Apolipoprotein E Regulates Injury-Induced Activation of Hippocampal Neural Stem and Progenitor Cells

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Abstract

Partial recovery from even severe traumatic brain injury (TBI) is ubiquitous and occurs largely through unknown mechanisms. Recent evidence suggests that hippocampal neural stem/progenitor cell (NSPC) activation and subsequent neurogenesis are responsible for at least some aspects of spontaneous recovery following TBI. Apolipoprotein E (ApoE) regulates postnatal neurogenesis in the hippocampus and is therefore a putative mediator of injury-induced neurogenesis. Further, ApoE isoforms in humans are associated with different cognitive outcomes following TBI. To investigate the role of ApoE in injury-induced neurogenesis, we exposed wild-type, ApoE-deficient, and human ApoE isoform-specific (ApoE3 and ApoE4) transgenic mice crossed with nestin-green fluorescent protein (GFP) reporter mice to controlled cortical impact (CCI) and assessed progenitor activation at 2 d post-injury using unbiased stereology. GFP+ progenitor cells were increased by approximately 120% in the ipsilateral hippocampus in injured wild-type mice, compared with sham mice ($p < 0.01$). Co-localization of GFP+ cells with bromodeoxyuridine (BrdU) to label dividing cells indicated increased proliferation of progenitors in the injured hippocampus ($p < 0.001$). This proliferative injury response was absent in ApoE-deficient mice, as no increase in GFP+ cells was observed in the injured hippocampus, compared with sham mice, despite an overall increase in proliferation indicated by increased BrdU+ cells (86%; $p < 0.05$). CCI-induced proliferation of GFP+ cells in both ApoE3 and ApoE4 mice but the overall response was attenuated in ApoE4 mice due to fewer GFP+ cells at baseline. We demonstrate that ApoE is required for injury-induced proliferation of NSPCs after experimental TBI, and that this response is influenced by human APOE genotype.

Key words: apolipoprotein E; hippocampus; neural stem/progenitor cells; neurogenesis; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a leading cause of morbidity and mortality in the United States.¹ However, some degree of spontaneous recovery occurs after TBI, suggesting activation of endogenous repair mechanisms. One such mechanism that has been recently identified to be important for recovery is injury-induced hippocampal neurogenesis.² After TBI, there is activation of early, nestin-expressing neural stem/progenitors (NSPCs) in the dentate gyrus of the hippocampus, which leads to generation of new neurons.^{3, 4} This neurogenic response has been shown to be necessary for recovery of spatial learning and memory after injury.² The functional significance and development of these newly-formed neurons continues to be a source of investigation. To better understand the mechanisms underlying injury-induced hippocampal neurogenesis, regulators of physiologic adult neurogenesis have been targeted for their putative role.

Normal postnatal neurogenesis occurs throughout adulthood in two distinct neurogenic niches—the subventricular zone (SVZ) of

the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus.^{5–7} In the SGZ, the self-renewing stem/early progenitor cell is the type 1 cell that expresses nestin and glial fibrillary acidic protein (GFAP).^{8,9} Type 1 cells give rise to type 2 cells, neural progenitors, that lose GFAP expression and initially express nestin as a type 2a cell but progressively lose nestin expression and gain doublecortin expression as a type 2b cell.^{6, 8,10} Neurons generated by these adult neural NSPCs are able to incorporate into the granular layer of the hippocampus and function similarly to embryologically-derived hippocampal granular neurons.^{11,12} Gene expression analysis of early, nestin-expressing NSPCs in the dentate gyrus identified several candidate genes for cell-autonomous regulation of development that were significantly upregulated or down-regulated at postnatal Day 28 (P28), compared with P7.¹³ Apolipoprotein E (ApoE) was upregulated more than four-fold at P28, making it a gene of interest for potential regulation of hippocampal neurogenesis.¹³

In the central nervous system, ApoE is produced and secreted primarily by astrocytes but also has been found to be expressed by

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neural progenitor cells during development and by neurons exposed to stress or injury.^{13–17} ApoE is involved in regulation of lipid transport, beta-amyloid clearance, and maintenance of the blood–brain barrier.^{16, 18–21} Recently, ApoE also has been found to play a key role in development. ApoE expression is temporally regulated during postnatal development, with expression in NSPCs emerging around P14 in a minority of type 1 cells and progressively increasing until more than 95% of type 1 cells express ApoE at two months of age.²² ApoE deficiency leads to depletion of the hippocampal neural stem/progenitor pool over time; however, ApoE-deficient NSPCs have a greater proliferative ability *in vivo*, suggesting its role as a growth suppressor that functions to inhibit overproliferation and resultant depletion of the stem/progenitor cell pool.²² ApoE also is necessary to maintain proper neuronal differentiation of neural stem cells in the hippocampus. Its deficiency shifts neural stem cell differentiation towards astrogenesis instead of neurogenesis.¹⁷

In humans, polymorphisms in the ApoE gene result in three common isoforms of the protein—ApoE2, ApoE3, and ApoE4. ApoE3 is considered the “wild-type” (WT) as it is the most prevalent isoform in the population and is not associated with a disease phenotype.²³ Human ApoE isoforms can influence hippocampal neurogenesis, as ApoE4 knock-in mice have been shown to exhibit abnormal neuronal maturation and a subsequent decreased population of mature neurons without an alteration in astrogenesis.¹⁷ ApoE4 also is associated with poorer outcomes after TBI in humans and in a mouse model of brain injury.^{24–26} Given this potential relationship between ApoE, neurogenesis, and recovery after injury, we wanted to investigate the role of ApoE in injury-induced neurogenesis and how this was affected by human ApoE isoforms. To do this, we used WT, ApoE-deficient (ApoE KO), and human ApoE-isoform transgenic mice crossed with nestin-green fluorescent protein (GFP) reporter mice to label progenitor cells and assess NSPC activation after controlled cortical impact (CCI) injury.

Methods

Animals

Experimental animals were humanely housed and cared for under the supervision of the Institute of Comparative Medicine, and all experimental procedures were conducted under the approval of the Institutional Animal Care and Use Committee at Columbia University. WT mice contained a nestin- δ -HSV TK-eGFP transgene, as developed and characterized previously,^{2, 3} which provided GFP labeling of nestin-expressing early neural progenitor cells. ApoE KO and glial fibrillary acidic protein (GFAP)-ApoE3 and GFAP-ApoE4 transgenic mice maintained on a murine ApoE-null background were obtained from Jackson Laboratory (Bar Harbor, ME). GFAP-ApoE mice express human ApoE alleles under the control of the GFAP. ApoE KO mice and GFAP-ApoE mice were crossed with nestin- δ -HSV TK-eGFP mice to generate ApoE KO or GFAP-ApoE heterozygous mice with GFP expression in nestin-expressing progenitor cells.

CCI injury

Experimental injury was induced in eight-week-old (26 males and 21 females) WT, ApoE KO, and GFAP-ApoE transgenic mice as previously described.² Briefly, mice were anesthetized with 4% isoflurane with supplemental 1 L flow of oxygen and placed in a stereotactic frame. Body temperature was maintained at 37°C using a heating pad (Adroit, Loudon, TN). Following a midline incision on the scalp, the soft tissues were reflected and a 5 mm by 5 mm

craniectomy was performed between bregma and lambda, to the left of the sagittal suture, to expose the underlying dura. A cortical contusion injury was delivered using a Leica One Stereotaxic Impactor device (Leica, Houston, TX) to impact the exposed dura with a 3 mm stainless steel impounder tip at a speed of 4.4 m/sec, deformation of 0.7 mm, and dwell time of 0.3 sec. After injury, the scalp was closed with sutures and the animals were removed from anesthesia and allowed to recover.

Fluorescent activated cell sorting (FACs)

Seventy-two h after CCI injury, mice were sacrificed and the hippocampus dissected from both hemispheres of nestin-GFP transgenic animals ($n=8$). Individual hippocampi were coronally sectioned into 600 μ m slices and the dentate gyrus extracted from all slices. The digestion media consisted of activated papain: 42 μ L of papain (Worthington Biochemical Corporation, Lakewood, NJ), 27 μ L of 100 mM Cystein-HCl and 6 μ L of 100 mM EDTA in 425 μ L of DMEM/F12. The single-cell suspension was sorted using a MoFlo cell sorter (Dako, Carpinteria, CA) with fluorescent activated cell sorting analysis. RNA was extracted from sorted cells with RNeasy Kit (Qiagen, Germantown, MD) according to the manufacturer's specifications. Total RNA was synthesized into complementary DNA (cDNA) with reverse transcriptase using iScript cDNA synthesis kit (Biorad, Hercules, CA). Real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sequences for real time PCR were (5'-3'): ApoE Forward, TCCTGTCCTGCAACAACATCC, ApoE Reverse, AGGTGCTTGAGACAGGGCC; GAPDH Forward, CCATTCTCGGCCTTGACTGT, GAPDH Reverse, CTCAACTACATGGTC TACATGTTCCA. All primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Tissue homogenization

Forty-eight h after CCI injury, mice were anesthetized with isoflurane and underwent transcardiac perfusion with 15 mL of ice-cold phosphate-buffered saline (PBS; $n=6$ for WT; $n=3-4$ for GFAP-ApoE3 and GFAP-ApoE4; $n=3$ for ApoE-KO mice). For comparison of protein levels in the ipsilateral versus contralateral hippocampi of injured CCI mice, individual hippocampi were dissected on ice from ipsilateral and contralateral hemispheres and placed in 250 μ L of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8, 1% IGEPAL, 10% glycerol, 1 μ g/mL leupeptin, 10 μ g/mL aprotinin). Tissue was manually homogenized in a 1.5 mL Eppendorf tube using a polypropylene pestle (Kimble Chase, Vineland, NJ), incubated on ice for 15 min, and centrifuged at 16,000 rpm at 4°C for 20 min. The supernatant was then collected.

Western immunoblot analysis

Protein concentration of hippocampal supernatant samples was determined by DC Protein Assay (Bio-Rad, Hercules, CA). Protein samples were resolved on a 10% polyacrylamide SDS-PAGE gel (Mini-PROTEAN TGX Gel; Bio-Rad), transferred onto a nitrocellulose membrane (Bio-Rad), blocked for 1 h in blocking buffer (5% Blotting-Grade Blocker (Bio-Rad) in Tris-buffered saline containing 0.1% Tween-20 [TBST]). Membranes were incubated overnight at 4°C with goat anti-ApoE antibody (1:5000; Millipore, Billerica, MA) or mouse anti-beta-actin antibody (1:5000; Invitrogen, Grand Island, NY) in blocking buffer. The membranes were washed three times in TBST, then incubated 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies anti-goat immunoglobulin G (IgG) (1:5000; Jackson ImmunoResearch, West Grove, PA) or anti-mouse IgG (1:5000; Jackson ImmunoResearch) in blocking buffer. The membranes were washed three times, and protein was detected

using Clarity Western ECL Substrate (Bio-Rad) and visualized on HyBlot CL film (Denville Scientific, South Plainfield, NJ). Due to similar protein size of ApoE and beta-actin, membranes were stripped with 0.02% NaN_3 in 5% blotting-grade blocker in TBST at room temperature for 1 h before beta-actin antibody binding. Protein bands were quantified by densitometric analysis using ImageJ software and normalized to actin (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

Forty-eight h following CCI or sham surgery, mice were injected intraperitoneally with 100mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) then, 2 h later, were deeply anesthetized with isoflurane and sacrificed by transcardiac perfusion with 15 mL ice-cold PBS followed by 40 mL ice-cold 4% paraformaldehyde (PFA) in PBS. Whole brains were extracted and underwent overnight fixation in 4% PFA. Post-fixation, whole brains were embedded in 4% agarose gels and serial 50 μm coronal sections were cut in a series of six sections through the rostro-caudal length of the hippocampus using a vibratome (VT1000S, Leica, Buffalo Grove, IL). For BrdU staining, a set of sections containing every sixth slice was washed with PBS three times, rinsed with water, and denatured with 1N HCl in a 37°C water bath for 45 min. Sections were then washed with 0.3% Triton X-100/1xPBS (PBST) three times and blocked with 5% normal donkey serum (NDS; Sigma-Aldrich) in PBST at room temperature for 1 h. Sections were incubated with mouse anti-BrdU antibody (1:500, BD Bioscience, San Jose, CA) and rabbit anti-GFP antibody (1:500, Invitrogen) in 5% NDS with PBST overnight at 4°C.

The following day, sections were washed with PBST three times and incubated with Alexa Fluor 488 anti-rabbit IgG (1:200, Jackson ImmunoResearch) and Alexa Fluor 594 anti-mouse IgG (1:200, Jackson ImmunoResearch) in 5% NDS with PBST for 3 h at room temperature. Sections were then washed with PBST twice, PBS twice, then mounted on slides with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) and sealed with coverslips. For ApoE staining, a series of one-in-six sections was prepared as above. Sections were washed in PBST three times, blocked in 5% NDS with PBST for 1 h at room temperature, then incubated with goat anti-ApoE (1:50,000; Millipore) in PBST with 0.02% NaN_3 at room temperature for 2 d. After 2 d, the sections were washed with PBST three times and with PBS three times, and incubated with biotinylated anti-goat IgG (1:8000; Jackson ImmunoResearch) in PBST with 0.02% NaN_3 at room temperature for 2 d. After 2 d, the sections were washed with PBS three times, incubated with ABC Reagent (VECTASTAIN; Vector Laboratories) at room temperature for 1 h, washed with PBS three times, incubated with horse-radish peroxidase-streptavidin and Alexa Fluor 568 tyramide (TSA Kit, Invitrogen) for 15 min, washed with PBS three times, then mounted on slides with mounting medium and sealed with coverslips. For ApoE with co-staining for GFAP or GFP, on Day 5 of the ApoE protocol, instead of mounting the sections, rabbit anti-GFAP antibody (1:200, Dako) or rabbit anti-GFP antibody (1:500, Invitrogen) in 5% NDS with PBST was incubated overnight at 4°C. The next day, slides were washed three times with PBST and incubated with Alexa Fluor 488 anti-rabbit IgG (1:200, Jackson ImmunoResearch) in 5% NDS with PBST for 3 h at room temperature. Sections were then washed with PBST twice and with PBS twice, then mounted on slides with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) and sealed with coverslips.

Quantification and statistics

To determine the number of GFP- and BrdU-expressing cells in the subgranular zone of the dentate gyrus, an unbiased stereological approach was used ($n=3-4$ per mice per group for sham; $n=5-6$

mice per group for CCI). A set of sections containing one in every sixth coronal slice, spanning the rostral 75–80% of the hippocampus, were immunostained and visualized using a Zeiss microscope (Zeiss Axio Imager M2, Thornwood, NY) with a Hamamatsu camera (Hamamatsu Orca-R2; Bridgewater, NJ). The cells were counted using an optical fractionator and stereological image analysis software (StereoInvestigator; MBF Bioscience, Williston, VT). The subgranular zone, our area of interest, was outlined under a 20 $\times$ objective lens. The stereological software selected sample frames (70 $\mu\text{m} \times 70 \mu\text{m}$) at random, and cells in the counting frames (40 $\mu\text{m} \times 40 \mu\text{m}$) were counted under a 63 $\times$ oil-immersion objective. Co-localization of GFP and BrdU also were performed with the 63 $\times$ oil-immersion objective. The cell number was estimated by the software using weighted section thickness to yield an absolute number of total cells within the rostral 75–80% of the dentate gyrus. Data was analyzed using GraphPad Prism 6 software (GraphPad Software, LaJolla, CA).

For cell counts in sham SGZ, CCI ipsilateral SGZ and CCI contralateral SGZ were compared using one-way ANOVA followed by post-hoc analysis with Tukey's multiple comparison test. Both ipsilateral and contralateral are included in SGZ cell counts because there was no statistical difference between ipsilateral and contralateral sides in sham-injured mice. For comparison of ApoE expression in ipsilateral and contralateral dentate gyrus by Western blot and real-time polymerase chain reaction (RT-PCR), paired *t*-test was performed. A *p* value of <0.05 was considered statistically significant. Images were obtained with a Leica Confocal microscope (Leica TCS SP5 MP, Buffalo Grove, IL).

Results

ApoE expression is attenuated in hippocampal neural stem/progenitor cells

Previous work demonstrated that ApoE is a negative regulator of hippocampal neurogenesis.²² In order to investigate the *in vivo* regulation of ApoE expression after injury, nestin-GFP mice were exposed to CCI, then ApoE and GFP expression were assessed 48–72 h later in the hippocampus both qualitatively by immunohistochemistry and quantitatively by Western blot and RT-PCR. Nestin-GFP mice have been used and characterized extensively by us and others and GFP expression in these mice is well known to be restricted to stem/progenitor cells and not expressed in reactive astrocytes after injury.^{2,3,13,27} In uninjured animals, we found that ApoE immunoreactivity co-localized with GFP-expressing progenitors in the dentate gyrus (Fig. 1, A-D). Following injury, however, GFP-expressing cells become activated and proliferate, as indicated by increased GFP-staining in cell bodies and processes, but expression of ApoE was attenuated (Fig. 1, E-H). Quantitative analysis of ApoE protein levels in the supernatant of hippocampal homogenates by Western blot confirmed an approximately 20% decrease in ApoE levels in the ipsilateral hippocampus after injury, compared with the contralateral hippocampus ($p<0.01$; Fig. 1, I-J). To evaluate ApoE expression specifically in GFP-expressing progenitors, RT-PCR was performed on fluorescent-activated cell (FAC)-sorted GFP-positive progenitors isolated from the dentate gyrus, and ApoE expression was found to be similarly down-regulated ($p<0.001$; Fig. 1K).

Proliferation of nestin-expressing and ApoE-expressing neural progenitors in response to CCI injury

Type 1 NSPCs of the dentate gyrus express ApoE, which regulates their postnatal development.²² We confirmed ApoE expression in Type 1 cells of the SGZ in our WT mouse (Fig. 2, A-C). To verify the previously described NSPC proliferative response to injury,^{3,4} nestin-GFP mice underwent CCI injury and BrdU injection 48 h after CCI before being sacrificed 2 h later. Serial brain

FIG. 1. Apolipoprotein E (ApoE) is expressed in neural progenitors and reduced after injury. (A–C) Within the subgranular zone of the dentate gyrus of 8-week-old wild-type mice, nestin- green fluorescent protein (GFP) expressing progenitor cells express ApoE. (D) High-power magnification of a representative section from the boxed area in (C) shows co-localization of nestin-GFP and ApoE. (E–G) Forty-eight h after controlled cortical impact (CCI) injury, nestin-GFP progenitors (green) in the subgranular zone are activated and have attenuated ApoE expression. (H) High-power magnification of a representative section from the boxed area in G shows co-localization of nestin-GFP and its attenuated ApoE signal. (I–J) Western blot for ApoE protein in hippocampal homogenate 48 h after CCI injury shows decreased ApoE protein in the ipsilateral hippocampus following injury, compared with contralateral ($n = 6$ mice). (K) Quantitative reverse transcription polymerase chain reaction of fluorescence-activated cell-sorted nestin-GFP progenitors from the dentate gyrus of 8-week-old mice at 3 d after CCI injury show decreased ApoE messenger RNA levels in the ipsilateral side, compared with contralateral ($n = 8$). Values are mean $\pm$ standard error of the mean. ** $p < 0.01$ and *** $p < 0.005$ by paired t -test.

sections were then stained for GFP and BrdU. By immunohistochemistry, we observed a proliferative response of nestin-expressing cells in the SGZ in injured mice, compared with sham, as indicated by increased GFP and BrdU staining (Fig. 2, D–L). Using unbiased stereology, we examined the SGZ of the dentate gyrus (the niche for nestin-expressing Type 1 and Type 2 neural progenitor cells) and quantified the number of GFP+, BrdU+, and double-positive (BrdU+GFP+) cells. GFP+ cells were increased in both the ipsilateral (120% increase; $p < 0.01$) and contralateral (89% increase; $p < 0.05$) SGZ at 48 h after injury, compared with sham (Fig. 2M). Cellular proliferation was increased overall in the ipsilateral dentate gyrus as indicated by increased BrdU incorporation and increased BrdU+ cell number (151% increase over sham; $p < 0.01$), which was localized to the side of injury (Fig. 2N). Co-localization of BrdU with GFP-expressing progenitors revealed that CCI more than doubled the number of GFP-expressing neural progenitors proliferating in the ipsilateral dentate gyrus in response to injury (238% increase over sham; $p < 0.001$; Fig. 2O).

Injury-induced proliferation of hippocampal neural progenitors is absent in ApoE-deficient mice

ApoE deficiency leads to an attenuation of the hippocampal NSPC pool in vitro, but an increased proliferative potential of NSPCs in vivo.²² To investigate the influence of ApoE-deficiency on the injury-induced proliferative response of NSPCs, nestin-GFP ApoE-deficient mice were exposed to CCI and injected with BrdU to label dividing cells 48 h later as described above. We confirmed the absence of ApoE in the hippocampus and GFP-expressing progenitors using immunohistochemistry and Western blot (Fig. 3, A–C). Immunohistochemistry for GFP and BrdU was performed on coronal sections from sham and injured mice (Fig. 3, D–L). While CCI caused an increase in GFP-expressing cells in WT mice, no change in the number of GFP-expressing progenitors in the injured hippocampus of ApoE-deficient mice was observed (Fig. 3M). While an overall increase in proliferation was observed in the ipsilateral dentate gyrus of ApoE-deficient mice (85% increase in

FIG. 2. Injury induces proliferation of nestin-expressing neural progenitors. (A–C) Nestin- green fluorescent protein (GFP) mice express GFP in neural stem/progenitor cells within the subgranular zone of the dentate gyrus and apolipoprotein E (ApoE) is co-expressed within nestin-GFP expressing stem/progenitor cells. (D–L) Representative confocal images of the subgranular zone in sham mice and 48 h after controlled cortical impact (CCI) injury in the ipsilateral and contralateral side. Coronal sections were immunostained for nestin-GFP and bromodeoxyuridine (BrdU). Merged images yielded double-positive BrdU + GFP+ cells (yellow). (M–O) Quantification of GFP+, BrdU+ and double-positive BrdU + GFP+ cells within the subgranular zone of sham and CCI mice by unbiased stereology. CCI causes an increase in GFP+, BrdU+, and double-labeled cells in the ipsilateral dentate gyrus ($n=4-6$ mice per group). Values are mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance with Tukey's post hoc analysis.

FIG. 3. Injury-induced proliferation of hippocampal neural progenitors is absent in apolipoprotein E (ApoE)-deficient mice. **(A-B)** In ApoE knock-out (KO) mice, nestin- green fluorescent protein (GFP) progenitor cells do not express ApoE. **(C)** Hippocampal homogenates contralateral and ipsilateral to injury are deficient in ApoE expression by Western blot. **(D-L)** Representative confocal images of the subgranular zone of the dentate gyrus in sham and controlled cortical impact (CCI) ApoE-deficient mice 48 h after injury. Serial coronal sections were immunostained for nestin-GFP and bromodeoxyuridine (BrdU). Merged images yielded double positive BrdU+GFP+ cells. **(M-O)** Quantification of GFP+, BrdU+ and double-positive BrdU+GFP+ cells within the sham, and injured ipsilateral and contralateral subgranular zone of the dentate gyrus by unbiased stereology is shown. The number of GFP+ progenitors or proliferating progenitors in the ipsilateral dentate gyrus is not increased after injury despite an increase in overall proliferation ($n=3-6$ mice per group). Values are mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance with Tukey's post hoc analysis.

BrdU+ cells, compared with sham and contralateral SGZ; $p < 0.05$; Fig. 3N) similar to WT mice; unlike WT mice, there was no increase in the population of GFP-expressing cells actively dividing in response to injury in ApoE-deficient mice (Fig. 3O).

ApoE is increased after injury in GFAP-ApoE3 and GFAP-ApoE4 transgenic mice

Using transgenic mice overexpressing either human ApoE3 or human ApoE4 on a murine ApoE-null background crossed to nestin-GFP mice (GFAP-ApoE mice), we examined the role of human isoforms on

injury-induced progenitor proliferation. Expression of human ApoE isoforms in these transgenics is under the control of the GFAP promoter, which is expressed in astrocytes and nestin-expressing NSPCs.^{9,28} Using immunohistochemistry, we confirmed co-localization of GFAP and ApoE in the hippocampus of GFAP-ApoE4 transgenic mice in morphologic astrocytes (Fig. 4, A-C) and co-localization of ApoE in nestin-expressing GFP+ NSPCs (Fig. 4, D-F). Similar staining was observed in GFAP-ApoE3 mice (data not shown). ApoE expression was increased in the ipsilateral hippocampus of GFAP-ApoE3 and GFAP-ApoE4 mice in response to injury as measured by Western blot ($p < 0.01$, compared with contralateral; Fig. 4G, 4H).

FIG. 4. Hippocampal apolipoprotein E (ApoE) is overexpressed after injury in transgenic glial fibrillary acidic protein (GFAP)-ApoE3 and GFAP-ApoE4 mice. (A-C) A representative confocal image of the dentate gyrus in 8-week-old GFAP-ApoE4 mice shows co-localization of GFAP and ApoE expression. (D-F) A representative confocal image showing green fluorescent protein (GFP) expression in a Type 1 nestin-GFP stem cell and co-localization with ApoE under high power magnification (merged). (G) Western blot for ApoE in hippocampal homogenate 48 h after controlled cortical impact (CCI) injury shows increased ApoE protein in ipsilateral hippocampus, compared with contralateral hippocampus. (H) Quantification of hippocampal ApoE level by densitometry shows a greater than three-fold increase in ApoE expression in the ipsilateral versus contralateral hippocampus after CCI ($n = 3-4$ mice per group). Values are mean $\pm$ standard error of the mean. ** $p < 0.01$ by paired t -test.

Injury-induced proliferation of neural progenitors is preserved in GFAP-ApoE3 mice

To examine the effect of ApoE3 on injury-induced progenitor proliferation, GFAP-ApoE3/nestin-GFP transgenic mice underwent CCI, received one dose of BrdU 48 h after injury, and were sacrificed 2 h later for immunohistochemistry (Fig. 5, A-I). Using stereology, we quantified the number of cells within the subgranular zone of the dentate gyrus positively labeled for GFP, BrdU, and co-localization of GFP and BrdU. While the overall number of GFP+ cells was not significantly increased in the ipsilateral SGZ following injury (30% increase over sham, non-significant; Fig. 5J), an increased proliferative response was noted

by increased BrdU uptake (96% increase over sham and contralateral; $p < 0.01$). Quantification of double-positive GFP+ BrdU+ cells indicated that dividing GFP+ progenitors were part of this proliferative response, with approximately 150% increase in proliferating progenitors in the injured ipsilateral dentate gyrus ($p < 0.01$, compared with sham; Fig. 5L).

Injury-induced proliferation is intact in GFAP-ApoE4 mice despite reduced neural progenitor number

Following CCI in GFAP-ApoE4/nestin-GFP mice, the number of GFP+ cells increased by approximately 120% in both the ipsilateral and contralateral dentate gyrus ($p < 0.05$, compared with

FIG. 5. Apolipoprotein E3 (ApoE3) retains injury-induced proliferation of hippocampal neural progenitors. (A-I) Representative confocal images of the subgranular zone of the hippocampus in sham and controlled cortical impact (CCI) injured glial fibrillary acidic protein (GFAP)-ApoE3/nestin- green fluorescent protein (GFP) mice. Coronal sections were immunostained for nestin-GFP and bromodeoxyuridine (BrdU). Merged images yielded double-positive BrdU+GFP+ cells. (J-L) Unbiased stereology quantification of GFP+, BrdU+ and double-positive BrdU+ GFP+ cells within the subgranular zone of the dentate gyrus in sham and CCI mice. In the subgranular zone ipsilateral to injury, there is increased BrdU uptake and an increased population of actively proliferating nestin-GFP cells, compared with the subgranular zone in sham mice and contralateral to injury ($n = 3-5$ mice per group). Values are mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$ by one-way variance of analysis with Tukey's post-hoc analysis.

sham; Fig. 6J). Overall proliferation was increased in the ipsilateral dentate gyrus as indicated by an increase in BrdU+ cells (180% increase over sham; $p < 0.01$; Fig. 6K) and a five-fold increase in double-positive GFP+ BrdU+ cells indicated active proliferation of progenitors after injury ($p < 0.05$, compared with sham; Fig. 6L).

Compared with WT mice, the ApoE-deficient and GFAP-ApoE3 mice had an attenuated injury-induced increase in the GFP+ cell population in the ipsilateral hippocampus, compared with sham, with a 17% and 30% increase in GFP+ cells, respectively, versus a 183% increase in WT and 128% increase in GFAP-ApoE4 mice (Fig. 7A). GFAP-ApoE4 mice increased their GFP+ population

after injury yet the sham-injured mice had fewer GFP+ cells (4167 ± 2451), compared with other genotypes: WT (6642 ± 3269); ApoE-deficient (8522 ± 2992); GFAP-ApoE3 (8122 ± 2273 ; Fig. 7A). Their overall increase in the GFP+ cell population was attenuated likely due to fewer NSPCs (Fig. 7A). All genotypes were able to mount a robust overall proliferative response (Fig. 7B); however, ApoE-deficient mice had no increase in the number of dividing progenitors (Fig. 7C). When evaluating the ratio of dividing NSPCs over the total NSPC population, we found that WT and GFAP-ApoE3 mice had an increased ratio ipsilateral to injury, which was not seen in GFAP-ApoE4 and ApoE-deficient mice,

FIG. 6. Apolipoprotein E4 (ApoE4) mice have reduced neural progenitors but intact injury-induced proliferation. (A-I) Representative confocal images of the subgranular zone 48 h following sham or controlled cortical impact (CCI) injury in glial fibrillary acidic protein (GFAP)-ApoE4/nestin-green fluorescent protein (GFP) mice. Coronal sections were immunostained for nestin-GFP and bromodeoxyuridine (BrdU). Merged images yielded double-positive BrdU+GFP+ cells. (J-L) Quantification of GFP+, BrdU+, and double-positive BrdU+GFP+ cells within the subgranular zone of the dentate gyrus by unbiased stereology is shown. GFP+ cell number is decreased in sham GFAP-ApoE4 mice, compared with other genotypes. However, GFP+ neural progenitors are increased in the ipsilateral and contralateral dentate gyrus following CCI. Proliferation is increased overall in the subgranular zone ipsilateral to injury as evidenced by increased BrdU+ cell number, and increased number of BrdU+GFP+ cells indicates active proliferation of nestin-GFP cells ($n = 4-5$ mice per group). Values are mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$ by one-way analysis of variance with Tukey's post hoc analysis.

FIG. 7. Graphical summary representing the effects of apolipoprotein E4 (ApoE) status on injury-induced proliferation. (A–C) Green fluorescent protein (GFP)+, bromodeoxyuridine (BrdU)+, and BrdU+GFP+ cell data from all genotypes. (D–E) Percentage of double-positive (BrdU+GFP+) over green fluorescent protein (GFP)+ (proliferating neural stem/progenitor cells [NSPCs]) as a percent of all NSPCs or BrdU+ (proliferating NSPCs) as a percent of all proliferating cells. Values are mean $\pm$ standard error of the mean. * $p < 0.05$, ** $p < 0.01$ by one-way analysis of variance with Tukey's post hoc analysis for within genotype comparison.

while the contribution of new and/or dividing progenitors varied after injury with genotype (Fig. 7D).

Discussion

Injury-induced neurogenesis has been described for multiple injury paradigms including ischemia, seizures, and mechanical injury,^{29–31} and it is now clear that activation of hippocampal stem/progenitor cells is required for replacement of vulnerable neuroblasts and more mature hippocampal neurons.^{2,3} Although many molecules have been implicated in normal adult neurogenesis, it is unclear whether the same mechanisms are active during injury.³² Recent data suggest that hippocampal neurogenesis following TBI is beneficial to recovery and therefore strategies directed towards mechanistic understanding of this process could potentially be therapeutic.^{2,33}

The presence of ApoE4 in humans has long been known to increase the likelihood of developing late-onset Alzheimer's disease but more recently also has been found to independently predict outcome severity after TBI, as well as increase the risk of developing Alzheimer's disease and dementia following TBI.^{24,34–36} ApoE has widespread brain expression and its role in brain injury may be mediated through multiple pathways. ApoE has been shown to play a key role in clearance of the Alzheimer's disease-associated peptide amyloid-beta,^{21,37,38} which accumulates in the brain following TBI^{39–42} and improves outcome when therapeutically-targeted after injury in animal models.^{43,44}

The blood–brain barrier, which when disrupted is a significant cause for secondary brain injury and cerebral edema, also has been shown to be regulated by ApoE in multiple animal models^{45,46} and human studies.^{47,48} In a recent study by Bell and colleagues, ApoE knock-out and ApoE4 mice had breakdown of tight junction proteins leading to permeable blood–brain barriers, which was mediated through the NF- κ B/MMP9 pathway.¹⁸ This effect was noted as

early as two weeks of age and preceded neuronal and synaptic dysfunction.¹⁸

ApoE also exerts developmental effects on the brain, which have been observed as young as infancy in the magnetic resonance imaging (MRI) of healthy infants. ApoE4 carriers had significantly different regional gray matter volume and myelination, compared with non-carriers, suggesting a developmental susceptibility to injury due to a reduction in gray matter or dysregulated myelin development.⁴⁹ This result also was observed in the MRI of children where ApoE4 carriers had a thinner cortex within the left entorhinal region than non-carriers.⁵⁰ The genetic effect of ApoE3, compared with ApoE4, after injury has been more broadly described by cortical and hippocampal microarray, which showed a myriad of potential repair pathways that were attenuated in ApoE4 when compared with ApoE3 mice.⁵¹

ApoE is expressed primarily in astrocytes and following injury, astrocytes become activated and astrocyte-specific proteins such as GFAP become highly expressed.^{52, 53} Interestingly, we found that although ApoE is expressed similarly to GFAP in both astrocytes and type 1 stem cells, its expression is down-regulated following injury in WT mice. This response may have a pro-proliferative effect that has been observed in vitro and in vivo in ApoE-deficient hippocampal neural stem/progenitor cells.^{17,22} However, while injury induced proliferation of progenitor cells in WT mice with ApoE intact, genetic ablation of ApoE in knock-out mice led to an absence of this response, likely due to the age-dependent depletion of neural stem/progenitor cells in ApoE deficient mice by two months of age, which we have observed previously.²²

Several other proteins expressed or secreted by astrocytes have been shown to influence different aspects of neurogenesis. The Notch signaling pathway helps maintain progenitors in an undifferentiated state via direct cell-cell contact with astrocytes expressing the Notch receptor Jagged 1.⁵⁴ Whether disruption of physical contact between cells occurs or a reduction in Notch

signaling after injury to allow for differentiation is unclear. Wnt has been shown to direct neuronal differentiation,^{55,56} as have multiple growth factors, including insulin growth factor^{57,58} and brain-derived neurotrophic factor,^{59,60} which also have been shown to be increased in reactive astrocytes after injury.⁶¹ Further, reactive microglia, excitotoxicity, and blood–brain barrier disruption after injury also contribute additional factors that have been demonstrated to effect neurogenesis,⁶² indicating that injury-induced neurogenesis is the result of activation of a multitude of signaling pathways. The strong phenotype observed here in ApoE deficient mice, however, may be due to the effect of ApoE on proliferation, which is an early process in injury-induced neurogenesis and upstream of other processes, such as neuronal differentiation and maturation. While ApoE has been shown to affect these processes, as well,^{17,22} whether this is true after injury has not been investigated.

In this study, we used GFAP-ApoE3 and GFAP-ApoE4 transgenic mice to determine how human isoforms might affect the progenitor proliferative process. Since these are transgenic mice, the expression of ApoE is regulated by the same regulatory system that controls GFAP expression in astrocytes and not endogenous ApoE.²⁸ We also found clear evidence of ApoE's expression in type 1 GFAP-expressing hippocampal stem cells in these transgenic mice, although others have not found it to be detectable in this model using standard immunohistochemistry.¹⁷ It was not surprising to observe that following injury, the expression of both ApoE3 and ApoE4 were increased, as GFAP is known to be up-regulated after injury.^{52,53} In addition, this could explain why we did not observe an overall change in the number of GFP-expressing progenitors after injury in GFAP-ApoE3 mice. Although ApoE3 functions as presumably the WT allele, its overexpression in the GFAP transgenic mice may have prematurely suppressed normal progenitor proliferation. Interestingly, GFAP-ApoE4 mice demonstrated a similar but attenuated proliferative response to WT mice after injury, despite having fewer progenitors at baseline. Further, the overall number of neural progenitors in the injured dentate gyrus was lower in GFAP-ApoE3 and GFAP-ApoE4 mice, compared with WT mice. It is possible that this could affect cognitive function after injury, as we have previously shown that this population of cells contributes to cognitive recovery.²

Despite the significance of these findings, there are a number of areas that need more in depth exploration. The model chosen here overexpresses ApoE3 and ApoE4 under injury conditions, which may not mimic accurately what occurs in humans. Although it is known that ApoE regulates hippocampal neurogenesis in an apparently cell autonomous manner, ApoE has other functions in the brain particularly after injury, such as regulating the blood–brain barrier.¹⁸ In addition, we did not perform detailed hippocampal-specific behavioral analyses on the transgenic models after injury. These transgenics are known to behave abnormally without injury. In a series of behavioral phenotyping experiments, GFAP-ApoE4 mice had working memory impairments, as demonstrated by their failure to learn the task required to complete acquisition training of the radial arm maze.⁶³ A model using cell-specific and temporally-regulated deletion or replacement of ApoE is needed in order to definitively attribute impairment in functional outcome to defects in injury-induced neurogenesis.

In this study, we observed ApoE's influence on hippocampal neurogenesis after CCI. Mice with WT ApoE mounted a robust neurogenic response, which was absent in ApoE-deficiency. Transgenic mice that express human ApoE isoforms, GFAP-ApoE3 and GFAP-ApoE4, also responded with proliferation of

NSPCs after injury; however, GFAP-ApoE4 mice had fewer NSPCs at baseline and an attenuated GFP+ cell population after injury. Given the multitude of pathways ApoE influences, further investigation into the mechanism influenced by ApoE deficiency and isoform differences will be needed to clarify this effect and to determine whether it influences integration of neurons into the injured hippocampal network.

Author Disclosure Statement

No competing financial interests exist.

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