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Serum Amyloid A is expressed in the brain after traumatic brain injury in a sex-dependent manner

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Abstract

Serum amyloid A (SAA) is an acute-phase protein upregulated in the liver after traumatic brain injury (TBI). So far, it has not been investigated whether SAA expression also occurs in the brain in response to TBI. For this, we performed a moderate controlled cortical impact injury in adult male and female mice and analyzed brain, blood, and liver samples at 6 hours, 1, 3, and 10 days post-injury (dpi). We measured the levels of SAA in serum and liver by western blot. We used immunohistochemical techniques combined with *in situ* hybridization to determine SAA expression in the brain. Our results revealed higher levels of SAA in the bloodstream in males compared to females at 6 h post-TBI. Liver and serum SAA levels showed a peak of expression at 1 dpi followed by a decrease at 3 to 10 dpi in both sexes. Both SAA mRNA and protein expression colocalize with astrocytes and macrophages/microglia in the cortex, corpus callosum, thalamus, and hippocampus after brain injury. For the first time, here we show that SAA was expressed in the brain after TBI. Collectively, SAA expression was predominant in males compared to females, and in association with the sex-dependent neuroinflammatory response after brain injury. We suggest that SAA represents a crucial protein associated with the acute neuroinflammatory

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response following TBI, not only for its hepatic and bloodstream levels but also for its expression in the injured brain.

Keywords

neuroinflammation; brain trauma; acute phase response; microglia; neutrophil accumulation

INTRODUCTION

Traumatic brain injury (TBI) occurs when a sudden impact causes damage to the brain. In the United States, an estimated 2.5 million people suffer from TBI annually (Centers for Disease and Prevention, 2013). The initial primary injury is followed by a phase of secondary damage, which results in the many sequelae. It is widely accepted that inflammation after TBI is the leading cause of this secondary injury (Das et al. 2012; Gyoneva and Ransohoff 2015; Hinson et al. 2015; Woodcock and Morganti-Kossmann 2013). Secondary response induces the release of proinflammatory cytokines and chemokines to the injured brain regions and helps attract immune cells to the brain parenchyma (Ziebell and Morganti-Kossmann 2010). The immune cells, including neutrophils, monocytes, and lymphocytes, migrate to the area of injury, release proinflammatory cytokines, and further exacerbate the inflammatory response (Ziebell and Morganti-Kossmann 2010). Neutrophils are known to be the first peripheral immune cells to cross the blood-brain barrier and infiltrate the brain post-TBI (Scholz et al. 2007). The activation of the infiltrated neutrophils contributes to tissue damage by the release of oxygen radicals, proteases, and proinflammatory cytokines (Neumann et al. 2008), making neutrophils a critical aspect of the acute inflammatory response (Yang et al. 2017). Activation of microglial cells along with the recruitment of peripheral macrophages are some of the principal producers of cytokines and chemokines after TBI and are also associated with the propagation of inflammation to the brain areas surrounding the site of the injury (Du et al. 2017).

Additionally, the release of cytokines and chemokines after TBI has been found to interact with peripheral organs, which may, in turn, propagate the systemic inflammatory response (Catania et al. 2009; Utagawa et al. 2008). Systemic inflammation activates the Acute Phase Response (APR) and involves multiple organs and subsequent signals that can contribute to further inflammatory responses. A key player in APR is the hepatic serum amyloid A (SAA), a small apolipoprotein, usually synthesized by hepatocytes (Ramadori et al. 1985b). SAA is highly inducible in response to inflammatory events such as systemic infection (McAdam and Sipe 1976) and is upregulated in response to cytokines and other regulatory factors. Overexpressing SAA produces an increase in the number of microglial cells, levels of proinflammatory cytokines, and impairs social recognition in an SAA transgenic mouse model (Jang et al. 2017). Clinical studies have also reported detectable SAA in the plasma of TBI patients (Anada et al. 2018; Carabias et al. 2019; Gao et al. 2014). High levels of SAA have a deleterious consequence in some patients with inflammatory conditions (Chae et al. 2009; Kuroda et al. 2017; Kuroda et al. 2002; Lane et al. 2017). Although the liver is the primary site of SAA synthesis, there is growing evidence that SAA production is also

extrahepatic (Upragarin et al. 2005). SAA has been found in cerebrospinal fluid and bound to cerebrovascular amyloid deposits as well as neurofibrillary tangles in Alzheimer's disease (AD) patients (Sundelof et al. 2009), demonstrating that SAA is locally produced in a neuroinflammatory environment (Liang et al. 1997). SAA is also an amyloid precursor protein and plays an alternative role in amyloidosis (Gorevic 2013; Guo et al. 2002). We have demonstrated in the previous study that upon APR post-TBI, serum, and hepatic SAA levels increase in a mouse model of acute TBI (Villapol et al. 2017). Several studies have shown that SAA can act as a chemoattractant for neutrophils and monocytes, and binds to receptors such as TLR-2/4 (Villalba et al. 2012), which are known to be highly expressed in inflammatory immune cells (Kinkley et al. 2006). However, the majority of pre-clinical studies in TBI have been focused on males, and only a few have employed both sexes on translational studies (Hughes 2007; Sandberg et al. 2015). Our laboratory and others have demonstrated sex differences in the neuroinflammatory response following TBI (Doran et al. 2019; Villapol et al. 2017), and it would be necessary to determine if there are also discrepancies between males and females in the SAA expression.

In the present study, we identified for the first time mRNA and protein SAA expression in the injured brain and its association with specific brain regions and cell types in a sex-dependent manner. We suggest that SAA may act as an inflammatory mediator in the brain following TBI. Thus, we conclude that sexually dimorphic response to TBI results from the rapid production of SAA in the liver and, release into the circulation, but also from SAA expression in the brain.

MATERIAL AND METHODS

Mice and controlled cortical impact (CCI) injury

Young adult (9–14 weeks) male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were kept under a 12:12 hour light and dark cycle with access to food and water *ad libitum*. We used random cycling of female mice to better reflect clinical applicability. We anesthetized mice with isoflurane (3% for induction, 1.5–2% for maintenance) and performed a moderate controlled cortical impact (CCI) injury on the left side of the brain at the primary motor and somatosensory cortices using an electromagnetically driven CCI device (Impact One stereotaxic impactor; Leica Microsystems, Buffalo Grove, IL). The site of impact was located at 2 mm lateral, and 2 mm posterior to Bregma, with a 3 mm diameter flat impact tip, impact velocity of 3.25 m/s, impact depth of 1.5 mm, and dwell time of 0.1 s. We have previously determined that these parameters can induce a strong and acute inflammatory response (Villapol et al. 2015; Villapol et al. 2014; Villapol et al. 2017). Sham mice received identical anesthesia and surgery without craniotomy or brain injury to serve as uninjured controls. After injury, the incision was closed with staples and the mice were allowed to recover fully from anesthesia before being transferred to their home cages. All animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at Georgetown University (Protocol number: 2016-1263) and Houston Methodist Research Institute (Protocol number: IS00004860), and were conducted following the NRC guide to the Care and Use of Laboratory Animals.

Tissue extraction and serum collection

We euthanized the CCI animals at 6 hours, and 1, 3, and 10 days post-injury (dpi) and sham mice at 1-day post-surgery. All mice were euthanized with carbon dioxide (20%) and perfused with phosphate-buffered saline (PBS). For serum collection, trunk blood was collected and allowed to clot for 30 minutes at room temperature, and serum was obtained by centrifugation (14,000 x g) at 4°C for 10 minutes and stored at -80°C. Livers were removed and stored at -80°C. Brains were removed, post-fixed in 4% paraformaldehyde overnight, and stored at 4°C in a 30% sucrose solution.

Western blot analysis for detection of SAA

Liver tissue was mechanically homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) supplemented with Pierce Protease and Phosphatase Inhibitor (Thermo Fisher Scientific, Waltham, MA). 30 µg of total protein from liver or a 1:5 dilution from serum samples were combined with 4x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and heated at 100°C for 10 min before loading into 12% Mini-PROTEAN TGX Stain-Free gels (Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Following transfer, membranes were blocked in 5% w/v skim milk powder in PBS-Tween 20 (PBS-T) blocking buffer for 1 h at room temperature. Membranes were incubated with goat anti-SAA primary antibody (1:500; AF2948, R&D Systems, Minneapolis, MN) overnight at 4°C and then with horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (1:3000; Thermo Fisher Scientific, Waltham, MA) for 1 h at room temperature. Membranes were developed with Clarity Western ECL (Bio-Rad Laboratories, Hercules, CA). A ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA) was used for the Stain-Free and the chemiluminescence imaging and densitometry quantification was performed using the ImageLab 6.0.1 software (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence analysis.

A sliding microtome (Microm HM 430, Thermo Fisher Scientific, Waltham, MA) was used to section the brains at 20 µm-thick in coronal orientation through the dorsal hippocampus. The brain sections were then cryoprotected in an antifreeze solution (30% glycerol + 30% ethylene glycol + 40% 0.01 M PBS) for storage at -20°C. Free-floating parallel brain sections were washed with PBS with 0.5% Triton X-100 (PBS-T) 3 x 5 min and blocked with 5% normal horse serum (NHS, #S2000, Vector laboratories, Burlingame, CA) in PBS-T for 1 h. Brain sections were incubated at 4°C overnight in PBS-T and 3% of NHS using the following primary antibodies: SAA, anti-rabbit TLR4 (1:200, sc-10741, Santa Cruz Biotechnology, Dallas, TX) for activated microglia/macrophages, anti-rat Ly-6B.2 alloantigen (1:100, MCA771GA, Bio-Rad Laboratories, Hercules, CA) for neutrophils, or anti-rat CD11b/c (OX-42, 1:200, Cat. 554862, BD Biosciences) is expressed on the surface of monocytes/macrophages, granulocytes, dendritic cells, and microglia in the brain.

After incubation, brain sections were washed in PBS-T and incubated with the corresponding anti-rabbit Alexa Fluor 568-conjugated or anti-mouse Alexa Fluor 488-conjugated IgG secondary antibodies (all 1:1000, Thermo Fisher Scientific, Waltham, MA)

for 2 h at room temperature. Sections were rinsed with PBS 3 x 5 min and incubated in PBS with DAPI solution (1:50,000, Sigma-Aldrich, St. Louis, MO) for counterstained nuclei. The sections were rinsed with distilled water and coverslipped with Fluoro-Gel with Tris Buffer mounting medium (Electron Microscopy Sciences, Hatfield, PA). Brain sections for negative controls were incubated without the primary antibody (Supplementary Fig. 1). For quantitative analysis of immunolabeled sections, we implemented unbiased standardized sampling techniques to measure tissue areas corresponding to the injured cortex showing positive immunoreactivity as we previously described (Villapol et al. 2015; Villapol et al. 2014). To quantify the number of Ly6B.2, TLR4 and SAA-positive cells in the injured cortex, an average of four coronal sections from the lesion epicenter (−1.34 to −2.30 mm from bregma) were counted and imaged for each animal, n= 5 mice per group. Within each brain region, positive cells were counted in each of 5 cortical fields (x20 objective, 151.894 mm²/field) around the impact area, as we have previously described (Villapol et al. 2017). All the investigators were blinded for the experimental groups. Images were acquired on a Nikon motorized fluorescence microscope (Eclipse Ni-U, Melville, NY) with a pco.Edge Scomos camera (4.2LT USB3) and analyzed using NIS-Elements software.

RNAscope® in situ hybridization combined with immunohistochemistry

Coronal brain sections were mounted on gelatin-coated glass slides (Superfrost Plus, Thermo Fisher Scientific) and stored at −80°C until use. Fluorescent in situ hybridization (FISH) was performed as per the manufacturer's instructions using RNAscope® Technology 2.0 Red Fluorescent kit (Advanced Cell Diagnostics (ACD), Hayward, CA) as we previously described (Fe Lanfranco et al. 2017; Villapol et al. 2017). Brain tissue sections were dehydrated by 50%, 70%, and 100% ethanol gradually for 5 min; boiled during 10 min with pretreatment 2 solution (citrate buffer), then they were incubated with pretreatment 3 solution (protease buffer) for 30 min before hybridization. Sections were then incubated at 40°C for 2 h with the following target probe for mouse: *Mus musculus Saa1* mRNA (Cat. No. 450191, ACD). In addition, the negative (Cat. No. 310043, ACD) and positive (Cat. No. 313911, ACD) control probes were applied and let hybridized for 2 h at 40°C. The amplification steps were performed according to manufacturer's directions. After FISH, slides were washed three times with PBS and blocking with PBST and 5% normal goat serum for 1 h. Immunofluorescence was performed using a mixture of primary antibodies incubated overnight at 4°C; polyclonal anti-rabbit Iba-1 (1:500, 019-9741, Wako Chemicals, Richmond, VA) for microglia/macrophages cells, monoclonal anti-mouse GFAP (1:500, AB5541, Millipore, IL) for astrocytes, and polyclonal anti-rat F4/80 (1:200, MABF152B, Sigma) for macrophage cells. Alexa Fluor 488-conjugated goat anti-rabbit, and anti-rat IgG (1:1,000, Invitrogen, Carlsbad, CA) were applied for 2 h at room temperature. Sections were rinsed with PBS three times and incubated in PBS with DAPI solution for counterstained nuclei. The sections were washed three times with distilled water and coverslipped with Fluoro-Gel with Tris Buffer mounting medium (Electron Microscopy Sciences).

Statistical Analysis

All data in this study are expressed as the mean ± standard error of the mean (SEM). $P < 0.05$ or less was considered statistically significant. For western blot, we used 2-way ANOVA with time after injury as one variable and sex as the other variable, then a post-hoc

test with Bonferroni multiple test correction. For immunohistochemistry analysis, the Mann-Whitney U test was used for groups of two. All statistics were performed with GraphPad Prism 5.0 software (GraphPad, San Diego, CA).

RESULTS

SAA protein levels in the blood are higher in males 6 hours after injury and peak at 1 day after TBI

Western Blot analysis was performed on serum collected from male and female mice at 6 hours and 1, 3, and 10 dpi as well as from sham mice. In the sham group, SAA expression was undetectable in all serum samples from female mice, whereas it was above detection levels in male mice (Fig. 1A, B). After TBI, both male and female mice showed an increase in SAA levels with a peak at 1 dpi that decreased at 3 dpi and was undetectable at 10 dpi. At 6 hours post-TBI, the males exhibited a significantly higher SAA expression when compared to the females, consistent with the trend described for the sham mice. We also analyzed the expression of SAA in the liver (Fig. 1C, D). Low levels of SAA were found both in male and female sham mice, with no significant differences between males and females. Similar to the serum, liver SAA increased in the CCI group, with the highest expression found at 1 dpi followed by a drop at 3 dpi. At 10 dpi, liver SAA expression returned to sham levels. Although there is a trend towards increasing in males, we did not observe statistically significant differences between male and female mice after injury, for any of the time points analyzed.

More neutrophils and inflammatory cells infiltrate the brain in males compared to females after TBI.

TBI induces rapid infiltration of blood-derived peripheral immune cells into the brain (Hsieh et al. 2013). We used specific antibodies for receptor Ly-6B.2 to identify infiltrated neutrophils into the brain at 1 dpi. Infiltrating neutrophils were higher in males compared to females (Fig. 2A, B). The TLR4 and CD11b/c are expressed in peripheral monocytes/macrophages, on dendritic cells, neutrophils, mast cells, granulocytes, microglia or B cells, and it is known to be a target of SAA (Villapol 2016). We quantified the total number of TLR4 and CD11b/c positive cells in the cerebral sensorimotor cortex after TBI, and we found that the injured cortex from males has more TLR4 (Fig. 2D, E) and CD11b/c (Fig. 2G, H) positive cells than females. The quantitative graphs show an increase of 56.85, 57.41 and 52.12% in the number of Ly-6B.2, TLR4, and CD11b/c positive cells respectively in the injured cortex from males compared to females (Fig. 2C, F, I). A correlation of these markers with the brain SAA expression was established (Fig. 2 c, f, i). In sham mice, we did not find Ly-6B.2, TLR4, or CD11b/c positive cells in the cortex, hippocampus or thalamus (data not shown).

Detection of SAA mRNA and protein in the mouse brain following TBI

To further investigate whether SAA was expressed in the brain, we performed immunohistochemistry and *in situ* hybridization targeting *Saa1* mRNA at several time points post-TBI. We observed fluorescent puncta of *Saa1* mRNA in the cortex, hippocampus, anterior thalamus, and corpus callosum after TBI. Fig. 3A shows a schematic representation

of the contralateral and the ipsilateral hemispheres with the cavity and pericontusional region at -2.06 mm from Bregma were *Saa1* mRNA was detected. Injured cortex, hippocampus, corpus callosum and anterior thalamus contained puncta staining in males and females (Fig. 3B, C). The contralateral hemisphere (Fig. 3D) and sham brains did not exhibit *Saa1* mRNA hybridization. SAA protein was more abundant in males compared to females in the cortex after TBI, with a significant difference at 1 dpi (Fig. 3, E, F, G), with a slight rise in SAA levels at 10 dpi. A correlation of SAA expressed in the brain and these inflammatory markers was shown in Fig. 3 e, f, g. In sham mice, neither *Saa1* mRNA puncta nor SAA expression in the brain was detected.

Microglia/macrophage expression of *Saa1* mRNA following TBI

Saa1 mRNA was detected in the injured cortex colocalizing with GFAP staining for astrocytes and microvessels (Fig. 4A, B). Images in Fig. 4 a1, b2-3 are showing that *Saa1* mRNA hybridization signal was detected inside of the microvessel, as evidenced by the colocalization of GFAP (green) and dapi (blue) at 6 h post-TBI. Within the hippocampus, *Saa1* mRNA was more abundant in glial cells, as GFAP positive cells (Fig. 4C, D, d1-2) and Iba-1 positive microglial cells (Fig. 4E, e1-2). We also identified *Saa1* mRNA inside of the macrophages in the cortex surrounding the lesion. *Saa1* mRNA puncta are also detected in areas containing fiber tracts at the corpus callosum (Fig. 4F). The corpus callosum (cc) contained more puncta aligned in parallel rows, predominantly around nuclei of cells as evidenced by the colocalization of dapi; however, it did not colocalize with Iba-1 marker (Fig. 4F, f1-3). The results obtained by the RNAscope in situ hybridization reveal which cells express *Saa1* mRNA in the brain, evidencing an extrahepatic production induced by TBI. The semi-quantitative analysis showed that males express more *Saa1* mRNA positive puncta in the cortex at 1 dpi than females. *Saa1* mRNA is and located mainly in vessels, astrocytes, and microglia/macrophages with a peak at 1 dpi (Table 1). However, SAA protein expression is prevalent in microglia/macrophages cells of males at 1 and 3 dpi compared to females (Table 1).

SAA protein expression in microglia/macrophages is more predominant in males at 1 day post-injury

SAA protein was observed in Iba-1 positive cells in the hippocampus (Fig. 5 A-C, c1-3) and in the border of the lesion in the primary somatosensory cortex (Fig. 5 D-F, f1-4) at 1 dpi. SAA protein colocalizes with microglia/macrophages with hypertrophy/bushy and pseudopodia morphology (Fig. 5c1-3), or with amoeboid round microglia at 1 dpi (Fig. 5 f1-4). Overall this suggests that activated microglia/macrophage cells express SAA predominantly in males in the injured cortex (Table 1).

DISCUSSION

To our knowledge, this is the first preclinical study that identifies SAA expression in the brain of male and female mice after TBI. Although the liver is the primary site of the production of SAA, there is growing evidence in animal models and cell culture experiments that SAA synthesis is also extrahepatic (Kovacevic et al. 2008; Ramadori et al. 1985a; Upragarin et al. 2005). Previously, we demonstrated that hepatocytes expressed high levels

of SAA as a consequence of TBI, and SAA was immediately released into the bloodstream (Villapol, 2015). Recently, we showed how serum levels of SAA protein correlate with the severity of brain injury, suggesting that SAA could be used as a novel biomarker for acute TBI (Wicker et al. 2019).

In this study, we analyze SAA expression in the liver, blood, and brain in males and females mice at several timepoints post-TBI. We have not selected females in a particular stage of the sexual cycle before TBI, because previous research has shown that random stages of the estrous cycle in female mice did not increase in neuroprotective and inflammatory effects (Bramlett and Dietrich 2001) or the variability in performance (Tucker et al. 2017; Tucker et al. 2016) compared to male mice. However, we observed that blood SAA levels have a sex-dependent production in response to brain damage. Moreover, *Saa1* mRNA and protein expression were found in the injured brain in a sex-dependent manner, at the same time that the neuroinflammatory response (Villapol et al. 2017). Our results demonstrate that SAA is expressed in brain regions around the cortical impact site. We used *in situ* hybridization associated with immunohistochemistry as a mean to detect cell-specific mRNA expression, as we have previously described (Fe Lanfranco et al. 2017; Lanfranco et al. 2017). *Saa* mRNA expression was located in vessels and microvessels in the cortex, as well in different glial types such as microglia and astrocytes, as indicated in Fig. 4. *Saa1* mRNA positive cells are challenging to quantify from their punctate detection. For this reason, we quantified the density of cells that express SAA protein in the damaged cortex at different times after TBI in males and females. We found that males exhibit more SAA protein expression in the injured somatosensory cortex than females.

Recently, we have shown female mice showed decreased inflammation and cell death after TBI, as compared to male mice (Villapol et al. 2017). We found that TBI causes less microglia activation (Iba-1+ cells) and infiltrated macrophages (F4/80+ cells) in females compared with the rapid and pronounced cortical microglia/macrophages activation and pro-inflammatory cytokines (IL-1 β and TNF α) in males. Besides, we have demonstrated that female mice show less enhanced astrogliosis, neuronal death, and increased lesion volume through 7 dpi compared to males (Villapol et al. 2017). In the present work, we showed that females show a lower number of inflammatory cells, neutrophils, and monocytes/macrophages than males at 1 dpi, supporting again that females are more protected against peripheral inflammation in response to an acute TBI. Other studies have also observed that females are resistant when compared to male to the effects of brain injury and showed a decreased microglial activation, reduced inflammatory gene response, and lower blood-brain barrier permeability after TBI (Loram et al. 2012; Santos-Galindo et al. 2011; Villapol et al. 2017).

These previous observations are associated with the results of this study. Male mice showed higher levels of SAA in blood hours after TBI compared to females, and a similar trend was also observed in the liver. Acute SAA levels following TBI are correlated with the strong glial activation and increased of infiltrated inflammatory cells. We have also shown how females have a decreased neuroinflammatory response compared to males after damage (Villapol et al. 2017), and this fact may affect the expression of SAA. Another critical role of SAA is its chemoattracted properties and stimulator of cell infiltration to injury sites (Uhlir

and Whitehead 1999a). Here, we found a correlation between the peak of SAA positive cells in the brain and the number of infiltrated neutrophils and inflammatory cells, higher in males than females (Fig. 2).

Moreover, we observed microglia and macrophages cells expressing SAA (mRNA and protein) in the brain and particularly predominant in the brains of males. Intriguingly, SAA production has been associated with microglial cells and their proinflammatory role in several cell culture studies (Facci et al. 2018; Yu et al. 2014). SAA stimulated the expression of proinflammatory cytokines in primary microglia cultures, and intracerebral injection of SAA also induced the activation of microglia (Barbierato et al. 2017; Facci et al. 2018; Yu et al. 2014).

Further, clinical studies have concluded that women fare better after TBI than men (Moore et al. 2010; Saverino et al. 2016; Slewa-Younan et al. 2008), and with lower rates of injury across all age groups and severity of injury (Cogan et al. 2019). However, others have shown that gender-dependent differences in the context of TBI are controversial concerning the vulnerability of damage, response to treatment, and outcomes (Liossi and Wood 2009; Mollayeva et al. 2018; Ratcliff et al. 2007).

Peripheral immune response contributes to the infarct volume associated with sex differences in animal models of stroke (Dotson et al. 2015). Sex differences were demonstrated in the recruitment of T lymphocytes, natural killer cells, and monocytes into the CNS (Santos-Galindo et al. 2011). The effects of SAA on regulating inflammatory cells through Toll-like receptor-2 in a model of sarcoidosis, suggests that SAA is produced, induced and/or released by these cells during inflammation (Chen et al. 2010). In addition to the pro-inflammatory properties of SAA, it has also been characterized as an amyloid precursor protein (Uhlir and Whitehead 1999b). SAA was detected in dementia and multiple sclerosis brains, together with pro-inflammatory cytokines (Elovaara et al. 1986; Kisilevsky 1997). SAA was found at high concentrations in human brain tissues together with brain inflammatory cytokines in dementia and AD patients (Chen et al. 2016; Liang et al. 1997), suggesting that SAA can be locally produced in a neuroinflammatory environment. Our results show for the first time that SAA is expressed in the brain after TBI, but its role remains unknown. We suggest that SAA expressed by microglia/macrophages may participate in the formation of A β peptides, AA amyloid fibrils, or in the synthesis of amyloid deposits years after TBI. We suggest that SAA may play an essential role in the link between TBI and the development of AD. Together, our findings provide evidence that acute SAA expression in the brain could be the bridging the gap between the neuroinflammatory response to TBI and the development of fibrinogen, fibril, fibrillar AA amyloid, or diffuse plaques observed chronically following brain injury. Importantly, SAA plasma levels could help to identify patients with an increased risk of developing neurodegenerative disorders.

In summary, our work sheds further light on the importance of the sex differences in the acute inflammatory phase, and SAA production post-TBI. We described that SAA is expressed in the brain as a consequence of TBI, predominantly in males. This suggests that the accumulation of SAA may aggravate the neuroinflammatory cascade and the initial stages of degenerative diseases associated with brain trauma. This sexual discrepancy has to

be considered when we are designing treatments to target inflammatory pathways, recognizing that the response of females is not as fast or reliable compared to males. Further studies will be necessary to establish whether the SAA expression in the brain enhances aggregation and persistence of amyloid protein. In conclusion, the impact of the potential role of SAA in the neuroinflammatory response after TBI merits further investigation. Our study opens the door for future studies to explore non-aggregating peptides or inhibitors of SAA for reducing brain inflammation after brain injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. SAA levels in blood and liver increase in response to brain injury.

Western blot analysis (A, B) showing SAA protein expression (12 kDa) in serum in male sham mice, but not detectable in females. At 6 h post-TBI, blood SAA levels were higher in males compared to females, and peak at 1 dpi with no differences between both sexes. SAA levels return to sham levels at 3 to 10 dpi. SAA expression in the liver has a similar pattern, but there are no significantly early sex differences (C, D). Quantification of SAA protein expression is normalized by using stain-free gels. Mean $\pm$ SEM, * p <0.05 males (M) vs. females (F), and ### p <0.001, ## p <0.01, peak at 1-dpi vs. rest of timepoints, N.D.=not detectable, n =5/group.

Figure 3. Distribution of SAA mRNA and protein positive cells in the injured brain. Schematic drawing of coronal brain section showing the injured regions after TBI corresponding to the cavity (brown) and the pericontusion (red) in the ipsilateral hemisphere, and non-injury contralateral hemisphere at -2.06 mm to Bregma (A). Distribution in the ipsilateral hemisphere *Saa1* mRNA puncta detected in the cortex (cx), hippocampus (hp) and thalamus (Th) of male and female brains (B, C). Representative brain section through the contralateral hemisphere denoting absence of *Saa1* mRNA puncta expression (D). Probe for negative (E) and positive controls (F and high magnification in f) for RNAscope. The quantitative graph (G) indicates the time course of SAA protein positive cells in the cortex

of sham and TBI mice, with a peak of expression at 1 dpi higher in males compared to females. Western blot analysis (H) showing SAA protein expression (12 kDa) in the brain in male and female mice, and in the liver from sham and injured brains as positive controls. SAA protein expression is located along the border of the pericontusional cortex and increases in males (I) compared to females (J) at 1 dpi. Dapi (nuclei, blue), and SAA protein and mRNA (red). Scale bar: 100 μm (B, C), and 50 μm (E, F). Mean $\pm$ SEM, ** $p < 0.01$ males vs. females, $n = 5/\text{group}$.

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Figure 4. Cell-specific localization of *Saa1* mRNA in the cortex, hippocampus and corpus callosum after TBI.

Representative images of coronal male brain sections show the distribution of red dots (*Saa1* mRNA) post-TBI. Positive *Saa1* mRNA (A) hybridization colocalized with GFAP (B) positive cells with vessels morphology (a1, b1-3) in the cortex at 6 h post-TBI, and hippocampus (C, D) displaying astrocytic morphology (d1, d2) at 1 dpi. Representative images show how *Saa1* mRNA positive cells in the hippocampus colocalize with Iba-1 (microglia/macrophages) at 3 dpi (E, e1, e2) and in the corpus callosum detected with red dots surrounding the nucleus (F, f1-f3). Panels are magnifications of the areas shown by

white boxes (a1, b1-3, d1, d2, e1, e2, f1-3). Scale bar: 100 μm (A, B), 50 μm (a1, b1, C, D, E, F), and 20 μm (b2, b3, d1, d2, e1, e2, f1-3).

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Figure 5. SAA protein expression is located in microglia/macrophages in the injured cortex and hippocampus after TBI.

Representative images show the injured hippocampus stained with SAA antibody (A, red) and with Iba-1 (B, microglia/macrophages, green) and counterstained with Dapi (blue) (C). SAA protein expression in the cortex (D) double-stained with F4/80 positive macrophages (green) (E, F) after 3 dpi in males. Panels are magnifications of the areas shown by white boxes (c1-3, f1-6). The Iba-1 and F4/80 positive cells colocalize with SAA and are displaying an activated (c1-c3) or phagocytic (f1-6) morphology. Scale bar: 50 μ m (A-C, D-F), and 20 μ m (c1-3, f1-6).

Table 1.

Colocalization of SAA with cell-type markers for microglia/macrophages (Iba-1) and GFAP (astrocytes/vessels) in the injured cortex of males and females.

MALES								
	Iba-1 (microglia/macrophages)				GFAP (astrocytes/vessels)			
	6h	1d	3d	10d	6h	1d	3d	10d
<i>mRNA Saa1</i>	+	+	++	+/-	+	+	++	+
<i>Protein SAA</i>	+	+++	++	+	+	+	+	-
FEMALES								
	Iba-1 (microglia/macrophages)				GFAP (astrocytes/vessels)			
	6h	1d	3d	10d	6h	1d	3d	10d
<i>mRNA Saa1</i>	+	+	-	+/-	+	+	+/-	-
<i>Protein SAA</i>	-	++	+	-	+/-	+/-	+	-

The degree of colocalization is graded as; +++ (high), ++ (moderate), + (occasional), +/- (in rare cases), and - (non existing).