# THE EFFECTS OF GENETIC POLYMORPHISMS ON RECOVERY AFTER REPEATED MILD TRAUMATIC BRAIN INJURY IN A MOUSE MODEL AND PERSONALIZED TREATMENT APPROACHES

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# ABSTRACT OF THE DISSERTATION

**The Effects of Genetic Polymorphisms on Recovery after Repeated Mild Traumatic Brain Injury in a Mouse Model and Personalized Treatment Approaches**

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Traumatic Brain Injury (TBI) is a serious and potentially life-threatening clinical problem. In 2013, there were 2.5 million TBIs in the United States, 50,000 of which led to death and 70,000 of which led to permanent neurological damage. Clinicians have long noticed that certain patients have worse recovery than others after TBI and identifying what makes some patients more susceptible is a vital step in understanding the underlying mechanisms through which TBI causes its deleterious effects. In this study, we sought to determine the effect of specific single nucleotide polymorphisms (SNPs) on recovery after TBI, and to investigate the underlying mechanisms that may be a factor. This knowledge can be used to explore personalized approaches to treatment of TBI. We have investigated cellular and behavioral outcomes in genetically engineered mice with either the Brain-derived neurotrophic factor (BDNF) Val66Met or ApoE4 polymorphism following repeated mild TBI (rmTBI) using a lateral fluid percussion injury model. We have found that relative to injured Val66Val and ApoE3 carriers, injured Val66Met and ApoE4 carriers have a larger injury volume and increased levels of neurodegeneration, apoptosis, p-tau, activated microglia, and gliosis in the cortex and/or hippocampus at 1 and/or 21 days post injury (DPI) as

well as altered levels of BDNF. As a result, we have concluded that the ApoE4 and the Val66Met genetic polymorphisms are a risk factor for poor outcomes after rmTBI. Using this information, we developed a personalized approach to treating genetically susceptible individuals by targeting the pathway altered in those genotypes. In the Val66Met mice, we used an AAV-BDNF virus to overexpress wildtype BDNF in the cortex and hippocampus and investigated outcomes at the 21 DPI timepoint. In the ApoE mice, we used Bryostatin-1, a PKCε activator that has been shown to rapidly increase BDNF levels, and investigated outcomes at the 1 DPI timepoint. We have shown that these interventions are able to improve cellular as well as motor and cognitive behavior outcomes at these timepoints. This study lays the groundwork for further investigation into the genetics that play a role in recovery after rmTBI and highlights the role that personalized therapeutics may be able to play in recovery for susceptible individuals.

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# Chapter 1: Introduction

## Traumatic Brain Injury: Definition, Prevalence, Current Diagnosis and Treatment

Traumatic Brain Injury (TBI) is a serious and potentially life-threatening clinical problem. It occurs when there is a force to the head, which results in a disruption of brain function. In 2013, there were 2.5 million TBIs in the United States, 50,000 of which led to death and 70,000 of which led to permanent neurological damage. The rates of TBI and hospitalizations have been increasing year by year and are expected to continue to increase. [1]

TBI can have wide ranging effects, which can result in symptoms that are cognitive, behavioral, or sensorimotor in nature. Many who are affected end up with either short term or long-term deficits in learning and memory, executive functioning, motor ability, sensory function, language skills, and alterations in emotion resulting in depression and anxiety. [1] Currently, diagnosis of TBI is done using a combination of physical exam and basic neuroimaging. There are no accepted gold standard treatments for TBI; instead, treatments tend to focus on treating the symptoms of TBI as they emerge.

## Repeated Mild Traumatic Brain Injury

Based on the neurological symptoms that occur after a TBI, the injury is classified either as mild, moderate, or severe. A mild TBI is defined as having a loss of consciousness for less than 30 minutes, a period of confusion and memory loss for less than 24 hours, and no findings on initial CT scan. [2] Mild TBI is the most prevalent form of TBI that occurs in

the United States. [3, 4] In 2013, there were approximately 1.125 million mild TBIs in the United States, with costs from mild TBI reaching $117 billion each year. [1] While the majority of patients who suffer from mild TBI tend to recover over time, it is estimated that around 15% of patients have symptoms that last longer than three months and develop into chronic disabilities. [5] This problem is further compounded when a person sustains a second mild TBI after an initial head injury. Sustaining a repeated mild TBI (rmTBI) can have devastating consequences; unfortunately, they are common in athletes and military personnel. [3] [4]. Currently, many groups are studying the link between undergoing rmTBIs and developing long-term neurodegenerative diseases. [6]

One of the reasons that rmTBIs can cause problems long after the injury has occurred is that they are not discrete events. There is a primary injury that occurs, due to a force acting upon the head, that causes an initial damage. However, the initial damage that occurs activates secondary mediators, which results in inflammation and a secondary injury process. Over time, regeneration can occur, and the brain can regain some of the function that may have been lost due to the injury. However, this process is further disrupted if a person sustains a second blow to the head while still recovering from the first injury. In the case of rmTBI there is an increased risk of developing long-term health problems, such as dementia. [7] It is becoming increasingly evident that those who have suffered from mild repeated brain injuries can end up developing neurodegenerative diseases such as chronic traumatic encephalopathy (CTE). [6, 8]

## Differential Outcomes after TBI

While some patients who suffer from TBI recover quickly and have no obvious long-term symptoms, other patients undergo a prolonged secondary injury phase and have a much harder time recovering. [9, 10] One of the reasons that not all individuals respond similarly to TBI could be the genetic differences that exist in the population, however specific genes that may affect outcomes after TBI have not been identified.

The investigation of the role that single nucleotide polymorphisms (SNPs) play in affecting the function of important proteins may prove to be an important piece of information to understanding differential outcomes. SNPs are the most common form of genetic variation between people and occur when a single nucleotide in the DNA sequence is altered. [11] When SNPs are in the coding regions of DNA, the amino acid sequence of the resulting protein is altered. This results in the same protein having a different structure, and potentially function, among different people.

## Role of Neurotrophins and their Receptors in Traumatic Brain Injury

Neurotrophins are a family of proteins known to play an important role in brain function, particularly with the ability of the brain to recover after injury. The Neurotrophin family includes factors such as brain-derived neurotrophic factor (BDNF), Nerve Growth Factor (NGF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4) [12] and SNPs in the genes that encode these factors have been associated with various clinical conditions. Neurotrophins act through two receptor families; tropomyosin receptor kinase (Trk) and p75. Mature neurotrophins bind with high affinity to their requisite Trk receptor, and with low affinity to the p75 receptor. On the other hand, the precursor pro-neurotrophins bind

with high affinity to the p75 receptor. [13] BDNF is a neurotrophin that plays an important role in brain functioning. [12, 14-16] It is one of the most highly expressed neurotrophins in the central nervous system [17, 18] and plays a particularly important role in the hippocampus. [19] The precursor proBDNF binds with high affinity to the p75 receptor which induces apoptosis and cell death, while the mature BDNF form binds with high affinity to the trkB receptor which promotes neuronal survival and synaptic plasticity. [13, 20]

BDNF has been shown to play an important role after injury. Early studies have shown that after injury, levels of BDNF protein and mRNA are upregulated however which isoform of BDNF increased was not known. [21-25] Our group went on to show that after TBI levels of proBDNF and proNGF, the p75 receptor, and the sortilin co-receptor are preferentially upregulated relative to mature BDNF and its trkB receptor (Chapter 6) and may explain why there is apoptosis and neurodegeneration after injury rather than cell survival and neural regeneration. [26] These results are consistent with what has been shown in the literature that after injury proBDNF is preferentially upregulated relative to mature BDNF. [25-27]

Recently, there has been an interest in using BDNF as a biomarker after injury. Studies in humans have been conducted to see if there are correlations between levels of BDNF and outcomes after TBI. One study investigated levels of BDNF in the serum after TBI in humans, and found that low serum levels of BDNF acutely after injury correlated with impaired memory, causing the authors to posit that after injury, BDNF plays an important role in the hippocampal-dependent memory recovery. [28] Other studies have seen a correlation between BDNF levels in the cerebrospinal fluid (CSF) [29] but not in the

plasma [30, 31], which correlate to poor outcomes after TBI in humans. These results suggest that low levels of BDNF in the plasma and CSF may be able to predict poor outcomes after injury.

In order to investigate more directly the role of BDNF after injury, animal studies have been conducted to look at BDNF and the signaling pathways that are affected after injury. These studies have shown that by activating the beneficial mature BDNF trkB pathway after injury, there can be improved recovery. One recent study looked at the effect that molecule called CN2097, an enhancer of trkB signaling, has on recovery after TBI. They found that by activating the trkB signaling pathway, they were able to decrease pro-inflammatory factors, improve hippocampal functioning, and auditory processing. [32] Another study used neural stem cells genetically modified to encode BDNF as a treatment method after a rat model of TBI. They showed that treating with BDNF in this form, they were able to increase synaptic protein levels through downstream signaling pathways of trkB, primarily MAPK/Erk1/2 and NRF2/Trx. [33] One study looked at the effect of physical exercise after TBI in a rat model of injury, and found that exercise increased BDNF and trkB level and contributed to the recovery of cognitive deficits. [34] Interestingly, one group looked at the effect that acupuncture has on recovery after TBI, and found that by using acupuncture they were able to maintain elevated levels of BDNF and trkB, as well as the downstream signaling molecules p-Akt and p-Erk1/2. They found that this treatment modality promoted the recovery of neurological impairment, an effect that was blocked when a specific inhibitor of trkB, K252a, was given. [35] These studies have shown that by activating the mature BDNF trkB signaling pathway that it is possible to improve outcomes after injury, making it a potential clinical treatment target.

These studies highlight the important role that BDNF plays in recovery after injury. However, they do not address the effect that all of the different isoforms of BDNF play on its role in recovery after injury, nor the effect that genetic differences in the BDNF protein may have. Our group previously showed that after TBI, it is the pro form of BDNF and its p75 and sortilin receptors that are upregulated relative to the mature form of BDNF and its trkB receptor (Chapter 6). [26] In addition, we also showed that by manipulating these pathways, we were able to alter recovery after TBI. We found that when we treated wild type (WT) mice after TBI with p75 antagonist TAT-Pep5 or the trkB agonist 7,8-DHF or mutant mice lacking the p75 receptor, that we were able to improve outcomes, such as decreasing cell death and neurodegeneration, as well as improving motor and cognitive function. [26] This was an important finding, and showed that the role of BDNF in recovery after injury is complex, and may be dependent on more factors than simple upregulation of total BDNF levels. For this reason, we decided to investigate the role that SNPs in *BDNF* play in its ability to improve recovery after injury.

## BDNF Genetic Polymorphisms

*BDNF* has a SNP site, rs6265, at the 66 amino acid position of the BDNF protein, and occurs when the wildtype Val is replaced with a Met. The reported frequency of the various BDNF genotypes ranges greatly depending on ethnicity and race (Val66Val 26-68%, Val66Met 27-53%, and Met66Met 3-20%). [36, 37] Since the Val66Met SNP is at the 66 amino acid position which is in the pro domain of the BDNF protein, when the prodomain is cleaved from the mature, the resultant mature BDNF protein will have no altered sequence. However, studies have shown that the genetic polymorphism in proBDNF leads to altered intracellular packaging, which affects the axonal transport of BDNF and results in decreased activity dependent secretion of BDNF at the synapse. [38, 39] Naturally, this

can become an issue in disease states such as after TBI, where there is a need for an increased mature BDNF signaling in order to stimulate repair and recovery. In addition, recent work has highlighted the importance of the prodomain itself that has been cleaved off from the mature BDNF protein. Previously thought to be inert, new research has shown that it is in fact an active ligand. Importantly, the 66Met substitution changes the structure of the prodomain, which causes acute growth cone retraction, and decreases Rac activity in the hippocampus relative to the 66Val prodomain. [40] These actions are thought to be due to the differential interaction of the 66Met prodomain with the SorCS2 receptor. [40] Newer studies have shown that the 66Met prodomain is also able to disassemble dendritic spines and eliminate synapses in hippocampal neurons, leading to impaired hippocampal-dependent fear extinction behavior. [41] These toxic effects of the 66Met, but not 66Val, prodomain may also play a role in differences in outcomes between Val66Val and Val66Met carriers but this has not yet been investigated.

The Val66Met genotype has been reported to be associated with poor outcomes in a number of disease states such as major depressive disorder [36], anxiety [42], stroke [43], and Alzheimer's disease [44], however clinical research in TBI patients is not consistent with whether the Met or the Val polymorphism confers worse outcomes. [28, 45-51] The Val66Met polymorphism results in diminished BDNF in dendrites due to alterations in the trafficking of the protein within the cell and therefore reduced amounts of BDNF released into synapse upon stimulation. [52] [53] In healthy populations, individuals with the Val66Met polymorphism have been shown to have impaired cognitive function. [38] It seems to follow logically that the Val66Met polymorphism would result in impaired neurocognitive performance after TBI [50], as well as being a risk factor for TBI in combat forces [47-49]. However, some recent studies in humans have shown that

counterintuitively, after injury, the Val66Val carriers actually have worse recovery compared to the Val66Met carriers. [28, 45, 46, 54] Specifically, in long-term studies of combat veterans, carriers of the Val66Met polymorphism had recovery of executive function back to baseline levels while the Val66Val carriers did not. [45, 46] On the other hand, other studies have found no effect of the polymorphism influencing outcomes after injury. [55-57] Thus there is controversy from the human studies as to which allele is the risk factor for worse outcomes.

To our knowledge there have been no studies investigating the role of the Val66Met SNP in mouse models of TBI, but there have been studies that have investigated another type of neural trauma, stroke. After stroke, there is evidence that humans with the Val66Met polymorphism do worse than those with the Val66Val polymorphism. [43, 58-60] However, in mouse models of stroke, the studies have been conflicting. In some studies, the time course of stroke recovery is thought to play a role, with the Val66Met allele shown to be the risk allele in motor ability acutely [61], and surprisingly, Val66Val allele shown to be the risk factor chronically. [62] Age and sex can also interact with genotype to determine outcomes after stroke, which complicates the human studies that attempt to find correlates between the genotype and outcomes and adds another factor of consideration to the mouse studies. [63]

In addition, there are other SNPs in the BDNF protein which may prove to play a role in recovery after injury. [64] For example, studies have shown that the BDNF SNP rs1157659 interacts with mild TBI to result in altered hippocampus size in humans. [65] The role of the BDNF SNP rs7124442 also has been investigated in conjunction with the rs6265, in order to determine risk factor status after TBI. [57, 66] These studies have found that the

rs7124442 SNP may interact with age, injury timing, and other BDNF SNPs to cause worse recovery after TBI but that there are complex relationships between these factors.

Thus, the research as to which BDNF allele is the risk factor for outcomes after TBI is not all in agreement, and could benefit from a bottom-up, controlled experimental research study as has been done to study other disease models where genetic polymorphisms may play a role.[67]

## Role of the ApoE Protein in Traumatic Brain Injury

ApoE has is another protein that has been shown to play a role in neural degeneration as well as neuronal repair after injury and has SNPs associated with differing outcomes in disease. The ApoE protein is an important protein responsible for the transport and clearance of lipids and cholesterol in the brain. [68] Under baseline conditions, ApoE is produced principally by astrocytes in order to support normal lipid transport and membrane repair. However, after injury neurons begin to synthesize ApoE, presumably to support the neuronal repair mechanisms. [69-71] Interestingly, there appear to be differences in how effectively the different isoforms of ApoE are able to function, and differences in proteolytic cleavage may contribute to long term neuropathology after injury.

## ApoE Genetic Polymorphisms

*ApoE* is a critical neuronal gene that contains two single nucleotide polymorphisms (SNP), at the 122 and 158 residue positions, also referred to as rs429358 and rs7412, which affect the structure and function of the resultant protein. [68] There are three isoforms that are frequently investigated; ApoE2, ApoE3, and ApoE4. ApoE4 has an Arg at both the 122

and 158 position, while ApoE3 has a Cys at the 122 position, and ApoE2 has a Cys at both the 122 and 158 positions. [72] ApoE3 is generally thought of as the wildtype allele with an allelic frequency ranging from 53.6%-89.8%, although there is considerable variation in allelic frequency across worldwide populations, with the ancestral ApoE4 allele having a frequency ranging from 5.2%-40.7%. [73] In this study, I have chosen to study the effect of ApoE3 and ApoE4, given their higher relative frequency within the population.

The changes in ApoE structure between isoforms has been shown to have clear effects on the function of the protein. The addition of the Arg at the 122 position in ApoE4 results in a protein that is less stable, has a decreased affinity for small lipoproteins, and tends to aggregate. [68, 74] These changes have led to ApoE4 being the highest known genetic risk factor for the development of late onset Alzheimer's disease. It is currently thought that ApoE4 contributes to the development of AD through a number of different pathways, some of them dependent on increased beta-amyloid deposition and others independent of it. [75] Carriers of the ApoE4 allele tend to have increased beta-amyloid deposition, increased tau phosphorylation, impaired neuronal plasticity, dysfunction of the medial temporal lobe, decreased hippocampal volumes, and greater memory impairments. [76-78]

Previous studies in humans have investigated the effect that the ApoE genetic polymorphisms have on outcomes after injury. [79] Early studies looked retrospectively at boxers and hockey players who had a history of repeated concussions, and worse long-term outcomes were correlated with being a carrier for ApoE4. [80, 81] And while some human studies have shown that having the ApoE4 allele is not a risk factor for sustaining a concussion, other studies have shown that it is a risk factor for sustaining multiple concussions. [82, 83] Although it seems like there is some support in the literature for

ApoE4 being a risk factor for poor outcomes after TBI, there is no consensus among studies. An early meta-analysis showed that carriers of the ApoE4 allele had worse long term outcomes 6 months after injury [84], while a more recent meta-analysis showed that there was no association with the ApoE4 status and poor cognitive outcomes after TBI. [85]

In order to attempt to elucidate the effect of ApoE SNPs on recovery after TBI, a number of animal studies have been done as well. In a closed head injury mouse model of injury, comparison of the ApoE4 polymorphism to the ApoE3 polymorphism has shown that ApoE4 mice were twice as likely to die from an injury compared to the ApoE3 mice. In the mice that did live, at 3 and 11 days post injury, the ApoE4 mice had significantly higher neurological severity scores than the ApoE3 mice. [86] However, the pathology behind these results is not well elucidated. While some reports that ApoE4 worsens axonal pathology after TBI but not Aβ deposition [87], others have shown that there is indeed Aβ accumulation after TBI in ApoE4 mice. [88] There are conflicting reports on how the ApoE4 allele causes these detrimental effects, and the role that type of injury, age, and other genetic alleles play are not well studied. [89-93]

Given the interest in how repeated mild TBI can lead to long term consequences, some researchers are choosing to study the effect of the ApoE polymorphism after rmTBI. One study attempted to investigate the role that ApoE4 has on recovery after a repeated mild CCI injury and found that there was no effect of the ApoE4 genotype on recovery in their model. However, given their parameters they used, which involved a very mild injury, it is possible that they were unable to distinguish any small differences that may be occurring. In addition, they used WT C57BL/6 mice as the control mice, which have the mouse form

of ApoE4, given that it is the ancestral allele, so is not an accurate study of the difference between the human ApoE3 compared to ApoE4 alleles. [94] Others found that after repeated blast injury, ApoE4 injured mice had more p-tau relative to ApoE3 injured mice that was regulated by synj1. [95] Another recent study looked at the effect of ApoE4 relative to ApoE3 in middle aged mice that underwent repeated mild injuries for a month. They found that ApoE4 was not a risk factor for increased astrogliosis or total microglia levels, but that it was for increased levels of MC1, a tau conformational marker. [96]

Consequently, the research investigating whether ApoE4 is a risk allele compared to ApoE3 after TBI is not all in agreement, and could benefit from a bottom-up, controlled experimental research study as has been done to study other disease models where genetic polymorphisms may play a role. [67] None of the studies that have been done has utilized a repeated mild LFP model where the human form of ApoE3 and ApoE4 are compared in young adult mice.

In addition, the pathway through which ApoE4 may be having its deleterious effects is unknown. Previous studies have postulated that it may be due to the role that ApoE plays in neural repair, with ApoE4 carriers having impaired ability to repair membranes after injury, while others have suggested that it is due to the neurotoxic fragments that are created when the neuronally produced ApoE4 is proteolytically cleaved. [69-71] Recent studies have begun to investigate the role that the ApoE4 genetic polymorphism has on neurotrophic factors in the brain. Studies have found that AD ApoE4 carriers have lower levels of Brain-Derived Neurotrophic Factor (BDNF) in the serum [97], and that mildly cognitively impaired ApoE4 carriers have impaired BDNF increase after exercise compared to non-carriers. [98] Interestingly, it has been found that ApoE4 causes reduced

BDNF release by increasing the nuclear translational of histone deacetylases (HDAC) 4/6. This action causes negative gene regulation, and results in less BDNF being produced in the cell and secreted by astrocytes. [99, 100] It is known that BDNF signaling plays an important role in recovery after injury [26, 101], making this a potential therapeutic target for ApoE4 carriers that has not yet been explored.

## Animal Models of Traumatic Brain Injury

One of the difficulties of studying TBI is that it is a complex and multifocal injury process. In humans, there are various types of injuries that are sustained, and numerous confounding factors. TBI has been called "the most complex disease in the most complex organ,". [102] For this reason, using animal models to study TBI disease processes may be illuminating. By using animal models, we are able to deliver replicable injuries, to limit confounding factors, and to study the cellular and molecule mechanisms that contribute to the pathology seen after TBI. [103] However, there is no single animal model of TBI that is able to completely recapitulate the damage seen in human TBI. For this reason, it is important to carefully choose the animal model of TBI that will be used, in order to determine which will be best suited for the study being conducted and the outcomes that will be assessed.

One of the important aspects to consider when choosing an animal model of TBI is whether the injury will be focal, diffuse, or mixed. Focal injuries are injuries where there is tissue damage at a local site, reminiscent of an injury that may be sustained from a blow to the head, such as when a person has undergone an assault. On the other hand, diffuse injuries occur more globally in the brain, and the damage is generally caused by acceleration forces which in particular can be damaging to white matter tracks. Finally, mixed injuries contain

aspects of both focal and diffuse injuries. Mixed injuries can be seen in falls, motor vehicle accidents, and military and athletic injuries. [103]

Depending on the injury model chosen, it may be possible to alter the parameters to best model the type of injury being studied. Groups may choose to study mild, moderate, or severe injuries. They may also choose to study these injuries in either a single or multiple paradigm. For example, in order to model the effect of an assault, one might choose to use a single moderate control cortical impact model. On the other hand, if one wants to mimic the effect of undergoing multiple concussions caused by playing a sport, the best option may be to use a repeated mild lateral fluid percussion injury model.

**Controlled Cortical Impact**

One of the most frequently used injury models is the controlled cortical impact injury (CCI) model. The CCI model is a focal injury model, useful for modeling injuries caused by ballistic damage or physical assault. In this method, the dura is surgically exposed, and a pneumatic rod is used to strike the exposed brain tissue. This creates a localized area of brain tissue damage, where the cellular and molecular mechanisms of injury can be studied. [104]

An updated CCI method does not expose the dura, and instead uses the rod to strike the surface of the skull. [105] The result of this injury paradigm is a closed head injury. This method is useful to investigate mild TBI, and in particular mild repeated TBI, such as might be seen in chronic boxing head injuries.

However, the CCI model has a number of drawbacks. Firstly, the small size of the impactor causes a focal injury, but does not create a diffuse injury. A diffuse injury which causes damage to axons is seen in humans who sustain a TBI and is an important factor to consider in a model. A CCI injury also usually does not create diffuse swelling which can result in brainstem injury and death in humans with more severe head trauma. Finally, a CCI impactor will often fracture the skull and penetrate the dura, causing a severe disruption of the tissue more reminiscent of a penetrating injury blow than a closed head injury seen in the majority of human cases. [106]

**Weight Drop Impact Acceleration**

The weight drop injury model has been developed by multiple teams in order to fit the needs of the investigator. Depending on which method is used, the injury is either focal or diffuse. In the initial method, the dura was surgically exposed, and a free-falling weight was allowed to strike the exposed brain tissue. This method created a focal injury that allowed for the investigation of cellular mechanisms of injury. [107] Later modifications discontinued the exposure of the brain and allowed the weight to be dropped directly onto the skull, in a closed head injury paradigm. [108] This method again created a focal injury, which allowed for the investigation of mild and repeated mild TBI.

While the previous two methods discussed allowed for investigators to alter the paradigms of their focal injury model, the final weight drop injury model we will discuss mimics a diffuse injury. Commonly referred to as the Marmarou weight drop injury model, in this method, a segmented brass weight is allowed to free fall through a plexiglass tube. In order to keep the weight from creating focal damage by directly impacting brain tissue, or from creating skull fractures by directing impacting the skull, the weight impacts a stainless steel

disk that is placed on the skull. [109] This method allowed for the force of the impact to manifest as an acceleration impact, creating a diffuse injury impact similar to what is seen in motor vehicle accidents.

Drawbacks of the weight drop model mirror the drawbacks seen in the CCI model; the creation of a focal injury does not accurately mimic the types of injury seen most commonly in humans. The modifications to the weight drop model attempt to remedy this, but result in a high mortality rate and great variability between injuries due to the indirect forces that create the injury. [106]

**Lateral Fluid Percussion**

Finally, there is the lateral fluid percussion (LFP) method of injury. The LFP model of injury is one of the best characterized methods of TBI in an animal model and is being increasingly used. [110] The strength of this model is that it fulfills the criteria for the three types of validity that animal models strive for; construct, face, and predictive validity. The LFP method has construct validity because it recreates the types of injuries that humans sustain. In particular, when the LFP injury pulse duration is ~20msec, it mimics the injuries seen in crash test stimulations for humans with mixed focal and diffuse components. [111] The LFP method also has face validity, meaning that the effects of the injury in the mice are similar to the effects of the injury in humans. For example, after LFP injury groups have shown that there is increased macrophage accumulation and astrogliosis that leads to the formation of a glial scar. [111-113] There has also been evidence that LFP injury creates sensorimotor and cognitive deficits similar to those seen in humans, due to the injury to the sensorimotor cortex and the underlying hippocampus. [114-116] Finally, the LFP method also has predictive validity. This means that interventions that improve outcomes in

humans are also shown to improve outcomes in mice that have undergone the LFP method. [111] This is an important aspect of animal models, because it allows us to test pharmacological and genetic manipulations with predictive ability for their effect in humans under similar conditions. For these reasons, the LFP model is one of the most reliable methods employed by labs studying TBI and is being increasingly used. Using a modified LFP paradigm is a novel approach to studying the mechanisms of repeated mild TBI.

## Outcomes after Traumatic Brain Injury in Humans and Animal Models

TBI is a complex disease that is variable by its inherent nature which can make understanding the disease process difficult. However, there are a number of useful methods that are employed to study the effect of TBI on humans and animals. [117]

### Imaging

Computed Tomography (CT) scans are the most frequency conducted imaging modality in human TBI cases due to their relative low cost, ease of use, and availability. CT scans are most useful in the staging of the TBI diagnosis in conjunction with the Glasgow coma scale, a measure of basic physiological function, in order to determine if a TBI is of mild, moderate, or severe quality. [118] CT scans are able to detect skull fractures, hemorrhages, and injuries to the parenchyma, and they are used frequently to identify the nature of focal injuries in preparation for neurosurgical intervention.

However, CT scans are of limited utility in the case of mild TBI. Although 90% of CT scans are negative, many patients still have severe symptoms that are seemingly

unexplained by the imaging that is routinely done. [119] When researchers have looked at patients over time, they have found that patients who were initially CT negative after a mild TBI actually do have observable pathology on Magnetic Resonance Imaging (MRI) [120] This is particularly relevant for patients who end up with repeated mild TBI.

MRI is a valuable tool that is currently in use in a number of hospitals and clinical settings around the world, although it is not currently the standard of care for TBI. Conventional MRI utilizes T1 and T2 weighted imaging, as well as utilizing fluid-attenuated inversion recovery (FLAIR) sequences. These methods allow for pathology information to be observed, such as the identification of epidural or subdural bleeding, contusions, microscopic bleeding, wound size, volumetric analysis, and skull fractures.

There are also advanced MRI techniques being developed. These include diffusion tensor imaging (DTI) and diffusion weighed imaging (DWI). DTI measures the Brownian movement of water molecules, and is a very sensitive tool to detect white matter pathology after TBI.

Positron emission tomography (PET) is currently being investigated as a novel imaging modality for TBI. PET scans use a radioactive tracer that is taken up by the tissues in the body, and then is able to be detected in the machine by the gamma decay given off by the tracer. The uptake is then placed at a location in the body, sometimes with the help of a CT scan in the case of the combined PET/CT scan. [121] A number of tracer ligands have been developed for TBI and are currently being tested, such as a glucose tracer [122], an amyloid tracer [123], and a tau tracer. [124] This method offers an emerging way to understand the

underlying pathology of injury in a mouse model in a longitudinal method that does not require sacrificing the animal. [125]

## Histological

Immunohistochemistry is a powerful tool that can be used to understand the effects of TBI on the brain at the level of cellular components in tissue sections. This method uses primary monoclonal or polyclonal antibodies that recognize an epitope on a protein of interest. After treatment with the primary antibody, a secondary antibody that is linked to a fluorophore is applied. [126] This method allows for visualization of the location and extent of the expression of the protein of interest and allows researchers to understand what is happening on a molecular level in disease states such as TBI.

### Cleaved Caspase 3:

Apoptosis is the process of controlled cell death that occurs either using the intrinsic or extrinsic pathways. In the intrinsic pathways, cells sense stress and activate the pathway themselves, while in the extrinsic pathway they receive the signals from other cells. Cleaved caspase 3 is a caspase protein that is downstream of both caspase 8 and 9 and is the point where the extrinsic and intrinsic apoptosis pathways converge. [127] For this reason, cleaved caspase 3 is frequently used as a marker to determine the activation in apoptosis in tissue in disease states. After TBI, there is often an increase in apoptosis, and quantitating the levels of apoptosis can help to determine the underlying pathophysiology of TBI. [128, 129]

After TBI, cell death can occur by either apoptotic or necrotic pathways. Generally after mild TBI, the apoptotic pathway is activated, while after severe TBI, the more toxic necrotic pathway is activated. [129] While some level of apoptosis after TBI may be protective after injury due to its ability to activate cell death with a lack of immune cell activation [129], excessive apoptosis has still been shown to lead to worse outcomes. [130] Quantifying levels of cleaved caspase 3 can inform us about the pathophysiological events occurring after TBI, and the extent of the tissue damage that is occurring.

**Fluoro-Jade C**

Following TBI, there is damage to the brain tissue via a number of different pathophysiological pathways. [131, 132] In addition to apoptosis, there is also degeneration of neurons that occurs. Neurodegeneration is hallmarked by the progressive loss of function of neurons, which can be caused by various types of insults. [133] In order determine neurodegeneration in tissue a Fluoro-Jade stain is used. [133-135]

The Fluoro-Jade C stain has been developed to create a sensitive probe for degenerating neurons, with a high signal to noise ratio. [135] It has been shown to stain the cell body, dendrites, and axons. Fluoro-Jade C is not a traditional immunohistochemical stain using antibody binding, but instead is an anionic fluorochrome dye that is able to selectively stain degenerating neurons due to its chemical structure. However, there is no clear understanding of the mechanism through which Fluoro-Jade C is able to accomplish this task. There has been speculation that it is due to electrostatic interactions of the acidic Fluoro Jade C molecule with a basic molecule expressed in the degenerating neurons, possibly a polyamine being expressed during neurodegeneration process due to cleavage of a larger biomolecule. [133, 135] Although the mechanisms by which Fluoro-Jade C is

able to stain degenerating neurons is not completely clear, it is a practical and frequently used method to visualize and quantitate neurodegeneration after TBI.

**Phosphorylated Tau:**

One specific way in which neurodegeneration can occur is the hyper phosphorylation of tau. Tau is a soluble protein that helps to stabilize microtubules in the brain under normal state. In neurodegenerative diseases such as Alzheimer's disease or after TBI, tau can dissociate from tubulin. When this dissociation occurs, phosphorylation sites are exposed. When the tau is hyper phosphorylated, it will bind to other tau proteins, and create aggregates. This is a disruption of normal functioning and can lead to long-term neurodegeneration. [94, 136, 137]

In particular, acutely after repeated mild TBI, hyper phosphorylation of tau has been shown to be present in high risk individuals. However, most studies have shown that signs of later stage neurodegeneration such as such as aggregations of the hyper phosphorylated tau referred to as neurofibrillary tangles (NFTs), are not usually seen. [8] This highlights the possibility that phosphorylated tau (p-tau) plays a role in the development of long-term outcomes after TBI, such as chronic traumatic encephalopathy and Alzheimer's disease. Quantifying the levels of p-tau after TBI can provide us with valuable information about the secondary effects of injury and may lead to elucidation of the pathways that cause long-term problems.

**Glial Fibrillary Acidic Protein:**

In additional to the importance of neurons in the brain, the support glial cells are vital to proper functioning. Astrocytes generally work to maintain homeostasis in the brain, and are known to play an important role in the response to TBI. [138] After TBI, astrocytes are activated and function to repair the damage that has been induced. While the actions of astrocytes at baseline tend to be beneficial, after injury the prolonged activation of astrocytes can lead to inflammation and secondary injury processes. In particular, the formation of a glial scar can impair neuronal regeneration and lead to worse long-term outcomes. [139]

Glial Fibrillary Acidic Protein (GFAP) is an intermediate filament expressed in astrocytes. After TBI, GFAP in astrocytes is upregulated in relation to the degree of injury. [140] Because of this, GFAP staining is often used as a way to assess the severity of the injury. While an initial increase in GFAP might be seen as a positive factor in recovery due to the beneficial effects short-term activated astrocytes can have, it becomes a large barrier for recovery when it is prolonged and starts to contribute to the secondary injury process. Quantifying the levels of activated astrocytes using GFAP staining is an established method that allows for a standardized assessment of injury severity. This method can be useful when evaluating if interventions have the therapeutic potential to limit the detrimental effects of increased reactive astrocytes due to injury. [141]

**Ionized Calcium-Binding Adapter Molecule 1**

The neuroimmune system plays an important role in the balance between recovery and secondary injury after TBI. Comparable to the activation of astrocytes after injury, there is activation of the resident neuroimmune cells in the brain, microglia. [142] Microglia are

specialized neuroimmune cells, which have both phagocytotic and antigen presenting abilities. At baseline, they are in a resting, or ramified form. [143] Along with astrocytes, microglia are responsible for initiating the inflammatory response after injury at which point they become activated. And similarly to the role of activated astrocytes after injury, while the initial response may be protective, if the activated microglia persist over time they will contribute to secondary injury processes and worse long-term outcomes. [142]

Ionized Calcium-Binding Adapter Molecule 1 (IBA-1) is a factor that is specifically found in microglia in the brain. After injury, IBA-1 expression is upregulated in microglia. [144] Because of this, staining for IBA-1 is a useful measure in order to determine the extent of the neuroinflammatory process that has been activated. In addition, by performing immunohistochemical staining with IBA-1, we can determine if the microglial cell is in a resting or activated state by morphology. By using this method, we can determine the extent of the microglia activation which may have important ramifications for long-term outcomes after injury.

## Behavioral

After TBI in humans there are often cognitive effects that can range from mild to severe in nature. These include deficits in visuospatial learning, working memory, and executive function. [145, 146] In order to test these functions in animal models, researchers have developed specific methods to test spatial learning and memory, recognition memory, fear learning and memory, as well as anxiety. In addition to cognitive effects, there are also sensorimotor deficits seen after TBI. [26, 111] A number of tests have been developed to examine these abilities in animals as well as described below.

**Morris Water Maze**

The Morris Water Maze (MWM) is one of the most reliable and valid tasks that has been developed to test spatial learning and memory in rodents. [147] Originally developed for rats, it has been since modified for mice. [148-150] While the MWM paradigm is called a maze, it is not a maze in the traditional sense. The testing arena consists of a plain circular pool filled with opaque water. The maze is split into four quadrants using imaginary bisecting lines to create northwest, southwest, southeast, and northeast quadrants. A hidden platform is placed in one of the quadrants, traditionally the northeast quadrant. The animals rely on hippocampal-dependent learning and memory to find and remember the spatial location of the hidden platform. In the most common format of the test, extramaze cues allow the animal to use spatial mapping ability to find the platform. There is typically a learning phase of three to six days where the animal gradually learns where the platform is located and decreases its latency to find the platform over time. In the learning phase, the animal is subjected to four to eight trials a day, with pseudo-random start placement alternating between the four different quadrants. Following the learning phase, a probe trial is often done to test memory. In the probe trial, the hidden platform is removed, and the animals' memory of where the platform used to be is measured by the time it spends swimming in the northeast quadrant. The probe trial may be done at the end of the learning phase to test short term memory, as well as at later time points to test long-term memory. Due to the fact that the probe trial is an extinction trial, caution must be taken not to repeat it an excessive number of times.

While hippocampal dependent learning has been shown to be vitally important to complete learning and memory tasks such as this one, studies have shown that injuries to other

regions of the brain can also influence performance in the MWM tasks. These regions include the cortex, the cerebellum, the striatum, and the basal forebrain. [147] This highlights the potential role for the coordinated actions of different brain regions in order for animals to effectively use spatial mapping in learning and memory.

While the MWM task has been shown to be a useful test for spatial learning and memory in rodents, it also has some shortcomings, particularly in respect to the testing of mice. Mice have a higher rate of so called "non-performers", wherein some mice will refuse to swim and simply float for the duration of the trials. Other mice make no effort to find the platform, but simply swim around the edge of the pool in large circles for the duration of the trials. These mice have the ability to confound the studies and their outcome data need to be dealt with appropriately if determined to be an outlier. [149] In addition, although most land-based minor motor deficits have not been shown to have a crossover effect on swim ability or speed in animals [149, 151, 152], there is a possibility that severe motor deficits that impact swim ability may affect the outcomes of the MWM task. These limitations must be taken into account when designing an experiment that uses that MWM paradigm.

**Novel Object Recognition Test**

The Novel Object Recognition (NOR) Test is a newer behavioral test that investigates recognition learning and memory in animals. Animals have a natural curiosity for novel objects and will tend to spend more time exploring a new item compared to a familiar object. This task was developed as a way to test learning and memory in animals in a way that utilizes their innate exploratory behavior in a low stress environment, thereby decreasing the confounding factors that may affect other behavioral tests. [153, 154] This

paradigm requires that the animal uses recognition learning and memory to remember the familiar object they have been previously exposed to.

This task consists of three phases; habituation, familiarization, and test. While there is significant variation in the timing and methods across different laboratories that employ the test, the general components remain the same. In the habituation phase, the animal is placed in an empty open field arena and allowed to explore for a set amount of time. Then, in the familiarization phase, the animal is introduced to two of the same objects in the open field arena and allowed to interact with them. This phase can be repeated over the course of a few days to ensure memory retention of the familiar objects. Finally, in the test phase, the animal is introduced to one familiar object and one novel object in the open field arena. The time spent with the novel object is compared to the time spent with the familiar object and can be recorded by metrics such as the discrimination index or preference index.

While this paradigm is certainly promising with its ability to test learning and memory in a manner that does not rely upon stress, fear, or reward, it also suffers from some problematic aspects. There is considerable variability in the manner in which testing is done, making it difficult to compare results across laboratories. In particular, the set up has been known to widely vary in the types of objects used, the arena conditions, and room lighting conditions. All of these factors have been shown to have an effect on animal behavior and may be confounding factors in the test. In particular, care must be taken in the selection of objects, in order to make sure that the animals are able to distinguish the objects are different, but to also ensure that they do not display a natural preference for one object over another. For example, coffee mugs are a common item used for this test. [155] However, in the testing phase of our NOR paradigm set up, our lab has found that the

animals displayed a natural preference for the handle of the mug which confounded our data analysis. The NOR paradigm has the potential to be a powerful method to analyzing learning and memory in animals, however it is particularly fastidious, and must be set up and run with care and attention to detail.

**Rotarod**

The rotarod (RR) test is used to test vestibulomotor, sensorimotor, and gross motor function. [111] This paradigm uses an accelerating rod that generally starts rotating at 4 rpm and accelerates at a rate of 20 rpm/min. The mouse is placed on the rotating rod and allowed to adjust to the movement for 10 seconds. If the mouse falls off during the time, it is picked up and placed back on the rod. After 10 seconds, the acceleration begins, and the latency to fall is measured. [156]

The rotarod test was developed as an assay to test motor ability in an objective automated manner that is reproducible across studies. It is able to detect motor differences in transgenic animals, animals that have been subjected to different disease states models such as Parkinson's disease or traumatic brain injury, or animals that have been dosed with drugs. [157] While generally a robust test of gross motor skills, there are a number of factors that can confound the data. Mice that are placed on the rod may fall off it immediately and fail to complete this test. If this occurs repeatedly, the animal will need to be excluded from the study. There are also some animals which will fall off the rod but remain clinging to it. This can be avoided by choosing appropriate materials for the rod. Finally, weight can act as a confounding factor in this study. Animals that are heavier have a harder time with this test, and this may skew the data, especially in the case of transgenic mice with weight difference and studies that use both male and female mice with

significant weight discrepancies. [157] Care should be taken to minimize these confounding factors when running the rotarod test.

**Balance Beam**

The balance beam test is used to assay balance and fine motor skills. [156, 157] In this test, a mouse is trained to walk across a flat narrow beam to a safe platform at the end. There are different modifications that can be made to make the task simpler or more difficult as needed. In order to determine balance and motor coordination, after training, a test day is conducted. On the test day, the time that it takes to cross the beam is recorded, as well as the number of falls, and the number of paw faults. This data is generally put into an algorithm deemed "Balance Beam Score", with higher scores reflecting worse fine motor ability.

The balance beam assay is a more sensitive assay of motor ability than the previously mentioned rotarod test is. The quantitation of these three metrics of motor ability allows for the detection of more subtle defects in balance and can be a valuable tool in the assessment of animal motor ability. However, due to the fact that this test requires a human observer, the scoring is of a subjective nature. Observers must be trained to be as reliable as possible, in order to ensure that the assay is replicable. Other confounding factors include the fact that mice must have trained to walk across the beam prior to test day. If significant training is not done, the mice may turn around on the beam mid trial or fail to complete the test for reasons not related to motor ability. If too much training is done, differences between groups may be eliminated. For these reasons, care must be taken when utilizing the balance beam test, and all efforts should be made to standardize the test across observers.

## Personalized Therapy for TBI

In 2015, President Obama announced the launch of the Precision Medicine Initiative. With this call to action, the National Institute of Health undertook the conduction of large-scale research efforts using precision medicine, defined as, "An emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person." [158]

As researchers continue to discover new things every day, one important lesson we are learning is how complex nature is. It is becoming evident that the solution to the multifaceted, complex problems that we study will not be solved with one simple broad tool, but will most likely require a nuanced understanding of the specific underlying factors that may vary from person to person. For this reason, we seek to understand disease processes at their cellular and genetic level, so that we can attempt to develop precision or personalized therapies for individuals based on their specific needs.

In this project, I specifically examined the role that different genetic polymorphisms have on outcomes after repeated mild TBI. In order to do this, I investigated the cellular responses to repeated mild TBI in different genetic polymorphisms, and where I saw differences, I hypothesized about the causes of these differences. In an attempt to initiate research into the development of personalized therapies for TBI, I explored three potential personalized treatments based on the differences in genotypic responses. While personalized approaches have been used in cancer treatment, there are currently no

approved personalized treatments for TBI. This translational study is one of the first to investigate using personalized medicine to treat TBI.

**BDNF-Adeno Associated Virus**

In BDNF Val66Met polymorphic mice, I found that in vulnerable genotypes, mature BDNF levels were decreased. In order to investigate whether targeted treatment of an increasing BDNF levels could rescue this vulnerability, I used an Adeno-Associated Virus (AAV) vector which overexpressed BDNF. Previous studies have found that BDNF neuropeptide mimetics can have positive effects after both spinal cord injury and TBI. [32, 35, 159] However, the effect of mimetics is transient, and requires continued administration of the drug in order to see long lasting benefits. The advantage of using the AAV-BDNF is that after the initial treatment there is persistent expression.

AAV is a small virus which can infect human cells and remain in an extrachromosomal state or integrate into the host cell genome. In this way, the AAV vector can use the host cell machinery to express the protein of interest encoded in it. [160] AAV vector therapy has emerged as a promising way to deliver precision medicine therapies [161] however whether BDNF overexpression in a specific genotype is effective has not yet been explored.

**Bryostatin-1**

Previous studies have shown that different ApoE genetic polymorphisms have altered secretion of BDNF at baseline. [99] In my studies, I have shown that they also have differential expression of BDNF after injury. Given this, another precision medicine

method of treatment could potentially be to increase endogenous levels of BDNF by activating the cellular pathways upstream of BDNF. Bryostatin 1 was first investigated as an anti-cancer drug, and was approved for use in clinical trials before being abandoned due to lack of efficacy. [162]

Interestingly researchers found that while high doses of Bryostatin 1 were useful in preclinical cancer trials, low doses seemed to be helpful for cognition. Bryostatin 1 is now currently in Phase II clinical trials for Alzheimer's disease (AD) treatment. [163] It functions as a protein kinase C epsilon (PKCε) activator, and has been shown to reverse synaptic loss in AD. Recently, it has begun to be investigated as a treatment for TBI, where it has been shown to improve blood brain barrier integrity after a blast induced TBI model in rats [164] however its role in repeated mild TBI and the mechanism by which it works is unknown.

Bryostatin 1 shows promise as a treatment for TBI due to its activation of PKCε. Interestingly, studies have shown that ApoE isoforms have differential effects on PKCε, which in turn can decrease BDNF levels in the vulnerable genotype. [100] By treating the vulnerable genotype with the PKCε activator Bryostatin 1, we may be able to develop a personalized therapy for TBI in carriers of this genotype.

**Encapsulated Human Mesenchymal Stem Cells**

Finally, another potential personalized treatment method for TBI is the use of human mesenchymal stem cells. MSCs are a promising potential therapy because they are able to react to their environment and stimulate it in positive ways. MSCs secrete a range of

cytokines and growth factors that can help repair after injury, and their secretome is modulated by their local microenvironment. [165, 166] For example, MSCs that have been transplanted into AD model brains were able to promote endogenous growth by their secretion of growth factors and cytokines. They decreased apoptosis, free radicals, and inflammation and promoted recovery. [167]

Initial studies investigating the use of MSCs as treatment for TBI have been encouraging. [168] MSCs that are injected after injury are able to migrate to the site of injury. [169] After they reach the site of injury, they have immunosuppressive effects which reduce the inflammatory secondary injury processes [170, 171] and facilitate tissue regeneration. [172, 173]

MSCs can ameliorate injury due to their cell replacement effect or due to their beneficial secretome. In addition, they have been shown to increase levels of neurotrophic factors such as BDNF after injury. [174, 175] Most studies where naked MSCs are injected into the brain show that they differentiate into both neurons and glia. [176] One potential downside of the use of MSCs is that the site of injury seems to induce MSCs to develop in astrocytes, which may contribute to the detrimental glial scarring seen after injury. This has led researchers to attempt to use the secretome of MSCs without implanting the cells themselves. [177]

In addition to the baseline beneficial effects that MSCs have, research is being done into ways to stimulate MSCs to secrete specific factors based on the disease state and need. [178] For example, the Yarmush lab at Rutgers University has been working on

encapsulating MSCs with alginate to increase the time that they stay viable, to influence their secretome to be a beneficial one, and to limit their movement from the site of injury. [179] Encapsulated MSCs have been shown to improve outcomes in spinal cord injury [180], and are beginning to be tested in TBI. [181] Some labs have embedded MSCs in hydrogel materials with or without other factors, such as BDNF peptide mimetics as a treatment for TBI. [182] If it is shown that vulnerable genotypes have altered levels of BDNF after TBI, MSCs may be provide a personalized treatment by secreting beneficial neurotropic factors, such BDNF.

## Importance of Mouse Model Studies for Translational Research:

Mouse studies allow us to test the effect of genetic polymorphisms in a much more controlled and precise manner than human studies. [183] They allow for the manipulation of variables, the direct study of the affected brain tissue, and the ability to give experimental treatments and determine the effects that it has on the disease state. For these reasons, doing these experiments in mouse studies is vital to advance our knowledge of the mechanisms underlying differential genetic vulnerability to TBI and treatment approaches.

## Hypothesis

In this study, I hypothesize that mice with the Val66Met or ApoE4 genotype will have an exacerbated response to repeated mild TBI relative to their Val66Val and ApoE3 counterparts, due to alterations in BDNF levels, and that genetically susceptible mice will show improved recovery following treatment with a source of trophic factors.

# Chapter 2: Materials and Methods

## Animals

### BDNF

Adult male and female mice aged 10-12 weeks were used in all studies. BDNF mice were generously provided by Dr. Francis S. Lee of Weil Cornell Medical College.[184] The mice were created utilizing a targeting vector with or without the point mutation (G196A) which is regulated by the endogenous mouse BDNF promoter. The colony was maintained by crossing $BDNF^{Val/Met}$ mice to each other, which yield offspring at Mendelian rates. Mice were housed in a 12 h light/dark cycle with food and water available ad libitum. All procedures described were performed in accordance to the NIH guidelines and were approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). A power analysis was used to determine the appropriate sample size for experiments to reach 80% power; for histology the group size n=5-8, for biochemistry the group size n=3-4, and for behavioral tasks n=8-12 were used to reliability detect changes of the magnitude we are examining ($\alpha = 0.05$) based on the difference seen between experimental groups in our previous publication. [26]

### ApoE

Adult male and female mice aged 10-12 weeks were used in all studies. ApoE mice were generously provided by Patrick Sullivan of Duke University. These targeted replacement mice were developed by Nobuya Maeda at the University of North Carolina by targeting the murine ApoE gene for replacement with the human APOE4/APOE3 allele. [185]

Resultant chimeras were backcrossed to C57BL/6 and the colony was maintained through mating of homozygotes. Mice were housed in a 12 h light/dark cycle with food and water available ad libitum. All procedures described were performed in accordance to the NIH guidelines and were approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). A power analysis was used to determine the appropriate sample size for experiments to reach 80% power; for histology the group size n=5-8, for biochemistry the group size n=3-4, and for behavioral tasks n=8-12 were used to reliability detect changes of the magnitude we are examining ($\alpha = 0.05$) based on the difference seen between experimental groups in our previous publication. [26]

**p75**

All studies employed male mice ages 10-12 weeks. Wild type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used for studies exploring receptor and ligand expression as well as pharmacological studies. B6.129-Ngfr$^{tm1Jae}$/J mice (Jackson Laboratories, Bar Harbor, ME) that had been backcrossed to C57BL/6J inbred mice were used for studies involving p75 mutant mice and their WT controls. p75 mutant mice have been reported to display abnormal sensory neuron innervation resulting in reduced sensitivity to heat and ulcers on their toes by 4 months [186], however since we used the mice prior to this age, we did not observe any phenotype. The procedures described were conducted in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee.

## Lateral Fluid Percussion Injury

Lateral fluid percussion injury uses a rapid fluid pulse to cause injury to the brain by the displacement of neural tissue. This process has previously been described in detail [111] but has been modified to create repeated mild injury. Briefly, mice were anesthetized using 4-5% isoflurane in 100% $O_2$ and maintained on 2% isoflurane throughout the procedure. They were placed in a stereotaxic frame, and a trephine-guide 3 mm plastic disc was attached with Loctite glue (444 Tak Pak, Henkel Corporation, Rocky Hill, CT). It was placed on the skull, halfway between lambda and bregma, laterally on the right hemisphere. A trephine (3 mm outer diameter) was used to perform a craniectomy. A rigid Luer-loc needle hub (3 mm inside diameter) was secured onto the skull over the opening that was made using cyanoacrylate adhesive and dental acrylic (Henry Schein, Dublin, OH). After a 60 min recovery period, the animals were re-anesthetized and connected to the fluid percussion injury device (Custom Design and Fabrication, Virginia Commonwealth University) through the Luer-loc hub.

For mTBI: Once the animals regained normal breathing, before sensitivity to stimulation, a ~0.8 ATM pulse (15 msec) was generated through the LFP device to strike the intact dura of the brain. Upon return of righting reflex (<4 minutes for mild injury) the hub was filled with saline and capped. 48 hours from the initial injury, a second injury was given. This occurred again at 96 hours from the initial injury. This experimental timeline was chosen based on previous studies which have sought to mimic human repeated mild TBIs in a mouse model which controls for the rodent life span. [94, 187-189] After the final injury, the hub and dental acrylic were removed and the scalp incision was closed with 3M Vetbond (Fisher Scientific, Waltham, MA). The animals were individually housed after

the injury and returned to normal housing conditions. At this repeated, mild level of injury, about 5% of animals died after the 3rd injury in the chronic post-traumatic period. This is a normal and anticipated feature of the LFP TBI model because it mimics human TBI. Mice that underwent the surgical procedure but not the injury were used as sham controls. Assignment of the mice to the LFP or sham group was randomized.

For single moderate TBI: Once a normal breathing pattern resumed, before sensitivity to stimulation, a ~1.5 ATM pulse (~15 msec) was generated through the LFP device. Upon return of righting reflex (4-10 minutes for moderate injury) the hub and dental acrylic were removed. The scalp incision was sealed with 3M Vetbond (Fisher Scientific, Waltham, MA) and the animals were returned to normal housing conditions. At this moderate level of injury, ~10% of animals died as a result of the injury within the acute post-traumatic period (15 min), generally from respiratory failure and pulmonary edema [190]. This is a normal and anticipated feature of the TBI model because it mimics human TBI [191]. Mice that undergo the surgical procedures but that were uninjured served as the sham controls. Assignment of mice to LFP or sham group was done in a random manner.

## MRI Imaging

Magnetic resonance imaging (MRI) was done in order to assess the injury volume as determined by increased relative intensity (ROI). The scans were done utilizing a fast spin echo sequence with a mouse brain coil. Scans were done in the axial position at 1, 7 and 21 days after the final injury. The region of interest was recorded as areas of higher intensity in the damaged location of the brain. Scans were done at the Rutgers University Molecular Imaging Center with the center's M2 Compact High-Performance MRI (1T).

## Immunohistochemistry

- To collect tissue for immunohistochemistry, mice were perfused with 0.9% saline, followed by 4% paraformaldehyde at 1 and 21 days after the final injury. After perfusion, the brains were cryoprotected with 30% sucrose for at least 3 days. Sectioning was done in 20 μm thick slices, in a 1:10 series throughout the length of the hippocampus, incorporating the area around the site of injury in the cortex. To measure apoptotic cell death, sections were pretreated with 0.01M Citrate buffer at 90$^{o}$ C. Anti-cleaved caspase-3 (1:1000, 9661, Cell Signaling, Danvers, ME) was then applied overnight, followed by Alexa Fluor 594 goat anti-rabbit (1:1000, Invitrogen, Waltham, MA). To measure astrogliosis, Glial Fibrillary Acidic Protein (GFAP) antibody was applied overnight (1:500, MAB3402, Millipore, Billerica, MA), followed by Alexa Fluor goat anti-mouse 488 (1:500, Invitrogen, Waltham, MA). To measure neuronal degeneration, sections were first treated with 1% NaOH and 0.06% $KMnO_4$, then 0.0005% Fluoro-Jade C (AG325, Millipore, Burlington MA)/0.0001% DAPI (D9564, Sigma, St Louis, MO) was applied for 20min. To measure microglial activation, IBA1 antibody was applied overnight (1:10,000, 019-19741, Wako Labs, Richmond, VA), followed by Alexa Flour goat anti-rabbit 488 (1:1000, Invitrogen, Waltham, MA). To measure levels of phosphorylated tau, AT8 antibody was applied overnight (1:500, MN1020, Pierce Antibodies, Waltham, MA), followed by Alexa Fluor goat anti-mouse 488 (1:1000, Invitrogen, Waltham, MA). All slides were incubated in 4',6-diamidino-2-phenylindole (DAPI) (1:1000 DAPI in PBS, Sigma, St Louis, MO). For double immunohistochemistry, anti-cleaved Caspase-3 (1:1000 9661, Cell Signaling, Danvers, ME) or p75 (1:1500 8232, Cell Signaling, Danvers, ME) antibody was applied overnight followed by secondary goat anti-rabbit 594 (1:250). Then NeuN (1:200 MAB377, Millipore, Billerica, MA) or GFAP (1:500 MAB3402, Millipore, Billerica, MA) antibody was applied overnight followed by secondary goat anti-mouse 488 (1:250). DAPI

(1:1000, Sigma, St. Louis, MO) was used to label nuclei and staining was visualized at 63X using a z stack on a LSM 510 Meta Zeiss Confocal. Slides were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL), except for the Flouro-Jade C slides which were mounted in DPX Mountant (44581, Sigma, St Louis, MO). Visualization of the fluorescent stains was done using a Leica microscope (Model DMIRB, Leica Microsystems, Buffalo Grove, IL). Five to eight animals per time point and treatment were analyzed. Sectioning of tissue was done using a Cryostat (Leica), and collected coronally in 1:10 series throughout length of the hippocampus. For each biological replicate, 6-12 sections of brain were counted.

Positive cells were counted in the hemisphere ipsilateral to the injury. In the cortex, for each section, six fields of 40X view (starting at the dorsal midline and moving laterally for three fields of vision, and then the three fields of vision just ventral to the first three) were counted. In the hippocampus, the dentate gyrus as well as the CA1-CA3 was used for quantitation of cells. Analysis was performed blind to experimental group and genotype.

## Vestibular Rotarod Test

In order to study the vestibular motor abilities of the mice after LFP, the rotarod test was conducted. The rotarod test utilized a 36-mm outer diameter, rotating rod whose velocity increased from 4 to 40 rpm over a maximum 180 sec interval. Balance and motor function were measured using the latency to fall. Each trial ended when the animal fell off the rotarod. Eight to ten mice per genotype and condition were used. Acclimation and baseline analysis was done one day prior to the first injury, using three trials separated by a one hour inter-trial rest phase. At 1, 7, and 21 days after the last injury, each mouse underwent three

trials separated by a one hour inter-trial rest phase. The same mice were used for each time point. The average latency to fall was compared between injured and sham groups.

## Balance Beam Test

In order to study fine motor function, the balance beam test was conducted. The beam apparatus consists of a 1 m long flat beam with a width of 20 mm, raised 30 cm above the table surface. A black box was placed at one end of the beam as the finish point. The mice were pretested on the beam apparatus for four days before the test day for training and baseline measurements. On test day, the mice were observed crossing the beam while the number of paw faults, falls, and relative time to cross were recorded manually. Mice were tested at 7 and 21 DPI, and the same mice were used for each time point. Eight to ten per genotype and condition were used. Values were imputed into a predetermined scale to evaluate outcomes with weighted values for the different traits analyzed in order to account for the severity of injury indicated by each. A score of 1 was standard for all mice, the number of falls was added after being multiplied by 2, the number of foot faults were added, and if the mouse crossed the beam in under 5 seconds, a score of 1 was removed from the final score. Analysis was done blinded to condition.

## Novel Object Recognition Test

In order to study recognition memory, the novel object recognition test was conducted. The task was conducted in an open field arena (100 cm x 100 cm) with opaque walls. The task was composed of a habituation phase, a familiarization phase, and a testing phase. During the habituation phase, the mice were allowed to explore the empty open field arena for 5 minutes. Twenty-four hours after the habituation phase concluded, the familiarization

phase began. In the familiarization phase, there were four familiarization trials separated by twenty-four hours, where the animals were exposed to two identical objects in the same open field arena. The mice were allowed to explore the objects in the arena for five minutes. The total time that the nose-point was in the object interaction zone, a 3 cm border around the object, was recorded. The testing phase began an hour after the last familiarization trial concluded. In the testing phase, one of the familiar objects was replaced by a novel object of roughly the same height and volume, but with a different visual appearance and shape. The time spent interacting with the familiar and novel object were recorded for each object. The data collected was used to compute the preference index (time spent with the novel object over the total time spent exploring objects) and the total distance traveled. Eight to ten per genotype and condition were used. Data was recorded using a video-tracking system (EthoVision XT; Noldus Information Technology, Leesburg, VA).

## Morris Water Maze Test

In order to study spatial memory, the Morris Water Maze (MWM) test was utilized. Mice were acclimated to the paradigm and tested for baseline response using a visible platform test three days prior to the start of the injury paradigm. The animals were placed in a circular pool (1 m diameter), filled with opaque water containing non-toxic white paint and a clear escape platform marked by a visible rod. To assess learning, the mice were tested using a hidden platform fixed in the northwest quadrant starting one day after the last injury. The testing was conducted using four trials a day for six days in a row. For the p75-/- studies mice were subjected to 8 trials/day for 3 consecutive days starting at either 1 or 21 days post injury (a separate set of mice was used for each of the two time points). On the seventh day, a probe test was completed to test memory, where the hidden platform

was removed, and the time spent exploring the northwest quadrant was recorded. Black and white distal extra-maze cues were placed on the walls of the room, and geometric shaped proximal extra-maze cues were placed above the walls of the maze. The mice were placed in pseudo-randomly varied quadrants throughout testing, and the time to locate the platform was recorded. Trials were run until the mouse found the platform or was placed there after the maximum trial time of 60 seconds. At the conclusion of the trial, the mouse was kept on the hidden platform for 15 seconds to consolidate learning, followed by removal from the pool and placement onto a heating pad for 10 minutes. Eight to ten mice per group and condition were used. Data was recorded using a video-tracking system (EthoVision XT; Noldus Information Technology, Leesburg, VA).

## Western Blot Analysis

The cortex and hippocampus on the ipsilateral side to the injury site were collected from mice at 1 and 21 dpi and flash frozen. Four mice per group and condition were analyzed at each timepoint. Tissue lysates were prepared using T-PER with protease inhibitors and EDTA (Pierce, Rockford, IL). Samples were homogenized for 30 seconds, and then centrifuged for 10 minutes. The protein content of the supernatant was determined using the bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce, Rockford, IL). Equal amounts of protein were loaded onto Bis Tris Gels. (Invitrogen, Grand Island, NY). The proteins were transferred onto polyvinylidene difluoride (PVDF)-filter Immobilon-P transfer membranes (Millipore, Billerica, MA). Following blocking in 5% BSA + 5% normal donkey serum overnight at 4°C, the primary antibody was applied overnight at 4°C. 40 μg of protein was run on a 12% Bis Tris gel and probed for pro and mature BDNF (1:500 BDNF Icosagen). p75, trkB and proNGF expression were determined by loading

40µg of protein on a 4-12% Tris-Glycine gel and probing with p75 (1:200 sc-8317, Santa Cruz, Biotechnology, Inc., Santa Cruz, CA), trkB (1:400 sc-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or proNGF (1:200 AN-005, Alomone Labs, Jerusalem, Israel) antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000, Biodesign, Saco, ME) was used as a loading control. Secondary anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated IgG antibodies were used (1:5000, GE Healthcare, South Plainfield, NJ). GAPDH protein was visualized by chemiluminescence using the Enhanced Chemiluminescence (ECL) detection kit (Perkin Elmer, Waltham, MA) and all others were visualized using the SuperSignal West Femto Maximum Sensitivity Substance (ThermoFisher Scientific). Levels of the immunopositive bands were quantified densitometrically using Quantity One version 4.2.1 software on a GelDoc 2000 (Bio-Rad, Hercules, CA). All data is normalized to the sample's own GAPDH and expressed as a fold change relative to the average of the time matched sham controls.

## BDNF Virus Infusions

An AAV9-CMV-GFP-2A-mouseBDNF construct at a titer of 4.5 $X10^{13}$ viral genomes/µL was purchased from Vector BioLabs (Malvern, PA). Mice were anesthetized using 4-5% isoflurane in 100% $O_2$ and maintained on 2% isoflurane throughout the procedure. They were placed in a stereotaxic frame, and a 32 G Hamilton Neuro syringe was used to deliver a volume of 0.75 µL at a speed of 0.25 µL/min into both the ipsilateral cortex (AP -1.9 mm, ML, -1.5 mm, DV -1.5 mm) and hippocampus (AP -1.9 mm, ML, -1.5 mm, DV -2.5 mm) of animals 5 minutes after the final LFP injury. The needle was left in place after injection for 5 minutes to allow from completion infusion of the drug. The control group received

the same injection protocol with a control AAV-CMV-GFP construct 4.5 $X10^{13}$ viral genomes/μL purchased from Vector BioLabs.

## Bryostatin Injections

Bryostatin 1 was purchased as a powder from Enzo Life Sciences (Farmingdale, NY) and was reconstituted in 100% ethanol (EtOH); 1 μg of drug was dissolved in 20 μL of EtOH to make the bryostatin 1 stock solution. The stock solution was further diluted 1:20 in PBS to make the final solution. For the 1 DPI timepoint, 5 minutes after the final injury, mice were given a dose of 20 ug/kg intraperitoneal (i.p.) injection of the bryostatin 1 solution or control PBS. 24 hours after the first injection, mice were given a second dose of 20 ug/kg i.p. injection of either bryostatin 1 or PBS. 3 hours after the second injection, mice underwent the perfusion or tissue collection protocol. For the 7 DPI timepoint, mice were given 5 doses of 20 ug/kg every 2 days beginning 5 minutes after that last injury. For the 21 DPI timepoint, a $6^{th}$ dose of 20 ug/kg was given at 20 DPI.

## Mesenchymal Stromal Cell Culture

Human mesenchymal stromal cells (MSCs) were purchased from the Institute for Regenerative Medicine (Texas A&M College of Medicine) at passage 1. Cells were cultured with Minimum Essential Medium α (αMEM) without deoxy- or ribonucelosides, supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1 ng/ml basic fibroblast growth factor (bFGF) (Gibco, Waltham MA), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). MSCs were plated at 5000 cells per $cm^2$ and allowed to proliferate to 70% confluence. MSCs were used in experiments at passages 2 through 5.

## Mesenchymal Stromal Cell Alginate Encapsulation

For assessment of encapsulated MSC secretion of BDNF, alginate-MSC microspheres 450-550 μm in diameter were created with an electrostatic bead generator using a conical encapsulator needle with an inner diameter of 0.35 mm (Nisco Engineering, Zurich, Switzerland). For *in vivo* studies, alginate-MSC microspheres 220-380 μm in diameter were generated using a conical encapsulator needle with an inner diameter of 0.17 mm (Nisco). Capsules were made with 25-75 cells per small capsule and 150-300 cells per large capsule, and experiments were conducted with $4\times10^6$ cells per ml. The capsules were allowed to rest in a crosslinking solution of 100 mM calcium chloride, 145 mM sodium chloride, and 10 mM MOPS in deionized water at a final pH of 7.2. The capsules were washed with PBS, and coated with poly-l-lysine (PLL) at 0.05% w/v, washed again with PBS, and suspended in fully supplemented α-MEM in a T25 flask. Viability of cells in the capsules was assessed with a DAPI, calcein acetomethoxy (AM) (live) and ethidium homodimer (dead) staining, which was visualized using an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan). Cells were counted using SlideBook image analysis software version 5.0 (Intelligent Imaging Innovations, Denver, CO).

## Encapsulated Mesenchymal Stromal Cell Injections

Using a stereotactic surgery set-up, encapsulated MSCs were injected ICV into the ipsilateral ventricle following injury. Coordinates used were anterior–posterior − 0.1 mm with respect to bregma; lateral, ± 1.0 mm; ventral, − 3.0 mm with respect to the surface of the skull. A 50uL Hamilton syringe with an inner barrel diameter of 1.03mm was used with a 20G needle that had an inner diameter of 0.5mm. For each animal, in order to reach a therapeutic dose, we injected $2 \times 10^3$ capsules in 25 uL for a total of $1 \times 10^5$ MSCs/mouse.

Injections were done at a speed of 1 μL/min, and after injection the syringe remained in place for 5 minutes to allow for total infusion.

## Encapsulated Mesenchymal Stromal Cell Imaging

Optical-fluorescent scans were conducted using two ranges of Emissions/Excitations: 480/700 and 520/600. Bright field and X-ray images were also captured so that the optical images could be overlaid. Scans were conducted on control, uninjured mice that were injected intracerebroventricularly with approximately $2x10^3$ capsules containing 6 mg/ml ChMNP. The scans were done using the FX-PRO at the Rutgers Molecular Imaging Center.

## Statistical Analysis

StatPlus software was used for all data analysis. Groups were compared using Student's two-tailed t-test or one-way ANOVA followed by Fisher's PLSD post-hoc analysis. $p<0.05$ is considered statistically significant.

# Chapter 3: BDNF Val66Met Genetic Polymorphism

## Introduction:

Traumatic Brain Injury (TBI) is a serious and potentially life-threatening clinical problem. It occurs when there is a force to the head, which results in a disruption of brain function. In 2013, there were 2.5 million TBIs in the United States, 50,000 of which led to death and 70,000 of which led to permanent neurological damage. [1] Based on the neurological symptoms that occur after a TBI, the injury is classified either as mild, moderate, or severe. Mild TBI is the most prevalent form of TBI that occurs in the United States, and in particular, athletes and military personnel tend to suffer from mild and repeated traumatic brain injuries. [3, 4] While the majority of patients who suffer from mild TBI tend to recover over time, it is estimated that around 15% of patients have symptoms that last longer than three months and develop into chronic disabilities. [5] This problem is only exacerbated when a person is subjected to repeated mild TBI (rmTBI). It is becoming more evident that some athletes and military personnel who have suffered from mild repeated brain injuries can end up developing neurodegenerative diseases. [6] TBI should be thought of as an ongoing disease process, rather than a discrete event. The primary injury occurs as the result of mechanical force to the brain. After the initial insult, a secondary injury process occurs as a result of inflammation and secondary mediators. While some patients who suffer from TBI recover quickly and have no obvious long-term symptoms, other patients undergo a prolonged secondary injury phase and have a much harder time recovering. [9, 10] One of the reasons that not all individuals respond similarly to rmTBI may be the genetic differences that exist in the population, however specific genes that may affect outcomes after rmTBI have not been identified.

A critical neuronal gene that contains a single nucleotide polymorphism (SNP) is *Brain–derived neurotrophic factor* (*BDNF*), a neurotrophin that plays a role in neuronal survival and synaptic plasticity.[14-16] *BDNF* has a SNP site, rs6265, at the 66 amino acid position of the BDNF protein, and results in the wildtype Val being replaced with a Met. The Val66Met genotype has been reported to be associated with poor outcomes in a number of disease states such as major depressive disorder [36], anxiety [192], stroke [43], and Alzheimer's disease [44], however clinical research in TBI patients is not consistent with whether the Met or the Val polymorphism confers worse outcomes. [28, 45-50] The Val66Met polymorphism results in diminished BDNF in dendrites and reduced amounts of BDNF released into the synapse upon stimulation. [52] [53] In healthy populations, individuals with the Val66Met polymorphism have been shown to have impaired cognitive function. [38] It seems to follow logically that the Val66Met polymorphism has been shown to result in worse neurocognitive performance after TBI [50], as well as being a risk factor for TBI in combat forces[47-49]. However, some recent studies in humans have shown that counterintuitively, after injury, the Val66Val carriers actually have worse recovery compared to the Val66Met carriers. [28, 45, 46, 54] In long-term studies of combat veterans, carriers of the Val66Met polymorphism had recovery of executive function back to baseline levels while the Val66Val carriers did not. [45, 46] On the other hand, other studies have found no effect of the polymorphism influencing outcomes after injury. [55-57] Thus, the clinical research as to which BDNF allele is the risk factor for outcomes after TBI is not all in agreement, and could benefit from a bottom-up, controlled experimental research study as has been done to study other disease models where genetic polymorphisms may play a role.[67]

The story is complicated by the fact that the BDNF protein has two important and varied forms, the pro form and the mature form. Mature BDNF binds to its receptor trkB and stimulates neurogenesis and cell survival. ProBDNF binds to the p75 receptor, and activates the apoptotic cascade. [20] We have previously shown that the pro form of BDNF and its signaling pathways are preferentially upregulated after TBI. [26] While other groups have shown that the Val66Met genetic polymorphism affects levels of total BDNF in dendrites without altering the relative levels of pro and mature BDNF, the effect that this polymorphism has on levels of pro and mature BDNF after injury and the effect this has on injury outcomes have not been elucidated. [39, 183]

In this investigation, we studied the effect of the BDNF rs6265 genetic polymorphism on cellular, biochemical, and behavioral changes after repeated mild lateral fluid percussion (LFP) brain injury in mice. The LFP model of TBI is a longstanding method used due to its reproducibility, and its ability to mimic injuries seen in humans, by mixing focal and diffuse components. We have shown that after rmTBI, compared to Val66Val injured mice, Val66Met mice have increased injury volume, cell death, neurodegeneration, p-tau, astrogliosis, and activated microglia at 1 and/or 21 DPI in the cortex and hippocampus. When investigating the relative levels of pro and mature BDNF after injury in these mice, we found that injured Met carriers have less total BDNF in the cortex at 21 DPI, and more pro-BDNF relative to mature BDNF in the hippocampus at 1 DPI compared to injured Val carriers. Finally, we have found that when Val66Met carriers are treated with an AAV virus vector to overexpress BDNF, that we can rescue the high levels of astrogliosis and activated microglia down to the levels observed in Val66Val injured mice. Treatment with AAV-BDNF also rescues learning and memory in Val66Met injured mice in the Morris Water Maze paradigm to the level observed in Val66Val injured mice. To our knowledge, this is

the first report showing that there is genotypical susceptibility to TBI that can be rescued by altering neurotrophic signaling.

## Experimental Results:

### rmTBI injured mice have decreased herniation and increased edema over time

To investigate the role that genotype plays on the volume of injury after our repeated mild lateral fluid percussion model, we used a 1T MRI to scan the brains of the mice at 1, 7, and 21 DPI. Utilizing T2 fast spin echo sequence imaging scans, we found that by 21 DPI, edema at the site of the craniectomy returned to pre-injury levels in sham condition mice when the same mice were imaged over multiple time points. (Fig 1) We therefore selected the 21 DPI time point in order to assess the effect of the BDNF SNP on injury volume.

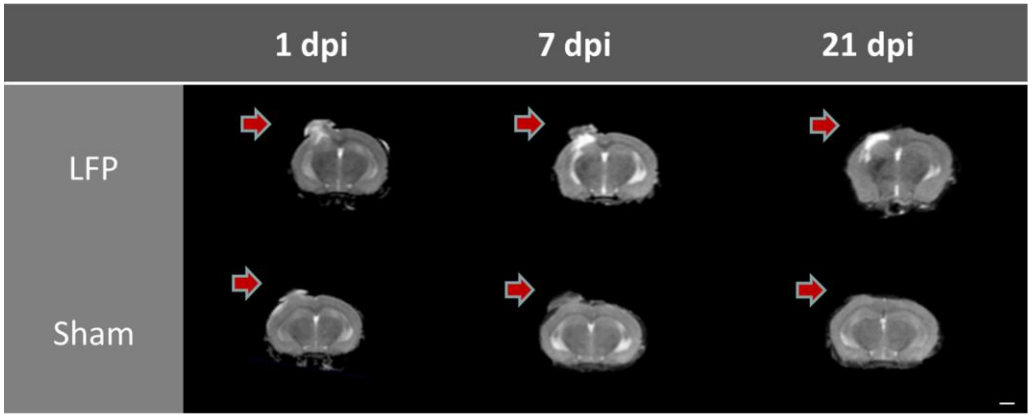



*Figure 1. Representative images of T2 magnetic resonance imaging scans of individual mice after 1, 7, and 21 days after rmTBI or sham procedure. Arrow indicates site of craniectomy and injury. Scans were conducted at the Rutgers University Molecular Imaging Center with the center's M2 Compact High-Performance MRI (1T). Scale bars = 3mm.*

### Val66Met injured mice have a larger injury volume compared to Val66Val injured mice at 21 DPI following rmTBI.

At 21 DPI there was a significant difference between the injury volume in sham and injured mice as determined from measuring hyperintensity volume of T2 MRI scans. Particularly of interest, we saw that Val66Met injured mice had a significantly larger injury volume

compared to Val66Val injured mice. (Fig 2). These data suggest that there are differences in the level of recovery occurring between these two genotypes at 21 DPI due to the increase in edema seen. However, it is not known which processes are affecting this difference.

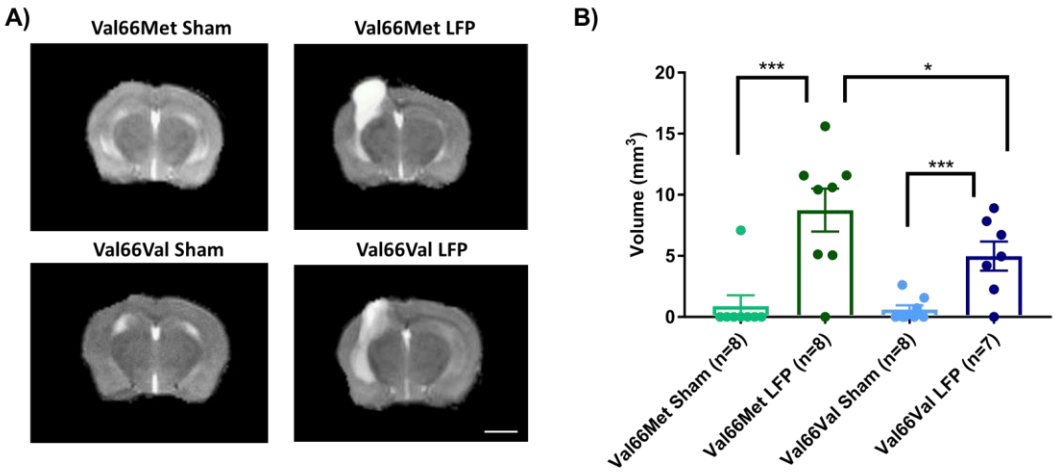



*Figure 2. Val66Met injured mice have greater injury volume than Val66Val injured mice.* ***(A)*** *Representative MRI images of mice subjected to LFP and sham at 21 days after the final injury.* ***(B)*** *Quantitation of injury volume in different genotypes, 21 DPI as determined by assessment of hyperintensity ROI. p * < 0.05, ** < 0.01, *** <0.001, ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =7-8. Scale bar = 3mm.*

**Levels of activated caspase-3 are higher in Val66Met injured mice compared to Val66Val injured mice at 1 DPI, but not at 21 DPI.**

To study the underlying cellular changes that contribute to the genetic difference in injury volume, we conducted immunohistochemical staining at both 1 and 21 DPI to assess the immediate as well as longer lasting effects of rmTBI on various cellular processes. As our lateral fluid percussion injury paradigm includes both focal and distal components, we analyzed the ipsilateral cortex to gain an appreciation of the focal components of injury as well as its possible effects on sensorimotor function. We also analyzed the ipsilateral hippocampus to investigate the slightly distal effects of injury and to gain insights into the possible effect on cognitive function. Given that the lateral fluid percussion model of injury

has both focal and diffuse components, instead of using the contralateral cortex and hippocampus as controls, we chose to use mice that have undergone craniectomy surgery but no injury (sham) and analyzed the ipsilateral hemisphere of those mice. Since increased levels of neuronal cell death are common following injury [26, 193], we used activated caspase-3 to assess levels of apoptosis. We found that at 1 DPI, both Val66Met and Val66Val injured mice has a significant increase in the number of activated caspase-3 positive cells relative to their sham controls indicating that apoptosis was at high levels at 1 DPI. Importantly, Val66Met injured mice had a significantly higher number of activated caspase-3 positive cells relative to the Val66Val injured mice, in both the ipsilateral cortex and hippocampus. (Fig 3A and B) suggesting that Val66Met mice have more cell death after rmTBI than Val66Val mice. However, by 21 DPI levels of cell death had decreased so that there was no difference between the injured mice and their sham controls (Fig 3C and E). These data suggest that while there are initial genotypic differences in apoptotic cell death after injury with Val66Met exhibiting worse outcomes than Va66Val, other cellular processes must be responsible for the deleterious processes occurring at 21 DPI.

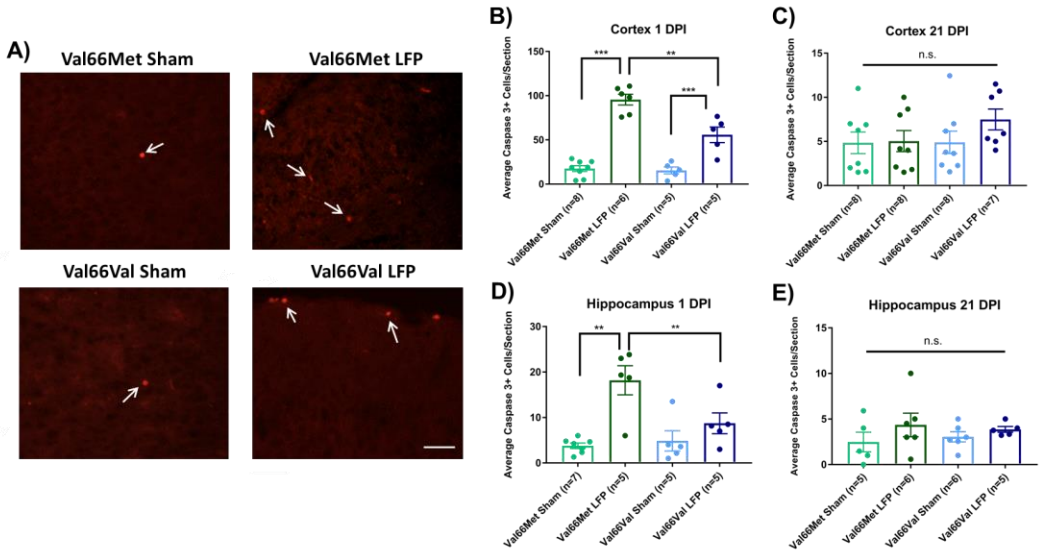



*Figure 3. Repeated mild LFP injury causes an increase activated caspase-3 positive cells in brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with activated caspase-3 (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of activated caspase-3 positive cells per cortical or hippocampal section ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Neurodegeneration is increased in Val66Met injured mice compared to Val66Val injured mice at 1 DPI and 21 DPI.**

Increased levels of neurodegeneration are common sequelae following injury to the brain. [26, 189] We used Fluorojade C (FLJC), a marker for neurodegeneration [135], in order to ascertain the level of neurodegeneration in the ipsilateral cortex and hippocampus. We found that at 1 DPI, Val66Met injured mice had significantly more FLJC positive cells in both the ipsilateral cortex and hippocampus relative to their sham controls. (Fig 4B and C) At this timepoint, Val66Val injured mice did not significantly differ from Val66Met injured mice in numbers of FLJC positive cells in the ipsilateral cortex, although Val66Val mice did differ from Val66Met mice in the hippocampus. Importantly, the Val66Met injured mice had more FLJC positive cells than the Val66Val injured mice in the ipsilateral cortex at 1 DPI, indicating that the Val66Met injured mice have more neurodegeneration

at this early timepoint. By 21 DPI, both the Val66Met injured mice and the Val66Val injured mice had significantly more FLJC positive cells than their sham controls in the ipsilateral cortex, and notably, the Val66Met injured mice had significantly more FLJC positive cells than the Val66Val injured mice as this time point as well. (Fig 4D) In the hippocampus at 21 DPI there was still a significant difference between the injured Val66Met mice and their sham controls, but no difference between the Val66Val mice and their sham controls. (Fig 4E) There was also no longer a difference between the Val66Met and Val66Val injured mice at this time point. These data suggest that the neurodegeneration process begins as early as 1 DPI, and that increased levels of neurodegeneration in response to injury may be sustained until at least 21 DPI in the Val66Met injured mice.

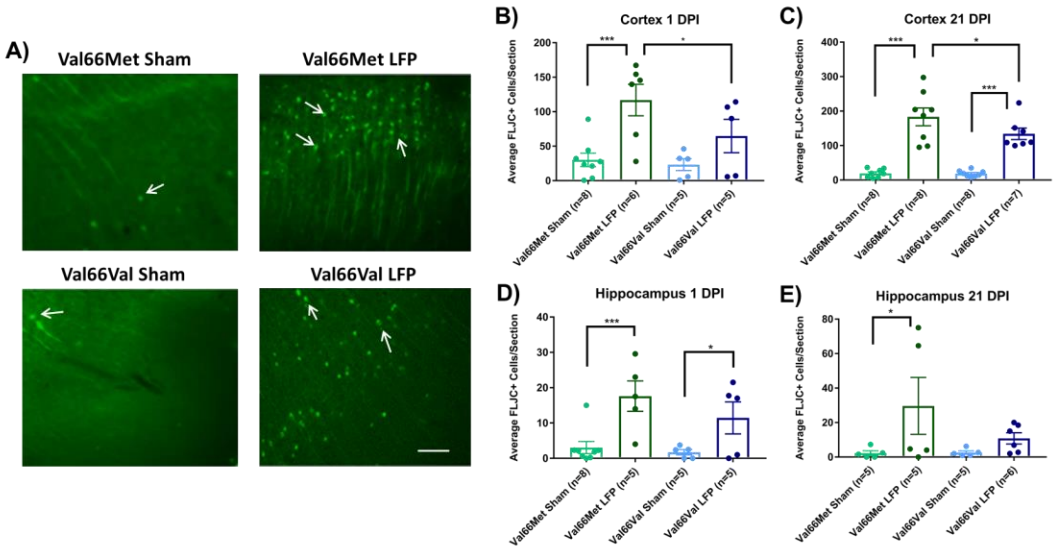



*Figure 4. Repeated mild LFP injury causes an increase in Fluoro-Jade C (FLJC) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 and 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with FLJC (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of FLJC positive cells per cortex or hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Phosphorylated tau is increased in Val66Met injured mice compared to Val66Val injured mice at 1 and 21 DPI.**

It has previously been shown that phosphorylated tau can contribute to long-term pathologies in the brain. [194] After injury, there are frequently higher levels of phosphorylated tau at the site of injury as well as in distally affected brain areas. [195] We investigated the levels of phosphorylated tau at 1 and 21 DPI in both the ipsilateral cortex and hippocampus. We found that there was a significant increase in levels of phosphorylated tau in both Val66Met and Val66Val injured mice compared to their sham controls in the ipsilateral cortex at both 1 and 21 DPI. (Fig 5B and C) Of note, the Val66Met injured mice had significantly more phosphorylated tau compared to Val66Val injured mice, seen at 1 DPI and sustained through to 21 DPI. This suggests that Val66Met injured mice may have an exacerbated phosphorylated tau reaction after injury that begins at 1 DPI and is sustained until 21 DPI. In the hippocampus, we found that the Val66Met injured mice had significantly more phosphorylated tau than their sham controls at both 1 and 21 DPI, while the Val66Val injured mice did not differ from their sham controls. (Fig 5D and E) These data suggest that after repeated mild injury, Val66Met mice are uniquely susceptible to mechanisms that result in increased levels of phosphorylated tau in the hippocampus.

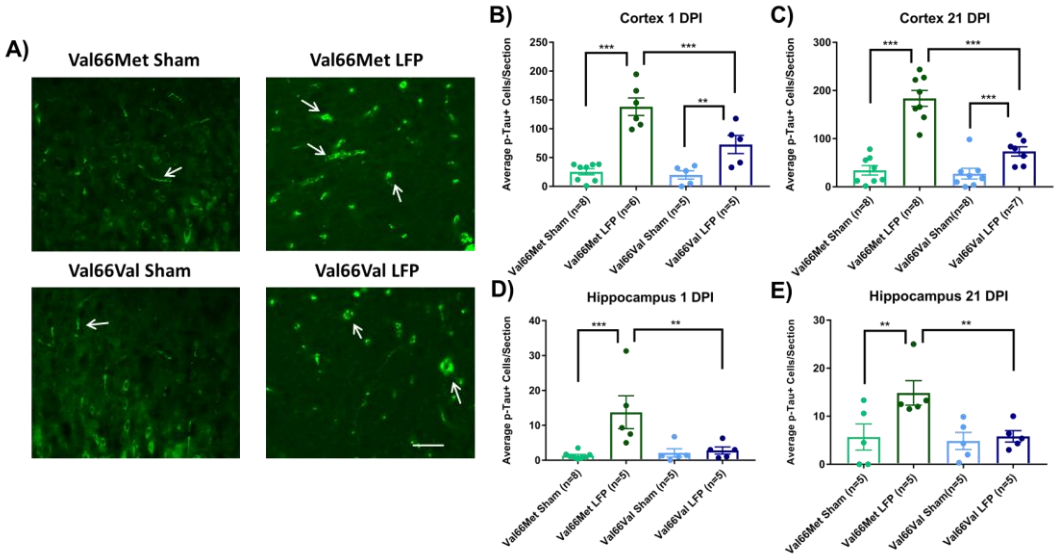



*Figure 5. Repeated mild LFP injury causes an increase in phosphorylated tau (p-tau) in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 and 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with activated AT8 (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of FLJC positive cells per cortex or hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

## Astrogliosis is increased in the ipsilateral cortex in Val66Met injured mice compared to Val66Val injured mice by 21 DPI, but not at 1 DPI

It is well documented that after injury there is proliferation of glia [26, 189] that can contribute to the formation of glial scarring, inhibiting the ability of neurons to regenerate, and preventing the injured brain from recovering normal morphology and function. [196] Using glial fibrillary acidic protein (GFAP), a marker for astrocytes, we quantified the level of gliosis after injury. We found that at 1 DPI, both Val66Met and Val66Val injured mice had more astrogliosis than their sham controls in the cortex and hippocampus. (Fig 6 B and D) Interestingly, we found that the Val66Met injured mice had more astrogliosis than Val66Val injured mice in the hippocampus at this 1 DPI, but not in the cortex, suggesting that the differential genotypic effects on astrocytes are more evident earlier in the time course in the more distal hippocampal region. By 21 DPI, both the Val66Met and Val66Val

injured mice had significantly more astrogliosis than their sham controls in the cortex, although by this timepoint only the Val66Met injured mice remained different from their sham controls in the hippocampus. (Fig 6 C and E) The injured Val66Met mice also had increased astrogliosis relative to Val66Val injured mice in the ipsilateral cortex and hippocampus at 21 DPI. These data suggest that activation of astrocytes after injury is variable based on genotype, and that Val66Met injured mice have impaired recovery compared to Val66Val injured mice.

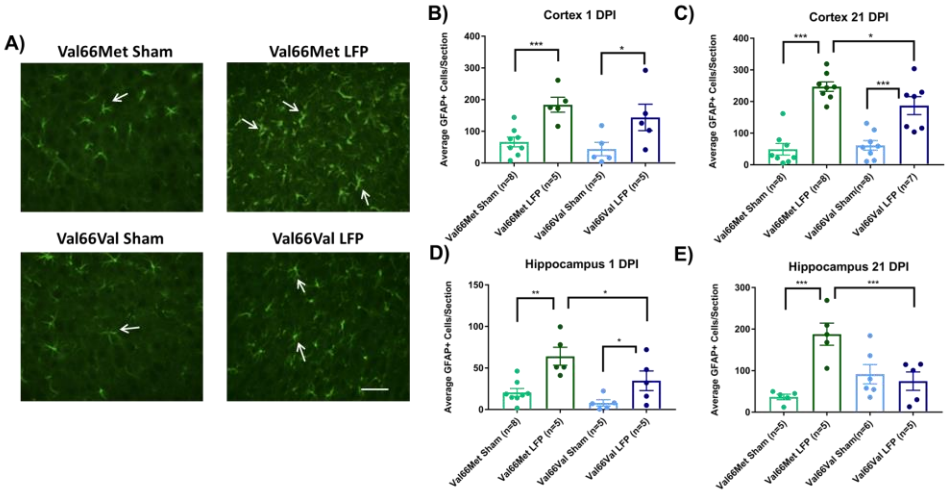



*Figure 6. Repeated mild LFP injury causes an increase in glial fibrillary acidic protein (GFAP) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with glial fibrillary acidic protein (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of GFAP positive cells per hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Activated microglia are increased in Val66Met injured mice compared to Val66Val injured mice, while non-activated microglia are consistent across all groups.**

To examine the effect that repeated mild traumatic brain injury has on the neuroimmune system, we analyzed activated microglia. Activated microglia are an important part of the secondary injury process, have been shown to persist for years after the initial injury, and contribute to long-term neurological dysfunction. [197] We used IBA1 as a marker for

microglia and we used morphology to distinguish activated microglia from non-activated microglia. Non-activated microglia were identified by their ramified appearance, while activated microglia were in either the reactive bushy state or the phagocytic ameboid shape.[198] We found that at 1 DPI Val66Met injured mice had significantly more activated microglia than their sham controls in both the ipsilateral cortex and the hippocampus. Notably, we found that at 1 DPI Val66Met injured mice had significantly more activated microglia than the Val66Val injured mice in the ipsilateral cortex or hippocampus. (Fig 7B and D) These data indicate that Val66Met injured mice have earlier microglial activation than the Val66Met injured mice. By 21 DPI, the Val66Met and Val66Val injured mice both had significantly more activated microglia than their sham controls. Again, we observe a significant difference in the levels of activated microglia in the Val66Met injured mice compared to the Val66Val injured mice at 21 DPI in both the ipsilateral cortex and hippocampus. (Fig 7C and E). These data suggest that Val66Met injured mice respond to repeated mild injury by activating microglia earlier than Val66Val injured mice, and this increased activation is sustained through 21 DPI.

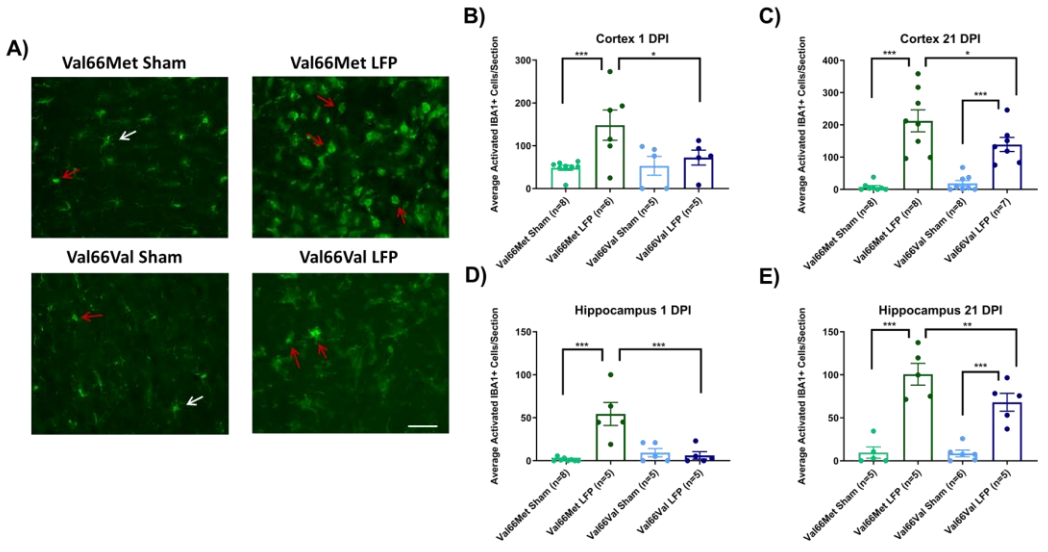



*Figure 7. Repeated mild LFP injury causes an increase in ionized calcium binding adaptor molecule 1 (IBA1) positive cells in the brains of injured Val66Met mice compared to injured Val66Val mice at 1 DPI.* ***(A)***

*Representative images of cortical sections at 1 DPI stained with IBA1. White arrows indicate resting microglia, red arrows indicate activated microglia. Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of IBA1+ positive cells, broken down into activated and resting categories by morphology, per cortex and hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Injured Met carriers have less total BDNF in the cortex at 21 DPI, and more pro-BDNF/mature BDNF in the hippocampus at 1 DPI compared to injured Val carriers**

Previous studies have shown that Val66Met and Met66Met mice have comparable levels of BDNF compared to their Val66Val counterparts at baseline [183], although they have less BDNF released from the dendrites. [39] However, to our knowledge no one has assessed the levels of pro and mature BDNF after repeated mild traumatic brain injury. Given our data showing that Met carriers appear to have worse recovery than Val carriers after injury, we hypothesized that after injury Met carriers would have less overall BDNF and more of the detrimental pro form of BDNF relative to the beneficial mature BDNF. Using Western Blot analysis, we have found that injured Met carriers did indeed have less total BDNF in the cortex at 21 DPI compared to injured Val carriers (Figure 8C). We have also found that at 1 and 21 DPI, injured Met carriers had more pro-BDNF/mature BDNF than Val carriers (Figure 8E and F). To quantify these results, we combined the mature bands at 14 kD and 16kD to account for the differences in molecular weight caused by the Met-His tag on the BDNF transgenic gene. These data support our conclusion that there is differential genotypic response to injury and highlight a potential pathway to target for therapies.

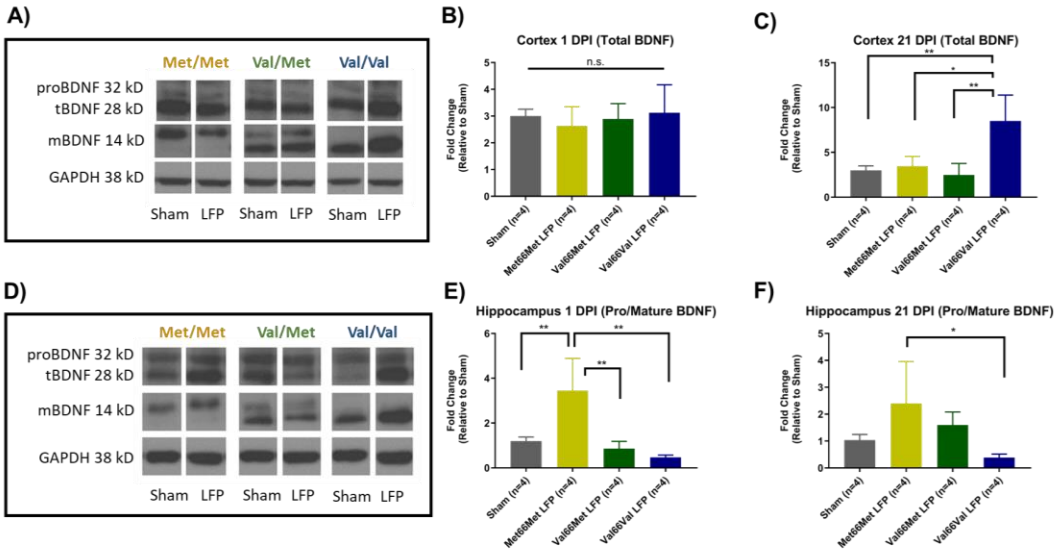



*Figure 8. Repeated mild LFP injury causes an increase in proBDNF/mature BDNF expression in Met carriers compared to Val carriers in the hippocampus at 1 and 21 DPI.* ***(A, D)*** *Representative Western Blot showing pro and mature BDNF expression in the hippocampus after injury. Each lane represents one animal.* ***(B-C)*** *Quantitation of protein levels in the cortex at 1 DPI and 21 DPI and* ***(E-F)*** *hippocampus at 1 DPI and 21 DPI. All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative to the average ± SEM of the time matched sham controls which are represented as a single bar in the graph* p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=4.*

## After AAV-BDNF injection there is expression in the cortex and hippocampus in both glia and neurons

Analysis of our data suggests that Val66Met injured mice have worse recovery after repeated mild lateral fluid percussion injury than their sham controls and Val66Val injured mice. Our Western Blot data suggest that there may be alterations in BDNF levels that are affecting outcomes after injury. Therefore, we injected an AAV-BDNF expressing vector to increase levels of BDNF in Val66Met injured mice and examined cellular and behavioral outcomes, using injections of AAV-GFP as the control. The AAV expressed in the cortex and hippocampus in glia. (Figure 9) We previously found that there were differences in both cellular and behavioral outcomes between injured and sham mice using our rmTBI model, but no differences between the Val66Met and Val66Val sham groups, so for our

rescue study we chose to focus on the differences we saw between the injured groups and the effect of the treatment on those groups.

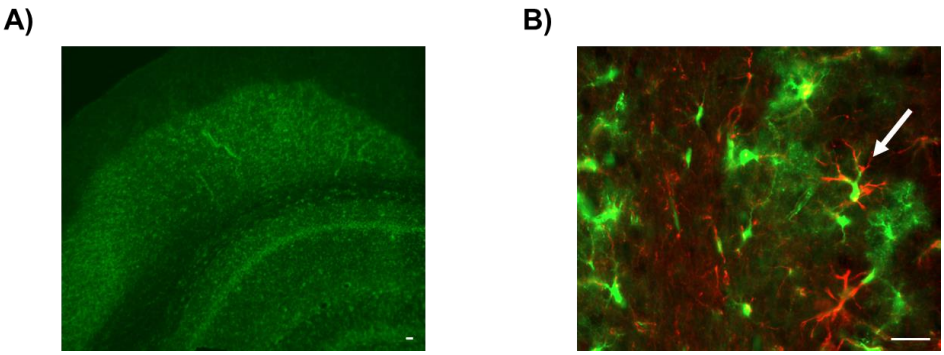
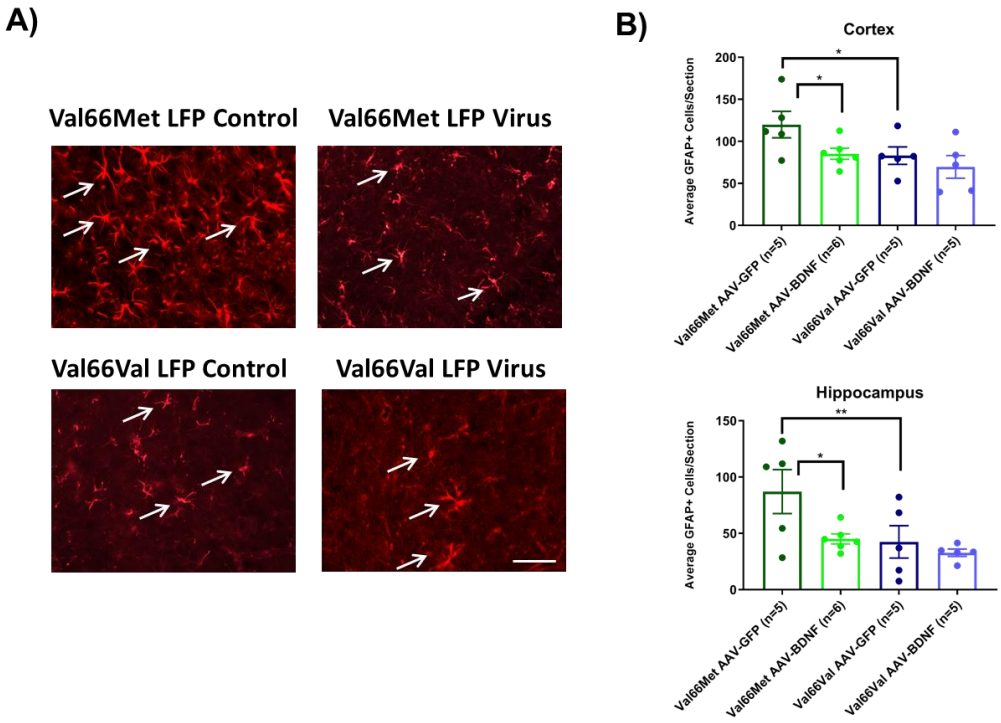



*Figure 9. AAV-BDNF GFP injection is expressed in the cortex and hippocampus, and in glia.* ***(A)*** *Representative image showing AAV-BDNF GFP expression in the cortex and hippocampus at 21 DPI. Green represents virus GFP expression.* ***(B)*** *Representative image showing AAV-BDNF GFP expression glial cells. Red indicates GFAP positive cells. Scale bars = 100μm.*

**Treatment of Val66Met injured mice with AAV-BDNF reduces the level of astrogliosis and activated microglia, but not p-tau, at 21 DPI to the levels seen in Val66Val injured mice.**

We chose to analyze astrogliosis, activated microglia, and p-tau at the 21 DPI timepoint since we had previously seen significant differences between the two injured genotypes in these markers. Val66Met injured mice treated with the control AAV-GFP had significantly higher levels of GFAP+ cells in the cortex and hippocampus and activated IBA1+ cells in the cortex relative to both the Val66Met injured mice treated with the AAV-BDNF and the Val66Val injured mice treated with either AAV-GFP or AAV-BDNF at 21 DPI. The AAV-BDNF treated Val66Met mice had levels of astrogliosis and activated microglia that were similar to levels seen in the Val66Val injured mice, both those treated with the control AAV-GFP and AAV-BDNF. (Fig 10A-D). We did not see any differences in p-tau levels in the Val66Met injured mice treated with AAV-GFP relative to the other groups. (Fig 10E-F) These data show that treatment of injured Val66Met injured mice with AAV-BDNF

can decrease astrogliosis and activated microglia relative to injured Val66Met mice treated with control AAV-GFP, indicating that the AAV-BDNF treatment is able to reduce inflammation after injury.



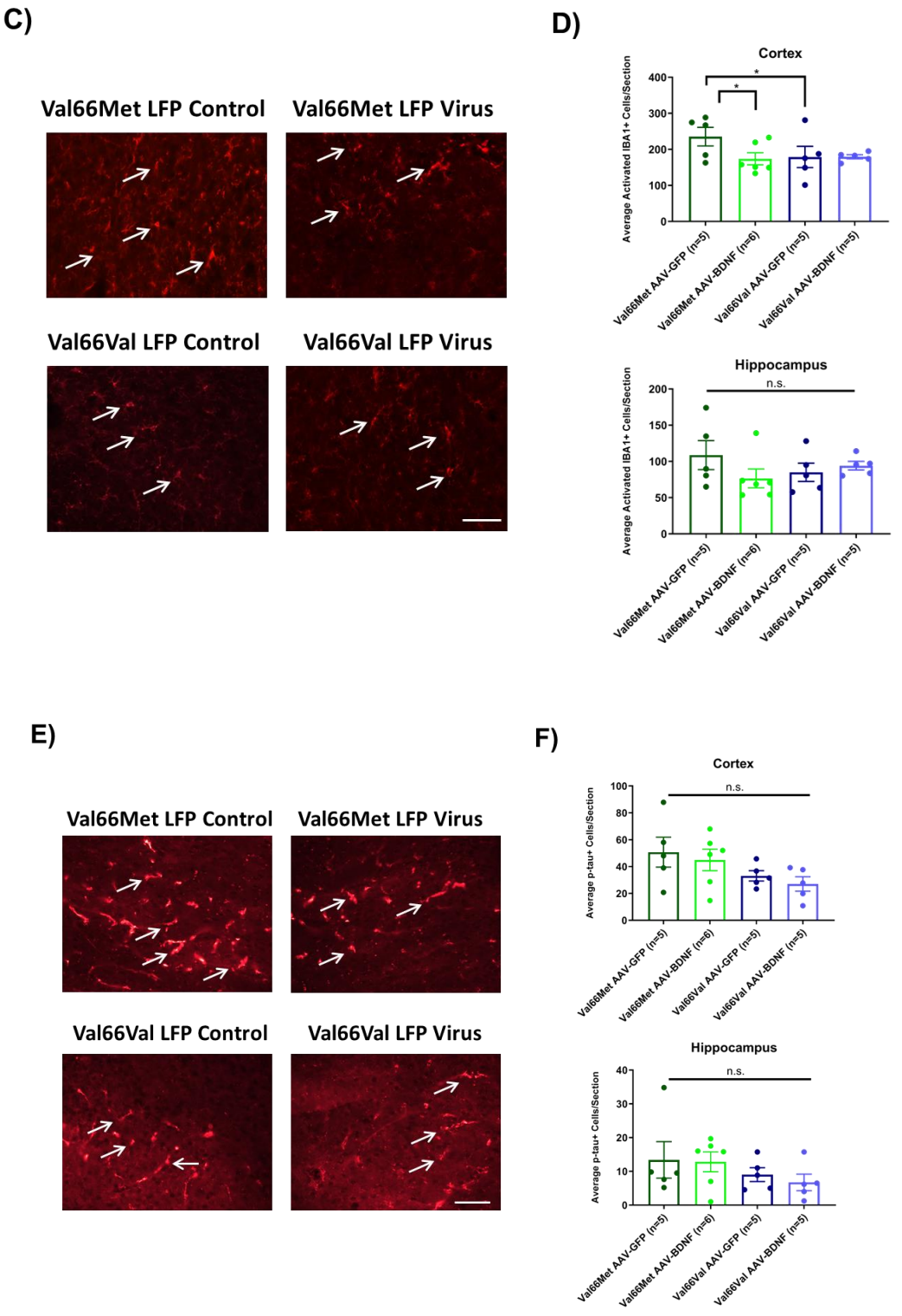



*Figure 10. Treatment of injured Val66Met with AAV-BDNF after repeated mild LFP injury causes a decrease in astrogliosis back to injured Val66Val levels in the cortex and hippocampus and activated microglia in the cortex but not p-tau at 21 DPI.* ***(A)*** *Representative images of cortical sections at 21 DPI stained with GFAP. (indicated by arrows). Scale bars = 100µm.* ***(B)*** *Quantitation of the average number of GFAP positive cells per*

*cortex and hippocampus ± SEM.* ***(C)*** *Representative images of cortical sections at 21 DPI stained with IBA1. (indicated by arrows). Scale bars = 100μm.* ***(D)*** *Quantitation of the average number of activated IBA1 positive cells per cortex and hippocampus ± SEM.* ***(E)*** *Representative images of cortical sections at 21 DPI stained with p-tau. (indicated by arrows). Scale bars = 100μm.* ***(F)*** *Quantitation of the average number of p-tau 3 positive cells per cortex and hippocampus ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=5-6*

**Treatment of Val66Met injured mice with AAV-BDNF improves learning at 16 and 17 DPI, but not memory at 21 DPI or motor function at 14 and 21 DPI, to the levels seen in Val66Val injured mice.**

We next examined the effect that our injury paradigm has on cognitive function using the Morris Water Maze which measures spatial learning and memory. It has been established that brain injury can have detrimental effects on cognition, especially in animals that have defects in the hippocampus. [26, 199] Here, we found that Val66Met injured mice treated with AAV-GFP had a longer latency to the hidden platform than Val66Val injured mice treated with AAV-GFP at 16 and 17 DPI. Importantly, when the Val66Met injured mice were treated with AAV-BDNF they found the hidden platform significantly faster than the Val66Met injured mice treated with the control AAV-GFP at 16 and 17 DPI, indicating that overexpression of BDNF was able to improve spatial learning at these timepoints. (Fig 11B)

In the Probe Test at 21 DPI, we found that the control AAV-GFP Val66Met injured mice spent significantly more time in the target NE quadrant than control AAV-GFP Val66Val injured mice, indicating improved spatial memory. However, the AAV-BDNF Val66Met injured mice did not spend significantly more time in the target NE quadrant than the AAV-GFP Val66Met injured mice, indicating that treatment with overexpression of BDNF did not improve spatial memory in these mice. (Fig 11C) These data suggest that in addition

to improving immunohistochemical differences seen after injury, that treatment with AAV-BDNF is able to rescue functional cognitive deficits as well.

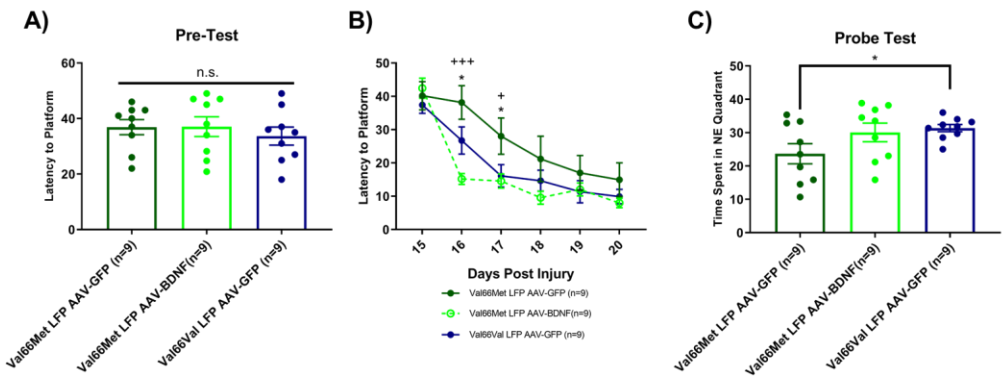



*Figure 11. Repeated mild LFP injury causes worse learning and memory in Val66Met injured mice relative to Val66Val injured mice. Treatment with AAV-BDNF in injured Val66Met mice improves learning, but not memory.* ***(A)*** *Average latency to platform in pretest phase ± SEM.* ***(B)*** *Average latency to platform ± SEM 15-20 DPI.* ***(C)*** *Average time spent in the target quadrant in the probe test ± SEM at 21 DPI. * p < 0.05, ** p < 0.01, *** p < 0.001 in Val66Met LFP Control relative to Val66Val LFP Control, + p < 0.05, ++ p < 0.01, +++ p < 0.001 in Val66Met LFP Control relative to Val66Met LFP BDNF. ANOVA Fisher's PLSD post-hoc test relative to indicated groups. n=9*

It has previously been shown that following brain injury, there can be deficits in motor ability. [199, 200] This is particularly true with our model of lateral fluid percussion, due to the damage that is done to the sensorimotor cortex at the site of injury. [201] In order to measure motor ability, we used the Rotarod Test for vestibular motor function and proprioception and the Balance Beam Test for more subtle differences in motor skills and balance. For the Rotarod Test, we found that at 15 DPI AAV-GFP treated Val66Met injured mice had a shorter latency to fall compared to AAV-GFP Val66Val injured mice, indicating impaired vestibular motor ability, but that treatment with AAV-BDNF did not shorten the latency to fall in the injured Val66Met mice (Fig 12B). At 21 DPI, we did not find any differences across groups in latency to fall in the Rotarod Test, indicating that the

vulnerable Val66Met injured mice had endogenously improved vestibular motor ability back to levels comparable with Val66Val injured mice. (Fig 12C)

For the Balance Beam Test at 21 DPI, we did not observe any difference between the injured Val66Met control AAV-GFP mice and either their Val66Met AAV-BDNF treated counterparts or the Val66Val control AAV-GFP injured mice (Fig 12D). These data suggest that while there may be differences in motor ability at earlier timepoints after injury, that by 21 DPI Val66Met control AAV-GFP mice that are acutely vulnerable have recovered back to levels comparable with the less vulnerable Val66Val control AAV-GFP mice. Therefore, unlike our single moderate LFP paradigm that creates a significant long-term motor deficit [26], our repeated mild LFP may create more subtle short-term motor deficits.

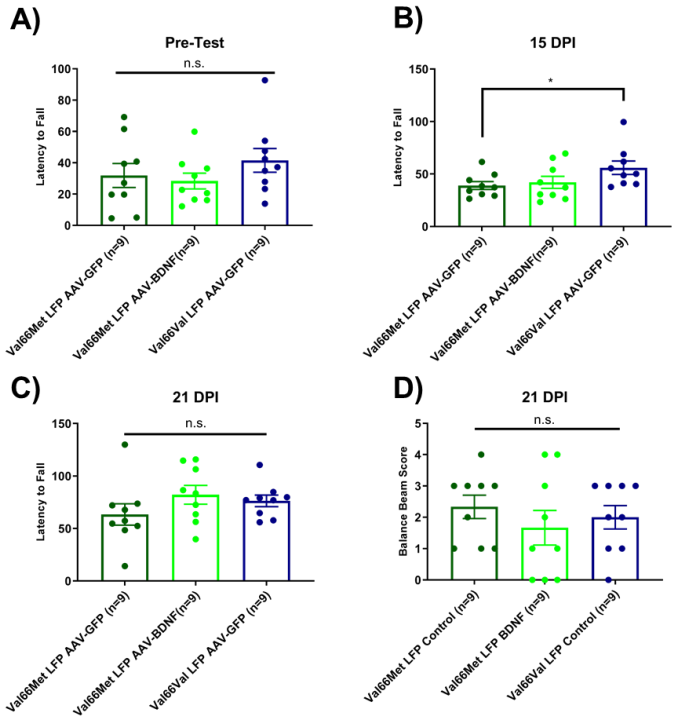



*Figure 12. Repeated mild LFP injury causes a deficit in vestibular motor function at 14 days in injured Val66Met mice relative to injured Val66Val. Treatment with AAV-BDNF does not significantly improve this*

*deficit.* ***(A)*** *Quantitation of latency to fall in the rotarod pretest assay ± SEM* ***(B)*** *Quantitation of the latency to fall in the rotarod assay ± SEM at 14 DPI and* ***(C)*** *21 DPI.* ***(D)*** *Quantitation of balance beam score ± SEM at 21 DPI. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =9*

**Treatment of Val66Met injured mice with AAV-BDNF increases levels of total and mature BDNF as well as p-trkB in the cortex and the hippocampus relative to Val66Met injured mice treated with control AAV-GFP.**

In order to determine how the AAV-BDNF treatment was working to improve function in these mice, we conducted Western Blot analysis on brain tissue from the cortex and hippocampus after 21 DPI. We determined that the AAV-BDNF virus does indeed elevate levels of BDNF, particularly mature BDNF, in both the cortex and the hippocampus (Fig 13A-C).

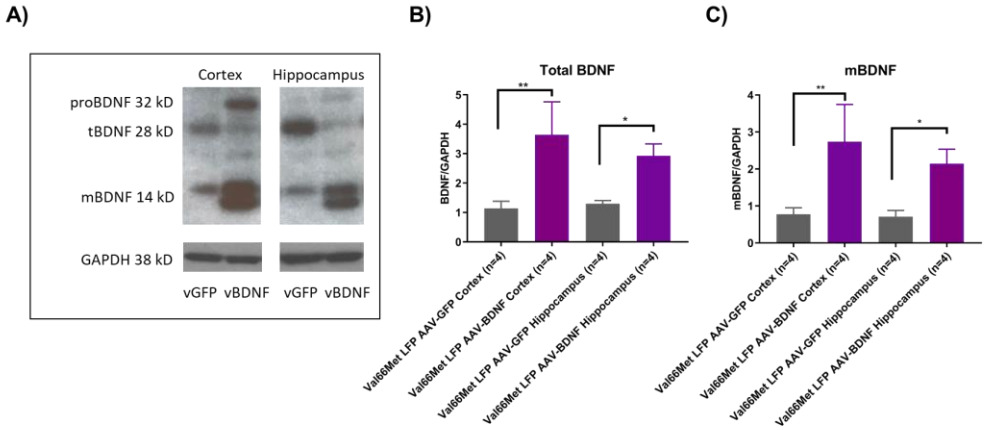



*Figure 13. AAV-BDNF injection causes an increase in total and mature BDNF expression relative to control AAV-GFP injection in the cortex and hippocampus at 21 DPI in injured Val66Met mice.* ***(A)*** *Representative Western Blot showing BDNF expression in the cortex and hippocampus after injury. Each lane represents one animal.* ***(B)*** *Quantitation of protein levels ± SEM at 21 DPI in the cortex* ***(C)*** *and hippocampus. All data is first normalized to GAPDH to control for protein loading ± SEM * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=6.*

In addition, we found elevated levels of p-trkB/trkB in AAV-BDNF treated Val66Met injured mice relative to control Val66Met injured mice in both the cortex and the hippocampus (Fig 14A-B), signifying increased activation of the mature BDNF trkB signaling pathway. Together, these results support the idea that disruptions in levels of BDNF play a role in the detrimental effects seen in the Val66Met injured mice and that targeted treatment to elevate BDNF can rescue the effects of repeated mild injury in this vulnerable genotype.

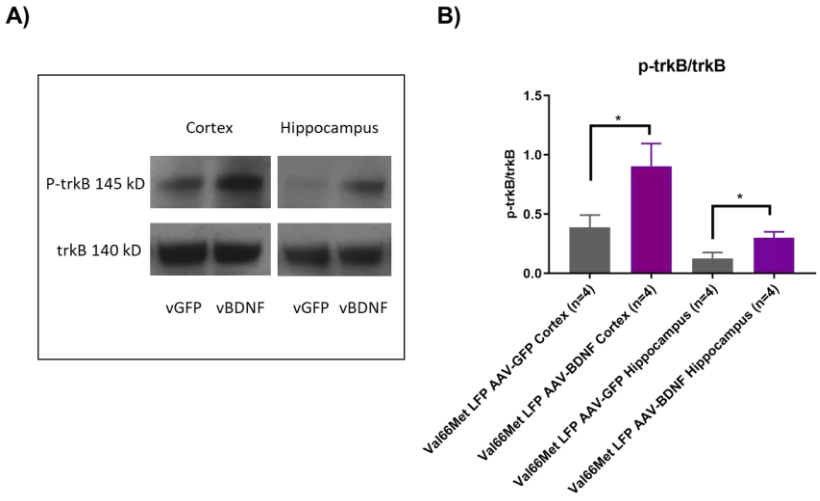



*Figure 14. AAV-BDNF injection causes an increase in p-trkB expression relative to control AAV-GFP injection in the cortex and hippocampus at 21 DPI in injured Val66Met mice.* ***(A)*** *Representative Western Blot showing BDNF expression in the cortex and hippocampus after injury. Each lane represents one animal.* ***(B)*** *Quantitation of p-trkB protein levels at 21 DPI in the cortex* ***(C)*** *and hippocampus. All data is first normalized to trkB to control for protein loading ± SEM * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=4.*

## Discussion of Results:

In this study, we show that the BDNF Val66Met genetic polymorphism results in worse recovery relative to Val66Val genotype following repeated mild lateral fluid percussion injury in mice. BDNF has been shown to play an important role after injury. Early studies

have shown that after injury, levels of BDNF protein and mRNA are upregulated. [21-25] Our group went on to show that after TBI levels of proBDNF and proNGF, the p75 receptor, and the sortilin co-receptor are preferentially upregulated relative to mature BDNF and its trkB receptor and may explain why there is apoptosis and neurodegeneration after injury rather than cell survival and neural regeneration (Chapter 6). [26] These results are consistent with what has been shown in the literature, that after injury proBDNF is preferentially upregulated relative to mature BDNF. [25-27] Other groups have confirmed that activating the mature BDNF trkB signaling pathways using molecules [32], neural stem cells [33], physical exercise, and acupuncture [35] can improve outcomes after TBI.

The LFP model of injury was used due to its ability to recreate injuries similar to those seen in the human population. [26, 110, 111, 202] Cellular changes were investigated at 1 and 21 DPI, in order to ascertain a more complete picture of the various biological processes that are activated at different time points after injury. We found that as the primary site of injury, effects were more dramatic in the cortex demonstrating the focal effects of injury, but that we also were able to identify diffuse effects of the injury in the hippocampus in the ipsilateral hemisphere. Thus the repeated mild lateral fluid percussion injury paradigm we have used is a good model of human rmTBI.

We show that Val66Met genetic polymorphism results in worse recovery following repeated mild lateral fluid percussion injury. The Val66Met mice had a larger injury volume by 21 DPI relative to Val66Val mice as assessed by MRI. At 1 DPI, there was also more cell death, neurodegeneration, phosphorylated tau, and activated microglia in the Val66Met injured mice compared to the Val66Val mice in the cortex and the hippocampus. By 21 DPI, the levels of cell death were reduced to sham levels, however the other markers

sustained their elevated levels, and in addition, astrogliosis became significantly elevated in the Val66Met injured mice relative to the Val66Val injured mice.

Taken together, these results indicate that the Val66Met injured mice respond differentially to a repeated mild injury than Val66Val injured mice starting as early as one day after the final injury. This is an important and novel finding, since previous research on the effect of the Val66Met genetic polymorphism has been done primarily in clinical studies, with some results concluding Val66Met is the risk factor, while others concluding that Val66Val is. While some clinical studies have shown that the Val66Met polymorphism results in impaired neurocognitive performance after TBI [50] and is a risk factor for TBI in combat forces [47-49], others have found that In long-term studies of combat veterans, carriers of the Val66Met polymorphism had recovery of executive function back to baseline levels while the Val66Val carriers did not [45, 46], while still others have shown no effect of the polymorphism influencing outcomes after injury. [55-57] Our study is the first to investigate the role of the Val66Met genetic polymorphisms on outcomes after rmTBI in a mouse model, and to find that it is the Val66Met genetic polymorphism that is the risk allele in this type of trauma. However, our data is supported by previous work investigating the role of the BDNF Val66Met genetic polymorphism in stroke and spinal cord injury. After stroke there is evidence that humans with the Val66Met polymorphism do worse than those with the Val66Val polymorphism. [43, 58-60]

We show here that in the acute phase, Val66Met mice have more cells undergoing apoptotic cell death than their Val66Val counterparts, which has been shown in other studies to contribute to worse outcomes. [130] However, after the acute phase of injury, levels of apoptosis often return back to baseline [203, 204], while other processes have

been set in motion that continue to have a pathophysiological effect. The degeneration of neurons can be seen in the acute post injury phases as well, but it can have longer lasting effects that persist up until at least 21 days after the final injury. [133] In this case, the injured Val66Met mice show higher levels of neurodegeneration which may contribute to the progressive loss of function in neurons in these mice. One specific way in which neurodegeneration can occur is the hyper phosphorylation of tau. When tau is hyper phosphorylated, it will bind to other tau proteins, and create aggregates. This is a disruption of normal functioning and can lead to long term neurodegeneration. One way in which tau phosphorylation can lead to neurodegeneration is by interfering with axonal transport. When this occurs, both retrograde trophic signaling as well as the process of autophagy of defective tau proteins is disrupted, leading to long-term problems in function. [94, 136, 137] Our findings are in agreement with other studies that have shown that acutely after repeated mild TBI, hyper phosphorylation of tau has been shown to be an issue, while later stages of tauopathies such as accumulations of hyper phosphorylated tau like NFTs are not yet seen. [8] We demonstrate that injured Val66Met mice have higher levels of p-tau than their Val66Val counterparts, which highlights the potential for phosphorylated tau (p-tau) to contribute to the differential development of long-term outcomes after TBI.

In addition to cell death and neurodegeneration after injury, it is also common to see inflammation and activation of the neuroimmune response. Astrocytes generally work to maintain homeostasis in the brain, and are known to play an important role in the response to TBI. [138] After TBI, astrocytes are activated, and function repair the damage that has been made. While the actions of astrocytes at baseline tend to be beneficial, after injury the prolonged activation of astrocytes can lead to inflammation and secondary injury processes. In particular, the formation of a glial scar can impair neuronal regeneration and

lead to worse long term outcomes. [139] We see that 1 day after the final injury, both Val66Met and Val66Val injured mice have an elevation of activated astrocytes, but by 21 days after the final injury the Val66Met injured mice have significantly more activated astrocytes than Val66Val injured mice, suggesting they may have impaired neuronal regeneration due to the formation of a glial scar. Microglia are the resident neuroimmune cells in the brain, which are activated as early as 1 day after the final injury and persist until at least 21 days after the final injury in our study. Along with astrocytes, microglia are responsible for initiating the inflammatory response after injury at which point, they become activated. And similarly to the role of activated astrocytes after injury, while the initial response may be protective, if the activated microglia persist over time they will contribute to secondary injury processes and worse long term outcomes. [142] We have shown that as early as 1 day after the final injury, the Val66Met injured mice have elevated activated microglia relative to Val66Val injured mice, and that this difference persists until at least 21 days after the final injury, perhaps contributing to differential long-term outcomes.

Our findings that the Val66Met polymorphism is a risk allele after rmTBI is consistent with recent reports in the literature. Recent human studies have shown that the Met allele is a risk factor after a single mild TBI in the areas of attention, executive function, memory, and overall cognition. [50] In a study that examined emotional symptoms after a single mild TBI, Met carriers were found to have more emotional symptoms than Val carriers. [205] The Met allele has also been found to be a risk factor after a single mild-moderate TBI in cognitive language processing speed. [56] Interestingly, previous studies had shown that the Met allele was actually protective in long term executive function after focal frontal TBI in combat veterans [45, 46], perhaps indicating the importance of the SNP

interaction with type of injury sustained, however the retrospective analysis was unable to eliminate confounding factors that may have played a role. In addition to the effects of the Val66Met polymorphism on outcomes TBI, there have also been reports of its effect after spinal cord injury and stroke. The Val66Met SNP has been shown to be a risk factor for a worse clinical presentation in cervical spondylotic myelopathy [206], impaired spinal cord plasticity [207], and reduced exercise induced serum BDNF levels after spinal cord injury [208] in humans. It is thought that after stroke Val66Met might be a risk factor for poor outcomes [43, 58-60], but that the time course of stroke recovery may be more complicated than originally thought, with the Val66Met allele shown to be the risk allele in motor ability acutely [61], and surprisingly, Val66Val allele shown to be the risk factor chronically. [62] While yet another study has posited that perhaps Val66Met carriers do not have worse overall recovery, but recover using different underlying brain pathways. [209]

To our knowledge, this is the first report that shows the effect of the Val66Met SNP on recovery after repeated mild TBI. Our results provide evidence that the Val66Met genetic polymorphism confers risk after repeated mild TBI. Furthermore, we have shown that the Val66Met genetic polymorphism alters the upregulation of BDNF that occurs after injury. Previously, our group as well as others, have shown that after TBI levels of proBDNF and its p75 receptor are preferentially upregulated relative to mature BDNF and its trkB receptor. [25-27] Other groups have shown that the Val66Met genetic polymorphism does not affect the relative levels of pro and mature BDNF at baseline, despite the fact that the SNP is located in the prodomain of the protein. [39, 183] However, importantly, the Val66Met SNP does decrease intracellular trafficking of BDNF and lowers the activity-dependent secretion of BDNF which can result in functional deficits. [38] Here, we show

that after repeated mild TBI, Met carriers have less total BDNF in the cortex at 21 DPI compared to Val carriers. We also show that in the hippocampus at 1 and 21 DPI, Met carriers have significantly more pro/mature BDNF compared to Val carriers. These data suggest that these alterations in BDNF levels after injury in Met carriers may contribute to the worse outcomes seen.

Given that the Val66Met genetic polymorphism appears to be a risk allele following repeated mild TBI and that altered BDNF levels may be a contributing factor, we attempted to rescue the Val66Met injured mice by injecting an AAV that overexpresses control BDNF in the cortex and the hippocampus after injury. Previous studies have employed delivery of BDNF through AAV in animal models as a potential treatment for various brain pathologies, such as depression [210], stroke [211, 212], Alzheimer's disease [213], and spinal cord injury [214, 215]. We delivered the AAV treatment 5 minutes after the last injury, and investigated the effect of our treatment on outcomes at 21 DPI, given that we observed worse outcomes in cellular markers in Met carriers at this time point, as well as significant differences in BDNF levels in the cortex and hippocampus at this time point. In addition, focusing on this later time point allows time for the AAV to express sufficiently and allows us to determine the treatment's efficacy in changing longer-term outcomes, which has more translational potential.

Here, we show that BDNF overexpression was able to decrease levels of astrogliosis, a well-known marker for poor outcomes after injury, in Val66Met injured mice back to the levels seen in Val66Val injured mice. BDNF overexpression was also able to reduce levels of activated microglia in injured Val66Met mice to levels equivalent to injured Val66Val mice, signaling a reduced pro-inflammatory post injury phase. We did not see a difference

in phosphorylated tau levels in injured Val66Met injured mice treated with AAV-BDNF, suggesting that another mechanism may be responsible for the increase seen in phosphorylated tau levels.

In addition to the differences in cellular markers that we observed, we also investigated the effect that AAV-BDNF has on motor and cognitive behavior outcomes. We found that AAV-BDNF was able to increase spatial learning in injured Val66Met mice at 16 and 17 DPI in the Morris Water Maze Test. However, we did not find that treatment with AAV-BDNF had any effect on motor outcomes at 15 DPI or 21 DPI in the Rotarod Test and Balance Beam Assay, signifying that there are potentially other factors driving the difference between injured Val66Met and injured Val66Val mice in terms of gross vestibular motor ability. These data suggest that the AAV-BDNF treatment had a more potent effect on rescuing hippocampal neurons than on sensorimotor neurons.

Given that previous studies have shown the importance of BDNF for hippocampal dependent processes [216], it is logical that increasing levels of BDNF in these mice improves their recovery in learning assays after injury and may be an important specialized treatment for Val66Met carriers who have lower levels of BDNF compared to their Val66Val counterparts after injury. We found that in these AAV-BDNF treated injured Val66Met mice that injection of the virus increased levels of total and mature BDNF in the cortex and hippocampus, as well as levels of p-trkB at 21 DPI, signifying that the mature BDNF pathway is more activated in AAV-BDNF treated mice relative to control treated mice. These results offer promising evidence that by manipulating the BDNF pathway, we may be able to develop targeted therapies for Met carriers who are more susceptible to poor outcomes after injury.

Since the Val66Met SNP is at the 66 amino acid position which is in the pro domain of the BDNF protein, when the prodomain is cleaved from the mature region, the resultant mature BDNF protein will have no altered sequence. However, due to the fact that the SNP is found in the full length proBDNF, before the protein is cleaved, studies have shown the Met SNP leads to altered intracellular packaging, which affects the axonal transport of BDNF and results in decreased activity dependent section of BDNF at the synapse. [38, 39] Naturally, this can become an issue in disease states such as after TBI, where there is a need for an increased mature BDNF signaling in order to stimulate repair and recovery. In addition, recent work has highlighted the importance of the prodomain itself that has been cleaved off from the mature BDNF protein. Previously thought to be inert, new research has shown that it is in fact an active ligand. Importantly, the 66Met substitution changes the structure of the protein and causes acute growth cone retraction and in the hippocampus, it decreases Rac activity, a mediator of synaptic plasticity. [40] Newer studies have shown that the 66Met prodomain is also able to disassemble dendritic spines and eliminate synapses in hippocampal neurons, leading to impaired hippocampal dependent fear extinction behavior. [41] These toxic effects of the 66Met, but not 66Val, prodomain may also play a role in differences in outcomes between Val66Val and Val66Met carriers. Given this knowledge, treatment with AAV-BDNF which supplies the 66Val form of BDNF, may be a useful treatment for other conditions that Val66Met carriers suffer from in addition to its ability to facilitate recovery after rmTBI.

Our study lays important experimental groundwork in the investigation of the genetic underpinning of the differential response to injury seen in the TBI patient population, and highlights the rs6265 SNP and the BDNF signaling pathway as a potential mediator of

these differences. However, our study was limited by several factors that future studies should attempt to incorporate. While the benefit of our study is that we could clarify the role of a single SNP, there may interactions of the rs6265 SNP with other SNPs that could play a role in determining genetic susceptibility. We also maintained strict control over the environment that the mice were in and investigated outcomes at the set timepoints of 1 and 21 DPI. Future studies should investigate the interaction of these genetic susceptibility with other factors such environmental influences and age. We used a very controlled and replicable repeated mild lateral fluid percussion injury method. Given that the human TBI data seems to suggest that genetic risk factors may vary based on the different types of injury sustained, future studies should investigate the role of the rs6265 in other forms of injury as well. In addition, we found that treatment with AAV-BDNF with the 66Val form can facilitate recovery after TBI, but we did not investigate the role that 66Met prodomain might be playing after injury. This would be an interesting pathway to investigate more thoroughly, potentially by analyzing signaling through the SorCS2 pathway.

Taken together, this study has investigated the role of the Val66Met genetic polymorphism on cellular markers and demonstrated the role that it plays on BDNF levels and signaling after repeated mild TBI. We have explored using overexpression of BDNF as a personalized therapy for the susceptible Met carriers, and highlighted the potential usefulness of targeting BDNF signaling pathways for treatment.

# Chapter 4: ApoE4 Genetic Polymorphism

## Introduction:

Traumatic Brain Injury (TBI) results when there is an insult to the head which causes a disruption of normal brain function. Injuries can range in severity from mild to severe, with mild TBIs, commonly called concussions, being the most common form. In 2013, there were approximately 1.125 million mild TBIs in the United States, with costs from mild TBI reaching $117 billion each year. [1] Mild TBIs can actually cause very serious health problems, and it is estimated that around 15% of patients have symptoms that last longer than three months and develop into chronic disabilities. [5] This problem is further compounded when a person sustains a second mild TBI after an initial head injury. Sustaining a repeated mild TBI (rmTBI) can have devastating consequences which is common in athletes and military personnel. [3] [4]. Currently, many groups are studying the link between undergoing rmTBIs and developing long term neurodegenerative diseases. [6] The ability to recover after undergoing rmTBI is variable; some are able to recover quickly and have no obvious long-term problems, while others go on to develop lifelong complications. [9, 10] One of the reasons for this differential response to rmTBI may be the genetic differences that exist in the population.

ApoE is a critical neuronal gene that contains two single nucleotide polymorphisms (SNP), at the 122 and 158 residue positions, also referred to as rs429358 and rs7412, which affect the structure and function of the resultant protein. [68] The ApoE protein is an important protein responsible for the transport and clearance of lipids and cholesterol in the brain. There are three isoforms; ApoE2, ApoE3, and ApoE4. ApoE4 has an Arg at both the 122 and 158 position, while ApoE3 has a Cys at the 122 position, and ApoE2 has a Cys at both

the 122 and 158 positions. [72] ApoE3 is generally thought of as the wildtype allele with an allelic frequency ranging from 53.6%-89.8%, although there is considerable variation in allelic frequency across worldwide populations, with the ancestral ApoE4 allele having a frequency ranging from 5.2%-40.7%. [73] Carriers of the ApoE4 allele have the biggest known genetic risk factor for the development of late onset Alzheimer's disease. [76-78]

In addition to its role in neurodegeneration, ApoE has been shown to play a role in neuronal repair after injury. Under baseline conditions, ApoE is produced principally by astrocytes in order to support normal lipid transport and membrane repair. However, after injury, neurons begin to synthesize ApoE, presumably to support the neuronal repair mechanisms. [69-71] There appear to be differences in how effectively the isoforms of ApoE are able to function.

Previous studies in humans have investigated the effect that the ApoE genetic polymorphisms have on outcomes after injury. [79] Early studies looked retrospectively at boxers and hockey players who had a history of repeated concussions, and worse long-term outcomes were correlated with being a carrier for ApoE4. [80, 81] Some studies have shown that having the ApoE4 allele is a risk factor for sustaining multiple concussions, while other studies have shown that the ApoE4 allele is not correlated with risk. [82, 83] In addition, an early meta-analysis showed that carriers of the ApoE4 allele had worse long term outcomes 6 months after injury [84], while a more recent meta-analysis showed that there was no association with the ApoE4 status and poor cognitive outcomes after TBI. [85] In order to attempt to elucidate the effect of ApoE SNPs on recovery after TBI, a number of animal studies have been done as well. In a closed head injury mouse model of injury, comparison of the ApoE4 polymorphism to the ApoE3 polymorphism have found

that ApoE4 mice were twice as likely to die from an injury compared to the ApoE3 mice. [86] In a very mild repeated mild CCI TBI model, one group found no differences between ApoE4 and wild type mice [94], while another study found that after a repeated blast injury ApoE4 mice had more p-tau than ApoE3 mice [95], and another group found that after a month of repeated mild injuries that ApoE4 mice had higher levels of MC1, a tau conformation marker [96]. Consequently, the research investigating whether ApoE4 is a risk allele compared to ApoE3 after TBI is not all in agreement, and could benefit from a reliable bottom-up, controlled experimental research study using the replicable lateral fluid percussion model.

Furthermore, the mechanism by which ApoE4 may increase the risk for poor outcomes after TBI is not known. Previous studies have postulated that it may be due to the role that ApoE plays in neural repair, with ApoE4 carriers having impaired ability to repair membranes after injury, while others have suggested that it is due to the neurotoxic fragments that are created when the neuronally produced ApoE4 is proteolytically cleaved. [69-71] Some studies have found that Alzheimer's disease (AD) ApoE4 carriers have lower levels of Brain-Derived Neurotrophic Factor (BDNF) in the serum [97], and that mildly cognitively impaired ApoE4 carriers have impaired BDNF increase after exercise compared to non-carriers. [98] Interestingly, it has been found that ApoE4 causes reduced BDNF release by increasing the nuclear translational of histone deacetylases (HDAC) 4/6. This action causes negative gene regulation, and results in less BDNF being produced in the cell and secreted by astrocytes. [99, 100] It is known that BDNF signaling plays an important role in recovery after injury [26, 101], making this a potential therapeutic target for ApoE4 carriers.

In this investigation, we studied the effect of the ApoE4 genetic polymorphism on recovery after repeated mild lateral fluid percussion (LFP) brain injury in mice. The LFP model of TBI is a longstanding method used due to its reproducibility, and its ability to mimic injuries seen in humans, by mixing focal and diffuse components. This study examined the effect of the ApoE4 genetic polymorphism on cellular, biochemical, and behavioral changes following repeated mild LFP injury. We have shown that after rmTBI, the ApoE4 SNP results in differential recovery. Injured ApoE4 mice have increased injury area, cell death, neurodegeneration, and activated microglia at 1 DPI in the cortex and/or hippocampus relative to injured ApoE3 mice. Injured ApoE4 mice also have increased levels of p-tau at 21 DPI compared to injured ApoE3 mice. When investigating the relative levels of BDNF after injury in these mice, we found that injured ApoE4 carriers have more total BDNF than injured ApoE3 carriers at 1 and 21 DPI. Finally, we have found that when injured ApoE4 carriers are treated with Bryostatin 1, a PKCε activator which has been shown to increase BDNF levels [163, 217, 218], that we can rescue the elevated levels of neurodegeneration and activated microglia back to the levels observed in ApoE3 injured mice at 1 DPI. Bryostatin 1 also is able to rescue spatial learning at 2 DPI and spatial memory and balance at 7 DPI. This report has important implications for ApoE4 carriers, and to our knowledge, is the first report showing that this genotypical susceptibility can be rescued by altering neurotrophic signaling.

## Experimental Results:

### ApoE4 injured mice have a larger injury volume compared to ApoE3 injured mice at 21 DPI following rmTBI.

To investigate the role that genotype plays on the volume of injury after repeated mild lateral fluid percussion model, we used a 1T MRI to scan the brains of the mice at 1, 7, and

21 DPI. Utilizing T2 fast spin echo sequence imaging scans, we found that by 21 DPI, edema, as measured by the volume of T2 hyperintensity, at the site of the craniectomy returned to pre-injury levels in sham condition mice by 21 DPI when the same mice were imaged over multiple time points. (Figure 1) We therefore selected the 21 DPI time point in order to assess the effect of the ApoE SNP on injury volume. At 21 DPI there was a significant difference between the injury volume in sham and injured mice. Particularly of interest, we saw that ApoE4 injured mice had a significantly larger injury volume compared to ApoE3 injured mice. (Fig 15) These data suggest that there may be differences in the recovery course occurring between these two genotypes after injury. However, the cellular processes and molecular mechanism underlying these differences are not known.

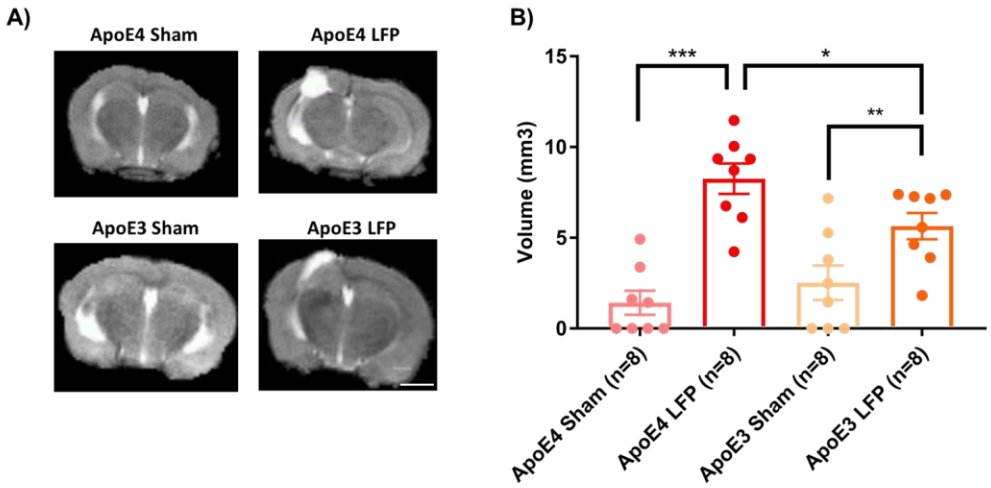



*Figure 15. ApoE4 injured mice have greater injury volume than ApoE3 injured mice.* ***(A)*** *Representative MRI images of mice subjected to LFP and sham at 21 days after the final injury.* ***(B)*** *Quantitation of injury volume in different genotypes, 21 DPI as determined by assessment of T2 hyperintensity ROI. p * < 0.05, ** < 0.01, *** <0.001, ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =8. Scale bar =3mm.*

**Levels of activated caspase-3 are higher in ApoE4 injured mice compared to ApoE3 injured mice in the cortex and hippocampus at 1 DPI.**

We conducted immunohistochemical staining at both 1 and 21 DPI to assess acute and longer-term outcomes after injury. Our lateral fluid percussion injury paradigm creates both focal and distal injuries, so we analyzed the ipsilateral cortex to gain an appreciation of the focal components and the ipsilateral hippocampus to investigate the slightly distal effects of injury. We used mice that have undergone craniectomy surgery but no injury (sham) as controls, given that the lateral fluid percussion model of injury has diffuse components which can have effects on the contralateral cortex and hippocampus. Since increased levels of neuronal cell death are common following injury [26, 193], we used activated caspase-3 to assess levels of apoptosis. We found that at 1 DPI, both ApoE4 and ApoE3 injured mice had a significant increase in the number of activated caspase-3 positive cells relative to their sham controls indicating that apoptosis is at high levels at 1 DPI. Importantly, ApoE4 injured mice had a significantly higher number of activated caspase-3 positive cells relative to the ApoE3 injured mice, in both the ipsilateral cortex and hippocampus. (Fig 16 A-B) suggesting that ApoE4 mice have more cell death after rmTBI than ApoE3 mice. However, by 21 DPI levels of cell death had decreased so that there was no difference between the injured mice and their sham controls (Fig 16A-B). These data suggest that while there are initial genotypic differences in apoptotic cell death after injury with ApoE4 exhibiting worse outcomes than ApoE3, that apoptotic cell death does not play a role by 21 DPI.

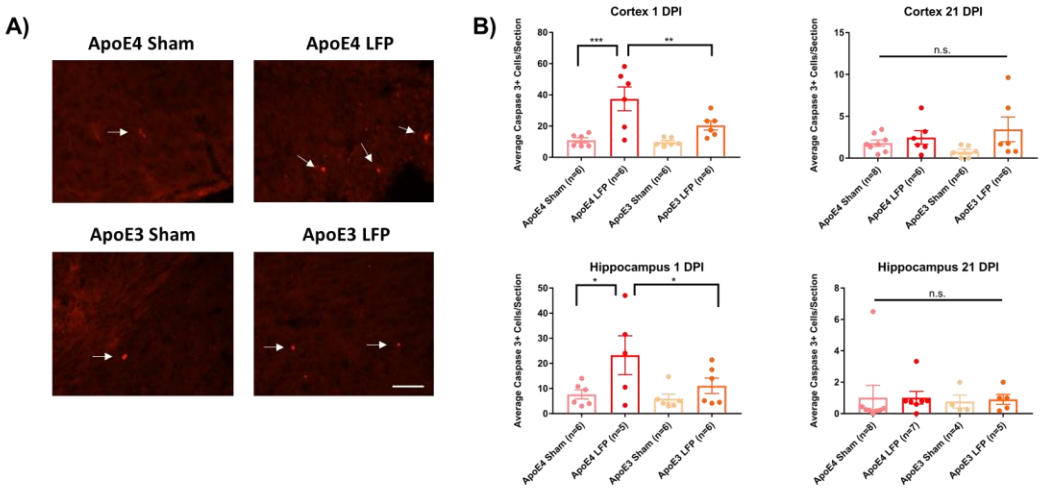



*Figure 16. Repeated mild LFP injury causes an increase activated caspase-3 positive cells in brains of injured ApoE4 mice compared to injured ApoE3 mice at 1 DPI in the cortex and the hippocampus but not at 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with activated caspase-3 (indicated by arrows). Scale bars = 100µm.* ***(B-E)*** *Quantitation of the average number of activated caspase-3 positive cells per cortical or hippocampal section ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

## Neurodegeneration is increased in ApoE4 injured mice compared to ApoE3 injured mice in the cortex and hippocampus at 1 DPI.

In addition to apoptosis, after injury there is known to be neurodegeneration. [26, 189] We used Fluorojade C (FLJC), a marker for neurodegeneration [135], in order to ascertain the level of neurodegeneration in the ipsilateral cortex and hippocampus. We found that at 1 DPI, ApoE4 injured mice had significantly more FLJC positive cells in both the ipsilateral cortex and hippocampus relative their sham controls. Notably, we found that ApoE4 injured mice also had significantly more FLJC positive cells in both the ipsilateral cortex and hippocampus relative to ApoE3 injured mice. (Fig 17A-B). By 21 DPI, levels of neurodegeneration had decreased back to baseline so that there was no difference between the injured mice and their sham controls in both the cortex and hippocampus. (Fig 17A-B). These data suggest that the neurodegeneration process begins as early as 1 DPI, but that levels of neurodegeneration in response to injury are returned to baseline by 21 DPI.

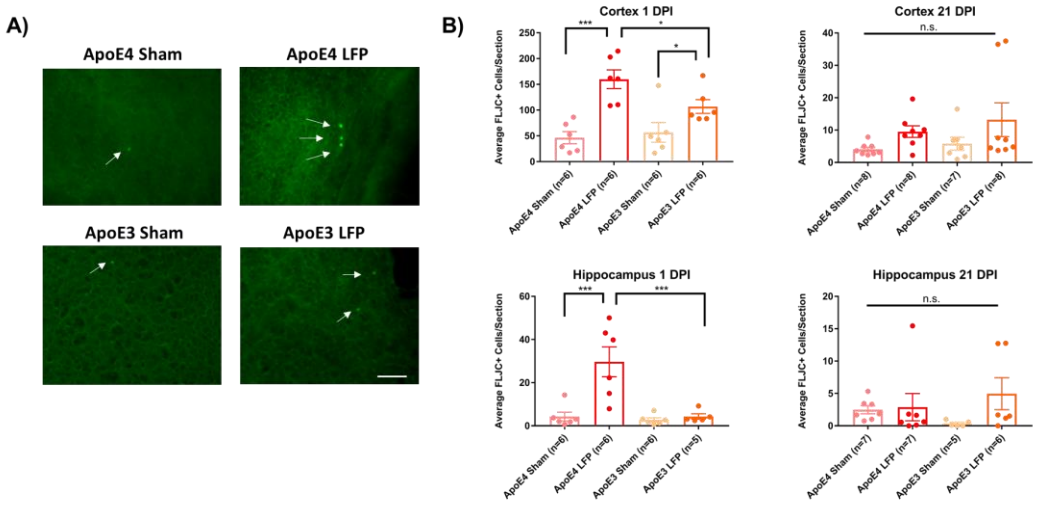



*Figure 17. Repeated mild LFP injury causes an increase in Fluoro-Jade C (FLJC) positive cells in the brains of injured ApoE4 mice compared to injured ApoE3 mice at 1 DPI in the cortex and hippocampus but not at 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with FLJC (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of FLJC positive cells per cortex or hippocampus ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

## Activated microglia are increased in ApoE4 injured mice compared to ApoE3 injured mice in the cortex and hippocampus at 1 DPI.

It has been shown that activated microglia are an important part of the secondary injury process and have been shown to persist for years after the initial injury and contribute to long-term neurological dysfunction. [197] To examine the effect that repeated mild traumatic brain injury has on the neuroimmune system, we analyzed activated microglia. We used IBA1 as a marker for microglia and morphology to distinguish activated microglia from non-activated microglia. Non-activated microglia were distinguished by their ramified appearance, while activated microglia were in either the reactive bushy state or the phagocytic ameboid shape.[198] We found that at 1 DPI ApoE4 injured mice has significantly more activated microglia than their sham controls in both the ipsilateral cortex and the hippocampus. Importantly, we found that at 1 DPI ApoE4 injured mice has significantly more activated microglia than the ApoE3 injured mice in the ipsilateral cortex

or hippocampus. (Fig 18 A-B) These data suggest that ApoE4 injured mice have earlier microglial activation than the ApoE3 injured mice. However, by 21 DPI both the ApoE4 and ApoE3 injured mice both had significantly more activated microglia than their sham controls, but the ApoE4 injured mice no longer had increased numbers of activated microglia relative to ApoE3 injured mice. (Fig 18 A-B)). These data suggest that ApoE4 injured mice respond to repeated mild injury by activating microglia earlier than ApoE3 injured mice, but by 21 DPI levels have decreased so that there is no difference between the ApoE4 injured mice and ApoE3 injured mice.

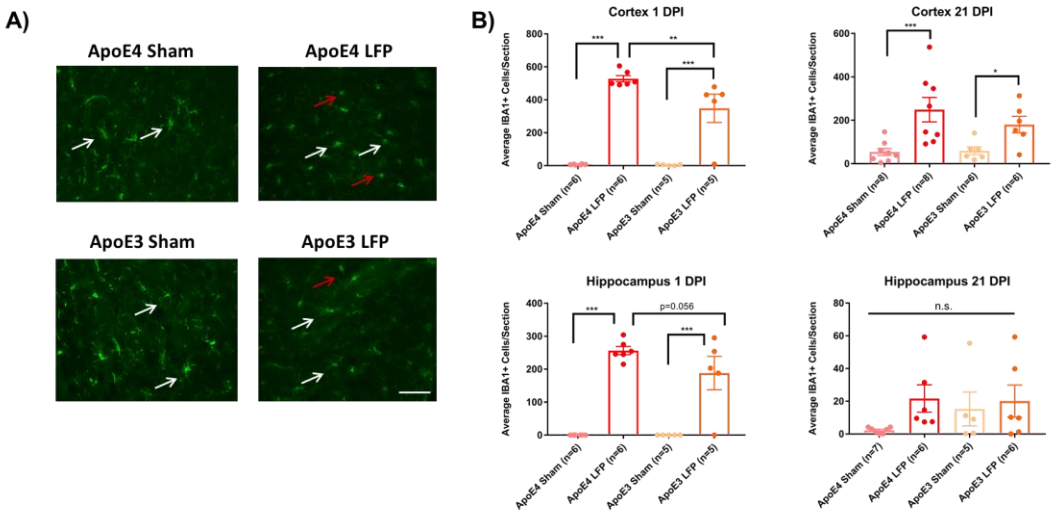



*Figure 18. Repeated mild LFP injury causes an increase in ionized calcium binding adaptor molecule 1 (IBA1) positive cells in the brains of injured ApoE4 mice compared to injured ApoE3 mice at 1 DPI in the cortex but not at 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with IBA1 (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of IBA1+ positive cells, broken down into activated and resting categories by morphology, per cortex and hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Phosphorylated tau is increased in ApoE4 injured mice compared to ApoE3 injured mice at 21 DPI.**

It has previously been shown that phosphorylated tau can contribute to long-term pathologies in the brain.[194] After injury, there are frequently higher levels of phosphorylated tau at the site of injury as well as in distally affected brain areas.[195] We investigated the levels of phosphorylated tau at 1 and 21 DPI in both the ipsilateral cortex and hippocampus. We found that there was a significant increase in levels of phosphorylated tau in both ApoE4 and ApoE3 injured mice compared to their sham controls in the ipsilateral cortex at 1 DPI. (Fig 19A-B) Yet, at 1 DPI there was no significant difference in phosphorylated tau levels between the ApoE4 injured mice and the ApoE3 injured mice. However, by 21 DPI in the cortex the levels of phosphorylated tau in the ApoE3 injured group had returned to their sham control levels, while the ApoE4 injured levels remained elevated and significantly different from their sham controls and the ApoE3 injured group. This suggests that ApoE4 injured mice have an exacerbated phosphorylated tau reaction which may lead to long term neurodegeneration. (Fig 19A-B)

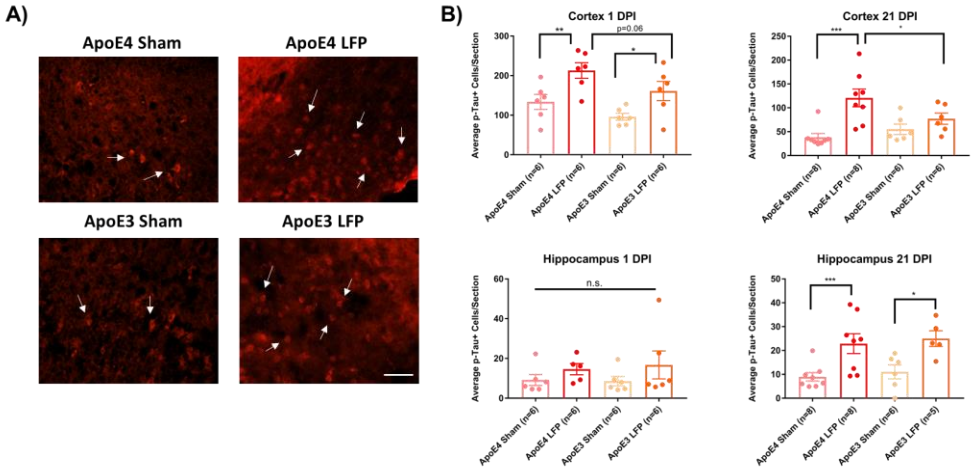



*Figure 19. Repeated mild LFP injury causes an increase in phosphorylated tau (p-tau) in the brains of injured ApoE4 mice compared to injured ApoE3 mice at 21 DPI in the cortex.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with activated AT8 (indicated by arrows). Scale bars = 100μm.* ***(B-E)*** *Quantitation of the average number of FLJC positive cells per cortex or hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Astrogliosis is increased after rmTBI, but there is no difference in levels between injured ApoE4 and ApoE3**

After injury, it is well documented that there is proliferation of glia [26, 189]. The proliferation of glia can contribute to the formation of glial scarring, inhibiting the ability of neurons to regenerate, and preventing the injured brain from recovering normal morphology and function. [196] Using glial fibrillary acidic protein (GFAP), a marker for astrocytes, we quantified the level of gliosis after injury. We found that after our rmTBI model, the injured groups had an increase in GFAP positive cells relative to their sham controls in the cortex and hippocampus, but that there was no difference between the two injured groups at 1 and 21 DPI. (Fig 20A-B) This suggests that any differences observed between the Val66Met injured mice and the Val66Val injured mice are not due to differences in levels of astrogliosis.

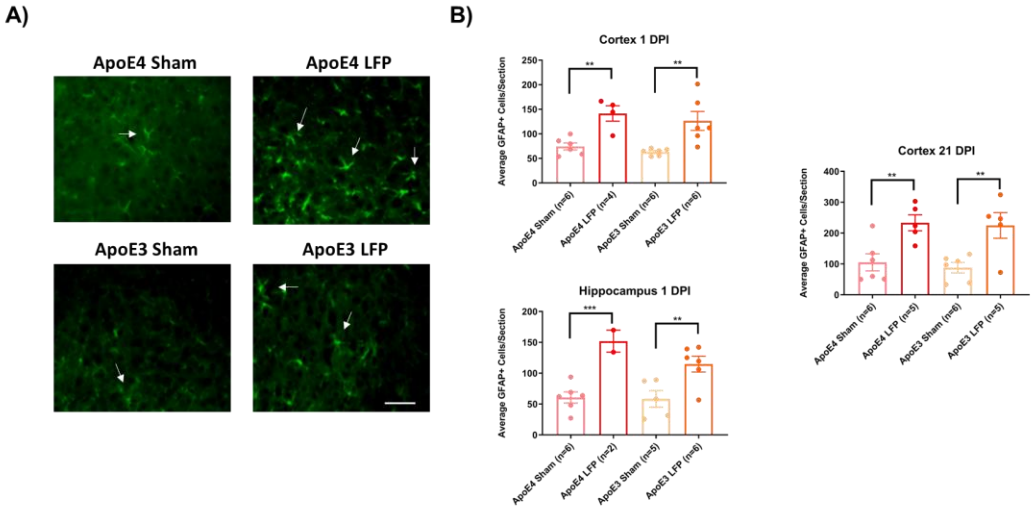



*Figure 20. Repeated mild LFP injury does not cause an increase in glial fibrillary acidic protein (GFAP) positive cells in the brains of injured ApoE4 mice compared to injured ApoE3 l mice at 1 DPI or 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with glial fibrillary acidic protein (indicated by arrows). Scale bars = 100µm.* ***(B-E)*** *Quantitation of the average number of GFAP positive cells per hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =5-8.*

**Injured ApoE4 mice have less total BDNF than injured ApoE3 mice in the cortex and hippocampus at 1 and 21 DPI.**

Previous studies have shown that ApoE4 carriers have less BDNF in the serum of patients with apathy [97] and in brain tissue in patients with AD [99]. It has also been shown that ApoE4 carriers have less BDNF secreted in primary hippocampal astrocyte cultures [99]. One proposed mechanism for this is that ApoE4 increases nuclear translocation of histone deacetylases, which has the downstream effect of reducing BDNF levels. [100] However, to our knowledge, no one has investigated levels of BDNF in the different ApoE genetic polymorphisms after rmBI. We examined levels of proBDNF (32 kD), truncated BDNF (28kD), and mature BDNF (14kD) after injury in order to determine levels of total BDNF in both ApoE4 and ApoE3 injured compared to their sham controls. We found that after injury, levels of total BDNF are significantly higher in ApoE3 injured mice relative to their sham control in the cortex and hippocampus at 1 and 21 DPI. (Fig 21A-B) Of importance, we also saw that there was a significantly more total BDNF in ApoE3 injured mice than there was in ApoE4 injured mice, and that all forms of BDNF (pro, truncated, and mature) were elevated in these mice. These data support our conclusion that there is a differential response to injury in ApoE3 compared to ApoE4 carriers and highlights a potential pathway to target to therapeutics.

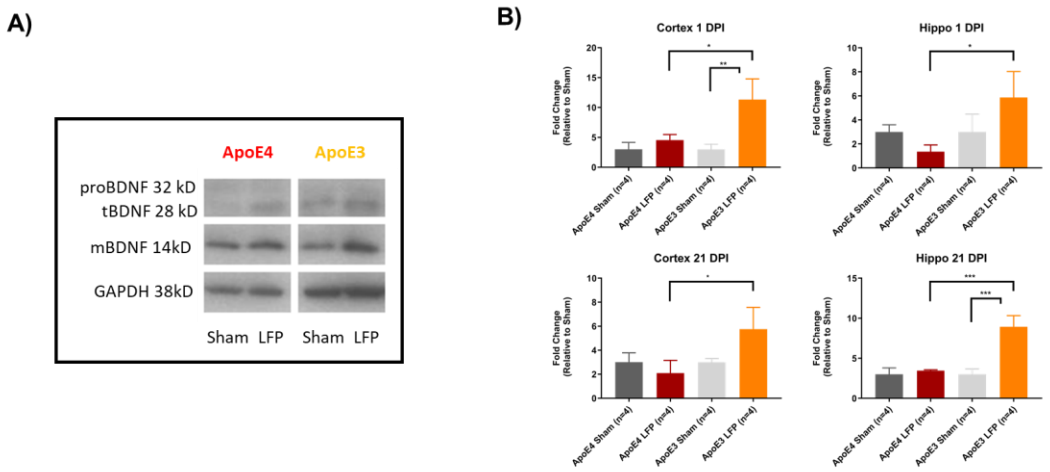
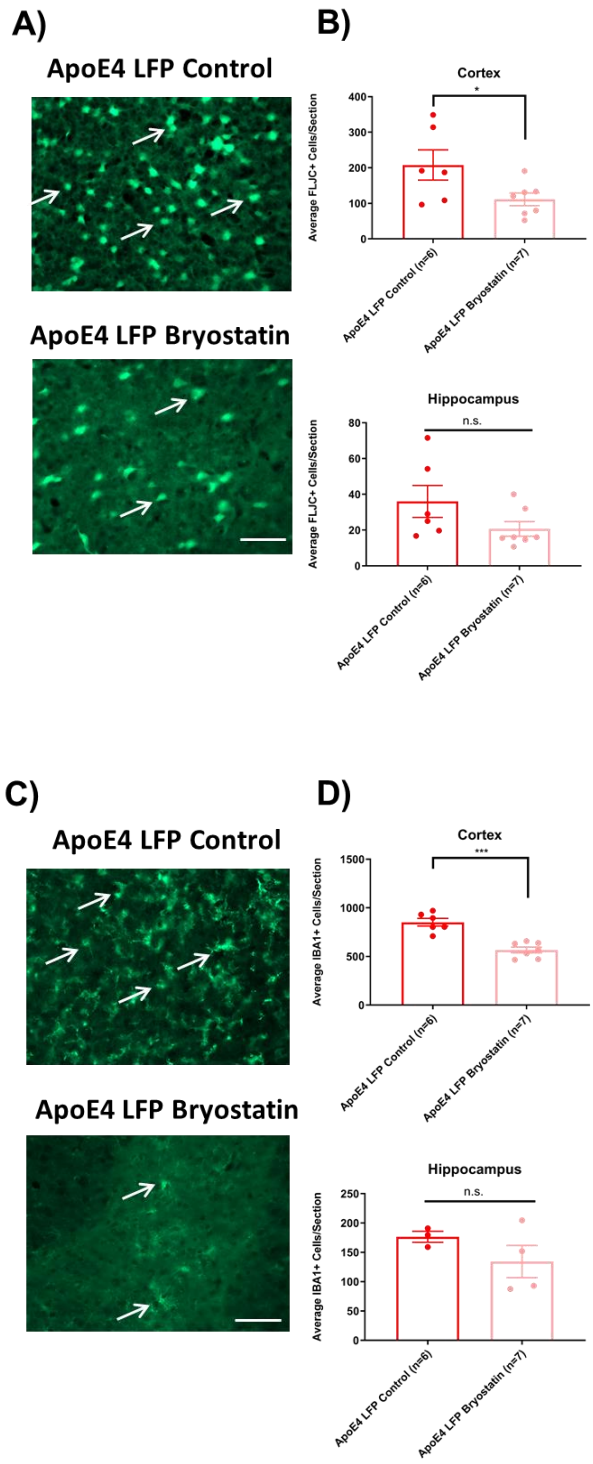



*Figure 21. Repeated mild LFP injury causes an increase in total BDNF expression in ApoE4 carriers compared to ApoE3 carriers in the cortex and hippocampus at 1 and 21 DPI.* ***(A)*** *Representative Western Blot showing total BDNF expression after injury. Each lane represents one animal.* ***(B)*** *Quantitation of protein levels in the cortex and hippocampus at 1 DPI and 21. All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative to the average ± SEM of the time matched sham controls. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=4.*

**Treatment of ApoE4 injured mice with Bryostatin 1 reduces the excessive neurodegeneration and activated microglia, but not apoptotic cell death, seen in the injured ApoE4 mice back to the levels seen in the injured Apoe3 mice at 1 DPI**

Analysis of our immunohistochemical data suggests that ApoE4 injured mice have worse recovery after rmTBI than their sham controls and ApoE3 injured mice. Our western blot data suggests that there are differences in levels of total BDNF that may be affecting outcomes after injury. In order to determine if we can treat ApoE4 carriers with a personalized therapy to ameliorate this risk, we used Bryostatin 1, a PKCε activator, which has previously been used to treat ApoE4 carriers with AD [163], after stroke [219], and after blast injury [164]. We found that when treated with 2 doses of 20 ug/kg that ApoE4 injured mice had significantly fewer FLJC+ cells and activated IBA1+ at 1 DPI in the cortex than ApoE4 injured mice treated with control PBS. (Fig 22A-D) We found no effect of Bryostatin 1 treatment in ApoE4 injured mice relative to ApoE control injured mice on

levels of activated caspase 3 (Fig 22E-F), suggesting that activating PKCε may have an effect on inflammation and neurodegenerative necrotic death but not the apoptotic controlled cell death pathway in the injured brain.



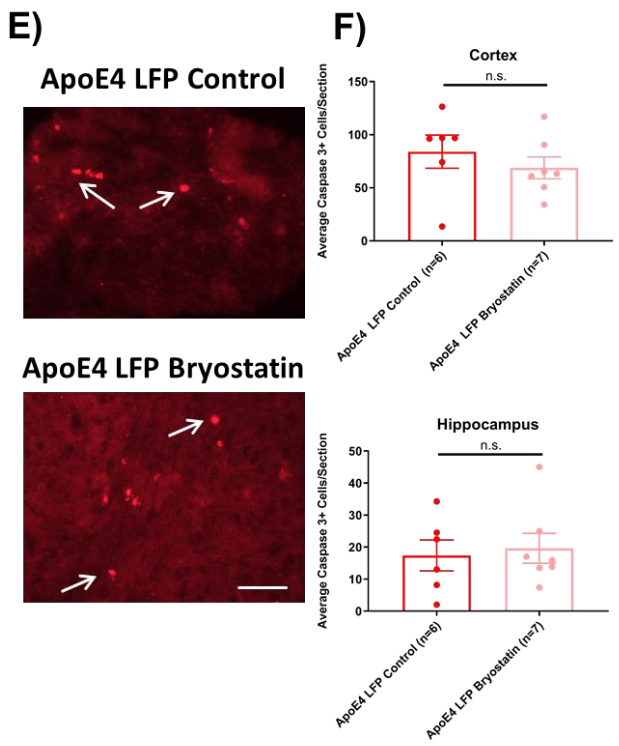



*Figure 22. Treatment of injured ApoE4 mice with Bryostatin-1 after repeated mild LFP injury causes a significant decrease in astrogliosis in the cortex and hippocampus and activated microglia in the cortex but not p-tau at 21 DPI.* ***(A)*** *Representative images of cortical sections at 1 DPI stained with FLJC. Scale bars = 100µm.* ***(B)*** *Quantitation of the average number of FLJC positive cells per cortex and hippocampus ± SEM.* ***(C)*** *Representative images of cortical sections at 1 DPI stained with IBA1. Scale bars = 100µm.* ***(D)*** *Quantitation of the average number of IBA1 positive cells per cortex and hippocampus ± SEM.* ***(E)*** *Representative images of cortical sections at 1 DPI stained with activated caspase 3. Scale bars = 100µm.* ***(F)*** *Quantitation of the average number of activated caspase 3 positive cells per cortex and hippocampus ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=6-7.*

## Treatment of ApoE4 injured mice with Bryostatin 1 improves learning at 2 DPI and memory at 7 DPI back to levels seen in ApoE3 injured mice

We next examined the effect that Bryostatin -1 treatment after injury in ApoE4 mice has on cognitive function using the Morris Water Maze to study spatial learning and memory. It has been established that brain injury can have detrimental effects on cognition, especially in animals that have defects in the hippocampus. [26, 199] Here, we found that ApoE4 control injured mice had a longer latency to find the hidden platform that ApoE3 control injured mice, suggesting an impairment in spatial learning, at 1 DPI and 2 DPI.

Importantly we found that when treated with 5 doses of 20 ug/kg of Bryostatin-1, that ApoE4 injured mice found the hidden platform in significantly less time than their ApoE4 control injured counterparts at 2 DPI. (Fig 23B) In the Probe Trial at 7 DPI, we found that ApoE4 control injured mice spent more time in the target NE quadrant than ApoE3 control injured mice, indicating impaired spatial memory. Importantly, we found that ApoE4 Bryostatin-1 injured mice spent significantly more time in the target NE quadrant than the ApoE4 control injured mice. (Fig 23C) These data suggest that treatment with Bryostatin-1 is able to improve learning and memory deficits in vulnerable ApoE4 injured mice following rmTBI in addition to improving immunohistochemical differences.

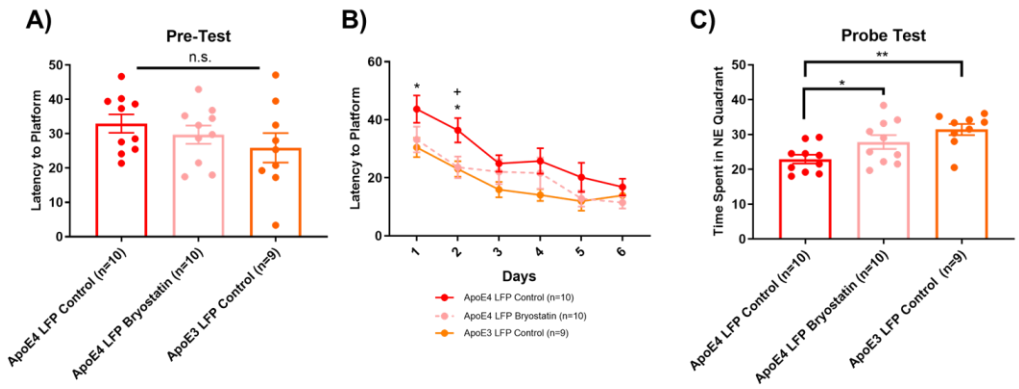



*Figure 23. Repeated mild LFP injury causes worse learning and memory in ApoE4 injured mice relative to ApoE3l injured mice. Treatment with Bryostatin in injured ApoE4 mice improves learning 1 and 2 DPI and memory at 7 DPI.* ***(A)*** *Average latency to platform in pretest phase ± SEM.* ***(B)*** *Average latency to platform ± SEM 1-6 DPI.* ***(C)*** *Average time spent in the target quadrant in the probe test ± SEM at 7 DPI. * p < 0.05, ** p < 0.01, *** p < 0.001 in ApoE4 LFP Control relative to ApoE3 LFP Control, + p < 0.05, ++ p < 0.01, +++ p < 0.001 in ApoE4 LFP Control relative to ApoE4 LFP Bryostatin. ANOVA Fisher's PLSD post-hoc test relative to indicated groups. n=9-10*

**<u>Treatment of ApoE4 injured mice with Bryostatin-1 improves fine motor balance at 7 DPI, but not gross vestibular motor function, back to levels seen in ApoE3 injured mice</u>**

It has previously been shown that following brain injury, there can be deficits in motor ability. [199, 200] This is particularly true with our model of lateral fluid percussion, due

to the damage that is done to the sensorimotor cortex at the site of injury. [201] In order to measure motor ability, we used the Balance Beam Test for more subtle differences in motor skills and balance and the Rotarod Test for vestibular motor function and proprioception. For the Balance Beam Test, we found that ApoE4 control injured mice had a higher balance beam score, indicative of worse balance, than the ApoE3 control injured mice at 7 DPI. By 21 DPI balance beam scores decreased so that there were no significant differences between groups. (Fig 24A-B) Of note, we found that ApoE4 control injured mice treated with 5 doses of 20 ug/kg of Bryostatin-1 had a significantly decreased balance beam score at 7 DPI, indicating that treatment with Brysoatin-1 was able to improve fine motor balance skills. For the Rotarod Test, we found that ApoE4 control injured mice had a shorter latency to fall relative to ApoE3 control injured mice, indicating impaired vestibular motor function at 1, 7, and 21 DPI. However, we found that ApoE4 injured mice treated with Bryostatin-1, that the latency to fall was not significantly different than their ApoE4 control injured counterparts, suggesting that treatment with Bryostatin-1 does not increase gross vestibular motor skills. (Fig 24C-E)

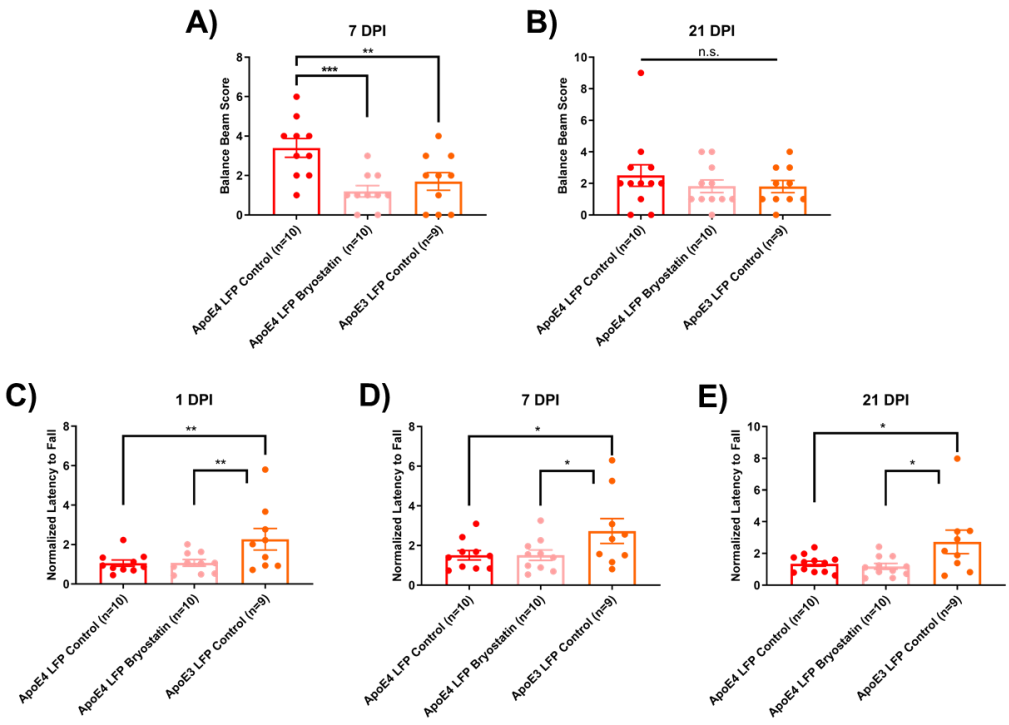



*Figure 24. Repeated mild LFP injury causes impaired fine motor balance and vestibular motor function in injured ApoE4 mice relative to injured ApoE3 mice. At 7 DPI, treatment of injured ApoE4 mice with Bryostatin improves balance but not vestibular motor function.* ***(A)*** *Quantitation of latency to fall in the rotarod pretest assay* ***(B)*** *Quantitation of the latency to fall in the rotarod assay at 14 DPI and* ***(C)*** *21 DPI.* ***(D)*** *Quantitation of balance beam score at 21 DPI. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n =9*

## Treatment of ApoE4 injured mice with Bryostatin-1 does not increase BDNF or p-trkB levels

In order to determine how treatment with Bryostatin-1 was improving recovery after rmTBI, we performed Western Blot analysis on Val66Met injured mice treated with Bryostatin-1 or control PBS at 1 DPI to investigate levels of BDNF and p-trkB, since previous research has shown that treatment with Bryostatin-1 increases BDNF levels. [163] We found no increase in BDNF or p-trkB levels in Bryostatin-1 treated Val66Met injured mice relative to control PBS treated Val66Met. (Fig 25A-F) This suggests that the results we see at 1 DPI are due to another pathway.

Taken altogether, these data provide evidence for the role of Bryostatin-1 as a potential treatment after rmTBI, particularly for ApoE4 carriers. These results highlight the role that activating PKCε by using Bryostatin-1 can play in genetically susceptible individuals to resolve neurodegenerative necrotic cell death and neuroimmune inflammation, as well as the effects it can have on learning, memory, and balance.

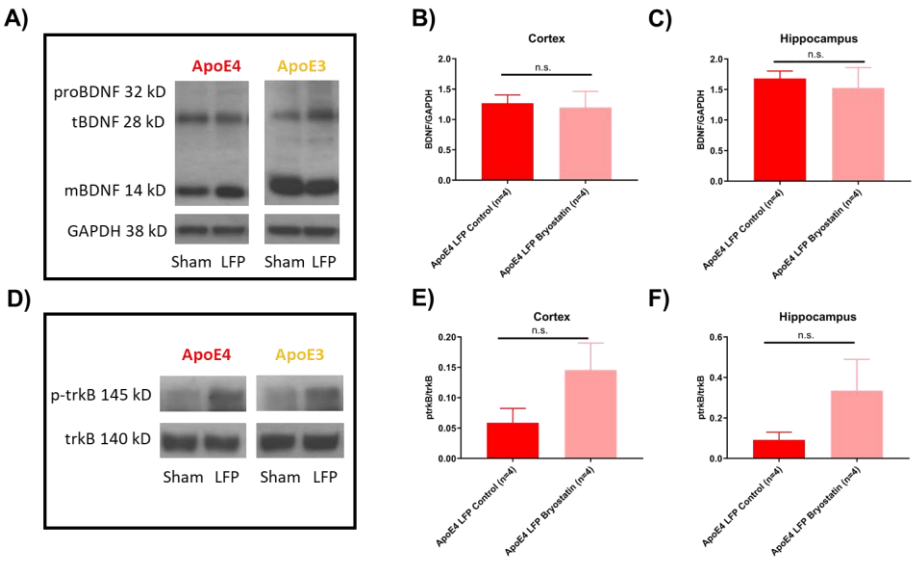



*Figure 25. Bryostatin treatment does not cause an increase in total BDNF or ptrkB expression relative to control in the cortex and hippocampus at 1 DPI in injured ApoE4 mice.* ***(A)*** *Representative Western Blot showing BDNF or* ***(D)*** *ptrkB expression in the cortex and hippocampus after injury. Each lane represents one animal.* ***(B)*** *Quantitation of BDNF protein levels at 1 DPI in the cortex* ***(C)*** *and hippocampus.* ***(E)*** *Quantitation of p-trkB protein levels at 1 DPI in the cortex* ***(F)*** *and hippocampus. All data is first normalized to GAPDH or trkB to control for protein loading ± SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA Fisher's PLSD post-hoc test relative to indicated groups, n=4.*

## Discussion of Results:

In this study, we report that the Apo4 genetic polymorphism impedes recovery relative to the ApoE3 genetic polymorphism after rmTBI in a mouse model. We used the LFP model of injury in our mouse study because it is able to recreate injuries similar to those seen in the human population, with both focal and diffuse components. [26, 110, 111, 202] We

show that at 21 DPI the ApoE4 injured mice have a larger injury volume as assessed by MRI than the ApoE3 injured mice. In order to gain an appreciation for the biological processes that are activated at different time points after injury, we investigated cellular changes at 1 and 21 DPI, in both the ipsilateral cortex, to examine the focal effects of injury, as well as the hippocampus, to consider the diffuse effects. We found that at 1 day after the final injury, there was more cell death, neurodegeneration, and activated microglia in the Apo4 injured mice than the ApoE3 injured mice in the cortex and hippocampus. By 21 days after the final injury, we found that levels of cell death and neurodegeneration were reduced back to baseline sham levels, and the levels of activated microglia in the ApoE4 injured mice were no longer significantly elevated relative to ApoE3 injured mice. However, we did find that levels of p-tau which were elevated in both ApoE3 and ApoE4 injured mice at 1 day after the final injury in the cortex relative to their sham controls, were reduced back to sham levels in the ApoE3 injured group, but remained elevated in the ApoE4 injured group. We saw the most dramatic effects in the cortex, due to the fact that this was the primary site of injury, while also seeing some effects in the hippocampus. These data show that the injured ApoE4 mice respond differentially to a repeated mild injury than injured ApoE3 mice starting as early as one day after the final injury and lasting until at least 21 days after the final injury.

Previous work has shown that ApoE plays an important part in brain recovery after injury, due to its support of neuronal repair mechanisms. [69-71] After injury, ApoE has been shown to play a role in the recovery of the blood brain barrier [220], the clearance of neurotoxic fragments [221], and suppressing pro-inflammatory factors [222]. In fact, studies have shown that ApoE mimetics are able to ameliorate detrimental neurodegeneration seen in an AD mouse model. [223] However, there appear to be

differences in how effectively the isoforms of ApoE are able to complete these tasks. Previous work has shown that in the brain, ApoE4 may perform these tasks less well than ApoE3, and that proteolytic cleavage of ApoE4 may actually contribute to the collection of neurotoxic fragments that can result in long-term pathology. [223] However, it is less well understood how these genetic polymorphisms play a role in acute recovery after rmTBI, and if there are any chronic effects that can be seen after this injury paradigm.

We have demonstrated that injured ApoE4 mice have more cells undergoing apoptotic cell death and neurodegeneration in the acute phase of injury than the injured ApoE3. Previous studies have shown that elevated levels of apoptosis and neurodegeneration are tied to worse outcomes after injury [130, 133, 135]. It has also been shown that ApoE4 results in elevated levels of apoptosis [224] and neurodegeneration [225] relative to ApoE3 acutely after injury. However, in the chronic phase of injury, levels of apoptotic cell death and neurodegeneration in the injured ApoE4 mice return back to sham baseline conditions. This is consistent with previous reports [203, 204], and indicates that other pathways may play a role at later timepoints in the development of chronic injury processes.

In addition to apoptosis and neurodegeneration, acutely after injury there is an increase in inflammation and the activation of the neuroimmune system. Commonly seen, is the activation of the resident neuroimmune cells of the brain, microglia, and astrocytes. Both astrocytes and microglia play an important role in maintaining homeostasis in the brain, and after injury they are activated in order to initiate the neuroimmune response to injury. [139, 142] Previous work has highlighted the role that increased activation of the immune system can play when interacting with the ApoE isoforms, with the ApoE4 isoform simulating pro-inflammatory factors [226] and the ApoE3 isoform promoting a reduced

M1 microglia activation. [227] We observed that as early as 1 day after the final injury, injured ApoE4 mice have elevated activated microglia relative to injured ApoE3 mice, but by 21 days after the final injury, levels of activated microglia decreased in both groups so that, although they were both still elevated relative to their sham controls, there was no difference between the two injured groups. This suggests that while levels of differential elevation of activated microglia may play a role acutely after injury, that neuroinflammation does not seem to persist to cause chronic differences between the two genotypes. Previous research has shown that there is a distinct difference in morphology of the microglia in primary microglial culture between the ApoE4 relative to ApoE3 mice. Specifically, ApoE4 cultures displayed more ameboid "activated" forms, while the ApoE3 mice had more ramified "resting" forms. [226] Similarly to our microglia results, we found at both at acute and chronic time points after injury, that levels of astrocytes were increased in the two injured groups relative to their sham controls, but that there were no differences between the two injured groups (data not shown), indicating that gliosis does not play a role in genotypic differences in outcomes from repeated mild lateral fluid percussion at these timepoints. Given that previous work has shown that gliosis can interact with the ApoE4 isoform to cause worse long-term neurodegeneration [228], we may be examining gliosis too early in the process to determine any significant differences between groups.

One important contribution to long term neurodegeneration is the hyper phosphorylation of tau. After injury, tau can become hyper phosphorylated, which allows it to bind to other tau proteins and form aggregates. These aggregates can disrupt normal functioning in the brain and lead to chronic neurodegeneration. [94, 136, 137] We have shown that injured ApoE4 mice and injured ApoE3 mice both have elevated levels of phosphorylated tau relative to their sham controls as early as 1 day after the final injury, but that by 21 days

after the final injury, the levels of phosphorylated tau in injured ApoE3 mice have been reduced back down to sham levels, while the levels of phosphorylated tau in injured ApoE4 mice remain elevated. These data highlight phosphorylated tau as a potential cause of long-term neurodegeneration in ApoE4 injured mice and differential outcomes between injured ApoE4 mice and injured ApoE3 mice. These results make logical sense, given that previous work has shown that ApoE4 carriers have impaired autophagy, and clear tau more slowly. [229] Previous work has also shown that proteolysis of the ApoE4 isoform leads to an increase in hyper phosphorylated tau in ApoE4 mice relative to ApoE3 mice, which can lead to neuronal deficits. [230] It is known that the hyper phosphorylation of tau increases with the combination of the ApoE4 isoform and age [231], highlighting the role it may play in the development of the neuronal deficits seen in Alzheimer's Disease. Importantly, we show here that rmTBI in young adults can also stimulate this relative increase in phosphorylated tau in injured ApoE4 mice compared to injured ApoE3 mice.

Based on the results presented in this study, we conclude that the ApoE4 genetic polymorphism is a risk allele after rmTBI. These data are consistent with previous literature about the role of ApoE4 genetic polymorphisms in rmTBI models. Previous research has found that after repeated blast injury, ApoE4 injured mice had more p-tau regulated by synj1, an important regulatory lipid phosphatase, relative to ApoE3 injured mice. [95] Another recent study examined the effect of ApoE4 relative to ApoE3 in 12-month-old "middle aged" mice that underwent repeated mild injuries for a month. They found that ApoE4 was not a risk factor for increased astrogliosis or total microglia levels, but that it was for increased levels of MC1, a tau conformational marker. [96] Another group attempted to investigate the role that ApoE4 has on recovery after a repeated mild CCI injury and found that there was no effect of the ApoE4 genotype on recovery in their model.

However, given their parameters they used, which involved a very mild injury, it is possible that they were unable to distinguish any small differences that may be occurring. In addition, they used WT C57BL/6 mice as the control mice, which have the mouse gene sequence of ApoE4 and since that mice have the ancestral allele which is homologous to ApoE3, that study is not an accurate comparison of the difference between the human ApoE3 and ApoE4 alleles. [94]

Given that there seems to be a genotypic difference between the two isoforms and how they recover after rmTBI, it is necessary to investigate the pathway through which these differences may be mediated. Previous studies have postulated that the mechanism may be due to the role that ApoE plays in neural repair, with ApoE4 carriers having impaired ability to repair membranes after injury, while others have suggested that it is due to the neurotoxic fragments that are created when ApoE4 is proteolytically cleaved. [69-71] Recent studies have begun to investigate the role that the ApoE4 genetic polymorphism has on neurotrophic factors in the brain. Studies have found that AD ApoE4 carriers have lower levels of Brain Derived Neurotrophic Factor (BDNF) in the serum [97], and that mildly cognitively impaired ApoE4 carriers have dampened BDNF increase after exercise compared to non-carriers. [98] Interestingly, it has been found that ApoE4 causes reduced BDNF release by increasing the nuclear translational of histone deacetylases (HDAC) 4/6. This action causes negative gene regulation, and results in less BDNF being produced in the cell and secreted by astrocytes. [99, 100] It is known that BDNF signaling plays an important role in recovery after injury [26, 101], making this a potential therapeutic target for ApoE4 carriers.

In order to determine the effect that the ApoE genetic polymorphism has on BDNF levels after injury, we conducted Western Blot analysis probing for BDNF in the cortex and the hippocampus at both 1 and 21 DPI. We found that injured ApoE4 mice have lower levels of total BDNF than injured ApoE3 mice. These data highlight the role that alterations in BDNF signaling may play in differential outcomes after injury seen in ApoE mice.

Currently, the investigation of Bryostatin 1, an activator of PKCε when given in lose doses over short time courses, is underway as a treatment option for AD. [163] Bryostatin 1 has been shown to increase BDNF levels and improve cognitive ability in mouse studies performed in an AD model, showing promise that it may be effective for improving outcomes in ApoE4 carriers after injury as well. For this reason, we chose to use Bryostatin-1 as a personalized treatment for injured ApoE4 mice.

We found that treatment with Bryostatin-1 was able to reduce the elevated levels of neurodegeneration and activated microglia, but not cell death, in injured ApoE4 mice relative to control injured ApoE4 mice. It is possible that we did not see effects of the Bryotstain-1 treatment on cell death because that pathway is activated very quickly after injury, and it's possible that Bryostatin-1 does not work fast enough, especially given that the first Bryostatin-1 injection was not given until after the final injury. Bryostatin-1 treatment also was able to increase learning 2 days after the final injury in the Morris Water Maze Test and memory 7 days after the final injury in the Morris Water Maze Probe Test. Treatment also improved fine motor balance in the balance beam test, but was unable to improve gross vestibular motor ability in the rotarod test. These data suggest that Bryostatin-1 is acting to increase positive gene regulation, potentially increasing BDNF levels, as well as other beneficial modulatory factors after injury to improve outcomes.

Importantly, our study was done at mice at 10-12 weeks, the equivalent of young adulthood in humans. We are able to see differences between the genotypes after rmTBI at this early age that may contribute to long term pathology which are usually not seen in mice until 6-7 months. [230] Therefore, this study lays the groundwork for understanding the differential outcomes seen after rmTBI in ApoE genetic polymorphic mice and highlights the potential that personalized therapies may be able to play in ameliorating symptoms and improving outcomes. However, our study was limited by a number of factors that future studies should attempt to improve upon. First of all, we choose the 1 and 21 DPI timepoints based on our previous studies and knowledge that they are times after injury where many important processes will be occurring. However, based on the fact that this is a rmTBI paradigm, there may be some value in investigating outcomes after only 1 or 2 hits, in order to gain a fuller picture of the cumulative effect of the repeated injuries. In addition, we looked at the 21 DPI as our longest timepoint, but given that effects of rmTBI in humans are often seen years after the fact, future studies may benefit from including longer term outcomes such as 6 months-1 year to determine if there are long term consequences to our rmTBI paradigm. Moreover, we chose to use a very controlled experimental paradigm to investigate the effect of one singular genetic polymorphism on outcomes after rmTBI. This afforded us benefits in limiting confounding factors but does not take into account the complex genetic background that many people who sustain rmTBI have. Future studies would be wise to investigate the role of other genetic polymorphisms in combination with ApoE4, such SNPs that exist in the BDNF gene like rs6265 to determine if these genetic polymorphisms together act to compound the damage seen after rmTBI.

To conclude, this study has investigated the role of the ApoE4 genetic polymorphism on outcomes after rmTBI such as injury volume, cellular markers, protein levels, and behavior. We have explored the use of Bryostatin- 1 as a personalized treatment method for these carriers and have shown that it is able to alleviate some of the damage seen after rmTBI. This report highlights ApoE4 as a risk factor for poor outcomes after rmTBI and the potential usefulness PKCε activators for treatment in this genotype.

# Chapter 5: Encapsulated Mesenchymal Stem Cells

## Introduction:

Human mesenchymal stem cells, or mesenchymal stromal cells, (MSCs) are stem cells that are specialized stem cells derived from the bone marrow in humans, that are able to differentiate into the mesodermal lineage. In addition to bone marrow, they have also been derived from adipose, amniotic fluid, endometrium, dental pulp, skin, the umbilical cord, and Wharton's jelly. [232] Importantly, MSCs are immunomodulatory, and have the ability to secrete anti-inflammatory molecules and cytokines. This fact makes them a potentially potent treatment source of anti-inflammatory molecules for a wide variety of conditions hallmarked by an increased activation of inflammatory and immune molecules, such as TBI.

MSCs are required to fit a minimum of three criteria in order to be classified as such. They must have plastic adherence, they must possess a specific set of cell surface markers (+ for CD73, D90, CD105 and – for CD14, CD34, CD45, and HLA-DR), and have the ability to differentiate into adipocytes, chrondrocytes, and osteoblasts in vitro. [233] There is some variation in cell subtype, based on the tissue from which the MSCs are derived, complicating the process of using them for cellular therapy.

Importantly, for MSCs to be able to differentiate into neuronal cells, they can come from any known source except the dermis. [234, 235] Studies have shown that when cultured with neural progenitor maintenance medium, within a few passages, cells are positive for neuronal nuclear antigen (NeuN), neuron specific enolates (NSE), Nestin, and glial fibrillary acidic protein (GFAP), signaling the differentiation into neural cells. [236]

MSCs are a potentially valuable cellular therapy due to the fact that they only illicit a weak immune response, owing to their lack of MHC class II markers. [237] They have been used as an experimental therapy in a number of neural disease states such as Alzheimer's disease [238], Huntington's disease [239], Parkinson's disease [240, 241], amyotrophic lateral sclerosis [242], stroke [243], spinal cord injury [244], and traumatic brain injury [168].

In addition, the ability of MSCs to be modified in order to influence their secretome represents a powerful cell transplantation therapy model. Groups have used pre-treatment with factors to influence the secretome of MSCs [245] or they have directly genetically engineered them [246] in order to achieve this. Interestingly, research has shown that MSCs at baseline secrete neurotrophic factors such as BDNF and NGF [247], and that by genetically modifying MSCs to secrete increased levels of these neurotrophic factors, they could be a powerful treatment option in disease states where low neurotrophin levels may be responsible for lack of neuronal repair and recovery. [240, 243, 246]

One method to modify MSCs is to encapsulate MSCs with alginate which has the effect of increasing the time that they stay viable, increasing their secretome to be a beneficial one, and limiting their movement from the site of injury. [179] Encapsulated MSCs have been shown to improve outcomes in spinal cord injury [180], and are beginning to be tested in TBI. [181] Some labs have embedded MSCs in hydrogel materials with or without other factors, such as BDNF peptide mimetics as a treatment for TBI. [182]

However, there is still a lack of experimental research on the use of MSCs as a treatment

for rmTBI in mouse models. [248] This important work will need to be done in order to test the safety and efficacy of MSCs for treatment of rmTBI in humans. In addition, there remains a significant amount of fine tuning to be done in terms of MSC treatment optimization. In this study, we investigated the potential of MSCs as a treatment after rmTBI and investigated ways to optimize the treatment for experimental effects in a mouse model.

## Experimental Results:

### Free MSCs secrete BDNF

To investigate the potential of MSCs for treatment after TBI, we began by evaluating the secretome of MSCs at baseline. Here, we show that at baseline, MSCs secrete BDNF. (Fig 26) BDNF is an important neurotrophic factor in the brain that stimulates repair and recovery after injury. [175]

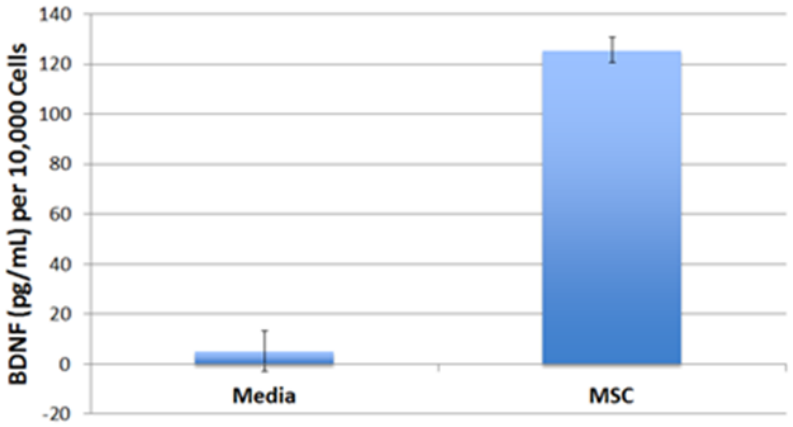



*Figure 26. Free MSCs secrete BDNF. The total amount of BDNF produced by the MSCs and secreted into the media was 125 pg/mL, or 1.5 pg/mL per 10,000 cells. N=3 replicates per condition. Error bars are ± standard deviation. *=p<0.0005.*

**Treatment with MSCs after TBI decreases injury volume at 21 DPI**

In order to determine the ability of MSCs to act as a treatment following TBI, we injected wild type C57BL/6J mice with 5 x $10^4$ free MSCs or control artificial cerebrospinal fluid (aCSF) intracerebroventricularly. We found that at 1 and 7 DPI there were no differences in the injury volume as assessed by MRI between the two injured groups, but by 21 DPI the control aCSF injured mice had a significantly larger injury volume than the MSC treated injured mice. (Fig 27A-C) This suggests that treatment with MSCs is able to decrease the secondary inflammation that occurs after injury, which may limit the long-term damage that occurs after injury.

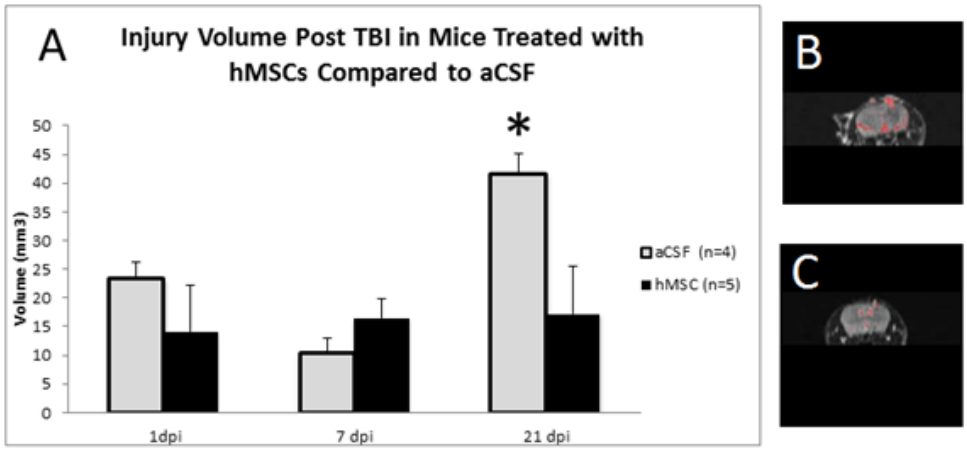



*Figure 27. Treatment with MSCs after TBI decreases injury volume at 21 DPI. A) WT C57BL/6J mice injected with 5 x $10^4$ free hMSCs intracerebroventricularly have a decreased injury volume compared to WT C57BL/6J mice injected with aCSF following TBI as assessed by MRI. Bars represent injury volume as determined by increased ROI ± SEM. * $p < 0.05$, ** $p < 0.01$, t-test relative to the aCSF control from that time point. B) Representative image of MRI scan performed at 21 dpi in an injured mouse treated with aCSF vehicle C) Representative image of MRI scan performed at 21 dpi in an injured mouse treated with hMSCs.*

**Treatment with MSCs after TBI decreases apoptosis in the cortex and hippocampus at 1 DPI**

Next, in order to determine the pathways through which treatment with MSCs is decreasing injury volume at 21 DPI, we examined cellular markers 1 DPI. We found that at 1 DPI,

injured MSC treated mice had significantly fewer cleaved caspase 3 positive cells relative to the injured control aCSF treated mice in both the cortex and the hippocampus. (Fig 28A-B) Levels of cleaved caspase 3 positive cells in injured MSC treated mice were reduced back to levels seen in both the control aCSF and MSC sham groups. These data indicate that treatment with MSCs was able to reduce apoptosis acutely after injury.

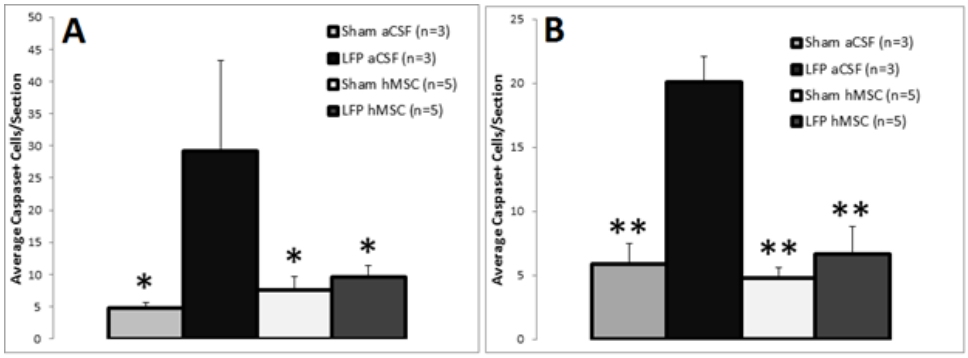



*Figure 28. Treatment with MSCs after TBI decreases apoptosis in the cortex and hippocampus at 1 DPI. WT C57BL/6J mice injected with 5 x $10^4$ free hMSCs intracerebroventricularly have fewer Caspase positive cells than WT C57BL/6J mice injected with aCSF following TBI. (A-B) Quantitation of the average number of Caspase positive cells per cortical and hippocampal section at 1 dpi ± SEM. * $p < 0.05$, ** $p < 0.01$ relative to LFP aCSF mice, ANOVA Fisher's PLSD posthoc test.*

**Treatment with MSCs after TBI decreases neurodegeneration in the cortex and hippocampus at 1 DPI**

We next chose to investigate the effect that treatment with MSCs had on levels of neurodegeneration after injury as assessed by the number FLJC positive cells. We found that at 1 DPI, injured MSC treated mice had significantly more FLJC positive cells relative to the injured control aCSF treated mice in both the cortex and the hippocampus. (Fig 29A-B) Levels of FLJC positive cells in injured MSC treated mice were reduced back to levels seen in the control aCSF and MSC sham groups. These data indicate that treatment with MSCs was able to reduce neurodegeneration acutely after injury.

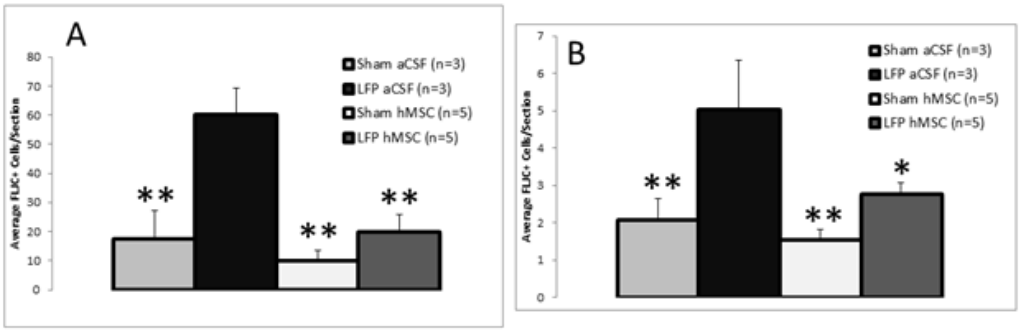



*Figure 29. Treatment with MSCs after TBI decreases neurodegeneration in the cortex and hippocampus at 1 DPI. WT C57BL/6J mice injected with 5 x $10^4$ free hMSCs intracerebroventricularly have fewer FluorojadeC positive cells than WT C57BL/6J mice injected with aCSF following TBI. (A-B) Quantitation of the average number of FluorojadeC positive cells per cortical and hippocampal section at 1 dpi ± SEM. * $p < 0.05$, ** $p < 0.01$ relative to LFP aCSF mice, ANOVA Fisher's PLSD posthoc test.*

## Pre-treatment of MSCs with forskolin increases BDNF secretion

Next, we wanted to investigate methods to increase the levels of BDNF secretion of MSCs in order to make them a more potent therapy for neural recovery after TBI. We investigated known compounds that are used in stem cell reprogramming protocols that have been shown to differentiate cells down the neuronal lineage pathway such as retinoic acid, forskolin and interleukin 1β. [249] Forskolin functions to stimulate adenylate cyclase, which increases cAMP. We found that treatment with 12.2 μM of forskolin significantly increased BDNF levels at the 18 hour timepoint. (Fig 30)

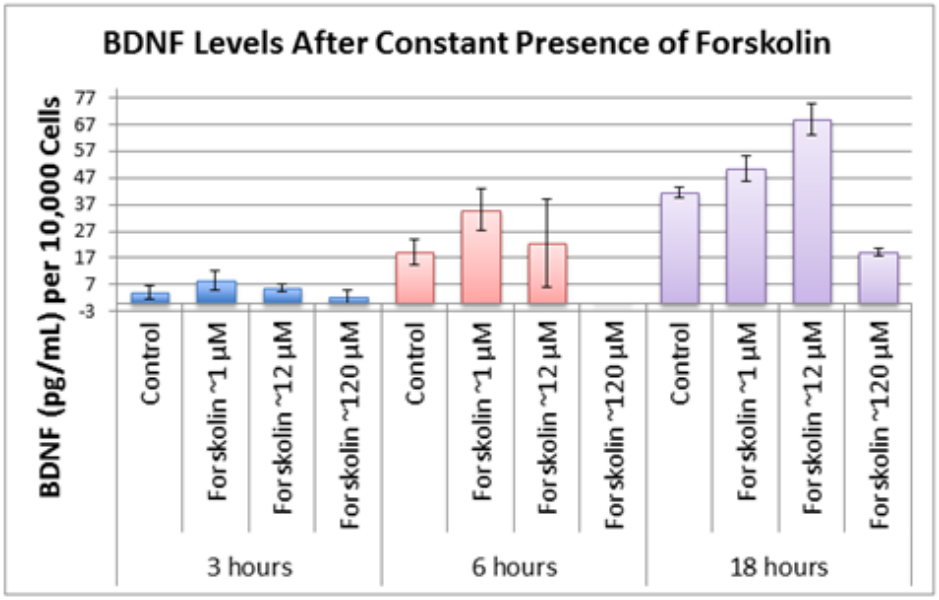



*Figure 30. Pre-treatment of MSCs with forskolin increases BDNF secretion. Levels of BDNF secreted by MSCs after continuous presence of forskolin in the media. At the 18-hour timepoint, the 12.2 µM dose of forskolin significantly increased BDNF levels. N=3 biological replicates for all conditions except Forskolin, 3 hours, 1.22 and 12.2 µM. Error bars are ± SEM. *=p<0.05.*

Treatment with retinoic acid (RA) and interleukin 1β (IL-1β) did not increase BDNF secretion at any of the concentrations or timepoints tested. (Fig 31)

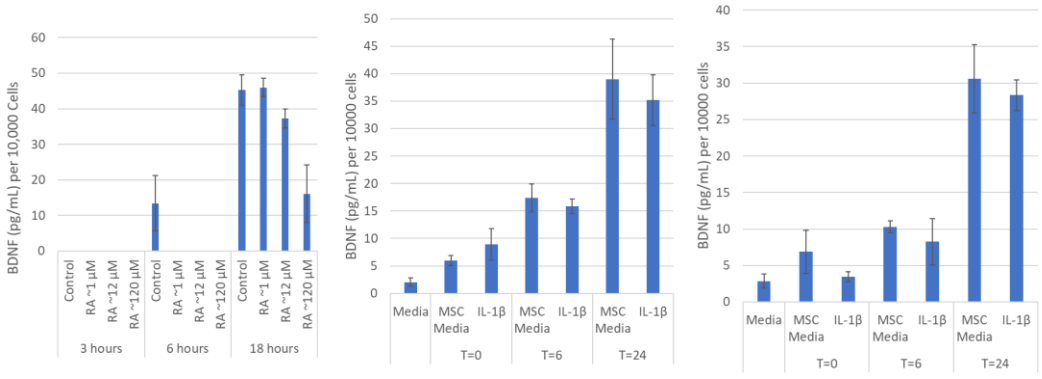



*Figure 31. Pretreatment of MSCs with retinoic acid or interleukin 1β do not increase BDNF levels. Pre-activation with 1 ng/mL retinoic acid or IL-1β does not significantly affect MSC BDNF secretion. N=3 biological replicates. Error bars are ± standard deviation. *=p<0.05. Constant presence of retinoic acid or IL-1β does not increase BDNF levels N=3 biological replicates. Error bars are ± standard deviation. *=p<0.05.*

**Encapsulation of MSCs with alginate results in 80% cell viability**

Another method of MSC manipulation is to encapsulate the cells in alginate. Alginate was chosen as the compound for encapsulation due to its chemical properties, and its ability to modulate the neuro-inflammatory processes. [250] Previous work has shown that alginate encapsulation can skew the secretome of MSCs to a more beneficial one. [179] We used a novel approach being developed by the Yarmush lab in conjunction with the Grumet lab at Rutgers University in order to create small diameter capsules for injection into a mouse model. [180] We show that using this new method, we are able to create alginate-MSC microspheres 220-380 μm in diameter with 25-75 cells per capsule and 80% cell viability at 24 hours post encapsulation. (Fig 32) These results demonstrate that we are able to create alginate encapsulated MSCs for experimental research in mouse models.

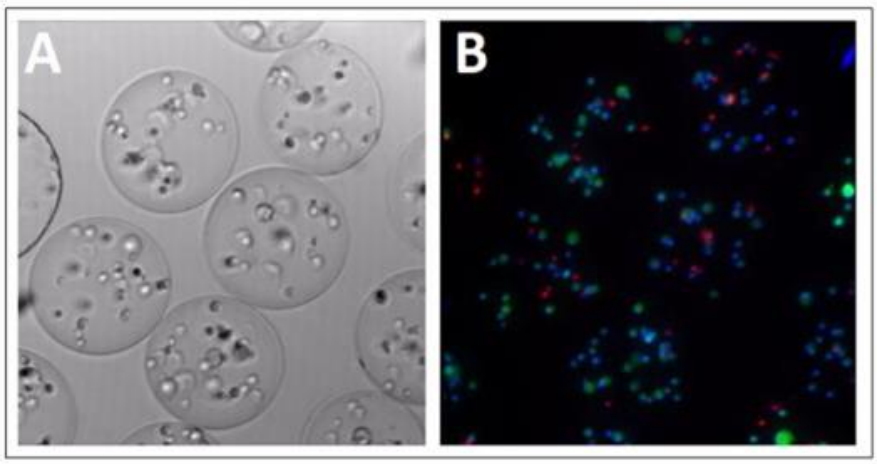



*Figure 32. Representative images of encapsulated MSCs. A. Encapsulated MSCs in white light B. Encapsulated MSCs stained for viability. Viability of cells in the capsules was assessed with a DAPI (cell body), calcein acetomethoxy (live) and ethidium homodimer (dead) staining, which was visualized using an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan). Cells were counted using SlideBook image analysis software version 5.0 (Intelligent Imaging Innovations, Denver, CO).*

**Alginate encapsulation of MSCs decreases the secretion of pro and mature BDNF**

Analysis of the secretome of the encapsulated MSCs for pro and mature BDNF found that relative to free MSCs, encapsulated MSCs have less pro and mature BDNF. (Fig 33) However, pro BDNF is more significantly reduced than the mature BDNF. These results suggest that alginate encapsulation may be a useful method to skew the neurotrophin profile of secreted proteins towards a more mature and beneficial pathway, however it does not seem to increase the overall release of neurotrophin factors. This suggests that alginate encapsulation should be done in conjunction with genetic manipulation of the MSCs if overexpression of BDNF is the intended target.

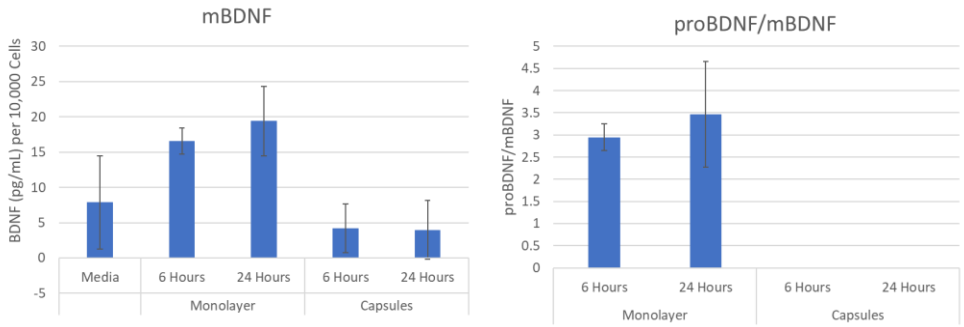



*Figure 33. Alginate encapsulation of MSCs decreases the secretion of pro and mature BDNF. However, the pro form of BDNF is more significantly reduced by this process, skewing the secretome towards a more beneficial, mature BDNF profile.*

**Creation of chitosan-coated magnetic nanoparticle (ChMNP) tagged microspheres allows for in vivo visualization of injected capsules**

In order to track the injected capsules in vivo in our mouse model, we created ChMNP tagged capsules. (Fig 34A) The capsules were injected intracerebroventricularly and imaged using MRI at 1 DPI, 18 DPI, and 3 months post injury (MPI). (Fig 34B) The tagged capsules appeared as an area of hypointensity in the in vivo MRI scans, and remained in

the area of injection until at least the 3 MPI timepoint. At 3 MPI, we conduced ex vivo optical fluorescent scans and positive signal confirmed presence of the capsules was confined to the brain and not found in other areas of the mouse, such as the liver or kidney. (Fig 34C)

These results indicate that we are able to use MSCs to improve outcomes after TBI, and that we are able to create alginate encapsulated MSCs that can be injected into the ventricles in a mouse model and persist there for at least 3 months post injury. These are the first steps to developing a targeted MSC based therapy for TBI that can be tested in an experimental mouse model.

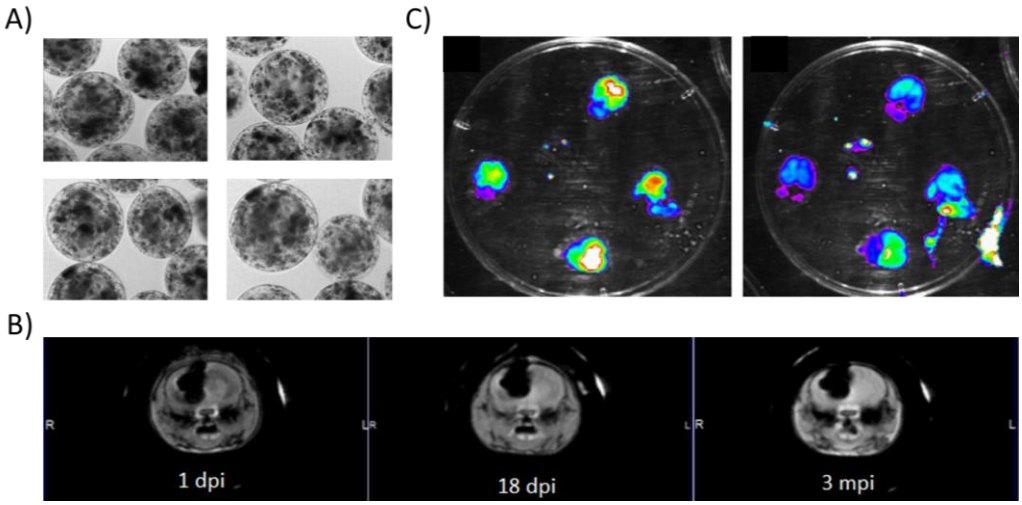



*Figure 34. Creation of chitosan-coated magnetic nanoparticle (ChMNP) tagged microspheres allows for in vivo visualization of injected capsules. A) Representative confocal microscopy images of capsules made of alginate containing chitosan-coated magnetic nanoparticles (ChMNP). B) Representative images from mice at 1 day, 18 days, and 3 months post injection. C) Optical-fluorescent scans were conducted using two ranges of Emissions/Excitations: 480/700 and 520/600. Bright field and X-ray images were also captured so that the optical images could be overlaid. The scans were done using the FX-PRO at the Rutgers Molecular Imaging Center.*

## Discussion of Results:

These results highlight the potential of MSCs to be an important stem cell therapy after

TBI. We show here that free MSCs are able to ameliorate some of the detrimental effects of TBI, such as decreasing levels of apoptosis and neurodegeneration at 1 DPI and decreasing injury volume by 21 DPI. We show that pre-treatment with factors such as forskolin can improve the secretome of MSCs to secretome BDNF, which may be beneficial to treat neural injury and stimulate repair. We also show that we are able to use alginate encapsulation of MSCs to skew their secretome toward the more beneficial mature BDNF over the detrimental proBDNF. Finally, we show that it is feasible to use encapsulated MSCs in a mouse model; small capsules of the appropriate diameter are able to be created, we are able to deliver a therapeutic dose to the mice within these small capsules, and these small capsules persist in the area of injection with no obvious negative effects on health in the mice for up to 3 months.

One of the reasons why MSCs have emerged as a potential therapeutic is because of their low immunogenic profile. This is an important feature of MSCs which means that when they are implanted for cellular therapy, due to their low expression of MHC I and no expression of MHC II, that they are able to avoid detection by the host immune natural killer (NK) cells and avoid stimulating a host immune rejection response . [251] However, while they were originally thought of as “immune-privileged”, due to the fact that they do not stimulate an immune response from host tissue when transplanted, recent research suggests that they are more properly thought of as “immune evasive”. [252] In order to avoid immune complications, an ideal therapy would use autologous MSCs, taken from the patient themselves. However, in many cases this is not feasible, and allogenic MSCs must be used. In these cases, recent research shows that although the MSCs may be immune evasive, there can still be an immune response to their transplantation [253]. Due to the fact that MSCs are able to cause an immune response in certain cases, future work should

make sure to characterize the immune response after injection of MSCs in order to more fully understand the long-term effects they can have on host tissue. In addition, studies have recently shown that there is donor variability in function between MSCs, which is an important factor to consider when designing experiments based upon this variable cell population. [245]

MSCs have the potential to be a viable treatment for TBI, due to their innate abilities and our ability to modify them. Previous work has investigated the use of MSCs for treatment after TBI. Groups have shown that using MSCs that overexpress interleukin-10 can promote a more beneficial inflammatory response in a rat model of TBI. [254] Another group has investigated the process of using autologous MSCs for treatment after TBI and showed that in a rat model this method was able to improve function outcomes such as learning in the Morris Water Maze. [255] Another method that is being investigated is the use of MSCs in combination therapy. MSCs in combination with propranolol has been shown to improve cognitive and memory at 120 DPI. [256] These results are promising, but there is still much work to be done before this treatment is brought to the clinic. Our work is unique, since we are attempting to use encapsulated MSCs to alter neurotrophic signaling after rmTBI in a mouse model. Encapsulation of MSCs has many benefits, and would be a novel approach to treating rmTBI.

Future research needs to investigate the best ways to optimize treatment while analyzing the safety profile of the novel treatment as well. Our mouse model provides a way for researchers to analyze these questions in different mouse disease models and using transgenic mouse strains.

# Chapter 6: BDNF Signaling after TBI

## Introduction:

Traumatic brain injury (TBI) is an alternation in brain function, or other evidence of brain pathology, caused by an external force [257]. In recent years there is increasing literature deciphering the neuropathology associated with TBI, however the molecular mechanisms that account for the lack of regeneration following TBI are not well understood. TBI is divided into two stages: acute and chronic. The acute phase of injury can be initiated by a direct contusion to the brain or from shearing and stretching forces causing displacement of brain tissue [258, 259]. The acute and subacute stage lasts for hours after initial trauma and immediately results in changes in cell structure that lead to apoptosis and cell death. The chronic phase of injury persists for days after the initial event and is a continuation of cell death and neuronal degeneration in regions both focal and distal to the original site of injury [260, 261]. In addition, astrogliosis leads to glial scar formation that may impede recovery [262]. These injuries affect many processes including motor function, memory and spatial learning [263-267]. For example, in humans, hippocampal cell death can lead to verbal memory deficits, retrograde and anterograde amnesia, and learning deficits [268, 269]. Motor deficits are also prevalent after TBI, although they are more likely to persist after severe TBI [270].

Among the numerous physiological outcomes following both spinal cord injury and TBI is the induction of mRNA and protein expression of the neurotrophin family of growth factors. In both humans and animal models, brain-derived neurotrophic factor (BDNF) has been found to be upregulated following TBI in neurons and glia [21-24, 271, 272]. For example, transient increases in expression of BDNF have been detected in the CSF of

children with severe TBI [273, 274]. However, because past studies of BDNF levels after TBI have mainly focused on the message levels or the protein levels as determined by immunocytochemistry or ELISA, these previous studies could not discriminate between the neurotrophin protein isoforms which are known to have important functional consequences. Mature neurotrophins bind with low affinity to the p75 receptor however, pro-neurotrophins including proBDNF, proNGF, proNT3 and proNT4/5 bind with high affinity to the p75 receptor to induce cell death [13]. Although proNT3 and proNT4 binding to p75 have been associated with apoptosis [275] most studies have focused on the role of proBDNF and proNGF binding to p75 to induce cell death [276]; [277] as well as long-term depression [278]. In contrast, mature neurotrophins bind tightly to the tropomyosin-receptor kinase (Trk) receptors to mediate cell survival [279]; NGF binds to TrkA, BDNF and NT4/5 bind to trkB, and NT3 binds primarily to TrkC [280]. In addition to cell survival, the interaction of mature BDNF with trkB promotes differentiation and long-term potentiation in the hippocampus [281]. Based on the approaches previously used to analyze neurotrophin expression, it is unclear whether neurotrophins are predominantly present in their precursor or mature form and therefore which receptor-signaling pathway is activated following injury. Although it is known that p75 is upregulated after various forms of CNS injury [282, 283], the consequence of reducing p75 signaling in lateral fluid percussion injury has not been explored. In addition, due to the high expression of BDNF and trkB in the hippocampus and cortex [284] and the well-described roles of BDNF in survival and plasticity, much of the focus for prior studies has been on BDNF. Although BDNF has been demonstrated to potentiate recovery after acute injury [285, 286] and a very recent study demonstrates that a trkB agonist increases activation of plasticity markers and enhances memory [287] however, conflicting studies show that mature neurotrophins have minimal beneficial effects [288, 289], indicating that the role of neurotrophins as well as the p75 and Trk receptor signaling following injury has not been definitely elucidated.

In this report we have systematically studied expression of different isoforms of neurotrophins and their receptors after moderate lateral fluid percussion injury in mice. Among the TBI models studied, LFP is the most reproducible and commonly used system to evaluate mixed focal and diffuse brain injury [190]. LFP recapitulates acute and chronic injuries observed in humans, rendering it clinically relevant, and allows for exploration of novel therapeutics for clinical translation [110]. We therefore examined the effects of genetic and pharmacologic manipulation of neurotrophin signaling on the cellular, biochemical and behavioural changes following LFP. To examine potential effects of both proBDNF and proNGF, we targeted the p75 receptor and to explore the role of mature BDNF, we activated the trkB receptor. Our findings indicate that intervention with p75 signaling reduces apoptosis, neuronal degeneration and astrocytosis and improves outcome on two major objective measures of neurological function following injury: the Morris Water Maze (MWM) and Rotarod test to examine spatial learning and sensorimotor function, respectively.

## Experimental Results:

### trkB and p75 receptor and pro and mature neurotrophin expression patterns following LFP injury

In this study we have used a moderate LFP injury procedure in mice [290]. LFP has been shown to destroy the blood brain barrier at the site of injury as well as cause slow and continuous white matter degeneration and an increase in glial fibrillary acidic protein (GFAP) concentration at the site of injury [291, 292]. These responses demonstrate that LFP mimics both acute and chronic injury in animal models. We performed moderate LFP at the sensory-motor cortex and that also affects the underlying hippocampus and corpus

callosum. Righting reflex times within 4-10 minutes were used to represent moderate injury resulting in deficits that hamper normal functioning and continued atrophy after the initial insult [293, 294]. Since studies have found that the craniectomy procedure performed prior to the LFP injury may also cause mild injury, we have included sham mice that have received craniectomies but no LFP injury as controls. We have also only analyzed the cortex and hippocampus ipsilateral to the injury. The advantages of these approaches are that they will reduce the risk of confounding variables [295].

In order to examine the expression of pro and mature forms of the neurotrophins and their respective receptors, p75 and trkB, tissue lysates from the cortical area surrounding the injury and the whole hippocampus were examined by Western blot analysis 1, 7 and 21 days post injury (dpi). Quantitation of protein levels in both regions showed an overall trend of reduced trkB levels at all time points following injury with statistical significance at 21 dpi in cortex and 1 dpi in the hippocampus (Fig. 35A-C) when data are normalized to sham animals from the equivalent time point. The protein levels for p75 revealed an overall increase at all the time points with statistical significance of about 2-fold increase at 1 dpi in cortex and 21 dpi in the hippocampus (Fig. 35E-G). The co-receptor for p75, sortilin, also demonstrated an upregulation in the cortex at 1 dpi (Fig. 35H-I). No effect of injury was seen for sortilin in the hippocampus (data not shown). Therefore, the overall effect of LFP injury on receptor expression is a downregulation of trkB as early as 1 dpi in the hippocampus and an upregulation of p75 as early as 1 dpi in the cortex.

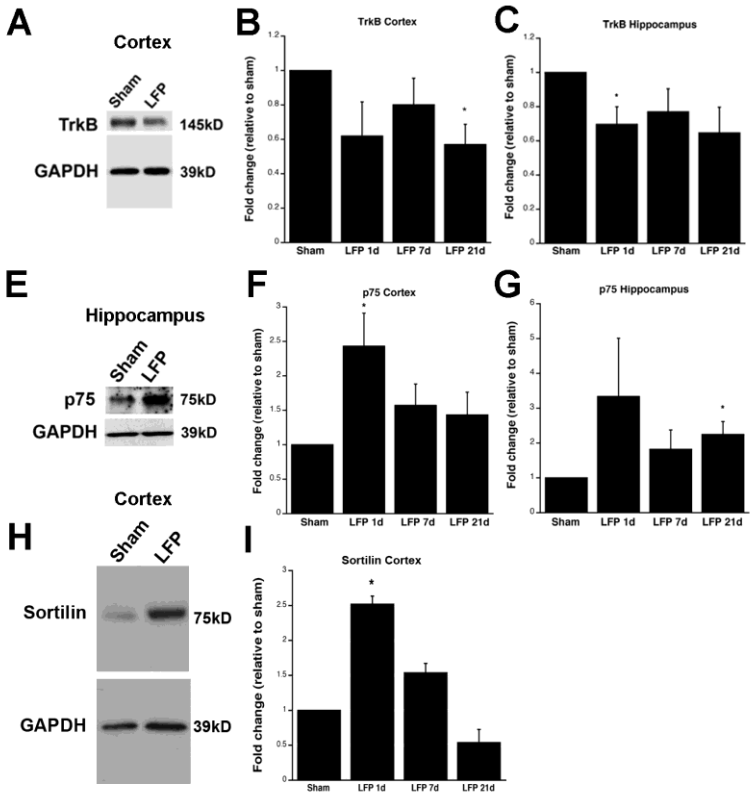



*Figure 35. trkB protein expression decreases and p75 and sortilin levels increase following LFP injury. (A, E, H) Representative Western blots showing trkB, p75 and sortilin expression in the ipsilateral cortex (A and H) and hippocampus (E) (each lane represents one animal). (B-C, F-G and I) Quantitation of protein levels in the cortex and hippocampus, respectively. All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative to the average ± SEM of the time matched sham controls which are represented as a single bar in the graph (n = 5-7 mice/group for trkB and p75. n = 3-4 mice/group for sortilin). * p < 0.05, t-test relative to the sham control from that time point.*

To assess the expression of pro and mature BDNF protein levels, the same lysates were subjected to Western blot analysis and probed with an antibody that recognizes the mature 14kD, truncated 28kD and pro 36kD isoforms of BDNF. Quantitation of the protein levels of each of the isoforms in cortex (Fig. 36B, D, and F) and hippocampus (Fig. 36C, E and G) showed an increase at specific days following injury when normalized to protein levels from animals subjected to sham surgeries from the equivalent time point. Specifically, the mature form of BDNF (14kD) showed a trend to an increase at 7 dpi in the cortex and at 7 and 21 dpi in the hippocampus. The 28kD truncated BDNF isoform showed little difference

in the cortex after LFP injury, while in the hippocampus both at 1 and 21 dpi there was a statistically significant increase observed. The 36kD proBDNF isoform increased at 7 dpi in the cortex but there was no obvious change in the hippocampus at any time point. Since proNGF also binds to the p75 receptor, the protein lysates were analyzed for this neurotrophin as well. There was a trend to an increase in proNGF at all time points after TBI when compared to sham animals in the cortex with a significant increase at 7 dpi (Fig. 36I) with no apparent trend in the hippocampus (Fig 36J). In sum, we observed an overall trend to an increase in expression of the various isoforms of BDNF and NGF with a significant increase in the levels of pro-neurotrophins as early as 1 dpi in the hippocampus and 7 dpi in the cortex. Increased mRNA and protein expression of BDNF has been described in spinal cord and traumatic brain injury [21-24, 271, 272], however this is the first report of a change in expression of different isoforms of BDNF and their receptors along with proNGF after LFP injury.

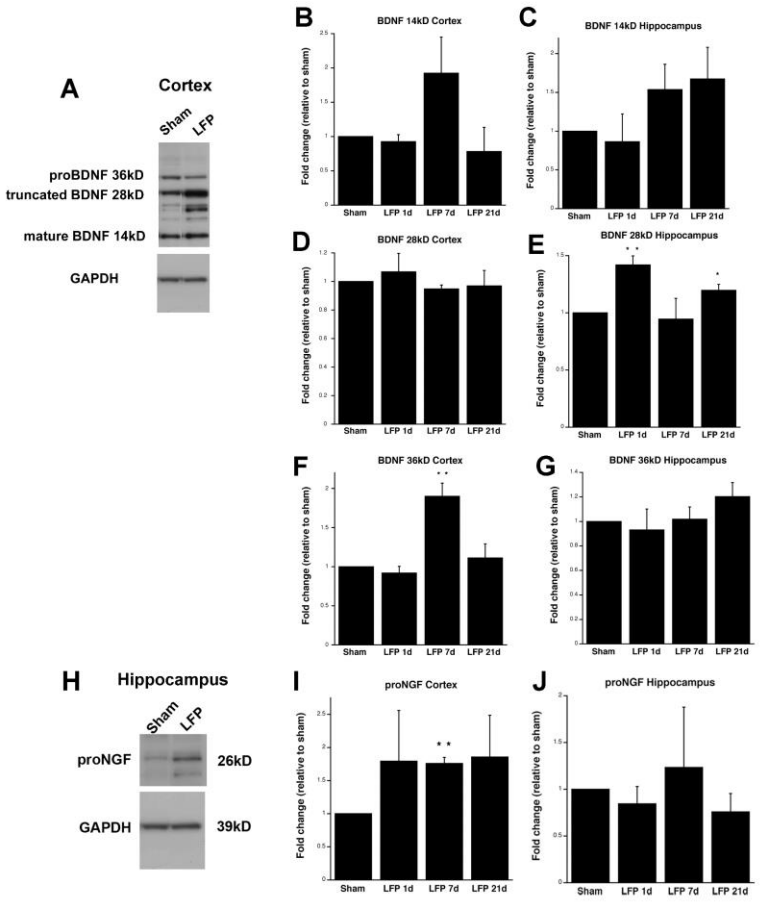



*Figure 36. pro-Neurotrophin expression increases following LFP.* ***(A, H)*** *Representative Western blots showing BDNF (mature 14kD, truncated 28kD and pro 36kD) and proNGF (26kD) expression in the ipsilateral cortex (A) and hippocampus (H).* ***(B-G, I-J)*** *Quantitation of protein levels in the cortex and hippocampus for BDNF (B-G) and proNGF (I-J). All data is first normalized to GAPDH to control for protein loading and then expressed as a fold change relative ± SEM to the average of the time matched sham controls which are represented as a single bar in the graph (n = 4,5 mice/group). * p < 0.05, ** p < 0.01, t-test relative to the sham control from that time point.*

## Effect of interventions with p75 signaling on neurodegeneration following LFP

To begin to explore the role of p75 signaling in the effects of LFP, we performed experiments on p75-/- mice and compared outcomes relative to WT mice. By using p75 mutant mice, we eliminated signaling from both proNGF and proBDNF ligands. To demonstrate the effect of these manipulations on neurodegeneration, Fluorojade C (FJC), a relatively new but widely used fluorescent dye [296, 297] was used to identify neuronal degeneration after LFP injury both in the cortex and hippocampus. FJC positive cells

appear smaller and slightly contracted when compared to normal neurons which is consistent with somatic degeneration. The number of FJC positive cells significantly increased subsequent to LFP injury in WT mice when compared to sham WT mice. The effect was observed as early as 1 dpi and persisted to 21 dpi although the effect was most apparent at 1dpi reflecting a decrease in the rate of cell death over time following TBI (Fig 37A-B, K-L). In p75-/- mice subjected to LFP injury, the number of FJC positive cells showed a significant decrease in the cortex relative WT LFP mice at 1 dpi (Fig. 37B, D, and K,). No effect of the p75 mutation on FJC+ cells after injury compared to WT mice was observed in the hippocampus at any time point (Fig. 37L). There was no effect of the p75 deletion on numbers of FJC+ cells in sham relative to WT mice implying that p75 does not play a role in baseline neurodegeneration.

Since the effect of the LFP injury on FJC+ cells was most evident at 1 dpi, analysis of pharmacological treatments on WT mice was performed at that time point. The effect of pharmacological intervention on neurotrophin signaling was examined using either the short peptide p75 receptor antagonist TAT-Pep5 (1.7 mg/kg) or the small molecule trkB agonist 7,8 Dihydroxyflavone (7,8 DHF) (5 mg/kg). The $p75^{NTR}$ inhibitor Pep5 (TAT-Pep5) is made cell-permeable by fusing it with the N-terminal protein transduction domain sequence (11 amino acids) from HIV protein TAT. TAT-Pep5 has been shown to act as an effective blocker of MAG- and Nogo-induced inhibition of neurite outgrowth in both dorsal root ganglion and cerebellar neurons [298, 299]. 7,8 DHF has been shown to have potent neurotrophic activities [300] and improves motor function in a mouse model of Huntington's disease [301]. Both compounds cross the blood brain barrier. Importantly, after LFP injury and treatment with 7,8 DHF or TAT-Pep5 the neurodegeneration as shown by FJC positive cells was significantly lower after 1 dpi in the cortex when compared to

vehicle treated animals (Fig. 37M and O). Although treatment with TAT-Pep5 did show a significant reduction after 1 dpi in the hippocampus (Fig. 37P), the reduction in FJC positive cells was not apparent after treatment with 7,8 DHF (Fig. 37N). Neither compound had an effect on baseline FJC levels in sham mice. In sum, interfering with the p75 pathway either through genetic deletion or pharmacological intervention prevents cell degeneration following LFP at 1 dpi, primarily in the cortex. Furthermore, activating trkB reduces cell degeneration in the cortex at 1 dpi.

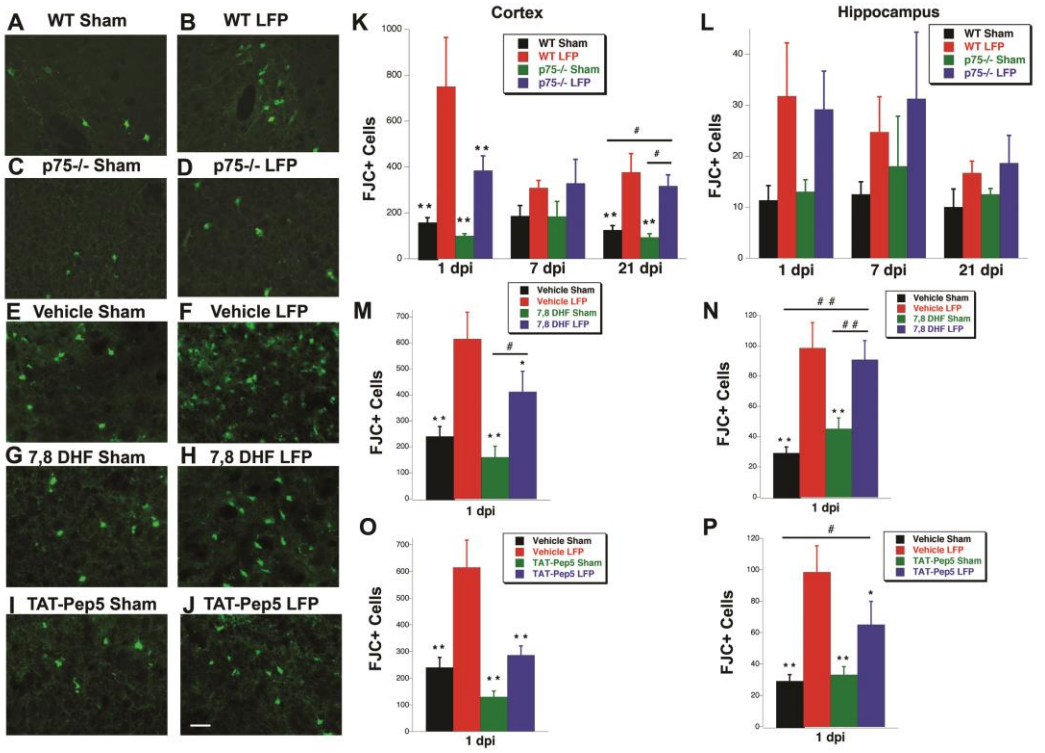



*Figure 37. p75-/- mice and mice treated with p75 antagonist, TAT-Pep5 or trkB agonist, 7,8 DHF have fewer FluorojadeC positive cells than WT or vehicle treated mice following LFP.* ***(A-J)*** *Representative images of cortical sections at 1 dpi stained with FJC. Scale bars = 100µm.* ***(K-P)*** *Quantitation of the total number of FJC+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group). * p < 0.05, ** p < 0.01, ANOVA Fisher's PLSD post-hoc test relative to WT LFP or Vehicle LFP. # p < 0.05, ## p < 0.01, ANOVA Fisher's PLSD post-hoc test relative to indicated groups.*

**Analysis of cell death following LFP in mice with reduced p75 or enhanced trkB signaling**

It is established that subsequent to TBI, there is neuronal loss by cell death and that apoptosis is associated with Caspase-3 activation [302]. We have thus quantitated activated Caspase-3 by immunohistochemistry following LFP injury in both in p75-/- mice as well as after treatment with pharmacological agents that affect neurotrophin receptor function. LFP injury results in a significant increase in the number of Caspase-3 positive cells when compared to sham or drug treated mice in both the cortex and hippocampus especially at 1 dpi (Fig. 38). There was an overall trend to fewer Caspase-3 positive cells in WT mice at 7 and 21 dpi in the injured cortex relative to the number of Caspase-3 positive cells at 1 dpi, again reflecting a decrease in the rate of cell death over time following injury. p75-/- mice exhibited significantly fewer Caspase-3+ cells relative to WT mice at 1, 7 and 21 dpi in the cortex and at 7 dpi in the hippocampus (Fig. 38K and L). There was no effect of the p75 deletion on baseline numbers of Caspase-3+ cells in sham mice.

The effect of pharmacological intervention was examined at 1 dpi given that the highest number of Caspase-3+ cells was observed at that time point. After treatment with either 7,8 DHF or TAT-Pep5, cell death as measured by Caspase-3 positivity was significantly lower after 1 dpi when compared to vehicle treated animals subjected to LFP injury in the cortex (Fig. 38M and O). However, the reduction in Caspase-3 positive cells was less pronounced in the hippocampus after treatment with 7,8 DHF or TAT-Pep5 (Fig. 38N and P). Neither drug had an effect on baseline Caspase-3+ levels in sham animals. Together these data imply that blocking the p75 pathway genetically or pharmacologically or activating trkB prevents cell death, primarily in the cortex, following LFP at 1 dpi.

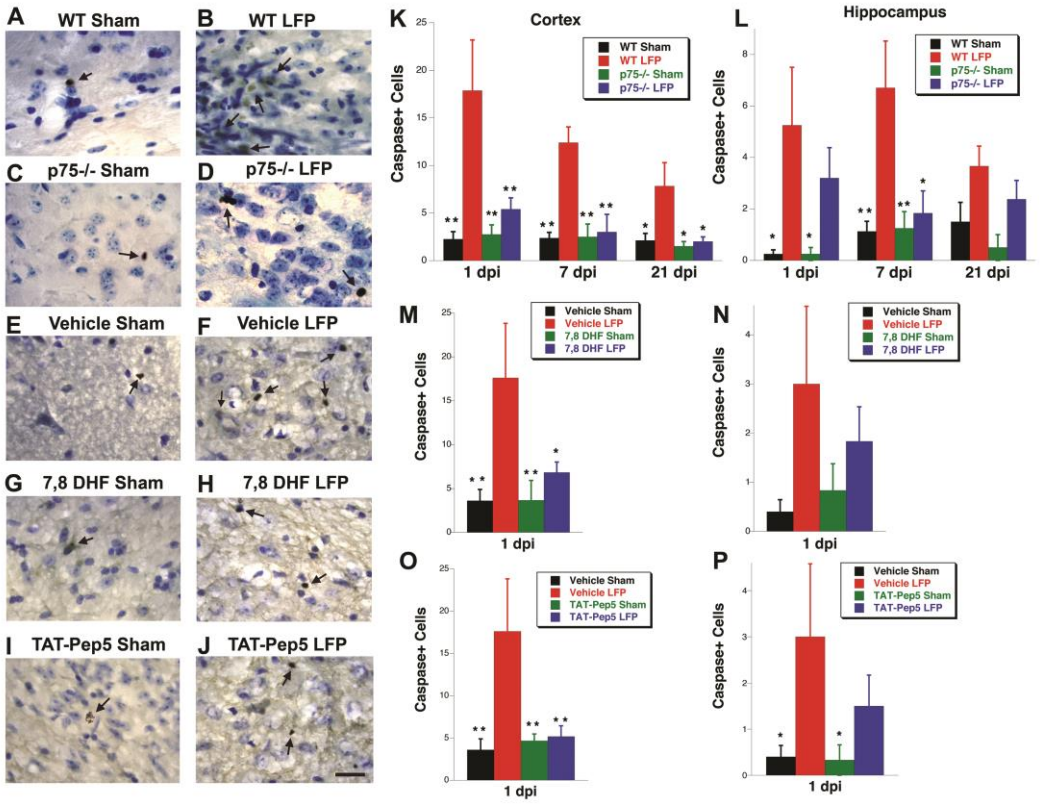



*Figure 38. p75-/- mice and mice treated with p75 antagonist, TAT-Pep5 or trkB agonist, 7,8 DHF have fewer Caspase-3 positive cells than WT or vehicle treated mice following LFP.* ***(A-J)*** *Representative images of cortical sections at 1 dpi (E-J) immunostained for activated caspase-3 (indicated by arrow). Scale bars = 100 µm.* ***(K-P)*** *Quantitation of the total number of caspase-3+ cells per cortex or hippocampus ± SEM for WT or p75-/- mice (K-L) and mice treated with Vehicle, 7,8 DHF (M-N) or TAT-Pep5 (O-P) subjected to Sham or LFP procedures (n = 8 mice/group * $p < 0.05$, ** $p < 0.01$, ANOVA Fisher's PLSD post-hoc test relative to WT LFP or Vehicle LFP. There was no significant difference between any of the other groups by ANOVA Fisher's PLSD post-hoc test.*

## Identification of cell type affected by LFP

To determine precisely which cells were undergoing apoptosis and which cells expressed p75, cortical tissue sections were stained with either activated Caspase-3 or p75 antibody and subsequently co-labeled with either NeuN or GFAP antibody to identify neuronal and glial cell types respectively. Both Caspase-3 and p75 positive cells also stained with NeuN (Fig. 39A and C) but not with GFAP (Fig. 39B and D) antibody after LFP injury. This finding suggests that the cells undergoing apoptosis after injury via activation of p75 expression and Caspase-3 signaling are most likely neurons rather than astrocytes.

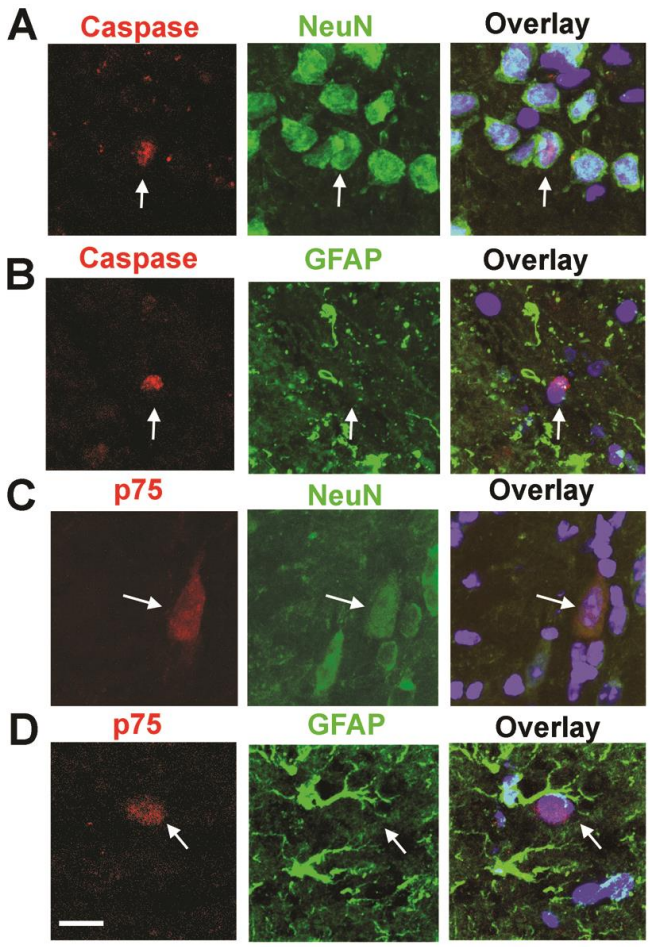



*Figure 39. Activated Caspase-3 and p75 co-localize with neuronal but not glial markers after injury.* ***(A-D)*** *Representative images of cells from the cortex of mice subjected to LFP****.*** *Cortical sections stained with antibodies against Caspase-3 (red indicated by arrow) (A-B) or p75 (red indicated by arrow) (C-D) and NeuN (green) (A and C) or GFAP (green) (B and D). The overlay images also include DAPI nuclear stain. There is co-localization of Caspase-3 and NeuN (A) as well as of p75 and NeuN (C) as indicated by the overlap between red and green but not of Caspase-3 and GFAP (B) or p75 and GFAP (D). Scale bar = 30 µm.*

**Effect of p75 deletion on astrocyte cell number following LFP**

Increased gliosis, as evidenced by cells expressing GFAP have been reported following TBI and these cells may be forming a glial scar, preventing regeneration [262]. GFAP is an intermediate filament protein distinctly localized to the cytoskeleton of mature astrocytes, the most abundant cell type in the central nervous system. Our evaluation of GFAP staining of cortical and hippocampal sections respectively showed a significant

increase in GFAP positive cells in WT injured mice when compared to sham WT animals at 1 dpi (Fig. 40E-F). Quantitation of GFAP positive cells in injured p75 mutant mice demonstrated a decrease in the number when compared to WT LFP mice. There was no effect of the p75 deletion on baseline levels of GFAP+ cells in sham mice. These results indicate that the mice lacking p75 exhibit less gliosis in the cortex and hippocampus following injury.

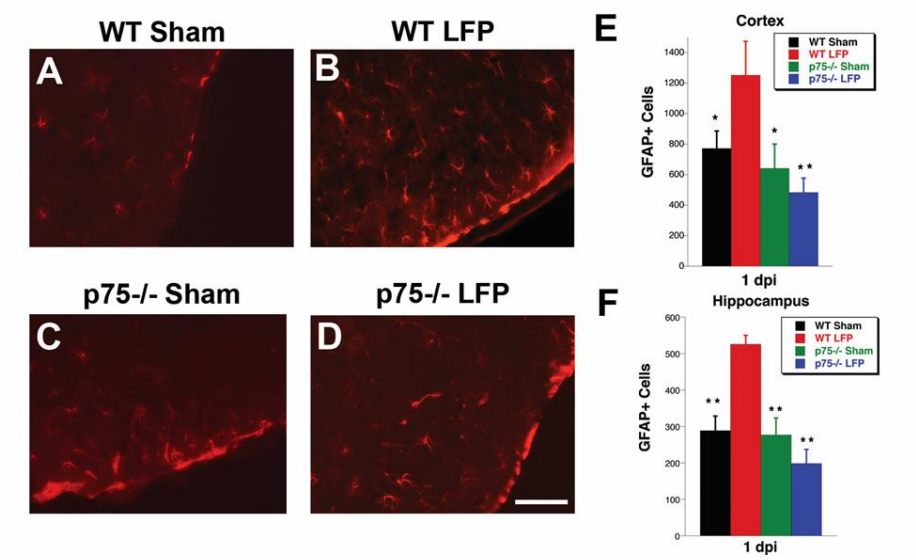



*Figure 40. p75-/- mice have fewer GFAP+ astrocytes than WT mice following LFP.* ***(A-D)*** *Representative images of cortical sections from WT (A-B) and p75-/-* ***(****C-D) subjected to sham and LFP injuries and immunostained for GFAP at 1dpi.* ***(E-F)*** *Quantitation of the average number of GFAP+ cells per cortical and hippocampal section ± SEM (n = 4,5 mice/group). Scale bar = 500 µm. * p < 0.05, ** p < 0.01, ANOVA Fisher's PLSD post-hoc test relative to WT LFP mice. There was no significant difference between any of the other groups by ANOVA Fisher's PLSD post-hoc test.*

## Analysis of learning in mice lacking p75 signaling

To assess the neurofunctional outcomes of LFP injury, cognitive and motor tests were performed including the Morris Water Maze (MWM). Animals with hippocampal damage perform significantly worse than intact animals on the MWM and have a longer latency to find the platform indicating that this paradigm is an excellent measure of studying the

effects of cell death on spatial learning memory [303]. We used the MWM test to measure impairments in spatial learning in mice subjected to LFP injury.

Latency in the ability to find the platform is increased in WT LFP mice when compared to WT sham mice when training is started at 1 dpi or 21 dpi in separate sets of mice (Fig. 41A-B). There is a progressive decrease in the latency to locate the platform over a three-day training period in all the groups with a leveling out in the last two days for the WT sham mice, indicating that learning is taking place. When training is started at 1 dpi, the p75-/- LFP mice have a shorter latency to platform than the WT LFP mice and are similar to the p75-/- sham mice on day 1 of training (Fig. 41A). At 21 dpi the LFP-injured p75-/- mice show a significant improvement in their ability to find the hidden platform relative to WT LFP mice on day 2 of training. The p75-/- sham mice had longer latency to platform relative to WT sham mice. These findings were consistent with the results of pretesting in the MWM prior to injury, where p75-/- mice exhibited a trend to increased latency to platform prior to injury (WT 28.57 ± 2.17, p75-/- 32.71 ± 2.08 sec, n = 14-21 mice/group, $p > 0.05$ ANOVA Fisher's PLSD post-hoc test). There was no significant effect of any of the treatment groups on swim speed following injury (WT sham 23.52 ± 1.63, WT LFP 23.67 ± 1.40, p75-/- sham 22.32 ± 1.11, 21.63 ± 1.46 cm/sec, n = 7-9 mice/group, $p > 0.05$ ANOVA Fisher's PLSD post-hoc test). Together these data indicate that the effect observed of improved learning in the p75-/- mice is not due to a shorter baseline time to platform because the p75 mutant mice have slightly impaired learning in the absence of injury.

For pharmacological assessments of neurotrophin receptor signaling, all training was initiated at 1 dpi. Vehicle controls were shared between the two treatment groups to reduce

the use of animals. Animals subjected to injury and treated with TAT-Pep5 demonstrated significantly shorter latencies relative to vehicle-treated LFP mice on multiple days of training (Fig. 41C). Conversely, when the injured mice were treated with the trkB agonist 7,8 DHF there was no improvement in cognitive function on any of the days of training (Fig. 41D). These data indicate that genetic or pharmacological intervention of p75 receptor signaling but not activation of the trkB receptor can result in cognitive functional recovery following LFP injury.

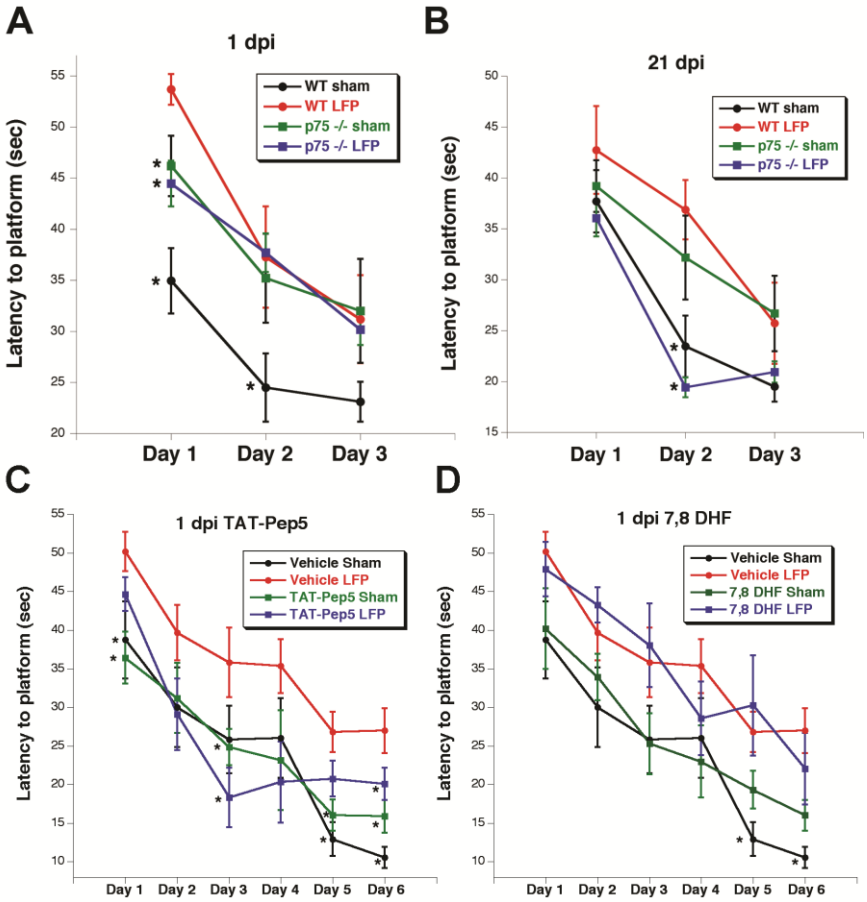



*Figure 41. p75-/- and TAT-Pep5 treated injured mice have a shorter latency to platform on the Morris Water Maze.* ***(A-B)*** *Average latency to platform per day ± SEM for p75-/- or WT mice subjected to sham or LFP injuries at 1dpi (A) and 21dpi (B) (n = 6-10 mice/group). Each subject underwent 8 trials/day for 3 consecutive days beginning at 1 or 21 dpi.* ***(C-D)*** *Average latency to platform per day ± SEM for WT mice treated with vehicle, TAT-Pep5 (C) or 7,8 DHF (D) following sham and LFP injuries and beginning training at 1 dpi (n = 8,9 mice/group). Each subject in this series underwent 4 trials/day for 6 consecutive days. All statistics are presented relative to WT LFP or vehicle LFP, * p < 0.05, ANOVA Fisher's PLSD post-hoc test.*

**Sensorimotor analysis of mutant or pharmacologically-treated mice following LFP**

Deficits in motor functioning are a consequence of TBI that causes damage to the sensorimotor cortices and can be measured using the Rotarod test [304]. The Rotarod test involves placing animals on a rotating cylinder and measuring the amount of time the animals can stay balanced without falling; longer times indicate better motor coordination. In this study, average latency to fall was measured in a set of mice subjected to LFP and assayed at 3 time points: 1, 7 and 21 dpi. As shown in figure 8A, WT LFP mice consistently showed a shorter latency to fall when compared to WT sham mice. The latency to fall in injured mice is the shortest at 1 dpi implying that there is partial recovery of motor function over time. Interestingly, when we tested the LFP p75-/- mice on the Rotarod device, we found that these mice exhibited longer latency to fall times when compared to WT LFP mice at 1 and 7 dpi (Fig. 42A). There is no difference in the latency to fall in sham WT and p75-/- mice. Moreover, there was no difference in the latency to fall between p75-/- and WT mice during pre-testing prior to injury (WT 52.54 ± 5.99, p75-/- 64.64 ± 6.32 sec, n = 16 mice/group, $p > 0.05$, t-test), suggesting that p75 does not play a role in baseline sensorimotor function. When the effect of the two pharmacological agents modulating neurotrophin receptor was measured after LFP on Rotarod performance, we saw that the p75 antagonist TAT-Pep 5 effectively increased the latency to fall when compared to vehicle LFP mice (Fig. 42B) at 1, 7 and 21 dpi. However, the trkB agonist 7,8 DHF did not result in an increased latency to fall relative to vehicle mice at any of the time points. In sum, sensorimotor function can be improved after injury in mice in which p75 signaling is inhibited.

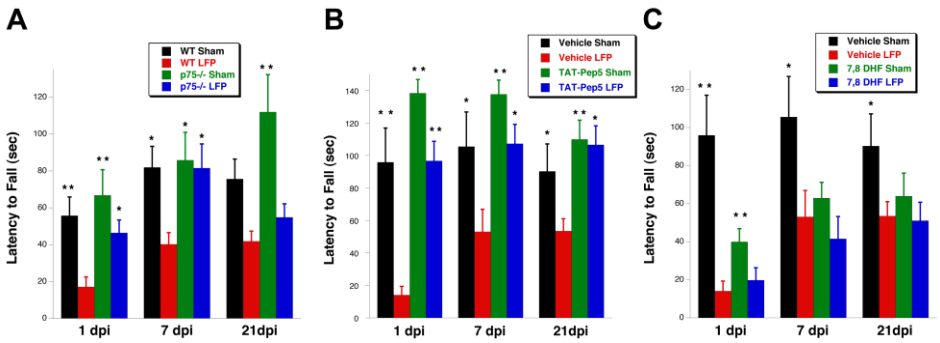



*Figure 42. p75 mutant and TAT-Pep5 treated LFP mice have a longer latency to fall relative to WT LFP or Vehicle LFP mice at 1 and 7 dpi. Each subject underwent 3 trials per day on the Rotarod device.* ***(A)*** *Bars represent average latency to fall per day ± SEM at 1, 7, and 21 dpi for WT and p75-/- mice subjected to sham or LFP injuries (n = 8-10 mice/group).* ***(B-C)*** *Average latency to fall per day ± SEM at 1, 7 and 21 dpi for mice treated with vehicle, TAT-Pep5 (B) or 7,8 DHF (C) following sham or LFP injuries (n = 8,9 mice/group) * p < 0.05, ** p < 0.01 relative to WT LFP or vehicle LFP, ANOVA Fisher's PLSD post-hoc test.*

## Discussion of Results:

In this study, we report that following LFP injury, p75 is upregulated and trkB is downregulated in the cortex and hippocampus with more variable changes in the levels of the neurotrophin ligands. p75 mutant mice as well as WT mice treated with the p75 antagonist TAT-Pep5 or the trkB agonist 7,8-DHF exhibited less neurodegeneration and cell death relative to control mice after injury. There was also less astrocytosis in mice lacking p75 expression. Behavioural experiments demonstrate that there is improved motor and cognitive function in p75 mutant mice and mice treated with TAT-Pep5 compared to control mice following LFP.

Increased mRNA and protein expression of BDNF has previously been described in traumatic brain injury and spinal cord injury [21-24], however this is the first report of a change in expression of different isoforms of BDNF and their receptors along with proNGF after lateral fluid percussion injury paradigm. The BDNF and trkB levels after TBI studied so far have focused mainly on the message [21, 25, 305, 306] or protein levels determined

either by immunocytochemistry or ELISA, which do not allow for discrimination of the isoforms of the neurotrophin expression.

In this report we have investigated the total protein changes at three time points, 1, 7 and 21 days post injury to include a wide time frame when different biochemical processes are activated. Our data indicated a change in expression of both neurotrophin receptors starting at 1-day post injury (1 dpi); p75 receptor is increased whereas trkB expression is reduced. This is consistent with previous findings that have determined as early as one day after initial injury there are distinct changes in cell structure leading to cell death [260, 261]. We have observed a reproducible change in receptor protein levels after lateral fluid percussion both in the cortex and the hippocampus indicating the effect of the external injury extends to distal regions including the hippocampus. It has been widely seen that after TBI, secondary injury can continue for days after the initial event and is associated with continuation of cell death in regions that are both focal and distal to the original site of injury [260, 261]. Although the change in protein levels measured at different time points showed an overall trend of a decrease in trkB levels and an increase in p75 amounts, there are some variations in the extent of protein alterations at different time points. This can be attributed to either differences between animals or the slight unpredictability of the extent of injury.

Conflicting studies have reported that trkB mRNA and protein levels either increase or decrease after TBI, however these studies differ with regard to timing and proximity to injury site of trkB expression [25, 305, 307, 308]. The upregulation of p75 after injury is more consistently observed. In spinal cord injury models, the expression patterns and timing of mRNA for p75 and Trk receptors is complex but p75 is increased as early as 6

hours after injury at sites proximal to the injury [309]. Moreover, a recent report using reverse phase protein microarray demonstrates that as long as 8 weeks after penetrating TBI, there is increase in p75 expression in the area surrounding the cavity [27]. Thus, p75 expression is induced quickly and is maintained for extended periods after injury. It has been shown that the concentration of the p75 receptor is increased in serum and CSF following TBI [274], suggesting that the upregulation of p75 could be used as a clinical biomarker. Moreover, p75 expression after other types of insults has also been reported including Alzheimer's disease, corticospinal axotomy, seizures and hyper-osmolar stress [310-314]. Recently, microRNA-592 was found to regulate the expression of p75 and apoptotic-inducing activity following neuronal ischemic injury [277]. Whether MiR-592 also plays a role in the upregulation of p75 following TBI remains to be explored.

p75 binds to its sortilin co-receptor and activates the caspases involved in the intrinsic apoptotic cascade [315, 316]. Our results showed that following LFP, sortilin levels significantly increased in the cortex at 1dpi. Corticospinal neurons are protected from death following a lesion in mice lacking the p75 co-receptor sortilin [317], suggesting that the p75/sortilin cell death pathway could be contributing to the damage following LFP.

We observed variability in protein expression patterns with regard to the different isoforms of neurotrophins following LFP, but the overall trend of increase for mature and pro-neurotrophins in both the cortex and hippocampus was evident. Some of the variability we observed between animals and isoforms may be caused by mild hypoxia resulting from brief apnea-like episodes that most mice experience immediately after the injury. We detected that the 14kD mature BDNF isoform increased by almost two fold after 7 dpi in the cortex and 7 and 21 dpi in the hippocampus. The 14kDa mature form, mBDNF has

high affinity for tropomyosin-receptor kinase (trkB) receptors [318, 319]. Upon binding to trkB, mBDNF potentiates neuron survival, differentiation, and plasticity [320]. A recent report indicates that intranasal administration of the protease tissue plasminogen activator results in functional recovery after TBI in rats, suggesting that cleavage of proBDNF to mBDNF by TPA enhances recovery [321]. In vitro, mBDNF protects against glutamate toxicity [322-324] and oxygen deprivation [325] and in vivo, BDNF pretreatment protects against chemical-induced injury [326, 327] and axotomy [328, 329]. In spinal cord injury, mBDNF increases the number of axons, promotes regeneration, increases the amount of myelin and improves motor function [330-333]. Experimental models of cerebral ischemia demonstrated that mBDNF reduced infarct size, protects hippocampal neurons, and improves memory and motor function [334-340]. Thus, although mBDNF is protective in some systems, its upregulation in LFP may be too late or at insufficient levels to promote survival.

Interestingly, in the cortex LFP injury did not affect the 28kD truncated BDNF isoform which results from proBDNF processing by a calcium-dependent serine proteinase known as membrane-bound transcription factor protease site 1 (MBTPS-1) [341], while in the hippocampus both at 1 and 21 dpi there was a statistically significant increase in the 28kD truncated form. The larger 36kD precursor proBDNF isoform, increased at 7 dpi in the cortex, with not much of a trend in the hippocampus. The p75 receptor also induces apoptosis when bound to proNGF [342, 343]. We show that proNGF levels are increased at all time points in the cortex. In sum, although proNGF is upregulated early, the increases in proBDNF occur slightly later than the upregulation of p75 which is detected as early as 1 dpi. Therefore the apoptotic pathway may be regulated by the enhanced receptor activation rather than the augmented presence of neurotrophin ligands. It would be of

interest to examine the levels of the proteases that cleave pro-neurotrophins to see if they are regulated by LFP and control how much of the various isoforms are available.

Although cell death and degeneration were increased by LFP in WT and vehicle treated mice relative to sham controls, p75 mutant mice and mice receiving the p75 antagonist TAT-Pep5 had reduced neuronal damage. The trkB agonist, 7,8 DHF was less effective, demonstrating that activating the trkB pathway may not be sufficient to prevent the toxic effects of p75 signaling. Alternatively, the treatment schedule or dose of 7,8 DHF may not have been optimal to produce an effect in our assays. One limitation of this study is that we cannot distinguish short-term (2 hr) versus longer-term (daily) effects of the compounds. Although the mice were given treatments on a daily basis, on the day of behavioral testing or perfusion for histological analysis, the dose of drugs was administered 2 hours prior to the testing. This acute dose could have been sufficient for the drugs' effects which can be explored in the future. In support of the effect of trkB activation improving recovery after TBI, a very recent study demonstrates that 7,8 DHF activates proteins related to plasticity and improves memory function after LFP which could be reversed by a Trk antagonist [287]. Moreover, another recent report demonstrates that TAT-Pep5 reduces lesion volume after controlled cortical impact to a similar degree as p75 deletion in mice [283]. Mature exogenous BDNF has proven protective during neuronal insult in some systems including spinal cord injury [330-333] and TBI [285]. In contrast, other reports reveal that mature neurotrophins have minimal beneficial effects [288, 289], suggesting mature neurotrophins are not sufficient for full recovery of neuronal function. Perhaps once the cell death pathway is activated by p75 signaling, it is not possible to reverse the apoptotic signaling by promoting survival mechanisms. Studies have indicated that p75 receptors can be present on neurons after injury before the onset of the apoptotic pathway

but trkB only appears after the caspase-3 pathway has been induced[344] thus lending support to the idea that p75 receptor activation overrides trkB activation and causes cell death during and after TBI. Moreover, proNGF can elicit apoptosis even when the Trk receptors are activated [313], again demonstrating that p75 can override the effects of Trk receptors.

The effects of the injury relative to sham on markers of apoptosis and axonal degeneration were most apparent at 1 dpi indicating that there is some endogenous recovery from the insult by 21 dpi. In parallel to these findings, the genetic and pharmacological interventions were most effective at 1 dpi for reducing cellular loss. Thus, early treatment can potentially inhibit the cellular and molecular pathways that cause long-term damage to the tissue. Furthermore, the cortical area demonstrated higher numbers of dying and degenerating cells relative to the hippocampus suggesting that the immediate site of injury is more prone to damage than slightly distal regions. Finally, the number of activated capase-3 and FJC+ cells in the p75-/- mouse and mice treated with the p75 antagonist were more robustly reduced in the cortex relative to the hippocampus indicating that the interventions are most effective at the site of the highest damage.

The cells undergoing apoptosis and the cells expressing p75 after injury appear to be neurons rather than glial as determined by immunohistochemistry. This is in contrast to a previous study which showed that p75 is expressed on astrocytes following seizure-induced injury [345]. These variations in findings may be explained by the different forms of injury. In support of our results, induction of p75 expression after injury mediates an apoptotic response in neurons but not astrocytes [346]. We did observe an increase in astrogliosis following lateral fluid percussion in both the cortex and hippocampus that is reduced in the

p75 mutant mice suggesting that there is some interaction between glia and the neurons that express p75. The glial scar is thought to prevent axonal regeneration [262]. Specifically, myelin-associated glycoprotein inhibits axonal outgrowth by interaction with the Nogo66 receptor and activation of the p75 receptor [347]. Therefore in the absence of p75 signaling, there may be less glial inhibition of axonal growth. The glial scar and increase in neurons expressing activated caspase-3 and p75 is likely to result in the cognitive and motor deficits observed following LFP relative to sham.

Cognitive function as assessed by the Morris Water Maze and sensorimotor function as determined on the Rotarod are both reduced following lateral fluid percussion injury although there is some natural recovery from the injury over time. There was a slightly longer latency to platform for the p75-/- mice prior to injury and after surgery the p75-/- sham mice had a longer latency to platform than WT sham mice. These observations are consistent with published findings that p75 mutant mice exhibit subtle behavioural impairments in MWM [348]. However, the p75-/- sham mice had a shorter latency to platform on day 1 of training relative to the WT LFP mice indicating that the injury paradigm is capable of exacerbating the effect of the p75 mutation. Thus our findings support the conclusion that the improved outcome in the p75-/- LFP mice relative to WT injured mice can be attributed to the absence of p75 signaling. Since swim speed was not altered by the injury in either WT or p75-/- mice, we can conclude that the injury induces less impairment in learning and memory and motor function when p75 signaling is silenced through either genetic or pharmacological treatment. However, the trkB agonist did not have an effect on cognition or motor skills consistent with its lack of effect on histological markers and suggesting that activating the trkB pathway is not sufficient to prevent damage due to the injury.

Several previous studies have manipulated neurotrophin and receptor expression levels to explore the effects on various forms of injury. BDNF+/- mice have improved motor function after stroke, suggesting that reducing BDNF levels enhances recovery [349] and while the authors attributed this to mature BDNF, it is possible that the effect could be due to a reduction in proBDNF protein. Moreover, when fibroblasts transfected with BDNF cDNA are implanted into a lesioned brain, they enhanced neuronal survival and increased axonal growth only to a small degree [350, 351] which could be due to the fact that both mature and proBDNF levels are increased following transfection. Other methods to reduce p75 signaling have proven beneficial following injury. For example, transplantation of p75-suppressed bone marrow stromal cells promotes functional behaviour in rat spinal cord injury [352], supporting our results that p75 inhibition is beneficial to motor recovery. In addition, histone deacetylase inhibitors downregulate p75 receptors and apoptotic factors after controlled cortical impact and these treatments are associated with increased stem cell and decreased glial cell marker expression in the injured tissue [353]. Furthermore, cells expressing mutant neurotrophins that have been engineered to have reduced binding affinity to p75 when grafted into mice subjected to spinal cord injury result in enhanced axonal growth [354]. Finally, posttraumatic increases in p75 expression are caused by enhanced GABAA-mediated depolarization activating Rho kinase and this cascade can be blocked by the loop diuretic bumetanide to improve outcome [355]. These studies above have attempted to alter p75 or pro-neurotrophin expression, in addition to using genetically mutant p75 mice, we have used a pharmacological approach to specifically inhibit p75 activation.

In support of our findings, a different p75 antagonist, LM11A-31, promotes improved motor function and coordination after spinal cord injury. Treatment with LM11A-31 following contusion injury results in an increase in the number of surviving oligodendrocytes and myelinated axons through inhibition of apoptosis via JNK3 [356]. The LM11A-31 compound also increases neurogenesis, inhibits neuronal death, and prevents activation of astrocytes and microglia following Controlled Cortical Impact model of TBI [357]. In contrast, LM11A-31 has been found to lack protective effects following pilocarpine-induced seizures [358]. Although our data indicate that p75 affect neuronal death and prevents glial activation, whether TAT-Pep5 affects other cellular processes such as myelination and neurogenesis remain to be explored.

The optimization of pharmacological agents that interfere with the p75 pathway should be performed in various models of TBI and spinal cord injury. Moreover, the effectiveness of blocking p75 signaling could be explored using other behavioural and cognitive tests. Although inhibiting the p75/pro-neurotrophin pathway appears to be more effective than activating the trkB pathway, future studies will also examine a combinatorial effect. In conclusion, our observations illustrate that targeting p75 can be a novel strategy that is non-invasive to counteract the damaging effects of TBI.

# Chapter 7: Ongoing Studies, Future Directions, and Conclusion

## Summary of Results:

Here, I report on the effect that single nucleotide polymorphisms (SNPs) in the *BDNF* and *ApoE* genes have on recovery after repeated mild TBI in a mouse model. I show that both Val66Met and ApoE4 are risk factors for poor recovery after repeated mild TBI relative to their Val66Val and ApoE3 counterparts which supports my hypothesis. I provide evidence that there are alterations in neurotrophin levels in these mice, and that personalized treatments aimed at remedying the deficits in the risk alleles are able to improve outcomes after injury which also supports my hypothesis.

## BDNF

I have shown that after rmTBI, compared to Val66Val injured mice, Val66Met mice have increased injury volume, cell death, neurodegeneration, p-tau, astrogliosis, and activated microglia at 1 and/or 21 DPI in the cortex and hippocampus.

| | | Caspase | FLJC | P-tau | GFAP | Iba1 | |
|---|---|---|---|---|---|---|---|
| | | | | | | Total | Activated |
| 1 DPI | Cortex | √ | √ | √ | √ | √ | √ |
| | Hippocampus | √ | √ | √ | √ | √ | √ |
| 21 DPI | Cortex | - | √ | √ | √ | √ | √ |
| | Hippocampus | - | √ | √ | √ | √ | √ |

*Table 1. Results summary from the immunohistochemical analysis of the BDNF genetic polymorphic mice. Green arrows represent statistically significant differences between injured Val66Met mice and injured Val66Val mice. Gray arrows indicate a trend between injured Val66Met mice and injured Val66Val mice. Gray dashes represent no differences between injured Val66Met mice and injured Val66Val mice.*

In addition, I have shown that treatment with AAV-BDNF in the injured Val66Met mice is able to improve outcomes at 21 DPI by decreasing levels of activated microglia and astrogliosis, improving learning, and increasing levels of BDNF and trkB signaling. (Fig 43)

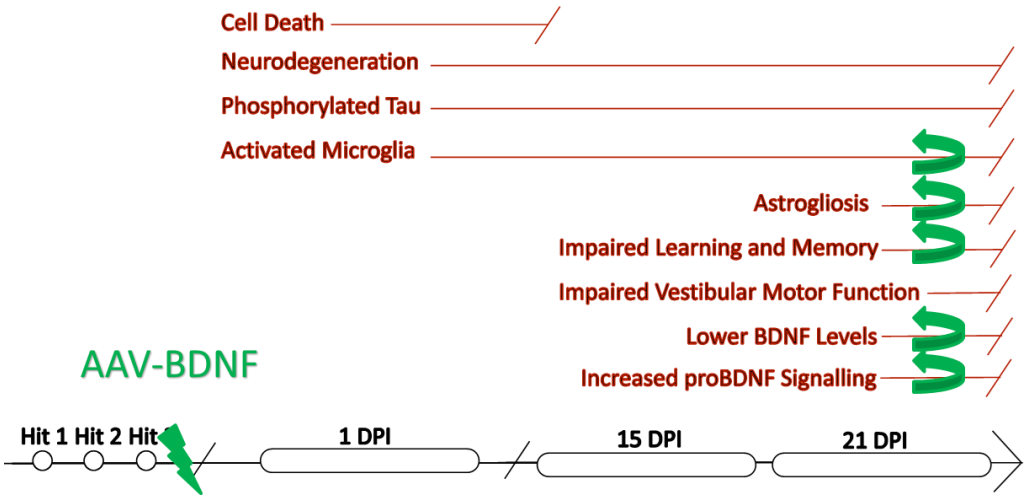



*Figure 43. Summary of BDNF results and the effects of rescue treatment with AAV-BDNF. Data shown represents outcomes where Val66Met injured mice had worse outcomes than Val66Val injured mice, and green arrows indicate where AAV-BDNF treatment was able to improve those outcomes.*

**ApoE**

I have shown that after rmTBI, injured ApoE4 mice have increased injury area, cell death, neurodegeneration, and activated microglia at 1 DPI in the cortex and/or hippocampus relative to injured ApoE3 mice. Injured ApoE4 mice also have increased levels of p-tau at 21 DPI compared to injured ApoE3 mice.

| | | Caspase | FLJC | Iba1 | p-Tau | GFAP |
|---|---|---|---|---|---|---|
| 1 DPI | Cortex | √ | √ | √ | √ | - |
| | Hippocampus | √ | √ | √ | - | √ |
| 21 DPI | Cortex | - | - | √ | √ | √ |
| | Hippocampus | - | - | - | √ | - |

*Table 2. Results summary from the immunohistochemical analysis of the ApoE genetic polymorphic mice Green arrows represent statistically significant differences between injured ApoE4 mice and injured ApoE3 mice. Gray arrows indicate a trend between injured ApoE4 mice and injured ApoE3 mice. Gray dashes represent no differences between injured ApoE4 mice and injured ApoE3 mice.*

In addition, I have shown that treatment with Bryostatin-1 is able to improve outcomes at 1-7 DPI in the injured ApoE4 mice by decreasing levels of neurodegeneration and activated microglia, improving learning and memory and fine motor balance, and possibly increasing BDNF levels. (Fig 44)

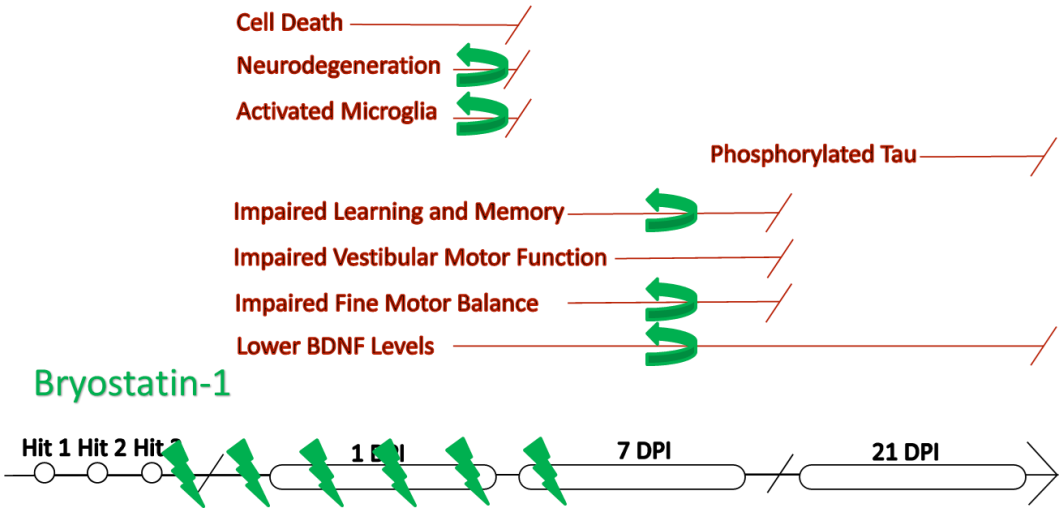



*Figure 44. Summary of ApoE results and the effects of rescue treatment with Bryostatin-1. Data shown represents outcomes where ApoE4 injured mice had worse outcomes than ApoE3 injured mice, and green arrows indicate where Bryostatin-1 treatment was able to improve those outcomes.*

## Ongoing Studies:

### BDNF

To date, I have shown that the Val66Met genetic polymorphism is a risk factor for poor recovery after rmTBI. I used MRI to analyze the injury volume at 21 DPI, immunohistochemical methods to analyze cellular markers at 1 and 21 DPI, and Western Blot to analyze the levels of proteins at 1 and 21 DPI. Using the information gleaned from these experiments, I hypothesized that treatment with AAV-BDNF would rescue the vulnerable Val66Met injured mice. In order to determine this, I analyzed outcomes after treatment using immunohistochemical methods at 21 DPI, Western Blot at 21 DPI, and cognitive and motor behavioral analysis 15-21 DPI.

I found that treatment of injured Val66Met mice with AAV-BDNF is able to rescue the increased astrogliosis and activated microglia detected at 21 DPI relative to their control injured Val66Met counterparts. Treatment with AAV-BDNF in these mice is also able to increase learning at 16 and 17 DPI relative to their controls. I determined that the AAV-BDNF treatment also increased total and mature BDNF levels and p-trkB in the cortex and hippocampus, signifying that treatment with AAV-BDNF activated the trkB signaling pathway.

Future studies in the lab will investigate the mechanisms through which the AAV-BDNF treatment is working. The AAV-BDNF treatment will be given in conjunction with a trkB antagonist, such as ANA-12 that can reduce trkB signaling back to normal levels. If the beneficial effects we see in these mice are caused by the overexpression of BDNF

activating the mature BDNF trkB receptor, then in the groups that are given AAV-BDNF treatment in conjunction with the trkB antagonist, the beneficial effects of the rescue treatment will be abolished. This experiment will help elucidate the mechanism through which the AAV-BDNF treatment is improving recovery after injury. If the overexpression effect is blocked by the trkB inhibitor, it will verify our hypothesis that altered neurotrophin signaling is responsible for the difference the two genotypes, and that it can be targeted with this therapy modality. In addition to using a trkB antagonist, we could consider such as using siRNA to block the action of trkB or crossing our mice with trkB knockout mice and observing the effects that blocking trkB signaling has on the effects we see after our AAV-BDNF treatment.

In addition, we plan to research the effect that the BDNF prodomain might be having in our experiments. Other labs have shown that the prodomain that is cleaved from mature BDNF is an active ligand, and that the 66Met form of the prodomain is particularly toxic. [40, 41] We expect that the 66Met prodomain may be responsible for some of the cell death and neurodegeneration that is seen after injury in our Val66Met mice, and that treatment with the AAV-BDNF which has the 66Val form may be acting to counter this damage. We plan to look at levels of the prodomain, as well as activation of its receptor, SorCS2, since previous studies have shown that even in at low levels the 66Met prodomain can have very detrimental effects. [40, 41]

We also can investigate the effect of other treatments that may be more easily translatable into the clinic, such as BDNF mimetics which can also activate trkB signaling [32] or the nasal administration of tPA, which cleaves proBDNF into mBDNF and has been shown to be effective in single moderate TBI in WT mice. [359] These may also be effective ways

to stimulate trkB signaling after injury in our vulnerable Val66Met injured mice.

Taken altogether, we can consider using therapies that take advantage of the sum of our knowledge about the BDNF signaling pathways and the effect that the Val66Met SNP has on them. Given that we have shown that we are able to improve outcomes after injury by stimulating the trkB pathway or inhibiting the p75 pathway [26], it is important to consider the effect that the Val66Met SNP may be playing in the overall signaling pathways (Fig 45) It has been shown that proBDNF is an apoptotic ligand that induces cell death by binding to the p75 and sortilin receptors [276], while mature BDNF has been shown stimulate neuronal survival by binding to the trkB receptor. [360] Interestingly, the pro-domain of BDNF has recently been shown to be an active ligand itself, binding to both the sortilin and SorCS2 receptors. Importantly, recent studies have shown that the 66Met and 66Val pro-domain differentially interact with the SorCS2 receptor. The 66Met SNP results in a distinct conformation change in the pro-domain, which causes it to have a differential engagement of the SorCS2 receptor, leading to an increase in the SorCS2 mediated growth cone retraction. [40] By taking these factors into consideration, we can develop better targeted therapies, and consider using a combination treatment which can activate trkB signaling while blocking p75, sortilin, and SorCS2 signaling. A combination therapy such as this one could potentially be a potent treatment after injury in these Val66Met injured mice.

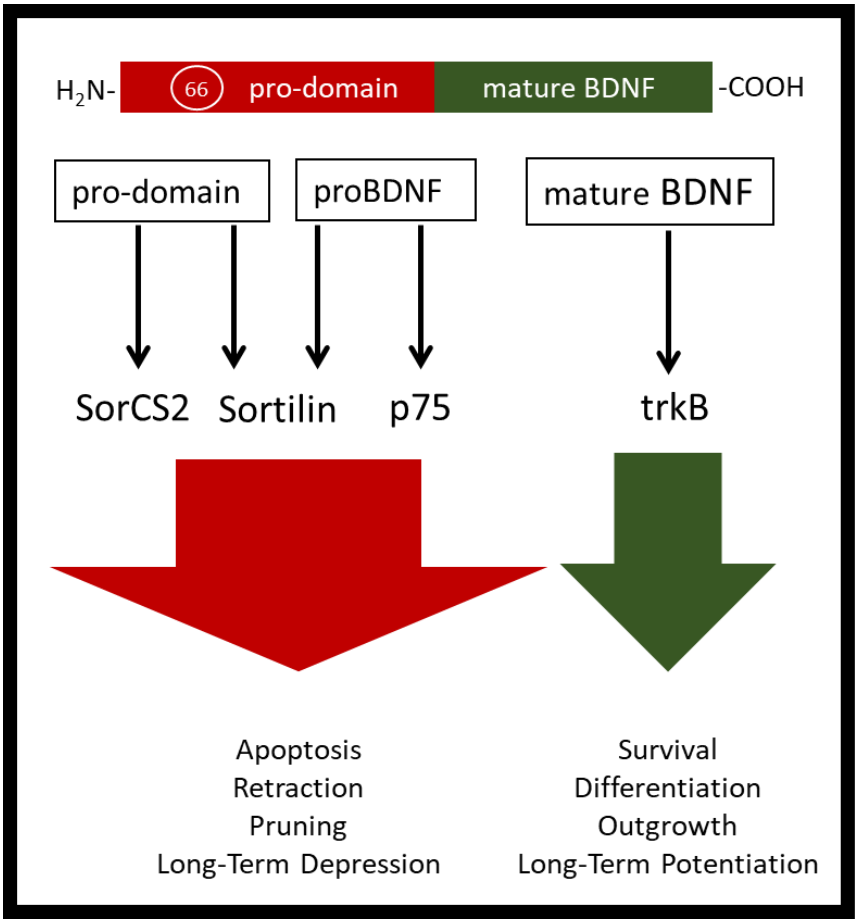



*Figure 45. Summary of BDNF Signaling Pathways*

**ApoE**

To date, I have shown that the ApoE4 genetic polymorphism is a risk factor for poor recovery after rmTBI. I used MRI to analyze the injury volume at 21 DPI, immunohistochemical methods to analyze cellular markers at 1 and 21 DPI, and Western Blot to analyze the levels of proteins at 1 and 21 DPI. Using the information gleaned from these experiments, I hypothesized that treatment with Bryostatin-1, a PKCε activator, would rescue the vulnerable ApoE4 injured mice. In order to determine this, I analyzed outcomes after treatment using immunohistochemical methods at 1 DPI, Western Blot at 1 DPI, and cognitive and motor behavioral analysis 1-7 DPI.

From these results, I have found that treatment of injured ApoE4 mice with Bryostatin-1 is able to rescue the increased neurodegeneration and activated microglia seen at 1 DPI relative to their control injured ApoE3 counterparts. Treatment with Bryostatin-1 in these mice is also able to improve learning at 2 DPI and memory at 7 DPI. However, when I analyzed levels of BDNF and p-trk B at 1 DPI after two doses of Bryostatin-1 in these mice, I did not see the increase in levels we had expected to detect. However, this is not completely surprising, since previous studies in the literature have shown that levels of BDNF increase with increased Bryostatin-1 dosing. [162-164, 218, 219] Specifically, previous studies have shown that BDNF levels peak in mice after 3 weekly injections of Bryostatin at 25 $\mu g/m^2$. [163] We plan to test our samples in a more sensitive BDNF ELISA assay, but if we find similar results, then the effects we are seeing at 1 DPI are likely due to another mechanism, rather than an increase in neurotrophin factors as I had hypothesized. In order to determine if the effects I see during our behavioral experiments might be affected by an increase in neurotrophin levels, future studies will analyze levels of BDNF and p-trkB signaling after the dosing regimen given to these mice culminating at 7 DPI. If we do see a difference in BDNF and p-trkB levels, we plan to then investigate if the BDNF signaling is necessary for the effects of Bryostatin-1 that we see at that timepoint. In order to do this, we will give Bryostatin-1 treatment with a trkB antagonist, such as ANA-12. If injured ApoE4 mice given treatment with both Bryostatin-1 and ANA-12 no longer show recovery, then we will know that BDNF signaling is necessary for the beneficial effects of Bryostatin-1. We also plan on repeating all rescue experiments at the 21 DPI timepoint to see if we can affect the longer-term effects, such as the increase in p-tau that ApoE4 mice exhibit after injury at this timepoint.

In order to more fully understand which pathways might by affected by treatment with

Bryostatin-1, we plan to work with our collaborator at Duke University, Dr. Patrick Sullivan, in order to perform sensitive multiplex ELISA assays. We will analyze BDNF levels, molecules in the PKCε signaling pathway, and pro-inflammatory factors from brain cortical and hippocampal brain lysates at 1 and 21 DPI. By conducting these experiments, we hope to understand more fully why ApoE4 mice have worse recovery than ApoE3 after rmTBI and learn how Bryostatin-1 is acting to facilitate recovery after rmTBI in these mice.

We can attempt to understand the pathways that may be affected by Bryostatin-1 treatment in ApoE4 injured mice by understanding the way that Bryostatin-1 interacts within the context of ApoE signaling. Based on previous research, we know that ApoE4 and ApoE3 differentially interact with PKCε. [100] ApoE4 inhibits the action of PKCε, which removes inhibition on HDAC 4/6. This allows HDAC 4/6 to translocate into the nucleus, where it engages in histone deacetylation and causes negative gene regulation. On the other hand, ApoE3 activates PKCε, which inhibits HDAC 4/6 so it does not translocate into the nucleus. Therefore, there is histone acetylation in the nucleus, and positive gene regulation. This positive gene regulation causes an increase in a number of factors, including BDNF. Bryostatin-1 is an activator of PKCε which acts to mimic the actions of ApoE3. (Fig 46) Treatment with Bryostatin-1 results in positive gene regulation, and others have shown it increases BDNF levels. [163] By understanding these interactions, we hope to be able to maximize our understanding of Bryostatin-1 as a treatment option for ApoE4 mice after injury.

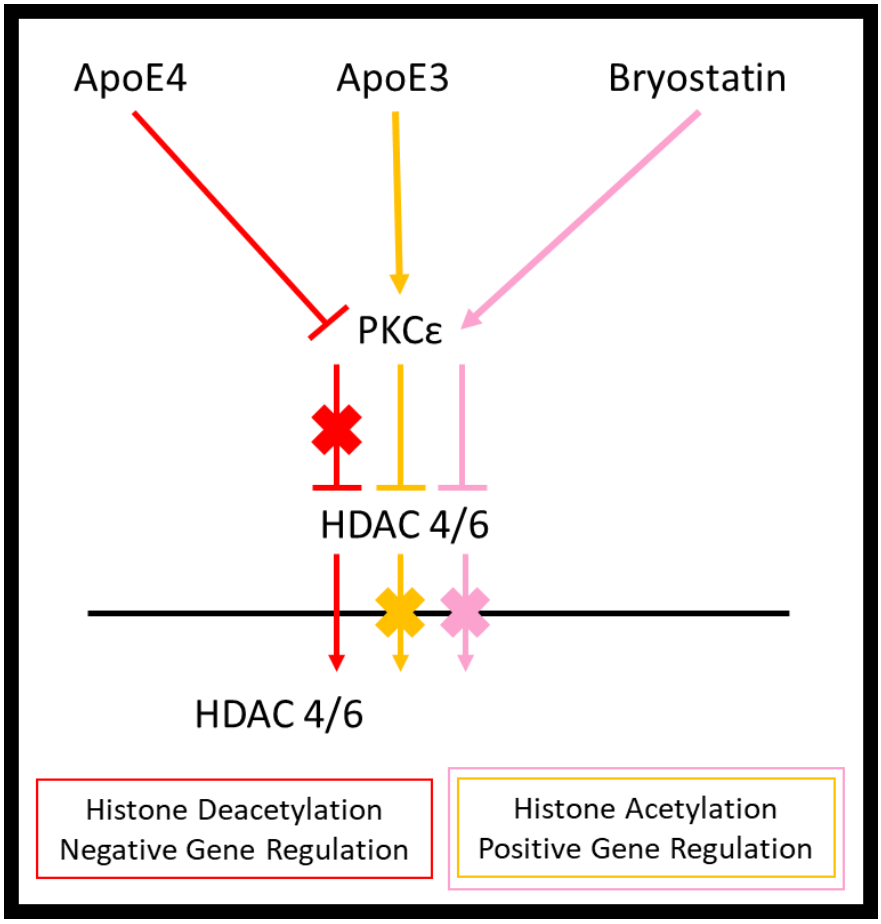



*Figure 46. Summary of the differential interaction of the isoforms of ApoE with PKCε*

## Future Directions

### Studying BDNF x ApoE

As mentioned in the Introduction, genetics in the human population are not as simple as we attempt to make them in experimental mouse studies. Future studies should consider the compounding effects that multiple risk alleles have on outcomes after rmTBI. One method to approach this issue would be to study whether there is a compound risk of having both the ApoE4 and BDNF Val66Met genotypes. Recent work has found that there is a compound effect of the ApoE4 and BDNF Val66Met on episodic memory in older adults [361] and they moderate the aβ related cognitive decline in preclinical AD. [362] However, there are no experimental mouse studies investigating the role that these two factors may

have on outcomes after TBI. The investigation of this question would advance our knowledge, and could lead to the development of specialized treatments for particularly susceptible individuals.

**Studying Other TBI Injury Models**

For our studies, we chose to use a repeated mild TBI injury model that employed three hits separated by 48 hours each. This injury pattern mimics a specific type of repeated mild concussion that is commonly seen in contact sport athletes and military personal, however the outcomes resulting from this pattern of injury may not be generalizable to all repeated mild TBIs that occur. Future studies should consider different patterns of repeated mild TBI. For example, in the general population, it might be more common to sustain two mild TBIs. In particularly violent contact sport positions, there may be daily subconcussive hits that never reach the threshold of what is considered a mild TBI. In addition, these repeated subconcussive hits may be more accurately assessed using a mild closed head model. Mimicking these injury patterns in a mouse model would allow for an increased understanding of the way that repeated mild TBI can cause damage in the human population.

In addition, we examined outcomes at 1 and 21 DPI, but it is becoming increasingly evident that rmTBI can cause long-term effects that last decades. Future studies should take this into account and analyze outcomes at time points such as 6 months and 1 year in the mice. We conducted our study in mice aged 10-12 weeks, which is used to represent young adulthood in humans, since that is the age during which these types of rmTBIs are generally sustained. Future studies should take this into account and study both younger and older mice, in order to determine if age is a compounding factor as well. Finally, we used both

male and female mice in our study. In our analysis of the data, we analyzed differences between male and female mice in order to consider the effect that sex might be having on outcomes. When we did this analysis, we did not see any statistical significance between the groups. However, our studies were not powered to investigate the differences between sex. Future studies should increase the power of the groups in order to determine if there are any differences that can be detected, since previous studies in humans have showed that there may be sex dependent differences in recovery after TBI. [363]

**Studying New Directions**

In this work, we have investigated outcomes using imaging, immunohistochemistry, biochemistry, and behavior. These are traditional basic science methods which can greatly help increase our knowledge of the pathophysiology of disease states. However, these methods, with the exception of the imaging, are not easily translatable into clinical findings. In order to increase the translational nature of the project, we collected serum and CSF in order to attempt to validate biomarkers after rmTBI. One way to explore whether biomarkers correlate with histological and behavioral changes and therefore represent useful markers for the different genotypes would be to conduct multiplex ELISA after injury. Future studies in the lab will investigate this.

We have shown in our work that by stimulating the trkB pathways and inhibiting the p75 pathway, we can improve recovery after TBI. Given that we have found that inhibiting the p75 pathway has a more dramatic effect, we hypothesize that inhibiting other detrimental signaling pathways, we may be able to increase recovery after TBI. Since it has been shown that the toxic 66Met interacts with SorCS2 to have detrimental effects, future studies in the lab can explore the use of combinational therapy targeting these three signaling pathways.

**Studying Encapsulated MSCs for TBI Treatment**

MSCs have the potential to be a powerful therapy, however there are a few things to consider when planning to use them as a treatment after TBI. As we showed here, MSCs are able to decrease inflammation, cell death, and neurodegeneration after injury. Other groups have showed that MSCs have powerful anti-inflammatory properties and are able to decrease the time it takes to make the transition from M1 to M2, a sign that recovery is occurring and that there is a shift from pro-inflammatory markers to anti-inflammatory markers. [168, 170, 181] However, long-term treatment with MSCs has proven to be difficult, since the cells migrate from the injury site and are phagocytized by the body.

One potential way to increase the action of MSCs is to encapsulate them in alginate, which has also been shown to influence their secretome positively. [180] However, when this is done, it is only the action of the secretome that is contributing to recovery after injury, there is no longer any possibility of cell replacement contributing to recovery. It also increases the difficulty of injecting them into a mouse model, due to the relative size of the capsules.

Treatment with MSCs relative to AAV-BDNF and Bryostatin-1 has the advantage that there is a generalized anti-inflammatory effect, and if encapsulated they can persist for an extended period of time at the site of injection. This is in contrast to the targeted nature of treatment with AAV-BDNF and the transient nature of treatment with Bryostatin-1. However, because MSCs are foreign cells, they also have a higher degree of unpredictability. In some cases, MSCs have had a number of serious side effects, and some have even grown into cancerous masses when injected. [364]

Here, we have also shown the feasibility of using MSCs for treatment after rmTBI. Future studies will work in collaboration with the biomedical engineering department in order to begin testing the encapsulated MSCs in our mouse model of TBI and classifying the effect that they have on recovery after injury.

**Using GWAS**

Although they are a vital part of the scientific inquiry process, experimental mouse studies are limited in the number of variables that they are able to consider. Future studies should consider leveraging the large amount of data that can be gleaned from big data genome-wide association studies (GWAS). Recently, there has been interest in investigating the role that the genome has on TBI and using a GWAS study is a particularly powerful to analyze the question of genetic influences on outcomes after TBI. [365] Here, we have used the candidate gene approach, which while it is able to uncover important information about the effect of these SNPs on recovery after TBI, is by its very nature a biased approach. The GWAS approach would be an unbiased approach, and may uncover previously unknown information about SNPs that are risk factors for poor recovery after TBI. In fact, initial studies have shown that GWAS may be beneficial in determining genomic risk factors for TBI, which can then by probed experimentally by mouse studies. [366, 367]

**Bench to Bedside, Bedside to Bench**

Translational medicine has the potential to unlock important findings that can change the way that healthcare is practiced and provide tangible benefits to patients. However, too

often researchers and clinicians are unable to communicate in an effective manner. Researchers attempting to study translational medicine need to have an understanding of the clinical aspects of care, and clinicians need to be able to communicate their daily experiences with patients back to researchers in a way that enables basic researchers to study these healthcare problems. In this study, I used my conversations with clinicians who treat brain injury on a daily basis in order to inform the way I did our experimental studies. It is my hope that in the future this dialogue will continue, and we will be able to combine the talents of researchers and clinicians in order to make meaningful progress in the understanding of the pathology of brain injury and to develop effective methods of treatment.

## Clinical Trials

Future studies investigating the role that genetic polymorphisms have on outcomes after injury and the development of targeted therapies will be most effective if they are able to bridge the translational gap discussed previously. The desired end goal would be to take this study into clinical trials. In order for this to occur, preclinical trials would need to have identified a number of different genetic polymorphisms thought to confer risk after TBI in mouse models, and the mechanism through which these differences occur would be investigated. These would be further investigated experimentally in pig and non-human primate studies. At the same time, case control and cohort studies would be done in athletes and the general population, enrolling people who have sustained TBIs and sex and age matched controls who have not. Genetic analysis would be done in these groups. When specific genetic polymorphisms have been implicated in these studies, enrollment for randomized control trials could begin. At this point, enrolled patients with TBI and specific genetic polymorphisms would be randomized to either a novel targeted treatment group,

based on the mechanisms previously investigated, or a control treatment plan. When there has been a significant amount of research done investigating these research questions, a meta-analysis would be done in order to determine what the consensus of the field states. At this point, if there have been genetic polymorphisms strongly identified to confer risk, and a targeted treatment option that shows promise in these studies of being able to improve outcomes, a clinical trial would commence. The clinical trial would mostly likely be run by a pharmaceutical company with a vested interest in the targeted therapy. Phase I would test the targeted therapy for safety and dosage in healthy individuals. Phase 2 would test the targeted therapy for efficacy in a small sample of the affected population. Phase 3 would test the targeted therapy for efficacy in a wider range of the affected patient population. After which point, the targeted therapy would be approved by the FDA and move into Phase 4, extended tests for safely and efficacy over time. In this way, translational research could help bridge the gap between basic research science, and the development of a therapy that is brought to clinical trials.

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