Received: 24 May 2023 | Revised: 16 August 2023 | Accepted: 12 September 2023

DOI: 10.1096/fj.202301035R

RESEARCH ARTICLE

The FASEB Journal

Acute stress modulates the outcome of traumatic brain injury-associated gene expression and behavioral responses

Johanna Christina Reiners[1] | **Laura Leopold**[1] | **Vera Hallebach**[1] | **Daniela Sinske**[1] | **Philip Meier**[2] | **Mattia Amoroso**[3] | **Dominik Langgartner**[3] | **Stefan O. Reber**[3] | **Bernd Knöll**[1]

[1]Institute of Neurobiochemistry, Ulm University, Ulm, Germany

[2]Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland

[3]Laboratory for Molecular Psychosomatics, Department of Psychosomatic Medicine and Psychotherapy, Ulm University Medical Center, Ulm, Germany

Correspondence
Bernd Knöll, Institute of Neurobiochemistry, Ulm University, Albert-Einstein-Allee 11, Ulm 89081, Germany.
Email: bernd.knoell@uni-ulm.de

Funding information
Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: 251293561, 406037611 and 441734479

Abstract

Psychological stress and traumatic brain injury (TBI) result in long-lasting emotional and behavioral impairments in patients. So far, the interaction of psychological stress with TBI not only in the brain but also in peripheral organs is poorly understood. Herein, the impact of acute stress (AS) occurring immediately before TBI is investigated. For this, a mouse model of restraint stress and TBI was employed, and their influence on behavior and gene expression in brain regions, the hypothalamic–pituitary–adrenal (HPA) axis, and peripheral organs was analyzed. Results demonstrate that, compared to single AS or TBI exposure, mice treated with AS prior to TBI showed sex-specific alterations in body weight, memory function, and locomotion. The induction of immediate early genes (IEGs, e.g., c-Fos) by TBI was modulated by previous AS in several brain regions. Furthermore, IEG upregulation along the HPA axis (e.g., pituitary, adrenal glands) and other peripheral organs (e.g., heart) was modulated by AS–TBI interaction. Proteomics of plasma samples revealed proteins potentially mediating this interaction. Finally, the deletion of *Atf3* diminished the TBI-induced induction of IEGs in peripheral organs but left them largely unaltered in the brain. In summary, AS immediately before brain injury affects the brain and, to a strong degree, also responses in peripheral organs.

KEYWORDS

acute stress, ATF3, HPA axis, immediate early gene, traumatic brain injury

Abbreviations: ACTH, adrenocorticotropic hormone; AS, acute stress; ATF3, activating transcription factor 3; BDNF, brain derived neurotrophic factor; CNS, central nervous system; contra, contralateral; Cort, corticosterone; HPA, hypothalamic pituitary adrenal axis; ipsi, ipislateral; IEG, immediate earyl gene; NMDA, n-methyl D-aspartate; NSS, neurological severity score; OF, open field; PTSD, posttraumatic stress disorder; PVN, paraventricular nucleus; SA, spontaneous alternation; TBI, traumatic brain injury; TF, transcription factor; wt, wildtype; YM, Y maze.

1 | INTRODUCTION

In developed countries, injuries occurring through, for example, car accidents are a main cause of death in young adults. In this age group, traumatic brain injury (TBI) is a major cause of disability, morbidity, and mortality.[1] However, in the majority of patients (approx. 70%–90%), TBI is considered mild (mTBI) with a Glasgow Coma Scale score of 13–15.[2,3] Following TBI, patients frequently present neuropsychiatric problems (e.g., depression, anxiety) and substance abuse as a post-TBI sequel.[4,5] In turn, previous mental health problems or substance abuse elevate a person's risk of experiencing TBI.[6–8]

So far, the impact of a patient's stress load before TBI has been largely neglected when predicting outcomes following mTBI. Such pre-TBI stress may interfere with recovery from mTBI, possibly predisposing patients to neuropsychiatric disorders in response to injury. Indeed, a recent study in human mTBI patients showed that stressful events (e.g., life-threating accidents and robberies) may predispose patients with mTBI to develop mental health issues.[8] Research in this field is of particular importance for the treatment of posttraumatic stress disorder (PTSD), a disorder frequently affecting combat veterans after experiencing extreme physical trauma.[9] Notably, the effects of stress on TBI are not always negative. In rodent models, unpredictable chronic stress during adolescence may have a protective impact on TBI-induced cognitive deficits.[10]

Currently, the molecular interaction between psychological stress and TBI is poorly understood. However, one mechanism by which these different stressors might be connected is through activation of the hypothalamic–pituitary–adrenal (HPA) axis, including cortisol release (corticosterone in mice) from the adrenal cortex. Indeed, rodent models have identified HPA axis modulation, altered corticosterone, and brain-derived neurotrophic factor (BDNF) release as factors mediating the interaction of stress and TBI.[11–15] Congruent with this, approximately one-third of people exposed to TBI develop neuroendocrine deficits, as shown, for example, by hypopituitarism.[16] Notably, as shown in rodent models, the interaction of stress and TBI can be targeted by cellular stress-modulating compounds. For instance, salubrinal, protecting from ER stress, can reduce impulsive-like behavior induced in a repetitive rodent TBI model.[17]

Currently, there is only limited research investigating molecular mechanisms behind the interplay of previous stress exposure and subsequent mTBI. In two reports, pre-exposure to severe stress aggravated TBI-associated long-term deficits (e.g., learning), such as learning in a rodent model.[18,19] While these few available animal studies address behavioral effects, changes in gene expression have not been investigated in great detail. Furthermore, there is limited knowledge on how individual stress or the combination of two stressors (AS and TBI) affects gene transcription in peripheral organs. In this study, such changes in gene expression were investigated in a mouse model combining acute (restraint) stress (AS) pre-exposure with a mTBI model. Previously, it was shown that the individual application of restraint AS or TBI induced a rapid (within a few minutes) transient upregulation of immediate early genes (IEGs) within few minutes in the brain.[20–25] IEGs encompass *c-Fos, FosB, Jun, Npas4, Arc*, and Egr family members (*Egr1, Egr2, and Egr3*). Furthermore, *Atf3*, encoding the activation transcription factor 3 (ATF3), is an injury- and stress-responsive IEG that is upregulated by both AS and TBI.[25–30] ATF3 provides neuroprotection in several trauma conditions, including TBI, stroke, and nerve injury, as revealed by *Atf3* mutant mice.[31–33] In the literature, *Atf3* is described as a regeneration-associated gene (RAG), and its upregulation is mainly associated with pro-regenerative functions.[31,32,34] Due to the neuroprotective functions of ATF3, *Atf3* mutant mice were expected to present an exacerbated phenotypes following TBI alone and in combination with AS. Therefore, *Atf3* mutant mice were analyzed to see whether this gene regulator provides a transcriptional link to connect stress integration in combination with TBI.

In neurons, IEG upregulation is indicative of neuronal activation and enhanced firing.[35,36] So far, IEG upregulation upon psychological stress or physical trauma in peripheral organs has not been demonstrated. Herein, we show that TBI upregulates IEGs in several brain regions and peripheral organs, which is modulated by prior AS exposure in mice. Furthermore, AS exposure before TBI affects TBI-associated behavioral changes and adrenal corticosterone release, which is in part regulated by ATF3. Interestingly, deletion of *Atf3* significantly diminished this IEG induction in peripheral organs but not as much in the brain, which further influences TBI-associated behavioral changes. In summary, this data shows that a single previous stress exposure can modulate TBI outcomes on a molecular and behavioral level not only in the brain but also in peripheral organs.

2 | MATERIALS AND METHODS

2.1 | Mice

Constitutive *Atf3* mutant mice ($Atf3^{-/-}$) on a C57Bl/6 background were a kind gift of Dr T. Hai.[37] The *Atf3* mutant allele does not produce any ATF3 protein, for

instance, in the liver[37] or brain.[27,32] For genotyping, a published protocol was followed.[37] As a control for genotype, offspring harboring two wildtype (wt) *Atf3* alleles (*Atf3*$^{+/+}$) were used. Wt and *Atf3* mutant animals were littermates derived from breeding of two *Atf3* heterozygous parents. All experiments were in accordance with institutional guidelines and German animal protection laws and were approved by the regional government authority (Number: 1423; Regierungspräsidium Tübingen, Germany). Mice were kept with free access to food and water in a pathogen-free animal facility at Ulm University, with a 12 h day-night shift (summer: 6 a.m.–6 p.m., winter: 5 a.m.–5 p.m.), appropriate temperature, and humidity. The health conditions of animals were checked every day.

2.2 | TBI model

A modified weight-drop-based TBI model[38,39] was applied, compared to our previous reports.[22,27,40] Here, wt and mutant mice of both sexes were used at the age of 14–15 weeks after birth. The average weight of animals was as follows: wt males (29.7 ± 2.6 g), *Atf3* mutant males (28.6 ± 2.5 g), wt females (23.5 ± 1.8 g), and *Atf3* mutant females (22.6 ± 1.7 g). Mice were anesthetized with sevoflurane inhalation, and autonomic reflexes were checked. Upon absence of reflexes, animals were injected with buprenorphine (Temgesic; 0.04 mg/kg). Next, the skin over the skull was shaved and cleaned with ethanol. After this, the skull was exposed by applying a skin incision with a scalpel (approx. 1 cm in length). The head of the animal was placed under the weight-drop apparatus, in which the skull was secured by a holding frame. Using the 3-axis mobile platform of the weight-drop apparatus, the impactor was positioned over the left cortical (ipsilateral) hemisphere (from the bregma, $x = +3.0$ mm, $y = -2.0$ mm, $z = 0.0$ mm). mTBI was applied by dropping the weight of the impactor (120 g, ∅ tip: 3 mm) from a height of 40 cm. To induce a mild TBI, we applied a spacer providing a mechanical stop to the impactor that allows for a maximum penetration depth of 2.5 mm. The overall exposure time of mice to sevoflurane did not exceed 15 mins. The skin was sutured with three stitches using a proline 6.0 surgical thread. The animals were then transferred to a recovery cage with ad libitum access to food and water. Buprenorphine was injected prior to the beginning of the dark phase and then again 12 h later. All surgeries were performed on a heating pad (pre-warmed to 37°C) and lasted approximately 10–15 min/mouse. For sham-treated animals exactly the same procedure (shaving, anesthesia, analgesia, skin incision, and skin suture) was employed except for the actual TBI impact. TBI was always performed in the morning between 9 and 12 a.m.

2.3 | Acute stress

Mice were split into two groups: Either a control or an acute stress group (AS). Control animals were kept in their home cage, while animals in the stress group were restrained in well-ventilated 50-mL falcon tubes and left undisturbed in the dark under an opaque box for 45 min.[25,41] After the restraint period, animals were transferred into their home cage for 20 min and thereafter received either a sham surgery (AS group) or TBI (AS + TBI group). AS was performed between 9 and 12 a.m.

2.4 | Behavioral tests and survival analysis

All behavior tests were performed between 9 a.m. and 1 p.m. Tests were performed in the order OF, 1 h break, NSS (odd days), or YM, 1 h break, and ladder walk (even days).

2.4.1 | Body weight

Body weight was measured before receiving AS or anesthesia, 6 h after, and then daily for 7 days (always after completion of the behavior tests).

2.4.2 | Neurological severity score

The neurological severity score (NSS) was performed as before.[40] The NSS includes 10 tests: 1. exit circle; 2. seeking behavior; 3. Paresis; 4. straight walk; 5. startle reflex; 6. beam balancing (7 mm × 7 mm); 7–9. beam walk (3 cm, 2 cm, and 1 cm wide stick); and 10. round stick balancing (5 mm diameter). On average, it took 5–10 min/mouse to complete all 10 tests. For a failed test, one point was recorded, while a passed test was counted as zero. Therefore, a NSS score of "0" indicates that the mouse passed all tests, whereas a score of "10" indicates maximum neurological impairment. The NSS was measured 2 days pre-TBI, as well as 6 h, 1 day, 3 days, and 5 days post-TBI or sham surgery.

2.4.3 | Y maze

The Y maze (YM) was made of opaque light gray plastic and had three identical arms (A, B, C) spread at 120° from each other.[41] Each mouse was placed at the end of one arm and allowed to explore the maze for 5 min. After each trial, the arena was cleaned with 70% ethanol and dried.

The percentage of spontaneous alternations (SA; e.g., A > B > C) among all moves was analyzed and calculated as a percentage of the total number of triplets. The Y maze was performed 1 day pre-TBI, 2 days, 4 days, and 6 days post-TBI or sham surgery.

2.4.4 | Open field

To determine locomotor and exploratory behavior, the open field (OF) was performed 2 days pre-TBI, 1 day, 2 days, 3 days, 5 days, 7 days post-TBI or sham surgery. Mice were placed in the middle of a square-shaped OF arena (50 × 50 cm) and tracked for 15 min with a video camera. The Viewer III software (Biobserve, Bonn, Germany) determined the overall track length as reported before.[22,25,41]

2.4.5 | Ladder walk

To assess the post-traumatic motor skills, a ladder walk test was performed at 1 day pre-TBI, 2 days, 4 days, and 6 days post-TBI as before.[22] In the ladder walk, mice walked over a horizontal ladder to reach the other side. The ladder walk had a length of 60 cm with a space of 1 cm between rungs. Each animal crossed the ladder three times while they were recorded with a Samsung NX1000 camera. The recorded videos were analyzed at ¼ of the original speed to count slips and the time needed to cross the ladder for each run. The average number of slips per second is depicted.

2.5 | Dissection and quantitative real-time PCR (qPCR)

Entire brains were cut into 1 mm-thick coronal sections using a tissue chopper apparatus. Tissue slices were transferred to a Petri dish with ice-cold PBS, and single brain sections were separated from one another under a microscope. Brain regions of interest, including the hypothalamus, cortex, and hippocampus, were dissected using tungsten needles and collected from different slices. Total RNA was isolated with the RNEasy Kit (Qiagen) according to the manufacturer's instructions. The reverse transcription was performed with 1 µg of RNA using reverse transcriptase (Promega) and random hexamers. qPCR was performed using the Roche Light Cycler 480 (Roche) by mixing 2 µL cDNA, specific primer pairs, and SYBR Premix Ex Taq (Tli RNase H Plus) PCR Master Mix (TaKaRa Bio Europe, Saint-Germain-en-Laye, France) in a total volume of 10 µL per well.[22,31] The LC480 II software was used to detect the cycle threshold values. Relative mRNA expression of each target gene was calculated relative to the housekeeping gene *Gapdh* (glycerinaldehyde-3-phosphate-dehydrogenase) with the ΔCt method. All experiments were performed in technical duplicates. Primer details are provided upon request.

2.6 | Proteomics

For the in-solution digest, 6 µg of plasma protein was reduced with 5 mM DTT (AppliChem, Darmstadt, Germany) for 20 min at RT and subsequently alkylated with iodoacetamide (Sigma-Aldrich, St. Louis, USA) for 20 min at 37°C. Trypsin (Thermo Scientific, Rockford, IL, USA) was added in a 1:50 enzyme-protein ratio and digested overnight at 37°C.

Employing an LTQ Orbitrap Elite system (Thermo Fisher Scientific, Bremen, Germany) online coupled to an U3000 RSLCnano (Thermo Fisher Scientific, Idstein, Germany), samples were analyzed as described previously.[42] Database search was performed using MaxQuant Ver. 1.6.3.4 (www.maxquant.org).[43] Employing the built-in Andromeda search engine,[43] MS/MS spectra were correlated with the UniProt mouse reference proteome set (www.uniprot.org). Carbamidomethylated cysteine was considered as a fixed modification, along with oxidation (M) and acetylated protein N-termini as variable modifications. False discovery rates were set on both, peptide and protein level, to 0.01. For statistical analysis, LFQ values were used and missing values imputed using the Perseus computational platform[44] with default settings. Regulated proteins were classified based on the Student's *t*-test (class I) and a significance B test.[43]

2.7 | Corticosterone (Cort.) measurement

Ex vivo ACTH stimulation of the adrenal glands was performed as described previously with minor modifications.[45] In brief, left adrenals were stored in ice-cold DMEM (DMEM/F-12, Life Technologies, Inc., Grand Island, NY) supplemented with 0.1% BSA. Afterwards, adrenal glands were halved, weighted, and each half was pre-incubated in 200 µL of DMEM/F-12 + 0,1% BSA (37°C, 95% O_2, 5% CO_2) for 1.5–2 h in one well of a 96-well plate. Then, medium was replaced by 100 µL of fresh DMEM/F-12 + 0,1% BSA and 25 µL of isotonic saline solution (basal: one half of left adrenal) or 25 µL of a 500 nM stock (final ACTH concentration per well: 100 nM; ACTH: other half of left adrenal) and adrenal

halves were incubated for 1 h at 37°C (95% O_2, 5% CO_2). After stimulation, supernatants were removed and stored at −20°C until further analysis. Samples were analyzed using a commercially available ELISA for Cort. (analytical sensitivity: <0.564 ng/mL, intra-assay and inter-assay coefficients of variation: ≤6.35%; IBL International, Hamburg, Germany). Cort. concentrations were calculated in relation to the respective weight of each adrenal explant.

2.8 | Statistics

All data were calculated and further compared in GraphPad Prism Software (v. 9.5.0). All values are expressed as mean ± SD unless otherwise indicated. Mann–Whitney test was applied to compare mean values in two groups. Kruskal-Wallis test with Dunn's multiple comparison was applied for single timepoint markers among multiple groups. Survival and Beam balancing were performed using the Fisher's exact test. Significance is indicated as: $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$.

3 | RESULTS

3.1 | AS modulates TBI-associated behavioral responses

The analysis of an AS–TBI interaction was started in wildtype (wt) mice grouped into four cohorts (sham, AS only, TBI only, and AS + TBI; Figure 1A). Of note, throughout the study, sham mice (gray) were subjected to sham surgery and pain medication (see materials and methods) thereby resulting in elevated baseline stress levels compared to 7 days after sham surgery (see Figure 3E) or completely untreated mice.[25] AS mice (green) only received a 45 min restraint in a falcon tube, followed by sham surgery, whereas TBI mice (red) received a TBI injury (Figure 1A). In the combined AS + TBI cohort (blue), mice were treated with AS for 45 min, followed by 20 min of rest, and TBI thereafter (Figure 1A). Mice were sacrificed at 1 h after TBI for qPCR studies or further analyzed using behavior tests starting at 6 h after TBI up until 7 days (Figure 1A).

In a first set of experiments, cohorts underwent the NSS. Here, a NSS of 0 reflects no neurological impairments (Figure 1B). In this study, a mild TBI was chosen, more closely reflecting the majority of TBI incidents in patients (see introduction). In agreement with this, mice from all four cohorts expectedly achieved scores close to 1 (Figure 1B), which is in contrast to our previously used more severe TBI model, where mice reached NSS values between 3 and 4.[22,31]

Next, changes in body weight after injury in relation to the starting weight were investigated (pre-injury, "pre"; Figure 1C). Sham mice of both sexes lost weight between 6 h and 7 days after sham treatment compared to pre-injury. This weight loss was increased in male and female mice subjected to AS. For mice receiving TBI alone, only female but not male mice lost more weight compared to the sham group (Figure 1C). The AS + TBI cohort showed a tendency to lose more weight, particularly at early post-TBI timepoints, compared to their sex-matched TBI groups (Figure 1C).

In the OF, AS mice demonstrated elevated activity as revealed by an increased relative track length (normalized to pre-injury; Figure 1D), in agreement with previous reports.[25] For TBI-treated animals, male and female animals behaved oppositely, with females decreasing and males increasing their activity after injury (Figure 1D). Of note, pre-exposure of female TBI animals to AS reversed the responses observed for TBI alone, with females now showing enhanced activity (Figure 1D).

The Y maze quantifies SAs indicative of cognitive deficits. In general, sham and AS cohorts enhanced the SA percentage over time (Figure 1E). In contrast, male and female mice subjected to TBI decreased SA in the Y maze at some timepoints, which was partially but not significantly improved in the AS + TBI cohort irrespective of sex (Figure 1E).

To asses for post-traumatic motor skills, the ladder walk was performed (Figure 1F). Compared to sham animals, AS-treated female animals slightly decreased the time to cross the ladder, while males were unaffected (Figure 1F). Male, but not female, TBI-treated animals responded with elevated crossing times compared to sham indicative of TBI-inflicted coordination impairments (Figure 1F). This was partially reversed when animals received AS before TBI. (Figure 1F).

3.2 | Induction of IEGs by trauma is modulated by previous AS exposure

Up until now, pre-exposure to AS altered TBI-associated behavioral responses (Figure 1). Both psychological (AS) and physical stress (TBI) are known to induce genes associated with neuronal activity, such as IEGs.[20–25] Hence, mRNA levels of typical IEGs were inspected in several brain regions (Figure 2). qPCR analysis was performed 1 h after TBI (see Figure 1A), a timepoint where IEG induction is typically observed after TBI.[22] In every brain region, the ipsilateral ("ipsi"; injury site) and contralateral

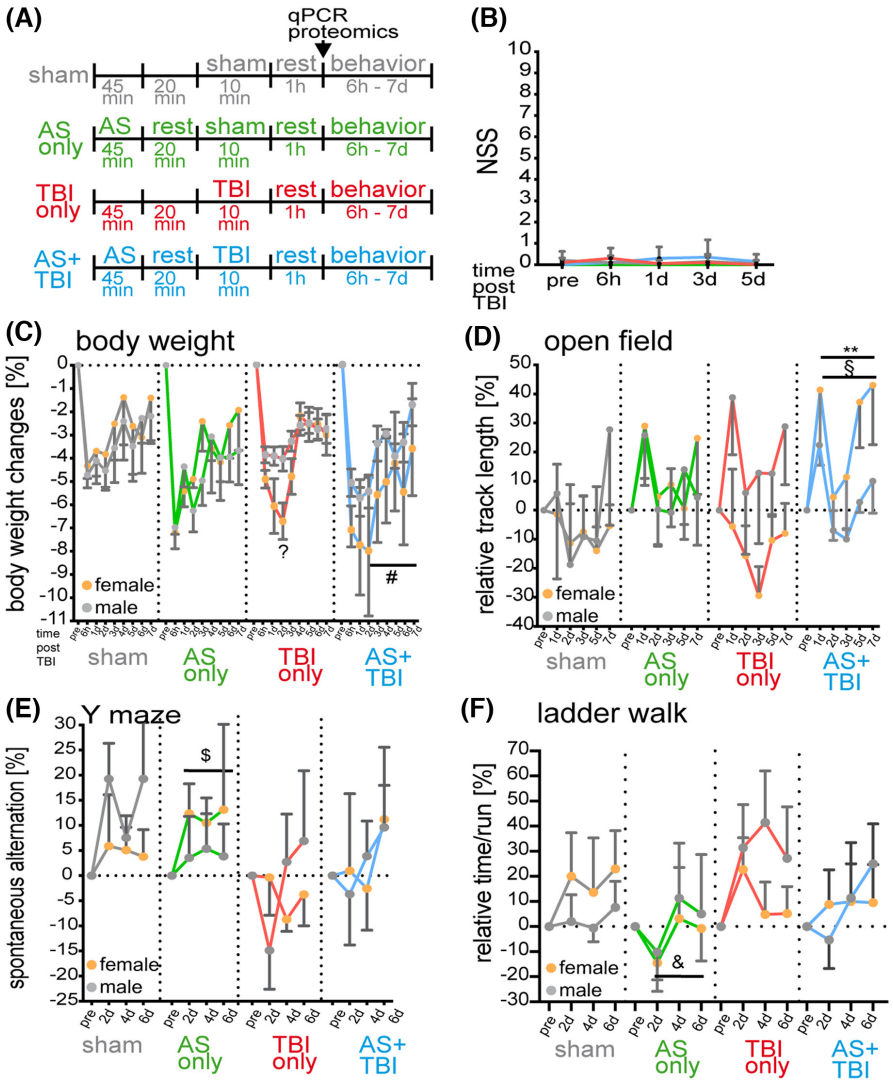

FIGURE 1 Previous acute stress (AS) affects traumatic brain injury (TBI)-associated behavior in wildtype mice. (A) Experimental scheme of the four cohorts (depicted with for different colors) employed. AS was applied for 45 min followed by 20 min of rest. After that TBI or sham surgery was applied and animals were sacrificed after 1 h (qPCR) or 7 days when they performed behavior tests at several timepoints between 6 h and 7 days. (B) In the neurological severity score (NSS) score all cohorts received scores between 0 and 1 (out of 10) indicative of a mild TBI. (C) Body weight was decreased in both females and males by AS alone and by TBI alone more strongly for females. Previous AS before TBI (AS + TBI) resulted in more pronounced weight loss compared to TBI alone particularly for females. (D) In the open field (OF), AS alone increased locomotor activity for both sexes whereas TBI only increased track length for males but decreased it for females. In females, AS + TBI resulted in increased OF activity compared to TBI alone. (E) In the Y maze, TBI treatment decreased the SA percentage compared to sham in both females and males. This decrease was slightly alleviated in AS + TBI animals. (F) In the ladder walk, TBI alone enhanced the crossing time in males but not females compared to sham. However, male mice treated with AS before TBI crossed the ladder walk faster. *N*-numbers were 9–10 animals/treatment (sham, AS, TBI, or AS + TBI) and 4-5/sex/treatment. # denotes significance between female AS versus female AS + TBI; * denotes significance between female TBI versus female TBI + AS; § denotes significance between female sham versus. female AS + TBI; $ denotes significance between females AS versus female TBI; & denotes significance between female sham versus female AS.? denotes significance between female TBI and male TBI. In (B–F) SEM is depicted.

("contra"; control site) hemispheres were investigated separately (Figure 2).

In the hippocampus, IEG induction of *c-Fos* (Figure 2A) and several other IEGs (heatmap, Figure 2B, top) was strongly induced by TBI. Typically for all IEGs in TBI mice, IEG mRNA levels were stronger on the ipsi- compared to the contralateral side (Figure 2B, bottom). Of note, in AS + TBI animals, we observed a reduction in IEG abundance for both the ipsi- and contralateral hippocampus, as exemplified by *c-Fos* (Figure 2A) but also other IEGs (Figure 2B). This highlights the important role of the hippocampus during stress processing and integration. In AS + TBI animals, almost no IEG response was observed on the contralateral side (see Figure 2A). Thus,

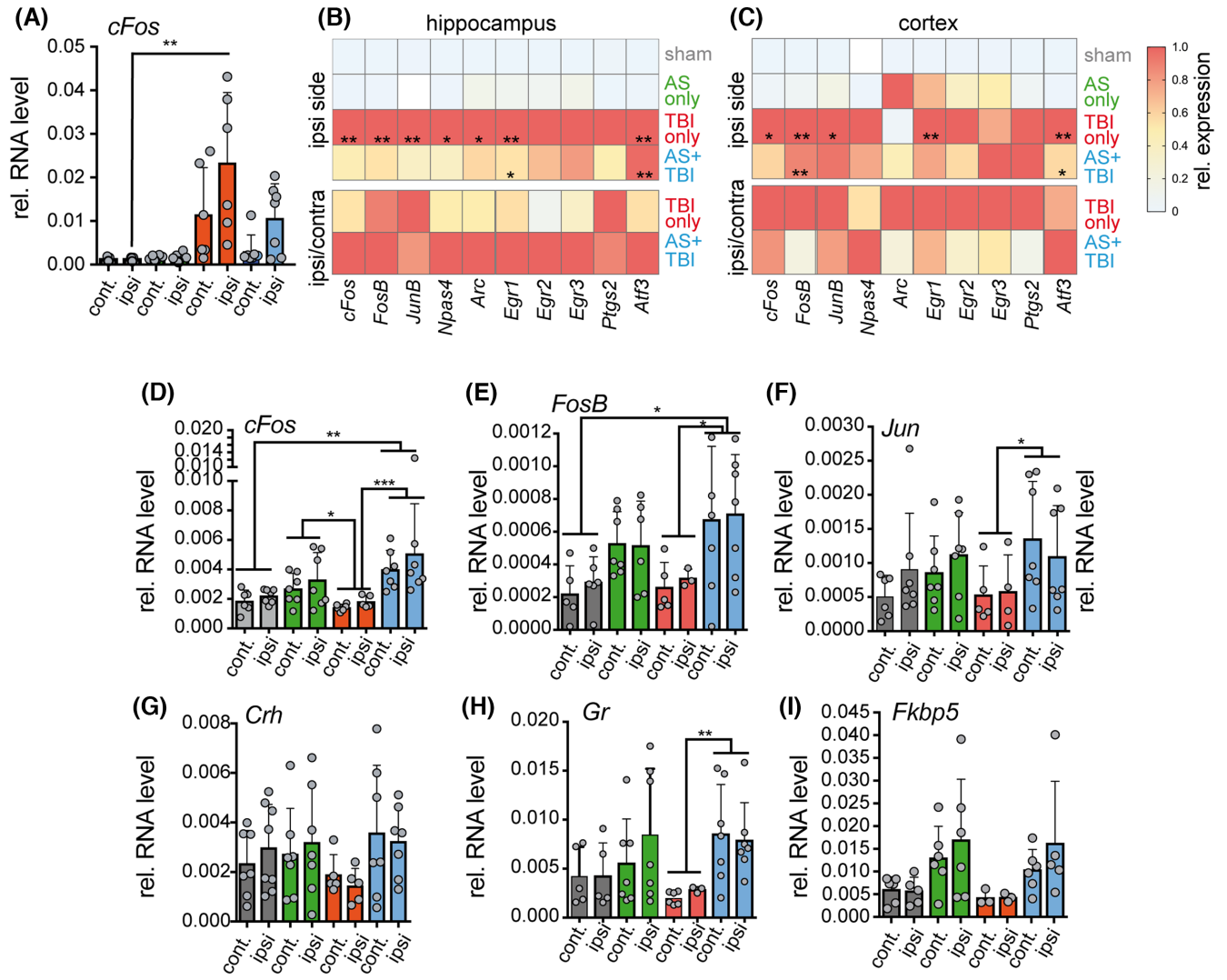

FIGURE 2 Interaction of acute stress (AS) with traumatic brain injury (TBI)-modulates immediate early gene (IEG) expression in different brain regions. (A, B) qPCR of IEG mRNA levels 1 h after TBI in the ipsi- and contralateral (cont.) hippocampus. *c-Fos* was induced by TBI alone more strongly on the ipsi- compared to contralateral site (A). This was reduced when TBI animals were pretreated with AS (A). Several IEGs were induced by TBI alone on the ipsilateral side but this was reduced by AS + TBI (B, top). When providing the ratio of mRNA levels between ipsi/contra, a higher ratio was noted for most IEGs for AS + TBI compared to TBI alone (B, bottom). (C) (top) In the cortex, AS alone induced some IEGs and TBI robustly induced the majority of IEGs. AS + TBI resulted in slightly weaker IEG levels compared to TBI alone. (bottom) The magnitude of IEG levels depicted by the ipsi/contra. Ratio was higher in TBI alone compared to AS + TBI. (D–I) In the hypothalamus 1 h after TBI, IEGs (D–F) were downregulated (D, F) or not affected (E) by TBI alone. AS + TBI upregulated those IEGs above sham levels in the ipsi and cont. site. Genes associated with glucocorticoid signaling (G–I) were reduced by TBI compared to sham whereas AS upregulated *Fkbp5* (I). AS + TBI elevated mRNA levels of all three genes compared to TBI alone. In (A, D–I) one circle depicts one animal analyzed. In (B, C) significance was calculated in relation to sham. *N*-numbers were as follows: $n = 6–7$ animals/treatment (hippocampus, cortex) and 4–7 animals/treatment (hypothalamus).

when calculating the ratio between ipsi/contralateral side for IEG induction, a stronger ratio was observed in AS + TBI compared to TBI-only animals (Figure 2B, bottom). The same tendency was observed in the amygdala (data not shown). In the hippocampus, AS alone did not induce IEGs in contrast to brain regions such as the hypothalamus (Figure 2D–F) or septal nucleus (data not shown). It also should be noted that AS-treated animals were sacrificed >2 h after AS onset (see Figure 1A). Thus, the peak of IEG induction (approx.1 h after AS onset) might have been exceeded or IEG expression is already receding as reported before.[25]

In the cortex (Figure 2C), TBI alone resulted in strong IEG induction, similar to the induction seen in the hippocampus (Figure 2B). Again, this was also reduced by a previous AS exposure. However, in the cortex the extent, of IEG reduction by AS + TBI was not as strong as in the hippocampus (Figure 2C). This emphasizes that AS is integrated

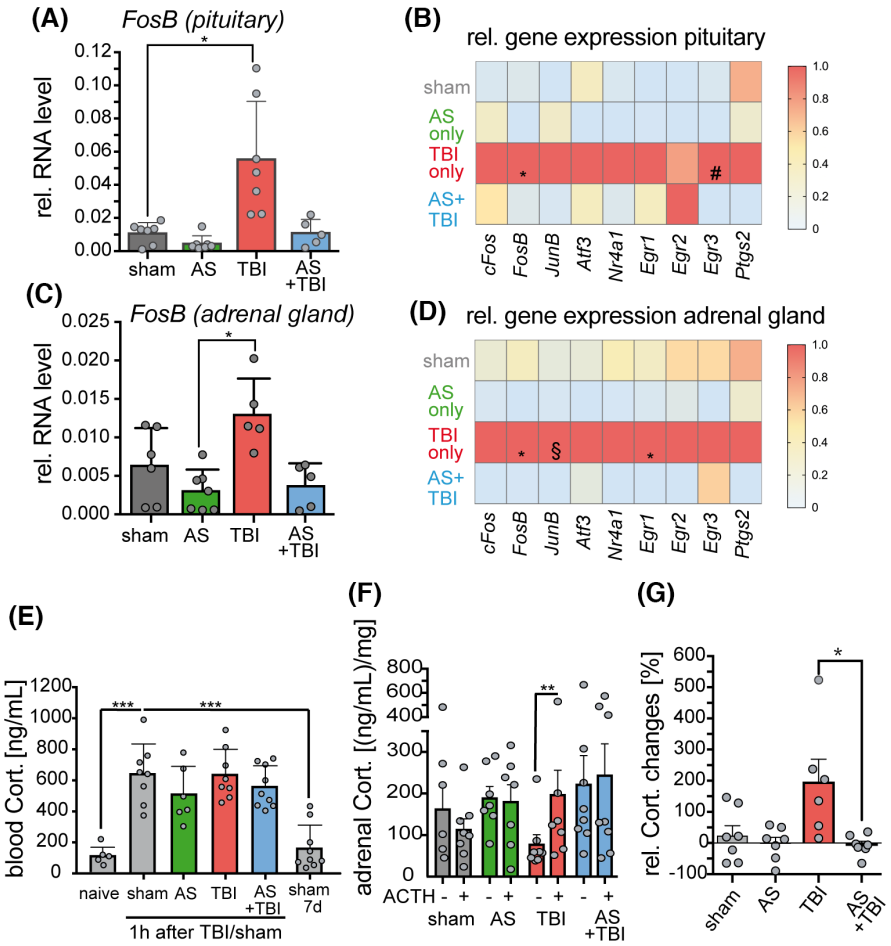

FIGURE 3 Traumatic brain injury (TBI) affects responses in hypothalamic–pituitary–adrenal (HPA) axis organs, which is modulated by acute stress (AS). (A–D) In the pituitary (A) and adrenal glands (C), *FosB* was induced at 1 h after TBI. AS alone reduced *FosB* levels in both organs compared to sham and suppressed TBI-induced *FosB* mRNA abundance (A, C). Similar to *FosB*, TBI induced several IEGs in the pituitary (B) and adrenal glands (D) which was diminished by previous AS except for *Egr2* in the pituitary (B). In the heatmaps, expression was normalized to sham condition. (E–G) Corticosterone (Cort.) levels were determined in the plasma (E) or in supernatants of ex vivo cultured adrenal glands (F, G) at 1 h after TBI if not indicated otherwise. In the blood, Cort. levels did not change between cohorts at 1 h after TBI. At 7 days post-TBI, Cort. levels went back to baseline indicating that also sham-treated animals were stressed at the 1 h timepoint (E). In (F, G) adrenal glands harvested at 1 h after TBI were cultured ex vivo in the presence of ACTH (+) or saline (−) and Cort. levels were measured in the supernatants. Only in TBI alone treated animals, adrenal glands responded with an ACTH-induced Cort. release whereas this was abolished by prior AS treatment (absolute values in F; relative Cort. induction in G). In (A, C; E–G) each dots reflects one animal. In (B, D) * depicts significance between sham versus TBI, # depicts significance between AS versus TBI and § depicts significance between TBI versus AS + TBI. In (E–G) SEM is depicted. *N*-numbers included 6–7 animals/treatment (adrenal gland and pituitary), 6–8 animals/treatment (CORT plasma), and 6–7 animals /treatment (adrenal stimulation).

and processed in a brain region-specific manner, suggesting that AS is not as strongly processed in the primary injury site of the cortex. Thus, AS is less effective in reducing the TBI-evoked IEG upregulation in the cortex.

Since HPA axis activity originates in the hypothalamus, IEGs (Figure 2D–F) and genes associated with HPA axis signaling (Figure 2G–I) were investigated. While IEGs were no longer upregulated at 2 h post-AS onset in the cortex and hippocampus, AS exposure resulted in some IEG induction in the hypothalamus, particularly obvious for *FosB* (Figure 2E). Furthermore, the combination of both stressors resulted in an amplification of the AS-induced response. TBI alone did not alter IEG expression in the hypothalamus (Figure 2D–F). Next, genes associated with HPA axis signaling, including Corticotropin releasing hormone (*Crh*), glucocorticoid receptor (*Gr*), and FK506 binding protein 5 (*Fkbp5*), were analyzed in the hypothalamus. AS alone only induced *Fkbp5* in both hemispheres (Figure 2I).

In contrast, *Crh*, *Gr*, and *Fkbp5* mRNA levels were not significantly altered between sham and TBI (Figure 2G–I). As noted for IEGs (Figure 2D–F), previous AS augmented expression of all genes in TBI animals compared to mice treated with TBI alone (Figure 2G–I).

In summary, TBI-associated gene expression in several brain regions is modulated by previous AS exposure. Furthermore, AS and TBI are processed and integrated in a brain region-specific manner.

3.3 | TBI induces IEGs in organs of the HPA axis

So far, analysis of TBI-associated gene expression responses has been predominantly restricted to the brain as the prime TBI target. In contrast, the immediate effects of TBI on peripheral organs are largely unexplored. Before, modulation of IEG abundance in the hypothalamus by AS alone and AS + TBI was observed (Figure 2). Next, it was of interest whether this gene expression response was proceeded to the remaining organs of the HPA axis (pituitary and adrenal gland) localized distantly from the TBI impact site (Figure 3).

In the pituitary, *FosB* levels were significantly elevated by TBI compared to sham (Figure 3A). Interestingly, as seen in the hippocampus (Figure 2A,B), AS exposure before TBI resulted in reduced *FosB* mRNA levels similar to sham levels (Figure 3A). This stress effect on TBI was observed for all IEGs except *Egr2* in the pituitary (Figure 3B).

Similar to the pituitary, the adrenal gland showed an almost identical expression pattern (Figure 3C,D). All nine IEGs were upregulated by TBI alone in the adrenal gland (Figure 3C,D). This suggests that TBI-induced propagation of an IEG response also reaches peripheral organs of the HPA axis, such as the adrenal glands. As seen before, when animals experienced AS before TBI, IEG mRNA levels were strongly reduced compared to TBI alone (Figure 3C,D). Of note, AS alone consistently lowered IEG levels compared to sham (Figure 3C,D), which is in line with the AS impact in the AS+TBI group.

3.4 | TBI effects on HPA axis activity

Given TBI's effect on IEG induction in the adrenal gland, we analyzed whether Cort. levels in the plasma (Figure 3E) or ex vivo Cort. release from the adrenal gland upon ACTH stimulation (Figure 3F,G) were affected. In the plasma, Cort. levels were identical in all four cohorts at 1 h after TBI/sham surgery (Figure 3E). Here, it should be mentioned that sham animals clearly showed a stress response (>600 ng/mL Cort.) compared to naive animals (approx. 132 ng/mL; Figure 3E; see also[46,47]) or sham animals at 7 d pi (100 ng/mL; Figure 3E).

In contrast to plasma Cort. levels (Figure 3E), TBI affected ACTH-induced Cort. release from the adrenal gland (Figure 3F,G). Here, ACTH was able to cause a significant elevation in the adrenal Cort. release exclusively in the TBI group. As seen for gene expression (Figure 3C,D), this response was not visible in AS + TBI animals; therefore, Cort. release by the adrenal gland was similar to sham- and AS-treated animals (Figure 3F,G). This suggests that TBI allowed the adrenal gland for Cort. release, whereas previous AS exposure before TBI reduced this response.

3.5 | TBI induces IEGs in peripheral organs

Next, it was of interest to analyze whether TBI and AS + TBI-treated animals also showed a response in other peripheral organs, including the heart and spleen, at 1 h after TBI (Figure 4).

Indeed, in the heart, TBI induced a consistent twofold to fourfold induction for several IEGs (Figure 4A–H). In AS-treated animals, in contrast, IEGs were no longer induced ~2 h after AS onset. Interestingly, when AS was applied before TBI (AS+TBI), IEGs could no longer be induced by TBI except for *ΔFosB* (Figure 4A–H). Furthermore, mRNA levels of the pro-inflammatory cytokine *Ccl2* were tested (Figure 4I). *Ccl2* was induced in TBI-treated mice but not in AS + TBI-treated mice, suggesting a reduced inflammatory response (Figure 4I). Furthermore, the stress-responsive gene *Fkbp5* showed a strong response to AS, independent of a subsequent TBI (Figure 4J). Last, markers associated with cardiac injury, including *Tpm2* (tropomyosin; Figure 4K) and *Anp* (atrial natriuretic peptide; Figure 4L), were analyzed.[48–50] Notably, both markers revealed a pattern that was similar to the pattern seen in IEG expression, with the lowest levels of both injury markers in the AS + TBI condition (Figure 4K,L). This might indicate a potential protective function of previous AS for subsequent TBI.

Besides the heart, AS and TBI also affected gene expression in the spleen (Figure 4M). Here, similar to the heart, TBI elevated the abundance of many but not all IEGs. Once more, co-application of AS and TBI lowered IEG induction by TBI alone except for *Atf3* (Figure 4M).

3.6 | Proteomics identifies proteins modulated by AS and TBI in the blood

So far, data suggest that previous AS modulates TBI-induced gene expression not only in the brain but also in peripheral organs (Figures 2–4). This implies a communication between the brain and peripheral organs, which might be accomplished by signaling proteins released from, for example, the brain and circulating in the bloodstream. To identify such signaling proteins, plasma

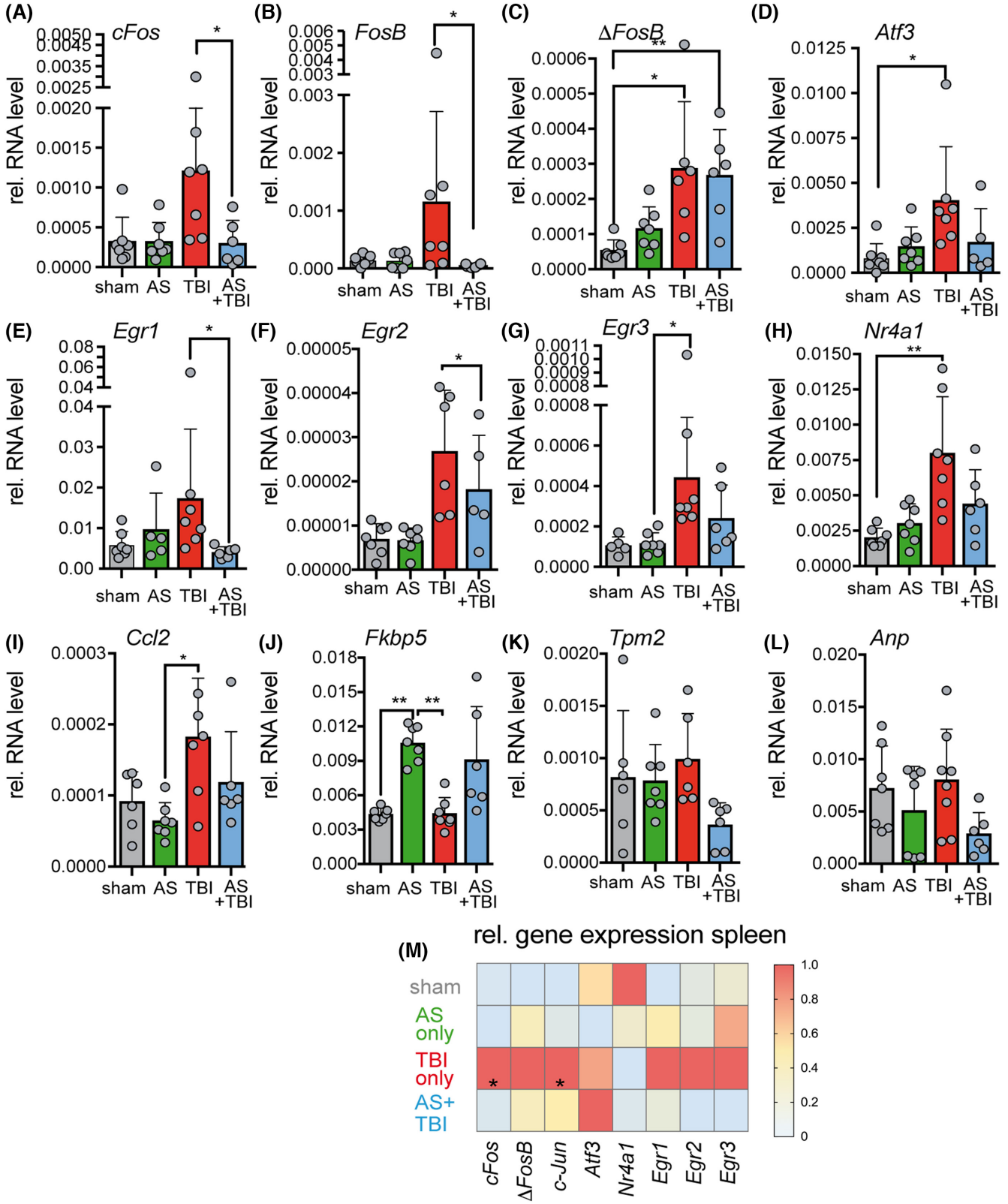

FIGURE 4 Traumatic brain injury (TBI)-induced gene expression in the heart and spleen is reduced by previous acute stress (AS). (A–L) qPCR was performed in the heart at 1 h after TBI/sham surgery. For all immediate early genes (IEGs) (A–H) and *Ccl2* (I), TBI alone resulted in upregulation of mRNA abundance. Prior exposure with AS before TBI (AS + TBI) resulted in reduced gene expression of IEGs (A–H) and *Ccl2* (I) compared to TBI alone. For AS alone, IEG levels were similar to sham. In contrast, *Fkbp5* was upregulated by AS and in AS + TBI mice (J). Two markers associated with cardiac integrity, *Tpm2* (K) and *Anp* (L) were lowest in AS + TBI-treated animals. (M) In the spleen many IEGs were upregulated by TBI alone 1 h after induction. This was reduced in AS + TBI mice with exception of *Atf3*. In (A–L) each dot reflects one animal (6–7 animals/treatment).

collected at 1 h after TBI/sham (see Figure 1A) from animals of all four cohorts ($N \geq 5$ animals/cohort) was subjected to proteomics analysis (Figure 5).

AS alone resulted in four proteins being either significantly upregulated (alpha-synuclein, Snca) or downregulated (vascular cell adhesion molecule 1, Vcam1; phosphatidylethanolamine binding protein 1, Pebp1; and mannosidase beta, Manba; Figure 5A,B). Treatment of animals with TBI alone resulted in a different protein set being significantly upregulated or downregulated (Figure 5C,D). The downregulated proteins included serine/threonine-protein kinase ATR (Atr, ataxia telangiectasia and Rad3-related) and Multimerin 1 (Mmrn1), a protein localized in the extracellular matrix.[51] TBI upregulated the abundance of nucleoside diphosphate kinase A (GM20390, NME1), angiotensin I converting enzyme (Ace), inter-alpha-trypsin inhibitor heavy chain H4 (Itih4), collagen type 1 alpha 2 (Col1a2), ADP-ribosylation factor 1 (Arf1), and proteoglycan 4 (Prg4; Figure 5C,D).

Co-treatment of mice with both AS and TBI resulted in four significantly regulated proteins (Figure 5E,F). One of them, Itih4 was also found in TBI alone (Figure 5C,D) thereby confirming in independent samples that this protein is present in the blood of TBI-treated animals. Besides Itih4, ferritin light polypeptide 1 (Ftl1) and serum amyloid a1 (Saa1) were upregulated by AS + TBI in relation to sham mice (Figure 5E,F). Of note, higher Saa1 levels were previously reported in TBI patients.[52] Apolipoprotein N (Apon) was downregulated approximately sixfold after AS + TBI (Figure 5E,F). Apon was also downregulated when comparing TBI- versus AS + TBI-treated animals (Figure 5G,H). When comparing TBI with AS + TBI, it was obvious that several proteasome subunits (Psmb3, Psma1, Psmb2) were more highly abundant in TBI-treated animals (Figure 5G,H) and this confirms previous data on proteasome subunits being upregulated by TBI.[53,54] Protein levels of proteasome subunits were reduced upon previous AS exposure in the AS + TBI cohort (Figure 5G,H). Finally, cadherin 1 (Cdh1) was also lower abundant in AS + TBI compared to TBI alone (Figure 5G,H).

Overall, proteomics identified several candidate proteins released by AS and/or TBI in the blood.

3.7 | ATF3 deficiency affects gene expression by AS and/or TBI

ATF3 is a transcription factor (TF) induced in neurons and other cells in rodent models by a variety of stressors.[27] Given that both AS and TBI target ATF3 abundance, it was analyzed whether ATF3 is a TF involved in regulation of AS and/or TBI-mediated IEG gene expression. So far, ATF3 was not identified as a major TF regulating IEGs in previous transcriptome studies.[32,55] In order to analyze the contribution of ATF3 during AS and/or TBI, several brain regions and peripheral organs were analyzed at 1 h after TBI/sham induction (Figure 6). For this, constitutive *Atf3* mouse mutants were employed,[32,37] in which *Atf3* is deleted from all cells (Figure 6). Indeed, in *Atf3* mutant animals, *Atf3* mRNA was strongly diminished in all tissues analyzed in this study (see all left columns in Figure 6A–E).

In the hippocampus (Figure 6A) and cortex (Figure 6B), a comparable gene expression pattern between wildtype (wt) and *Atf3* mutant mice ($Atf3^{-/-}$) was observed. Here, AS alone only induced selected IEGs in the cortex but not hippocampus of wt and *Atf3* mutant animals (Figure 6A,B). TBI alone resulted in a robust IEG induction in the hippocampus of wt animals (Figure 6A; see also Figure 2), which was similar for most IEGs in ATF3 deficient mice. In the hippocampus, AS reduced TBI-induced IEG induction in wt and $Atf3^{-/-}$ animals (Figure 6A). In contrast, TBI alone and AS + TBI resulted in comparable IEG induction in the cortex of wt and *Atf3* mutant animals (Figure 6B). Nevertheless, the extent of IEG induction in hippocampus and cortex for TBI and AS + TBI was slightly diminished in mutant animals compared to wt but never reached statistical significance (Figure 6A,B).

In previous results, TBI-induced gene expression in peripheral organs was modulated by previous AS (Figures 3 and 4). Thus, in the next step, the impact of ATF3 ablation on AS- and/or TBI-induced IEG induction was analyzed in the pituitary, adrenal gland, and heart (Figure 6C–E). In wt mice, TBI alone resulted in IEG induction in all three organs tested (Figure 6C–E; see also Figures 3 and 4). In contrast, TBI exposure did not induce IEG expression in all three organs of ATF3 deficient animals (Figure 6C–E). This points toward an important role of ATF3 in mediating TBI-associated IEG induction.

In summary, ATF3 deletion strongly interfered with TBI-associated IEG expression in peripheral organs.

3.8 | Behavioral changes associated with AS and/or TBI are partially ATF3 dependent

So far, ATF3's contribution in AS or TBI-associated behaviors or the interplay of both has not been studied. Therefore, several behavioral tests already applied in wt mice (Figure 1) were also applied in ATF3 deficient animals (Figures 7 and 8).

First, TBI-associated mortality occurring in the first 5 min after TBI was quantified (Figure 7A). In female and male wt mice, previous AS did not affect TBI-associated

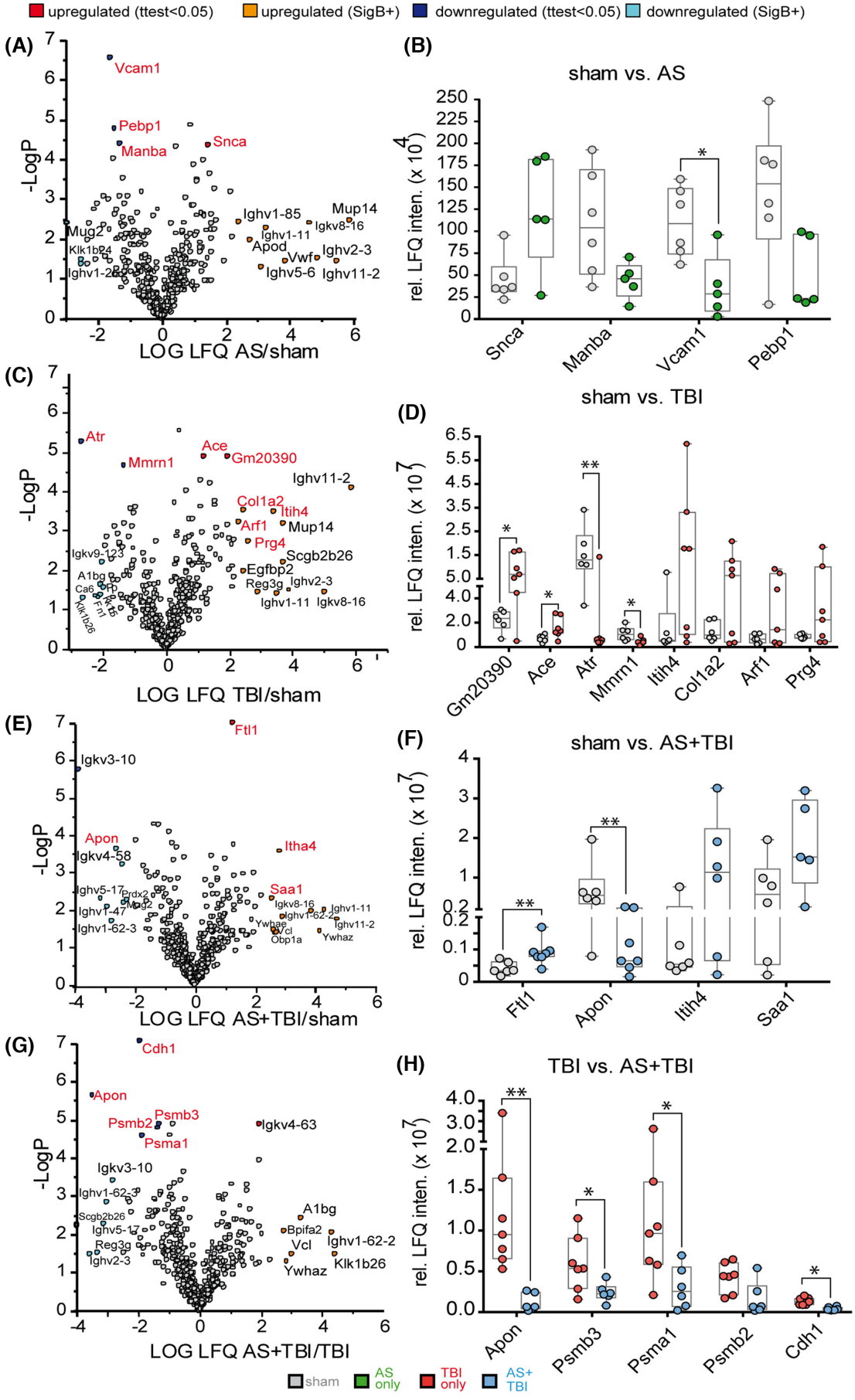

FIGURE 5 Proteins upregulated or downregulated by traumatic brain injury (TBI) and acute stress (AS) were analyzed by proteomics in the blood. Proteomics was performed in plasma samples taken from mice of all four cohorts at 1 h after TBI/sham injury. Vulcano plots (A, C, E, G) showed significantly upregulated (red and orange) or downregulated (dark and light blue) proteins. Selected proteins were separately depicted with LFQ intensities of individual mice (circles) displayed (B, D, F, H). Each circle reflects one mouse ($n = 5–7$ animals/treatment). (A, B) Comparison of sham versus AS-treated animals. Snca was upregulated whereas Manba, Vcam1, and Penp1 were downregulated by AS in relation to sham. (C, D) Comparison of sham versus TBI-treated animals. Gm20390, Ace, Itih4, Col1a2, Arf1, and Prg4 were upregulated, whereas Atr and MMrn1 were downregulated by TBI in relation to sham. (E, F) Comparison of sham versus AS + TBI-treated animals. AS + TBI resulted in Ftl1, Itih4, and Saa1 upregulation compared to sham. In contrast, the apolipoprotein N (Apon) was less abundant in AS + TBI versus sham-treated animals. (G, H) Comparison of TBI versus AS + TBI-treated animals. AS exposure before TBI resulted in down-regulation of several proteasome subunits (Psmb3, Psma1, Psmb2) and Cdh1 in relation to TBI alone. As before (F) when compared to sham, Apolipoprotein N was also downregulated by AS + TBI compared to TBI (H).

mortality. In ATF3 deficient animals, TBI-associated mortality (with or without AS) was higher compared to wt (Figure 7A), which is in line with previous results.[27] In *Atf3* mutant animals, previous AS affected TBI-associated mortality in a sex-specific manner (Figure 7A). In female mice, previous AS reduced TBI-associated mortality, whereas it enhanced mortality in male animals (Figure 7A). In mice surviving TBI, hematoma formation was analyzed immediately after TBI (Figure 7B). In wt, 80% of mice developed a hematoma, which was reduced to 39% in AS + TBI mice (Figure 7B). In contrast, in *Atf3* mutant mice no such reduction in the AS + TBI cohort was observed (Figure 7B).

As in wt (Figure 1), the NSS was used to assess neurological impairments in *Atf3* mutant animals. ATF3 deficient animals had a slightly higher NSS after TBI compared to wt, which was further exacerbated by previous AS (Figure 7C). Nevertheless, in ATF3 deficient animals, the NSS score never exceeded a value of 2; therefore, still showing that a rather mild TBI model was used. One parameter of the NSS that was particularly influenced by ATF3 deletion was beam balancing. While pre-exposure to AS reduced the TBI-induced inability to balance on a beam from 30% to 0% in wt, it increased it from 30% to 50% in *Atf3* mutant animals (Figure 7D). This again emphasizes a role of ATF3 during the integration of two stressors.

Next, relative body weight changes were analyzed separately for female and male mice (Figure 8A,B). In wt females, AS and TBI alone resulted in more weight loss compared to sham treatment. This weight loss was further exacerbated following AS + TBI (Figure 8A; see also Figure 1). Of note, AS-induced weight loss was less pronounced in female *Atf3* mutant mice than in female wt mice, particularly at early timepoints (6 h and 1dpi; Figure 8A). Pooling of male and female mice, resulted in significantly decreased AS-induced weight loss in Atf3 mutants compared to wt mice at 6hpi (Figure 8B). TBI- and AS + TBI-treated animals did not show obvious differences between genotypes in females (Figure 8A). In contrast, in males, ATF3 ablation resulted in more pronounced weight loss in AS + TBI animals compared to wt (Figure 8A).

In wt females, previous AS enhanced track length in the OF after TBI, whereas TBI alone decreased locomotor activity compared to sham (Figure 8C; see also Figure 1). In female *Atf3* mutant mice, sham-treated animals moved greater distances in the OF at several timepoints compared to wt (Figure 8C). Such enhanced locomotor activity was also seen in TBI- and AS + TBI-treated female *Atf3* mutant animals (Figure 8C). While female *Atf3* mutant mice responded with an increase in track length compared to wt females, male *Atf3* mutant mice behaved contrarily and showed a decrease in track length compared to wt (Figure 8C). This result showed the importance of including both, female and male animals, in animal research.

In the Y maze (Figure 8E,F; see also Figure 1), the SA percentage is used to quantify cognitive deficits. In both, wt females and males, previous AS before TBI slightly enhanced SA percentage compared to TBI alone (Figure 8E). In contrast, female and male *Atf3* mutant mice did not show this improvement in SA following AS + TBI (Figure 8E,F). Furthermore, *Atf3* mutant females and to some extent males had worse SAs following TBI (independent of AS exposure) than wt mice.

Taken together, ATF3 deletion had an impact on AS or TBI-associated behavioral changes and also on the combination of both.

4 | DISCUSSION

4.1 | TBI-induced gene responses in the brain are modulated by prior AS exposure

Mild TBI represents the majority of TBI cases in patients. Previously, severe TBI was reported to induce gene responses, such as IEGs, in mice, but data on mTBI are sparse. Here, it was shown that even mild TBI (NSS < 1; Figure 1) induces a robust IEG response in the mouse brain at 1 h after injury (Figure 2), in line with data from mTBI patients.[21]

In this study, divergent gene expression profiles in different brain subregions were reported (Figure 2). Close to

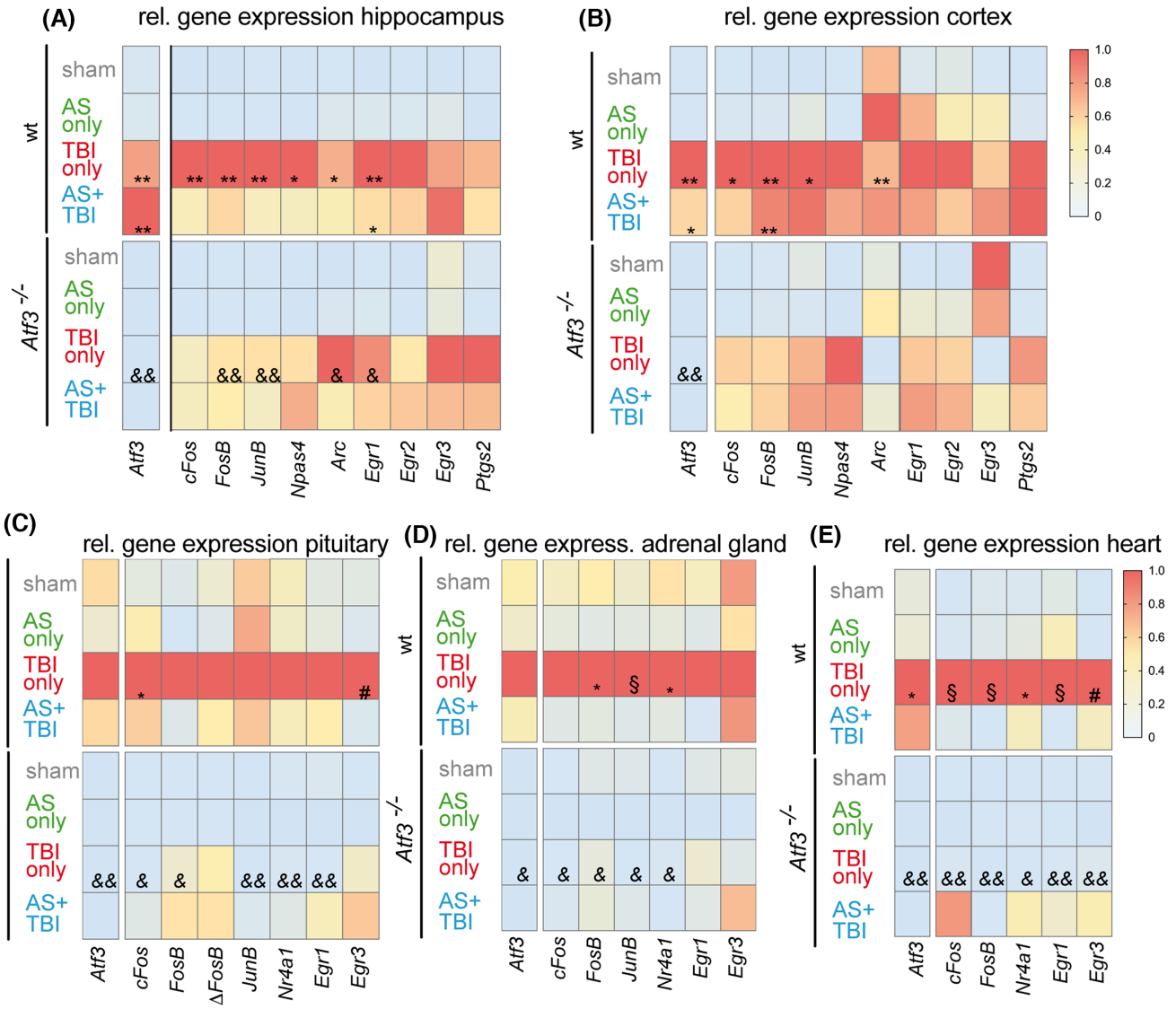

FIGURE 6 ATF3 ablation has a systemic impact on gene expression modulated by the acute stress (AS) and traumatic brain injury (TBI) interplay. (A–E) Heatmaps of immediate early gene (IEG) mRNA abundance assessed by qPCR in several organs of wildtype (wt) (upper rows) and constitutive *Atf3* mutant (lower rows) mice at 1 h after TBI/sham surgery. Data for wt animals are comparable to Figures 2 and 3. In *Atf3* mutant animals *Atf3* mRNA was removed from all organs investigated (left column in panels A–E). (A, B) In the hippocampus (A) or cortex (B) of wt mice, TBI alone induced IEGs, which was reduced in AS + TBI mice in the hippocampus but less pronounced in the cortex. In *Atf3* mutant mice, the IEG mRNA abundance in TBI and AS + TBI cohorts was slightly lower as in wt (A, B, bottom). However, similar to wt, AS + TBI also resulted in lower IEG abundance compared to TBI alone in the hippocampus (A). (C–E) In peripheral organs, pituitary (C), adrenal gland (D), and heart (E), TBI resulted in IEG upregulation at 1 h after TBI of wt animals (top rows) in all three organs, whereas this was reduced upon prior AS exposure (AS + TBI). In contrast to wt, in $Atf3^{-/-}$ mice, TBI alone only weakly induced IEGs in all three organs compared to sham treatment. In AS + TBI-treated ATF3 deficient mice, IEG levels were slightly higher in the pituitary (C) and heart (E) compared to TBI alone, which was different from wt. *Reflects significance between wt/sham versus wt/TBI or wt/AS + TBI; # reflects significance between wt/AS and wt/TBI; § reflects significance between wt/TBI versus wt/AS + TBI; & reflects significance between wt/TBI versus ko/TBI. *N*-numbers were as follows: hippocampus/cortex = 5–6 animals/treatment; pituitary = 5–6 animals/treatment; adrenal gland = 2–4 animals/treatment; heart = 5–6 animals/treatment.

the impact site (hippocampus and cortex), TBI upregulated several IEGs. In contrast, AS-treated mice showed no persistent IEG upregulation at 1 h post-injury. This can be explained by the extended time (>2 h) that passed after AS onset, so that IEGs peaked in between but declined when mice were sacrificed.[25] Interestingly, AS pre-exposure reduced the TBI-mediated IEG upregulation (Figure 2A–C). In the brain, this reduction was most prominent in the hippocampus, which is a brain region greatly involved the perception of stress and termination of stress responses. IEG upregulation (e.g., *c-Fos*) highlights neuronal activation by many external stimuli.[35,36] Thus, mTBI alone

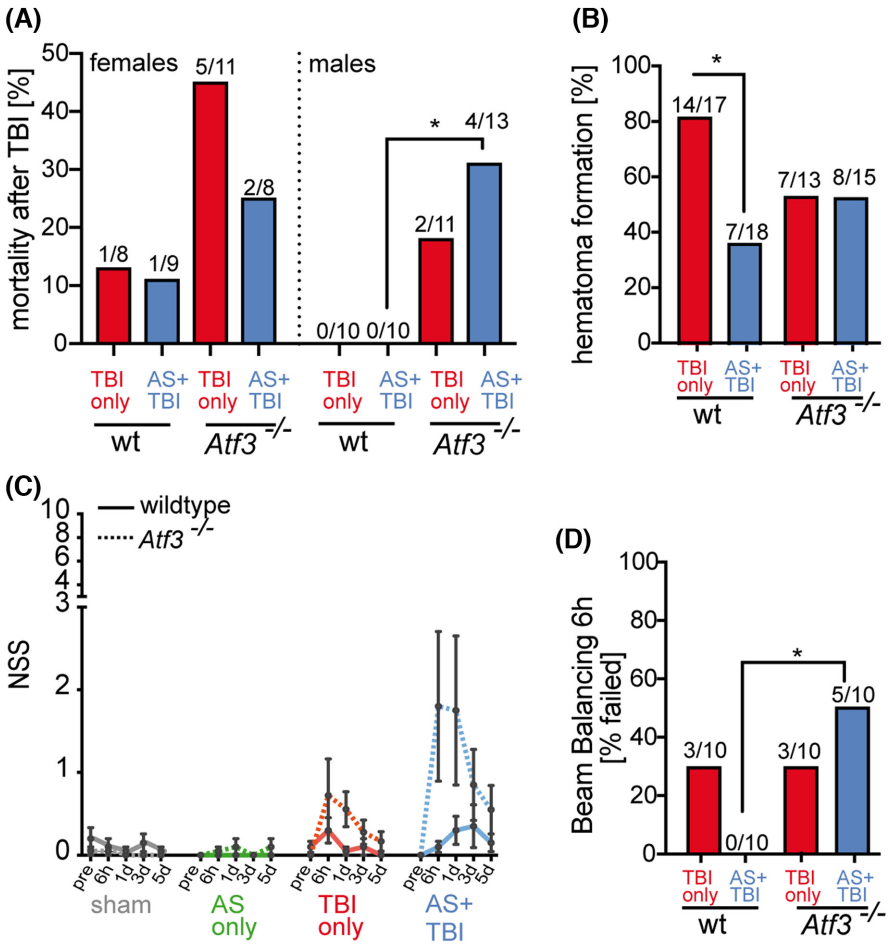

FIGURE 7 Acute stress (AS) and traumatic brain injury (TBI)-associated mortality and hematoma formation is modulated by ATF3. (A) TBI-associated mortality was higher in *Atf3* mutant compared to wildtype (wt) animals. In wt, AS did not change TBI-associated mortality whereas it did in *Atf3* mutant mice. (B) In wt mice TBI-induced hematoma formation was reduced by previous AS, which was not seen in *Atf3* mutant mice. (C) The neurological severity score (NSS) was slightly higher in ATF3 deficient mice treated with TBI and AS + TBI compared to wt. (D) AS reduced mistakes in beam balancing in wt animals with TBI whereas this was elevated upon ATF3 ablation. *N*-numbers of animals/treatment (A, B, D) are indicated in the bars. In (C) 9–10 animals/treatment (4–5/sex) are employed.

might induce a neuronal hyperexcitability as revealed by IEG induction in mice. AS exposure before TBI is partially restricting this IEG induction, which might reflect less neuronal activation. Therefore, although speculative at this stage, AS before TBI might dampen the brain's potential for neuronal activation.

It is known that acute restraint stress induces the activation of the HPA axis.[25,41] Therefore, IEG expression in the hypothalamus was investigated. Interestingly, IEG levels were highest in the cohort where two stressors (AS + TBI) were combined (Figure 2D–F). This was also observed for several genes encoding for HPA axis-associated glucocorticoid signaling (Figure 2G–I). The activation of the HPA axis and the release of CRH from neuroendocrine cells are limited to the paraventricular nucleus (PVN). In this study, the entire hypothalamus, consisting of several nuclei, was analyzed. Thus, effects on *Crh* abundance might be affected by a dilution effect provided by these other nuclei.

In summary, the hypothalamus, which is more distantly localized to the injury site, responded differently compared to, for example, the hippocampus (Figure 2). This emphasizes that AS, TBI, and their combination are integrated in a brain region-specific manner.

4.2 | TBI induces systemic gene expression responses modulated by a preceding AS

So far, knowledge on systemic responses after TBI is limited. Currently, only some potential interactions between brain injury and peripheral organs have been described[56] including effects on liver and muscle metabolism.[57–60] However, comprehensive studies of peripheral organs are missing. Herein, systemic responses in several peripheral organs focusing on gene expression were addressed (Figures 3 and 4). In the spleen and heart, TBI resulted in a robust IEG induction (Figure 4), whereas AS-treated animals showed no elevation of IEGs 1hpi. Since the spleen is crucial for immune responses, these findings indicate modulation of inflammation by TBI. Surprisingly, when AS preceded TBI, no TBI-induced induction of IEGs was present anymore. This was very similar to what we observed in the hippocampus (Figure 2). Thus, AS exposure closely before TBI is able to dampen gene responses associated with cellular activation in several organs. This was further corroborated when inspecting the pituitary and adrenal glands (Figure 3). Here, pre-exposure to AS reduced and partially even completely blocked the

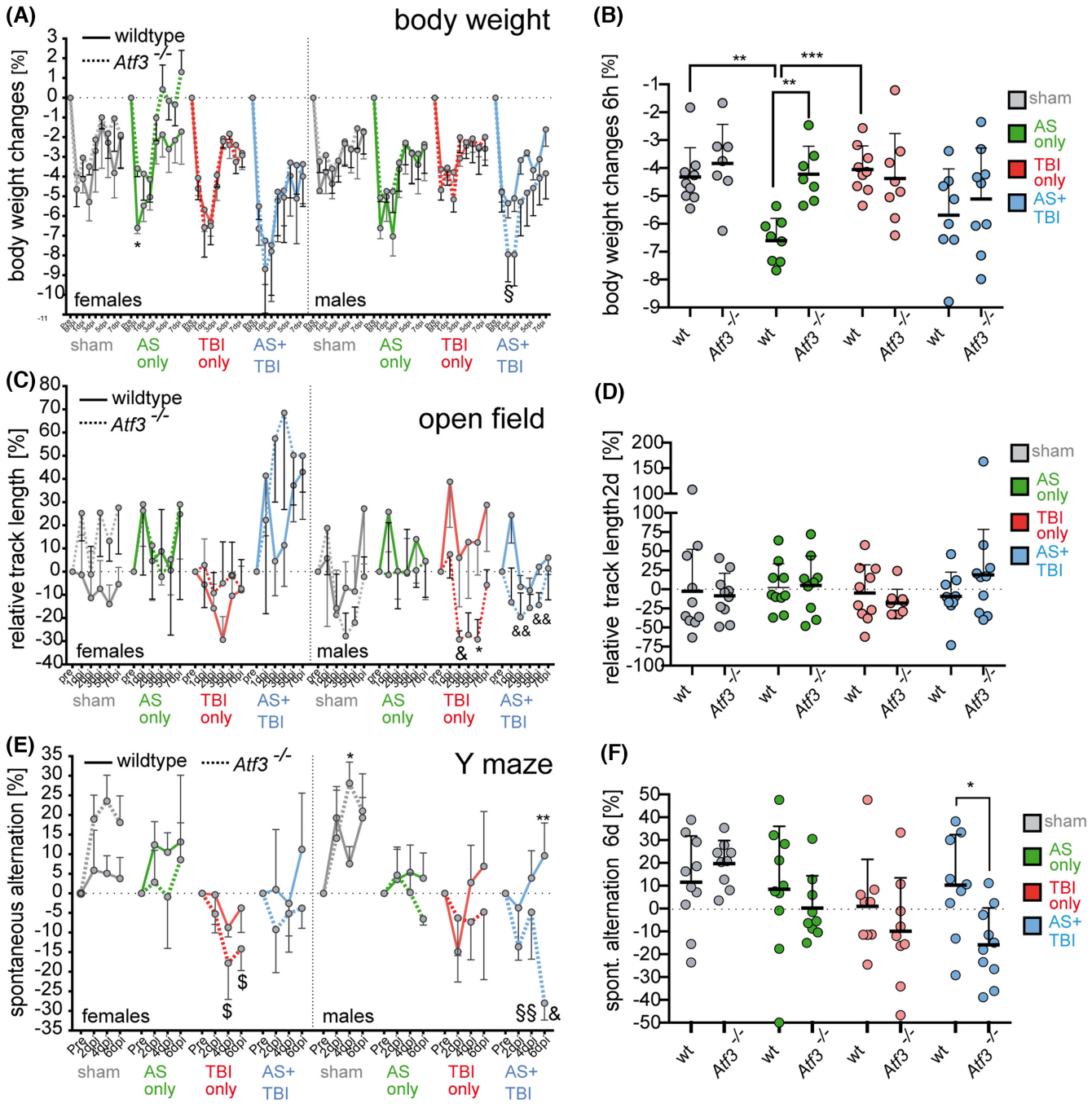

FIGURE 8 Acute stress (AS) and traumatic brain injury (TBI)-associated behavior is modulated by ATF3 ablation. (A, B) Body weight was measured at several days for wildtype (wt) and *Atf3* mutant animals separated by sex (A) or males and females combined at 6 h (B). In *Atf3* mutant mice, AS-induced weight loss was reduced compared to wt (6 h, 1 and 3 dpi; A, B). In males, ATF3 ablation resulted in enhanced weight loss in AS + TBI animals compared to wt (A). (C, D) In the OF, female *Atf3* mutant mice showed enhanced locomotor activity in TBI and AS + TBI compared to wt (C). In contrast, *Atf3* mutant males were rather less active in the OF compared to wt (C). At 2 days no differences between cohorts and genotypes were observed (D). (E, F) In the Y maze, AS previous to TBI slightly enhanced SA percentage compared to TBI alone for both wt females and males before TBI (E). In contrast, this enhancement seen in AS + TBI treatment was not seen in female and male *Atf3* mutant mice at several timepoints (E). This was significant when combining sexes at 6 days (F). In (B, D, F) circles reflect individual animals. In (A, C, E) SEM and in (B, D, F) SD is presented. *Denotes significance between wt and ko. § denotes significance between sham and AS + TBI. & denotes significance between males and females. $ denotes significance between sham vs. TBI. *N*-numbers were 9–10 animals/treatment (sham, AS, TBI or AS + TBI) and 4-5/sex/treatment.

TBI-induced expression of IEGs. The latter, at the first glance, is in line with a significantly increased ACTH induced (delta ACTH—basal values) ex vivo adrenal corticosterone secretion in the TBI versus AS + TBI group (Figure 3G). However, the lack of an adequate ACTH induced increase in adrenal ex vivo corticosterone secretion in all groups except the TBI group is most likely the consequence of a very high basal corticosterone production in the Sham, AS, and AS + TBI group, not allowing any further response to ACTH.

Data on gene expression suggest a systemic impact of TBI on several peripheral organs. Since the underlying mechanism of this interaction might involve communication through proteins released to the bloodstream, proteomic analysis of plasma samples was employed (Figure 5). Currently, it is difficult to decipher the cellular source of these proteins detected. Several proteins are associated with the extracellular matrix (Itih4, Multimerin, Col1a2, Prg4, Saa1) and are released in a rather organ-specific manner such as Snca in the brain and Itih4 from the liver. Among the proteins identified in sham versus AS (Figure 5A,B), alpha-synuclein (Snca) is associated with Parkinson's disease, but elevated blood Snca levels were recently described in other neurodegenerative diseases.[61] Our data show elevated Snca levels after AS in line with a previous study likewise reporting altered Snca levels in a rodent chronic stress model.[62] In TBI-treated mice, the regulation of several proteins previously reported as TBI markers was confirmed (Figure 5C,D). For instance, the inter-alpha-trypsin inhibitor heavy chain 4 (Itih4), an acute response protein that is secreted by the liver, was upregulated by TBI (and AS + TBI) (Figure 5C,D), as shown before.[63] Similarly, serum amyloid 1 (Saa1) was upregulated by TBI as well as AS + TBI (Figure 5E,F). Notably, Saa1 was recently identified as a novel biomarker in TBI patients, and the Saa1-TLR4 (toll-like receptor 4) axis provides an important link between (peripheral) inflammation and the outcome of TBI patients. Besides Saa1, Ace was previously found to be upregulated in the blood of TBI patients.[64] Since the renin-angiotensin system (RAS) is an important modulator of inflammation (de Barros et al.[64]), increased Ace levels in the plasma might imply a systemic inflammatory response. Interestingly, Ace levels were only increased in the plasma of TBI-treated but not AS + TBI-treated mice. Finally, proteomic analysis revealed that several components of the 20S proteasome were decreased in the plasma of AS + TBI-treated mice compared to TBI-treated mice (Figure 5G,H). Previously, it was demonstrated that TBI modulates the composition and function of the proteasome in the CNS of rodents[53,54] and elevates the activity of the circulating proteasome in the blood of TBI patients.[65] Taken together, our data are in line with models showing that the impact of central brain trauma is not restricted to the CNS but also affects peripheral organs. Of note, peripheral trauma (e.g., lung trauma) also affects brain functions,[66] pointing to an important role for deciphering brain-periphery communication in the future.

4.3 | AS exposure modulates TBI-associated behavior

Approximately every second, TBI patient encounters physical and/or psychological impairments in their daily life post-TBI.[67] Stress encountered before TBI might negatively or positively affect the outcomes and recovery after TBI.[68] Of note, sham mice received sham surgery, anesthesia, and analgesia (see materials & methods) thereby raising baseline stress levels (Figure 3). Thus, compared to completely untreated mice, sham mice might already have some behavioral alterations (e.g., weight loss, track length; Figure 1). In this study, AS pre-exposure negatively influenced the TBI-induced weight loss, while it positively affected beam balancing (NSS) and the time per run in the ladder walk in both sexes (Figure 1). Thus, AS pre-exposure influences TBI-induced behavioral changes in both a beneficial and unfavorable manner.[68]

Interestingly, in the OF and Y maze, the influence of AS on a subsequent TBI was sex-specific (Figure 1D,E). In the OF, both sexes responded to AS + TBI by increasing their track length in the OF. However, when compared to sex-matched TBI-treated mice, pre-exposure to AS increased the overall track length in females, while if slightly decreased locomotor activity in TBI-treated males. In the Y maze, AS exposure improved cognitive deficits in TBI-treated males at 2 dpi, while SA of females stayed unchanged. Such sex-specific behavioral responses were in contrast to IEG induction, which in a previous study,[22] and this study (Figures 2 and 3) were comparable between male and female mice. Taken together, this study emphasizes the importance of analyzing sex-specific behavioral responses, which is currently missing in the majority of reports. Of note, besides sex-specific differences it will be important that future studies also investigate rodent models of pediatric TBI since young children (0–4 years old) and adolescents (15–19 years old) are the age group most frequently affected by TBI.[69]

4.4 | ATF3 deletion impairs AS and TBI responses in the periphery

ATF3 is a TF providing neuroprotection in rodent models upon several insults.[27,32,34,70] ATF3 forms homodimers

but also heterodimers with further IEG-encoded TFs such as c-Jun and c-Fos.[71] So far, several transcriptomic studies[32,70] have failed to observe transcriptional regulation of IEGs by ATF3. Herein, it was shown that TBI-associated IEG induction in peripheral organs (heart, pituitary, and adrenal glands) requires ATF3. In this study, constitutive *Atf3* mouse mutants were used. Therefore, effects observed in peripheral organs are most likely due to cell-autonomous ATF3 function and not due to an indirect effect of brain-resident ATF3 deletion. In summary, ATF3-mediated gene regulation provides an important role during the TBI-induced communication between the brain and the periphery.

4.5 | ATF3 deletion affects AS and TBI behavioral responses

Previously, a higher TBI-associated mortality in *Atf3* mutant mice was observed,[27] which was confirmed in this study. Furthermore, ATF3-deficient mice displayed greater neurological impairments (NSS; Figure 7), which is in line with the already described neuroprotective function of ATF3 following injury in wt mice.[31,32] Interestingly, while the combination of AS + TBI exposure improved beam balancing in wt animals, it was significantly worsened in *Atf3*-deficient mice (Figure 7D). Likewise, pre-exposure to AS reduced hematoma formation in wt animals, which was not observed upon *Atf3* deletion (Figure 7B). ATF3 was previously associated with the regulation of body weight.[72] Congruent with this, ATF3 ablation altered body weight responses, particularly those associated with AS and, to some extent, with AS + TBI (Figure 8). Furthermore, ATF3 deficiency resulted in enhanced activity in the OF in females but lower locomotor activity in males, irrespective of treatment (Figure 8). Finally, AS + TBI-modulated cognitive performance in the Y maze was negatively modulated by ATF3 deletion. These results support the neuroprotective ATF3 function during injury previously reported. ATF3 might exert such neuronal protection through the reduction of extrasynaptic NMDA receptor-induced glutamate excitotoxicity.[73] Therefore, the results presented in this report suggest an involvement of ATF3 as a neuroprotective factor not only in TBI alone but also in the integration of multiple different stressors.

AUTHOR CONTRIBUTIONS

Bernd Knöll and Stefan O. Reber conceived the study and supervised the project. Johanna Christina Reiners, Vera Hallebach, Laura Leopold, Daniela Sinske, Philip Meier, Mattia Amoroso, and Dominik Langgartner performed experiments and data analysis. Bernd Knöll prepared figures and drafted the manuscript. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) through the grant No. 443642953 to BK. BK and SOR are funded by the DFG—Project-ID 251293561—SFB 1149. BK is further funded through individual grants by the DFG (Project number: 406037611 and 441734479). The authors thank U. Binder for his technical assistance and help in measuring plasma and supernatant corticosterone concentrations as well as in performing the ex vivo adrenal explant stimulations. We thank Sebastian Wiese and Reinhild Rösler of the Core Unit Mass Spectrometry and Proteomics at Ulm University for mass spectrometry and subsequent data analysis. Sebastian Wiese was supported by the Deutsche Forschungsgemeinschaft through SFB1074. Open Access funding enabled and organized by Projekt DEAL.

DISCLOSURES

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

ORCID

Stefan O. Reber https://orcid.org/0000-0002-1583-7618
Bernd Knöll https://orcid.org/0000-0001-7685-3796

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How to cite this article: Reiners JC, Leopold L, Hallebach V, et al. Acute stress modulates the outcome of traumatic brain injury-associated gene expression and behavioral responses. *The FASEB Journal*. 2023;37:e23218. doi:10.1096/fj.202301035R