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Gene co-expression networks identify *Trem2* and *Tyrobp* as major hubs in human APOE expressing mice following traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is strongly linked to an increased risk of developing dementia, including chronic traumatic encephalopathy and possibly Alzheimer's disease (AD). *APOEε4* allele of human Apolipoprotein E (*APOE*) gene is the major genetic risk factor for late onset AD and has been associated with chronic traumatic encephalopathy and unfavorable outcome following TBI. To determine if there is an APOE isoform-specific response to TBI we performed controlled cortical impact on 3-month-old mice expressing human APOE3 or APOE4 isoforms. Following injury, we used several behavior paradigms to test for anxiety and learning and found that APOE3 and APOE4 targeted replacement mice demonstrate cognitive impairments following moderate TBI. Transcriptional profiling 14 days following injury revealed a significant effect of TBI, which was similar in both genotypes. Significantly upregulated by injury in both genotypes were mRNA expression and protein level of ABCA1 transporter and APOJ, but not APOE.

To identify gene-networks correlated to injury and APOE isoform, we performed Weighted Gene Co-expression Network Analysis. We determined that the network mostly correlated to TBI in animals expressing both isoforms is immune response with major hub genes including *Trem2*, *Tyrobp*, *Clec7a and Cd68*. We also found a significant increase of TREM2, IBA-1 and GFAP protein levels in the brains of injured mice. We identified a network representing myelination that correlated significantly with APOE isoform in both injury groups. This network was significantly enriched in oligodendrocyte signature genes, such as *Mbp* and *Plp1*. Our results demonstrate unique and distinct gene networks at this acute time point for injury and APOE isoform, as well as a network driven by APOE isoform across TBI groups.

1. Introduction

Traumatic brain injury (TBI) is one of the leading causes of death and disability in the United States. Approximately 2 million people sustain a TBI and 50,000 TBI-related deaths occur in the United States every year. Currently, there is no treatment for TBI, patients are only given supportive care for which the cost is approximately $60 billion annually. TBI can either be caused when the head violently impacts with another object or when an object pierces the skull and enters the brain tissue. Studies show that following the acute phase, over the long-term, patients may develop changes in cognition, and increases in both anxiety and depression (Perez-Garcia et al., 2016; Ghroubi et al., 2016). The high level of variability in injury outcomes suggests, to a significant extent, a strong role for genetic influence on brain susceptibility and recovery (Draper and Ponsford, 2008; Whitnall et al., 2006).

TBI is strongly linked to increased risk of developing dementia, including chronic traumatic encephalopathy and possibly Alzheimer's disease (AD) (Jordan, 2007; Jordan, 2014; McKee et al., 2016). The *APOEε4* allele of human apolipoprotein E (*APOE*) gene is the major genetic risk factor for late onset AD and has been associated with chronic traumatic encephalopathy and unfavorable outcome following TBI. Multiple studies have identified worse outcomes following TBI based on the inheritance of *APOEε4* allele (Alexander et al., 2007; Diaz-Arrastia et al., 2003). The role of APOE in neuronal survival and repair and in overall response to TBI, however, is not well understood. It has been suggested that APOE4 is less stable and catabolically degraded more quickly than the other APOE isoforms, possibly due to its lower lipidation level (Kim et al., 2009). In mice, studies have identified APOE-genotype and brain-region specific genomic changes using mRNA microarrays after controlled cortical impact (CCI) (Crawford et al., 2009). Patients, carriers of *APOEε4*, experiencing TBI demonstrated worse memory performance

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in a verbal learning test and verbal fluency measured 6 months post-injury (Crawford et al., 2002). In contrast, other in vivo data did not find or confirm a role for APOE4 in TBI (Mannix et al., 2011; Mannix et al., 2016; Moran et al., 2009; Willemse-van Son et al., 2008). For example, in adult patients with moderate to severe TBI assessed 3, 6 and 12 months post-injury, APOE4 patients did not have poorer cognitive performance, functional outcome or slower improvement (Ponsford et al., 2008). There is a uniform agreement that more studies are needed to clarify the role of *APOEε4* allele in TBI. Mechanical stress placed on the brain due to the impact is considered the primary injury. Following the impact, a secondary injury occurs leading to additional damage and cell death, worsening the outcome. Mechanisms of secondary injury include neuronal excitotoxicity, edema, oxidative stress, and neuroinflammation. The inflammatory state in the brain can persist for many years following the injury; chronic neuroinflammation following TBI was closely associated with neuronal death and impaired cell proliferation both immediately adjacent to, and locations more distant from, site of injury (Acosta et al., 2013). Multiple inflammatory molecules are upregulated after TBI and are believed to contribute to these processes, as well as blood brain barrier dysfunction. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), chemokine C motif ligand 2 (Ccl2), and chemokine CX3C motif receptor 1 (Cx3cr1) are among those that have been intensely studied for their impact on brain pathology following TBI (Dalgard et al., 2012; Ferreira et al., 2014; Gyoneva and Ransohoff, 2015; Hua et al., 2011). Studies have shown that the expression levels of the majority of those inflammatory factors are associated with severity of injury and outcome in TBI patients, the reduction of neurobehavioral impairments and injury volume as well as the survival rates in rodent models (Morganti et al., 2015; Yang et al., 2013).

To our knowledge, transcription profiling of APOE expressing mice following TBI using Next Generation Sequencing has not yet been performed. The aim of this study was to determine if there is an interaction between APOE isoform and the response to TBI affecting phenotype and the transcriptome. We performed controlled cortical impact on 3-month-old mice expressing human APOE3 or APOE4 isoform and following the injury, tested for anxiety and learning. Transcriptional profiling of hippocampal and cortical tissue from the injury site was performed using mRNA-sequencing (mRNA-seq). We hypothesized that there is APOE isoform-specific response to injury and APOE4 mice would have worse cognitive outcomes and higher inflammatory gene expression following TBI. We found that APOE genotype, while a significant variable in both behavioral tests, did not modulate the changes in transcriptome seen two weeks post injury. To correlate the transcriptome to the phenotype we used network-based approach and applied Weighted Gene Co-expression Network Analysis (WGCNA). This analysis not only connects the genes within networks and identifies the most connected members of a given pathway, but elucidates the relevance of the networks to the experimental findings. Thus, we identified that TBI significantly affected immune response, with *Trem2* and *Tyrobp* being highly ranked within the interconnected gene network.

2. Methods

2.1. Animals

All animal experiments were approved through the University of Pittsburgh Institutional Animal Care and Use Committee and carried out in accordance with PHS policies on the use of animals in research. We used human APOE4$^{+/+}$ and APOE3$^{+/+}$ targeted replacement mice on a C57BL/6 background (Fitz et al., 2012). Experimental male and female APOE3 or APOE4 mice were kept on a 12 h light-dark cycle with ad libitum access to food and water. Mice at 3 months of age were randomly assigned to either sham or controlled cortical impact (CCI) experimental group and initially were handled for 2 days (5 min per day). Following surgical procedures, mice were allowed to recover for 3 days before starting behavioral testing. All materials were purchased through Thermo Fisher Scientific, unless otherwise noted.

2.2. Controlled cortical impact

CCI model of brain injury was performed according to previous published methods (Brody et al., 2007). Following induction of anesthesia with 5% isoflurane, the mouse was moved to the stereotaxic frame, where the head was secured, core body temperature maintained at 37 °C using a heating pad and anesthesia continued with 1.5% isoflurane. The head was shaven, surgical site sterilized with two separate iodine - alcohol washes, a 50% mixture of bupivacaine and lidocaine applied to the surgical site and ophthalmic ointment applied to the eyes. The scalp was opened with a midline incision exposing the dorsal aspect of the skull and the skull leveled. A 4.5 mm diameter craniotomy was performed over the left parietal cortex using a dental drill. Once the bone flap was removed, mice in the CCI group received a single impact at 1.0 mm depth with a 3.0 mm diameter metal tip onto the cortex (3 m/s, 100 ms dwell time; Impact One, Leica). Sham mice received identical anesthesia and craniotomy, but did not receive impact and are considered negative controls. Following the impact, the surgical site was sutured, triple antibiotic cream applied, Buprenex (0.1 mg/kg; IP) provided for analgesia, and sterile saline administered for rehydration. Mice were allowed to recover on heating pad, until freely mobile, before returning to their home cage.

2.3. Elevated-plus maze

The elevated plus maze (EPM, San Diego Instruments) test was performed 4 days post-injury as described previously (Washington et al., 2012). The maze consists of 4 arms in the shape of a "+". All arms are the same length (30.5 cm) with a central square (10 × 10 cm); 2 arms are open on the sides, and 2 have 16 cm high walls. The entire maze is raised 40 cm off the ground. The elevated plus maze tests anxiety-related behavior by utilizing rodent's fear of open and elevated spaces. Mice are placed into the maze within the center square facing a closed arm and are allowed to explore for 5 min. Percent time spent in each arm was tracked using the ANY-maze software (Stoelting Co.) from a camera positioned over the maze. 50% of body area within an arm was established in ANY-maze for definition of entry.

2.4. Morris water maze

Spatial navigational learning and memory retention were assessed using Morris water maze (MWM) as described previously (Fitz et al., 2010; Lefterov et al., 2010); with testing performed on days 6–12 post-injury. Briefly, in a circular pool of water (diameter 122 cm, height 51 cm, temperature 21 ± 1 °C), we measured the ability of mice to form a spatial relationship between a safe but invisible platform (submerged 1 cm below the water level; 10 cm in diameter) and several visual extra maze cues surrounding the pool of water. On day 6 post-injury, mice received a habituation trial, during which the animals were allowed to explore the pool of water without the platform present. Beginning the next day, they received four daily hidden platform training (acquisition) trials with 5-min inter-trial intervals for five consecutive days (days 7–11 post-injury). The platform remained in the center of one of the four quadrants of the pool (target quadrant). Animals were allowed 60 s to locate the platform and 20 s to remain there. Mice that failed to find the platform were lead to the platform by the experimenter and allowed to rest there for 20 s. Performance was recorded using Any-maze software (Stoelting Co.) during all trials. During the acquisition trials, escape latency (time to reach the platform) was subsequently used to analyze and compare the performance between all groups.