Experimental Traumatic Brain Injury Induces Rapid Aggregation and Oligomerization of Amyloid-Beta in an Alzheimer's Disease Mouse Model

Patricia M. Washington,¹ Nicholas Morffy,¹ Maia Parsadanian,¹ David N. Zapple,² and Mark P. Burns¹

Abstract

Soluble amyloid-beta ($A\beta$) oligomers are hypothesized to be the pathogenic species in Alzheimer's disease (AD), and increased levels of oligomers in the brain subsequent to traumatic brain injury (TBI) may exacerbate secondary injury pathways and underlie increased risk of developing AD in later life. To determine whether TBI causes $A\beta$ aggregation and oligomerization in the brain, we exposed triple transgenic AD model mice to controlled cortical impact injury and measured levels of soluble, insoluble, and oligomeric $A\beta$ by enzyme-linked immunosorbent assay (ELISA) at 1, 3, and 7 days postinjury. TBI rapidly increased levels of both soluble and insoluble $A\beta$ 40 and $A\beta$ 42 in the injured cortex at 1 day postinjury. We confirmed previous findings that identified damaged axons as a major site of $A\beta$ accumulation using both immunohistochemistry and biochemistry. We also report that soluble $A\beta$ oligomers were significantly increased in the injured cortex, as demonstrated by both ELISA and Western blot. Interestingly, the mouse brain is able to rapidly clear trauma-induced $A\beta$, with both soluble and insoluble $A\beta$ species returning to sham levels by 7 days postinjury. In conclusion, we demonstrate that TBI causes acute accumulation and aggregation of $A\beta$ in the brain, including the formation of low- and high-molecular-weight $A\beta$ oligomers. The formation and aggregation of $A\beta$ into toxic species acutely after injury may play a role in secondary injury cascades after trauma and, chronically, may contribute to increased risk of developing AD in later life.

Key words: adult brain injury; axonal injury; beta amyloid

Introduction

TRAUMATIC BRAIN INJURY (TBI) increases the likelihood of developing Alzheimer's disease (AD) in later life.^{1–3} Though the underlying mechanism is not known, TBI does cause accumulation of amyloid-beta ($A\beta$) in axons of the injured brain^{4–6} as well as acute deposition of insoluble $A\beta$ plaques in severe TBI cases.^{7–9} Because accumulation and aggregation of $A\beta$ in the brain is hypothesized to play a major role in AD pathogenesis,^{10,11} induction of these processes after injury may explain increased risk of AD after TBI. Further, formation of toxic $A\beta$ species after injury may contribute to secondary injury cascades and contribute to neuronal cell death post-trauma.

$A\beta$ peptides are generated by sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretases.¹² Monomeric forms of $A\beta$ can aggregate to form oligomers, protofibrils, and, finally, the fibrils that deposit as amyloid plaque.¹⁰ Although $A\beta$ plaques are the hallmark pathology of AD, it is now widely accepted that plaques are not the pathogenic, disease-causing spe-

cies.¹³ Instead, overwhelming evidence suggests that it is soluble $A\beta$ oligomers, the intermediate species formed during aggregation of monomeric soluble $A\beta$ peptides into plaque, that are the pathogenic species causing neurotoxicity and synaptic dysfunction in AD.^{13–15} Indeed, the soluble pool of $A\beta$, containing soluble monomers and oligomers, correlates better with AD symptoms and severity than $A\beta$ plaque levels.^{16,17} Further, soluble $A\beta$ oligomers impair synaptic transmission in mouse models of AD^{18,19} and are toxic to neurons *in vitro*.^{20–22} In addition to the potential long-term implication that aggregation can have on increasing the risk of AD pathogenesis, the formation of toxic soluble oligomers may contribute to secondary injury processes after TBI. Indeed, a recent study detected the presence of a high-molecular-weight (HMW) $A\beta$ oligomer species in human cerebrospinal fluid (CSF) acutely after TBI,²³ and the presence of these oligomers appears to predict patient outcome after injury.²³

Several groups, including ours, have reported increased levels of soluble $A\beta$ in the brain after experimental TBI using either wild-type (WT) or AD transgenic (Tg) mouse models.^{4,24–31} To study

¹Laboratory for Brain Injury and Dementia, Department of Neuroscience, and ²Advanced Research Computing (ARC), Georgetown University Medical Center, Washington, DC.

insoluble A β species and A β plaque formation after injury, the use of Tg mouse models overexpressing human APP is required because human A β is more aggressive at forming fibrils than mouse A β .³² Studies on insoluble A β species in Tg mice have focused primarily on amyloid plaque formation, and the results have been equivocal—with independent reports of experimental TBI increasing,^{4,26,30,33,34} decreasing,^{35,36} or having no effect^{37–39} on insoluble A β levels or amyloid plaque formation. To date, we are not aware of any studies on the formation of A β oligomers after injury in animal models of TBI.

Because many of the animal studies referenced above focused either on a single time point after injury, or on multiple time points after injury separated by long periods, greater temporal resolution may be necessary to determine A β dynamics after TBI. Therefore, to determine whether TBI causes acute A β aggregation and formation of soluble oligomers in the brain, we exposed triple Tg (3 $\times$ Tg) AD model mice to controlled cortical impact (CCI) injury and measured levels of soluble, insoluble, and oligomeric A β in the injured cortex at 1, 3, and 7 days after injury.

Methods

Controlled cortical impact model of experimental traumatic brain injury

All procedures were carried out in accord with protocols approved by the Georgetown University Animal Care and Use Committee (Washington, DC). CCI injury was induced using a Leica Impact One Stereotaxic Impactor device (Leica Microsystems, Richmond, IL), as previously described.⁴⁰ Briefly, mice were anesthetized with isoflurane (induction at 4% and maintenance at 2%) evaporated in oxygen and administered through a nose mask. Anesthesia depth was monitored by assessing respiration rate and pedal withdrawal reflexes, and mice were placed on a custom-made stereotaxic frame with a built-in heating bed to maintain body temperature at 37°C. The head was mounted in the stereotaxic frame, and the surgical site was clipped and cleaned with Nolvasan and ethanol scrubs. A 10-mm mid-line incision was made over the skull, and the skin and fascia were reflected to make a 4-mm craniotomy on the central aspect of the left parietal bone. The 3.5-mm-diameter impactor tip of the injury device was lowered to the surface of the exposed dura until contact was made. The impactor tip was then retracted and lowered 2.0 mm before inducing impact (impact velocity, 5.25 m/s) to cause a moderate injury. After injury, the incision was closed with staples, anesthesia was terminated, and the animal was placed in a heated cage to maintain normal core temperature for 45 min postinjury. Sham injury consisted of exposure to anesthesia, stereotaxic mounting, skin and fascia reflection, and closing of incision with staples.

Animals

AD disease model mice (3 $\times$ Tg) were originally generated by microinjection of human APP(Swe) and human tau(P301L) transgenes into the PS1(M146V) knock-in mouse.⁴¹ The colony at Georgetown University was established in 2004 and has been previously characterized.⁴² This colony has seen substantial drift from the pathology originally described by Oddo and colleagues⁴¹ and does not deposit any amyloid plaque until at least 18 months of age.⁴² We used two cohorts of mice for our studies, both at preplaque ages: Time-course experiments were performed in 10-month-old male mice (sacrificed 1, 3, or 7 days after sham or CCI injury; $n=4$ /time point), and a second cohort of 8-week-old male mice was sacrificed 1 day after sham or CCI injury ($n=3$ /time point). We chose two different time points to determine whether older mice would have a greater affinity toward A β aggregation after TBI.

Tissue homogenization and sequential extraction of proteins

Mice were anesthetized and transcardially perfused with ice-cold 1 $\times$ phosphate-buffered saline (PBS). The extracted brain was rapidly dissected into ipsi- and contralateral hemispheres, and a 5-mm-diameter punch of cortical tissue was taken from the ipsilateral side in TBI samples, centered around the lesion, and from the corresponding area in shams and contralateral cortex. The fimbria and surrounding white matter (WM) tracts were also dissected from the ipsilateral side before collecting cortical samples in the time-course experiment. Samples were snap-frozen and kept at -80°C until homogenization. Proteins were sequentially extracted from samples to obtain separate fractions containing proteins of differing solubility. Samples were homogenized in 10 volumes of ice-cold tissue homogenization buffer, containing 250 mM of sucrose, 20 mM of Tris base, 1 mM of ethylenediaminetetraacetic acid (EDTA), and 1 mM of ethylene glycol tetraacetic acid (pH 7.4), using a ground glass pestle in a Dounce homogenizer. After six strokes, the homogenate was mixed 1:1 with 0.4% diethylamine (DEA) in a 100-mM NaCl solution before an additional six strokes and then centrifuged at 135,000g at 4°C for 45 min. The supernatant (DEA-soluble fraction containing extracellular and cytosolic proteins) was collected and neutralized with 10% of 0.5 M of Tris-HCl (pH 6.8). The pellet was resuspended in 1 $\times$ radioimmunoprecipitation assay (RIPA) buffer, containing 50 mM of Tris-HCl, 150 mM of NaCl, 0.25% deoxycholic acid, 1% nonyl phenoxy-polyethoxyethanol, and 1 mM of EDTA (pH 7.4), by sonication for 10 sec and spun at 135,000g at 4°C for 45 min. After collecting the supernatant (RIPA-soluble fraction containing membrane-bound proteins), a third sequential extraction in formic acid was performed to obtain insoluble proteins. The RIPA-insoluble pellet was resuspended in one volume of 70% formic acid, sonicated, and spun at 14,000 rpm for 15 min at 4°C. The supernatant was collected (formic acid fraction containing insoluble proteins) and neutralized in 20 volumes of neutralization buffer containing 1 M of Tris base and 0.5 M of Na₂PO₄. DEA, RIPA, and formic acid fractions were frozen and kept at -80°C until needed for biochemistry. All buffers were prepared fresh using chemicals from Sigma-Aldrich (St. Louis, MO), and mammalian tissue protease inhibitor cocktail was added before use. All procedures were carried out on ice.

Measurement of amyloid-beta by enzyme-linked immunosorbent assay

Human A β 40 and A β 42 were individually measured in DEA (soluble A β) and formic acid (insoluble A β) fractions using commercially available kits from Invitrogen (Carlsbad, CA) per the manufacturer's instructions. These kits utilize neopeptide-specific antibodies (Abs) to specifically detect either human A β 40 or A β 42 and have no cross-reactivity with full-length APP.^{43,44} All readings were converted to pg per mg protein, except for A β measured in the formic acid fraction, which is expressed as pg per mL.

Oligomeric A β was measured using a commercially available kit (IBL International GmbH, Hamburg, Germany). This ELISA uses the same N-terminus-specific anti-A β Ab for both capture and detection of A β (82E1), requiring at least a dimer to be present to generate a signal.⁴⁵ This ELISA cannot differentiate between oligomers of different sizes, and because the result is calculated in molarity standardized on A β dimers, it is not in direct proportion to the weight or number of detected oligomers. The results are expressed as pmol/L.

Western blot analyses of proteins

Protein levels of APP, beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), and APP carboxyl terminal fragment (CTF; α and β) were measured in the RIPA fraction, as previously

described.²⁸ Briefly, protein concentration was first determined by bicinchoninic acid assay (Pierce, Rockford, IL) to prepare samples of equal protein concentration. Samples were prepared in Laemmli buffer containing beta-mercaptoethanol (BME) or lithium dodecylsulfate and reducing agent and then resolved on either 7% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels or 4–12% precast Bis-Tris gels (Invitrogen), transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and blocked for a minimum of 1 h in blocking buffer (5% nonfat dry milk in 1 $\times$ PBS containing 0.05% Tween 20 [PBS-T]) before incubation overnight at 4°C in primary Ab prepared in PBS-T containing 1% nonfat dry milk. The following primary Abs were used: C1/6.1 to detect full-length APP and APP CTFs (monoclonal Ab against the C-terminus of APP, a kind gift from Dr. Paul Mathews, Nathan S. Kline Institute, Orangeburg, NY), BACE1 (AB5940, polyclonal; Millipore, Billerica, MA), and myelin basic protein (SMI-99P, monoclonal; Covance, Princeton, NJ). Membranes were washed five times for 5 min in 1 $\times$ PBS-T and incubated in the appropriate HRP-conjugated secondary Ab (anti-mouse immunoglobulin G [IgG] or anti-rabbit IgG, 1:10,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Membranes were washed again (5 $\times$ 5 min in 1 $\times$ PBS-T) before visualizing protein complexes using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Protein bands were quantified by densitometric analysis of scanned film using QuantityOne Basic software (Bio-Rad). Data are presented as percent of sham.

Immunohistochemical detection of amyloid-beta

Mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) and brains were drop-fixed in 4% PFA overnight before cryoprotection in 30% sucrose. Frozen brains were sliced into 50- μ m floating coronal sections using a microtome. Before immunostaining, sections were incubated in 0.3% H₂O₂ to quench endogenous peroxidases, blocked with 3% goat serum in 0.25% Triton X and 1 $\times$ PBS, and incubated overnight in rabbit anti-A β 42 (1:500, catalog no. 700254; Invitrogen) or rabbit anti-APP (1:1000, catalog no. 51-2700; Invitrogen). After three 5-min washes in 1 $\times$ PBS, sections were incubated in goat anti-rabbit secondary Ab at a 1:1000 dilution made in 0.25% Triton X and 1 $\times$ PBS, followed by adenosine-triphosphate-binding cassette ABC (1:400, catalog no. PK-6100; Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (Sigma-Aldrich) substrate to visualize primary Abs.

Western blot detection of amyloid-beta oligomers

Non-reduced DEA samples (100 μ g of protein/sample) were prepared in Laemmli buffer without the addition of BME and separated by SDS-PAGE on five-well 12% Bis-Tris gels. As a positive control, A β oligomers (100 mM) were prepared *in vitro* from human A β 42 peptide, as described in Stine and colleagues.⁴⁶ Briefly, lyophilized A β 42 peptide (American Peptide Company, Sunnyvale, CA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich) until clear and then air-dried overnight to allow the HFIP to evaporate and a peptide film to form, which was stored at -20°C until use. Peptide films were brought to room temperature before preparing 5 mM of A β stock by resuspension in anhydrous dimethyl sulfoxide (Sigma-Aldrich) at room temperature. This was diluted in ice-cold Opti-MEM® (Invitrogen) for a final concentration of 100 μ M of A β , vortexed for 15 sec, and then kept at 4°C for 24 h. A final concentration of 50 mM of A β oligomer preparation was loaded as the positive control. Western blotting was carried out as described above, with the exception that blots were incubated overnight at 4°C with the monoclonal anti-human A β antibody, 6E10, recognizing amino acid residue 1–16 of A β (1:1000, catalog no. SIG-39320; Covance) and probed

with HRP-conjugated anti-mouse secondary Ab (1:10,000; Jackson ImmunoResearch).

Slot blot detection of amyloid-beta oligomers

For detection of oligomers in their native form by the oligomer-specific Ab, A11 (AHB0052; Invitrogen, and AB9234; Millipore), 5 μ L of DEA brain homogenate was loaded into the Whatman Minifold II System slot blot apparatus (GE Healthcare Life Sciences, Piscataway, NJ) to apply sample directly to nitrocellulose membrane. The membrane was treated as described above, except it was incubated in A11 primary Ab (1:1000) and rabbit secondary Ab (1:10,000).

Statistical analysis

Data obtained from independent measurements are presented as the mean $\pm$ standard error of the mean and were analyzed using either one-way analysis of variance (ANOVA) followed by post-hoc analysis by Dunnett's multiple comparison test, comparing all 1-, 3-, and 7-day time points to sham (for time-course experiments), or by unpaired *t*-test for comparison of sham versus injured (for characterization studies with only one time point). All statistical tests were performed using GraphPad Prism statistical software (version 5.0c; GraphPad Software, Inc., San Diego, CA). Differences were considered significant when *p* < 0.05.

Results

Traumatic brain injury causes increased amyloid precursor protein processing, production of amyloid-beta, and aggregation of amyloid-beta into insoluble species

We have previously reported that TBI causes an increase in APP processing and A β production in C57/Bl6 mice.^{27,28} Here, we repeat those findings in 10-month-old 3 $\times$ Tg AD model mice. APP processing can occur through either an α -secretase- or a β -secretase-mediated pathway, with the latter pathway leading to production of A β . We found that TBI caused a significant increase in both α - and β -mediated processing of APP (Fig. 1A). Levels of the β -secretase enzyme, BACE1, and the APP cleavage product, β -CTF, were increased in the injured cortex at 1 and 3 days after TBI (342% and 196% increase above sham levels, respectively; *p* < 0.05), indicating activated β -secretase-mediated cleavage of APP at both of these time points (Fig. 1A). Levels of α -CTF, generated by α -secretase cleavage of APP in the nonamyloidogenic pathway, were also increased by 86–93% at 1, 3, and 7 days after injury (*p* < 0.05; Fig. 1A).

The increased processing of APP through the β -secretase pathway after TBI causes increased production of A β . We found that injury caused a significant increase in levels of soluble A β 40 (166% increase, compared to sham; *p* < 0.001) and soluble A β 42 (58% increase, compared to sham; *p* < 0.05) in the ipsilateral cortex at 1 day postinjury (Fig. 1B), but both species had returned to sham levels at 3 days postinjury. We also extracted insoluble A β species using formic acid as part of a multi-step extraction procedure. We found that TBI caused a 120% increase in insoluble A β 40 (*p* < 0.001) and a 112% increase in insoluble A β 42 (*p* < 0.01) in the injured cortex, peaking at 1 day postinjury (Fig. 1B). Insoluble A β 40 levels returned to sham levels by 3 days after injury, similar to the time course of soluble A β . However, insoluble A β 42 remained elevated at 3 days postinjury (74% increase over sham levels; *p* < 0.05), which may indicate a slower clearance of this insoluble species.

FIG. 1. TBI increases APP processing and both soluble and insoluble Aβ in the ipsilateral cortex of 10-month-old 3×Tg mice. (A) Quantification of amyloid precursor protein (APP), beta-site APP cleaving enzyme 1 (BACE1), and APP C-terminal fragments (APP-CTF) from Western blots. Representative blots are shown. (B) Levels of soluble and insoluble Aβ40 and Aβ42, quantified by ELISA. Percentage of insoluble Aβ indicates the percent of insoluble Aβ in the entire brain homogenate and was calculated from raw Aβ levels (pg/mL). ANOVA with Dunnett's post-hoc analysis comparing 1, 3, and 7 days to sham. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ ($n = 4$ mice/time point). TBI, traumatic brain injury; Aβ, amyloid-beta; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

We further analyzed our data by performing a pools analysis to determine whether the increased levels of Aβ after TBI is caused by aggregation of soluble Aβ into insoluble Aβ or whether the changes merely reflect increased Aβ levels in all pools (Fig. 1B). Before injury, the majority of Aβ is available in the soluble fraction (79% of total Aβ40 and 84% of total Aβ42), with only the remainder in the insoluble fraction (21% of Aβ40 and 16% of Aβ42). After injury, however, the percentage of Aβ40 and Aβ42 that is in the insoluble pool increases over time as excess soluble Aβ is resolved, so that by 7 days postinjury 33% of the total Aβ40 pool is insoluble ($p < 0.01$). The shift in Aβ42 occurs earlier, with the percentage of Aβ42 in the insoluble pool increasing from 16% in sham mice to 32% at 3 days ($p < 0.01$) and remaining increased at 7 days (33%; $p < 0.01$). These data indicate that although total Aβ levels may return to sham levels by 7 days postinjury, an imbalance remains in

the ratio of soluble to insoluble Aβ species, with insoluble Aβ species making up a greater proportion of total Aβ 1 week postinjury.

Amyloid-beta peptides aggregate in the brain after traumatic brain injury in young triple transgenic mice

To examine whether increased aggregation after TBI in our 10-month-old 3×Tg mice is related to their advanced age, we also measured Aβ after injury in a cohort of 8-week-old 3×Tg mice. Levels of soluble Aβ40 (155% increase, $p < 0.001$; Fig. 2) and Aβ42 (122% increase, $p < 0.05$; Fig. 2) and insoluble Aβ40 and Aβ42 (248 and 153% increase, respectively, $p < 0.05$; Fig. 2) were all significantly increased in the injured cortex at 1 day postinjury (Fig. 2), indicating that acute injury-induced aggregation was not dependent on age. Comparison of ipsi- and contralateral cortices

FIG. 2. TBI increases levels of soluble and insoluble A β 40 and A β 42 in the injured cortex of young triple transgenic (3 $\times$ Tg) mice. Quantification of soluble and insoluble A β 40 and A β 42 from brains of 8-week-old 3 $\times$ Tg mice 1 day postinjury. Ipsi- and contralateral cortices are shown. Unpaired *t*-test for comparison of sham versus injured. **p* < 0.05; ****p* < 0.001 (*n* = 3 mice/group). TBI, traumatic brain injury; A β , amyloid-beta.

found that the TBI-induced increase in A β occurred in the ipsilateral side only.

Amyloid-beta 42 accumulates in damaged axons after injury

Immunohistochemistry (IHC) was performed using a cleavage-site-specific Ab against A β 42 on coronal slices from injured and sham animals. To ensure that the A β 42-specific Ab was not cross-reacting with APP, we performed IHC on adjacent sections of 3-day post-TBI tissue. We found that the APP-stained tissue presented an abundance of immunoreactive swellings in the ipsilateral corpus callosum and injured cortex (Fig. 3A). In contrast, there were only a small number of A β 42-positive accumulations in the corpus callosum (Fig. 3A). These data demonstrate that the site-specific A β 42 Ab is not detecting full-length APP.

At 1 day postinjury, we found increased intracellular accumulation of A β 42 and diffuse accumulations in the ipsilateral cortex near the area of lesion in injured mice (Fig. 3B). However, although diffuse plaque was detected in the ipsilateral cortex of 1 injured mouse, this was a rare occurrence and was observed in only 1 of 7 injured mice at this time point.

We found that the majority of A β 42 staining occurred in WM tracts in areas of traumatic axonal injury, with the most intense staining in the ipsilateral external capsule and in the fimbria of the hippocampus (Fig. 3C). A β -positive swellings were visible along the lengths of damaged axons, with multiple varicosities visible within a single axon. We verified this increase in WM A β by measuring soluble A β 40 levels in dissected WM by ELISA, showing that levels of soluble A β 40 are increased by 144% at 1 day after injury (*p* < 0.01), but return to sham levels by 3 days (Fig. 3D). Myelin basic protein levels were measured in the RIPA fraction of WM and ipsilateral cortical samples from the same animals to verify enrichment of axons in the WM dissection.

Traumatic brain injury increases levels of soluble amyloid-beta oligomers in the brain

Oligomerization of A β monomers is an obligatory, intermediate step in A β aggregation. To determine whether injury increases A β oligomerization in the brain, we first looked for the presence of soluble A β oligomers by Western blot and found multiple bands immunoreactive for the anti-A β Ab, 6E10, in our injured TBI samples that were not present in our sham samples or in the negative control

FIG. 3. A β accumulates in damaged axons after injury. (A) Comparison of anti-APP and neopeptide-specific anti-A β 42 antibodies on contiguous sections from a 3-day post-TBI mouse show large amounts of APP staining, but very little A β 42 staining. Scale bar, 100 μ M. (B) In the injured cortex 1 day postinjury, there was A β 42-positive plaque-like staining, along with intracellular A β accumulation in cells directly adjacent to the lesion. Scale bar, 20 μ M. (C) Intense A β 42 immunoreactivity in damaged axons of the external capsule and fimbria. Scale bar, 20 μ M. (D) Representative Western blots for myelin basic protein (MBP) confirm successful isolation of white matter tracts from cortical tissue from sham and TBI brain. These white matter homogenates were probed for soluble A β 40 by ELISA. One-way ANOVA with Dunnett's post-hoc analysis comparing 1, 3, and 7 days to sham. ** $p < 0.01$. ($n = 4$ mice/time point). A β , amyloid-beta; APP, amyloid precursor protein; TBI, traumatic brain injury; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

lacking primary Ab (Fig. 4A). To confirm this, we measured levels of A β oligomers in the DEA fraction by ELISA and found that levels of soluble A β oligomers were increased in the injured cortex by 53% at 1 day after injury, compared to sham ($p < 0.05$; Fig. 4B). Soluble A β oligomer levels also returned to sham levels by 3 days after injury.

Another technique commonly used for detecting A β oligomers is probing unresolved proteins on a dot or slot blot with the oligomeric-specific Ab, A11. We used A11 purchased from both Invitrogen and Millipore, but found that the slot blot technique could not be used for detection of A β oligomers in our samples because of the presence of endogenous autoAbs produced after TBI. These endogenous autoAbs appear to bind to both anti-mouse and -rabbit secondary Abs, producing immunoreactivity in the TBI samples that can be mistaken for a specific signal. So, although we saw an increase in A11 immunoreactivity in our TBI samples, these blots exactly resembled the negative control, indicating that the presumed A11 immunoreactivity was actually the result of binding of secondary Ab to endogenous TBI-induced Abs.

Discussion

We and others have previously demonstrated that experimental TBI increases levels of soluble A β in the brain using either WT or human APP Tg mice.^{4,24–31} Here, we repeat and expand upon those findings, using 3 $\times$ Tg AD model mice to show that TBI increases levels of both soluble and insoluble A β in the brain and alters the ratio of insoluble to soluble A β , so that even after total levels return to baseline, a greater proportion of A β in the injured brain is insoluble. For the first time, we also report the presence of soluble oligomeric A β species after TBI. Our data demonstrate that the accumulation of soluble A β acutely after TBI can lead to the oligomerization and fibrilization of A β in the mouse brain—however, the mouse brain does appear to be particularly proficient at clearing these abnormal A β accumulations within 7 days of injury.

There is a large amount of evidence indicating soluble oligomers as the pathogenic species of A β in AD.¹³ Recently, application of nanomolar concentrations of soluble A β oligomers to hippocampal slice cultures for 30 min was shown to induce synaptotoxicity and impair hippocampal synaptic transmission.⁴⁷ Given the rapid ability of oligomers to induce these changes, it is possible that increased levels of soluble A β oligomers acutely after TBI could contribute to synaptic breakdown and induction of secondary injury pathways. Interestingly, a recent study of human CSF of TBI

patients found that an HMW soluble A β oligomer is detectable in a subset of patients within 72 h of injury.²³ Further, this group found that patients with higher levels of A β oligomers had poorer outcome 6 months after injury, compared to patients with high levels of just A β 42 monomers in CSF, suggesting a relationship between A β oligomer formation and impairment after injury.

Here, we report the accumulation of A β oligomers after experimental TBI. We identified oligomers using two distinct methods: SDS-PAGE coupled with Western blot analysis and an oligomer-specific ELISA. SDS-PAGE is one of the most common methods used for A β oligomer size determination, and combining this with Western blot detection of A β with 6E10 Ab enhances sensitivity

B Soluble A β Oligomers by ELISA

FIG. 4. TBI increases levels of A β oligomers in the injured cortex of 3 $\times$ Tg mice. (A) Representative Western blot of 100 μ g of nonreduced DEA-soluble protein resolved on 12% gels and probed with the anti-A β antibody, 6E10. Multiple 6E10-positive bands are present in 1-day TBI samples, compared to sham samples. These bands correspond to multiple bands from the positive control, which contains A β oligomers prepared *in vitro*. The negative control consists of 100 μ g of 1-day TBI DEA homogenate probed with secondary antibody alone. (B) Quantification of soluble A β oligomers by ELISA confirms that soluble A β oligomers are increased in the ipsilateral cortex at 1 day post-injury. (C) Representative slot blot showing attempt to detect A β oligomers in unresolved samples by slot blot assay and the oligomer-specific antibody, A11, and the corresponding negative control blot that did not contain A11 antibody. ANOVA with Dunnett's post-hoc analysis comparing 1, 3, and 7 days to sham. * $p < 0.05$ ($n = 4$ mice/time point). A β , amyloid-beta; DEA, diethylamine; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

and resolution. In our blots, we can visualize the presence of numerous low-molecular-weight (LMW) and HMW 6E10-positive bands. The strongest of these appear to be a band below 10 kDa, which corresponds to a mono- and dimeric A β band, and a second band just above 10 kDa that corresponds to A β trimers on our positive control. We can also detect higher molecular weight species at approximately 30 kDa and between 45 and 100 kDa. Because the addition of SDS to samples may accelerate A β aggregation during the SDS-PAGE procedure,⁴⁸ we confirmed the presence of oligomers in our samples using a second method. The A β oligomer-specific ELISA uses the 82E1 N-terminus-specific Ab for both capture and detection and so requires two or more epitopes to be present in the same peptide sequence to generate a positive signal.⁴⁵ Similar to our Western blot data, we found that A β oligomers were significantly increased at 1 day postinjury. However, the brain was able to rapidly resolve the excess oligomers, which returned to sham levels by 3 days post-trauma. Finally, we wanted to use an oligomer-specific Ab, A11, to detect oligomers in brain homogenate after TBI. A11 is an oligomeric confirmation-specific Ab that will detect oligomeric species of several amyloidogenic peptides (including prion peptide, human insulin, α -synuclein, polyglutamine, lysozyme, and islet amyloid polypeptide in addition to A β) once they undergo a conformational change⁴⁹ and was demonstrated to not cross-react with soluble LMW A β or A β fibrils.⁴⁹ We used a slot blot assay that has been commonly employed to detect oligomers in nondenatured samples in conjunction with A11 Ab⁴⁸ and probed the blots with A11 sourced from two different companies. We found that both Abs showed a strong increase in signal in our TBI samples. However, despite the secondary Ab being anti-rabbit, these data were proven to be a false positive when we probed blots with just secondary Ab alone. We include these data here because this technique is commonly used in the AD field; however, as our data demonstrate, care should be taken when using this type of assay to probe TBI samples.

Previous studies of A β aggregation after experimental injury have focused primarily on the formation of amyloid plaque, because the principal objective has been to replicate the acute formation of plaque after TBI reported in humans. Initial studies in non-Tg rodents did not detect plaque formation after injury^{50–56}; however, it should be noted that levels of A β in mouse brain, even after injury, are well below the normal thresholds for detection by IHC. This lack of plaque formation in WT mice may also be the result of the fact that rodent A β has a lower ability to aggregate at low peptide concentrations, compared to human A β .⁵⁷ This problem is circumvented by the use of mice overexpressing human APP. Whereas TBI does not induce acute plaque formation in these mice,³⁴ it may accelerate chronic plaque deposition after a single³³ or repeat TBI.³⁰ Unfortunately, it is difficult to draw definitive conclusions because experimental TBI has also been reported to decrease,^{35,36} or have no effect on,^{37–39} insoluble A β or amyloid plaque formation.

A potential confounding factor in previous animal studies may be the length of time between injury induction and A β analysis, because different single time points ranging from acute to chronic have been explored, depending on the study. Data from human TBI studies indicate that plaques can appear as rapidly as 24 h after injury,⁷ and an increased number of TBI brains have plaques present many years after a single TBI, compared to noninjured controls.⁵⁸ Understanding A β dynamics acutely, chronically, and in the intermediate phase after injury are critical to our understanding of how the occurrence of a single TBI might be leading to the development of AD in later life. In this study, we have limited our investigations to the acute phase after TBI to obtain a more complete picture of A β production, ac-

cumulation, and aggregation in the mouse brain. We chose this time period because plaque development occurs very rapidly in the human brain after TBI,^{7–9} and it is also the time frame in which we have observed peak increases in soluble A β in WT TBI mouse brain.^{27,28} This peak in A β production occurs because of increased β -secretase cleavage of APP, as indicated by an accumulation of BACE1 and β -CTF. Interestingly, this occurs simultaneously with an increase in α -secretase cleavage of APP. This simultaneous trafficking of APP through both the α -secretase and β -secretase cleavage pathways is not normal, because, usually, levels of APP remain constant and the proportion of APP sent through either secretase pathway is inversely related. This can be explained by the accumulation of APP in the injured brain, which would result in both α - and β -mediated processing. In our study, we found no increase in total protein levels of APP at 7 days postinjury; however, we do see a large accumulation of APP in injured axons throughout the entire ipsilateral cortex, hippocampus, and thalamus. We believe this abnormally accumulating APP is acting as the excess substrate required to produce excess α - and β -CTF simultaneously.

Previous studies have identified axons as primary sites of A β accumulation after TBI in both humans^{5,6} and animals.^{4,34,56,59} Colocalization of A β with accumulated APP, BACE1, and presenilin 1^{4,6} within axons indicate axons as a hot spot for A β generation after injury. Here, we provide additional evidence that axons appear to be a major site of A β accumulation after injury using both IHC and ELISA data from isolated WM. Rapid generation of A β after injury causes accumulation of soluble A β 40 and A β 42 peptides, generation of soluble A β oligomers, and formation of insoluble A β species. We did not detect any fibrillar A β deposits in the majority of our mice, but did observe a cloudy A β 42-positive accumulation in just one mouse cortex. There was insufficient tissue to fully determine whether this deposition was thioflavin S positive. One other study investigated levels of insoluble A β in the injured cortex of 3 $\times$ Tg mice 24 h after TBI and found an increase in insoluble A β 40, but not soluble A β 40, at 24 h after injury.³⁴ However, the study did not report any amyloid plaque deposition.

Our data reveal that the mouse brain rapidly resolves excess levels of TBI-induced soluble, oligomeric, and insoluble A β within 3–7 days after injury, suggesting that A β clearance pathways are very active in the mouse brain after TBI. This occurs despite the continued elevation of both BACE1 and β -CTF at 3 days postinjury, indicating increased A β production at 3 days. This robust A β clearance in mouse brain may explain why there has been such difficulty reproducing acute TBI-induced amyloid deposition in rodents.

One factor that could influence A β clearance is apolipoprotein E (APOE) genotype. A retrospective study of human TBI postmortem brains revealed a strong influence of APOE4 genotype on plaque formation after injury.⁶⁰ Whereas only 10% of non-APOE4 brains had amyloid plaques, 35% of heterozygous APOE4 brains and 100% of homozygous APOE4 brains had amyloid deposits after TBI. In animal studies, the presence of the human APOE4 gene in PDAPP mice caused an increase in amyloid burden 6 months after experimental TBI.³³ However, although another study using similar mice did not report a significant effect of APOE4 genotype on A β levels after TBI by ELISA, there was an increase in intracellular A β staining in APOE4 TBI mice, compared to APOE3 TBI mice.²⁶

A potential limitation of our study is that we are investigating acute aggregation of A β after injury, whereas it is chronic accumulation and aggregation of A β that is thought to cause AD. Investigation of chronic time points after injury in 3 $\times$ Tg mice will be needed to determine whether TBI causes pathological and cognitive changes associated with AD in these mice, such as A β deposition,

neurofibrillary tangle formation, synaptic dysfunction or loss, and memory deficits. However, our data report the detection of soluble A β oligomers in an animal model of TBI for the first time, supporting the finding of these oligomers after human TBI. Increased levels of soluble A β oligomers in the brain during the acute phase after injury is relevant outside of their potential role in AD development, because the particular toxicity of this A β species may contribute to secondary injury after TBI.

Acknowledgments

The project described above was supported by R01NS081068 and R03NS067417 (to M.P.B.) from the National Institute of Neurological Disorders and Stroke (NINDS) and T32NS041218 (to P.M.W.; principal investigators, Drs. Jean Wrathall and Kathleen Maguire-Zeiss), the Cosmos Club Foundation's Young Scholars Grant Program (to P.M.W.), and the Georgetown University Medical Center Graduate Student Organization's Student Research Grants Program (to P.M.W.). The authors thank Drs. R. Scott Turner and Hyang-Sook Hoe for providing 3 $\times$ Tg AD model mice and Dr. Paul Mathews (Nathan S. Kline Institute, Orangeburg, NY) for C1/6.1 Ab.

Author Disclosure Statement

No competing financial interests exist.

References

- Mortimer, J.A., van Duijn, C.M., Chandra, V., Fratiglioni, L., Graves, A.B., Heyman, A., Jorm, A.F., Kokmen, E., Kondo, K., Rocca, W.A., Shalat, S.L., Soininen, H., and Hofman, A. (1991). Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group. *Int. J. Epidemiol.* 20, Suppl. 2, S28–S35.
- Plassman, B.L., Havlik, R.J., Steffens, D.C., Helms, M.J., Newman, T.N., Drosdick, D., Phillips, C., Gau, B.A., Welsh-Bohmer, K.A., Burke, J.R., Guralnik, J.M., and Breitner, J.C. (2000). Documented head injury in early adulthood and risk of Alzheimer's disease and other dementias. *Neurology* 55, 1158–1166.
- Schofield, P.W., Tang, M., Marder, K., Bell, K., Dooneief, G., Chun, M., Sano, M., Stern, Y., and Mayeux, R. (1997). Alzheimer's disease after remote head injury: an incidence study. *J. Neurol. Neurosurg. Psychiatry* 62, 119–124.
- Chen, X.H., Siman, R., Iwata, A., Meaney, D.F., Trojanowski, J.Q., and Smith, D.H. (2004). Long-term accumulation of amyloid-beta, beta-secretase, presenilin-1, and caspase-3 in damaged axons following brain trauma. *Am. J. Pathol.* 165, 357–371.
- Smith, D.H., Chen, X.H., Iwata, A., and Graham, D.I. (2003). Amyloid beta accumulation in axons after traumatic brain injury in humans. *J. Neurosurg.* 98, 1072–1077.
- Uryu, K., Chen, X.H., Martinez, D., Browne, K.D., Johnson, V.E., Graham, D.I., Lee, V.M., Trojanowski, J.Q., and Smith, D.H. (2007). Multiple proteins implicated in neurodegenerative diseases accumulate in axons after brain trauma in humans. *Exp. Neurol.* 208, 185–192.
- Ikonomic, M.D., Uryu, K., Abrahamson, E.E., Ciallella, J.R., Trojanowski, J.Q., Lee, V.M., Clark, R.S., Marion, D.W., Wisniewski, S.R., and Dekosky, S.T. (2004). Alzheimer's pathology in human temporal cortex surgically excised after severe brain injury. *Exp. Neurol.* 190, 192–203.
- Roberts, G.W., Gentleman, S.M., Lynch, A., and Graham, D.I. (1991). beta A4 amyloid protein deposition in brain after head trauma. *Lancet* 338, 1422–1423.
- Roberts, G.W., Gentleman, S.M., Lynch, A., Murray, L., Landon, M., and Graham, D.I. (1994). Beta amyloid protein deposition in the brain after severe head injury: implications for the pathogenesis of Alzheimer's disease. *J. Neurol. Neurosurg. Psychiatry* 57, 419–425.
- Hardy, J., and Selkoe, D.J. (2002). The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353–356.
- Hardy, J.A., and Higgins, G.A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* 256, 184–185.
- Selkoe, D.J. (1998). The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell. Biol.* 8, 447–453.
- Walsh, D.M., and Selkoe, D.J. (2007). A beta oligomers—a decade of discovery. *J. Neurochem.* 101, 1172–1184.
- Benilova, I., Karran, E., and De Strooper, B. (2012). The toxic Abeta oligomer and Alzheimer's disease: an emperor in need of clothes. *Nat. Neurosci.* 15, 349–357.
- Selkoe, D.J. (2002). Alzheimer's disease is a synaptic failure. *Science* 298, 789–791.
- McLean, C.A., Cherny, R.A., Fraser, F.W., Fuller, S.J., Smith, M.J., Beyreuther, K., Bush, A.I., and Masters, C.L. (1999). Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann. Neurol.* 46, 860–866.
- Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E., and Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. *Am. J. Pathol.* 155, 853–862.
- Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., and Selkoe, D.J. (2002). Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416, 535–539.
- Shankar, G.M., Li, S., Mehta, T.H., Garcia-Munoz, A., Shepardson, N.E., Smith, I., Brett, F.M., Farrell, M.A., Rowan, M.J., Lemere, C.A., Regan, C.M., Walsh, D.M., Sabatini, B.L., and Selkoe, D.J. (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med.* 14, 837–842.
- Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., and Klein, W.L. (1998). Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc. Natl. Acad. Sci. U. S. A.* 95, 6448–6453.
- Dahlgren, K.N., Manelli, A.M., Stine, W.B., Jr., Baker, L.K., Krafft, G.A., and LaDu, M.J. (2002). Oligomeric and fibrillar species of amyloid-beta peptides differentially affect neuronal viability. *J. Biol. Chem.* 277, 32046–32053.
- Deshpande, A., Mina, E., Glabe, C., and Busciglio, J. (2006). Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. *J. Neurosci.* 26, 6011–6018.
- Gatson, J.W., Warren, V., Abdelfattah, K., Wolf, S., Hynan, L.S., Moore, C., Diaz-Arastia, R., Minei, J.P., Madden, C.S., and Wigginton, J.G. (2013). Detection of beta-amyloid oligomers as a predictor of neurological outcome after brain injury. *J. Neurosurg.* 118, 1336–1342.
- Abrahamson, E.E., Ikonomic, M.D., Ciallella, J.R., Hope, C.E., Paljug, W.R., Isanski, B.A., Flood, D.G., Clark, R.S., and Dekosky, S.T. (2006). Caspase inhibition therapy abolishes brain trauma-induced increases in Abeta peptide: implications for clinical outcome. *Exp. Neurol.* 197, 437–450.
- Abrahamson, E.E., Ikonomic, M.D., Dixon, C.E., and Dekosky, S.T. (2009). Simvastatin therapy prevents brain trauma-induced increases in beta-amyloid peptide levels. *Ann. Neurol.* 66, 407–414.
- Laskowitz, D.T., Song, P., Wang, H., Mace, B., Sullivan, P.M., Vitek, M.P., and Dawson, H.N. (2010). Traumatic brain injury exacerbates neurodegenerative pathology: improvement with an apolipoprotein E-based therapeutic. *J. Neurotrauma* 27, 1983–1995.
- Loane, D.J., Pocivavsek, A., Moussa, C.E., Thompson, R., Matsuoka, Y., Faden, A.I., Rebeck, G.W., and Burns, M.P. (2009). Amyloid precursor protein secretases as therapeutic targets for traumatic brain injury. *Nat. Med.* 15, 377–379.
- Loane, D.J., Washington, P.M., Vardanian, L., Pocivavsek, A., Hoe, H.S., Duff, K.E., Cernak, I., Rebeck, G.W., Faden, A.I., and Burns, M.P. (2011). Modulation of ABCA1 by an LXR agonist reduces beta-amyloid levels and improves outcome after traumatic brain injury. *J. Neurotrauma* 28, 225–236.
- Mannix, R.C., Zhang, J., Park, J., Zhang, X., Bilal, K., Walker, K., Tanzi, R.E., Tesco, G., and Whalen, M.J. (2011). Age-dependent effect of apolipoprotein E4 on functional outcome after controlled cortical impact in mice. *J. Cereb. Blood Flow Metab.* 31, 351–361.
- Uryu, K., Laurer, H., McIntosh, T., Pratico, D., Martinez, D., Leight, S., Lee, V.M., and Trojanowski, J.Q. (2002). Repetitive mild brain trauma accelerates Abeta deposition, lipid peroxidation, and cognitive impairment in a transgenic mouse model of Alzheimer amyloidosis. *J. Neurosci.* 22, 446–454.

31. Wang, H., Durham, L., Dawson, H., Song, P., Warner, D.S., Sullivan, P.M., Vitek, M.P., and Laskowitz, D.T. (2007). An apolipoprotein E-based therapeutic improves outcome and reduces Alzheimer's disease pathology following closed head injury: evidence of pharmacogenomic interaction. *Neuroscience* 144, 1324–1333.
32. Fraser, P.E., Nguyen, J.T., Inouye, H., Surewicz, W.K., Selkoe, D.J., Podlisy, M.B., and Kirschner, D.A. (1992). Fibril formation by primate, rodent, and Dutch-hemorrhagic analogues of Alzheimer amyloid beta-protein. *Biochemistry* 31, 10716–10723.
33. Hartman, R.E., Laurer, H., Longhi, L., Bales, K.R., Paul, S.M., McIntosh, T.K., and Holtzman, D.M. (2002). Apolipoprotein E4 influences amyloid deposition but not cell loss after traumatic brain injury in a mouse model of Alzheimer's disease. *J. Neurosci.* 22, 10083–10087.
34. Tran, H.T., Sanchez, L., Esparza, T.J. and Brody, D.L. (2011). Distinct temporal and anatomical distributions of amyloid-beta and tau abnormalities following controlled cortical impact in transgenic mice. *PLoS One* 6, e25475.
35. Nakagawa, Y., Nakamura, M., McIntosh, T.K., Rodriguez, A., Berlin, J.A., Smith, D.H., Saatman, K.E., Raghupathi, R., Clemens, J., Saido, T.C., Schmidt, M.L., Lee, V.M., and Trojanowski, J.Q. (1999). Traumatic brain injury in young, amyloid-beta peptide overexpressing transgenic mice induces marked ipsilateral hippocampal atrophy and diminished Abeta deposition during aging. *J. Comp. Neurol.* 411, 390–398.
36. Nakagawa, Y., Reed, L., Nakamura, M., McIntosh, T.K., Smith, D.H., Saatman, K.E., Raghupathi, R., Clemens, J., Saido, T.C., Lee, V.M., and Trojanowski, J.Q. (2000). Brain trauma in aged transgenic mice induces regression of established abeta deposits. *Exp. Neurol.* 163, 244–252.
37. Conte, V., Uryu, K., Fujimoto, S., Yao, Y., Rokach, J., Longhi, L., Trojanowski, J.Q., Lee, V.M., McIntosh, T.K., and Pratico, D. (2004). Vitamin E reduces amyloidosis and improves cognitive function in Tg2576 mice following repetitive concussive brain injury. *J. Neurochem.* 90, 758–764.
38. Murai, H., Pierce, J.E., Raghupathi, R., Smith, D.H., Saatman, K.E., Trojanowski, J.Q., Lee, V.M., Loring, J.F., Eckman, C., Yonkin, S., and McIntosh, T.K. (1998). Twofold overexpression of human beta-amyloid precursor proteins in transgenic mice does not affect the neuromotor, cognitive, or neurodegenerative sequelae following experimental brain injury. *J. Comp. Neurol.* 392, 428–438.
39. Smith, D.H., Nakamura, M., McIntosh, T.K., Wang, J., Rodriguez, A., Chen, X.H., Raghupathi, R., Saatman, K.E., Clemens, J., Schmidt, M.L., Lee, V.M., and Trojanowski, J.Q. (1998). Brain trauma induces massive hippocampal neuron death linked to a surge in beta-amyloid levels in mice overexpressing mutant amyloid precursor protein. *Am. J. Pathol.* 153, 1005–1010.
40. Washington, P.M., Forcelli, P.A., Wilkins, T., Zapple, D., Parsadanian, M., and Burns, M.P. (2012). The effect of injury severity on behavior: a phenotypic study of cognitive and emotional deficits after mild, moderate and severe controlled cortical impact injury in mice. *J. Neurotrauma* 29, 2283–2296.
41. Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kaye, R., Metherate, R., Mattson, M.P., Akbari, Y., and LaFerla, F.M. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron* 39, 409–421.
42. Hirata-Fukae, C., Li, H.F., Hoe, H.S., Gray, A.J., Minami, S.S., Hamada, K., Niikura, T., Hua, F., Tsukagoshi-Nagai, H., Horikoshi-Sakuraba, Y., Mughal, M., Rebeck, G.W., LaFerla, F.M., Mattson, M.P., Iwata, N., Saido, T.C., Klein, W.L., Duff, K.E., Aisen, P.S., and Matsuo, Y. (2008). Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model. *Brain Res.* 1216, 92–103.
43. Luo, Y., Bolon, B., Kahn, S., Bennett, B.D., Babu-Khan, S., Denis, P., Fan, W., Kha, H., Zhang, J., Gong, Y., Martin, L., Louis, J.C., Yan, Q., Richards, W.G., Citron, M., and Vassar, R. (2001). Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nat. Neurosci.* 4, 231–232.
44. Savage, M.J., Trusko, S.P., Howland, D.S., Pinsker, L.R., Mistretta, S., Reaume, A.G., Greenberg, B.D., Siman, R., and Scott, R.W. (1998). Turnover of amyloid beta-protein in mouse brain and acute reduction of its level by phorbol ester. *J. Neurosci.* 18, 1743–1752.
45. Xia, W., Yang, T., Shankar, G., Smith, I.M., Shen, Y., Walsh, D.M., and Selkoe, D.J. (2009). A specific enzyme-linked immunosorbent assay for measuring beta-amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease. *Arch. Neurol.* 66, 190–199.
46. Stine, W.B., Jr., Dahlgren, K.N., Krafft, G.A., and LaDu, M.J. (2003). In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 278, 11612–11622.
47. Li, S., Jin, M., Zhang, D., Yang, T., Koeglsperger, T., Fu, H., and Selkoe, D.J. (2013). Environmental novelty activates beta2-adrenergic signaling to prevent the impairment of hippocampal LTP by Abeta oligomers. *Neuron* 77, 929–941.
48. Pryor, N.E., Moss, M.A., and Hestekin, C.N. (2012). Unraveling the early events of amyloid-beta protein (Abeta) aggregation: techniques for the determination of Abeta aggregate size. *Int. J. Mol. Sci.* 13, 3038–3072.
49. Kaye, R., Head, E., Thompson, J.L., McIntire, T.M., Milton, S.C., Cotman, C.W., and Glabe, C.G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300, 486–489.
50. Ciallella, J.R., Ikonovic, M.D., Paljug, W.R., Wilbur, Y.I., Dixon, C.E., Kochanek, P.M., Marion, D.W., and DeKosky, S.T. (2002). Changes in expression of amyloid precursor protein and interleukin-1beta after experimental traumatic brain injury in rats. *J. Neurotrauma* 19, 1555–1567.
51. Hoshino, S., Tamaoka, A., Takahashi, M., Kobayashi, S., Furukawa, T., Oaki, Y., Mori, O., Matsuno, S., Shoji, S., Inomata, M., and Teramoto, A. (1998). Emergence of immunoreactivities for phosphorylated tau and amyloid-beta protein in chronic stage of fluid percussion injury in rat brain. *Neuroreport* 9, 1879–1883.
52. Laurer, H.L., Bareyre, F.M., Lee, V.M., Trojanowski, J.Q., Longhi, L., Hoover, R., Saatman, K.E., Raghupathi, R., Hoshino, S., Grady, M.S., and McIntosh, T.K. (2001). Mild head injury increasing the brain's vulnerability to a second concussive impact. *J. Neurosurg.* 95, 859–870.
53. Lewen, A., Li, G.L., Olsson, Y., and Hillered, L. (1996). Changes in microtubule-associated protein 2 and amyloid precursor protein immunoreactivity following traumatic brain injury in rat: influence of MK-801 treatment. *Brain Res.* 719, 161–171.
54. Masumura, M., Hata, R., Uramoto, H., Murayama, N., Ohno, T., and Sawada, T. (2000). Altered expression of amyloid precursor proteins after traumatic brain injury in rats: in situ hybridization and immunohistochemical study. *J. Neurotrauma* 17, 123–134.
55. Pierce, J.E., Trojanowski, J.Q., Graham, D.I., Smith, D.H., and McIntosh, T.K. (1996). Immunohistochemical characterization of alterations in the distribution of amyloid precursor proteins and beta-amyloid peptide after experimental brain injury in the rat. *J. Neurosci.* 16, 1083–1090.
56. Stone, J.R., Okonkwo, D.O., Singleton, R.H., Mutlu, L.K., Helm, G.A., and Povlishock, J.T. (2002). Caspase-3-mediated cleavage of amyloid precursor protein and formation of amyloid beta peptide in traumatic axonal injury. *J. Neurotrauma* 19, 601–614.
57. Otvos, L., Jr., Szendrei, G.I., Lee, V.M., and Mantsch, H.H. (1993). Human and rodent Alzheimer beta-amyloid peptides acquire distinct conformations in membrane-mimicking solvents. *Eur. J. Biochem.* 211, 249–257.
58. Johnson, V.E., Stewart, W., and Smith, D.H. (2012). Widespread tau and amyloid-beta pathology many years after a single traumatic brain injury in humans. *Brain Pathol.* 22, 142–149.
59. Smith, D.H., Chen, X.H., Nonaka, M., Trojanowski, J.Q., Lee, V.M., Saatman, K.E., Leoni, M.J., Xu, B.N., Wolf, J.A., and Meaney, D.F. (1999). Accumulation of amyloid beta and tau and the formation of neurofilament inclusions following diffuse brain injury in the pig. *J. Neuropathol. Exp. Neurol.* 58, 982–992.
60. Nicoll, J.A., Roberts, G.W., and Graham, D.I. (1995). Apolipoprotein E epsilon 4 allele is associated with deposition of amyloid beta-protein following head injury. *Nat. Med.* 1, 135–137.

Address correspondence to:

Mark P. Burns, PhD

Department of Neuroscience

Georgetown University Medical Center

New Research Building-WP22a

3970 Reservoir Road NW

Washington, DC 20007

E-mail: mpb37@georgetown.edu