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Cutaneous burn injury induces neuroinflammation and reactive astrocyte activation in the hippocampus of aged mice

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Abstract

Background: By 2050, one in six people globally will be 65 or older. Confusion and delirium are significant complications after burn injury, especially in the elderly population. The etiology is still unknown, however complications may be driven by pro-inflammatory activation of astrocytes within the hippocampus (HPC) after burn injury. Reduced levels of phosphorylated cyclic-AMP response binding element (pCREB), caused by elevated systemic pro-inflammatory cytokines, could lead to cognitive decline and memory impairment.

Methods: To examine the effects of remote injury on neuroinflammation in advanced age, young and aged mice were subjected to a 15% total body surface area scald burn or sham injury, and euthanized after 24 hours. Expression of *ccl2* and *tnfa* were measured by qPCR in the whole brain and HPC. Astrocyte activation was measured by immunofluorescence within the HPC. pCREB was measured by immunohistochemistry in the dentate gyrus.

Results: We saw an 80-fold increase in *ccl2* and a 30-fold elevation in *tnfa* after injury in the whole brain of aged mice compared to young groups and aged sham mice ($p<0.05$ and $p<0.05$, respectively). Additionally, there was a 30-fold increase in *ccl2* within isolated HPC of aged injured mice when compared to sham injured animals ($p<0.05$). When investigating specific HPC regions, immunofluorescence staining showed a >20% rise in glial fibrillary acidic protein (GFAP) positive astrocytes within the cornu ammonis 3 (CA3) of aged injured mice when compared to all other groups ($p<0.05$). Lastly, we observed a >20% decrease in pCREB staining

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Declaration of competing interest

None.

Credit authorship contribution statement

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by immunohistochemistry in the dentate gyrus of aged mice compared to young regardless of injury ($p<0.05$).

Conclusions: These novel data suggest that remote injury in aged, but not young, mice is associated with neuroinflammation and astrocyte activation within the HPC. These factors, paired with an age related reduction in pCREB, could help explain the increased cognitive decline seen in burn patients of advanced age. To our knowledge, we are the first group to examine the impact of advanced age combined with burn injury on inflammation and astrocyte activation within the brain.

Keywords

Brain; traumatic injury; inflamm-aging; dentate gyrus; cytokine; chemokine; advanced age

1. Introduction:

The global population is aging, as the numbers of people aged >65 years of age has risen steadily since 2010. In the United States alone, the aged population accounts for 16.5% of all residents, amounting to 54 million people[1]. Burns amongst the aged population only represent 16.9% of all cases reported from 2009 to 2018, but the injuries are more fatal, with 52% of these patients succumbing to their injuries compared to just 14% of patients between 20–60 years of age[2]. Not only is advanced age an independent risk factor for burn injury, it also contributes to heightened morbidity, mortality[3], and neurocognitive complications[4] following insult. Additionally, when compared to male burn patients, females with equivalent burns have shown higher levels of mortality[5, 6], lower mental health status[7], and increased psychological distress[7].

The neurocognitive decline observed after burn injury involves multiple factors. Cutaneous burn injury triggers the release of cytokines, chemokines and danger-associated molecular patterns (DAMPs) from the injured tissue, contributing to a systemic “cytokine storm,” which promotes systemic inflammation[8]. Burn-induced systemic inflammation contributes to neurocognitive impairment[4, 9] in the aged population due to increases in neuroinflammation[10, 11], but the mechanisms behind this are still unknown. In the absence of burn or other insult, the blood-brain barrier (BBB) restricts access to the brain to pro-inflammatory cytokines and other potentially harmful factors[12]. Although the exact mechanisms remain elusive, heightened levels of IL-6 and IL-1β in the circulation and the brain of mice after burn injury have been linked to disruption of the BBB[13]. Burn injury also impairs the integrity of the BBB by reducing protein expression of tight junctional proteins in the cerebral endothelium and increasing transcytosis[13]. Disruption in the ability of the BBB to act as a barrier leads to increased pro-inflammatory cytokines and chemokines, such as CC motif chemokine ligand 2 (CCL2) and tumor-necrosis factor alpha (TNFα), within the brain[14], which contributes to neuroinflammation-induced neurological decline, confusion, delirium in patients[11]. The incidence of confusion, delirium, or memory loss after burn injury implicates damage to the HPC, which plays a vital role in learning, memory formation and recall[15, 16].

Burn related systemic inflammation activates microglia in young mice[14]. Microglia are the tissue resident macrophages of the brain and are maintained in a homeostatic state by the cytokine milieu of the brain[17]. Microglia can be activated[18] by illness or injury to secrete IL-1α and TNFα, which induce the activation of reactive astrocytes[19]. Prior to activation, astrocytes play a vital role in maintaining the blood-brain barrier, as well as ionic and bioenergetics homeostasis[20–22]. These active astrocytes change their phenotype into reactive, glial fibrillary acidic protein positive (GFAP+) astrocytes[11, 20] that can induce neuronal death through the secretion of a yet to be identified soluble toxin[19, 23]. Importantly, the presence of reactive astrocytes is an early feature of Alzheimer's Disease[24, 25], and may contribute to the progression of Alzheimer's Disease through impaired clearance of ameloid beta plaques[26].

Taken together, systemic inflammation caused by age[27], injury, or illness[28] activates astrocytes, which can contribute to excessive inflammation and neuronal damage. The dentate gyrus (DG) is the region of the HPC that is tasked with compiling and encoding sensory input to begin encoding into memories[29]. The activation of cyclic adenosine monophosphate response element binding-protein (CREB) by phosphorylation to form pCREB in the brain is necessary for a host of functions, including neuronal proliferation, cell differentiation, and neuronal survival[30]. In the DG, pCREB has a well-studied role in the formation of memories[31, 32] and is reduced in memory related neurocognitive disorders, such as Alzheimer's Disease[33]. If burn injury in aged patients triggers neuroinflammation and reactive astrocytes in the HPC, the region of the brain responsible for the encoding, formation, and recall of memories, it may explain the neurocognitive decline, confusion, and delirium seen in this patient cohort.

Using our well-documented, clinically relevant model of scald burn injury, we conducted a comparative study of neuroinflammation in young and aged mice. Scald burn was selected due to its prevalence, accounting for approximately 34% of all burn injuries requiring medical attention in the United States[2], compared to chemical burns, which account for 3%[2]. Importantly, scald burn injury induces a cytokine storm[8] that is similar to that seen in other traumatic injuries[34]. Here, we sought to examine the effects of advanced age and burn injury on pCREB, reactive astrocytes and expression of pro-inflammatory cytokines and chemokines in the brain, and, specifically, the HPC. We hypothesized that increased age would exacerbate neuroinflammation in the HPC and would lead increased activation of reactive astrocytes.

2. Materials and Methods

2.1. Mice and murine model of scald injury

Young (4–5 months of age, 35 year old human equivalent) and aged (18–22 months of age, 65 year old human equivalent) female BALB/cBy and C57BL/6 mice were obtained from the National Institute of Aging (NIA) Colony (Charles River Laboratories, Wilmington, MA). C57BL/6 mice were used for pCREB visualization, BALB/cBy mice were used for all other experiments. Mice were housed in sterile conditions in the University of Colorado Anschutz Medical Campus vivarium. All protocols were approved by the University of Colorado Denver Anschutz Medical Campus Institutional Animal Care and Use Committee

prior to conducting experiments. We utilized our well-established clinically relevant murine model of burn injury as previously described[35–37]. Briefly, aged and young mice, weighing approximately 25–30 g, were randomly divided into two groups (burn or sham). Higher numbers of mice were included in burn groups because of increased mortality, especially among aged mice. Mice were anesthetized (25 mg/kg of ketamine and 2.5 mg/kg of xylazine with constant isoflurane) (Webster Veterinary) and dorsa were shaved. Anesthetized mice were subjected to a 12–15% total body surface area (TBSA) scald burn injury by using a template and exposing the shaved dorsum to a 92–93°C water bath for 8 seconds, resulting in an insensate full-thickness skin injury. Sham-injured animals were exposed to room temperature water. Animals received 1mL of resuscitation fluids immediately post burn in addition to pain medication (1.0 mg/kg buprenorphine-SR LAB, Zoo Pharm). Animals received an additional volume of resuscitation fluids equal to 10 μL/g per mouse the following morning. At 24 hours after burn, a time point at which we have measured appreciable markers of inflammation in circulation[35] and multiple organ systems[37–41], mice were euthanized using CO_2 followed by exsanguination.

2.2. RNA extraction and quantitative RT-PCR.

Whole brains or isolated cortex and HPC were lysed in TRIzol (cat#15596018, Life Technologies) and RNA was extracted using chloroform (cat#C2432, Sigma-Aldrich) and isopropanol (cat#BP2618–1, Thermo-Fisher) precipitation. cDNA was then synthesized using iScript kit according to manufacturer protocol (Bio-Rad) as previously described[38]. Quantitative RT-PCR was performed using TaqMan probes *ccl2*(Mm00441242_m1), *il1b*(Mm00434226_m1), *il6(*Mm00446190_m1), *il10*(Mm01288386_m1), *il22*(Mm01226722_g1), *tnf*(Mm00443258_m1) (Applied Biosystems) and TaqMan Universal PCR Mastermix (cat#4364340, Applied Biosystems) run on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). Gene expression was quantified using the ΔΔCt algorithm[42] with *gapdh* as the endogenous control (cat#4352339E, Applied Biosystems).

2.3. Immunohistochemistry and Immunofluorescence

Mice were transcardially perfused with PBS under isoflurane anesthesia[43] prior to brain collection. Brains were fixed in 4%-paraformaldehyde (cat#47340, Thermo-Fisher) and cut into 50μm coronal sections using a Leica SM2010R freezing microtome (Leica Biosystems). For immunohistochemistry, sections were fixed with acetone prior to antigen retrieval (cat#H-3301, Vector), quenching of endogenous peroxidase activity, and blocked with animal-free blocking solution (cat#15019L, Cell Signaling). pCREB was visualized by incubation with 1:500 dilution of primary antibody (#ab32096, Abcam), 1:1000 dilution of HRP-conjugated secondary antibody (cat#7074s, Cell Signaling), 1:10000 dilution of SA-HRP (cat#3999s, Cell Signaling), and then incubated with 3,3′-Diaminobenzidine according to manufacturer's protocol (cat#8059, Cell Signaling). For immunofluorescence[44, 45], free-floating sections were washed, blocked, and incubated with primary antibody against GFAP (cat#Z0334, Agilent) overnight at 4°C.The next day sections were incubated with fluorescent conjugated secondary antibody (cat#712–545-150, Jackson ImmunoResearch) and Hoechst (cat#5117, Tocris) prior to being mounted and imaged using a Zeiss microscope. Image analysis was performed by obtaining pixel intensity using ImageJ

software. Each image underwent auto-thresholding with identical parameters for each image. Mean pixel intensity was measured for each positive cell. The mean pixel intensities for each cell were then averaged across each image, resulting in an average mean pixel intensity for every image. %GFAP pixel intensity within the CA3 region was determined by pixel intensity of GFAP within the CA3 region divided by the total image pixel intensity of GFAP, multiplied by 100 to receive a percentage. All immunohistochemical analysis, including selection of sections, fields of study, cells, and quantitation were performed by an investigator blinded to the experimental groups that the samples originated from.

3. Results

3.1 Post-burn neuroinflammation is exacerbated in aged compared to young mice

Increased neuroinflammation has been reported independently in age[11, 15, 16, 46] and burn injury[4, 9, 10, 13, 47] in humans and rodent models. Here, we assessed whether there is heightened neuroinflammation in the brains of aged mice, relative to young mice, subjected to cutaneous burn injury. In order to measure inflammatory responses in the brain, whole brains were collected from young and aged sham- and burn-injured mice and mRNA levels of *ccl2* and *tnfa* were measured. Our results show no difference in the expression of both *ccl2* and *tnfa* after burn injury in young mice (Fig. 1). Additionally, we observed no change in both *ccl2* and *tnfa* in aged sham-injured mice when compared to young sham-injured mice. Importantly, there was a significant increase in the expression of *ccl2* in aged burn-injured mouse brains when compared to young sham, young burn, and aged sham mice (80.8-, 15.4-, and 21.6-fold higher, respectively [$p<0.05$]). Expression of *tnfa* in the brains of aged burn-injured mice was similarly heightened when compared to all other groups (30.2-, 10.5-, and 7.5-fold, respectively [$p<0.05$]). Interestingly, we did not see changes in expression of other pro-inflammatory cytokines that were tested, namely interleukin-1β (*il1b*) or interleukin-6 (*il6*). Lastly we failed to detect differences in expression of anti-inflammatory cytokines interleukin-10 (*il10*) or interleukin-22 (*il22*) in any experimental group (data not shown).

3.2 Inflammatory response in the hippocampus is heightened in aged mice

The HPC is vital for integrating sensory input into memories, and the encoding and recall of those memories[48]. Since memory deficits, confusion, and delirium can occur in the aged population after burn injury, we decided to assess levels of expression markers of inflammation in isolated HPC of young and aged mice. Here, we measured *ccl2* expression in the isolated HPC and cortex of mice after burn injury (Fig. 2). There was no difference in *ccl2* within the HPC when comparing young sham-injured mice to either young burn-injured or aged sham-injured mice. However, there was a marked elevation in *ccl2* expression in the HPC of aged burn-injured mice, which was 30-fold above that of young sham-injured ($p<0.05$ from all groups). Within the cortex, a small increase in *ccl2* was noted in both young and aged animals after burn injury, but this increase was not statistically significant. These results reveal that burn injury in aged animals can increase markers of inflammation, which we found to be specifically located within the HPC.

3.2 Increased glial fibrillary acidic protein (GFAP) positive astrocytes in the cornu ammonis 3 (CA3) region of the hippocampus in aged burn-injured mice

We next sought to measure whether aged burn-injured mice had increased amounts of activated reactive astrocytes in their HPC by utilizing immunofluorescence relative to comparably treated younger mice (Fig. 3). The total amount of GFAP+ signal, normalized to total Hoechst, did not change significantly between groups (Fig. 3B), although we noted a trend toward an elevation in GFAP in the young burn group. Quantitation of the amount of GFAP+ signal specifically in the CA3 region of the HPC (Fig. 3A,C) of aged burn-injured mice, revealed a 23.7%, 51.2%, and 21.4% rise in signal over young sham, young burn, and aged sham mice respectively ($p<0.05$). The CA3 region assists in new memory encoding through pattern separation[49]. Additionally, the CA3 region is responsible for spatial rapid one-trial learning, pattern completion, and spatial short-term memory[50–52]. Reactive astrocytes within this region may damage neuronal cells and lead to neurocognitive decline. Interestingly, the percentage of GFAP+ cells within the young burn group fell to 13.4% of total GFAP, perhaps as a consequence of the observed trend towards an increase in GFAP+ reactive astrocytes within the rest of the HPC while being mostly excluded from the CA3 region.

3.4 Reduction in phospho-cyclic adenosine monophosphate response element binding-protein (pCREB) in dentate gyrus of aged mice

We sought to examine whether pCREB in the dentate gyrus was altered by age and/or burn injury (Fig. 4). The dentate gyrus is one of few brain sites where neurogenesis can occur in adult mammals[53], and this process is dependent on the presence of pCREB within its granular neurons[54]. We found that pCREB was reduced by 21.2% in the aged sham-injured mice and 26.5% in aged burn-injured animals, when compared to young sham-injured mice. However, there was no effect of burn on pCREB expression within the dentate gyrus, signifying that age is the primary determining factor in pCREB expression (Fig. 4B).

4. Discussion

Burn patients of advanced age carry a higher risk of post-burn complications, including neurological decline[4, 9, 10], which is associated with worse post-burn prognosis when compared to younger individuals who sustain comparable injuries[2, 3, 55]. While it is known that both increased age[15, 16, 27] and burn injury[4, 8–10, 13, 47] are associated with heightened neurological complications, the mechanisms behind burn-related neurological decline in advanced age are not well understood. The aim of this study was to determine whether there are differential responses in astrocyte activation and markers of inflammation in the brain of young and aged mice after remote burn injury using our well-documented, clinically relevant murine model of scald burn injury[35, 36, 39–41, 56–62]. We investigated whether burn injury differentially activated astrocytes in the brains of young and aged mice. We found that burn injury in older mice is associated with heightened neuroinflammation when compared to young burn-injured mice. This was achieved by documenting the levels of mRNA expression of *ccl2* and *tnfa* in whole-brain tissue (Fig. 1) and *ccl2* expression within the HPC (Fig. 2). CCL2 is a known monocyte chemoattractant[63] that is produced by neurons after injury and contributes to microglial activation[64]. TNFα is a highly pleotropic cytokine

that contributes to neuro-cognitive decline[65]. In addition to the direct effects of CCL2 on microglial activation, increased levels of neuroinflammation in the brain can give rise to activated microglia,[66] which in turn secrete pro-inflammatory cytokines that activate reactive astrocytes[19], causing further neurodegeneration. After burn injury, CCL2, TNFα, and a number of other pro-inflammatory cytokines and chemokines are elevated systemically and can impair the BBB[13]. The mechanisms behind neuroinflammation after burn injury remain elusive in young animals, and evidence demonstrating enhanced neuroinflammation in aged animals, prior to this study, has been lacking. However, leakiness at the BBB may induce neuroinflammation through less restricted entry of systemic pro-inflammatory cytokines[14] and bacterial products such as LPS[18] into the brain, which are able to activate reactive astrocytes.

To further investigate this neuroinflammatory phenotype, we isolated the HPC, which is vital for memory formation and recall[48]. We found that *ccl2* was significantly increased within the HPC of aged burn-injured mice when compared to young mice (Fig. 2). While it is known that age[15] or traumatic brain injury[67] alone can result in damage to the HPC, this study is the first to interrogate the intersection of age and remote burn injury in this brain region. This region specific increase in neuroinflammation may lead to increased neurodegeneration within the HPC, directly damaging the region associated with memory formation and recall, and thus causing the confusion, delirium, and memory deficits observed in burn patients of advanced age[68].

Using immunofluorescence, we visualized GFAP+ astrocytes within the HPC and observed that there was an increase in activated reactive astrocytes within the CA3 region of aged burn-injured mice when compared to young and sham groups (Fig. 3). The CA3 region of the HPC is directly connected to the dentate gyrus and assists in new memory encoding through pattern separation[49]. Additionally, the CA3 region is responsible for spatial rapid one-trial learning, pattern completion, and spatial short-term memory[50–52]. Given our data, reactive astrocytes in the CA3 region of older patients who sustain a burn injury may be contributing to site-specific neurodegeneration, thereby causing deficits in short term memory loss and confusion/delirium. To our knowledge, there is no evidence of CA3 specific damage after burn injury, in either young or aged mice. The CA3 region has been shown to experience more neuronal loss than the CA1 region after models of traumatic brain injury[67, 69]. Previous literature has suggested that this disparity may be due to differential levels of physical tissue strain experienced in these regions during injury[70, 71]. However, since traumatic brain injury induces neuroinflammation[69], our data would suggest that the differences in damage may be due to differential activation of reactive astrocytes within different regions of the HPC, especially in aged individuals. Further, traumatic brain injury has been shown to sensitize astrocytes to a secondary insult[72], such as burn injury, which could lead to further CA3 region damage and neurological consequences. This damage to the hippocampal CA3 region, combined with the observed age related deficit in pCREB in the dentate gyrus (Fig. 4), may explain the neurological complications in burn patients of advanced age. Together, our novel studies increase our understanding of the effects of aging and burn injury on neuroinflammation. Future work based on this study will examine the mechanistic underpinnings of this neuroinflammation and ultimately identify therapeutic

interventions that will target the specific pathways that contribute to clinical neuro-cognitive decline.

Further study will be required to determine why reactive astrocytes are found more often within the CA3 region of aged mice than young after burn injury. Perhaps the region specific increase in *ccl2* expression is of sufficient magnitude to trigger microglia activation[64] and subsequent activation of reactive astrocytes[19]. Increased *ccl2* may also lead to recruitment of blood monocytes[61, 63], which could, in turn, be activated in the brain by cleaved gelsolin[47], perpetuating the pro-inflammatory state. Unraveling the mechanisms responsible for excessive neuroinflammation and activation of reactive astrocytes after burn injury in aged mice will allow treatments to be developed to specifically target these pathways for therapeutic benefit. Finally, understanding these mechanisms will extend beyond just burn-injury to other forms of trauma and lead to a broader understanding and better treatment of trauma in the aged population.

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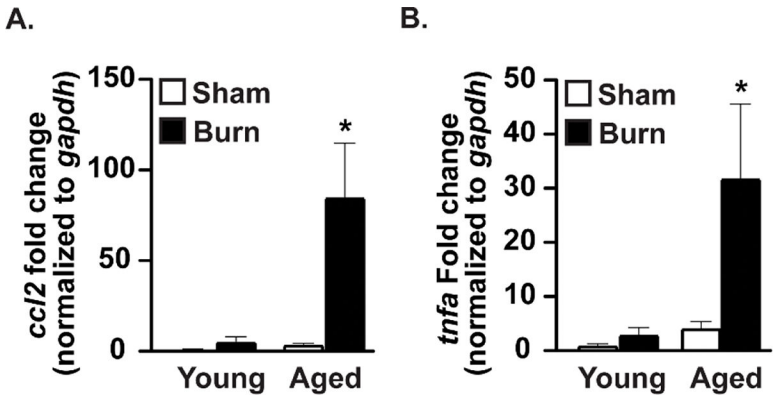

Figure 1. Dramatic elevation in markers of neuroinflammation in whole brains of aged burn-injured mice relative to other groups.

Brains were collected after sham or burn injury. mRNA expression of (a) *ccl2* and (b) *tnfa* across all groups are shown as mean fold-change relative to *gapdh* ± Standard Error of the Mean (SEM). *p<0.05 compared to all other groups by one-way Analysis of Variance (ANOVA), Tukey's multiple comparison test. Graphs shown are from a single representative of two independent experiments. n = 6 (sham) and 9 (burn) mice per group for *ccl2,* n=3 (sham) and 6 (burn) mice per group for *tnfa* in this representative experiment.

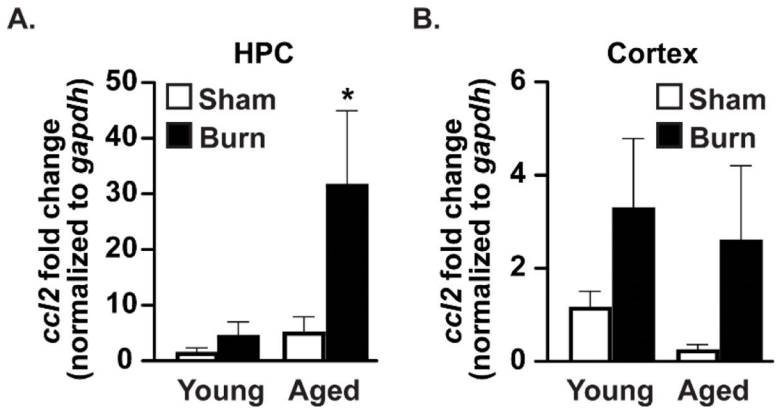

Figure 2. *ccl2* expression is elevated in the hippocampus, but not the cortex, of age burn-injured mice.

mRNA expression of *ccl2* in the (A) hippocampus and (B) cortex by RT-qPCR after burn injury across all groups shown as mean fold-change relative to *gapdh* ±SEM. *p<0.05 compared to all other groups by one-way ANOVA, Tukey's multiple comparison test. Graphs shown are from a single representative of two independent experiments. n=3 (sham) and 5 (burn) mice per group in this representative experiment.

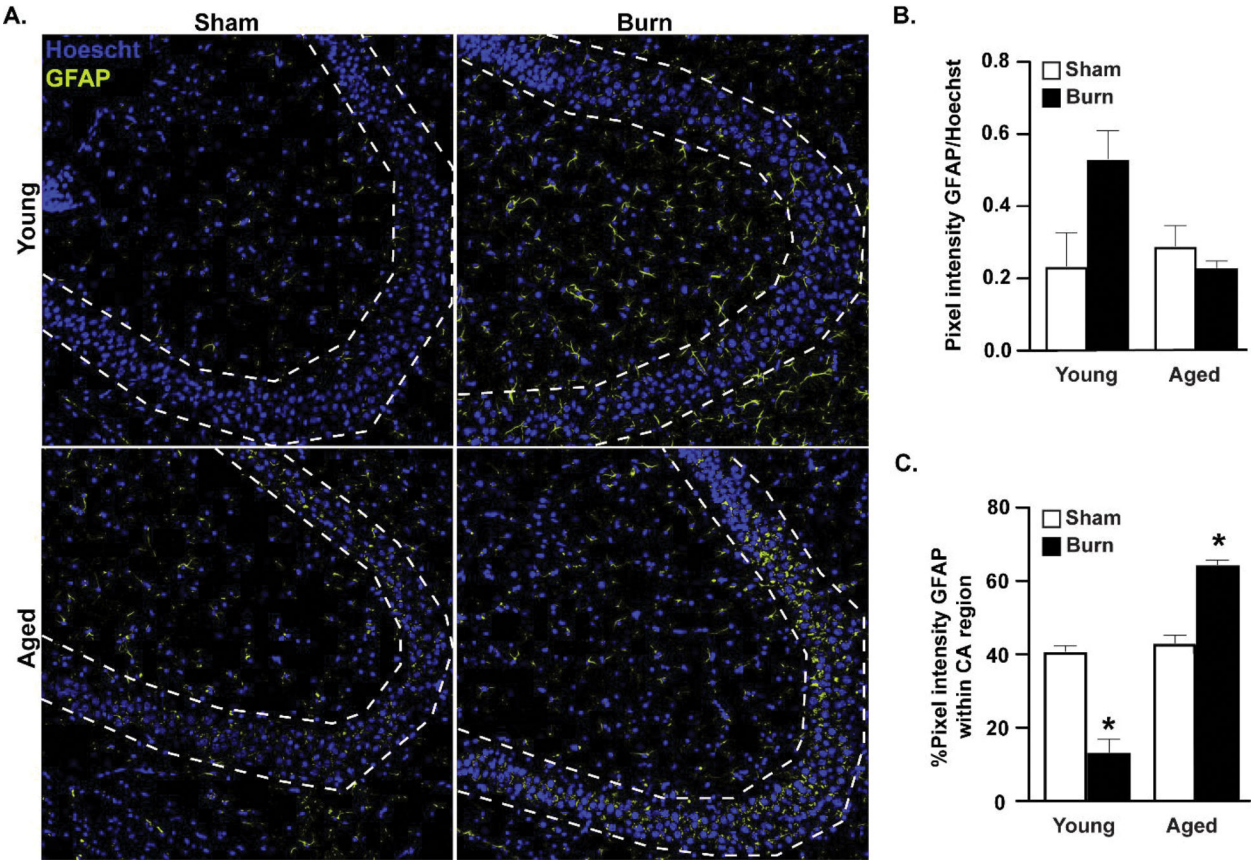

Figure 3. Increased GFAP+ reactive astrocytes in the CA3 region of the hippocampus of aged burn-injured mice.

(A) GFAP+ reactive astrocytes identified by immunofluorescence in perfused brains after burn injury. In aged burn-injured mice, GFAP+ cells increase their localization to the CA3 region (outlined in dotted line). Representative IF shown, n=3–4 mice per group, images shown from a representative of two independent experiments. (B) GFAP+ cells analyzed with ImageJ and presented as mean pixel density±SEM, normalized to Hoechst. n=3(sham)-4(burn) mice per group, experiment shown is a representative of two independent experiments. (C) Percentage of total GFAP+ signal that is within the CA3 (dotted lines) region reported as mean percentage±SEM. *p<0.05 from all other groups by ANOVA, Tukey's post-hoc test. Graphs shown are from a single representative of two independent experiments. n=3 (sham) and 4 (burn) mice per group in this representative experiment.

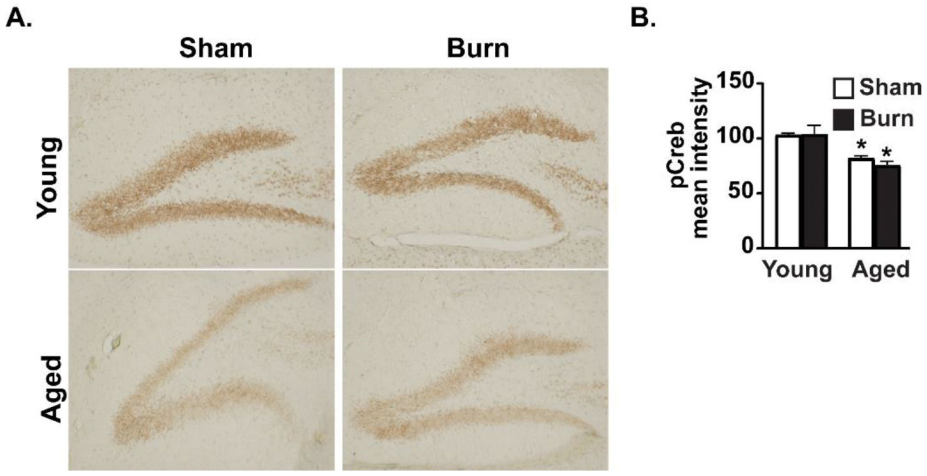

Figure 4. pCREB is reduced in aged mouse dentate gyrus regardless of burn injury.

(A) pCREB was measured by immunohistochemistry 3,3′-Diaminobenzidine staining in all groups after burn injury. (B) Quantification indicates that age, but not burn injury, reduces pCREB staining. pCREB mean intensity analyzed using ImageJ and reported as mean percentage ± SEM. *$p<0.05$ from young groups by ANOVA, Tukey's post-hoc test. Graphs and histology shown are from a single representative of two independent experiments. n=3 (sham) and 4 (burn) mice per group in this representative experiment.