Subacute pathobiological response following chronic repetitive mild traumatic brain injury in an aged preclinical model of amyloid pathogenesis

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Author contributions
JO and FC conceived the project and, FC and JO directed the project. FC and JO planned the experiments in the whole study. JO, and FC were primarily involved in the preparation of the manuscript. FC, JO, CB and BM participated in the establishment of the animal models. JO, CL, PL, MA performed the experiments. JO and FC participated in the analysis of the experimental data. WS and MM are active consultants for our TBI projects including this work. All authors contributed to the manuscript.

ABSTRACT

Repetitive mild traumatic brain injury (r-mTBI) is a high risk factor for Alzheimer's disease (AD). The precise nature of how r-mTBI leads to, or precipitates, AD pathogenesis remains unclear. In this study we explore subacute effects of chronic r-mTBI (12-impacts) administered over 1-month in aged-PS1/APP mice and littermate controls. We investigate specific mechanisms that may elucidate the molecular link between AD and r-mTBI, focusing primarily on amyloid and tau pathology, amyloid processing, glial activation states and associated clearance mechanisms. Herein, we demonstrate r-mTBI in aged PS1/APP mice does not augment, glial activation, amyloid burden, or tau pathology (with exception of pS202+Tau) 1-month after exposure to the last-injury. However, we observed a decrease in brain soluble Aβ42 levels without any appreciable change in peripheral soluble Aβ42 levels--this was accompanied by an increase in brain insoluble to soluble Aβ42 ratio in injured PS1/APP mice compared to sham-injury. A parallel reduction in phagocytic receptor, TREM-2, was also observed. This study demonstrates very subtle subacute effects of r-mTBI on a pre-existing amyloid pathology background, which may be on a continuum towards a slow and worsening neurodegenerative outcome compared to sham-injury, and, therefore, have many implications, especially in the elderly population exposed to TBI.

Key words: mTBI, amyloid pathology, glia, PS1/APP, protein clearance, age-related proteinopathy

INTRODUCTION

Numerous documented reports have demonstrated that exposure to repetitive mild traumatic brain injury (r-mTBI) is considered to be one of the greatest epigenetic risk factors for the development of later-life dementias, such as Alzheimer's disease [AD] (1-8). In sports-related concussions such as American Football, retired professional players exposed to recurrent concussions have been strongly linked to a high risk for late-life cognitive impairment (9). Some of the first clues linking TBI and AD originated from studies that found increased amyloid beta protein levels in up to 30% of patients in all age groups including children, who die acutely following TBI, and in peri-contussional tissue surgically excised from survivors of TBI (10-13). This has also been confirmed in neuroimaging studies, where the amyloid sensitive Pittsburgh B (PiB) compound was shown to increase in the gray matter and striata of TBI survivors exposed to injuries over the last year (14). PiB levels in TBI survivors, occasionally equaled those with MCI, who go on to develop AD (14).

The precise nature of how repetitive mild-TBI leads to or precipitates AD pathogenesis is not currently understood; our knowledge of the specific pathophysiological mechanisms common to TBI and AD is greatly limited. Animal models could be key to deciphering these pathophysiological mechanisms, as they could facilitate insight into the poorly understood role that TBI plays in the onset/progression of AD, and also enable identification of novel intervention therapies that can be translated from bench to bedside.

Several animal models demonstrating AD hallmark (i.e. amyloid pathology) have been used to study repetitive TBI. Some of these studies have reported augmentation of amyloid pathology at acute to sub-acute time points post-TBI (15-18), while some other reports show a regression in age-related amyloid pathology, especially at chronic time points post-injury (19-21). In human cases of repetitive mTBI the role of amyloid pathology also remains controversial since approximately 50% of the confirmed chronic traumatic encephalopathy (CTE) brains that display hallmarks of perivascular tau -- in neurons and astrocytes in the depths of the sulci -- show amyloid pathology (22). In addition, the relationship between amyloid and tau pathology also remains unclear in TBI (7, 22-24).

To date only a few preclinical studies have tried to investigate the molecular link between repetitive mTBI and AD, with some of these studies showing impairment in underlying neuroinflammatory processes, oxidative stress and protein clearance mechanisms (25-27). Most of these studies however, have only focused on low frequency of impacts at the pre-amyloid pathology stage, and only a few have explored time points beyond a few days or weeks post-injury. Also, little is currently known about the effects of prolonged repetitive mTBI on aged animals with pre-existing amyloid and how this may precipitate tau pathology, as this can be vital in understanding the risk for the development of AD in elderly patients exposed to TBI (for example, in incidence of repeated falls).

In this study we explore the sub-acute effects of chronic repetitive mTBI (12 impacts) over a period of one month in an aged mouse model with pre-existing amyloid pathology. We use the PSAPP mouse model [PS1(M146L), APP (K670N, M671L)], a very well characterized model of AD (28-30) that exhibits progressive age-dependent development of amyloid pathology in an accelerated manner compared to other models such as the Tg2576 mice that carry one (Swedish) mutation

alone. We also investigate specific underlying molecular mechanisms that may bridge the gap between repetitive mTBI and increased AD risk, focusing primarily on markers of tau pathogenesis, amyloid processing and catabolism, glial activation and glial mediated scavenger and phagocytic clearance. We demonstrate herein that repetitive mTBI in aged PSAPP mice does not augment amyloid burden, extent of cerebral amyloid angiopathy (CAA), glial activation or levels of tau species (with exception of pS202+ tau), two months after exposure to the first injury. However, we observed a reduction in soluble Abeta 42 levels (without any appreciable changes in soluble plasma Abeta 42) in cortical brain homogenates of injured aged PSAPP mice compared to sham counterparts, and this correlated with a significant increase in insoluble to soluble Abeta 42 ratio. A parallel reduction in phagocytic clearance receptor, TREM-2 was observed with injury in aged PSAPP mice. This study demonstrates very subtle effects of repetitive mTBI on aged mice with pre-existing amyloid pathology that may be on a continuum towards worsening neuropathological outcome.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with Office of Laboratory Animal Welfare and National Institutes of Health guidelines under a protocol approved by the Roskamp Institute Institutional Animal Care and Use Committee (IACUC – R056). The mouse model used transgenic (PSAPP) mice expressing APP KM670/671NL (Swedish), PSEN1 M146L on a C57BL/6 background [generated as previously described – (28)]. Mice were around 16 months of age at the start of this study. Animals were housed in standard cages under a 12-hour light/12-hour dark schedule at ambient temperature controlled between 22°C and 23°C under specific pathogen free conditions. Animals were given food and water ad libitum and maintained under veterinary supervision throughout the study. There was no evidence of disease among the colony. Mice of both sexes were randomly assigned to experimental groups. For the behavioral experiments, groups consisted of Wild type sham [n=6], Wild type TBI [n=9], PSAPP sham [n=5], and PSAPP TBI [n=6]. For histopathology and immunoblotting studies, groups consisted of N=5-6/group, except for immunoblotting studies where sham PSAPP mice consisted of N=3-4 due to statistically significant outliers. All analyses were carried out blind to study group assignment.

Experimental mTBI

The experimental TBI methods were performed as previously described (31, 32). Briefly, mice were anesthetized with 1.5 L per minute of oxygen and 3% isoflurane for 3 minutes. After shaving of the injury site, mice were transferred into a stereotaxic frame (Just For Mice Stereotaxic, Stoelting, Wood Dale, IL) mounted with an electromagnetic controlled impact device (Impact One Stereotaxic Motorized Impactor, Richmond, IL). Heads were positioned and fixed in the device, which prevented lateral movements as the impact was delivered. All mice were placed on a heating pad to maintain their body temperature at 37°C. A 5-mm blunt metal impactor tip attached to the electromagnetic motorized device was zeroed on the scalp and positioned above the midsagittal suture before each impact using the NeuroLab controller. On satisfactory positioning, the tip was retracted and the depth was adjusted to the desired level. The scalp was gently stretched by hand to restrict lateralization of the impact and to prevent the rod from delivering an inadequate trauma

load at an irregular angle. Injury parameters were 5 m per second strike velocity, 1.0 mm strike depth, 200 milliseconds dwell time, and a force of 72N. This sub-lethal impact does not cause direct tissue damage to the injury site, and there is no development of skull fracture or subdural hemorrhage, even after repetitive injuries. Mice in the r-mTBI group received 3 impacts every week (Monday, Wednesday, Friday) for one month (12 impacts), an inter-injury time of 48-72hrs. Repetitive sham control mice received anesthesia's of the same frequency (x12) and duration (~3mins per session) as their r-mTBI counterparts. Animals were grouped as repetitive shams or repetitive injury. After each impact was delivered, the mice were allowed to recover on a heating pad set at 37°C to prevent hypothermia. On becoming ambulatory, mice were returned to their cages and carefully monitored for any abnormalities.

Three chamber test for social novelty recognition test

Neurobehavioral tests were conducted one months post last injury. Social memory was quantified using a rectangular three-chamber test that includes a middle chamber with two doors leading to two separate (left and right) chambers, each containing a steel cage enclosure. After 5 minutes of habituation in the three chamber compartment, each mouse (experimental subject) was placed in the middle chamber and allowed to freely explore for 5 minutes, with the right chamber empty but an unfamiliar congener (Stranger I) held in the steel cage enclosure in the left chamber. To measure social memory (novelty recognition) a new novel stimulus mouse (stranger II) was subsequently placed in the previously empty right chamber and preference of the experimental subject for stranger I or stranger II was determined by measuring the number of entries and time the experimental subject spent in chambers holding Stranger I vs Stranger II.

Brain Tissue preparation and Western Blotting

One month post last injury brain tissue was collected following transcardial perfusion by gravity drip with phosphate buffered saline (PBS). One hemisphere was extracted and parietal cortex region was dissected, flash frozen in liquid nitrogen and kept at -80^{o}C for antibody based/biochemical analyses. The other hemisphere was post-fixed in 4% paraformaldehyde (PFA) for histological/immunohistochemical (IHC) analyses. For western blotting analyses, the dissected parietal cortex from each hemisphere were homogenized in 150µl of MPER buffer (pH 7.4) containing proteinase inhibitor cocktail, using a probe sonicator. Homogenized samples were spun in a centrifuge at 20,000rcf for 30 minutes and tissue supernatants were collected. Supernatant fractions were denatured at 100^{o}C by boiling in Laemmli buffer (Bio-Rad, CA, USA). Samples were then subsequently resolved on 4–20% gradient polyacrylamide criterion gels (BioRad, CA, USA) or 4-12% gradient NuPAGE novex Bis-Tris precast polyacrylamide gels (Life Technologies, Grand Island, NY). After electrotransferring, polyvinylidene difluoride (PVDF) membranes were blocked in 5% milk or normal rabbit serum (for only goat primary antibodies) made in tris buffered saline (TBS), and subsequently immunoprobed for different brain specific primary antibodies overnight (see Supplementary Table T1). After a rigorous washing step, membranes were probed with horseradish peroxidase (HRP) linked secondary antibodies (see Supplementary Table T1). For primary antibodies raised in mouse host species, a 20% superblocking buffer was used in the secondary antibody solution. Immunoblots were analyzed by using a housekeeping gene – GAPDH (or total protein levels) to quantify the amount of proteins electrotransferred, and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad, CA, USA).

ELISA

Preparation of detergent soluble Abeta and resolubilization of insoluble Abeta with Guanidine for PSAPP mice: briefly, brains were homogenized at 4°C in MPER reagent (Thermo Fisher Scientific Inc., IL, USA) containing 1 mM PMSF and 1X protease/phosphatase inhibitors cocktail (Thermo Fisher Scientific Inc., IL, USA) and centrifuged at 15,000 g for 30 min at 4°C. The supernatant containing detergent solubilized Ab was collected and denatured in 5M guanidine isothyocianate in 50mM Tris-HCL pH8 for one hour before dilution with the sample diluent provided in the ELISA kit and assessed for soluble Ab42 levels according to the manufacturer's protocol (Thermo Fisher Scientific Inc., IL, USA). The pellet containing detergent insoluble material was resuspended in 5M guanidine isothyocianate in 50mM Tris-HCL pH8 and left for an hour before dilution with the sample diluent provided in the ELISA kit for assessment of insoluble Ab42 levels. Soluble Abeta 40 levels were measured using ELISA method. Brain homogenates diluted in MPER buffer were centrifuged at 21,000 rcf for 30 minutes, and collected supernatant fractions at 1/5 and 1/20 dilutions for wild type and PSAPP mice were run on the Abeta 40 ELISA kits as instructed by the manufacturer's guide (Wako, Japan). Protein concentrations were measured in the guanidine extracts using the BCA method (Life Technologies, CA, USA) and results of brain Ab40and Ab42levels were calculated in ng/ml per µg protein.

Immunohistochemistry (IHC)/immunofluorescence(IF)

Half brain hemispheres (left side) were embedded in 4% paraformaldehyde for 24-48hrs followed by paraffin embedding. Series of 6µm-thick sagittal sections were cut throughout the extent of the cortex and hippocampus guided by known bregma coordinates using a microtome (2030 Biocut, Reichert/ Leica, Buffalo Grove, IL). Cut sections were mounted onto positively charged glass slides (Fisher, Superfrost Plus, Pittsburgh, PA).

Sections were deparaffinized in histoclear and rehydrated in a decreasing gradient of ethanol before the immunohistochemical procedure. Sections for IHC were rinsed in distilled water and subsequently incubated at room temperature in a solution of endogenous peroxidase blocking solution, containing 3% hydrogen peroxide diluted in PBS for 15 minutes. For primary antibodies requiring antigen retrieval sections were treated by microwave irradiationwith citrate buffer solution (pH 6) for 8 minutes. Following antigen retrieval sections were blocked for 30mins to 1hr in either (i) Dako protein serum free protein block (Dako, Carpinteria, CA), (ii) normal blocking serum to which secondary antibody was raised or (iii) mouse immunoglobulin G blocking reagent mouse on mouse[MOM] Kit (Vectors Laboratories, Burlingame, CA). Sections were stained in batches with primary antibodiesmade up in supersensitive wash buffer or antibody diluent background-reducing agent. Primary antibodies used can be found in Supplementary Table T1. For IF staining cocktail of primary antibodies were prepared in supersensitive buffer. After overnight incubation, sections were rinsed with PBS and transferred to a solution containing the appropriate secondary antibody (Millipore Billerica, MA;Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 30mins to 1hr depending on the specific requirement of the antibody protocol. For antibodies raised in a mouse host, secondary antibodies were diluted in 20% superblock blocking buffer (Pierce, Thermo Fisher Scientific, Rockford, IL). For IF staining, secondary antibodies were raised from the same host and were mixed as a cocktail. After rinsing in water, sections were incubated with avidin-biotin horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) solution for 30mins.

For IHC staining immunoreactivity was visualized with DAB (3,3-diaminobenzidine). Development with the chromogen was timed and applied as a constant across batches to limit technical variability before progressing to quantitative image analysis. The reaction was terminated by rinsing sections in copious amounts of distilled water. Mounted sections were progressed through a graded series of alcohols (dehydrated), cleared in xylene, and coverslipped with permanent mounting medium. The IHC procedure was previously validated for most antibodies used by including negative control sections, whereby the primary mouse monoclonal antibodies were omitted and replaced, with either blocking agent or biotinylated secondary antibodies alone, to eliminate issues surrounding nonspecific immunoreactivity or detection of mouse immunoglobulin reacting with anti-mouse secondary in the tissue sample. Immunoreacted sections were viewed using a motorized Olympus (BX63) upright microscope, and photographs were taken using the high resolution DP72 color digital camera.

Histology

Series of sections were deparaffinized and processed for histological stains. The Bielschowsky silver impregnation method was performed according to the manufacturer's guidelines to reveal amyloid plaques (Hitobiotec, Kingsport, TN). Congo red staining for amyloid plaque was performed using manufacturer's instructions (SIGMA, St. Louis, MO). To detect microhemorrhages in the brain tissue a Prussian blue staining was also performed according to the manufacturer's guidelines (ENG Scientific Inc, Clifton, NJ). Sections were subsequently processed through a descending gradient of alcohol, xylene, and coverslipped with Permount mounting medium. Thioflavine-S staining (SIGMA, St. Louis, MO) to visualize amyloid plaques were performed by incubating sections after IF staining in 0.1% Thioflavine-S solution dissolved in 70% Ethanol.

Image Analysis

Immunoreactivity for microglia and astrocyte cellular markers, APP and 4G8 were measured by quantitative image analysis (optical segmentation). Rigorous staining protocols were applied to ensure consistency of immunostaining and accuracy of image analysis. Analysis was performed blind to experimental conditions using coded slides to avoid bias in evaluation. Multiple regions of interest were analyzed in standardized fashion for each antibody. First, a survey of immunostained tissue sections was performed independently to verify specific immunoreactivity that was subsequently progressed to quantitative image analysis. Briefly, non-overlapping red, green, blue (RGB) images were digitally captured randomly within the defined areas from each section (comprising an average of 3 sections per animal for each marker). A 10-15 microscopic fields (x40 or x60 magnification) were analyzed per region per animal. Immunoreacted profiles that were optically segmented were analyzed using CellSens morphometric image analysis software (Olympus, Center Valley, PA). A semi-automated RGB histogram-based protocol (specified in the image analysis program) was used to determine the optimal segmentation (threshold setting) for immunoreactivity for each antibody. Immunoreactive profiles discriminated in this manner were used to determine the specific immunoreactive % area. Data were separately plotted as the mean percentage area of immunoreactivity per field (denoted ''% area'') ± SEM for each region and grouping. Amyloid plaque density and cerebral amyloid angiopathy (CAA)+ vessels were obtained on the same images of 4G8 captured above. Clearly discernable compact

neuritic plaques and vessels with positive 4G8 staining were counted on serial sections and images captured and expressed as density estimates per area square.

Statistical Analysis

The relationships between mTBI and sham animals for western blotting, IHC and ELISA, were assessed by using either a parametric One-way ANOVA or non-parametric Kruskal-Wallis Test with a predefined criterion of $p<0.05$ to assess group differences. For tau immunoblots a t-test was used for analyses between sham vs injured PSAPP or WT mice. Neurobehavioral data was analyzed using non parametric Man-Whitney U test for comparison between time spent in stranger I vs II chambers. Data were analyzed by the Kolmogorov-Smirnov test to evaluate if data sets fitted the normal Gaussian distribution. All analyses were performed with SPSS 21.0 (IBM Corp., Armonk, NY) and Graph pad prism (La Jolla, CA) statistical software.

RESULTS

Neurobehavioral outcome in aged transgenic AD mice after chronic repetitive mTBI

Aged WT and PSAPP mice performed poorly in the social novelty recognition test used to evaluate social memory (Supplementary Figure S1). Injured aged WT mice also showed an impairment in social memory, however, this was not significantly worse than aged WT or PSAPP sham mice (Supplementary Figure S1). Intriguingly chronic repetitive mTBI did not augment impairment in social memory in aged PSAPP mice, injured animals performed better than sham mice with stranger II spending 30% more time than stranger I chamber (Supplementary Figure S1).

Tau modification in aged transgenic AD mice after chronic repetitive mTBI

We examined total tau (DA9), phospho-tau (RZ3-Thr231, PHF1-S396/404, CP13-s202), tau conformer species (MC1), tau oligomers (TOC1), N terminal phosphatase activation domain of tau (TNT-1) and caspase cleaved tau (tau C3) in the parietal cortex of WT and PSAPP mice by immunoblotting. No injury dependent effect was observed for most tau antibodies in both WT and PSAPP mice compared to sham counterparts (Figure 1A-I). A significant effect was observed for CP13 levels in injured compared to sham PSAPP mice ($P=0.03$) (Figure 1D). Immunohistochemistry of CP13, PHF1 (Figure 2 A-H [see inset A'B' and E'F']), RZ3, and TauC3 (Figure 3 AA'-DD') in PSAPP mice showed a widespread peri-amyloid plaque staining of seemingly dystrophic neurites. Corresponding with the immunoblotting data no notable changes were observed in the immunoreactivity levels of most of these tau antibodies between sham and injured PSAPP mice. Only CP13 immunoreactivity levels appeared to be mildly increased in the cortex and CA3 pyramidal neurons of some injured PSAPP compared to sham animals (Figure 2AC vs 2BD).

Amyloid pathology in aged transgenic AD mice after chronic repetitive mTBI

We examined APP+ white matter pathology, amyloid plaque burden, amyloid plaque and CAA+vessel density, soluble Abeta40 levels and soluble Abeta 42 levels in the brain and plasma, and insoluble Abeta42 in the brain. There was a notable significant increase in APP immunoreactivity in PSAPP compared to WT mice (Figure 4A). However, APP immunoreactivity in the white matter was not statistically different between sham and injured animals in both aged WT and PSAPP mice (Figure 4A; see Figure 5A, B). No change was

observed in APP immunoreactivity in the cortex of sham vs injured aged PSAPP mice (Figure 5 C, D). Both amyloid plaque burden (Figure 4B), amyloid counts (Figure 4C) and CAA+ vessels (Figure 4D) were unchanged in all brain regions examined in sham vs injured aged PSAPP mice. This is supported by micrographs from 4G8 (Figure 6 A-B) and Bielschowsky staining (Figure 6 C-F) showing no overt or significant difference in the representative images presented.

A trend towards increase in soluble Abeta40 levels in the parietal cortex was observed in injured PSAPP mice compared to shams, however this did not reach statistical significance (Figure 4E). No change was observed in soluble Abeta40 levels between sham and injured aged WT mice (Figure 4E). Soluble Abeta 42 was significantly reduced in the parietal cortex of injured compared to sham PSAPP mice, and a trend towards reduction was also present in the hippocampus, however this did not reach statistical significance (Figure 4F). Insoluble levels of Abeta42 were both significantly unchanged in the parietal cortex and hippocampus between sham and injured PSAPP mice (Figure 4G), no change was also observed in soluble Abeta42 levels in the the plasma (Figure 4H). We expressed the levels of soluble Abeta42 to 40 ratio and insoluble to soluble Abeta42 ratio in the brain. Our observations show a marginally significant reduction (75%) in soluble Abeta 42 to 40 ratio in the parietal cortex of injured compared to sham PSAPP mice (Figure 4I). A significant increase in insoluble: soluble Abeta 42 levels was demonstrated in the parietal cortex of injured compared to sham animals (Figure 4J), no statistically significant effect was seen in the hippocampus (Figure 4J).

Astrocyte and microglia cellular markers in aged transgenic AD mice after chronic repetitive mTBI

Astrocyte activation was examined using GFAP immunoreactivity. PSAPP mice showed a significant increase in % area of GFAP immunoreactivity compared to WT mice in the cortex, hippocampus and corpus callosum (Figure 7A; Figure 8 A,B,E,F,I,J vs C,D,G,H,K,L). GFAP+ astrocytes were found around amyloid plaques in the brain parenchyma (Figure 8C inset) and blood vessels (Figure 8K inset) in PSAPP mice. Chronic repetitive mTBI did not alter % area of GFAP immunoreactivity in either PSAPP or WT mice (Figure 7A; Figure 8 A,E,I vs B,F,J and Figure 8 C,G,K vs D,H,L; see DAB staining in supplementary Figure S2). Cellular markers IBA1, CD45, MHCII, CD68, F4/80 were used to examine microglial activation patterns and their population dynamics following chronic repetitive mTBI in aged WT and PSAPP mice. PSAPP mice showed a notable significant increase in all microglia cellular markers compared to WT mice; microglia in PSAPP mice had a larger cell soma, short thick processes and amoeboid-like phenotype surrounding amyloid plaques and also present in the white matter regions of the corpus callosum (Figure 7B-E; Figure 9 A,B,E,F,I,J,M,N vs C,D,G,H,K,L,O,P; Figure 10 A,B,E,F,I,J,M,N vs C,D,G,H,K,L,O,P; Figure 11 A,B,E,F,I,J,M,N vs C,D,G,H,K,L,O,P).

We did not observe any significant differences in any of the microglia markers in either WT or PSAPP mice between sham and injured animals (Figure 7B-F; Figure 9 A,E,I,M vs B,F,J,N and C,G,K,O vs D,H,L,P; Figure 10 A,E,I,M vs B,F,J,N and C,G,K.O vs D,H,L,P; Figure 11 A,E,I,M vs B,F,J,N and C,G,K,O vs D,H,L,P).

Prussian blue staining was conducted after CD45 staining to demonstrate evidence of microhemorrhages or iron in brain tissue after injury. No evidence of Prussian blue reactivity or haemosiderin laden macrophage were observed in the brain tissue examined (data not shown).

Amyloid processing and clearance markers in aged transgenic AD mice after chronic repetitive mTBI

We examined by immunoblotting the expression levels of markers of amyloid processing (BACE1), enzymatic degradation of amyloid (IDE, neprilyisn), scavenger and phagocytic receptors (SRA1, MARCO, LOX-1, TREM-2). BACE1 levels were increased in aged PSAPP mice compared to WT sham mice, however no injury dependent effects were observed between aged WT and PSAPP mice (Figure 12 A,H). IDE and neprilysin levels were not significantly changed between aged PSAPP and WT mice, no injury effects was observed in both aged WT and PSAPP mice (Figure 12 B,C,H). Scavenger receptors SR-A1 and MARCO levels were consistently unchanged in both genotypes and no injury effect was observed both aged WT and PSAPP mice (Figure 12 D,E,H). LOX-1 expression was unchanged between aged PSAPP and WT sham mice, an injury dependent effect was significantly observed in aged WT mice, however, this injury effect was not present in aged PSAPP mice (Figure 12F,H). Phagocytic receptor TREM2 levels were significantly increased by four-fold in aged PSAPP mice compared to WT mice, a significant reduction (50%) in TREM2 levels were observed in injured aged PSAPP mice compared to their sham counterparts, however this effect was not seen in WT mice (Figure 12 G, H).

DISCUSSION

For over the last two decades researchers have investigated the idea that blows to the head could increase the levels of intra-cerebral expression of Abeta (10,11, 33, 34). Neuropathological studies have confirmed rapid and early appearances of diffuse amyloid plaque pathology in young adolescents after injury (11, 13). However, fifteen years ago, Nakagawa and colleagues reported that aged (24month-old) PDAPP mice bearing pre-existing amyloid pathology resulted in the regression of amyloid burden four months post-injury (21). To date, the specific role that TBI plays in altering the course and progression of pre-existing AD remains a debate. Here we attempt to address this question by expanding on the neuropathological observations of Nakagawa et al (21). We use an aged PSAPP mouse model of repetitive mTBI, with pre-existing amyloid pathology. Contrary to the findings by Nakagawa et al. (21), we observed no change in glial activation, amyloid burden, including levels of most tau species (except pS202+ tau), one-month post-injury. But, we show a significant reduction in soluble Abeta 42 levels in parallel with an increase in insoluble to soluble Abeta 42 ratio. This was also accompanied by the downregulation in phagocytic receptor, TREM2.

Amyloid pathology after repetitive mTBI

Biochemical observation from previous studies utilizing 5-7month old amyloid bearing transgenic animal models have demonstrated an increase in insoluble Abeta levels from 7days (35, 36) to 2 months (27) post-injury. This trend towards an increase in insoluble Abeta levels could be accounted for by aggregation of Abeta 42 species, as the soluble forms of Abeta 42 are prone to oligomerization, and are predominantly expressed in amyloid plaques. The reduction we observed in soluble brain Abeta 42 levels in correlation with a lack of persistent change in APP levels or their processing enzyme BACE1, therefore suggests that previously available pools are undergoing oligomerization into insoluble Abeta 42 aggregated material in the brain. This is supported by the higher insoluble to soluble brain Abeta 42 ratio that we observed in parallel with our data showing no appreciable change in soluble plasma Abeta42 levels. Additionally,

reductions in soluble Abeta42 could also explain the trend towards improvement in social memory, this is because soluble Abeta42 is known to be neurotoxic and can impair synaptic plasticity/function and learning and memory processing. In accordance with our biochemistry results, clinical studies have shown a decrease in soluble Abeta levels in the cerebrospinal fluid [CSF] (37) and interstitial fluid (ISF) in correlation with worsening outcome (38), and this has likewise been confirmed in a cortical contusion injury (CCI) model in wild-type mice and three different lines of transgenic AD mice (39). However, it is also worth mentioning that other studies have conversely reported elevations in soluble Abeta levels following TBI. For example, reports of an immediate increase in soluble Abeta42 levels in the CSF (33, 40, 41) and ISF have been shown in patients following severe TBI and diffuse axonal injury (42) respectively. Moreover, in young-adult animal models that develop AD pathology, elevations in brain Abeta levels in ipsilateral impact sites have been reported as rapid as 24hrs, and in some cases 6 weeks post-TBI (17, 43-45). Similar trends in young-adult non-transgenic AD rodents and porcine swine models have also been confirmed, showing a surge in brain Abeta accumulation from few hours to days following injury (15, 45-49), and in one case up to 6 months (45).

There are several possible explanations behind some of these lack of coherent findings. It is possible that in animal models or clinical studies where there is a surge in Abeta levels, widespread increase in production of APP and/or their enzymatic cleavage may be ongoing. Accumulation of APP along the length of axons or axonal bulbs is a common feature of multifocal axonal pathology. Large reservoirs of APP in the injured axons have been suggested as a source of amyloid plaques following cleavage by BACE1 (or secretase enzymes) to Abeta (24). Upregulation of BACE1 has been shown in humans and several animal models of TBI, including those mentioned above which show a surge in Abeta levels after injury (45, 50-52). Conversely, in those studies showing a reduction in Abeta 42 levels, it is possible that the consequences of the experimental TBI may have minimal effects on widespread levels of APP (i.e. focal rather than diffuse), their processing and/or biogenesis of Abeta, and this can result in the oligomerization of available pools of soluble Abeta 42 into insoluble species. Additional factors which may alter Abeta dynamics post injury, include but are not limited to: mutant specific post-injury effects, choice of transgenic mouse model, type of experimental TBI, severity of injuries, chronicity of impacts (single vs repetitive), sensitivity of detection methods and choice of samples (brain region, type of fluid) examined. Thus, it appears that TBI can augment the oligomerization of Abeta 42 species, albeit at a different pace, ultimately resulting in a gradual sink of aggregated material that has the potential to mature overtime into denser, neuritic plaques typical of advanced AD. In our advanced aged PSAPP model, we speculate that the process of oligomerization and augmentation of amyloidosis post-injury compared to age-matched un-injured counterparts occurs at a slower pace compared to younger animals (with pre/peri-onset pathology), considering that these aged mice have lower comparative levels of available soluble Aβ42 levels, and full blown amyloid pathology. We anticipate that examination of more chronic extended time points in our advanced aged model may thus have revealed a continuum towards an increase in amyloid burden.

Enzyme mediated catabolism of amyloid proteins after r-mTBI

Given the changes we observed in soluble and insoluble Abeta protein levels, we sought to examine markers related to the catabolism of amyloid beta protein in our model. Neprilysin is a metallo-endopeptidase and has emerged as a significant degrading enzyme in vivo capable of degrading monomeric and potentially oligomeric forms of Abeta extracellularly (53-55).

Neprilysin knockout mice accumulate Abeta40 and 42 in high levels, and sporadic AD patients show 50% reduction in neprilysin mRNA levels in areas with plaque pathology (56, 57). Intriguingly, a relationship between a polymorphism in neprilysin and acute Abeta plaque pathology following TBI has been described (58), and this has been postulated as one of the mechanisms contributing towards the generation of plaques post-injury. Our analysis failed to show any significant effect on neprilysin levels following injury. Further investigations of an intracellular Aβ catabolic enzyme, insulin-degrading enzyme (IDE) also showed no significant change between sham and injured mice. Thus, it appears that mTBI has minimal long-term effects on these enzymes and their clearance of amyloid -- at least in our animal model. These data, however should be interpreted with caution, because alterations in the activity of enzymes without any appreciable increase in their levels can influence enzyme kinetics and reaction.

Tau specific changes after repetitive mTBI in a transgenic AD model

Increase tau levels in the CSF and plasma has been consistently reported in living patients exposed to single or repetitive blows to the head (59-63). A neurodegenerative tauopathy termed chronic traumatic encephalopathy (CTE), which evolves following repetitive blows to the head, has been confirmed in the brains of professional athletes exposed to recurrent concussions (22, 64-66). In this study, we did not observe any significant changes in most of the tau species that we examined in the brain, except a very mild increase in pS202+ Tau. The involvement of tau following experimental TBI remains to be a controversial issue (27), with some studies showing an increase in intracerebral tau levels after TBI in WT and variety of transgenic mice (35, 36, 68-71), whilst others do not (32, 72-74). Consistent with our studies herein, in a similar TBI model and hit paradigm which investigated tau pathology in triple transgenic mice expressing age-related tau and amyloid pathology, the authors failed to show significant changes in any of the tau species analyzed after TBI at chronic time points even up to one year after injury (74). At first glance, these studies do not seem to support a role for amyloid in seeding tau pathology in the context of TBI. However, it is noteworthy to mention that in our mouse model which expresses murine tau, the biological conditions do not seem to favor a path towards tau pathology in vivo. Moreover, tau seeding and aggregation is a slow process, which may require many months or years before it is evident. Nonetheless, these data are consistent with some of our studies in young wild-type mice which have murine tau background, and do not show any TBI dependent tauopathy at 24hrs, 6, 12 and 24 months' post exposure (31, 32). Our previous studies seem to suggest that chronicity of impacts and presence of human tau isoforms along with underlying age-related tau pathology may be the necessary factors required to generate TBI dependent tau pathology in vivo (71, 75). It is worth mentioning that the impact of the slight increase in pS202+ tau species is unknown, and further time points will be needed to fully explore whether this change may be on a continuum towards worsening and persistent tau pathogenesis.

Glial cell function following TBI in a transgenic AD model

Glial activation has been reported as a common and persistent feature following mTBI in both humans (24) and a variety of animal models (27, 32, 74, 75). In this study, we hypothesized that repetitive mTBI in aged PSAPP mice with pre-existing amyloid pathology and inflammation would augment glial activation states and increase amyloid pathology. We had previously shown a dramatic increase in CD45 immunoreactivity three-weeks post-injury in aged 18month old hTau mice (71). However, contrary to our initial hypothesis, repetitive mTBI in both aged WT

and PSAPP mice showed no significant changes in any of the astrocyte (GFAP) and microglia (CD68, MHCII, CD45, IBA1, F4/80) activation markers analyzed. The presence or absence of amyloid pathology did not influence the direction of glial reactivity. These findings are intriguing considering that glial cells in aged brains are thought to be in a primed state, where they gradually lose their threshold of tolerance and can become vulnerable to subsequent stimuli (76, 77). It appears therefore that the aged glial cells may be more resilient to mTBI than previously anticipated. It is also worthy of note that changes at acute time points (hrs to days) were not examined in this model, and our knowledge about the possibility of an early transient and robust glial cell response to injury is limited.

Glial cells express scavenger and phagocytic receptors involved in clearance of cellular debris and protein aggregates. These receptors have been implicated in some neurodegenerative diseases (78), however their role following repetitive TBI remains elusive. In our analyses, we observed no significant change in scavenger receptors SRA1, CD68 or MARCO in both WT or PSAPP mice with injury. Notably we observed an increase in scavenger receptor, LOX-1 in WT mice following TBI, but this change was not observed in PSAPP mice. Repetitive mTBI significantly reduced levels of phagocytic receptor TREM2 in PSAPP mice. This study is one of the first to report changes in both LOX-1 and TREM2 after repetitive mTBI. LOX-1 is a class E scavenger receptor expressed on microglia (and also endothelia) in the brain. It appears that upregulation in their levels may be a normal response to TBI as seen in WT mice. The lack of change in LOX-1 levels seen in PSAPP mice could indicate an impairment in LOX-1 receptor mediated clearance mechanisms, and suggests that reparative mechanisms in microglia (and endothelia) pre-exposed to amyloid are not adequately employed after injury. TREM2 is a phagocytic receptor thought to be expressed on microglial cells, although some have confirmed a neuronal origin (79). They have been suggested to act as off signals for aberrant microglial activation by stimulating the M2 pathways and regulating pro-inflammatory genes (80). Genetic studies have demonstrated mutations in TREM2 associated with a higher risk factor for AD (81-82). An increase in TREM2 is a typical feature of AD (83-83), and is thought to signal an overwhelmed TREM2 dependent phagocytic machinery. This is corroborated in our study showing an increase in TREM2 in PSAPP compared to WT mice. The down regulation in their levels with TBI suggests that there is a dysfunction in the transcriptional regulation of these receptors to injury. The long-term consequence of a downregulation in this receptor could be detrimental for the clearance of amyloid protein. Notably, studies looking at TREM2 deficient mice in TBI and AD have demonstrated an increase in macrophage activation (85) and amyloid burden (86) respectively. A recent clinical study also reported concentrations of 3.50±1.91ng/mL and 4.50±1.74ng/mL for CSF soluble TREM2 levels (sTREM2) in 68 former NFL players with a minimum of 12 years in organized football and 21 aged-matched controls with no history of TBI or participation in contact sports (87). Although this data was not statistically significant between both groups, a simultaneous equation regression model did reveal that CSF sTREM2 levels in former NFL players significantly impacted on the relationship between cumulative head impact index and total-tau CSF levels (87), thus implicating the role of microglial activation (sTREM2 levels) in the pathogenesis of long-term neurological consequences associated with repetitive head impacts.

Conclusions

In summary, we report that exposure to repetitive mTBI in aged PSAPP mice, does not augment amyloid burden, cerebral amyloid angiopathy, glial activation and the majority of tau

species examined one month following last injury. We however, did observe a significant increase in insoluble to soluble Abeta42 ratio accompanied by a reduction in phagocytic clearance receptor, TREM-2 in injured PSAPP mice, suggesting a slow but progressive ongoing neurodegeneration. Our findings have many implications especially in the elderly population, given the high incidence of falls and their increased risk of developing age-related neurodegenerative diseases. Further detailed characterizations with longitudinal chronic time points, biochemical studies of mechanism implicated in amyloid beta biogenesis, and comparison with a younger cohort of mice will be needed to confirm the role that TBI has to play in precipitating pathological ageing, and whether amyloid pathology is a permanent, progressive or reversible component. This repetitive mTBI model will help lay a platform to explore these questions and help identify targets to ameliorate pathological consequences of TBI.

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LEGENDS

Figure 1: Tau protein changes in parietal cortex of aged PSAPP and WT mice one month after repetitive mTBI

We analyzed 8 different tau specific antibodies (normalized to GAPDH): total tau (A: DA9), phospho-tau (B: RZ3-Thr231, C: PHF1-S396/404, D: CP13-s202), tau conformer species (MC1), tau oligomers (TOC1), N terminal phosphatase activation domain of tau (TNT-1) and caspase cleaved tau (tau C3) in the parietal cortex (located beneath the impact site). We did not observe any statistically significant changes in 7 of the different tau species analyzed in the injured PSAPP or WT animals compared to their sham counterparts (A-H). A significant effect (P=0.03) was noticeable in CP13 levels between sham and injured PSAPP animals (D). Immunoblots are presented in I. Data were analyzed using a t-test. Graphs are presented as a percentage of respective shams as they were conducted on separate immunoblotting membranes. Abbreviation: WT – wild-type; rmTBI – repetitive mild traumatic brain injury. Asterisk denotes *P<0.05.

Figure 2: Phospho-tau (CP13, PHF1) staining in PSAPP mice one month after repetitive mTBI

Micrographs depict CP13-s202 (A-D) and PHF1-s396/404 (E-H) immunoreactivity in PSAPP mice from the cortex and hippocampus. Both CP13 and PHF1 immunoreactivity were observed in PSAPP mice in dystrophic neurites, primarily localized to the surrounding vicinity of amyloid plaques (see punctate staining in A'B' and E'F'). There appeared to be a slightly significant increase in positively stained dystrophic neurites in injured vs sham mice with CP13 staining in the cortex (AA'-BB'). There was a general paucity in CP13 and PHF1 immunoreactivity in neuronal cell bodies or dendrites. Some injured animals showed modest CP13 immunoreactivity levels in pyramidal neurons of the CA3 hippocampus (C, D). And there appeared to be a modest increase in injured vs sham mice in the hippocampus with CP13 staining (C vs D). No notable differences were observed with PHF1 immunoreactivity in the cortex or hippocampus of sham vs injured PSAPP mice (EG vs FH).

Figure 3: TauC3 and RZ3 staining in aged PSAPP mice one month after repetitive mTBI

Micrographs depict RZ3-Thr231 (A,B) and cleaved caspase Tau C3-Asp421 (C,D) immunoreactivity in PSAPP mice.

+ RZ3 immunoreactivity was observed in the grey matter of PSAPP mice in dystrophic neurites, primarily localized to the surrounding peri-plaque region (see punctate staining in AA' and BB'). Atypical distribution of Tau C3 immunoreacitivity was found in the hippocampus of all PSAPP mice in discrete regions as peri-plaque dystrophic neurites (CC',DD'). No notable differences in both RZ3 and Tau C3 immunoreactivity were observed in the grey matter of sham vs injured PSAPP mice (A,C vs B,D).

Figure 4: Amyloid pathology in the brain of aged PSAPP mice one month after repetitive mTBI

We examined APP immunoreactivity in the corpus callosum (A), amyloid plaque burden (B), and density (C), CAA+ vessels (D), soluble Abeta 40 (E), and soluble Abeta 42 (F), insoluble Abeta 42 (G), and plasma Abeta 42 levels (H), soluble Abeta42 to Abeta 40 ratio (I), insoluble to soluble Abeta 42 levels (J). Transgenic PSAPP mice showed a significantly higher level of APP immunoreactivity in the corpus callosum compared to wild-type mice, however there were no injury specific effects on APP immunoreactivity in either wild-type or PSAPP mice (A). Amyloid plaque burden (B) and counts (C) were not significantly different between injured and sham PSAPP mice in any of the regions analyzed. CAA+ vessel density was also unchanged between sham and injured PSAPP mice. We did observe a 2.9fold increase in soluble Abeta 40 levels in the parietal cortical homogenates in injured PSAPP (1.03±0.38pmol/L) compared to sham animals (0.549±0.15pmol/L), however this did not reach statistical significance; no changes were found in WT sham (0.258±0.012pmol/L) vs WT injury (0.238±0.03pmol/L) mice (E). There was a significant decrease in soluble Abeta 42 levels in the parietal cortex of injured compared to sham mice (F), a 54% reduction was also observed in the hippocampus, however this did not reach statistical significance (F). Insoluble Abeta 42 levels remained unchanged in the hippocampus of PSAPP sham and injured mice (G), a 1.6fold increase was observed in the parietal cortex, however this did not reach statistical significance (G). Plasma Abeta 42 levels were unchanged between both sham and injured PSAPP mice (H). Ratio of soluble Abeta 42 to 40 and insoluble to soluble Abeta 42 in PSAPP mice are expressed in I and J respectively. A 75% reduction in soluble Abeta 42 to 40 ratio was observed in the parietal cortex of injured compared to sham animals (I). A significant 64% reduction was observed in the ratio of insoluble to soluble Abeta 42 in the parietal cortex (J), no significant change was observed in the hippocampus (J). Figure E is presented as a percentage of respective sham animals as data from WT and PSAPP mice were obtained from separate ELISA quantitation. Data were analyzed using either non parametric Kruskal-Wallis test (A) or Mann-Whitney U test (B-J). Data represent mean±sem. Asterisk denotes *P<0.05. Abbreviation: APP – amyloid precursor protein; CAA – cerebral amyloid angiopathy; CC – corpus callosum; P-CTX – parietal cortex; F-CTX – frontal cortex; HP or Hippo – hippocampus; THL – thalamus; WT – wild-type; rmTBI – repetitive mild traumatic brain injury.

Figure 5: APP staining in PSAPP mice one month after repetitive mTBI

Micrographs depict APP immunoreactivity in PSAPP mice. The cortex (A,B), and white matter (C,D) showed widespread APP+ amyloid plaque pathology in both sham and TBI mice. No notable differences were observed in the cortex of sham and injured PSAPP mice (A vs B). In the injured animals, amyloid plaques were lightly stained with APP in the corpus callosum, and appeared to be smaller in size compared to the sham mice (C,D).

Figure 6: Amyloid pathology in aged PSAPP mice one month after repetitive mTBI
Low power micrographs (A, B) depict 4G8+ amyloid plaque pathology in PSAPP mice. The Cortex and hippocampus and corpus callosum showed widespread compact amyloid plaque pathology in both sham and TBI mice (A, B). No difference in 4G8 immunoreactivity was noted between sham and injured PSAPP mice. Higher power micrographs (C-F) depict Bielschowsky silver staining for amyloid plaque pathology in PSAPP mice. The hippocampus (C,D) and cortex (E,F) showed widespread amyloid plaque pathology in both sham and TBI mice. No overt differences were observed in the silver staining between sham and injured PSAPP mice. Abbreviation: CTX – cortex; CC – corpus callosum; Hippo – hippocampus.

Figure 7: Effect of repetitive mTBI in aged WT and PSAPP mice on astroglial and microglial cellular markers
We analyzed astroglial (GFAP) and microglial (IBA1, CD45, MHCII, CD68, F4/80) cellular markers in different brain regions one month after repetitive mTBI in aged WT and PSAPP mice. There was a significant increase in the expression of these markers in all brain regions of sham PSAPP mice compared to sham WT mice (A-E). No significant effect was observed in the % area of GFAP (A), IBA1 (B), CD45 (C), MHCII (D), and CD68 (E) immunoreactivity, in either wild type or PSAPP injured mice compared to their counterparts. F4/80 immunoreacitivity was mainly detected in PSAPP mice, however also no effect was observed between sham and injured PSAPP mice (F). Data were analyzed using non parametric Kruskal-Wallis test (A-E) or Mann-Whitney U test (F). Data represent mean±sem. Mean sample size is n=5. Asterisk denotes ***P<0.001. Abbreviation: ir – immunoreactivity; APP – amyloid precursor protein; CC – corpus callosum; CTX – cortex; HP – hippocampus; THL – thalamus; WT – wild-type; rmTBI – repetitive mildtraumatic brain injury.

Figure 8: GFAP immunoreactivity in aged WT and PSAPP mice one month after repetitive mTBI
Micrographs in A-D were taken from the cortex, E-H from the corpus callosum, and I-L from the CA3 hippocampus region. Astrocytes in the PSAPP mice (C-D; G-H; K-L) showed a higher immunoreactivity for GFAP compared to wild-type mice (A-B, E-F, I-J) in all brain regions examined. Astrocytes from PSAPP mice had thicker processes, larger cell soma, and were seen to surround the vicinity of DAPI/thioflavine S+ amyloid positive plaques (C inset; G, H) and cerebral amyloid angiopathy (K inset). Cortex of wild-type mice was almost devoid of GFAP+ astrocytes (A,B). No injury dependent effects were observed in both WT and PSAPP genotypes, GFAP immunoreactivity and astrocyte phenotypes appeared similar to their sham counterparts (A,E,I vs B,F,J and C,G,K vs D,H,L). Abbreviation: Thio-s – thioflavine S; BV – blood vessel.

Figure 9: IBA1 and CD45 immunoreactivity in aged WT and PSAPP mice one month after repetitive mTBI
Micrographs in A-D were taken from the corpus callosum, and E-H from the cortex and stained with IBA antibody. IBA1+ microglia in the PSAPP mice showed a higher immunoreactivity for IBA-1 compared to wild-type mice (A,B vs C,D and E,F vs G,H) in all brain regions examined. IBA1+ microglia from PSAPP mice had shorter and thicker processes, larger cell soma, and amoeboid-like microglia were seen to surround the vicinity of amyloid positive plaques (G, H). No injury dependent effects were observed in both WT and PSAPP genotypes; IBA1+ immunoreactivity and microglia phenotypes appeared similar to their sham counterparts (A,E vs

B,F and C,G vs D,H). Micrographs in I-L were taken from the corpus callosum and M-O from the cortex and stained with CD45 antibody. CD45+ microglia in PSAPP mice showed a dramatic increase in CD45 immunoreactivity compared to wild-type mice (I,J vs K,L and M,N vs O,P) in all brain regions examined. CD45+ microglia from PSAPP mice had a prominent CD45+ cell soma with absent or shorter and thicker cell processes (K,L). Amoeboid-like microglia were seen around the vicinity of amyloid positive plaques in PSAPP mice (K,L,O,P). Cortex and corpus callosum of WT mice showed very little or no CD45+ immunoreactivity. No injury dependent effects were observed in both WT and PSAPP genotypes; CD45+ immunoreactivity and microglia phenotypes appeared similar to their sham counterparts (I,M vs J,N and K,O vs L,P).

Figure 10: MHCII immunoreactivity in aged WT and PSAPP mice one month after repetitive mTBI

Micrographs in A-H were taken from the cortex and I-P from the corpus callosum. Microglia in the PSAPP mice showed a dramatic rise in MHCII immunoreactivity compared to wild-type mice (A,B,E,F vs C,D,G,H and I,J,M,N vs K,L,O,P) in all brain regions examined. PSAPP mice had a very high density of activated microglia, located around amyloid plaques, and exhibiting very prominent MHCII+ cell soma with shorter and thicker cell processes (see higher power micrographs in G and H). There were numerous amoeboid-like microglia stained with MHCII in PSAPP mice. Some blood vessels (BV) appeared to be stained with MHCII in PSAPP mice (C). Cortex and corpus callosum of WT mice showed very little or no MHCII+ immunoreactivity (A,B,E,F and I,J,M,N). No injury dependent effects were observed in both WT and PSAPP genotypes; MHCII+ immunoreactivity and microglia phenotypes appeared similar to their sham counterparts (A,E,I,M vs B,F,F,J,N and C,G,K,O vs D,H,L,P).

Figure 11: CD68 immunoreactivity in aged WT and PSAPP mice one month after repetitive mTBI

Micrographs in A-H were taken from the cortex and I-P from the corpus callosum. Microglia in the PSAPP mice showed a dramatic increase in CD68 immunoreactivity compared to wild-type mice (A,B,E,F vs C,D,G,H and I,J,M,N vs K,L,O,P) in all brain regions examined. Microglia from PSAPP mice had a very prominent CD68+ cell soma with shorter and thicker cell processes in the cortex (see higher power micrographs in G and H). Amoeboid-like microglia were mainly seen in the corpus callosum in PSAPP mice (K-P). Blood vessels were also stained in PSAPP mice with CD68 (C). Cortex and corpus callosum of WT mice showed a paucity in CD68+ immunoreactivity (A,B,E,F,I,J,M,N). No injury dependent effects were observed in both WT and PSAPP genotypes; CD68+ immunoreactivity and microglia phenotypes appeared similar to their sham counterparts (A,E,I,M vs B,F,J,N and C,G,K,O vs D,H,L,P).

Figure 12: Effects of repetitive mTBI in aged WT and PSAPP mice on specific amyloid processing and clearance markers

We examined BACE1 for amyloid processing (A), insulin degrading enzyme - IDE and neprilysin for enzymatic degradation of amyloid protein (B, C), scavenger receptor type I - SRA1 (D), macrophage receptor with collagenous structure – MARCO (E) and lectin-like oxidized low density lipoprotein receptor – LOX1 (F) for scavenger receptors, and triggering receptor expressed on myeloid cells 2 - TREM-2 (G) for phagocytic clearance mechanisms. A significant increase was observed in BACE 1 expression in sham PSAPP compared to sham WT mice, however no injury effect was observed in either WT or PSAPP mice (A). No

significant effect was observed in IDE, neprilysin, SRA1 and MARCO in aged PSAPP sham compared to WT sham mice, and there was no effect of injury in both aged WT and PSAPP mice compared to sham counterpart (B-E). A significant increase in LOX1 was shown in injured WT mice compared to shams, however, no effect was observed between sham and injured PSAPP mice, moreover we did not observe any change between both genotypes (F). There was a significant increase in TREM-2 levels in PSAPP compared to WT sham mice (G). No significant increase was observed in WT sham vs injured animals, however there was a significant reduction in injured PSAPP mice compared to sham mice (G). Representative immunoblots are in Figure (H). Data were analyzed using non parametric Kruskal-Wallis test. Data represent mean±sem of each marker normalized to housekeeping marker. Asterisk denotes *P<0.05; ***P<0.001; ****P<0.0001. Abbreviation: WT – wild-type; rmTBI – repetitive mild traumatic brain injury.

Supplemental Table T1: List of antibodies used in this study

Supplementary Figure S1: Social Memory in aged PSAPP and WT mice one month after repetitive mTBI

Aged wild-type and PSAPP mice were not able to distinguish between stranger I and stranger II mice in the social novelty recognition (SNR) test. Injured aged wild-type mice also showed a similar impairment in the SNR test, and did not perform significantly worse than their wild-type sham counterparts. Injured PSAPP mice spent almost twice as much time in stranger II chamber compared to stranger I. However, this trend was not significant after statistical analysis. Data were analyzed using non-parametric Mann Whitney U test (WT sham N=6; WT TBI N=9; PSAPP sham N=5; PSAPP TBI N=6). One outlier was removed from the PSAPP sham group. Abbreviation: WT – wild-type; TBI – Traumatic brain injury.

Supplemental Figure S2: GFAP immunoreactivity in aged WT and PSAPP mice one month after repetitive mTBI

Micrographs in A-D were taken from the corpus callosum, E-H from the cortex, and I-L from the CA3 hippocampus region. Astrocytes in the PSAPP mice (C-D; G-H; K-L) showed a higher immunoreactivity for GFAP compared to wild-type mice (A-B, E-F, I-J) in all brain regions examined. Astrocytes from PSAPP mice had thicker processes, larger cell soma, and were seen to surround the vicinity of amyloid positive plaques. Astrocytes from wild type mice in the corpus callosum (A, B) appeared activated with high cell density, however these features were less prominent when compared to PSAPP mice (C,D). Cortex of wild-type mice was almost devoid of GFAP+ astrocytes (E,F). No injury dependent effects were observed in both WT and PSAPP genotypes, GFAP immunoreactivity and astrocyte phenotypes appeared similar to their sham counterparts (A,E,I vs B,F,J and C,G,K vs D,H,L).

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