January 2014

Brainstem Auditory Evoked Potentials And Network Dysfunction In Mild Traumatic Brain Injury

Theresa Lynn Williamson
Yale School of Medicine, theresa.williamson@Yale.edu

Brainstem Auditory Evoked Potentials and Network Dysfunction in Mild Traumatic Brain Injury

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By

Theresa Lynn Williamson

2014

I. Abstract

Brainstem Auditory Evoked Potentials and Network Dysfunction in Mild Traumatic Brain Injury

Theresa L. Williamson BS[1,2], Amanda R. Rabinowitz PhD[1], Victoria E. Johnson MD[1], John A. Wolf PhD[1], Michael L. McGarvey MD[3], Douglas H. Smith MD[1] University of Pennsylvania Department of Neurosurgery Philadelphia, PA 19104[1], Yale School of Medicine New Haven, CT 06511[2], University of Pennsylvania Department of Neurology Philadelphia, PA 19104[3]

Introduction:
Mild traumatic brain injury (mTBI) challenges clinicians as symptoms do not map in a lesion-specific manner and there is no objective diagnostic measure. Diffuse axonal injury is a main mechanism of injury in mTBI [1, 2]. Injury to axons is proposed to alter the brain's networks and underlie common symptoms such as slow processing speed, poor concentration and memory. Clinical studies show that the auditory network is also commonly disrupted in mTBI and therefore the auditory pathway is a useful surrogate for study to understand network dysfunction as it relates to axonal pathology and signal processing speed.

Methods:
Decades of research using a rotational acceleration injury model in pigs scaled to the known mechanical loading conditions in humans demonstrates multi-focal swelling of axons [1]. This study utilizes a known model of mTBI to relate diffuse axonal injury to the physiologic functioning of a network. The technique is to record latency, amplitude and morphology of the auditory evoked potential response before, immediately after, and three days after injury as well as conduct a histopathologic investigation of the brainstem auditory pathway for evidence of axonal injury.

Results:
We have identified increased latency and morphologic changes of the brainstem auditory evoked potential waveforms in swine following injury that correspond to pathology in regions in the upper brainstem, immediately after and at three days post-injury as compared to a pre-injury control measurements. Additionally, we have identified axonal pathology, indicated by amyloid precursor protein positive axonal swellings, in the region of the lateral lemniscus and inferior colliculus.

Conclusions:
This data shows that in a clinically relevant model of mild traumatic brain injury, damage to axons in a pathway corresponds to functional delay in the pathway's processing. Identifying a link between axonal pathology and function in the auditory pathway is useful to represent network injury throughout the brain shedding light on mTBI's diffuse nature that underlies a group of symptoms that are both difficult to diagnose and treat.

II. Acknowledgements

I would like to extend special thanks to my thesis advisors, Dr. Douglas Smith at the University of Pennsylvania and Dr. Hal Blumenfeld at the Yale School of Medicine as well as the thesis chair for the department of neurology, Dr. Gordon Buchanan. Additionally, I would like to thank the office of student research for their support of my research throughout my medical school career. Finally, I would like to thank the mentors I worked closely with at the University of Pennsylvania to complete my thesis work: Dr. Amanda Rabinowitz, Dr. Victoria Johnson, Dr. John Wolf, and Dr. Michael McGarvey and technician Susan McNicholls for all of their support. Thank you to the National Institute of Health/National Institute of Neurological Disorders and Stroke Diversity Supplement Grant and the Yale School of Medicine Office of Student Research for funding my work.

III. Table of Contents

a. Introduction

Mild Traumatic Brain Injury: A large and abstruse public Health Problem

Mild traumatic brain injury (mTBI) is a significant public health problem. Mild traumatic brain injury, often referred to as concussion, is a rotation or force to the head that causes a change in brain functioning but does not result in prolonged loss of consciousness, intracranial hemorrhage, or prolonged post-traumatic amnesia[3]. It exacts a large toll on society due to its high incidence, 1.2-1.3 million injuries in the United States per year [4], unmatched by poor diagnostic tests and intervention strategies. Although, many patients suffering mTBIs recover fully, anywhere from 10-80% [5, 6] of patients proceed to persistent dysfunction lasting from six months to two years. Early intervention to prevent long-term symptoms is elusive, as there are few indicators of prognosis at the time of initial injury. Therefore, numerous research efforts in mTBI focus on improving diagnosis and prognosis of mTBI as a step towards developing therapeutic interventions.

Due to the non-fatal and non-surgical outcomes that define mTBI, non-invasive detection of injury severity, distribution, and temporal evolution are necessary in order to plan clinical trials and communicate prognostic information to patients. Currently, there is no objective measure of the pathophysiology of mTBI. Clinical diagnosis is difficult due to heterogeneity of clinical presentation, which can include cognitive, emotional, headache, balance, auditory, and visual complaints. Various mechanisms of injury and pre-morbid states may also play a role in this heterogeneity. Additionally, several consensus definitions of concussion or mild traumatic brain injury co-exist, such as the Zurich Consensus, Center for

Disease Control, and several smaller groups, leading to inconsistent selection criteria among large-scale studies of mTBI outcomes.

Proposed mTBI mechanisms

Although initial mild head injury triggers neurometabolic and inflammatory responses[7], the triggering of downstream channelopathies and resulting axonal damage likely underlies persisting brain network dysfunction in post-concussion syndrome[8, 9].

The concept of diffuse axonal injury (DAI) underlying symptoms of brain injury first arose while studying patients with characteristically severe presentations but limited underlying brain imaging changes. Post-mortem analysis of these patients revealed axonal damage and swelling of the white matter [10, 11]. mTBI similarly injures the brain without imaging findings, leading researchers to investigate DAI as a potential mechanism for injury. Large-scale post-mortem studies of mTBI patients have been difficult to conduct, due to the non-fatal nature of the injury. However, the post-mortem studies performed on the, albeit small, population of mTBI patients with other causes of death have shown histopathologic evidence of poor axonal transport due to breaks in its structural integrity [12].

The rotational acceleration biomechanics causing mTBI in events such as assaults, motor vehicle accidents, and sports-related injuries, result in stretching and shearing of axons in the white matter that are poorly designed to withstand such conditions[13].

In order to confirm and further study a strong hypothesis about the pathophysiology of mTBI, primate and swine models of rotational acceleration head injury were devised. The gyrencephalic, large animal models, allowed for direct replication of rapid acceleration/deceleration injury that occurs in human mTBI and the brains in these animals revealed significant axonal injury with a diffuse pattern of distribution [1, 2, 14]. These models made the study of the process of axonal injury possible, leading to the identification of axonal damage stages that can lead to long-term white matter degeneration[15]. The development of a large animal model of mTBI shed significant light on the previously mysterious pathophysiology and led researchers to pursue DAI.

Global injury to axons best explains persistent dysfunction as well as the wide range of clinical sequelae observed in mTBI patients. Global white matter injury and its relation to the symptomatology of mTBI have led to a new phase in mTBI research: the network dysfunction hypothesis.

mTBI and non-invasive diagnostics

Traditional imaging data has provided little insight into mild traumatic brain injury with uncommon and non-definitive changes in the white matter such as edema and microhemorrhage[16]. However, with the advancement of imaging techniques, including diffusion tensor imaging (DTI), there is increased understanding of the extent and characteristics of damage to the brain after mTBI[17]. DTI takes advantage of the movement of water between axons and the surrounding space and can elucidate changes in the structural integrity of an axon

track [17]. In mTBI patients as well as in animal models, DTI data points to structural damage in axon tracks as the primary injury in mTBI that persists and evolves over time[17, 18]. DTI provides researchers with a tool to reveal a previously invisible pathology, diffuse axonal injury. Blood biomarkers, such as GFAP and UCH-L1, elevated in post-injury patients, also indicate the presence of axonal injury and may be helpful in predicting functional outcome[19, 20]. For now, functional outcome is determined by neuropsychological and clinical evaluation. A major gap in mTBI knowledge remains: there is no method to link the burden of diffuse axonal injury to the functioning of the brain's networks. Because electrophysiological recording provides a direct measure of neuronal functioning, we argue for the pursuit of electrodiagnosis, specifically utilizing brainstem auditory evoked potentials, for diagnosis and prognosis of mTBI.

Functional Assessment of Diffuse Axonal Injury: Combining Mechanism and Diagnostics to Derive Meaning

How do we define injury to a network in mTBI? This new stage of research focuses on the connectivity between brain regions and how alterations to the brain's wiring result in dysfunction. Electrodiagnosis is particularly promising in this regard, as it is a direct measure of axonal functioning within a network. The onset of electrophysiology in mTBI work occurred with the measurement of conduction across axons from injured animal models in vitro. Small animal in-vitro models of traumatic axonal injury allow for considerable control over biomechanical inputs and extensive evaluation of the effects of axon deformation[14, 21]. Recently, a

study led by Greer, identified abnormal electrophysiology in both axotomized and non-axotomized neural soma after injury, suggesting that there are subtle changes to an axon's ability to process information despite remaining physically connected to it's target after injury[22]. Additionally, Baker and colleagues showed that in the rat model of TBI, axonal pathology correlated with decrease compound action potential across a white matter track, the corpus callosum[23]. This is important evidence to support the hypothesis that accumulation of subtle axonal injury can cause global network dysfunction.

Just as electrophysiologic changes reflect axonal damage, axonal recovery is measurable via conduction improvements as is persistent dysfunction. Reeves' group showed that myelinated axonal populations recover function better than unmyelinated axons in the rat model of mTBI [24]. Additionally, they found a rostrocaudal distribution of pathophysiology [25].

Traumatic brain injury is a diffuse disease that alters brain function in subtle ways that are not conspicuous upon gross inspection of brain structure. This has led many to believe that mTBI dysfunction is best studied through analysis of the brain's connections. Electrophysiology in small animal models can shed light on pathway damage, distribution of injury, and recovery process. This model of network evaluation is translatable to large animal models, in which the distribution of injury may be more relevant to networks as they are affected in human mTBI.

The ability to determine a network's functional disruption non-invasively after mTBI with electrophysiology has appealed to researchers and clinicians for decades, however limited progress has been made in this field. A prominent

electrophysiological method is the evoked potential. In this paradigm, a stimulus is presented to probe a specific network, for example, sound for the auditory network, at which point, recording electrodes track the waveforms, representing the signal as it is conducted across axon tracks and synapses at nuclei, to reach the appropriate cortical area.

Functional Assessment of The Auditory Network and mTBI: A model for understanding the Effects of DAI

The auditory network provides a promising window into the often subtle and clinically missed cerebral dysfunction post-mTBI. MTBI patients commonly report auditory dysfunction. The incidence of tinnitus and hyperacusis reaches up to 40-60% in several studies of military and non-veteran mTBI patients [26]. The extent of auditory dysfunction correlates with the severity of a traumatic brain injury, suggesting that the auditory pathway meaningfully represents the nature of brain injury [27]. Additionally, the discrete, predictable, and well-conserved neuroanatomy and well-known connectivity of the auditory pathway, increases the attractiveness of the network as a research and clinical tool[28].

In terms of electrophysiology, auditory evoked potentials are well classified based on lesion models and wavelets correspond to segments of the pathway between the cochlea, brainstem (brain stem auditory evoked responses [BAERs]), and Heschl's gyrus of the temporal lobe [28]. The neuroanatomy, electrophysiology, and symptom profile of the auditory network make it an ideal region of study in mTBI.

Historically, clinicians could not ignore the presence of auditory symptoms in mTBI patients nor the pathway's overall significance in the disease process. In 1969, Barber and colleagues gave a detailed report of their frequent experience with audiologic and vestibular findings in head injury [29]. After several similar papers, researchers such as Greenberg studied the auditory function in its entirety after severe head injury using multi-modality evoked potentials, including brainstem auditory evoked potentials, showing abnormalities [30]. Studies in severe brain injury demonstrated that brainstem evoked potentials are sensitive to pathology in severe injury. The tool was next translated to mild head injury in order to test detection of more subtle changes.

Animal studies and post-mortem human mTBI studies demonstrate significant axonal pathology in the brainstem [1]. A significant portion of the auditory pathway, including the cochlear nerve, lateral lemniscus, and inferior colliculus, as well as auditory radiations to the thalamus exist in the brainstem and therefore should be affected by such pathology. Indeed, brainstem auditory evoked potentials identify abnormalities in both amplitude and latency in mTBI patients. Schoenhuber and colleagues found changes in acoustic conduction after "minor head injury" in the early 1980s [31]. Following their reports there were a few small, similar studies prior to a renewal of efforts in 1995. A study by Aleksanov et al showed increased latency of an auditory signal traveling through the brainstem measured in short-latency brainstem auditory evoked potentials in patients with concussion [32]. Slowing signal processing or processing speed is a hallmark clinical presentation of mild traumatic brain injury and this data provided

an objective measure of that phenomenon in a discrete pathway. Further studies by Soustiel correlated changes in initial brainstem evoked potential latency to neuropsychological outcome at 3 months and up to a year respectively, showing potential predictive value in post-concussive syndrome [33]. A study by Drake et al identified amplitude changes in the brainstem auditory evoked responses (BAERs) waves of concussed patients, suggesting a decrease in signal strength after injury [34]. Soustiel's paper discussed the changes in latency as a potential marker of axonal change and in many ways expressed a pioneering view at the time identifying the connection between axon damage, pathway disruption as measured on electrophysiology, and persistent dysfunction seen in post-concussive syndrome: an early look into the role of BAERS as part of the network hypothesis underlying the symptom clusters in mild traumatic brain injury.

Interestingly, the promising initial BAERs studies did not lead to an expansive research program on their utility as a diagnostic in mTBI. Until recently, few studies explored the initial clinical data with further measures. There are several explanations for the suppression of BAERs-related mTBI research. Firstly, initial research efforts suggest that mTBI-related latency and amplitude changes are small. Without a dramatic effect, such as that seen in other conditions like coma, acoustic neuroma or multiple sclerosis, clinical interpretation of the BAERs is challenging in mTBI patients. Additionally, mTBI patient presentation is notoriously heterogeneous making establishing normative data with which to compare injured patients against difficult to obtain and quantify. Perhaps most importantly, the BAERs reflects only changes to a network within the brainstem. Although the

brainstem is clearly affected by rotational head acceleration injury, few mTBI patients experience prolonged loss of consciousness by definition; therefore the brainstem may not be the most promising anatomical region to look to in order to gain understanding into mTBI's pathophysiology. Finally, although there are many auditory symptoms of mTBI, these could be influenced by both peripheral and higher cortical mechanisms in addition to brainstem pathology, hence the relationship between brainstem dysfunction and auditory symptoms may not be direct.

Although we would not argue that brainstem pathology is responsible for the global, cognitive and somatic syndrome in mild traumatic brain injury, we do believe that it can serve as an excellent model for study. Furthermore, brainstem disruption could be a helpful index of more generalized pathology, as suggested by the initial studies of Soustiel, showing statistically significant correlations between changes in brainstem auditory evoked potentials and persistent clinical dysfunction [33]. Grossly, changes in latency and amplitude represent either decreased number or efficiency of axons. Therefore, BAERs provides a method for quantifying the extent of axonal damage. Hence, the electrophysiological functioning of the early auditory network provides a model for understanding how networks responds to injury more generally. Furthermore, the non-invasive technique allows for survival and serial assessment, which can help elucidate the temporal evolution of recovery or persistent dysfunction.

Temporal progression of mild traumatic brain injury is well represented by the auditory system. Post-concussive syndrome (PCS) often includes audio-

vestibular symptoms. In one small study of PCS, greater than half of patients complained of transient hearing loss for hours to two days immediately after injury and thirty-five percent of patients continued to have tinnitus or hyperacusis one year post-trauma [26]. This data suggests that the axonal injury sustained during a concussion affects the central auditory pathway in a way that in some cases recovers and in others persists. Linking this to the axonal damage underlying PCS, electrophysiology of the auditory system is an excellent model for temporal evolution after mild traumatic brain injury, significant due to the measurable effect of axon injury and repair.

Brainstem Auditory Evoked Potentials

BAERs are composed of five waveforms each with anatomically distinct generators, creating a map of the auditory pathway. The cochlear nerve corresponds to wave one, followed by wave two upon synapse with the cochlear nucleus. Wave three generates from the lateral lemniscus and the synapses between the inferior colliculi and medial geniculate nucleus of the thalamus generate waveforms four and five [28]. Together this map of the auditory pathway within the brainstem characterizes the distribution of axonal damage, as the latency and amplitude of each waveform can be affected differentially.

Utilizing BAERs in mTBI

Creating a model system for network dysfunction in mTBI based on the brainstem auditory network, requires combining the non-invasive, inexpensive, and

facile BAERs with sophisticated new stimulation and analysis tools in order to fully understand the distribution and extent of dysfunction. Munjal *et al* commenced analysis of this type with a study of patients after mild, moderate, and severe closed head injury using tympanometry, otoacoustic emissions, and pure tone and speech audiometry in conjunction with BAERs and middle latency auditory evoked responses. This study demonstrated changes in the BAERs associated with head injury and they were able to differentiate peripheral versus central auditory dysfunction [27], a step along the way to a greater understanding of distribution of injury in the auditory pathway.

Mapping the distribution and extent of diffuse axonal injury using brainstem auditory evoked potentials, requires validation of waveform abnormalities through evidence of underlying dysfunction. The majority of human cases of mTBI are non-fatal and therefore, histopathologic evidence is unavailable. Therefore, large animal models with gyrencephalic brains are the best candidates to study BAERs changes in relation to axonal pathology.

b. Statement of Purpose (specific hypothesis and specific aims of the thesis)

Specific Hypothesis:

Post-injury changes in amplitude and latency on brainstem auditory evoked potential recording will reflect underlying axonal injury in the clinically relevant swine mild traumatic brain injury model.

Specific Aims:

1. To evaluate the sensitivity of brainstem auditory evoked potentials in detecting diffuse axonal injury.

2. To use the neuroanatomically discrete, brainstem auditory pathway to model the effect of diffuse axonal injury on the functional integrity of the network's signal processing (Figure 1).

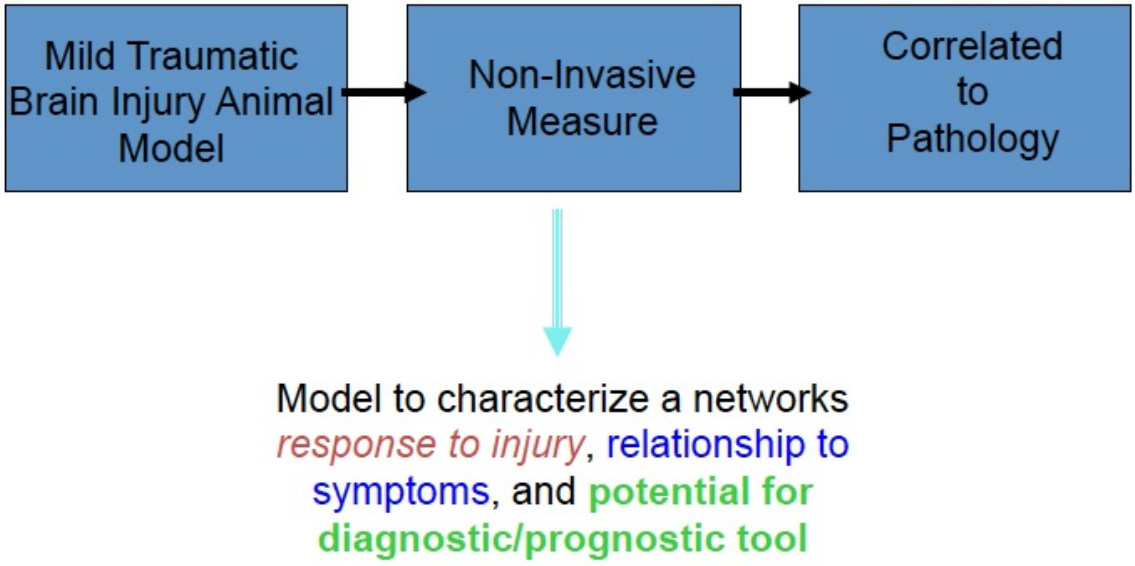


Figure 1. Explanation of the model of brainstem auditory evoked potentials, a non-invasive measure, correlated with underlying pathology in characterizing a network's response to injury.

c. Methods

Animal Model and Individual Animal Information

Previously published reports demonstrate the Smith laboratory's well-established, swine model of mild traumatic brain injury, scaled to represent injury characteristics in human concussion [1]. In accordance with the University of Pennsylvania Institutional Animal Care and Use Committee and National Institute of Health's policies for the humane and ethical treatment of animals, miniature pigs were selected to participate in the study protocol outlined in detail below.

Hanford pigs were matched by age and maintained under similar conditions for the purpose of study control (table 1). Pig 278 aged 2.5 months, was unique from the three pigs at six months, which was taken into account in the final analysis. Maintenance of miniature pigs was conducted by myself in association with the Smith laboratory large animal specialist, Kevin Browne, and the University of Pennsylvania large animal veterinarians and veterinary technicians.

Name	Age	Strain
Pig 2	6 months	Hanford Miniature Pig
Pig 3	6 months	Hanford Miniature Pig
Pig 4	6 months	Hanford Miniature Pig
Pig 278	2.5 months	Hanford Miniature Pig

Table 1. Hanford miniature pig characteristics.

Rotational Acceleration Injury and mTBI Conditions

To examine the electrophysiological and pathological changes in mTBI in swine before and after mild traumatic brain injury, we used a model of head rotational acceleration injury to induce mechanical loading conditions scaled to

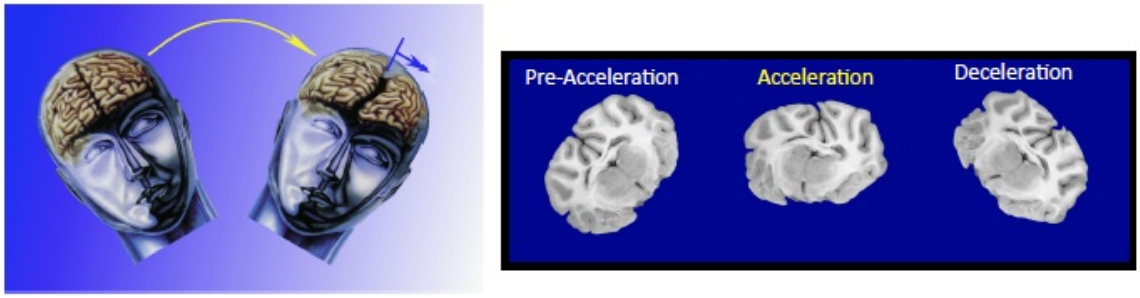
those found in human mTBI. Scaling was based on the mathematical association between mass, velocity, and acceleration (given the average miniature pig brain mass equals 80-90 grams versus human brain mass averages between 1000 to 1400 grams). Calculations made by biomedical engineers: Dr. David Meaney and Dr. Kacy Kullen, along with Dr. Douglas Smith, estimated an angular velocity range with a peak of 290 radians/second (rad/s) necessary to induce shear stress on the swine brain equivalent to that of concussive mechanics in humans previously documented in the literature [35, 36]. Of note, based on our laboratory's experience, acceleration is scaled four to six times that in the human literature in order to produce adequate sheer injury and reproduce relevant pathologic findings. Our mTBI model simulates clinical subjective criteria to the best of our ability including no evidence of prolonged loss of consciousness post-injury and absence of intracranial hemorrhage.

Unlike higher loading conditions that have been shown to cause prolonged coma and more severe gross pathologic findings including intracranial hemorrhage [1], a mild level of rotational acceleration in the coronal plane is associated with brief or absent loss of consciousness and evidence of axonal damage that mimics human findings, as indicated by accumulation of amyloid precursor protein in axonal swellings [14, 37](figure 2). Therefore we used the previously established scale for mild rotational acceleration injury and recorded the parameters of injury to ensure accuracy (Table 2). The injury protocol is controlled and the device operated by biomedical engineering expert, Dr. Kacy Kullen and his laboratory team.

Table 2	Plane of Acceleration	Direction of	Rotational Velocity (rad/s)

		Acceleration	
Pig 2	Coronal	Left	285
Pig 3	Coronal	Left	295
Pig 4	Coronal	Left	247.7
Pig 278	Coronal	Left	241

Table 2. Miniature pig injury characteristics scaled to rotational velocities within characterized parameters for the mild traumatic brain injury model.



A **B**

Figure 2. Rotational acceleration injury, proposed mechanical cause of mTBI (A) is replicated via rapid acceleration/deceleration of swine brain (B) causing shearing injury of axons (Figure generously provided by Dr. Douglas Smith).

mTBI Procedure and Animal Care

Animals (*n*=4) were fasted for 12 hours. Anesthesia was induced via intramuscular injection of midazolam (0.4 milligrams/kilogram). Upon sedation, minipigs were endotracheally intubated with an appropriately sized tube, and deep anesthesia was initiated with five percent (%) isoflurane/2 liter(L) oxygen (O_2). Following anesthesia initiation, anesthesia was maintained at 1.5-2% isoflurane/2L O_2. Vital signs including: heart rate, respiratory rate, oxygen saturation, and temperature were monitored continuously and adjustments were made as needed to ensure the animal's safety. Anesthesia was maintained by myself along with a team including my mentor, Dr. Amanda Rabinowitz and research specialist, Kevin

Browne. Anesthesia was maintained prior to the time of injury during pre-injury BAERs recordings, during the injury, and after the injury during post-injury BAERs recordings.

At the time of injury, the miniature pig's heads were secured to a padded snout clamp, which in turn was attached to a pneumatic actuator that converts linear motion into angular motion to deliver rotational acceleration (Figure 3). Triggered release of pressurized nitrogen drives the linkage assembly. The actuator was set to deliver a velocity of less than 300 rad/s in the coronal plane. Immediately prior to induction of the injury, the anesthesia tubing was disconnected from the endotracheal tube. The animals' heads were released following injury. Anesthesia was immediately reinstated post-injury in order for pain management of the animals and due to the fact that it was previously reported that animals awaken without prolonged loss of consciousness status post the stated injury [1]. Upon completion of the injury, the animals were observed for any signs of injury and if necessary assessed by a veterinarian, and transported back to their cages for post-injury monitoring. The miniature pigs were given access to food and water as tolerated. Daily assessments were made to ensure that there were no adverse events during recovery. Typically, animals recovered well within several hours.

For pigs two, three, and four, on day three after injury, the above steps to induce and maintain anesthesia were performed. Upon maintenance of anesthesia, the three day post-injury recordings were performed. Immediately following recordings, the animals were euthanized by transcardial perfusion using heparinized saline followed by 4% paraformaldehyde solution.

For pig 278, anesthesia was induced and maintained for a three day post-injury recording and was additionally followed by the same procedure on the sixth day post-injury after which, the animal was euthanized using the same procedure at six days. The animal was maintained for six days to begin investigation of longer-term effects of rotational acceleration injury on the BAERs.

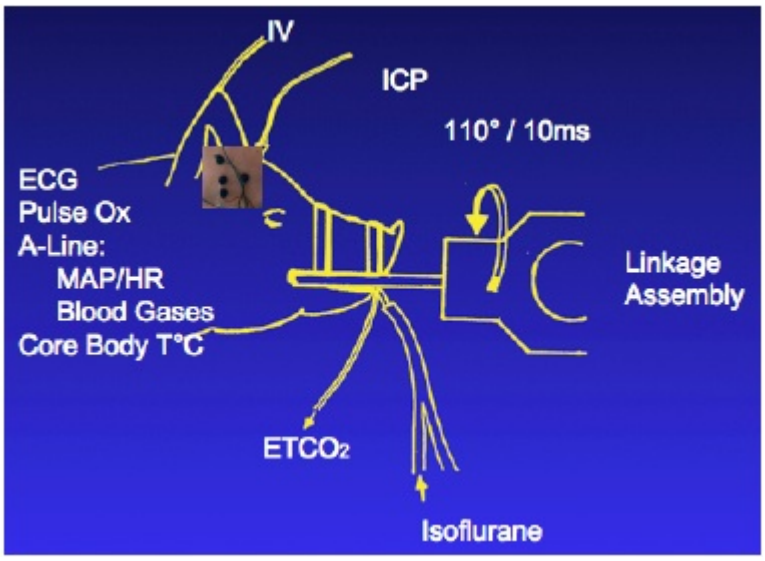


Figure 3. Device set-up for rotational acceleration injury in swine including demonstration of BAERs leads which are present during injury (image generously provided by Dr. Douglas Smith).

Brainstem Auditory Evoked Response Recording Parameters

All tests were performed in a quiet room to the best of our ability. Sub dermal needle electrodes were applied in a consistent fashion with recording electrodes (referred to as A1 and A2) adjacent to the left and right ears, respectively, at the junction with the palpable lateral crests of the skull. The vertex electrode was placed in front of the recording electrodes at midline, palpable by the sagittal crest and the vertex electrode was placed anterior to the reference electrode, also at midline. A ground electrode was placed on the back of the pig (Figure 4).

Electrodes were then plugged in to the endeavor system's receiving device in order that responses would be transmitted to the endeavor software for observation and analysis of the BAERs recording. Assistance with BAERs setup and recording was provided by neurologist, Dr. Michael McGarvey and electroencephalography technician, Susan McNicholls at the University of Pennsylvania.

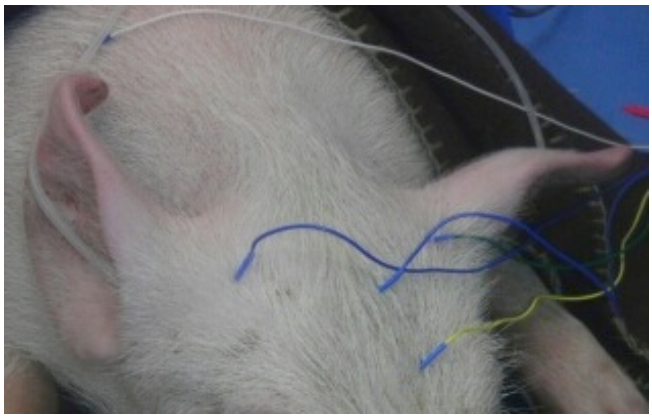


Figure 4. Example of BAERs electrode placement in miniature pig. Note presence of microphone ear plugs bilaterally which deliver auditory click stimulus.

A cylindrical earphone, covering a click sound generator was placed deep in each ear canal of the miniature pigs. Auditory click stimuli were delivered at an intensity of 103 decibels (dB) and a pulse rate of 11.1 hertz during each recording. 103db was chosen due to the recommendation in clinical brainstem recordings to stimulate at an intensity 20db greater than the average hearing threshold in order to generate each waveform [38]. Contralateral masking stimuli were employed to avoid misinterpretation of the stimulus between the ipsilateral and contralateral sides. The Endeavor CR system and amplifier were used at the same settings for each individual recording. Recordings were performed such that a fifteen millisecond (ms) window of brainstem response to the auditory clicks was acquired

and the recording was complete when 2000 responses were averaged over this period of time. Recordings were performed immediately prior to injury, immediately after injury, and at 3 days post injury (in pig 278 a fourth recording was performed at six days at well). Recording pre and post-injury allowed for internal control of an uninjured and injured condition.

Analysis of BAERs

Upon completion of brainstem auditory evoked response recordings, the averaged wave of 2000 runs was downloaded in image (jpeg) format and imported into matlab for further analysis. In matlab, a curve function was written to fit the wave and identify peaks and troughs with the help of Dr. Andrew Voyiages. Adequate waves were selected based on shape of waveform, reproducibility, and reversibility in the contralateral channel. A1 recordings represented the left auditory brainstem responses (LABR) and A2 the right auditory brainstem response (RABR). With the assistance of neurologist, Dr. Michael McGarvey, peaks were identified qualitatively as waves I-V in the BAERS, a method well-documented in the BAERs literature (such as when using BAERs in autism spectrum disorder research [39]). Waveforms were considered absent when no peak was identified in the appropriate region for the wave to occur, based on clinical judgment [38]. Waveforms four and five were considered merged when a single peak was present in place of two individually distinct waveforms [40].

Latency was measured as the horizontal (x) coordinate of the mid-peak of the waveform, or length of time from initiation of the response until the occurrence of

the peak in milliseconds. Amplitude was measured as difference between the vertical (y) coordinate of the peak and that of the following trough.

Data was exported to Microsoft Excel. Absolute latencies were compared as averages across subjects and standard deviation between subjects calculated. Amplitudes were also compared across subjects using a repeated measures ANOVA test to determine the effect of time (pre versus post versus three days post-injury) on the individual waveform amplitudes across miniature pigs. Amplitude for pig 278 was excluded from the analysis due to varying age, which is a factor associated with change in amplitude of the BAERs response.

In order to analyze shifts in waveforms and to decrease the variance between animals, interpeak latencies (IPL), a commonly used clinical entity [41, 42], were calculated by subtracting the latency of waveform five from each prior waveform. Interpeak latencies were compared across pigs using standard deviation between pigs and a repeated measures ANOVA statistical analysis to determine the effect of time (pre versus post versus three days post-injury) on the IPL, therefore evaluating if there is an injury effect on the interpeak latencies.

Generous assistance with statistical analysis was provided by Dr. Amanda Rabinowitz.

BAERs "Case Study" and Pig 278

In order to evaluate the future directions of the study parameters including measuring the change in response at various intensities and pulse rates to test for hearing thresholds and temporal acuity after injury, pig 278 was also evaluated as

above with stimuli parameters of varying decibels (75db to 103db) and at 7.1hz and 11.1hz. Interpeak and absolute latencies were calculated at each condition and qualitatively observed.

Histologic Processing and Analysis

After perfusion, the whole brain was removed, photographed, and inspected for evidence of gross hemorrhage or swelling. Tissue was post-fixed in 4% paraformaldehyde and blocked in paraffin. For pigs two and three, special blocks were created at the level of the inferior colliculi in order to specifically evaluate this region's pathway. Additionally, the medial geniculate nucleus of the thalamus was blocked. Finally, the pons was dissected out in the area parallel and directly inferior to the inferior colliculi to assess the region in which the lateral lemniscus resides. Pigs four and 278 were embedded coronally in paraffin in order to demonstrate global pathology if present. Subsequently, the blocks were cut into 10-micron-thick sections on a rotary microtome.

Several slides from each of the brainstem and thalamic regions were selected for staining. The sections were deparaffinized and rehydrated. A slide from each region of pigs two and three was selected for hematoxylin and eosin staining. Additionally, slides from each region were selected and placed into a protocol for antibody staining of amyloid precursor protein (APP), (Browne 2011) to evaluate for axonal injury (Figure 5). Endogenous peroxidase activity was blocked using five percent hydrogen peroxide in methanol. Antigen unmasking was performed using high temperature epitope retrieval. Sections were then blocked in two percent

normal horse serum and incubated in APP antibody (1:75,000) overnight at four degrees. The following day the sections were washed, incubated in secondary antibody, and washed again. Visualization of the antigen of interest was achieved after five minutes of exposure to diaminobenzidine (DAB; Vector Laboratories).

The slides were examined using a Nikon microscope equipped with a Canon digital camera.

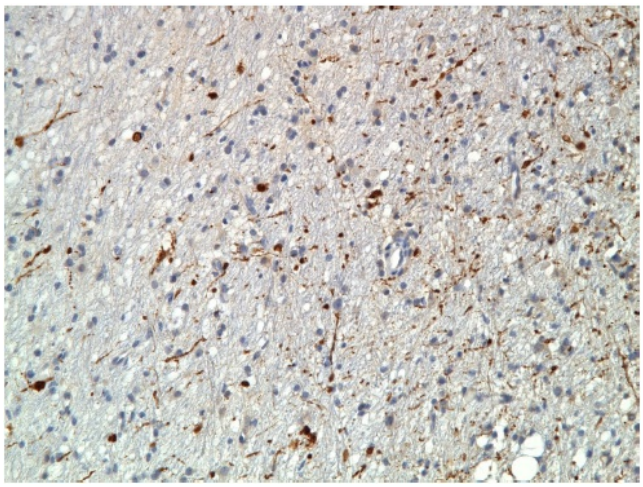


Figure 5. Sample of APP staining in pig tissue demonstrating accumulations and axonal swellings. Image generously provided by Dr. Victoria Johnson.

d. Results

Qualitative Observations in Brainstem Auditory Evoked Responses

The analysis of brainstem auditory evoked potentials begins with clinical judgment including assessment of the individual waveforms, checking for reproducibility of the wave, and identifying a reciprocal wave in the contralateral channel. After determining these factors, in conjunction with mentor, Dr. Michael

McGarvey, observational analysis revealed abnormalities in the morphology and timing of the waveforms between pre and post-injury time points. Individual waveform generators have been mapped using lesional models both based on human pathology such as tumors or multiple sclerosis lesions of known location as well as animal lesion models [28]. Waveforms are generated when the nerve impulse traveling from the peripheral ear and cochlea changes travel media, for example, synapsing at a nucleus or transferring between a peripheral (cochlear nerve) and central region (figure 6).

Figure 6. See Aminoff's Electrodiagnosis in Clinical Neurology Figure 24-16 [28]. Depiction of cochlear and brainstem generators of BAERs waveforms. Waveforms one through five (I-V) are generated prior to the medial geniculate nucleus of the thalamus. X axis represents latency or time until the waveform occurs and y axis, amplitude, relative strength of the signal. Figure adapted from [28].

A representative BAERs sample is displayed in figure 7. Initial visual inspection revealed dampening of the wave post-injury as indicated by the qualitatively decreased amplitude. Additionally, the waveforms, especially in the later waves, appear to occur later in the post-injury wave.

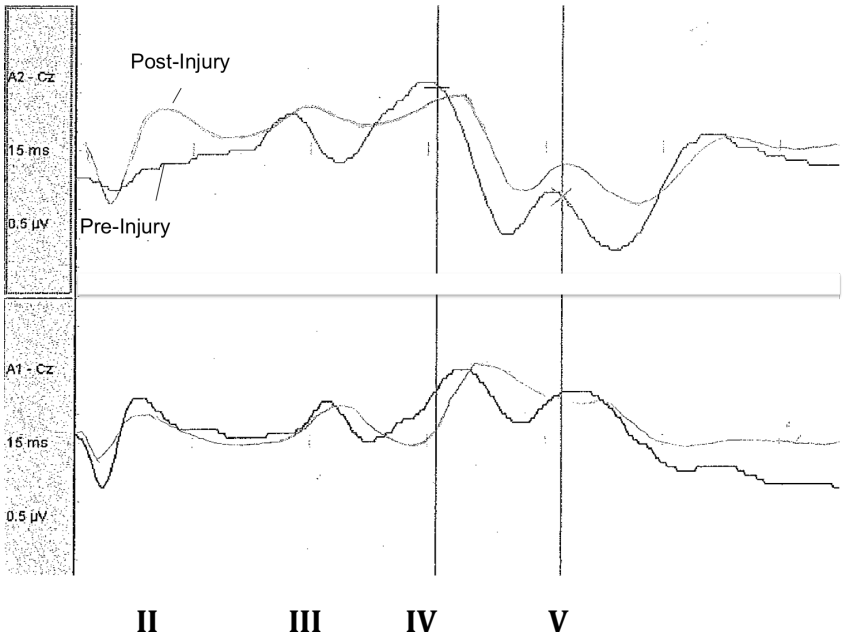


Figure 7. Representative sample of the differences between pre and post-injury brainstem auditory evoked response waves. Lines drawn through waveforms IV and V mark positions at which waveform dampening and/or latency prolongation are noted. A2 represents the ipsilateral channel for the right brainstem auditory evoked potential. X axis is 15ms and y is 0.5 microvolts (uV). A1 represents the contralateral, left channel, and contains a reciprocal wave.

Quantitative Analysis of Pre and Post-Injury Absolute Latencies

Pre- and post-injury waveforms were compared with both quantitative and qualitative analyses. Quantitative analysis included comparisons of peak latencies and peak amplitudes across three time-points for each animal: 1) pre-injury, 2) immediate post-injury, and 3) 3-day post injury. Absolute latency is the most commonly employed technique to clinically evaluate for BAERs abnormalities [38], as it is sensitive to numerous pathologies. However, absolute latencies are typically compared to normative data based on a large number of representative subjects, and no such norms are available in swine. It is a challenge to investigate the differences pre and post injury across our swine population due to variability

among animals, and the lack valid reference data. Figures 8 and 9 represent the left and right BAERs absolute latencies for each waveform averaged across swine, respectively. The effect of time on absolute latency was not statistically significant for either the left or right side based on standard deviation.. Although there is no difference between injury condition and waveform, it is reassuring to note that each waveform latency average is reproducible, a confirmation of effective recording and analysis methodology.

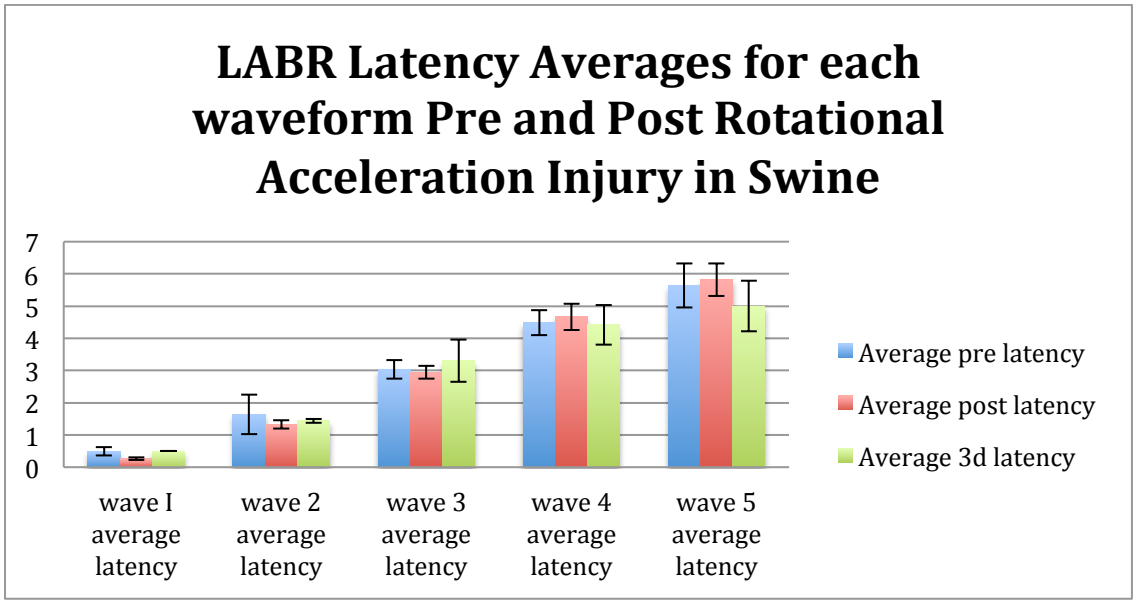


Figure 8. LABR absolute latency in milliseconds for each waveform (I-V). Error bars = standard deviation.

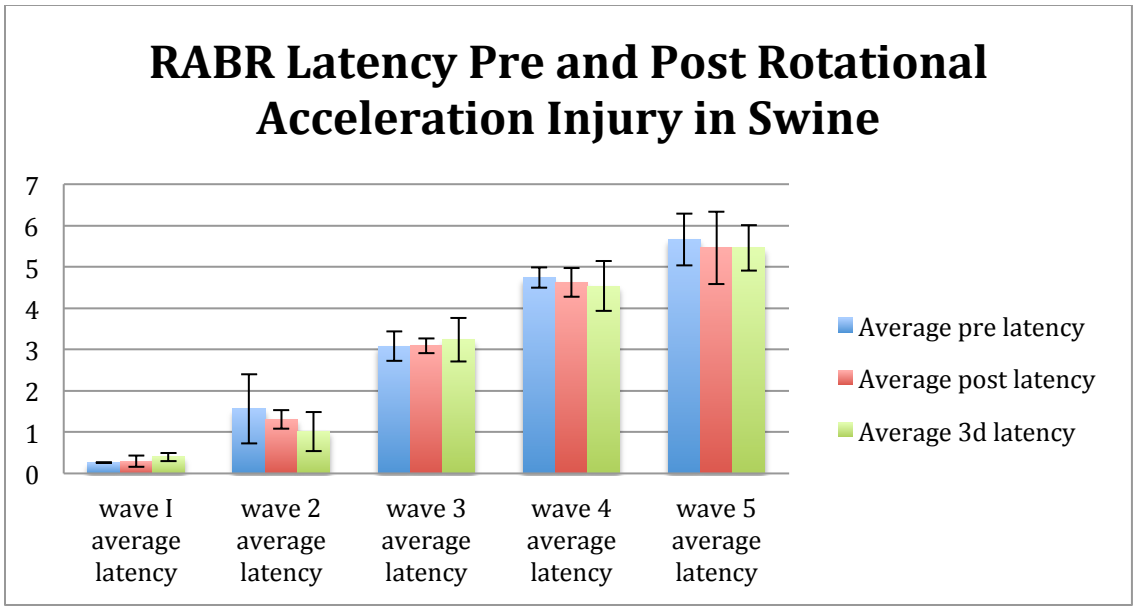


Figure 9. RABR absolute latency in milliseconds for each waveform (I-V). Error bars = standard deviation.

Analysis of Pre and Post-Injury Interpeak Latencies (IPLs)

Interpeak latencies were calculated next and can be interpreted as "conduction time" indicate at which point in the wave, if any, a delay in transmission of signal is occurring, as the I-V IPL represents the entire pathway whereas the III-V and IV latencies represent only the central, brainstem portions of the brainstem auditory pathway. It is important to note that in cases in which a waveform was not identified, the interpeak latency for that particular entity was not calculated and the remaining values across subjects were utilized.

All quantitative analyses were conducted using the R software environment for statistical computing (2012). LABR interpeak latency averages between each of the first four waveforms (i.e. waves I, II, III, IV) and wave V across pigs 2, 3, 4, and 278 are represented in figure 10. Visual inspection of the data revealed a pattern in

which post-injury interpeak latency is prolonged, particularly in the II-V, III-V, IV-V (brainstem regions) intervals [28]. In order to test the statistical significance of this effect, a repeated measures analysis of variance (ANOVA) model predicting LABR IPL values. Time-point (1 = pre injury, 2 = immediate post injury, and 3 = 3 day post injury), beginning and endpoint waves, and the time-point by wave interaction were entered as predictors. Results revealed a statistically significant effect of time on IPL ($F_2 = 9.12$, $p < 0.001$), suggesting that across LABR waveforms, IPL changed in response to injury and/or subsequent recovery. Inspection of the group means shows that the effect was such that IPL increased immediately after injury, and then decreased to near or below pre-injury levels by 3 days post injury (table 4). A possible decrease in IPL at three days may have been related to morphological changes in wave forms, which will be discussed in latter sections.

There was also a significant effect of beginning and endpoint wave on IPL ($F_4 = 28.20$, $p < 0.001$). This is not surprising, as the time elapsed between waves I and V will, by definition, consistently be greater than the time elapsed between waves II and V, which will always be greater than the time elapsed between waves III and V, etc. The wave by time point interaction was also significant ($F_8 = 2.16$, $p < 0.05$), suggesting that the injury effected IPL differently for different beginning and endpoint waves. Inspection of group means suggests that the effect of time on IPL was most robust for IPL II-IV and IPL III-V, both of which showed a decrease in three-day post-injury IPLs relative to pre-injury levels.

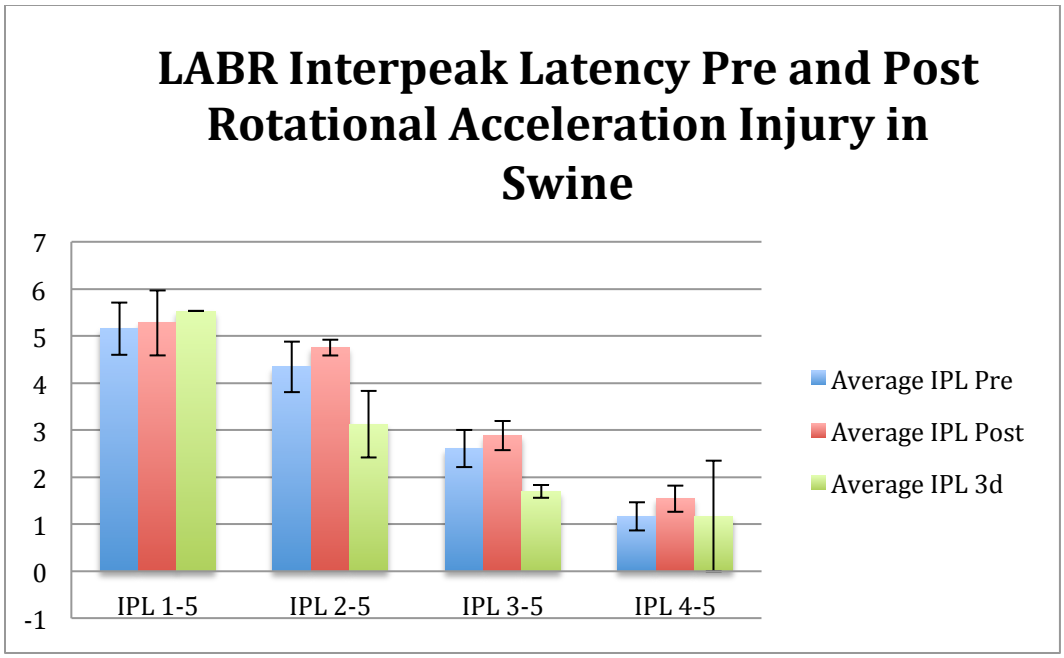


Figure 10. Interpeak latency (IPL) for each LABR waveform. Error bars = standard deviation.

LABR	IPL 1-5	IPL 2-5	IPL 3-5	IPL 4-5
Average IPL Pre	5.1520825	4.3453	2.608925	1.1616
Average IPL Post	5.27705	4.751	2.88275	1.5416
Average IPL 3d	5.5294	3.125	1.697575	1.1715

Table 3. LABR IPL averages for waveforms I-V.

LABR

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
time	2	0.0589	0.02943	9.118	0.000354 ***
waves	4	0.3642	0.09104	28.201	4.13e-13 ***
time:waves	8	0.0557	0.00697	2.158	0.044046 *

Table 4. LABR IPL repeated measures Anova. Test is the effect of time on waves, the IPL. Modeling test:waves reveals a significant P value of 0.044046 where Df is 8 and F value is 2.158.

RABR interpeak latency averages between each waveform and wave five across pigs 2, 3, 4, and 278 are represented in figure 11. Visual inspection of these data showed a less consistent pattern of results as compared with the left-sided

data. As was done on with the LABR, a repeated measures ANOVA model was constructed predicting IPL for the RABR. Time-point (1 = pre injury, 2 = immediate post injury, and 3 = 3 day post injury), beginning and endpoint waves, and the time-point by wave interaction were entered as predictors. Results revealed a statistically significant effect of time on IPL ($F_2 = 6.94$, $p < 0.01$), suggesting that across RABR waveforms, IPL changed in response to injury and/or subsequent recovery. Inspection of group means showed that, across waves, RABR IPLs showed a tendency to increase over time. As was the case with the left-sided data, there was also a significant effect of beginning and endpoint wave on IPL ($F_4 = 111.52$, $p < 0.001$). For the RABR, the time by wave interaction was not significant ($F_6 = 0.50$, $p = 0.80$) (Table 6).

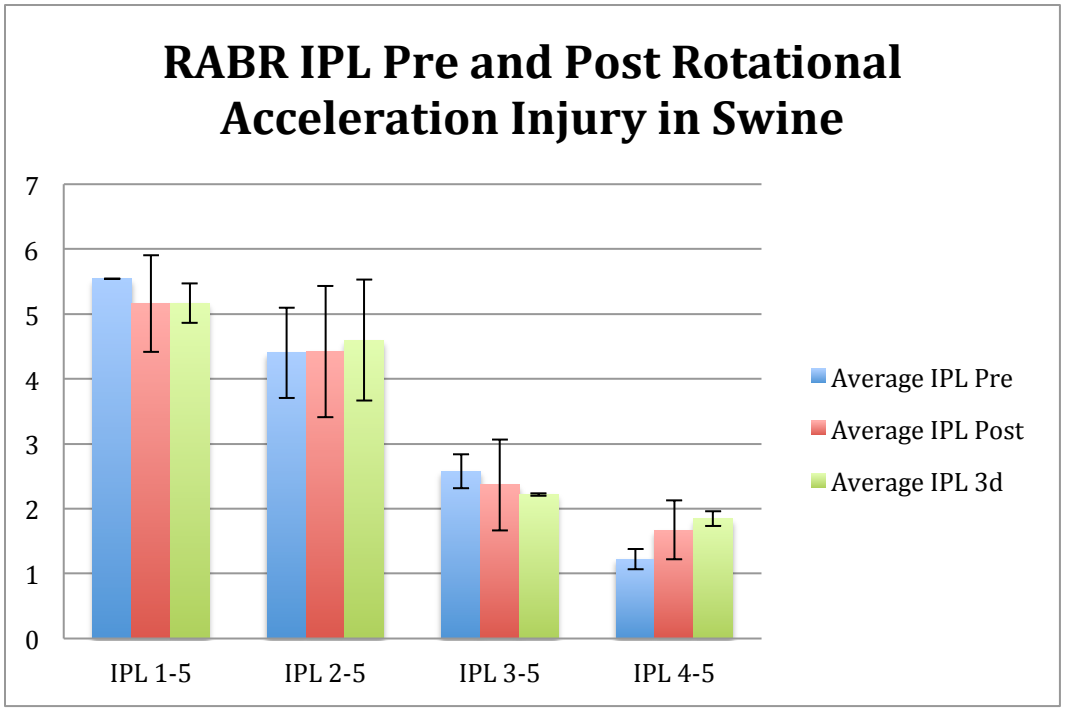


Figure 11. Interpeak latency (IPL) for each RABR waveform demonstrates a trend of increased IPL in the central brainstem waveforms. Error bars = standard deviation.

	IPL 1-5	IPL 2-5	IPL 3-5	IPL 4-5
Average IPL Pre	5.5431	4.4005	2.574475	1.225066667
Average IPL Post	5.159205	4.422533333	2.3666	1.6719
Average IPL 3d	5.167605	4.59755	2.22015	1.84535

Table 5. RABR IPL averages for waveforms I-V.

RABR

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
time	2	3.05	1.526	6.943	0.00513 **
waves	3	73.56	24.519	111.523	1.18e-12 ***
time:waves	6	0.66	0.110	0.500	0.80096

Table 6. RABR IPL repeated measures Anova. Test is the effect of time on waves, the IPL. Modeling test:waves reveals a significant P value of 0.00513 where Df is 2 and F value is 1.526.

Analysis of Pre and Post-Injury Amplitudes

Waveform amplitude, a measure of strength of an impulse at a generator is interesting albeit challenging to interpret due to vulnerability to outside factors. Because wave amplitudes are greatly influenced by the age of the animal, and pig 278 was younger than the other subjects, this animal was excluded from analysis of amplitude [28]. For the remaining animals, relative amplitudes were calculated according to the following formula: amplitude at the peak minus amplitude at subsequent trough [43].

LABR amplitudes pre, post, and three days post-injury are represented in figure 12. Visual inspection of these data showed a decrease in amplitude post-injury, particularly prominent in waveforms III, IV, and V. A repeated measures ANOVA model was constructed predicting LABR amplitude. Time-point (pre-injury, immediate post-injury, and 3 day post-injury), wave, and time by wave interaction

were entered as predictors. Results revealed that there was a significant effect of time on LABR amplitude ($F_2 = 11.89$, $p < 0.001$). Inspection of group means shows that amplitudes dropped immediately post-injury, and then increased at 3 days post-injury to near or above pre-injury. Of note, standard deviations for the 3-day post injury amplitudes are high, suggesting that 3-day post-injury trends should be interpreted with caution. The effect of wave on amplitude was also significant ($F_3 = 76.92$, $p < 0.001$), which is not surprising, as amplitude differences across the waves of the ABR have been well characterized [43]. The wave by time-point interaction was not significant ($F_6 = 0.73$, $p = 0.63$) (Table 7).

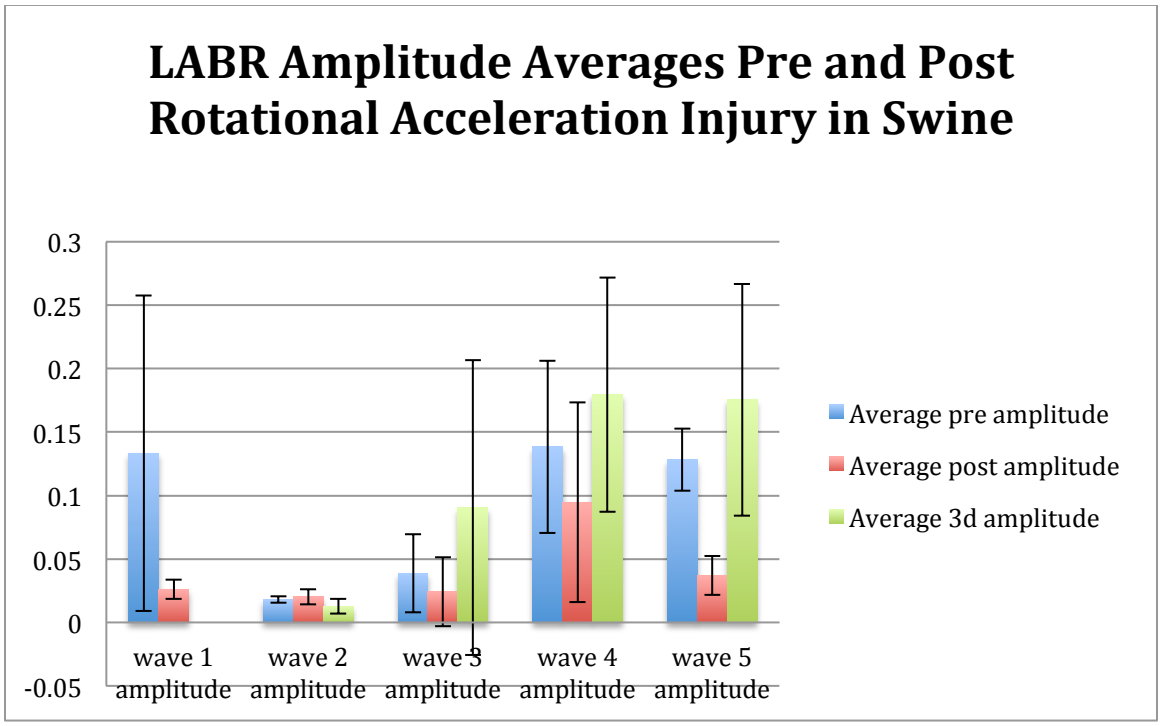


Figure 12. Amplitude for each LABR waveform. Error bars = standard deviation. Wave V pre to post-injury difference reaches statistical significance based on standard deviation.

LABR

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
time	2	7.89	3.943	11.894	0.000315 ***

waves	3	76.50	25.501	76.915	8.07e-12 ***
time:waves	6	1.46	0.243	0.733	0.628148

Table 7. LABR amplitude repeated measures Anova. Test is the effect of time on waves, the amplitude. Modeling test:waves reveals a significant P value of 0.000315 where Df is 2 and F value is 11.894.

Visual inspection of the RABR amplitude measurements showed a less consistent pattern. A repeated measures ANOVA model was constructed predicting RABR amplitude. Time-point (pre-injury, immediate post-injury, and 3 day post-injury), wave, and time by wave interaction were entered as predictors. Results revealed that there was a significant effect of time on RABR amplitude ($F_2 = 6.94$, $p < 0.01$). Inspection of group means shows no consistent pattern of change between the pre-injury and immediate post-injury time-points. However, there was a tendency for decreased amplitude 3-day post injury, relative to the other two measurement occasions. As was the case with the LABR data, the effect of wave on RABR amplitude was also significant ($F_3 = 111.52$, $p < 0.001$). The wave by time-point interaction was not significant ($F_6 = 0.50$, $p = 0.80$) (figure 13).

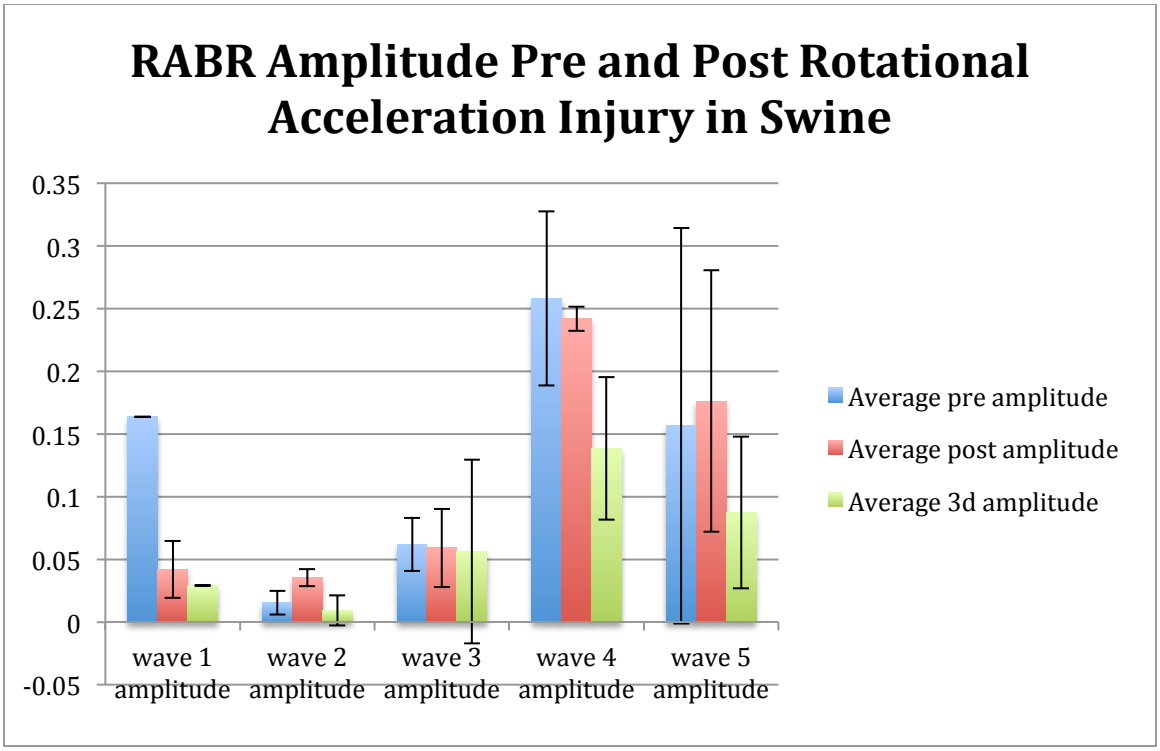


Figure 13. Amplitude for each RABR waveform. Error bars = standard deviation. Wave I pre to post-injury difference reaches statistical significance based on standard deviation.

RABR

Df	Sum Sq	Mean Sq	F value	Pr(>F)	
time	2	3.05	1.526	6.943	0.00513 **
waves	3	73.56	24.519	111.523	1.18e-12 ***
time:waves	6	0.66	0.110	0.500	0.80096

Table 8. RABR amplitude repeated measures Anova. Test is the effect of time on waves, the amplitude. Modeling test:waves reveals a significant P value of 0.00513 where Df is 2 and F value is 3.05.

Morphologic Analysis of Waveforms and Waveform Dropout

Waves I, III, and V are most consistently identified across normal subjects and therefore, changes in the presence or morphology of these waves is a useful surrogate for damage affecting the corresponding region of the pathway [28].

As previously described, waveform absence can occur in BAERs response

and a waveform is considered absent if no peak (identified using a matlab function), is marked in the expected region for it to occur. A qualitative assessment was performed to determine if there was a change in the percentage of wave I presence across all four mini pigs after injury. In the LABR, the percentage of wave I dropout increased post injury (figure 13) from zero to fifty percent immediately post injury to seventy-five percent three days post-injury. The increase in wave I dropout is indicative of injury early in the auditory pathway.

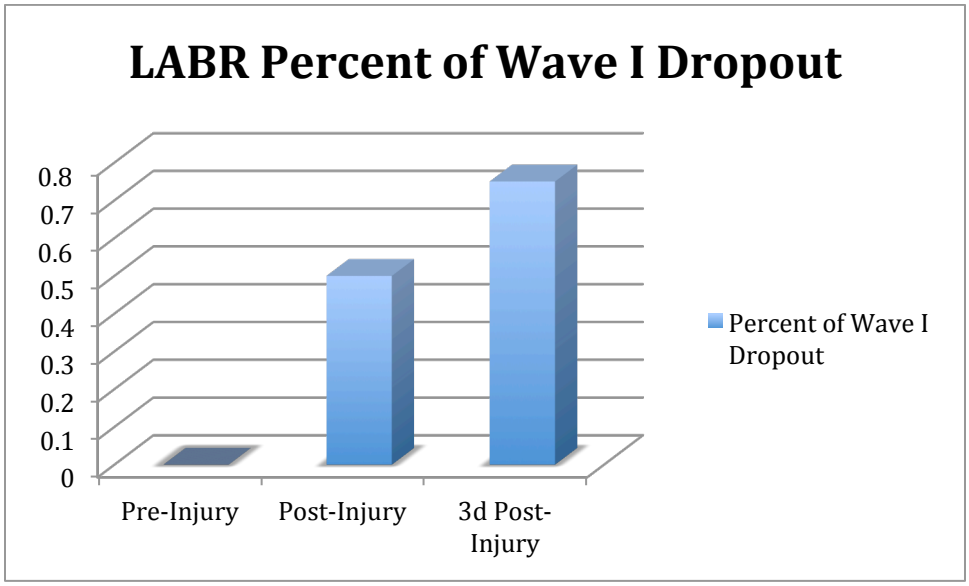


Figure 13. Percent of wave I dropout post injury and 3d post-injury in LABR recordings.

The incidence of wave I dropout was similar across pre-injury and 3-day post-injury measurement occasions with no qualitative increase in wave I dropout after injury (figure 14).

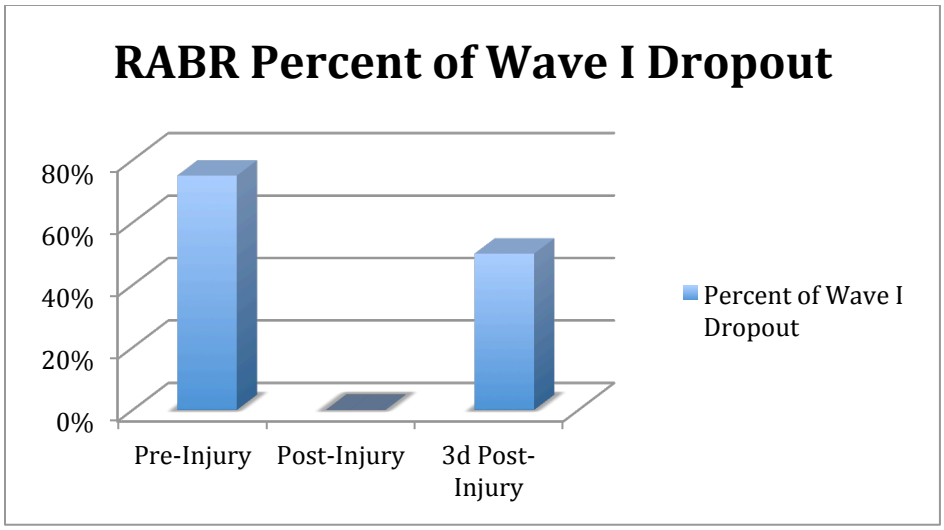
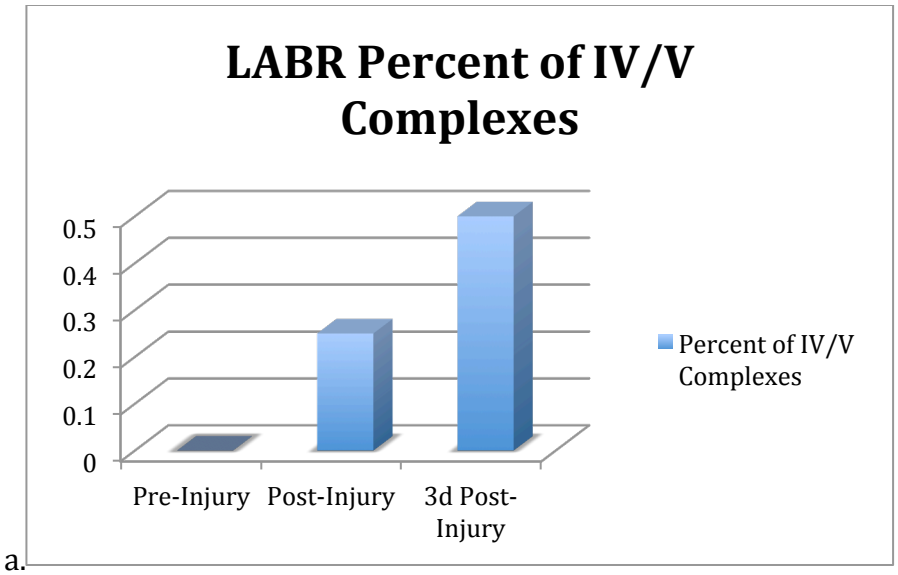
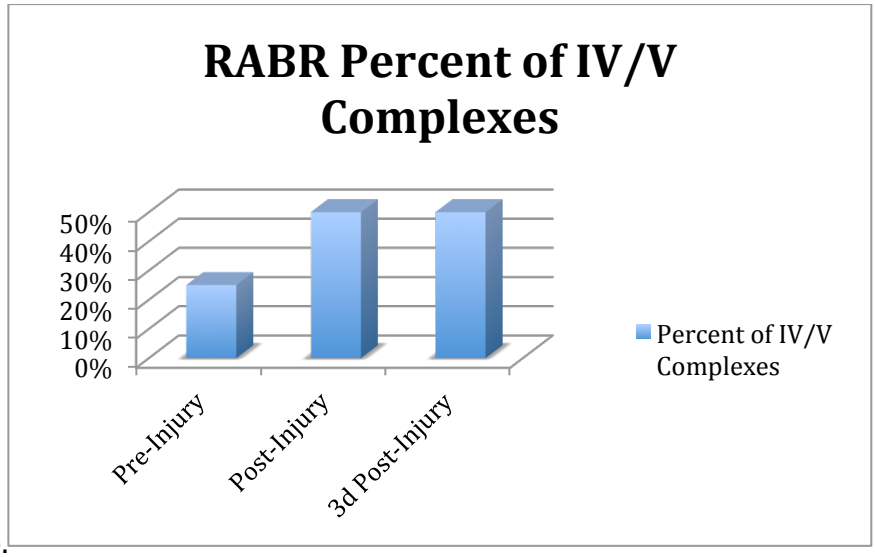


Figure 14. Percent of wave I dropout post injury and 3d post-injury in RABR recordings.

An additional morphologic assessment demonstrated a post-injury trend of increased number of merges of waveform IV and V on both the left and right BAERs (figure 15a, b). Waveform IV and V merges were defined as overlap of the two peaks such that only one peak is identifiable in the region of interest. The change in waveform IV and V is the only one conserved bilaterally although the change is less on the right side (twenty-five percent to fifty percent of dropout on the right versus zero to twenty five to fifty percent on the left).



a.



b.

Figure 15a, b. Percent of morphologic changes in the IV/V waveforms to form complexes increases post-injury from zero to twenty five to fifty percent post and three days post-injury respectively on the left (a) and from twenty-five percent pre-injury to fifty percent at post and three days post injury on the right side (b).

Pig 278 "Case Study": The qualitative effect of varying intensity and pulse rate on BAERs pre and post-injury.

Temporal frequency and intensity of auditory stimuli are known to influence the quality and magnitude of the BAERs [38]. Hence, experimental manipulation of

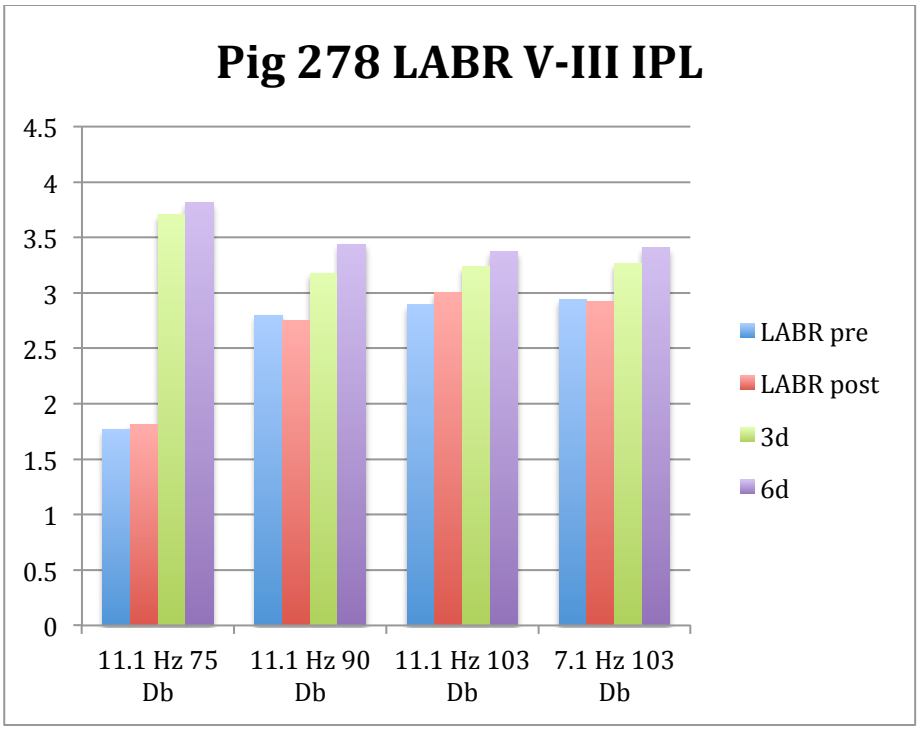
the auditory stimulus may show certain frequency and intensity levels with varying sensitivity to injury [38]. Varying intensity (decibel level) of sound and the pulse rate (frequency) at which the sound clicks are delivered are two methods that are used to determine the response with varying stimulus parameters. Therefore, a "threshold estimation", is detected at which point sounds become less audible as indicated by an absent or delayed latency response. Figure 16 a, b represent an example case of threshold estimation in which intensity is varied from 75 decibels to 103 decibels and pulse rate is either 7.1 hertz or 11.1 hertz.

At louder intensities of 90 and 103 decibels, the difference between pre and post-injury waveform III to V IPL is less appreciated than it is at 75 decibels. A softer sound is more difficult to hear and thus the injury effect is more appreciable, especially at the three and six day time points.

Temporal acuity or the ability to hear sounds based on the separation of pulses, pulse rate, affected the IPLs less. The 7.1 and 11.1 hertz trials elicited similar responses. This may indicate that the margin of varying pulse rate was not wide enough to elicit a robust change on IPL. However, the IPL increases from pre to immediately post-injury at a faster pulse rate of 11.1hz, arguably a slightly more difficult sound to distinguish sound that would elicit a subtle post-injury latency delay.

Although qualitative, this case study represents the additive value of threshold estimation to drawing out the effect of injury on the BAERs.

a.



b.

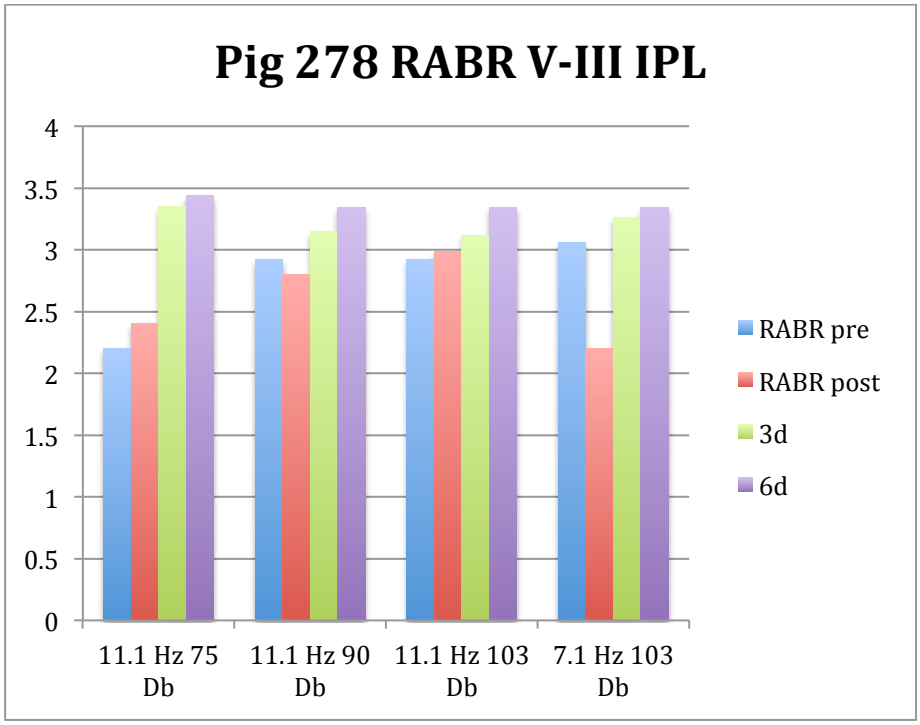


Figure 16a,b. Threshold estimation in LABR (a) and RABR (b) at pre, immediately post, 3 days post, and 6 days post-injury. Decibels increase from 75 to 90 to 103db from left to right with the last set representing a change in pulse rate to 7.1 Hz at

103 db.

Histologic Analysis of the Brainstem Auditory Pathway

In order to correlate change in BAERs with underlying axonal pathology, post-mortem tissue was analyzed histopathologically for evidence of axonal damage that would result in latency delays, decreased amplitudes, and morphologic waveform changes.

Amyloid precursor protein (APP), accumulates when axonal transport is disrupted due to shearing and stretching injury that occurs with rapid rotational acceleration mechanisms such as in these minipigs and in human concussive force [12]. Processed tissue was incubated in APP antibody according to protocol. Sections of pig 2 were dissected to reveal areas of the brainstem auditory pathway that correspond to the wave generators in the BAERs. Axonal pathology marked by APP was distributed bilaterally and throughout the brainstem region containing the lateral lemniscus and the pathway between the inferior colliculi (Figure 17a-d).

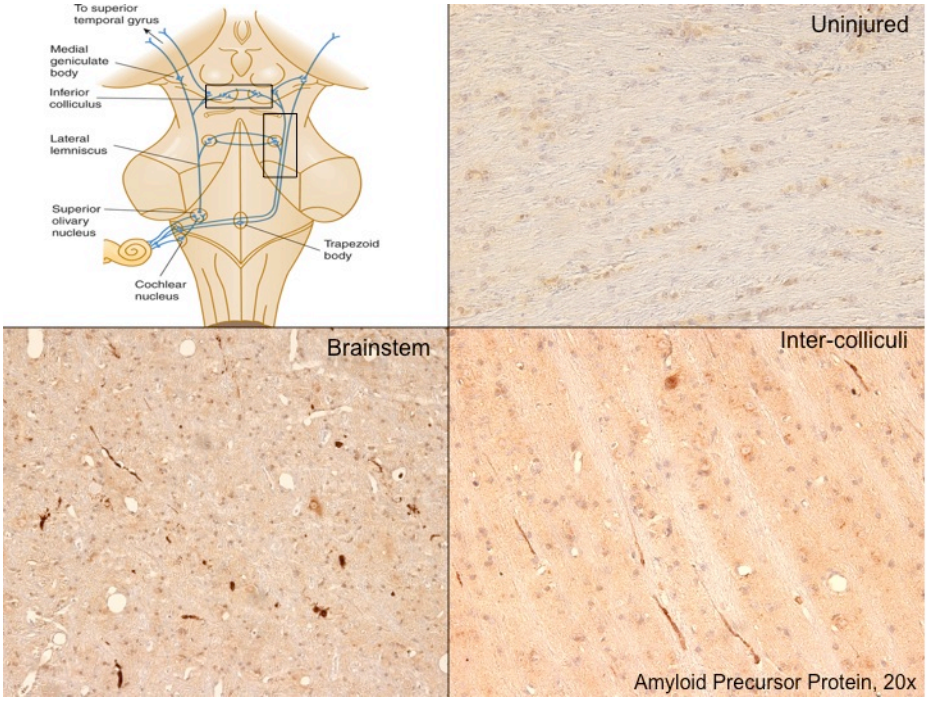


Figure 17a-d. (a, upper left) A schematic representing dissected areas (image adapted from [28]. (b) Negative control tissue demonstrates no axonal accumulations. (c,d)APP staining (1:75,000) of brainstem auditory pathway in pig 2, demonstrates axonal pathology at the levels of the inter-colliculi and parallel pons that contains the lateral lemniscus.

e. Discussion

BAERs Changes as an Indicator of Auditory Network Dysfunction

Evoked potentials are an attractive modality for analyzing network dysfunction due to both their non-invasive nature and ability to relay information about neuronal firing and conduction. Therefore the use of brainstem auditory evoked potentials in mild traumatic brain injury is a model to identify regions in the brainstem auditory pathway that are particularly affected by axonal pathology.

Correlating these regions with histopathologic evidence of axonal shear and stretch injury further solidifies this concept.

It is well described that a brief acoustic stimulus such as the clicks used in this experiment, creates a response across the nervous system that generates electrical signals or waveforms, as it travels through the pathway over time [28]. Previous animal injury, intra-operative and tumor studies created a map of the brainstem auditory regions by correlating waveform changes consistently associated with a lesion location. As detailed in figure 6, the earliest waveform, I, represents the electrical impulse of sound traveling across the cochlear or eighth cranial nerve. Following the path from the cochlear nerve, waveform II is generated at the synapse of the cochlear nucleus, waveforms III through V represent the path between the cochlear nucleus, superior olivary complex, inferior colliculus, and finally the medial geniculate nucleus of the thalamus [28].

Short-latency responses, those representing the brainstem auditory pathway, primarily represent change in action potential as sound stimuli conduct over the pathway [28]. Reeves and others demonstrated that traumatic axonal injury, the central mechanism in mild traumatic brain injury results in delayed action potentials in single neuron rat models [24]. Possible mechanisms underlying the delay in action potential post shear or stretch injury to an axon include immediate post injury influx of ions, especially calcium [44]. This single-cell principle is likely applicable to an entire network's response as a network is a compilation of axons forming a track between nuclei. The purpose of a network is to transport information, in this case, external auditory stimuli to the cortex for processing.

To determine which portions of the auditory axon tracks coursing through the brainstem were affected in a way that altered signal processing, brainstem auditory evoked potentials were measured immediately after and three days after injury and compared to pre-injury baseline. The clinical precedent of using BAERs, set by clinicians evaluating the effects of diseases such as: multiple sclerosis[45], vestibular schwannomas [28], and autism spectrum disorders [39], uses primarily waveforms I, III, and V [28].

Interpretation of Latency Changes following Rotational Acceleration Head Injury

Absolute latency is a commonly employed clinical tool for assessing the brainstem auditory evoked response for abnormality. Latency indicates speed of conduction between generators [38]. In this study, there was no statistically significant difference in absolute latency at any waveform, bilaterally. Latency for each waveform was however qualitatively conserved for both ears, indicating reliability of the BAERs recording and analysis.

Interpeak latencies are both clinically and neurophysiologically telling for two reasons: they are less affected by individual variability in absolute latency and theoretically, IPLs reflect the time required for a nerve impulse to travel along axons from one generator to the next [43]. Waveform I to V, and III to V interpeak latencies are commonly employed in clinical practice [38].

At the time point immediately after rotational acceleration injury, there was a statistically significant effect of injury time point on interpeak latency on the left side. It is important to note that anesthesia should have no effect on BAERs analysis

based on previous data [46]. Inspection of this data revealed the largest delay in the II-V, III-V, and IV-V intervals, with a slight delay appreciated in the I-V IPL or central transmission time (CTT). It is important to note that waveform V generators receive contralateral input due to crossing of pathways, therefore the interpeak latencies are not direct sums of all of the IPL components [28].

Delay in latency in the later IPL intervals indicates that central brainstem pathology is the main contributor to conduction slowing on the left side [38]. A subsequent decrease in IPL at three days post-injury occurred at both the II-V, III-V, and III-V intervals. Although a decrease is unexpected, its association with waveform morphologic changes (to be discussed in latter sections), suggests that it is consistent with central brainstem pathology.

As with the left side, there was a statistically significant change in IPL immediately post and three days post-injury via ANOVA analysis. Visual inspection and numerical means showed the largest increase in IPL in the IV-V IPL with a trend of increased IPL over time. The right-sided IPLs changed less with time than the left-sided IPLs for waveforms I-III. Together the IPL data on both sides revealed a delay immediately post-injury and changes at three days post-injury, most pronounced upon visual inspection in later, central regions, generated between the lower pons and the mesencephalon.

Interpretation of Amplitude Changes following Rotational Acceleration Head Injury

Abnormalities in amplitude ratios reflect dysfunction across the auditory pathways [28]. A pool of neurons firing generates a waveform and if less neurons

fire or a less intense signal reaches the point of the generator, amplitude is diminished. Although a popular tool for assessing dysfunction in the BAERs due to the supporting physiologic theory, amplitude changes are subject to significant variability and therefore must be interpreted with caution [38]. In order to best control the amplitude studies, aged-matched animals were included and stimulus parameters were consistent across subjects.

LABR amplitude data is consistent with findings in the IPL domain in which, central regions of the pathway are most affected immediately post-injury. In a repeated measures ANOVA model, waveform amplitude was significantly affected by time point, describing a meaningful change in response after injury. Visual inspection revealed changes in the III, IV, and V waveforms, consistent with central pathology. Additionally, waveform I largely decreased in amplitude immediately following injury and was not identified at three days post-injury. The large change in waveform one may indicate additional cranial nerve pathology that was not appreciated in the IPL analysis, or may be consistent with central pathology. Audiologists hypothesize that descending inhibition from the inferior colliculi changes with central lesions, resulting in early waveform amplitude changes [47, 48]. This typically would result in an increase in wave one amplitude rather than a decrease however, making the waveform I amplitude data more consistent with peripheral pathology.

At three days post-injury, the data must be interpreted with caution due to large standard deviations and high variability. However, the trend demonstrated increase in amplitude post-injury. This finding may represent recovery at three

days, but based on morphologic analysis at that time point is unlikely. Other hypotheses include: morphology changes distorting amplitude measurements and in increase in noise in the pathway after injury resulting from decreased inhibition.

The right-sided amplitudes, although showing a significant effect of injury time point on their values, demonstrated a less consistent pattern of change with the most conserved change a decrease in amplitude three days following injury, in waves I, III, IV, and V. Decrease in amplitude in waveforms III, IV, and V again indicates pontine to mesencephalic pathology and diminished waveform I response represents peripheral nerve pathology. Wave II amplitude increased immediately post-injury which could be interpreted as a decrease in central inhibition from descending, central pathways [48].

On the right side, the trend of decreased amplitude occurred later (at three days) than on the left. An inconsistent pattern of laterality in the BAERs is not surprising and likely represents real differences in underlying pathology as a unilateral BAERs is thought to predominantly reflect the activity of the ipsilateral ascending auditory tracks [28]. Lateralization and asymmetry in the BAERs is documented in other disease processes and in normal subjects [49]. Asymmetry in the pathologic distribution of the axonal injury in mild head injury is also documented in previous histopathologic swine studies [1]. Additionally, the leading side of the injury rotation, the left in this case, may affect the distribution of pathology causing laterality of effects.

Interpretation of Morphology Changes following Rotational Acceleration Head Injury

Presence versus absence of a waveform component and waveform appearance also provides important information about network function. An auditory evoked potential response is a sum of a large number of neurons firing together and pathologic changes to this group can cause a variety of phenotypes. The first, as discussed prior is that if the pool is displaced in unison, a latency delay will occur; the second change is that a decreased pool reaching a generator at the same time decreases amplitude. Desynchrony of the neuronal population due to non-uniform pathology, can cause change in waveform morphology or absence of the waveform. The mechanism of this change is such that it can be interpreted in a similar manner to that of a delay in the region of the waveform generator [28, 38].

A qualitative analysis looking at wave I, showed that the absolute number of waveforms identified decreased after injury on the left side. Increased wave I dropout after injury indicates cochlear pathology. Previous literature on wave I abnormality is related to cochlear infarction, ischemia, or acoustic tumors [50]. Based on mechanistic knowledge of rotational acceleration injury, an explanation for cochlear nerve injury in this case is: axonal injury following stretch and shear in the portion of the nerve traveling between the cochlea, through the skull base, and to the cochlear nucleus. Of note, the dropout of wave I also helps to explain the only minimal change in the I-V IPL as the IPL was not calculated when a component waveform was missing.

The right-sided data showed initial morphology variance with a large portion of wave I dropout prior to injury followed by a decrease after injury. Although there was a qualitative shift after injury, it is unexpected to see a decrease in waveform I

dropout. The increase in number of identifiable waveforms I after injury could be related to decreased central inhibition due to central injury and increased presence of firing from neural generators in the early brainstem regions. Central injury on the right side is supported by the IPL data. It is important to note that the absence of wave I in the right-sided data pre-injury may also demonstrate baseline individual and left versus right-sided variability that makes the right-sided data difficult to interpret.

An additional observation upon visual inspection was an increase in complex formation between waves IV and V following injury, both immediately and three days following injury, bilaterally. Changes in waveform complexes and shape are used to identify important changes including auditory pathway development [51]. Minimal data is available on the meaning of IV/V complex formation in terms of clinical disorders and this is a normally described variant, however theoretically, waveform merge is due to a decreased distinction between generators and a change from baseline may represent a significant, injury-related distinction. Desynchrony in either or both generators (IV at the lateral lemniscus and V at the level of the inferior colliculus) could result in decreased distinction between the two, already closely adjacent regions and a subsequent merge. Additionally, a decreased signal from either generator, resulting in waveform dropout could explain the observation of a single peak waveform at the expected location of both waveforms IV and V. These findings further support the observation of central brainstem abnormalities on the BAERs following rotational head acceleration injury. Additionally, wave IV and V complexes, particularly at three days post-injury effect the measurement of

IPL and amplitudes as the peak of the complex was used to determine the difference in latency between that point and the peak of waveform I, and the same peak was also used in amplitude measurement. This suggests that decreased IPL seen at three days post-injury is unlikely due to recovery, and more likely a result of change in morphology and an indicator of ongoing disease.

Pathologic Meaning of the BAERs Results and Correlation with Histopathology

The BAERs data detected a change in amplitude, interpeak latency, and waveform pathology both in the early, peripheral portion, and the late pontine and mesencephalic regions of the pathway. The distribution of abnormal findings shows the diffuse nature of mild traumatic brain injury. Previous literature demonstrates diffuse axonal injury as a mechanism in mild traumatic brain injury and this data preliminary demonstrates detection of axonal injury, through non-invasive, audiologic assessment. The BAERs, when analyzed by region and taking into account the mechanism of waveform generation, directly assesses pools of neuronal function following injury. In order to avoid misinterpretation of findings, it is important to note that the changes following injury are subject to interpretation, and one hypothesis is that axonal injury underlies these changes. This hypothesis is supported by the fact that the pattern of central and peripheral injury demonstrated in swine after mild head injury is similar to that seen in demyelinating diseases such as multiple sclerosis [28, 45].

A second hypothesis, supplementary to the first, is that neurometabolic changes underlie the BAERs abnormalities. This would be especially supported at

the initial time point following injury when acute decreases in amplitude and delays in IPL are appreciated. This is because previous animal and human studies show damaging neurometabolic cascades and inflammatory responses immediately following injury [7].

In order to further link axonal pathology and the BAERs changes, each animal was euthanized following injury and assessed for presence of axonal pathology as indicated by the accumulation of amyloid precursor protein along the track. In corresponding levels to waveforms III-V, figure 17 demonstrates scattered axonal swellings in the pons and inferior colliculi, not present in uninjured control. Visual inspection of APP stained tissue revealed similar pathology profiles bilaterally, but due to small n, further quantitative analysis is pending future studies. Cochlear nerve pathology was unavailable due to the inaccessibility of the nerve within the think skull base of minipigs. The observation of axonal damage in the central pons, however, confirms that the mild injury produced axonal injury that can be correlated to the pre-mortem non-invasive assessment.

It is important to think about non-invasive functional assessment of mTBI for two reasons: 1) Pathology is not available for use to provide information to patients based on their typical survival of injury and 2) staining with APP shows the breakdown of transport protein function following axonal injury but assessing the neuron's ability to function after injury provides a deeper story about the extent and localization of injury. Brainstem auditory evoked potentials are useful in giving information in the operating room, in patients with tumors, or other diseases, and

also provide key information in mild traumatic brain injury as a marker of network dysfunction.

Brainstem Auditory Evoked Potential mTBI Abnormalities and Patient Symptoms

Analyzing the BAERs in a large animal model in which abnormalities can be correlated with underlying pathology sheds light on the historical studies that found a correlation between post-concussive syndrome and BAERs changes [33]. Initial studies often found it difficult to link the brainstem auditory pathway to the unique constellation of symptoms mTBI patients encounter. A study such as this makes burden of axonal pathology the link and BAERs an indicator of the burden. Due to an inability to measure auditory symptoms in swine, it is not currently possible to directly correlate BAERs findings and axonal pathology in swine with auditory phenotype, however this study provides a conceptual and mechanistic link between the BAERs studies of the past and one of now, incorporating the importance of thinking of mTBI as a disease of network dysfunction.

Challenges in this Study

The main challenge presented by a brainstem auditory evoked potentials study in swine is the small n of a large animal study paired with large inter and intra-individual variability. This can make brainstem auditory evoked potentials difficult to interpret, especially given the diffuse nature of mild head injury. Therefore, in order to truly understand the localization and mechanism of injury

using the BAERs, human studies would need to establish normative values across large samples and use the technology most efficiently.

BAERs technology, although simple, allows for complicated analysis when using overlapping modalities to find subtle abnormalities. For example, in addition to latency and amplitude evaluation, morphology changes can be used to better interpret numerical results. Also, as shown in the single animal 278 study (figure 16), using threshold estimation by altering temporal acuity and intensity of sound stimulus further elucidates deficits. This technique of varying threshold is employed in much of the human literature and also in large animal studies such as using BAERs to detect bovine encephalopathy [52-54].

Future Directions

This study represents an important step in building a conceptual framework for linking the mechanism of axonal injury to axonal dysfunction to network dysfunction, using the auditory network. Future studies should include further assessment of the electrophysiology of the pathway as well as linking the function of the pathway with its structural integrity, for example using the technique of diffusion tensor imaging.

The eventual goal is to provide mTBI patients and their families with meaningful information about the nature of their disease. This will require breaking down a very diffuse process by mechanism, likely axonal and therefore network dysfunction. Although not a trivial task, functional assessment of neuronal function

using electrophysiology accompanied by other modalities provides hope towards understanding a challenging disease process.

REFERENCES

1. Browne, K.D., et al., *Mild traumatic brain injury and diffuse axonal injury in swine.* J Neurotrauma, 2011. **28**(9): p. 1747-55.
2. Longhi, L., et al., *Temporal window of vulnerability to repetitive experimental concussive brain injury.* Neurosurgery, 2005. **56**(2): p. 364-74; discussion 364-74.
3. Menon, D.K., et al., *Position statement: definition of traumatic brain injury.* Arch Phys Med Rehabil, 2010. **91**(11): p. 1637-40.
4. Center for Disease Control and Prevention, N.C.f.I.P. 2011 2013 [cited 2014.
5. Sigurdardottir, S., et al., *Post-concussion symptoms after traumatic brain injury at 3 and 12 months post-injury: a prospective study.* Brain Inj, 2009. **23**(6): p. 489-97.
6. Margulies, S., *The postconcussion syndrome after mild head trauma: is brain damage overdiagnosed? Part 1.* J Clin Neurosci, 2000. **7**(5): p. 400-8.
7. Giza, C.C. and D.A. Hovda, *The Neurometabolic Cascade of Concussion.* J Athl Train, 2001. **36**(3): p. 228-235.
8. Wallesch, C.W., et al., *Outcome after mild-to-moderate blunt head injury: effects of focal lesions and diffuse axonal injury.* Brain Inj, 2001. **15**(5): p. 401-12.
9. Smith, D.H., R. Hicks, and J.T. Povlishock, *Therapy development for diffuse axonal injury.* J Neurotrauma, 2013. **30**(5): p. 307-23.
10. Adams, J.H., et al., *Brain damage in fatal non-missile head injury in relation to age and type of injury.* Scott Med J, 1989. **34**(1): p. 399-401.
11. Saatman, K.E., et al., *Classification of traumatic brain injury for targeted therapies.* J Neurotrauma, 2008. **25**(7): p. 719-38.
12. Johnson, V.E., W. Stewart, and D.H. Smith, *Axonal pathology in traumatic brain injury.* Exp Neurol, 2013. **246**: p. 35-43.
13. Graham, D.I., et al., *Tissue tears in the white matter after lateral fluid percussion brain injury in the rat: relevance to human brain injury.* Acta Neuropathol, 2000. **99**(2): p. 117-24.
14. Smith, D.H., et al., *Protein accumulation in traumatic brain injury.* Neuromolecular Med, 2003. **4**(1-2): p. 59-72.

15. Povlishock, J.T., D.E. Erb, and J. Astruc, *Axonal response to traumatic brain injury: reactive axonal change, deafferentation, and neuroplasticity.* J Neurotrauma, 1992. **9 Suppl 1**: p. S189-200.
16. Mittl, R.L., et al., *Prevalence of MR evidence of diffuse axonal injury in patients with mild head injury and normal head CT findings.* AJNR Am J Neuroradiol, 1994. **15**(8): p. 1583-9.
17. Wilde, E.A., et al., *Serial measurement of memory and diffusion tensor imaging changes within the first week following uncomplicated mild traumatic brain injury.* Brain Imaging Behav, 2012. **6**(2): p. 319-28.
18. Smith, D.H., et al., *New magnetic resonance imaging techniques for the evaluation of traumatic brain injury.* J Neurotrauma, 1995. **12**(4): p. 573-7.
19. Siman, R., et al., *A panel of neuron-enriched proteins as markers for traumatic brain injury in humans.* J Neurotrauma, 2009. **26**(11): p. 1867-77.
20. Czeiter, E., et al., *Brain injury biomarkers may improve the predictive power of the IMPACT outcome calculator.* J Neurotrauma, 2012. **29**(9): p. 1770-8.
21. Tang-Schomer, M.D., et al., *Partial interruption of axonal transport due to microtubule breakage accounts for the formation of periodic varicosities after traumatic axonal injury.* Exp Neurol, 2012. **233**(1): p. 364-72.
22. Greer, J.E., J.T. Povlishock, and K.M. Jacobs, *Electrophysiological abnormalities in both axotomized and nonaxotomized pyramidal neurons following mild traumatic brain injury.* J Neurosci, 2012. **32**(19): p. 6682-7.
23. Baker, A.J., et al., *Attenuation of the electrophysiological function of the corpus callosum after fluid percussion injury in the rat.* J Neurotrauma, 2002. **19**(5): p. 587-99.
24. Reeves, T.M., L.L. Phillips, and J.T. Povlishock, *Myelinated and unmyelinated axons of the corpus callosum differ in vulnerability and functional recovery following traumatic brain injury.* Exp Neurol, 2005. **196**(1): p. 126-37.
25. Reeves, T.M., et al., *Unmyelinated axons show selective rostrocaudal pathology in the corpus callosum after traumatic brain injury.* J Neuropathol Exp Neurol, 2012. **71**(3): p. 198-210.
26. Lew, H.L., et al., *Auditory dysfunction in traumatic brain injury.* J Rehabil Res Dev, 2007. **44**(7): p. 921-8.
27. Munjal, S.K., N.K. Panda, and A. Pathak, *Relationship between severity of traumatic brain injury (TBI) and extent of auditory dysfunction.* Brain Inj, 2010. **24**(3): p. 525-32.
28. aminoff, m.J., *Aminoff's Electrodiagnosis in Clinical Neurology*, in *Brainstem Auditory Evoked Potentials: Methodology, Interpretation, and Clinical Application*, a. legatt, Editor. 2012, Elsevier: China. p. 519-560.
29. Barber, H.O., *The diagnosis and treatment of auditory and vestibular disorders after head injury.* Clin Neurosurg, 1972. **19**: p. 355-70.
30. Greenberg, R.P. and D.P. Becker, *Clinical applications and results of evoked potential data in patients with severe head injury.* Surg Forum, 1975. **26**: p. 484-6.
31. Schoenhuber, R., M. Gentilini, and A. Orlando, *Prognostic value of auditory brain-stem responses for late postconcussion symptoms following minor head injury.* J Neurosurg, 1988. **68**(5): p. 742-4.

32. Aleksanov, N.S., S. Shchigolev Iu, and S. Gizatullin, *[Short-latency brain-stem auditory evoked potentials in patients with a brain concussion].* Zh Vopr Neirokhir Im N N Burdenko, 1995(2): p. 17-20.
33. Soustiel, J.F., et al., *Trigeminal and auditory evoked responses in minor head injuries and post-concussion syndrome.* Brain Inj, 1995. **9**(8): p. 805-13.
34. Drake, M.E., Jr., S.J. Weate, and S.A. Newell, *Auditory evoked potentials in postconcussive syndrome.* Electromyogr Clin Neurophysiol, 1996. **36**(8): p. 457-62.
35. Greenwald, R.M., et al., *Head impact severity measures for evaluating mild traumatic brain injury risk exposure.* Neurosurgery, 2008. **62**(4): p. 789-98; discussion 798.
36. Pellman, E.J., et al., *Concussion in professional football: location and direction of helmet impacts-Part 2.* Neurosurgery, 2003. **53**(6): p. 1328-40; discussion 1340-1.
37. Blumbergs, P.C., et al., *Staining of amyloid precursor protein to study axonal damage in mild head injury.* Lancet, 1994. **344**(8929): p. 1055-6.
38. Association, A.S.-L.-H. *Short Latency Auditory Evoked Potentials (Relevant Paper)*. 1987 [cited 1987 2014]; Available from: http://www.asha.org/policy.
39. Kallstrand, J., et al., *Abnormal auditory forward masking pattern in the brainstem response of individuals with Asperger syndrome.* Neuropsychiatr Dis Treat, 2010. **6**: p. 289-96.
40. Gauly, M., et al., *Brainstem auditory-evoked potential assessment of auditory function and congenital deafness in llamas (Lama glama) and alpacas (L. pacos).* J Vet Intern Med, 2005. **19**(5): p. 756-60.
41. Fowler, C.G. and D. Noffsinger, *Effects of stimulus repetition rate and frequency on the auditory brainstem response in normal cochlear-impaired, and VIII nerve/brainstem-impaired subjects.* J Speech Hear Res, 1983. **26**(4): p. 560-7.
42. Eggermont, J.J., *Temporal modulation transfer functions for single neurons in the auditory midbrain of the leopard frog. Intensity and carrier-frequency dependence.* Hear Res, 1990. **43**(2-3): p. 181-98.
43. Starr, A. and J. Achor, *Auditory brain stem responses in neurological disease.* Arch Neurol, 1975. **32**(11): p. 761-8.
44. Lusardi, T.A., et al., *Effect of acute calcium influx after mechanical stretch injury in vitro on the viability of hippocampal neurons.* J Neurotrauma, 2004. **21**(1): p. 61-72.
45. Barajas, J.J., *Evaluation of ipsilateral and contralateral brainstem auditory evoked potentials in multiple sclerosis patients.* J Neurol Sci, 1982. **54**(1): p. 69-78.
46. Banoub, M., J.E. Tetzlaff, and A. Schubert, *Pharmacologic and physiologic influences affecting sensory evoked potentials: implications for perioperative monitoring.* Anesthesiology, 2003. **99**(3): p. 716-37.
47. Musiek, F.E. and D.W. Hoffman, *An introduction to the functional neurochemistry of the auditory system.* Ear Hear, 1990. **11**(6): p. 395-402.

48. Musiek, F.E., *Neuroanatomy, neurophysiology, and central auditory assessment. Part III: Corpus callosum and efferent pathways.* Ear Hear, 1986. **7**(6): p. 349-58.
49. Levine, R.A., J. Liederman, and P. Riley, *The brainstem auditory evoked potential asymmetry is replicable and reliable.* Neuropsychologia, 1988. **26**(4): p. 603-14.
50. Arnold, S.A., *Objective versus visual detection of the auditory brain stem response.* Ear Hear, 1985. **6**(3): p. 144-50.
51. Ichiyama, T., T. Hayashi, and S. Furukawa, *Developmental changes of contralateral brainstem auditory evoked potentials: evaluation of brainstem maturation.* Brain Dev, 1995. **17**(1): p. 49-51.
52. Arai, S., et al., *Brainstem auditory evoked potentials in experimentally-induced bovine spongiform encephalopathy.* Res Vet Sci, 2009. **87**(1): p. 111-4.
53. Pakarinen, S., et al., *Measurement of extensive auditory discrimination profiles using the mismatch negativity (MMN) of the auditory event-related potential (ERP).* Clin Neurophysiol, 2007. **118**(1): p. 177-85.
54. Kirby, A.E. and J.C. Middlebrooks, *Auditory temporal acuity probed with cochlear implant stimulation and cortical recording.* J Neurophysiol, 2010. **103**(1): p. 531-42.