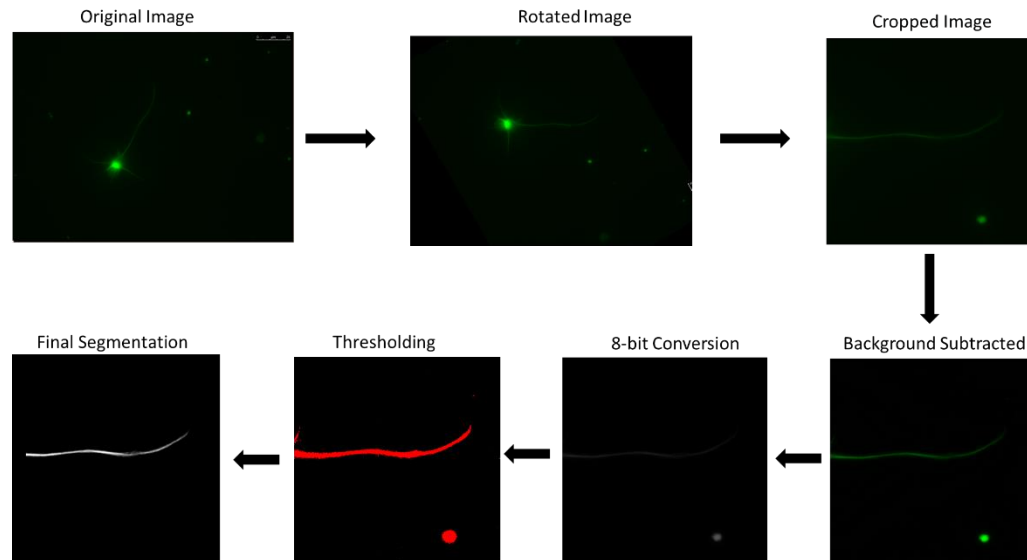


Figure 76. Axon rotation and segmentation in ImageJ before creation of the plot profile.

The java package MIJ was used to exchange images between Matlab and ImageJ. Creating the plot profile in Matlab was very similar to the beading program and consisted of finding and pruning the spine, finding the slope at every point on the spine, and using the overlapping line perpendicular to the slope to find the width of the axon at that point (Figure 77). The average fluorescence intensity of all pixels along that overlapping line was used to find the average intensity at that point. The values were oriented from proximal to distal and were used to create the plot profile of tubulin intensity along the axon.

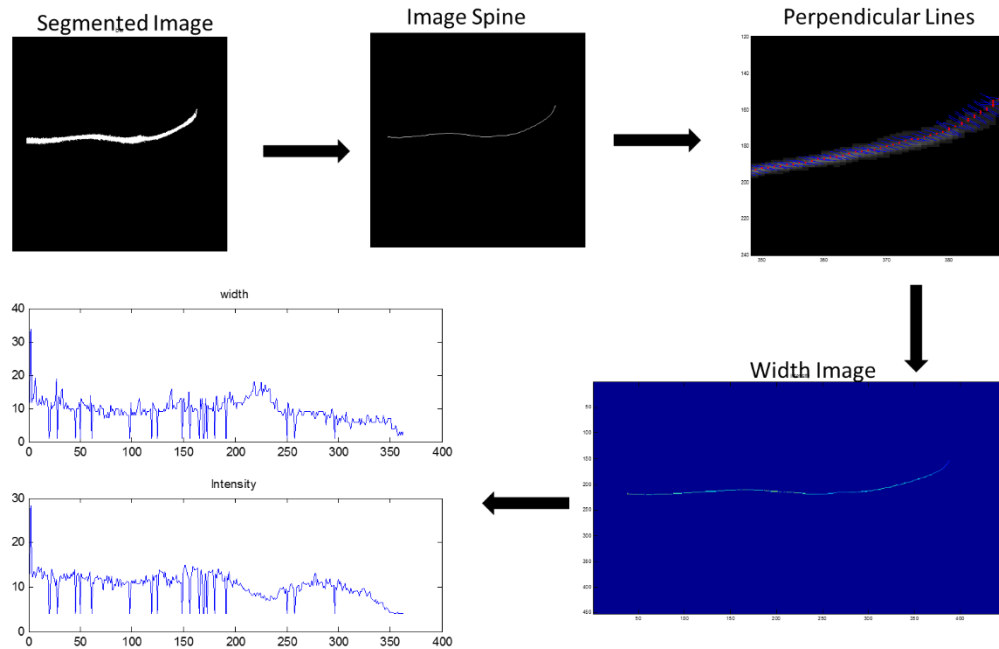


Figure 77. Matlab processing to create plot profile of microtubule intensity

The values for the plot profile were then analyzed in a separate program to find focal disruptions by finding local minima in fluorescence intensity. The intensity of the values along the axon was smoothed and filtered using a low-pass filter. All local minima and maxima were identified, and areas of microtubule disruption were chosen from the local minima based on a manual threshold. The final graphs are then presented to the user along with an image of the axon with the focal disruptions highlighted (Figure 78).

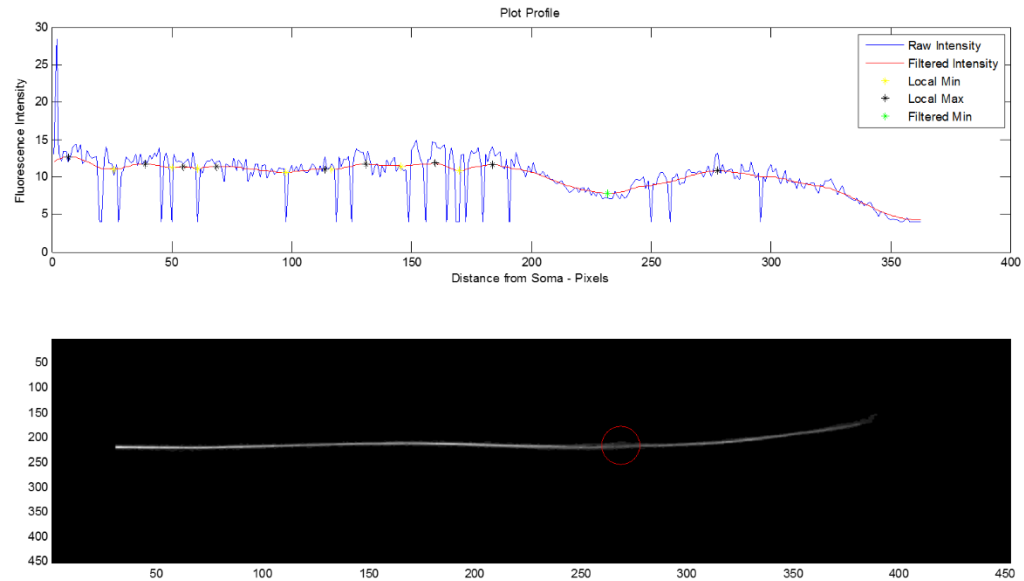


Figure 78. Plot profile filtering to identify focal disruptions in microtubules

Code

```
% javaaddpath 'C:\Program Files\MATLAB\R2014a\java\ij.jar'
% javaaddpath 'C:\Program Files\MATLAB\R2014a\java\mij.jar'
% MIJ.start
% load image from imagej

close all
axon = MIJ.getCurrentImage;

imagesc(axon)
title(axon)
%%this image is all black and white, except for where the data points are
%%
bw = logical(axon);
imshow(bw)
title('bw')
figure()
%get skeleton
skel = skeleton(axon);

%%plot overlay of skeleton and axon

imagesc(axon)
hold on
[S1, S2] = findPoints(skel);
plot(S1, S2, 'r')
%get slope array
slope = getSlope(skel);
% plot slope array
```

```

plotSlope(axon, skel, slope);

%find the average intensity at each point on the axon
[intensityImage, widthImage, stdIntensityImage] = IntensityCalc(axon,
bw,skel, slope);

%%
figure()
imagesc(intensityImage)
title('Intensity')
figure()
imagesc(widthImage)
title('width')

%click on point closest to
traced = traceSpine(skel);
hold on;
plot(traced(1,2), traced(1,1), 'or'); %%plot starting point

%%make backup image of traced values, so you can correlate with location
loc = zeros(size(axon));
for i = 1:length(traced)
loc(traced(i,1), traced(i,2)) = i;
end

%now get values for width and intensity from starting point

width = zeros(1, length(traced));
Intensity = zeros(1, length(traced));
stdIntensity = zeros(1, length(traced));
x = 1:length(traced);

for i = 1:length(x)
width(i) = widthImage(traced(i, 1), traced(i, 2)) ;
Intensity(i) = intensityImage(traced(i, 1), traced(i, 2)) ;
stdIntensity(i) = stdIntensityImage(traced(i, 1), traced(i, 2)) ;
end
%%

%Save Images so that it can be added to cell
Results = {axon, skel, traced, widthImage, intensityImage,
stdIntensityImage, width, Intensity, stdIntensity};

subplot(2, 1, 1)
plot(width)
title('width')
subplot(2,1,2)
plot(Intensity);
title('Intensity')

clear S2 S1 loc i Cbw x

```

```

function skel = skeleton(axon)
imshow(axon)

% smooth the axon
se = strel('disk',10);
axon = imclose(axon,se);
skel = bwmorph(axon, 'thin', Inf);
skel = bwmorph(skel, 'spur');
%%now prune
skel = prune(skel, 20);

skel = bwmorph(skel, 'spur');
skelb = bwmorph(skel, 'fatten');
skel = bwmorph(skelb, 'skeleton', 'inf');
end

function skel = prune(skel, lengthresh)
branchpoints = bwmorph(skel, 'branchpoints');
branchpoints = bwmorph(branchpoints, 'thicken');
b = imsubtract(skel, branchpoints);
b(b<0) = 0;

l = bwlabel(b);
figure()
imshow(l)
title('bwlabelled')
f = [];
for i = 1:max(max(l))
    if length(find(l==i))<lengthresh
        f = [f i]
    end
end

remove = ismember(l,f);
skel = skel-remove;
skel(skel<0) = 0;
%remove anything that is not the longest segment

l = bwlabel(skel);
l1 = zeros(max(max(l)),1);
for i = 1:max(max(l))
    l1(i) = length(find(l==i));
end
skel = ismember(l, find(l1==max(l1)));
end

function [S1, S2] = findPoints(bw)
[S2,S1]= find(bw==1);

end

```

```

function slope = getSlope(skel)
[S1, S2] = findPoints(skel);
r = 1;
slope = zeros(length(S1), 1);
for k = 1:length(S1)
    %draw a box around that point
    sx = S2(k); %x and y coordinates
    sy = S1(k);
    disk = skel(sx-r:sx+r, sy-r:sy+r);
    %find all the x and y points in the box
    [x, y] = find(disk);

    %calculate the slope
    coeff = polyfit(x, y,1);

    sk = coeff(1);
    sk = -1/sk;
    %%now figure out the ones that are extremes, so either 0 or inf (Nan)
    nnn = isnan(sk);
    slope(k) = sk;
    if abs(slope(k))<0.000001
        slope(k) = 100000;
    end
    if abs(slope(k))>1000000
        slope(k) = 0;
    end

    if(nnn== 1)
        slope(k) = 10000;
    end

end
end

function [intensityImage, widthImage, stdIntensityImg] =
IntensityCalc(img, axon,skel, slope)
[S1, S2] = findPoints(skel);
%for each point on the spine, find the overlapping points of a line
l = 50;
m = slope;

for i = 1:length(S1)

    xCenter = S1(i);
    yCenter = S2(i);
    %use the pythagorean theorem to find the range of x values

    lengthx = round(sqrt(((l/2)^2)/(1+m(i)^2))); %length on each side of
centerpoint
    if lengthx < 3 %in the case of a flat line
        lengthx = round(l/2);
    end
    xp = 1:2*lengthx+1;
    yp = xp*m(i);

```

```

%center the middle point of the x axis at S1(i)

xd = S1(i) - xp(lengthx);
xp = xp+ xd;

%raise the middle point of the y-axis to match the center point of
S2(i)

yd = S2(i) - yp(lengthx);
yp = yp+yd;

if (abs(m(i))==0) %%in case of a horizontal line
    xp = -lengthx:lengthx;
    xp = xp+S1(i);
    %and there will only be one y-value
    for j = 1:length(yp)
        yp(j) = S2(i);
    end

end

if (abs(m(i))>50) %%in case of a vertical line

    yp = -lengthx:lengthx;
    yp = yp+S2(i);
    %and there will only be one x-value
    for j = 1:length(yp)
        xp(j) = S1(i);
    end

end

%%also check the minimum and maximum ranges of yp
if(min(yp)<1 || max(yp) > length(axon))
    for j = 1:length(xp)
        yp(j) = yd;
    end
end
yp = round(yp);
C = improfile (axon, xp, yp);
intensityLine = improfile(img, xp, yp);

%the number of 1s in C will be the width at that point (in pixels);
%it is possible that the line will cross the axon at mutlipe
locations, so
%look for the region that contains the center point

Cbw = bwlabel(C);
if max(max(Cbw)) > 1
    np = max(xp == S1(i));
    Cbw = ismember(Cbw, np);
end
points = find(Cbw);

intensityValues = intensityLine(points);
Intensity(i) = mean(intensityValues);

```

```

        stdIntensity(i) = std(intensityValues);
        Width(i) = length(points);
        assignin( 'base', 'Cbw', Cbw);
    end
    intensityImage = zeros(size(img));
    widthImage = zeros(size(img));
    for i = 1:length(S1)
        intensityImage(S2(i),S1(i)) = Intensity(i);
        widthImage(S2(i),S1(i)) = Width(i);
        stdIntensityImg(S2(i),S1(i)) = stdIntensity(i);
    end
end
function traced = traceSpine(skel)

[xp yp] = findPoints(skel);
%get endpoints
endPoints = bwmorph(skel, 'endpoints');
[xEnd yEnd] = findPoints(endPoints);
%have the starting point be the one with the smallest xValue
minI = find(xEnd == min (xEnd));
P = [yp(minI) xp(minI)];
b = bwtraceboundary(skel,P(1,:), 'E', 8, length(find(skel)), 'clockwise');
traced = b;
end

function [nfoc nfocnorm] = groupFilter(Data)
close all;
for k = 1:length(Data)
    current = Data{k};
    [B,A] = butter(1, .05, 'low');
    [axon, ~, traced, ~, ~, ~, ~, Intensity, ~]=ExtractResults(current);

    %remove zero's from intensity values
    izeros = (Intensity <=1);
    Intensity(izeros) = nan;
    figure
    subplot(2,1,1)
    plot(Intensity)
    xlabel('Distance from Soma - Pixels')
    ylabel('Fluorescence Intensity')
    title('Plot Profile')

    IntensitySmooth = filtfilt(B,A, smooth(Intensity));
    Intensitydet = detrend(IntensitySmooth);
    dfIntensity = diff(IntensitySmooth);
    aIntensity = diff(dfIntensity);
    Intensity = smooth(IntensitySmooth);

    hold on;
    plot(Intensity, 'r')
    xlocPeaks = filterIntensity(Intensity)
    plot(xlocPeaks, Intensity(xlocPeaks), 'y*')
    [~, xlocmax] = findpeaks(Intensity);
    plot(xlocmax, Intensity(xlocmax), 'k*')

    %%for each minima, find surrounding maxima and see if it is within the

```

```

%%tolerance
xmin = [];
for i = 1:length(xlocPeaks)
    y1 = 0;
    y2 = 0;
    minx = xlocPeaks(i);
    miny = Intensity(minx);
    for j = 1:length(xlocmax)-1
        if (xlocmax(j)<minx)
            y1 = Intensity(xlocmax(j));
            y2 = Intensity(xlocmax(j+1));
        end
    end
end

%%now filter based on percentage difference
tol1 = 15;
tol2 = 5;

percentageDifference1 = round((y1-miny)/y1*100);
percentageDifference2 = round((y2-miny)/y2*100);

%%make sure it is not near the last 20 points
if (minx > length(Intensity) - 20)
    percentageDifference1 = 0;
end

if (percentageDifference1>tol1)

    if (percentageDifference2>tol2)
        xmin(end+1) = minx;
    end
end
plot(xmin, Intensity(xmin), 'g*')
legend('Raw Intensity', 'Filtered Intensity', 'Local Min' , 'Local Max'
, 'Filtered Min')

end

subplot(2,1,2)
imagesc(axon)
colormap(gray)
hold on;

plot(traced(xmin,2),traced(xmin,1), 'or', 'MarkerSize',30);
nfoc(k,1) = length(xmin);
nfocnorm(k,1) = length(xmin)/length(traced);
end

%%change places where intensity=0 to intensity = NA;
end

```


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