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ACI-35 AND AADVAC1 ACTIVE IMMUNOTHERAPY AS PREVENTATIVE TREATMENT OPTIONS FOR CHRONIC TRAUMATIC ENCEPHALOPATHY

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ACI-35 AND AADVAC1 ACTIVE IMMUNOTHERAPY AS PREVENTATIVE TREATMENT OPTIONS FOR CHRONIC TRAUMATIC ENCEPHALOPATHY

by

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One of the most common, as well as one of the most dangerous injuries amongst athletes today is mild traumatic brain injury (mTBI), commonly known as concussion. Aside from physical symptoms such as nausea, dizziness, and headaches; concussions have can have long-term effects on brain physiology. A common neurological disease that can result from multiple concussions is Chronic Traumatic Encephalopathy (CTE), characterized by symptoms such as severe depression, anxiety, confusion, and aggression; amongst others. On the cellular level, CTE is classified by a unique pathway that leads to the hyperphosphorylation of tau protein and subsequent clumping of tau-containing neurofibrillary tangles (NFTs).

Tau hyperphosphorylation and aggregation is also a key characteristic of a similar neurological condition, Alzheimer’s Disease (AD). Emerging active immunization treatment methods, including the ACI-35 and AADVac-1 vaccines, have been clinically tested in Alzheimer's patients with the goal of reducing hyperphosphorylation of tau and its effects. Because of the similarity between the two diseases, this paper takes a look at the possibility of applying this active immunotherapy treatment to patients who are likely to develop CTE.

KEY WORDS: Concussion, Mild Traumatic Brain Injury (mTBI), Chronic Traumatic Encephalopathy (CTE), Tau, Hyperphosphorylation, Active Immunotherapy
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Chapter 1: Concussions

Introduction

The term concussion has become very familiar in society today, but most people are unaware of the severity, pathophysiology, and long-term effects that a concussion may deliver. The term concussion is essentially synonymous to Traumatic Brain Injury, or TBI. By general definition, a TBI occurs when an external force is applied directly or indirectly to the brain, causing structural damage or overall brain impairment. TBI can refer to an injury as miniscule as a slight bump to the head, or as extreme as a life-ending brain impact. Concussions are often categorized on the milder end of the TBI scale, and are therefore commonly referred to as mild traumatic brain injuries (mTBI).

Although concussions are categorized as mild TBIs, no impact or blow to the head should ever be dismissed. The head houses arguably the most important organ in the body, the brain. The brain is a major component of the body’s central nervous system (CNS), meaning that it receives and processes information from all areas of the body and formulates the proper response. It is the reason that humans can feel pain, think thoughts, have emotions, move, breathe, and speak; amongst many other functions. It is because of these vital functions and roles that each head impact should be taken seriously and given appropriate time to heal.

Protecting the Brain

Because a healthy brain is so vital to human survival, there are many anatomical barriers in place to protect this organ. The most evident of these is the skull, a complex structure composed of 22 bones in total, 8 of which form directly to encase the brain in a structure known as the neurocranium. Thickness of the skull varies between individuals, but typically falls within
0.9-3 mm thick. The skull essentially acts as a helmet around the brain to protect from minor external bumps and blows.

In addition to the skull, there are many internal barriers to keep the brain free from harm. A few of these internal barriers include the blood brain barrier (BBB), blood-cerebrospinal fluid barrier (BCB), and the meningeal brain barriers. The BBB is a membranous layer composed of tightly regulated blood vessels that isolate the brain’s blood supply from the blood circulating in the human body. That way, if an impurity or cancerous cell type invades the human body and enters the bloodstream, the brain would remain secluded and therefore safe from the infection. This fixed barrier is formed by endothelial cells that line the walls of blood vessels in the BBB. These endothelial cells form tight junctions that greatly limit the free flow of ions and other molecules that would otherwise easily penetrate the bloodstream. Compared to regular endothelial cells, endothelial cells of the BBB possess a larger number of mitochondria to help facilitate active transport of only certain molecules across this barrier. In addition, endothelial cells of the BBB contain a much lower concentration of leukocyte adhesion molecules (LAMs), which help to limit the amount of immune cells that pass through the barrier. The BBB ultimately provides the brain with a great layer of protection, which, in one sense, is very helpful for protecting the brain against harmful or fatal infections. On the other hand, this hedge of protection does make it rather difficult to administer target drugs directly to the brain due to the inability of the regular bloodstream to deliver any unknowns to the cerebral bloodstream.

Another feature in place to safeguard the brain lies within the cerebrospinal fluid (CSF) of the blood-cerebrospinal fluid barrier, or BCB. The BCB is located in the choroid plexus, a highly vascularized tissue that fills the space between the 4 ventricles of the brain.
Figure 1, this region is located in the central section of the brain. The CSF alone also forms a thin layer between the brain and the skull.\textsuperscript{12} The BCB contains a system of capillaries enclosed in differentiated ependymal epithelial cells that are held together by tight junctions.\textsuperscript{10} However, unlike the extreme tightness of the epithelium in the BBB, the cellular junctions in the BCB are more permeable and allow movement of small molecules.\textsuperscript{13,14} The molecules that pass through the BCB due to its permeability do not go into the brain’s blood supply, but into the CSF, because this barrier is in place to separate the blood from the CSF. Therefore, damage to this barrier results in an increase in protein concentration in the CSF, not the blood.\textsuperscript{15} The CSF is produced and secreted by the ependymal epithelial cells of the BCB. In addition to its role as a barrier, the CSF also provides a buoyancy around the brain to absorb the blow from head trauma and allows room for swelling if an injury were to occur.\textsuperscript{13}

Lastly, the meningeal brain barrier is a protective membrane that works to keep the brain and CNS safe from injury. This layer is formed on the outer side of the arachnoid membrane, and
is comprised of three layers of cell types to act as a physical barrier between the outer CSF and the deeper dural layers of the brain.\textsuperscript{16,17} The dura mater in particular lacks a BBB, so the layers of meningeal cells are in place to protect this area of the brain by forming tight junctions to prevent passage of unwanted molecules into the brain.\textsuperscript{16} The meninges essentially anchor the brain within the skull to prevent excessive movement.\textsuperscript{17} This is especially important in times of injury such as whiplash-- if the brain was not anchored strongly to the skull, worse injury would likely occur.

The brain is contained in more protection than any other organ in the human body. By this fact alone, it can be concluded that the brain is of utmost importance in the operation of the human body. Although the brain is protected by a plethora of physical and biological barriers, concussions still have the ability to deeply resonate and have deleterious effects. It is for this reason that concussions in athletes are taken so seriously and each unique case is scrutinized individually in order to ensure the most effective recovery.

\textbf{The Dangers of Contact Sports}

According to some of the most recent statistics from the Center of Disease Control (CDC) concerning concussions, there were 2.87 million hospitalizations, emergency department visits, or deaths due to TBI in the United States in 2014.\textsuperscript{18} This huge number excludes all of those who likely suffered concussions but did not go to a hospital or emergency room to get it examined. This indicates that the number of concussions suffered that year is presumably higher. Another study conducted by the CDC in 2017 states that 15.1\% of high school student athletes reported to have had at least one concussion, and 6.0\% have reported having two or more concussions. Therefore, approximately 2.5 million high school students alone reported that they have had at least one concussion, and over 1.0 million reported having 2 or more.\textsuperscript{19} The sheer
number of young people that contribute to the total number of concussions suffered in the United States is tremendous. By suffering one or more concussions before the age of 18, high school students are placed at a huge risk for neurological problems down the road. Why? Because after suffering at least one TBI at a young age, if they were to suffer another later in life, it would not be the first, but rather the second, third, or fourth because of the high exposure of head impact in their high school years. Not to mention, those who suffered multiple concussions in high school alone have placed their brain at higher risk for later developing neurological disease.20

In addition, the numbers in the second survey account only for high school athletes, therefore excluding college or professional. According to the NCAA official website, there are over 460,000 participants involved in college athletics.21 This does not number does not include the number of athletes involved in NAIA, Junior College, or professional sports. The bottom line is that there are millions of young people competing in different sports year-round in the United States, thus creating increased exposure and risks for head injury. The amount of reported concussions amongst athletic programs has been on the rise over the past decade. One possible cause for increase is improved detection methods, but as athletes continue to grow bigger, stronger, and more competitive, it is likely that the number will continue to rise.

One obvious reason that athletes are at higher risk for concussion is because they are exposed to high impact situations on a daily basis. Contact sports such as hockey, lacrosse, wrestling, and football rank amongst the highest in percentage of athletes to suffer one or more concussions (Figure 2).22 However, athletes in nearly every sport are at risk for concussion and should be made aware of the potential effects. According to a study done by the University of Pittsburgh, approximately 300,000 sports-related concussions occur annually, and the likelihood
of experiencing a concussion in a season of a sport is estimated to be nearly 19%.\textsuperscript{23} This statistic is an estimate and varies depending on the sport, but it highlights the necessity of playing with caution and mindfulness.

Playing a sport does significantly increase the chance of suffering an mTBI.

**Diagnosis and Protocol**

Studies have shown that athletes returning to play before fully recovering from a concussion, which is typically 7-10 days minimum, are at high risk for undergoing a second one.\textsuperscript{24} This is because resting symptoms may rapidly decrease during this initial time period after mTBI, but are likely to return upon vigorous activity. Concussions are unique in that no two are exactly the same; they may have varying symptoms and recovery time depending on the type of impact suffered. It is this fact that makes concussions so difficult to accurately diagnose.
There is no universal scale to classify the severity of concussions or how long an athlete must be out before returning to play. Different literatures stand by different scales to measure the severity of a concussion. For example, one scale proposed by Physical Therapist Mark Lundblad, breaks concussions down into 5 subgroups: acute, classic, amplified, focal, and prolonged. By this definition, an acute concussion has symptoms lasting less than 10 days; classic, amplified, and focal concussions have symptoms lasting from 10-90 days; and prolonged concussions have symptoms lasting for over 90 days. This scale is based on the physical therapy management of sports-related concussions, and treatment plans are based on the length that symptoms persist as well as how consistent the symptoms are. However, a different scale known as the Glasgow Coma Scale (GCS) rates all TBIs from concussions to coma based on a numeric system from 1-15. Here, a score of 13-15 is given for mild injuries, 9-12 indicates a moderate injury such as temporary loss of consciousness, and anything less than 8 indicates severe injury which includes coma or amnesia for over 24 hours. Although a single, universally adopted concussion grade scale may be beneficial for diagnosing, each head injury is still different and unique and therefore should be evaluated individually and assessed with proper care.

The general rule for head injuries as determined by the Second International Conference on Concussion in Sport is that a concussion is classified as “simple” if symptoms persist for less than 10 days, and “complex” if symptoms last longer than that. Because concussions are so difficult to definitively diagnose, indirect methods are often used to measure the severity of cognitive and physical abilities following a head injury. Some of the most common indirect methods include comparing neurological abilities to a pre-injury baseline, measurement of balance deficits, and severity of symptoms.
Many athletes are now required to take a test that measures brain functions such as memory and concentration before participating in sports when their brain is healthy. When an athlete suffers a head injury, they can be given the same test, and results can be compared to the pre-injury baseline. A significant decrease in performance on this test could indicate a loss of brain function due to injury, and therefore greatly assist with the diagnosis process.

Balance is another factor commonly tested following a concussion because studies have shown that postural deficits are often present in the 24 hours following a concussion. One balance test that is often used is called the Dynamic Gait Index. This is a comprehensive test performed directly following injury that involves walking in a straight line, changing speeds, and navigating simple obstacles. Again, this test would be rather easy for a healthy individual to complete, but much more difficult in the case of dizziness due to a head injury suffered.

Lastly, a common method to evaluate injury is an assessment of symptoms known as the Post-Concussion Symptom Scale (PCSS). This scale features 22 symptoms that are common to concussions (Figure 3). In this assessment, student athletes are asked to rank their symptoms from 0-6, with increasing number signifying increased severity. A study done by Jen-Kai Chen took this a step further and looked at athletes’ responses to this test compared to their functional MRIs (fMRI). The fMRIs of post-concussive athletes were taken to measure brain activity compared to a
control, healthy brain. Results of his study showed that athletes that did rate themselves higher on the PCSS, meaning more symptomatic, also showed less brain activity. Results of Chen’s study are extremely beneficial to the diagnosis of concussions for a few reasons. First, these results verify that the self-reported PCSS can be an accurate method of measuring the severity of a concussion after head impact. Athletes that ranked themselves as having worse symptoms also had decreased brain activity, and therefore worse head injury. This study is also beneficial in that it shows that fMRI of the brain post-injury is a sensitive enough tool to track and diagnose the severity of a concussion. The use of fMRI could be very valuable in providing a standard for athletes on length of recovery before returning to play, since it does provide images and specific data on brain activity. Although this is a huge step in the right direction, each concussion is still unique, and should be assessed individually before allowing athletes to return to play.

**Pathophysiology**

Each concussion is unique and affects each person differently, but on a cellular level, the brain experiences a similar response to each mTBI. Directly upon any biomechanical impact to the head, an influx of neurotransmitters, primarily excitatory amino acids (EAAs), are released leading to a shift in ionic channels. Neurotransmitters are chemicals that are released at presynaptic nerve terminals and travel throughout the body to play many roles such as communication, development of the brain and nervous system, memory storage, learning, motor control, cardiovascular function, amongst others. Excitatory neurotransmitters, glutamate in particular, bind to receptors that open Na+ and K+ channels. Opening of these channels allows a free flow of ions, leading to the depolarization of membranes and increased action potential
firing. In other words, the effect of increased neurotransmitter release in response to impact is an overall escalation of energy in cell-to-cell signaling. This rapid energy shift causes cells to work in overdrive in order to combat the unusually high signal. The rapid depolarization of membranes also leads to a large increase of calcium ions in the cells and efflux, or decrease of potassium ions in the cell. Calcium ions are essential to the normal function of a cell, as they play a role in cell signaling, homeostasis, and the production of ATP in the mitochondria. However, when the concentration of calcium ions within the cell is rapidly increased, cell metabolism within the mitochondria is accelerated greatly, which can ultimately cause swelling of organelles and eventual apoptosis, or cell death.

In attempts to combat the influx of intracellular calcium ions and resulting depolarized cell membranes, sodium-potassium pumps begin working more quickly to increase amounts of ATP to meet the post-injury energy demands. The rise in ATP causes a rapid rise in glucose metabolism in a process known as hypermetabolism. During this period of hypermetabolism of glucose following a head impact, cerebral blood flow (CBF), or the amount of blood that flows through the brain per unit of time, is greatly diminished. Although it is not entirely understood why this occurs, it is suggested that CBF is tightly linked to glucose metabolism and therefore, when the rate of glucose metabolism is rapidly altered in the case of mTBI, so is cerebral blood flow.

Cerebral blood flow is very important because it delivers proper amounts of oxygen and enzyme substrates to the brain, while also removing waste products out of the brain. Reduced CBF is rather dangerous and is marked with symptoms such as dizziness due to the lack of oxygen availability, and can be detected by a method of MRI imaging known as arterial spin labelling. Arterial spin labelling in a noninvasive way to track CBF by using radio frequencies
and magnetic properties to tag blood coming into the brain as a way to quantify the total amount that passes through from the initial point.\textsuperscript{39} Since technologies like this are available, and reduced CBF is a common sign of concussion, with varying reductions indicating length of recovery, this tool can potentially be used as a standard detection of a concussion.\textsuperscript{42}
Chapter 2: Chronic Traumatic Encephalopathy (CTE)

Introduction

Although the NCAA and the NFL have both existed for nearly a century, neither organization has placed strict guidelines on concussion recovery and protocol until the past decade. The NCAA Board of Directors did not instate legislation requiring sports’ medical staff to have a set concussion protocol until 2010, 43 104 years after the NCAA program began. A similar concussion protocol system was not developed in the NFL until 2011. Concussion and TBI research have been ongoing since the late 1800s, so why did it take these two organizations so long to establish required concussion protocol as part of their legislation? 44, 45

The answer to this question lies within the competitive nature of coaches and players along with the disbelief that head injury would have any sort of long-lasting effect. A New York Times article writes that in 2000, Jerry Jones, owner of the Cowboy’s NFL team, would push one of their stars, Troy Aikman, to ignore concussion concerns during the playoffs because of the importance of the game. Jones supported this comment by claiming that there was no significant data to support that concussions had long term effects. 46 Although there was some evidence at the time suggesting long term effects of concussions, many players and coaches overlooked them in pursuit of getting their athletes back in the game as soon as possible.

Throughout the first century of the NCAA and NFL’s existence, players were often urged to play through head dings, even if it left them dizzy or tingling. However, news reports began appearing in the early 2000s that some former NFL stars such as Terry Long of the Pittsburgh Steelers, Dave Duerson of the Arizona Cardinals, Junior Seau of the San Diego Chargers, amongst many others, were committing suicide. 47 The overall percentage of former NFL players that committed suicide is not substantial. Of the 3,439 NFL players that participated in 5 or more
seasons of football by 2013, only 12 committed suicide. However, these reports still begged the question, “Was the exposure to constant head impact while playing football the reason for these traumatic deaths? If so, are all athletes that are exposed to head injury at risk for developing this extreme depressive and psychotic behaviors?” Research in the past decade and a half is beginning to explain why the answers to these questions is most likely yes.

**The Discovery of CTE**

A breakthrough study by Dr. Bennet Omalu in 2002 was done in response to these suicide reports. Dr. Omalu performed an autopsy on former NFL player Mike Webster, who died 12 years after his professional career. According to the results of the autopsy, Webster’s body and brain looked relatively normal from the outside. Omalu expected to see an outwardly deteriorated-looking brain because he suspected Webster to have had developed Alzheimer’s Disease (AD). Instead, he found that the brain looked healthy from the outside. However, further MRI studies revealed the presence of cortical amyloid plaques and tau-positive neurofibrillary tangles in the neocortex of the brain. These findings were similar to that of AD, however, the outward appearance of the brain as well as the distribution of neurofibrillary tangles found were not fully consistent with the diagnosis of AD. They were, however, consistent with a much lesser known neurological pathology that Dr. Omalu went on to name Chronic Traumatic Encephalopathy, or CTE.

The first ever case of pathological diagnosis of this condition was published in 1954 by German scientists concerning a former boxer. This publication revealed a histological report characterized by Aβ plaques and was termed ‘dementia pugilistica’ at the time. This name essentially translated to ‘punch drunk,’ which was the common name for the neurological
condition that boxers felt after being punched numerous times and experienced neurological symptoms such as ataxia, memory impairment, and personality changes; amongst others.\textsuperscript{52} For the next 35 years, this condition was only recognized in boxers, until a news report in 1990 was released stating that a woman was admitted to the hospital with multiple injuries, including bruises to the head. This woman died 10 months after being admitted to the hospital and her symptoms, as well as the autopsy report, made this the first case of ‘dementia pugilistica’ that was not a boxer.\textsuperscript{53} This case revealed that this neurological disease was not exclusive to boxers, and pointed towards the fact that the development of what is today called CTE is influenced by physical blows to the head.

Aside from these observations throughout the century, CTE as it is known today was not officially termed and discovered until 2002 when Dr. Omalu performed the autopsy on Mike Webster.\textsuperscript{50} Over the past decade and a half, a great deal of research has been done on CTE. However, it is still a fairly recently discovered disease, and there is a great need for more investigation in this area.

**What is CTE?**

Chronic Traumatic Encephalopathy is defined as a neurological disease that is suspected to be onset by repetitive head traumas.\textsuperscript{50} However, because of how new CTE is to the field of medicine, it is still not certain if the onset of this disease is linked solely to individuals with a history of repetitive TBIs. It is still unclear whether or not other factors such as a history of substance abuse, epilepsy, and amyotrophic lateral sclerosis, amongst others, impact the likelihood of developing CTE.\textsuperscript{54} Although these factors may play a role in increasing the speed or development of this neurological disease, research still explains that multiple mTBIs are a
direct cause of CTE, and development is often more severe in the case of more severe or more frequent head impacts. Whether CTE is linked to other neurological conditions or not, there is medical evidence revealing that individuals that have suffered even a single mTBI are 20% more likely to develop depression or other neurological disease later in life, and this percent chance only increases when the number of concussions suffered rises.

Some key symptoms of CTE include headaches, memory loss, personality changes, aggression, depression, and suicidal behavior, many symptoms similar to that of Parkinson’s Disease. However, symptoms such as these do not begin showing up until 8-10 years following repetitive head impacts. For instance, most retired NFL players do not experience symptoms of CTE until years after their retirement from the game. Therefore, prevention of the onset of CTE is rather difficult because of the asymptomatic nature of the development over a number of years.

This disease is also unique due to its own specific tauopathy, or progression on the cellular protein level. In short, CTE is characterized by hyperphosphorylation of the tau protein along with neurofibrillary tangles (NFTs), which are highly accumulated regions of hyperphosphorylated tau (p-tau). These features are very similar to those of a more common neurological disorder known as Alzheimer's disease. Alzheimer’s is also characterized by p-tau and NFTs, but the major difference between the two is how and where these two develop. In AD, NFTs are typically found in the middle frontal gyrus, superior and middle temporal gyri, and hippocampus region of the brain, as pointed out in Figure 4. However in CTE, NFT development is seen primarily around blood vessels and in the cortical sulci, or depressions or
grooves throughout the brain.\textsuperscript{58,59} This unique tauopathy of CTE will be discussed much more depth in Chapter 3.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The locations of p-tau and NFTs in Alzheimer’s Disease. The left shows the gyrus, and the right displays the hippocampus.}
\end{figure}

\textbf{Diagnosis of CTE}

Another roadblock in the diagnosis and therefore prevention of CTE is the fact that, like concussions, it is currently impossible to give a certain diagnosis of CTE to a living patient.\textsuperscript{60} As mentioned previously, the first real case of CTE was diagnosed by Dr. Omalu on the brain of Mike Webster in an autopsy after he had passed.\textsuperscript{50} Although many symptoms may present during a person’s life that would suggest that they had developed CTE, autopsies are currently the only way to provide a confirmed diagnosis of this disease.\textsuperscript{61}

To properly diagnose, pathologists will first examine the subject’s medical and social history. This includes items such as sports played throughout life, mood changes, or other
potential signs or symptoms that would have led them to believe that the subject had developed CTE.\textsuperscript{62} If signs and symptoms from his/her medical history point to the suspicion that CTE may have developed, the autopsy of the brain is further studied. When examining autopsy results, there are 4 basic criteria that must be present in the brain to confirm a diagnosis of CTE. These are: 1) Signs that p-tau containing NFTs and astrocytic tangles (ATs) are located perivascularly, or around blood vessels in the brain; 2) p-tau NFTs and ATs are irregularly distributed at the depths of cerebral sulci; 3) NFTs in the cerebral cortex are mostly in the superficial layers such as the temporal cortex; and 4) Potential clusters of ATs in the cerebral cortex, mostly at sulcal depths.\textsuperscript{63} In an autopsy, the brain is sectioned off and each part is analyzed. There is often visible atrophy in patients with confirmed diagnosis of CTE (Figure 5).\textsuperscript{64} In Figure 5, the numbered regions all denote areas that experienced shrinkage due to CTE, and the darker areas indicate larger presence of p-tau containing NFTs. To further investigate and ensure the presence of p-tau protein accumulation, brains are immunostained to highlight areas containing highly accumulated p-tau.\textsuperscript{60}
Potential Biomarkers

This unique tauopathy of CTE is seen and diagnosed in postmortem patients, and there is still no way to clearly diagnose this disease in a living. However, understanding the role that tau protein plays in developing CTE is crucial because it allows doctors to identify biomarkers in living patients that could reveal a confirmed diagnosis, as well as allow them to track the progression of this disease. Unfortunately, the technology to research in this specific area is rather expensive which is why advancements regarding the early diagnosis of CTE have been slow.65

Although technology and imaging systems to identify potential biomarkers are not fully developed, some studies have revealed potential biomarkers that may suggest that a patient is developing CTE. These biomarkers would be found in either the blood or cerebrospinal fluid.66 Taking and analyzing blood samples is quite simple and inexpensive, however, taking samples from the CSF is much more invasive to the central nervous system and should be avoided if at all possible.65 Therefore, the discovery of blood-borne biomarkers for CTE is crucial in the steps to provide a positive diagnosis in a living patient, as well as to begin to work towards possible preventative treatments for this neurological disease.

A particular biomarker that is being studied is the ratio between ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP). UCH-L1 is a neuron-specific protein that have the role of adding or removing ubiquitin from proteins that are to be metabolized, and GFAP is a structural protein in astrocytes that is released in the case of cellular disintegration or apoptosis. This ratio between the two, called the glial neuronal ratio (GNR) is important because it tends to be much higher in patients who have experienced a focal injury rather than a diffuse one. This means that those who experienced a localized TBI rather
than an a more widespread injury showed higher levels of GNR. This biomarker did not reveal any significant differences between gender or age, as it was only increased in patients with focal injury, and even more so increased after death. Therefore, the presence of higher GNR levels could be useful in diagnosing patients with TBI. However, it is still difficult to apply this to the certainty of developing CTE because it remains unknown whether one severe concussion or multiple mild concussions are the leading cause of CTE. Further research into GNR in accordance with confirmed diagnosis of CTE after death could help to reveal a confirmed diagnosis of CTE in a living patient.

An additional biomarker that is currently being researched as a potential diagnostic tool for CTE is a protein known as neuron-specific enolase (NSE). NSE is an enzyme involved in glycolysis in both neural cells and blood cells. It has been shown previously that elevated levels of NSE are typically correlated to severe TBI. NSE initially sparked interest in this area because it was found to be present in high amounts even after 2 months of rest from the sport. These results suggest that head injury was sustained for a substantial amount of time after initial impact. Therefore, NSE has the potential to be an early indicator of the development of CTE. Research in this area is expanding, but more should be done to link these biomarkers officially to the development of CTE.

**Prevalence Amongst Athletes**

So does this mean that every athlete who has experienced one or more concussions eventually develop CTE? The answer to this is no, but chances of developing CTE as an athlete are much higher than those who are not involved in sports. This is because athletes, especially those involved in high contact sports such as football or boxing, are constantly involved in taking
blows to the head. A review done in 2009 investigated the 51 confirmed cases of CTE at the time. Of the 51 cases, 46 of those were found to be in athletes, thus supporting the statement that it is more likely to develop CTE as an athlete.\textsuperscript{1} In addition to this, a hallmark study on CTE was done in 2017 on 202 deceased football players, and the results were astonishing. Of the 202 former football players, 177 of them were neuropathologically diagnosed with CTE. Even more shocking was that 111 of these former football players were members of the NFL. Of the 111 former NFL players, 110 of them were confirmed to be diagnosed with CTE. That is, 99\% of the former NFL players studied had developed CTE.\textsuperscript{64} This percentage is astounding in regards to the prevalence of CTE onset in athletes, and gives great insight to reveal that the odds of developing CTE are strongly linked to the amount of concussions suffered.

Because of these extremely high statistics, some athletes are encouraged to take time off or quit athletics altogether if they suffer 3 concussions or more. This is because at that point, they are at a very increased risk for developing long term neurological damage, such as CTE. However, sports medicine doctor Elizabeth Pieroth spoke out against this rule, saying that it may deter athletes from admitting symptoms of head injury because they do not want to be at risk for having to quit the sport that they love.\textsuperscript{70} It is for reasons such as this that preventing CTE in athletes is such a difficult task. The only absolute way as of now to prevent CTE in athletes is to prevent them from playing sports altogether--and it is fairly evident that that will never happen. Sports, especially football, are an essential part of the American culture, and potential scare of long-term brain injuries is not enough to convince athletes not to play.
Chapter 3: The Role of Tau in CTE

Introduction

As discussed previously, CTE is a neurological disease that is caused by multiple TBIs and characterized by symptoms such as memory loss, anger issues, and depression; amongst others. Many of these symptoms are similar to that of other neurological conditions such as Parkinson’s disease and Alzheimer’s Disease (AD). However, this particular disease differs from the others in the unique pathway of tau protein dissociation and aggregation in the brain throughout the development of CTE.

Tau is an important protein involved in the assembly and stability of microtubules in the brain. It is an essential and harmless molecule in a healthy human brain. However, in diseases such as AD and CTE, tau dissociates from microtubules, unfolds, and proceeds to form aggregates and tangles throughout the brain, restricting proper function. A further look into the specific tauopathy involved in CTE could reveal drug targets and possible preventions for the onset of this disease.

Tauopathy of CTE

Tau is a very hydrophilic and therefore soluble protein primarily found in axons of the central nervous system. This protein consists of two domains-- the “assembly domain” and the “projection domain.” The assembly domain binds to microtubules and assists with their formation, whereas the projection domain does not bind to microtubules, but instead projects off. These projection domains play a role in keeping enough space between axonal microtubules to prevent tangles. Because of its primary role in the production and maintainence of microtubules, tau is often called microtubule-associated protein tau, or MAP-tau.
A key characteristic of tau that is important when looking at it in relation to CTE is the post translational phosphorylation mechanism that modifies this protein. Phosphorylation is essentially the addition of a phosphate group to a specific site on the protein to give the protein unique properties and capabilities. Phosphorylation of tau in a normal brain helps to regulate the binding ability of tau to microtubules and membranes, thus highly governing its function. In the case of a blow to the head, this process amplifies to cause detrimental effects.

As mentioned, tau is a protein located on nerve axons. When a severe impact, such as TBI, occurs, axons are stretched, torn, and sheared. This disruption therefore causes the breakdown of microtubules and dissociation of tau that was associated with their stability, as well as negatively affects intracellular transportation and signaling. This initial pathway in response to head impact is known as diffuse axonal injury (DAI). Once the tau is segmented and separated from its initial state due to impact, multiple phosphorylation sites are exposed and the phosphorylation process works in overdrive, thus causing hyperphosphorylated tau. The specifics of the exact process of hyperphosphorylation of tau in CTE as shown in Figure 6 are still not entirely understood. However, it has been noted that

![Figure 6](https://www.frontiersin.org/files/Articles/461345/fneur-10-00980-HTML-r1/image_m/fneur-10-00980-p001.jpg)
once tau is hyperphosphorylated, it can no longer rebind to microtubules.\textsuperscript{56,73} This is where the largest problem lies. When tau is hyperphosphorylated and therefore detached from microtubules, it begins to cluster with other normal tau molecules to form aggregates. The process of hyperphosphorylation causes p-tau to go from highly soluble, to completely insoluble, thus causing aggregates to form. These aggregates favor a helical shape and are known as paired helical filaments (PHFs), as seen in \textit{Figure 6}.\textsuperscript{56,77} As a result, these PHFs become too large to work in axons and begin to build up further into neurofibrillary tangles (NFTs).\textsuperscript{77}

A very similar process is observed in the pathology of AD. Although this mechanism is most likely onset genetically and driven by the amyloid-β peptide in AD and is onset biomechanically with rare findings of amyloid-β peptide in cases of CTE, the hyperphosphorylation and aggregation up to this point are relatively similar. Both diseases are marked by an increase in hyperphosphorylated tau aggregating into PHFs and then forming NFTs.\textsuperscript{78} During this time, tau phosphatases actively work to dephosphorylate the hyperphosphorylated masses, but there becomes a time in which NFTs grow too large to be effectively dephosphorylated. This causes NFTs to grow and mature to a point in which their breakdown becomes nearly impossible.\textsuperscript{77}

The hyperphosphorylation of tau and buildup of NFTs is the largest problem in both CTE and AD. However, one large difference between these two neurological conditions is where NFTs begin developing. As mentioned before, NFTs in CTE develop closely to blood vessels in the cerebral cortex and spread to the neocortex, medial temporal lobe, diencephalon, basal ganglia, and brainstem as the condition worsens.\textsuperscript{56,79} In AD, NFTs develop first in the brainstem and entorhinal cortex, then proceed to spread to the medial temporal lobe, and eventually to the neocortex.\textsuperscript{56} Because of the distinctness of dispersion of NFTs in CTE vs. AD, these two
conditions are classified as two separate diseases. However, the two diseases are still similar enough to invoke the inclination to attempt to apply an experimental AD treatment to a patient with CTE in hopes of similar, beneficial effects.

**Role of Microglial Activation in Tau Hyperphosphorylation**

The above pathway of aggregation of tau occurs in response to a single head impact, and there is limited research to conclude that sustaining multiple head impacts is ultimately worse in regards to long-term effects. However, some theories point to the fact that NFT accumulation does get worse as more head injuries occur. As discussed, there is evidence that axons do experience abrasion in response to a single head impact. Aside from the hyperphosphorylation of tau due to DAI, excitatory neurotransmitters are also released in this event, as discussed in Chapter 1.

One particular study theorizes that in response to neurotransmitter release after one concussion, microglial cells of the brain are “primed” for subsequent injury. Microglial cells are the major immune cell of the central nervous system, and play a huge role in maintaining its homeostasis throughout a lifetime. Because they are involved in immune and inflammatory response, microglial cells are “primed,” or further activated, in response to concussion. This is because injuries such as these require the proper immune response.

Activated microglial cells can secrete a number of pro- and anti-inflammatory molecules to combat injury. However, in the case of repetitive TBI and therefore multiple inflammatory responses as denoted in Figure 7, these microglial cells can become hyperactive, secreting a dangerous amount of cytokines in the brain. Cytokines are small molecules that trigger the release of excitotoxins such as glutamate as discussed in Chapter 1. Excitotoxins have the ability
to inhibit the work of phosphatases. Again, phosphatases are the molecules responsible for dephosphorylating proteins. Therefore, in the case of repetitive concussions and large increase of excitotoxin release, there becomes a lesser chance that hyperphosphorylated tau will be dephosphorylated and therefore return to their normal, healthy state. This means that, according to this very plausible theory, more concussions lead to a lesser breakdown of NFTs, and consequently more severe progression of CTE. 

**Figure 7 - Microglial activation**
The 2nd pathway here illustrates the priming of microglial cells in response to TBI. In the case of another injury, or “immune challenge,” microglial cells may take on a permanently hyperactive state.


**Astrocytosis**

In addition to the activation of microglial cells, astrocytes, another cell type in the glial family, are also activated in response to head injury. Astrocytes are very common molecules in
the brain; they far outnumber the amount of neurons present and work to connect and hold the entire central nervous system together.\textsuperscript{82} In a healthy system, astrocytes help to maintain homeostasis by keeping glutamate levels low at nerve synapses. They also function in cell-to-cell transport and are linked to blood vessels to transport molecules in that way.\textsuperscript{83}

Unlike microglial cells, astrocytes are always active in a healthy brain, as they are constantly interacting with synapses and blood flow. However, in the case of brain injury, astroglial cells undergo profound structural changes due to the buildup of hyperphosphorylated tau within these cells.\textsuperscript{84} As mentioned previously, tau protein is typically exclusively located on axons to support microtubules, and therefore not present within astrocytic cells.\textsuperscript{71} However, in the case of neurodegenerative disease or head injury, high volumes of hyperphosphorylated tau have been seen to accumulate within astrocytes.\textsuperscript{85} Because it is known that astrocytes do not produce tau protein, but only uptake extracellular molecules, it can be inferred that if tau is present in astrocytes, there is a high volume of tau aggregates extracellularly.\textsuperscript{86}

When astrocytic cells are flooded with large amounts of hyperphosphorylated tau, they tend to inherit a thorn-like shape.\textsuperscript{85} This new and distinct shape taken on by astrocytes causes them to lose some function and cluster.\textsuperscript{87} Therefore, not only is CTE characterized by neurofibrillary tangles (NFTs) laced with hyperphosphorylated tau, but also by astrocytic tangles (ATs) laced with hyperphosphorylated tau. ATs have been seen to have many pathological effects such as inhibiting axonal regeneration after injury and overproduction of cytokines, causing enhanced swelling and worse injury.\textsuperscript{88} As CTE progresses, as does the presence of ATs throughout the brain, ultimately leading to more detrimental neurological effects.
**Tauopathy and The Four Stages of CTE**

In general, CTE is categorized into 4 stages of increasing severity. These stages are known as Stage I, Stage II, Stage III, and Stage IV, with Stage I being the least symptomatic, and Stage IV being the most severe. These stages were proposed by Dr. Ann McKee in 2013 in attempts to universalize the system for diagnosing CTE. A consensus panel run by the National Institute of Biomedical Imaging and Bioengineering 2 years after this proposal confirmed the categorization of the 4 stages of CTE.

![Brain autopsy slices and microscopic images from each stage of CTE.](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6616127/)

**Figure 8**

Brain autopsy slices and microscopic images from each stage of CTE.

Stage 1 is the least extreme level of CTE as the brain appears to be rather normal from the exterior (Figure 8). In this stage, phosphorylated tau protein is found in small amounts in
the sulci of the cerebral cortex of the brain. P-tau and neurofibrillary tangles (NFTs) are microscopic and rarely scattered about at this point. These NFTs are seen as the red specs in the microscopic images in Figure 8. Clearly, in Stage I, NFT distribution is the most sparse. Stage I of CTE is often asymptomatic, as the disease has not progressed very far at this point. However, on occasion, Stage I features symptoms such as headaches and short term memory loss.

Stage 2 is a slightly more developed level of CTE. It is in this stage in which there is some visible mild enlargement of the frontal horns and other areas of the brain that are highlighted in Figure 9. There is a much larger presence of p-tau and NFTs perivascular in regions such as the frontal, temporal, parietal, insular and septal cortices in Stage II; and, as opposed to Stage I, the NFTs in Stage II appear more often at superficial layers rather than only at depths of the cortical sulci. The most common symptoms seen in Stage II CTE are mood swings, depression, headaches, and short term memory loss.

Progressing from Stage II to Stage III, macroscopic changes are not especially noticeable. The major macroscopic change going into Stage III is the reduction of brain weight due to atrophy of the frontal and temporal lobes. In this stage, NFTs are more widespread in areas...
highlighted in Figure 9. It is in this stage that NFTs and astrocytic tangles (ATs) begin to be found in the amygdala and brainstem.\textsuperscript{85} The most common symptom of Stage III CTE is memory loss, followed by explosivity, concentration issues, depression, mood swings, executive dysfunction, and aggression.\textsuperscript{58, 90}

The final and most severe stage of CTE is Stage IV. The autopsy alone in Figure 8 reveals that the most visible atrophy to the brain is macroscopically seen in Stage IV.\textsuperscript{89} Brains with Stage IV CTE have a dramatic loss of weight due to severe atrophy. There is also a major loss of myelin and neuronal tissue due to the atrophy at this point.\textsuperscript{2} Severe tau deposition and NFT presence scattered throughout almost all areas of the brain are characteristic of Stage IV, as seen in Figure 9.\textsuperscript{85} Symptoms of Stage IV CTE are very severe, including memory loss to the point of complete dementia. Other symptoms include paranoia, extreme problems with concentration, suicidal depression, language difficulties, and psychotic tendencies.\textsuperscript{58, 90}
Chapter 4: ACI-35 and AADVac Active Immunotherapy

Introduction

The link between multiple concussions and development of CTE has not been proven for certain, but the majority of research up to date has proposed the likelihood of this correlation. The best method of preventing CTE, therefore, is by avoiding head injuries altogether. However, head dings will unavoidably occur and CTE will inevitably develop. This being said, preventative methods and potential CTE treatment must be researched further. As of now, there is no approved medicinal treatment or cure for this disease.

This paper proposes a method of treatment for the potential prevention of CTE. As mentioned previously, the tauopathy of CTE is strikingly similar to that of Alzheimer’s Disease (AD). One approach that has been used and tested recently in AD is the use of active immunotherapy; a method of stimulating a natural immune response to pathogenesis. Because of the similarity of CTE to AD, similar active immunotherapy techniques should be applied to limit or prevent symptoms and onset of CTE. Two active vaccines in particular, ACI-35 and AADVac1, have been tested in AD patients and are designed to invoke an immune response to pathological tau protein in AD. Due to the similar tauopathy of CTE, there is potential that these vaccines could be effective against both neurological diseases.

Active Immunotherapy

As mentioned previously, active immunotherapy is a method in medicine that is utilized to stimulate the body’s immune response to produce certain antibodies against disease. The active immunotherapy technique is most commonly utilized in routine vaccinations such as the MMR vaccine, rotavirus, and the chickenpox vaccine; amongst others. In such vaccinations,
patients are injected with a live virus that grows and replicates within them. The amount of virus in a live attenuated vaccine is often too miniscule to cause symptoms of the disease itself, but large enough for the body to develop a proper antibodies against it.\textsuperscript{94} An active vaccine is comprised of an antigen combined with an adjuvant.\textsuperscript{92} The antigen is the disease-producing component of the vaccine. The body recognizes viral antigens as foreign and therefore begin to produce antibodies as a protective response.\textsuperscript{92, 95} Adjuvants are typically added to live vaccines as well to enhance immune responsivity to the antigen by initiating the innate immune response.\textsuperscript{96}

This idea of active immunotherapy has recently been applied to the treatment of AD.\textsuperscript{92} In AD, the tau protein is affected by hyperphosphorylation at certain serine residues by GSK3 kinases.\textsuperscript{97} The body does not respond properly to the hyperphosphorylation, hence the progression of AD. In order to combat this progression, active immunotherapy methods are utilized to create synthetic peptides that are phosphorylated on the same residues as hyperphosphorylated tau, forcing the body to produce antibodies against this irregularity.\textsuperscript{97} As mentioned previously, the tauopathy of AD is very similar to that of CTE. Therefore, this paper hypothesizes that a similar active immunotherapy technique could be used as a treatment for CTE to produce similar beneficial effects.

**ACI-35**

One current active immunotherapy vaccine that has been created and clinically tested on AD patients is ACI-35.\textsuperscript{3} ACI-35 is a liposome based vaccine that contains a synthetic 16-mer peptide that corresponds to the human tau protein amino acid sequence 393–408, with phosphorylated residues S396 and S404.\textsuperscript{97} This is the chosen sequence for this vaccine because
serine 396 and serine 404 are primary sites of hyperphosphorylation in the pathology of AD. S396 and S404 are responsible for the polymerization of tubulin, making these sites some of the most crucial residues to the protein as a whole.\textsuperscript{97, 98} The hyperphosphorylation of these serines in AD disables tau from polymerizing tubulin and stabilizing microtubules, thus leading to tau aggregates and neurofibrillary tangles.\textsuperscript{98}

The purpose of the ACI-35 vaccine therefore is to expose the body to a milder form of phosphorylated tau so that it can make antibodies against it.\textsuperscript{3, 97} The ultimate goal of ACI-35 is to produce antibodies to hyperphosphorylated tau without invoking a B and T cell autoimmune reaction against healthy, naturally occurring tau within the brain.\textsuperscript{3} A study done on the effects of ACI-35 on mice solidified that this vaccine would produce a large amount of highly specific antibodies against phosphorylated tau.\textsuperscript{97} ACI-35 has recently completed phase 1b of clinical trials in human subjects with AD in Finland and the United Kingdom, with results not yet published.\textsuperscript{99} According to the FDA, less than 12\% of candidate medicines make it into phase 1 of clinical trials, so the fact that ACI-35 is in this stage is quite exciting for the future of AD and similar neurological conditions.\textsuperscript{100}

\textit{Application of ACI-35 to CTE}

To recap, the ACI-35 vaccine designed for the treatment of AD consists of a synthesized peptide corresponding to tau residues 393-408 with phosphorylated residues S396 and S404.\textsuperscript{97} Studies up to date reveal that this treatment method has been very effective in producing an antibody response to pathological tau protein.\textsuperscript{3, 97} Just as hyperphosphorylation at serine residues 396 and 404 are highly characteristic of AD, hyperphosphorylation at serine residue 422 has been found to be diagnostic of CTE.\textsuperscript{101} Phosphorylation at S422, seen in both AD and CTE, has
been associated with cognitive decline.\textsuperscript{101} Therefore, an active vaccine similar to that of ACI-35 could be used as a potential treatment in the early stages of CTE. The only change in catering the vaccine to CTE specifically would be that the synthetic peptide would encompass residues slightly past 422, with phosphorylation on S422. Applying this active immunotherapy treatment to patients who are likely to develop or have already developed CTE would allow for a better buildup of immunity and production of proper antibodies against the irregularly phosphorylated S422 residue on the tau protein.

One study done in 2012 tested an active immunotherapy against phosphorylated S422 in AD.\textsuperscript{102} Results from this study revealed less amounts of tau aggregates in vaccinated mice due to the fact that the vaccination decreased the total amount of insoluble tau material.\textsuperscript{102} Although research in this area is relatively limited as of now, these few published results indicate a promising potential for using active immunization to target the S422 residue in CTE.

AADVac1

Another forth-coming active immunotherapy treatment in the field of AD is the AADVac1.\textsuperscript{3} Unlike ACI-35, AADvac1 targets nonphosphorylated tau.\textsuperscript{103} The main goal of this vaccine is to target the misfolded nature of tau in AD.\textsuperscript{91, 103} It consists of a synthetic protein derived from the amino acid sequence 294-305 of a naturally occurring misfolded and truncated tau protein.\textsuperscript{99} It is coupled with keyhole limpet hemocyanin (KLH), and administered with aluminum hydroxide as an adjuvant.\textsuperscript{3, 104} KLH is an immunogenic protein that is utilized in the AADVac1 vaccine to help promote a further immune response to the misfolded protein.\textsuperscript{105}

AADVac1 has been tested in both a rat model, and clinically in human AD patients.\textsuperscript{3} The rat trial in 2014 modeled human AD tauopathy, with 5 administrations of the AADVac1 by
injection starting at 2 months of age, with another every 3 weeks thereafter. The key results from this study indicated that the AADVac1 vaccine (1) induced a strong antibody response, (2) was specific in that it only targeted one antigenic site on the tau protein, (3) was selective in that it targeted only pathogenic tau and not physiological tau, (4) was safe in that T-cell epitopes were derived only from the KLH, (5) was effective in that it reduced the amount of pathological tau and pathological tau oligomers, and (6) improved neurobehavioral parameters that were studied. Essentially, this vaccine proved to be extremely effective in the rat model and was therefore quickly approved for clinical trials.

As of now, Axon Neuroscience SE has completed Phase I and Phase II of clinical trials for AADVac1 in AD patients. The main goals of the most recent Phase II trials were to test for safety and efficacy of the AADVac1 vaccine. Out of the 185 patients enrolled in Phase II, not one had to drop out due to adverse effects such as disease or harm due to the vaccine, therefore confirming the safety of it. As for efficacy of the vaccine, 98.2% developed antibodies against the misfolded tau, and AADVac1 overwhelming slowed the increase in blood levels of neurofilament light chain, a marker for neurodegeneration. Although this vaccine does not completely cure AD, it does substantially slow down the progression of this disease and its corresponding tauopathy.

Application of AADVac1 to CTE

The overall idea of AADVac1 is to target misfolded tau and its corresponding neurodegenerative effects in AD. CTE pathology is also marked with misfolded tau and its consequential aggregation. Because AADVac1 does not target a specific residue like ACI-35 does, there is more potential for this vaccine to be used as-is for other neurological disorders such as CTE. According to a 72-week follow-up to Phase I trials of AADvac1, the antibodies
produced by this vaccine were responsive to not only AD tauopathies, but non-AD tauopathies as well. This finding suggests that this vaccine would be effective in slowing down the tauopathy of CTE as well as improving symptoms.
Chapter 5: Proposed Research

Specific Aim 1

Test the efficacy of modified ACI 35 vaccination and AADVac 1 in preventing CTE development following 2 concussions.

Injury Groups

To model CTE, a rat repeat concussion model will be utilized using methods similar to that in Thomsen et al.\textsuperscript{109} For this aim, a total of 60 rats will be used, separated into 5 groups outlined in Table 1. Injured groups 2A, 3A, 4A, and 5A will be administered bilateral TBI once a week for 2 weeks beginning 9 weeks postnatal. The week-long intervals of injury are designed to allow the rats to recover from acute effects of TBI before given another. The TBI will be administered with a controlled cortical impact (CCI) device, mounted at an angle of 10 degrees from the vertical. Before receiving injury, the rats will be anesthetized with 2.5% isoflurane and positioned on a cloth pad, using a nose cone for stability, but no bodily restraint. The CCI device will be delivered with a velocity of 6 m/s, a depth of 4 mm, and a dwell time of 0.2 seconds, and centered at the bregma and located 3 mm lateral of the midline. The first impact will be delivered on the left side followed by a second impact on the right side 15 seconds later. Directly following injury, rats will be placed on their backs in an empty cage on a heating pad. The time taken for the rat to wake up and return to normal position with all 4 limbs on the ground of the cage will be noted. The time taken to wake up and reorient will be used to distinguish between time of loss of consciousness due to injury verses due to anesthesia alone.

Group 1A rats will be a sham control. These rats will be placed under the same anesthetic procedure and handling as Groups 2A, 3A, 4A, and 5A, but will not be given the CCI injury.
### Table 1

<table>
<thead>
<tr>
<th>Group 1A</th>
<th>SHAM</th>
<th>10 Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2A</td>
<td>Control 1 (Concussions without Treatment)</td>
<td>10 Rats</td>
</tr>
<tr>
<td>Group 3A</td>
<td>Control 2 (Concussion with saline injection)</td>
<td>10 Rats</td>
</tr>
<tr>
<td>Group 4A</td>
<td>ACI 35 treated post 2nd concussion</td>
<td>15 Rats</td>
</tr>
<tr>
<td>Group 5A</td>
<td>AADVac1 treated post 2nd concussion</td>
<td>15 Rats</td>
</tr>
</tbody>
</table>

**Behavioral/Motor Skills Assessment**

**Rotarod**

Each group of rats will be taken through a series of behavioral assessments. The first of these will be the rotarod to test balance and motility. Each rat will be placed on a slowly rotating 3 mm wide rod for 210 seconds per trial. The speed will begin at 3 revolutions/minute for the first 30 seconds, and then steadily increase to 30 revolution/minute at the end of the test. Each rat will complete this test 3 times on each test day, with trial separated by 30 minutes. Times of each test will be averaged. The groups will complete 3 total rounds of rotarod testing. The first test day will be one week pre-injury to serve as a baseline, the second will occur the day of second injury, and the third will be 24 weeks post-injury to assess the efficacy of the treatment groups compared to controls.

**Beam Walking**

The second assessment will be beam walking to test balance. A 2 m long, 2.5 cm wide beam elevated 60 cm above a foam cushion will be used in this test. Rats will be timed from the time they are placed on the far side of the beam until they successfully make it to the other side. A 60-watt light will be shined over the start side to serve as an aversive stimulus. Again, this test
will be conducted 3 times on each test day, with trials separated by 30 minutes and times will be averaged. This test will be performed 1 week pre-injury, the day of second injury, and 24 weeks post-injury.

**Grip Strength**

This assessment will be used to test grip strength using a grip strength apparatus purchased from Bioseb Instruments. Each rat will be held by the tail and lowered onto the horizontal metal of the apparatus. When the rat grabs the bar, it will be pulled horizontally away from the apparatus. The peak tension that is applied to the apparatus will be recorded. Again, this test will be conducted 3 times on each test day, with trials separated by 30 minutes and times will be averaged. This test will be performed 1 week pre-injury, the day of second injury, and 24 weeks post-injury.

**Forced Swim Test**

This assessment will be used to test the onset of depression-like behavior. In this assessment, a cylindrical container with diameter of 20 cm and height of 50 cm will be filled 30 cm high with water. A video camera will be pointed at the container. For the test, each rat will be placed in the water for 5 minutes as the video camera is recording. After 5 minutes, the rat will be taken out and placed in a drying cage under a heat lamp.

To analyze the video content for the behavioral assessment, there are 3 movements that will be coded and recorded: 1) Time that rat is immobile, or floating with the no movement, 2) time that the rat is struggling or attempting to climb out of the container, and 3) time that the rat is swimming in forelimbs or hind limbs are paddling. These movements are behaviorally analyzed based on the assumption that immobility is a measure of psychological despair,
whereas attempt to swim and climb are normal behavior. This test will be performed 1 week pre-injury, the day of second injury, and 24 weeks post-injury.

*Barnes Maze*

The last assessment that will be used is the Barnes Maze to test spatial memory. This test will utilize methods similar to that of Meconi et al.\textsuperscript{112} This test will consist of an elevated circular platform with a diameter of 122 cm, that has 20 holes 10 cm in diameter evenly spaced around the perimeter. One of the holes will lead to an escape box, and this hole will remain in the same place throughout every trial of the experiment. The maze will be placed in a room that has distinct visual cues to assist rats on memorization of the escape hole. All rats will be trained on the Barnes Maze Apparatus for 2 days before the initial baseline test. Training will consist of 4 trials the first day, and trials on day 2 will be repeated until criteria is reached. Training criteria will be considered as met when the rat is able to locate the specified escape hole within 1 error in 2 trials. Before training, the rats will be enclosed in the escape box to get acclimated with it for 2 minutes. It will then be moved to the center of the maze and be given 5 minutes to locate the escape hole. If they are unable to find the hole within 5 minutes, they will be led by a researcher to the proper hole, and the test will be repeated until the rat is properly acclimated with the maze.

In the test trials, the mice will not begin in the escape box, they will immediately be placed in the center of the maze from their home cage. The distance travelled and the number of errors made before finding the escape hole will be measured for this assessment. An error would include if rat moved its head over any portion of a hole that was not the proper escape hole. Aside from the training that will begin 2 days prior to the first official test, all groups will complete 3 rounds of the Barnes Maze one week pre-injury, the day of second injury, and once every 2 weeks for 24 weeks following injury.\textsuperscript{112}
Treatment Groups

Two vaccinations will be used in this study. The first vaccine that will be used is ACI-35, mutated to encompass tau residues 410-425, with phosphorylation on S422 rather than original vaccine that is phosphorylated on S396 and S406. This vaccine will be purchased from Janssen Pharmaceuticals. The other vaccine that will be used in this study is AADVac1, and it will be purchased from Axon Neuroscience.

Directly following the Day 0 post-injury behavioral assessment, Group 4A will begin treatment with a subcutaneous injection of 200 μl modified ACI-35, and Group 5A will receive a subcutaneous injection of 200 μl AADVac1. Each group will be given a total of six doses of their respective treatment, with one dosage every 4 weeks. Groups 1A and 2A will not be given any treatment. Group 3A will be given 200 μl saline (0.9% NaCl) subcutaneous injection. This group will serve as a control to identify if rats with injection site have varying performance to Group 2A, controls with concussion only.
Experimental Timeline of Specific Aim 1

Tissue Analysis

24 weeks post-injury directly following the final behavioral assessment, all rats will be humanely euthanized via a ketamine/xylazine cocktail administration followed by transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde. Brain tissue will then be collected and postfixed in paraformaldehyde and stored in 30% sucrose overnight.

Collection and Analysis of P-Tau

Volume of p-tau concentrations will be measured from the cortex and corpus callosum of all brains used in this experiment using a method of quantitative stereological analysis as
described in Thomsen et al.\textsuperscript{109} To collect tau sample, 5% donkey serum and 0.25% Triton X-100 will be added to a brain section and will be placed in phosphate-buffered saline for one hour at room temperature to block nonspecific antibody binding. The p-tau localization will then be detected by incubation overnight at 4°C with mouse anti-AT8 antibody, purchased from Thermo Fisher Scientific.

Three 30 μm sections, 720 μm apart, beginning 1 mm posterior to the bregma will be cut. The specific number of p-tau cell types in each section will be quantified using the optical fractionator method, a process of immunostaining and microscopy to count the total number of selected cells in a given area. For the cortex region, a counting frame of 100 μm X 100 μm and grid size of 500 to 650μm X 500 to 650μm will be used, and for the corpus callosum, a counting frame of 200 to 300 μm 200 to 300 μm will be used.

*Cortex and Corpus Callosum Thickness Analysis*

The thickness of the cortex and corpus callosum of each brain section of all groups in this experiment will be analyzed based on photographs input into ImageJ software. 5 brain sections per rat, spaced 210 μm apart will be used. 3 thickness measurements per section per hemisphere will be averaged to give a statistical analysis of the thickness of each hemisphere of each rat.\textsuperscript{109}

*ELISA Test for Specific Antibodies*

5 specific ELISA tests will be done postmortem to analyze the concentration of 5 different antibodies. ELISA kits will be purchased from MyBioSource.com.\textsuperscript{115} The antibodies that will be tested for are S422 phosphorylated tau-specific IgG antibody, which should be naturally synthesized in response to ACI-35, and IgG antibody against axon 108, IgM antibody against axon 108, anti-KLH antibody, and anti-pathological-tau antibody, which should all be naturally synthesized in response to AADVac1.
**Intended Outcomes**

Group 4A receiving the modified ACI-35 vaccination and Group 5A receiving AADVac1 following 2 concussions should perform similarly to the SHAM control group on the 6-month post-injury behavioral assessment, due to the intended full recovery from injury in this time frame. Groups 4A and 5A should also produce specific antibodies to their respective vaccines: S422 phosphorylated tau-specific IgG antibody for the ACI-35 treatment group, and IgG antibody against axon 108, IgM antibody against axon 108, anti-KLH antibody, and anti-pathological-tau antibody for the AADVac1 treatment group. In addition, Groups 4A and 5A should have a reduction of p-tau when compared to Group 2A control rats, as well as thicker cortex and corpus callosum than those in Group 2A, and more similar to Group 1A measurements due to the anti-deterioration nature of the treatment. All injury groups are expected to perform worse on all behavioral assessments that are done on the day of second injury, but scores should all improve by the time of the 24 week post-injury behavioral assessment, as they are given a substantial amount of time to heal and should no longer face post-concussive effects.
Specific Aim 2

Test the efficacy of modified ACI 35 vaccination and AADVac 1 in preventing CTE development following 5 concussions

Injury Groups

The injury groups to test specific aim 2 will be the exact same as in specific aim 1. The one exception is that groups will undergo CCI injury once a week for 5 weeks as opposed to 2, meaning that rats will be given a total of 5 concussions. Groups are outlined in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Group 1B</th>
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<th>10 Rats</th>
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<tbody>
<tr>
<td>Group 2B</td>
<td>Control (Concussions without Treatment)</td>
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</tr>
<tr>
<td>Group 3B</td>
<td>Control 2 (Concussion with saline injection)</td>
<td>10 Rats</td>
</tr>
<tr>
<td>Group 4B</td>
<td>ACI 35 treated post 5th concussion</td>
<td>15 Rats</td>
</tr>
<tr>
<td>Group 5B</td>
<td>AADVac 1 treated post 5th concussion</td>
<td>15 Rats</td>
</tr>
</tbody>
</table>

Behavioral/Motor Skills Assessment

Rotarod, beam walking, grip strength, forced swim, and Barnes Maze tests will all be done in this specific aim as well. There will be a total of three test days: one week prior to first injury, the day of fifth injury, and 24 weeks post-injury. Barnes Maze will be performed once every two weeks following injury.
Treatment Groups

The treatment groups will also be the same as those used in specific aim 1. The only exception will be that Groups 4B and 5B will receive their first dosage of their respective treatment on the day of their 5th injury. Group 3B will receive its first saline injection on the day of 5th injury as well.

Experimental Timeline of Specific Aim 2

Tissue Analysis

The tissue will be analyzed in the same ways as in specific aim 1, with the exception that the rats will be euthanized 24 weeks post 5th-injury.
Intended Outcomes

The likelihood of developing CTE after 5 concussions is very high, as the hallmark study done by Dr. Ann McKee revealed that 110 out of 111 brains of former NFL players were positive for CTE.\(^6\) The application of the modified ACI-35 vaccination and AADVac1 following 5 concussions should produce specific antibodies mentioned previously that should limit CTE pathology, such as hyperphosphorylation of tau in perivascular neurofibrillary tangles. These rats in Groups 4B and 5B should have less p-tau buildup, as well as thicker cortex and corpus callosum layers compared to Groups 2B and 3B that were given injury without treatment. Rats given 5 concussions without any immunotherapeutic treatment will see long-term deficits in performance as well as tau buildup.

Groups 2B-5B are likely to perform worse than Group 1B Sham control in the 2nd round of behavioral assessments that will happen the day of the 5th concussion. Balance and strength should be negatively affected by the injury. As for the 24-week follow up behavioral assessment, Groups 2B and 3B should have the worst results on all assessments. The greatest outcome would occur if Groups 4B and 5B performed similarly to Group 1B in the 24-week follow up behavioral assessment, because this would reveal that the vaccines fully prevented the symptoms of CTE onset.
Conclusion

High contact sports are one of the leading causes of concussions in young adults in the world today, second only to motor-vehicle accidents.\(^{116}\) As long as sports such as football continue to be an integral part of culture, head injuries will inevitably continue to occur. An estimated 300,000 sports-related concussions occur annually, and an individual is 1-2 times more likely to receive a second concussion after one, 2-4 times more likely to receive another concussion after 2, and 3-9 times more likely to receive a fourth after 3.\(^{20}\) This phenomena, known as second-impact syndrome, contributes to the susceptibility of young athletes to develop Chronic Traumatic Encephalopathy down the road. The stigma in competitive sports to “play through the pain” is a huge factor in the high prevalence of CTE amongst former athletes.

Because getting athletes to retire before severe injury and further consequences is difficult to do, this paper proposes the use of active immunotherapy after 2 and after 5 concussions to prevent CTE before it fully develops. Active immunotherapy techniques have been used to treat a variety of neurological diseases such as Alzheimer’s disease, Parkinson’s disease, prion disease, and multiple sclerosis.\(^{117}\) In addition, active immunotherapy has been utilized in the treatment of cancers such as solid tumors and malignant blood-based cancers.\(^{118}\) The ultimate idea behind active immunotherapy techniques is to prime the body’s immune system to protect itself against specific foreign invaders. The minimally invasive approach to utilize a natural system that is built into the body is the reason why active immunotherapy has become such a widespread treatment option. Not only is the active immunotherapy technique approved over a wide variety of diseases, but it has been clinically shown to be a very effective method of treatment. It is hopeful that a similar method of active immunotherapy can be utilized in the prevention of CTE.
Future Directions

Going forward, there is still much research to be done regarding CTE. The development of this disease as it does not typically present with symptoms until years after athletes retire is perplexing. Further research looking into potential biomarkers and uncovering a method of diagnosing CTE in live patients. Confirmed diagnoses in a live patient would greatly help with treating this neurological disease as it is onset. In addition, early detection or confirmed diagnosis of CTE in living patients could prevent them from committing suicide or homicides down the road. As mentioned, CTE has severe mental effects, causing individuals to become extremely depressed, experience personality changes, and occasionally act erotically to the point of harming themselves or others. As of current, there is no medical treatment for CTE, and the only prevention of this neurological disease is to avoid head injury or concussion. Active immunotherapy techniques provide looks towards a promising outcome in preventing CTE, and models should be tested to verify the best plan of action concerning this detrimental neurological disease.
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