Interference of neuronal activity-mediated gene expression through serum response factor deletion enhances mortality and hyperactivity after traumatic brain injury

Philip Förstner | Bernd Knöll

Institute of Physiological Chemistry, Ulm University, Ulm, Germany

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

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Abstract
Traumatic brain injury (TBI) is one of the most frequent causes of brain injury and mortality in young adults with detrimental sequelae such as cognitive impairments, epilepsy, and attention-deficit hyperactivity disorder. TBI modulates the neuronal excitability resulting in propagation of a neuronal activity-driven gene expression program. However, the impact of such neuronal activity mediated gene expression in TBI has been poorly studied. In this study we analyzed mouse mutants of the prototypical neuronal activity-dependent transcription factor SRF (serum response factor) in a weight-drop TBI model. Neuron-restricted SRF deletion elevated TBI inflicted mortality suggesting a neuroprotective SRF function during TBI. Behavioral inspection uncovered elevated locomotor activity in Srf mutant mice after TBI in contrast to hypoactivity observed in wild-type littermates. This indicates an SRF role in modulation of TBI-associated alterations in locomotor activity. Finally, induction of a neuronal activity induced gene expression program composed of immediate early genes (IEGs) such as Egr1, Egr2, Egr3, Npas4, Atf3, Arc, Ptgs2, and neuronal pentraxins (Nptx2) was compromised upon SRF depletion. Overall, our data show a role of neuronal activity-mediated gene transcription during TBI and suggest a molecular link between TBI and such post-TBI neurological comorbidities involving hyperactivity phenotypes.

KEYWORDS
C-Fos, Egr, hematoma, hyperactivity, immediate early gene, transcription

Abbreviations: ADHD, attention-deficit hyperactivity disorder; ANOVA, analysis of variance; CREB, cAMP response element-binding protein; DAB, 3,3′-Diaminobenzidin; DMC, Dunn’s multiple comparisons test; DuMC, Dunnet’s multiple comparisons test; GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase; IEG, immediate early gene; NMDA, N-methyl D-aspartate; NSS, Neurological severity score; OF, open field; PTSD, post-traumatic stress disorder; qPCR, quantitative real-time PCR; SRF, serum response factor; TBI, traumatic brain injury; TFs, transcription factors; TMC, Tukey’s multiple comparisons test.
INTRODUCTION

Traumatic brain injury (TBI) is one of the most frequent CNS injuries accounting for high accident-related mortality rates in young adults. TBI patients, including many contact-sport related injuries, suffer from cognitive decline and frequently further neurological impairments including epilepsy, neurodegenerative diseases, and attention-deficit hyperactivity disorder (ADHD). Immediately after impact, TBI induces a strong glutamate efflux that may contribute to the acute neuronal network dysfunction. Particularly within the first hours after TBI, an increased neuronal excitability and reduced GABAergic inhibitory transmission were reported. Such early neuronal hyperexcitability involves NMDA receptor subunit upregulation and a function of, for example, GluN2B as injury mechanoreceptors. Taken together, previous reports describe enhanced neuronal activity at early stages after TBI.

Enhanced neuronal transmission results in nuclear activation of activity-driven transcription factors (TFs) triggering neuronal activity directed gene expression programs. So far, CREB (cAMP response element-binding protein) was considered one of the major TFs involved in propagation of neuronal activity-mediated gene transcription. In TBI, CREB phosphorylation, indicative of activation, is both down and upregulated which may depend on the brain area and timepoint. However, up until now, no functional role for CREB in TBI has been reported in neurons, for example, using mouse mutagenesis. In contrast, in astrocytes, enhanced CREB activation mediates neuroprotective TBI functions. Thus, the connection between TBI and neuronal activity directed gene transcription has currently not been investigated in great detail.

In this study, we aim at identification and functional characterization of such a neuronal activity dependent TF during TBI. For this, we analyzed a further prototypical neuronal activity-driven TF, the serum response factor (SRF). SRF responds to neuronal activity, for instance through calcium influx via NMDA receptors. After activation, SRF propagates in neurons an immediate early gene (IEG) response resulting in rapid (within minutes) but transient upregulation of target genes including cFos, Egr1, Egr2, Egr3, and, for example, Arc. Since many SRF effector genes encode TFs, this results in a second gene expression wave involved in modulation of learning and memory, synaptic plasticity as well as neuro-behavioral changes such as hyperactivity and modulated anxiety. So far, selected IEGs such as cFos, cJun, and Egr1 were found upregulated by TBI in humans and in rodent TBI models. However, a functional role of SRF or one of these IEGs in TBI has not been described. In this study we employ neuron restricted SRF deletion in mice to show a first SRF function in a rodent TBI model. In previous reports, such SRF deficient mice revealed impaired stress responsiveness, a hyperactivity and decreased anxiety phenotype, altered seizure propagation and impaired long-term potentiation and depression. For instance, challenging mice with a novel environment, acute stress, electroconvulsive shocks, or epileptic seizures upregulates IEGs in wild-type (wt) mice whereas this was strongly diminished for several IEGs after SRF ablation. This suggests a pivotal role for SRF as the TF mediating a neuronal activity-driven IEG response. Furthermore, mouse injury models for axon and myelin regeneration have shown that SRF exerts a neuroprotective function in wt mice. Herein, using a mouse TBI model, we expand on such neuroprotective SRF functions. We show that SRF in mice limits TBI inflicted mortality and SRF deficiency modulates neuro-behavioral changes elicited by TBI such as a hyperactivity phenotype not observed in wt mice. Finally, SRF directs expression of a neuronal activity-associated gene set consisting of several IEGs immediately after the TBI impact.

MATERIALS AND METHODS

2.1 Srf deficient mice and housing conditions

In this study, previously described neuron specific Srf mutant mice were used. We used male and female offspring derived from breeding pairs of parental mice harboring the CreERT2 allele and one “floxed” and one wild-type Srf allele (for more details see “Experimental details,” below). To induce a neuron specific deletion of Srf, CreERT2 positive mice with two “floxed” Srf alleles were daily ip injected with tamoxifen (2 mg dissolved in 10% ethanol in peanut oil, Sigma) for 5 consecutive days. After tamoxifen treatment, SRF deficient animals were designated as Srf ko. As wild-type control, CreERT2 positive littermates carrying two wild-type (wt) Srf alleles were used and treated with the same tamoxifen injection schedule. Thus, all animals (wt and ko) analyzed were treated with the same tamoxifen protocol (see Figure 1A). Of note, wt mice treated with or without tamoxifen before TBI were indistinguishable with regard to open field distance and center visits, NSS score, exit circle time, and ladder walk traversing time as well as slip number (data not shown). All mice were kept under standard laboratory housing conditions (12 hours light/12 hours dark cycle, lights on at 07.00 am, 22°C, 60% humidity) and had free access to water and standard mouse diet. All animal experiments were in compliance with international regulations for the care and use of laboratory animals (ARRIVE guide-lines and EU Directive 2010/63/EU for animal experiments).
in this study were approved by the local governmental authority for animal experimentation (Regierungspräsidium Tübingen, Germany).

2.2 | TBI model

A weight-drop TBI model was applied to induce TBI in mice 12 days after the last tamoxifen injection. In all experiments, mice had an age of 12-14 weeks. The TBI induction was performed as described earlier. In brief, mice were anesthetized by 5% sevoflurane inhalation (Sevorane, AbbVie, Wiesbaden, Germany). The head was shaved, cleaned with ethanol, and a skin incision was performed with a scalpel. Afterwards, the mouse was fixed on a cushion of modeling clay and the tip (Ø of 3 mm) of the metal rod (weight: 333 g) was positioned over a rostro-lateral target area of the left cortical hemisphere. The falling height of the bar was adjusted to 2.3 cm for males or 2 cm for females and dropped on the closed skull. Directly after the hit, the bar was retracted. Until regular breathing was restored, 100% of oxygen was given for 1 to 2 minutes and the skin was sutured. For analgesia, Buprenorphin (Temgesic, Indivior, Virginia, USA; 0.03 mg/kg) was injected s.c. shortly before the TBI induction and every 8 hours for the first 24 hours post-TBI in all mice (wt and ko, sham and TBI treated). As control, sham operated animals, treated exactly the same as TBI mice (i.e., sevoflurane inhalation, skin incision, suturing, analgesia), except for the actual impact of the falling rod were included.

2.3 | Hematoma size

For the assessment of the hematoma, a picture of each freshly dissected brain was taken with a Samsung NX1000 camera. The hematoma area on the surface of the ipsilateral cortex was measured in pixel with the lasso tool of the
graphic software paint.net (dotPDN LLC, Washington State University, USA) and the size calculated as a percentage relative to the measured whole surface area of the ipsilateral cortex.

2.4 | Quantitative real-time PCR

From freshly dissected brains of TBI-treated or sham-operated mice, cortical tissue with approximately 5-6 mm in diameter was dissected from the target area of the ipsilateral cortex as well as of the equivalent area of the contralateral side with the following coordinates: from Bregma to 3.5 mm lateral and from Bregma to −4 mm posterior. In addition, the ipsilateral and contralateral hippocampi were dissected. After total RNA was isolated with the Isolate II RNA/DNA/Protein kit (Bioline, Luckenwalde, Germany), cDNA synthesis was performed with 0.75 µg RNA, random hexamers (Biomers, Ulm, Germany), and the M-MLV reverse transcriptase (RT, Promega). RT-quantitative real-time PCR (qPCR) was performed with 2 µL of 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 10 µL reaction volume/well of a 96-well plate in a Roche Light Cycler 480 (Roche). The Ct value of a target gene was detected with the LC480 II software and the relative mRNA level of the target gene was calculated relative to the measured Ct value of the house-keeping gene Gapdh (glycerinaldehyd-3-phosphat-dehydrogenase) with the ΔCt method for the hippocampal tissue (Figure 6A-L): relative mRNA level of the target gene was calculated relative to the measured Ct value of the house-keeping gene Gapdh

2.5 | Biochemistry

Protein lysates were prepared with the Bioline kit and Western blots were performed as previously described. The membranes were incubated with the following primary antibodies: anti-SRF (rabbit, 1:500; a kind gift of Prof. Dr. A. Nordheim, Tübingen University, Germany) or anti-GAPDH (mouse, 1:60 000; Acris Origine Europe, Herford, Germany; ACR001P) overnight at 4°C. For detection horseradish peroxidase conjugated secondary antibodies (1:2000; Santa Cruz, Heidelberg, Germany) were applied in combination with ECL Western Blotting Substrate (Merck Millipore, Burlington, MA, USA). Band intensities were quantified with the FIJI software (Gels tool). For SRF, the combined intensity of both bands was measured and normalized on the intensity of the GAPDH signal.

2.6 | Immunohistochemistry

Dissected mouse brains were fixed in 4% formaldehyde in PBS, dehydrated, and embedded in paraffin. 5 µm slices were prepared with a microtome (MICROM HM355S, Thermo Fisher Scientific, Waltham, MA, USA) and immunohistochemistry was performed with the following primary antibodies: anti-SRF (rabbit, 1:500; Santa Cruz; sc-335; Lot K2514), anti-Egr1 (rabbit, 1:500; Santa Cruz; sc-110; Lot L239), anti-ATF3 (rabbit, 1:500, Sigma-Aldrich, Taufkirchen, Germany; HPA001562; Lot B103815), anti-c-Fos (rabbit, 1:500; Santa Cruz; sc-253; Lot D2815), anti-Iba1 (rabbit, 1:1000; Wako Chemical, Neuss, Germany; 019-19741; Lot LKN5648), anti-GFAP (mouse, 1:1000; Santa Cruz; sc-33673; Lot H2919), anti-NeuN (mouse, 1:500; Merck Millipore; MAB377; Lot 2716741), anti-Neurofilament H non-phosphorylated (Smii32; mouse, 1:2000; Covance, Princeton, New Jersey, USA; SMI-32P), and anti-P-cofilin (rabbit, 1:500; Santa Cruz; sc-21867; Lot G1912). Preabsorption of the P-cofilin antibody with the available blocking peptide (Santa Cruz; sc-365882 P) strongly diminished the signal (data not shown) thereby supporting the specificity of this antibody. For detection, either fluorescently labeled secondary antibodies with Alexa 488 or Alexa 546 (goat, 1:500; Life Technologies, Carlsbad, CA, USA) were applied, or a peroxidase-based detection system with Biotin conjugated secondary antibodies (goat, 1:500; Vectorlabs, Burlingame, CA, USA) was used in combination with the ABC complex (Vectorlabs) and the substrate DAB.

2.7 | Behavior

2.7.1 | Neurological severity score with exit circle test

The Neurological severity score (NSS) was performed as described before. The NSS includes the following 10 tests: (a) exit circle, (b) seeking behavior, (c) paresis, (d) straight walk, (e) startle reflex, (f) beam balancing (7 × 7 mm), (g-i) beam walk (3, 2, and 1 cm wide stick), and (j) round stick balancing (5 mm diameter). In the NSS only male mice were used. On average it took 10 minutes for one mouse to complete all 10 tests. For a failed test one point was awarded, while a passed test was counted as zero. An NSS score of “0” indicates that the mouse passed all tests whereas a score of “10” indicates maximum neurological impairment. The NSS was measured 1 day pre, as well as 1, 3, and 5 days post-TBI. The NSS includes the exit circle test where a mouse is located in the middle of a circular arena (Ø 30 cm) with a small exit whole (5 × 5 cm; see Figure 5). We measured the time to exit the
circle, which for a healthy mouse is typically under 3 minutes.\(^\text{36}\) The NSS also includes a test for the paresis formation. Here, the mouse was lifted by the tail and the mouse’s capability to hold on to a pole was tested. Paresis formation was additionally analyzed at 4 hours after injury.

### 2.7.2 | Open field test

To determine the locomotor activity, anxiety, and exploratory behavior, the Open field (OF) was performed 2 days pre-TBI, 6 hours post-TBI, and then daily until 7 days post-TBI. In the OF only male mice were used. To measure the locomotor activity, mice were placed in the middle of a square shaped OF arena (50 × 50 cm) and tracked for 15 minutes with a video camera. In the arena center, the light intensity was 30 lux. The Viewer III software (Biobserve, Bonn, Germany) determined the overall track-length in the 15 minutes trial as read-out for the locomotor activity as reported before.\(^\text{47}\) To analyze anxiety, the number and time span of visits within 15 minutes in a virtual square (20 × 20 cm) predefined by the Viewer III software in the center of the OF arena was determined by the Viewer III software.

### 2.7.3 | Ladder walk

To assess the posttraumatic motor coordination, a ladder walk test was performed 2 days pre-TBI, 4 hours and then daily until 5 days post-TBI. For this test only male mice were used. In the ladder walk, mice had to walk over a horizontal ladder to reach their home cage. The ladder was built of two transparent plates (square: 69.5 × 15 cm) and cylindrical ladder rungs (8 cm long, Ø of 2 mm). The ladder walk had a length of 60 cm with a space of 1 cm between rungs (see Figure 5). Before the first ladder walk test, each mouse accomplished one test run over the ladder. At each time-point each mouse had to cross the ladder three times, while they were recorded with a Samsung NX1000 camera. The recorded videos were analyzed at 1/4 of the original speed to count slips and time needed to cross the ladder for each run. The average slip number or time out of the three runs is depicted. Typically, this test was completed in 5 minutes by each mouse.

### 2.8 | Acquisition of mouse body weight

The body weight was measured 30-60 minutes before TBI and 6 hours after TBI followed by a daily measurement between 2 and 5 pm until day seven post-TBI. The weight loss of the mice after TBI was calculated as a percentage relative to the measured body weight before TBI.

### 2.9 | Microscopy and image analysis

Images of the stained brain slices were acquired with a BIORÉVO BZ-9000 microscope (KEYENCE, Neu-Ilsenburg, Germany) applying a 4x or 10x objective lens (Plan APO, Nikon) and merged with the software BZ-II Analyzer (version 2.2, Keyence). For image analysis for each staining one stained tissue slice was analyzed per mouse. The SRF positive area (Figure 1) was measured in a squared area (300 × 200 µm) of the upper leaf of the granule cell layer of the dentate gyrus with the hybrid cell count tool of the BZ-II Analyzer software. The positive area of EGR1, ATF3, or cFos (Figure 7) was measured in a squared area (300 × 100 µm) of the upper leaf of the granule cell layer of the dentate gyrus with the hybrid cell count tool of the BZ-II Analyzer software. Thereby, for all stainings the same threshold was used for all samples to distinguish the specific signal from background. The lesion area was measured in brain slices stained with anti-NeuN with the FIJI software (ImageJ 1.51s, National Institutes of Health, NIH, USA). The BZ-II Analyzer software was used to manually count the NeuN positive cells or Iba1 positive cells. NeuN positive cells were counted in a circular area (Ø 300 µm) positioned next to the lesion area in the cortical layers 2/3. Iba1 positive cells were counted in three circles (Ø 200 µm) positioned in one line next to the lesion area in cortical layers 2/3 (Figure 3C). The GFAP positive area was measured in the same three circles with the FIJI software. The threshold to distinguish positive area from background was set for each stained cohort (including TBI treated or sham operated wt and Srf ko mice) to 50% of the measured intensity of strongly GFAP positive cells from TBI-treated wt and Srf ko mice. The intensity of P-cofilin in the cerebral peduncles was measured with the FIJI software. For the quantification of Smi32 positive signals in the cerebral peduncles the hybrid cell count tool of the BZ-II Analyzer software was applied. Here, the same threshold was used for all stained slices.

### 2.10 | Experimental design and statistical analysis

Wt and Srf ko mice were randomly distributed in three groups. Induction of TBI was always performed between 9.00 and 11.00 am. All behavioral testing was conducted between 1.00 and 7.00 pm. Animals performed two (ladder walk and open field) or three (ladder walk, open field, NSS) tests at the same day. Mice were allowed to move freely in their home cages for 1 hour between two tests. For mortality quantification (Figure 2), all TBI-treated mice with a skull fracture (31 wt mice: 25 males, 6 females; 36 Srf ko: 29 males, 7 females) were included. For statistical analysis a Fisher’s exact test was applied.
**Figure 2**  SRF deletion enhances TBI-induced mortality in mice. A-C, Mortality was quantified irrespective of body-weight (A) and two different weight cohorts for males (B) and females (C). TBI inflicted mortality was elevated in SRF deficient mice for both males and females (A-C) as well as when accounting for weight differences (B, C). D, At 4 h post-TBI, SRF deficient mice had a slightly higher paresis incidence at three of the four extremities. E and F, Wt and Srf mutant mice exhibited comparable extent of hematoma formation on the ipsilateral cortex (arrows in E). Each dot in (F) depicts one animal. Black dots represent male whereas grey filled dots depict female animals. Data are depicted as mean ± SD.

G, Weight loss after TBI was comparable for wt and Srf mutant animals. Data are depicted as mean ± SEM. *P < .05 for TBI ko versus Sham ko; ##P < .01 for TBI ko versus sham ko; ++P < .01 for TBI wt versus sham wt. H, The overall NSS score was comparable between wt and Srf mutant TBI animals. A score of “0” signifies no impairments whereas a score of “10” reflects maximally impaired animals. I, The 10 individual tests comprising the NSS are depicted with the percentage of mice failing to complete a test. Data are depicted as mean ± SEM. *P < .05 for TBI ko versus sham ko; +P < .01 for TBI wt versus sham wt (G, H). Scale-bar (E) = 0.5 cm.
In the first experimental group, only male mice were used. The following number of male mice was used to assess paresis, hematoma, weight loss, NSS (Figure 2D-I), OF, exit circle, ladder walk (Figure 5), and histology (Figures 1D,E,G, 3A-F, and 4): 7 wt TBI, 8 Srf ko TBI, 5 wt sham, and 4 Srf ko sham. Here, mice without a cortical lesion were excluded to analyze animals with a comparable TBI impact only. Weight loss (Figure 2G) and behavior data (Figures 2H and 5B,D,F-I) are presented as mean ± SEM and data were analyzed with a two-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (TMC; α = 0.05). The statistical power for these data sets was 99.9%. In Figure 5B we additionally performed a two-way ANOVA with Dunnett’s multiple comparisons (DuMC) test to compare each timepoint with the pre-TBI baseline for each experimental group. OF data pre-TBI (Figure 5A,C,E) are depicted as mean ± SD with a Mann-Whitney test (Figure 5A) and an unpaired t-test (Figure 5C). Histology data (Figures 1G, 3E,F, and 4M-P) are presented as mean ± SD with a Kruskal–Wallis test and Dunn’s multiple comparisons test (DMC; α = 0.05). For Smi32 and P-cofilin staining (Figure 4), the number of Srf ko TBI mice was seven instead of eight.
The animals of the second experimental group were sacrificed 1 hour after TBI to measure the hematoma size (Figure 2E,F), gene expression (Figures 1B, 3G-I, and 6). This group included four wt sham (two males and two females), four Srf ko sham (two males and two females), seven wt TBI (five males and two females), and six Srf ko TBI (four males, two

**Figure 4** TBI-associated neurodegeneration is not affected by SRF ablation. A-D, Loss of NeuN positive neurons in the lesion center and close proximity 7 days after TBI treatment in wt (C) and Srf mutant (D) animals (arrows point at lesion center). The dashed circle in (C) shows the area for quantification depicted in (N). (A′-D′) are higher magnifications of boxed areas in (A-D). E-H, Induction of Smi32 reactivity by TBI in the cerebral peduncle of wt (G) and SRF deficient (H) animals in relation to sham treatment (E, F). (E′-H′) are higher magnifications of boxed areas in (E-H). I-L, P-cofilin is upregulated in SRF deficient sham (I) and TBI-treated (L) animals in the cerebral peduncle whereas P-cofilin levels remained low in wt sham (I) or TBI-(K) treated animals. (I′-L′) are higher magnifications of boxed areas in (I-L). M-P, Quantification of the lesion area by the lack of NeuN signal (M), neuronal cell loss (N) in the area depicted in (C), Smi32 positive areas (O) and P-cofilin (P). Each dot depicts one animal. Data are depicted as mean ± SD. †P < .05 for TBI versus respective sham; ‡P < .05 for TBI versus respective sham **P < .001 for wt TBI versus Srf ko TBI. Scale-bar (A-D) = 500 μm; (A′-D′) = 250 μm; (E-L) = 500 μm; (E′-L′) = 250 μm
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FIGURE 5  SRF ablation induces a hyperactivity phenotype after TBI. A-F, Open field analysis measuring distance travelled (A, B) or visits to the arena center (C-F). Before TBI, Srf mutant mice are hyperactive compared to wt mice (A). After TBI, wt sham and more pronounced wt TBI mice are hypoactive and slightly recover over time. In contrast, SRF deficient mice show hyperactivity exceeding pre-TBI levels from 4 days post-TBI onwards (B). Srf mutant mice show more visits to the arena center before TBI (C, E). Following TBI, Srf mutant mice visit the OF center more frequently compared to the other three cohorts (D, F). Each dot depicts one animal (A, C, E). Black dots with gray border are sham mice whereas completely black dots are mice that were subsequently subjected to TBI (A, C, E). In (C, D) absolute numbers of center visits are depicted whereas in (E, F) the time spent in the OF center is depicted. G. In the open circle, wt mice required much longer to exit the circle after TBI compared to Srf mutant mice. H and I, In the ladder walk, wt and Srf mutant mice had comparable slip numbers (H). The time required to cross the ladder was shorter for Srf mutant compared to wt animals treated with TBI (I). Data are depicted as mean ± SD (A, C, E) or SEM (B, D, F, G-I). Black symbols depict two-way ANOVA with TMC *P < .05 for TBI wt versus TBI ko; **P < .01 for TBI wt versus TBI ko; ***P < .001 for TBI wt versus TBI ko; ^P < .05 for TBI ko versus sham ko; &P < .05 for wt sham versus ko sham (B, D, F). Blue and red symbols depict two-way ANOVA with DuMC for wt TBI and ko TBI in relation to the pre-TBI data, respectively with *P < .05 and **P < .01 (B)
are shown as mean ± SD. For statistical analysis of data, a one-way ANOVA with TMC (α = 0.05; Figures 1B, 3G-I, and 6A-L), a Kruskal–Wallis test with DMC (Figure 1F), Mann-Whitney test (Figure 6M), or unpaired t-test (Figure 2F) was used. The statistical power for the gene expression data was 99.9%.
FIGURE 6 Impaired neuronal activity-driven gene expression after TBI upon SRF ablation. A-L, qPCR analysis of wt and Srf mutant sham treated hippocampal samples as well as ipsi and contralateral hippocampi collected 1 h after TBI of both genotypes. In wt mice, TBI upregulated all genes tested except for β-actin (I). Upon SRF ablation, induction of indicated genes was reduced for Egr1 (A), Egr2 (B), Egr3 (C), Npas4 (D), Arc (E), Aft3 (F), Ptgs2 (G), and Nptx2 (H). In contrast, TBI-associated cFos (J), ΔFosb (K), and JunB (L) upregulation was SRF independent. For statistical analysis, a one-way ANOVA (*P* values of multiple comparison depicted by asterisks; see below). M, qPCR analysis in the cortex of wt and Srf mutant animals 1 h after TBI. Values were normalized to respective sham values of wt or Srf mutant mice. Each dot depicts one animal. Black dots show male whereas gray filled dots represent female animals. Data are depicted as mean ± SD. *P* < .05; **P < .01; ***P < .001.

Animals of the third experimental group were killed 2 hours after TBI for immunohistochemistry (Figure 7). This group included two wt sham (one male, one female), five wt TBI (two males, three females), and three Srf ko TBI (one male, two females) animals.

Statistical analysis was performed with the Prism7 software (Graph Pad Software, San Diego, CA, USA).

3 RESULTS

3.1 Neuron-restricted conditional Srf mutagenesis in mice

In this study we employed neuron-restricted SRF deletion by using previously reported Srf^lox/lox;CaMKCreERT2 mice. Tamoxifen injection (see scheme in Figure 1A) resulted in robust downregulation of Srf mRNA (Figure 1B) and protein (Figure 1C-G) in Srf mutant (Srf^+/−;CaMKCreERT2 referred to as “Srf ko”) mice compared to control mice (Srf^+/+,CaMKCreERT2 referred to as “wt”).

Since SRF can regulate its own expression we analyzed whether TBI upregulates Srf mRNA levels 1 hour after TBI in the hippocampus (Figure 1B). As control, sham operated mice (“sham”), that is, receiving anesthesia, analgesia, and suturing were analyzed. Further, we employed both the ipsilateral (“TBI ipsi”) and contralateral brain hemisphere (“TBI contra”), not directly hit by the TBI impact. Srf mRNA abundance was significantly elevated by TBI compared to sham-treated animals and the contralateral hemisphere (wt TBI ipsi vs wt sham: P = .0061, Mann-Whitney test; wt TBI ipsi vs wt TBI contra: P = .0006, Mann-Whitney test; Figure 1B). At this 1 hour post-TBI timepoint, SRF protein abundance was also slightly elevated by TBI (Figure 1C), however statistical significance was not fully reached (P = .057; Figure 1F).

After 7 days of TBI, SRF protein was uniformly expressed in both the ipsi (arrow in Figure 1D) and contralateral hemisphere of wt mice (quantified in Figure 1G). In SRF deficient mice, SRF protein abundance was clearly diminished (Figure 1E-G).

In summary, at an early timepoint after TBI (ie, 1 h), SRF abundance increased after TBI whereas at a later timepoint (ie, 1 week post-TBI) this was not observed anymore.

3.2 SRF deletion enhances TBI-induced mortality in mice

TBI is one of the leading causes of mortality in young adults, particularly males. Hence, molecular identification of neuroprotective factors reducing TBI-associated death is an important topic in TBI research and animal TBI models might help in this respect.

When wt and Srf mutant mice were subjected to weight-drop based TBI we noted an immediate difference in post-TBI survival between cohorts (Figure 2A-C). In wt mice we obtained a mortality rate of 6.5% (Figure 2A) with males being slightly more affected than females. Typically, animals died within 5 minutes after TBI most likely involving the respiratory failure due to compression of the brainstem by the weight impact. In contrast to wt mice, more than one third of all Srf mutant animals died immediately after TBI with females (42.9%) slightly more affected than males (37.9%; Figure 2A).

Since weight differences in adult mice might account for alterations in mortality we grouped animals in several weight ranges. Still, when controlling for weight differences (Figure 2B,C) we noted that lighter Srf mutant animals of both sexes (<28 g males, <23.5 g females) were experiencing higher mortality rates of 57.1% (males) and 50% (females) compared to weight matched wt littermates (14.3% males, 0% females; Figure 2B,C). The same holds true for animals with more than 23.5 g (females) or 28 g (males) bodyweight (Figure 2B,C). Also, the changes in mortality are most likely not due to differences in weight or brain size before TBI since only subtle differences in body weight were observed between wt and Srf mutant animals (wt males: 29.5 ± 1.9 g; ko: 28.1 ± 2.0 g; P = .46; wt female: 24.03 ± 0.8 g; ko: 23.5 ± 1.4 g; P = .46; Figure 2E-G).

Next, we analyzed paresis prevalence at 4 hours (Figure 2D) and at several timepoints after TBI (Figure 2F) in those animals surviving TBI. In general, forelimbs were more frequently affected than hindlimbs in both genotypes. Also, extremities on the right side, contralateral to left brain hemisphere hit by the weight-drop, were more frequently affected than left extremities, particularly in Srf mutants (Figure 2D). Comparing wt and Srf mutant mice, the paresis formation was slightly higher after SRF ablation at three out of the four limbs at 4 hours after TBI (Figure 2D; for later timepoints see also I).
The weight hitting the skull resulted in hematoma formation in the ipsilateral cortical layers (Figure 2E,F). Quantification of hematoma size on the cortical surface did not result in a statistically significant difference between cohorts ($P = .97$, unpaired t-test; Figure 2F). Thus, on average the hematoma size was 10% of the entire ipsilateral cortical hemisphere regardless of genotype (Figure 2F).

Since TBI results in decreased body-weight in subsequent days, weight loss of wt and Srf mutant animals either sham or TBI-treated was quantified over 7 days post-TBI (Figure 2G). Sham-treated animals experienced weight loss of approximately 7%, most likely due to stress, anesthesia and performance of sham surgery with suturing (Figure 2G). In TBI treated animals, weight loss was more pronounced and peaked at 12%-15% at 1 day after TBI. Subsequently, animals gained weight in the following days (Figure 2G). Weight loss in TBI treated mutant compared to wt animals was slightly enhanced, however not reaching statistical significance (two-way ANOVA: $P \leq .0023$; $F_{time \times group} = 2.18$; with TMC for eg, wt TBI vs Srf ko TBI: adjusted $P = .32$ (6 h), $P = .51$ (1d); Figure 2G).

**FIGURE 7** SRF regulates EGR1 and ATF3 protein abundance after TBI but not c-Fos. A-C, EGR1 was upregulated in the hippocampus and cortex 2 h after TBI in wt (B) compared to sham-treated animals (A). In SRF deficient animals, EGR1 expression was lower after TBI (C). D-F, ATF3 was induced by TBI in wt TBI-treated animals (E) whereas only low ATF3 expression was present in sham animals (D). ATF3 expression was reduced after TBI in SRF deleted animals (F). G-I, c-Fos was upregulated to comparable extent in wt (H) and Srf mutant (I) animals after TBI. In contrast, in sham animals c-Fos abundance is low (G). A′-I′, are higher magnifications of boxed areas in (A-I). Scale-bar (A-I) = 1 mm; (A′-I′) = 50 μm.
Above, we noted that wt and SRF depleted animals slightly differed in paresis prevalence early after TBI (Figure 2D). In order to address whether both genotypes had comparable motor and neuro-behavioral outcome in the following days after TBI, the Neurological Severity Score (NSS; Figure 2H,I) was performed. The NSS consists of 10 individual tasks and failure to complete one task is given a score of “1”. Thus, animals with scores of “0” or “10” have succeeded or failed in all tests, respectively. After 1 day post-TBI the average NSS score reached 2.5 irrespective of genotype indicating an overall moderate TBI severity. When taken together all 10 tests of the NSS, mice of both genotypes recovered almost identically thereafter reaching an NSS of 1.5 at 5 days post-TBI (two-way ANOVA with TMC: $P \leq .06$; $F_{\text{time} \times \text{group}} = 1.96$; Figure 2H). However, we noted that wt and Srf mutant mice after TBI differed in individual NSS tests (Figure 2I). For instance, wt mice after TBI more often failed to exit the circle (Figure 2I; see also Figure 5G). In contrast, the percentage of Srf mutant mice having paresis at 3 and 5 days post-TBI and failing the round stick balancing was elevated (Figure 2I).

Overall, our data suggest that SRF deficiency results in higher mortality immediately after TBI. However, after TBI there was no major difference with regard to weight loss and overall NSS performance.

3.3 | Neuroinflammation and neurodegeneration after TBI is not affected by SRF deficiency

The mechanical impact during TBI induces morphological and anatomical changes to neurons, particularly in cortical layers. This is accompanied by a neuroinflammatory response resulting in astrocyte and microglia activation. To find out whether SRF ablation affects those processes we performed histology of wt and Srf mutant mice at 7 days post-injury (Figures 3 and 4).

Activation of brain resident immune cells such as astrocytes and microglia was analyzed with GFAP and Iba1 directed antibodies, respectively (Figure 3). Indeed, a statistically significant astrocyte and microglia activation in the cortex of TBI compared to sham-treated wt animals was noted ($P = .012$ for astrocytes in area “1”; .017 for microglia in area “1”; Kruskal–Wallis test with DMC; Figure 3E,F). Similarly to wt TBI-treated animals, both glial cell types were activated in SRF deficient animals after TBI ($P = .09$ for astrocytes in area “1”; .049 for microglia in area “1”; Kruskal–Wallis test with DMC; Figure 3E,F). These data suggest no overt interference of SRF with immune cell activation after 7 days of TBI.

We further corroborated these findings by quantification of chemokine and interleukin mRNA abundance 1 hour after TBI in the cortex (Figure 3G-I). TBI upregulated mRNA levels of Ccl2 (Figure 3G), Ccl3 (Figure 3H) and interleukin 1 beta (Il1b; Figure 3I) in wt and to a similar extent in Srf mutant animals. This further underlines no major function of SRF in regulating the TBI associated immune response within the first 7 days post-TBI.

The lesion size and neuron loss in the cortex was assessed by NeuN directed antibodies recognizing all neuronal cell bodies (Figure 4A-D,M,N). TBI induced a cortical lesion (arrows in Figure 4C,D) of approximately 0.5-0.7 mm² in the cortex irrespective of the genotype (Figure 4M). Since the actual lesion center is devoid of neurons (see Figure 4C,D) we quantified neuronal loss at 7 days post-TBI in direct vicinity to this lesion core (see dashed circle in Figure 4C). At this position there was a statistically significant reduction in NeuN positive neurons of approximately 20% in both wt and Srf mutant TBI treated animals compared to intact cortices of sham-treated animals ($P = .007$ for wt TBI vs wt sham and $P = .049$ for for Srf ko TBI vs Srf ko sham; Kruskall-Wallace with TMC; Figure 4N). Besides neuronal cell loss, mechanical injury during TBI induces axonal degeneration. We used the established degeneration marker Smi32 recognizing non-phosphorylated neurofilament H that is upregulated in conditions of axonal degeneration (Figure 4E-H,O). Here, we focused on the cerebral peduncle, an axonal trajectory connected to cortical neurons. In sham-treated animals of either genotypes, no signs of axonal degeneration were expectedly observed (Figure 4E,F,O). In contrast, 1 week after TBI, Smi32 levels were highly upregulated in axons of wt and Srf mutant animals (Figure 4G,H,O). As observed for neuronal loss (Figure 4A-D,M,N) there was no difference between wt and mutant animals indicating no obvious modulation of neuronal and axonal degeneration after TBI by SRF.

Finally, we analyzed whether cofilin, an actin severing factor whose activity depends on SRF was modulated by TBI (Figure 4I-L,P). For this, P-cofilin levels—inindicative of inactivated cofilin—was measured in the cerebral peduncle of sham- and TBI-treated animals. In wt animals, cofilin was not phosphorylated in both sham- and TBI-treated animals suggesting no obvious modulation of neuronal and axonal degeneration after TBI by SRF.

3.4 | SRF ablation induces a hyperactivity phenotype after TBI

Others and we previously described hyperactivity, memory impairments, and decreased anxiety in Srf mutant
mice including the mouse strain used in this study.\textsuperscript{33,37,40} Independently of these reports, experiments performed in this study confirmed hyperactivity and less anxiety in the open field (OF) test before TBI (Figure 5A-F). Thus, before TBI SRF deficient animals almost ran twice the distance in the OF compared to wt animals ($P = .0001$ for; Mann-Whitney test; Figure 5A). Since TBI typically induces hypoactivity early after TBI we investigated the locomotor activity of wt and Srf mutant animals daily in the OF over 7 days post-TBI (Figure 5B). We observed that wt TBI-treated animals reduced locomotor activity at 6 hours, 1, and 2 days after TBI to about half the activity measured pre-TBI (two-way ANOVA with TMC or DuMC: $P ≤ .0001$; $F_{\text{time} \times \text{group}} = 2.75$; Figure 5B). In part, this hypoactivity was also observed in wt sham-treated animals which particularly at later timepoints might be due to habituation to the daily encounter of the OF environment as shown before.\textsuperscript{52} In contrast, sham-treated Srf mutant animals showed less habituation and revealed rather constant activity scores along the entire 8 days of measurement (red dashed line Figure 5B). The most striking changes were observed in TBI-treated Srf mutant animals. First of all, in the early timepoints after TBI (ie, 6 h, 1, and 2 d) they did not reveal the hypoactivity observed in wt animals. Second, at later timepoints post-TBI (ie, 4-7 days post-TBI), Srf mutant animals even further increased their initial pre-TBI hyperactivity and now reached track lengths statistically significantly exceeding those measured pre-TBI (eg, adjusted $P = .011$ for Srf/k0 TBI vs Srf/k0 sham at 6d; Two-Way ANOVA with TMC; eg, $P = .01$ for Srf/k0 post-TBI vs Srf/k0 pre-TBI at 6d; Two-Way ANOVA with DuMC; with a statistical power of 99.9%; Figure 5B). For instance, at 5 and 6 days post-TBI, the track length of Srf mutant animals was elevated by approximately 35%-40% compared to baseline levels. Thus, SRF ablation results in hyperactivity already before brain jury and this is further augmented by TBI.

Notably, before TBI, prospective sham (black dots with gray border) and TBI-treated (black dots) wt and Srf mutant animals were indistinguishable in OF activity (Figure 5A; see also Figure 5C for center visits). Thus, the pre-TBI hyperactivity did not differ between designated sham and TBI-treated Srf mutant groups and therefore cannot account for enhanced hyperactivity in TBI-treated Srf mutant animals.

In the OF, elevated numbers of entries in the arena center zone are indicative of decreased anxiety.\textsuperscript{52} Similar to locomotor activity (Figure 5A,B) we observed that TBI-treated Srf mutant animals had more center visits pre (Figure 5C) and also at various timepoints post-TBI (Figure 5D,F) compared to the other three experimental cohorts. This was observed at early timepoints after TBI (ie, 6 h, 1, and 2 d) and also at the later timepoints, that is, 3-7 days after TBI (two-way ANOVA with TMC: $P = .45$; $F_{\text{time} \times \text{group}} = 1.00$; Figure 5D,F). However, at later timepoints mice might habituate to the OF challenge due to the daily testing and therefore interpretations regarding anxiety should be taken with some caution for these later timepoints.

The observed hyperactivity phenotype was confirmed by additional tests including open circle exit (Figure 5G) and ladder walk (Figure 5H,I). In the open circle, part of the NSS (Figure 2H, I), we noticed that wt TBI-treated animals required approximately 100 seconds to exit the circle (two-way ANOVA with TMC: $P = .065$; $F_{\text{time} \times \text{group}} = 1.92$; Figure 5G). In contrast, Srf mutants needed significantly less time resulting in circle exit after approximately 10 seconds (Figure 5G). In the ladder walk, TBI elevated the number of slips for wt and Srf mutant TBI animals almost identically (Figure 5H). However, once again a hyperactivity phenotype became apparent when stopping the traversing time (Figure 5I). Immediately after TBI (ie, at 4 h), animals of both cohorts needed the same time to cross the ladder (Figure 5I). However, at later timepoints after TBI, Srf mutant animals were faster and reached levels comparable to sham-treated animals of either genotype although statistical significance was not reached (Figure 5I).

In summary, SRF deficient mice show altered locomotor and anxiety related behavior after TBI.

### 3.5 | Impaired neuronal activity-driven gene expression after TBI upon SRF ablation

Above, we described induction of a hyperactivity phenotype after TBI upon SRF deficiency (Figure 5). SRF regulates several neuronal activity encoding IEGs including Npas4, Egr (Egr1, Egr2, Egr3) and Fos (cFos, ΔFosb) family members, pentraxins\textsuperscript{53} and cyclooxygenase (Pigs2\textsuperscript{54}). Of note, those IEGs were reported to induce hyperactivity phenotypes upon deletion in mice.\textsuperscript{55-59} In order to test whether altered regulation of such effector genes by SRF ablation might underlie TBI inflicted behavioral phenotypes we measured mRNA (Figure 6) and protein (Figure 7) abundance of several neuronal activity related genes.

For this, hippocampal (Figure 6A-L) or cortical (Figure 6M) tissue of wt and SRF deficient animals was subjected to qPCR at 1 hour after TBI. As control we included contralateral hemispheres of TBI animals as well as sham-treated animals (Figure 6A-L). In general, mRNA levels of all genes tested were low in these control tissues and no obvious difference between genotypes was observed. In contrast β-actin, a known SRF target gene, was highly expressed in control and TBI treated hippocampi and mRNA levels were significantly reduced upon SRF deletion ($P = .014$ for Srf/k0 TBI vs wt TBI; Mann-Whitney test; Figure 6I).

Besides β-actin all other genes investigated were robustly upregulated by TBI in wt mice. In contrast, we noted that several neuronal activity regulated genes were significantly less induced in Srf mutant hippocampi including Egr1, Egr2, Egr3.
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(P = .001, .001, .008, respectively Mann-Whitney test; Figure 6A-C), Npas4, Arc and Atf3 (P = .035, .001, .05, respectively Mann-Whitney test; Figure 6D-F). We also analyzed two novel SRF target genes, neuronal pentraxin 2,53,60 a gene involved in enhancing synaptic inhibition,61 and cyclooxygenase 2 (Ptgs2; Figure 6G), a gene regulated by SRF in muscles to regulate inflammation.54 Interestingly, both genes revealed a significant SRF dependency and were only weakly induced by TBI in SRF deficient animals (P = .005, .014, respectively Mann-Whitney test; Figure 6G, H). In opposite to the aforementioned genes, we noted that Fos and Jun family members were induced by TBI in an SRF independent manner (Figure 6J-L).

We also analyzed mRNA levels in the cortex (Figure 6M) and observed a similar tendency as described in the hippocampus above. Here, significant SRF-mediated gene induction by TBI for wt versus Srf mutant cortices was achieved for Npas4, Arc, Ptgs2, cFos, and Nptx2 (P = .008, .035, .001, .014, .035, respectively; Mann-Whitney test; Figure 6M). For other genes, that is, Egr1, Egr2, and Egr3 a similar SRF dependency was observed as in the hippocampus, however without reaching significance (P = .37, .53, .14, respectively; Mann-Whitney test; Figure 6M).

For gene expression we included male and female mice (black and gray dots, respectively; Figure 6). As with mortality (Figure 2), gender did not obviously alter the TBI outcome since TBI-induced gene transcription gave similar results in male and female animals (Figure 6).

Finally, qPCR data were corroborated on protein level for the three potential SRF targets Egr1, ATF3 and c-Fos (Figure 7). Indeed, we noted that 2 hours after TBI, EGR1, and ATF3 were induced in the hippocampus and cortex of wt mice (Figure 7B,E) compared to sham animals (Figure 7A,D). In the absence of SRF, the protein abundance of EGR1 and ATF3 was reduced after TBI (Figure 7C,F). For example, in the upper leaf of the dentate gyrus granule cell layer 2.87 ± 1.75% of the area was EGR1 positive whereas only 0.35 ± 0.43% of the area was positive in Srf mutant animals (for ATF3: wt TBI: 4.00 ± 5.53% vs Srf ko TBI: 0.99 ± 1.45%). As seen for mRNA (Figure 6J), c-Fos was comparably induced in both wt and SRF deficient TBI animals (Figure 7H, I; wt TBI: 25.5 ± 6.03% vs Srf ko TBI: 19.85 ± 7.4%) compared to only weak c-Fos levels found in wt sham animals (Figure 7G).

Taken together, we observed an SRF-dependent induction of several neuronal activity encoding genes after TBI.

4 | DISCUSSION

TBI triggers glutamate release resulting in neuronal dysfunction accompanied by enhanced network activity.62,63 Here, we investigated SRF, a neuronal activity-driven TF in the context of TBI. SRF deletion affected the TBI outcome on three levels: i) mortality, ii) behavior, and iii) gene expression.

4.1 | SRF is a neuroprotective factor in TBI-associated mortality

TBI is a leading cause of mortality in young adults.2 Herein, we observed a fourfold to sixfold elevated mortality upon SRF ablation compared to wt mice (Figure 2). This was irrespective of gender and provides data also for female animals typically neglected in many studies mainly focusing on males only. So far, only few animal studies identified molecules affecting TBI induced mortality. Repression of neuronal NF-κB signaling increases the acute posttraumatic mortality rate to 40%67 thus reaching similar rates as observed for Srf mutants in this study (Figure 2). In contrast, other mouse mutants for example, of Atf3,53 p53,64 complement receptor CR2,65 TNF receptors,66 Nogo receptor,67 or neurodegeneration-inducing superoxide dismutase68 affected mortality not at all or only by few percent. Thus, in relation to these factors, SRF emerges as strong modifier and protective factor of TBI-associated mortality in wt mice.

In our experiments, death occurred within 5 minutes after TBI. Given this short time window, it is unlikely that SRF confers protection from mortality via direct gene transcription since de novo mRNA production and subsequent protein translation might take too long to intervene in this protective mechanism. The only obvious correlation our data provide is a connection between the high mortality rate and the pre-TBI hyperactivity in Srf mutant mice (Figure 5A). The existing literature does not provide any obvious connection between hyperactivity and TBI-associated mortality in animal models or human patients. Thus, our data on SRF provide a first precedence and, although speculative at this stage, suggest that hyperactivity most likely associated with neuronal network changes (see below) might be a confounding factor in TBI-associated mortality.

4.2 | Induction of a post-TBI hyperactivity phenotype upon SRF ablation in mice

Previous reports showed that SRF deletion in glutamatergic33,69 and dopaminoceptive37 neurons produces hyperactivity in mice without any injury or stimulation. In this study we observed that brain injury by TBI further augmented hyperactivity in Srf mutant whereas wt mice responded with hypoactivity (Figure 5). Of note the “basal” hyperactivity in Srf mutant mice in the absence of any exogenous injury/stimulus (Figure 5A) is modulated differently depending on the subsequent stimulus type: in TBI, the initial hyperactivity was further enhanced (Figure 5)
whereas after acute stress, Srf mutants reacted with hypoactivity.\textsuperscript{31} This discrepancy might reflect differential involvement of brain regions in mechanical injury versus stress processing. In any case, this basal hyperactivity in Srf mutants (Figure 5A) has to be taken carefully into account when interpreting data on Srf mutant mice additionally challenged with stress or TBI.

The higher locomotor activity after TBI suggests that SRF ablation might actually enhance recovery after TBI. Indeed, locomotor activity particularly of those Srf mutants starting with an immediate post-TBI paresis (Figure 2) accelerated very quickly. Thus, for selected aspects of post-TBI regeneration such as locomotor activity, SRF deletion might be beneficial. However, SRF might exert a dual function in TBI since—as discussed above—SRF dysfunction clearly raises the risk in mice to die immediately after TBI. Besides IEGs (discussed below), one downstream effector of SRF possibly mediating hyperactivity during TBI is the actin severing factor cofilin (Figure 4). Cofilin mouse mutants display a hyperactivity phenotype\textsuperscript{20} similar to cofilin inhibition achieved in Srf mutant mice in our study (Figure 4).

As previously discussed by others,\textsuperscript{37} the hyperactivity observed in Srf mutants recapitulates one aspect of the wide behavioral spectrum often altered in ADHD patients. A mutual interaction between ADHD in patients and TBI is established\textsuperscript{6} and ADHD is frequently a sequel of brain injury.\textsuperscript{6} Clearly, ADHD patients present several behavioral alterations which most likely cannot all be recapitulated properly in animal models. Nevertheless, work in rodents by others reported induction of hyperactivity 3 weeks after a mild TBI\textsuperscript{71} whereas at earlier timepoints post-TBI (ie, first 3-5 days) wt animals still showed hypoactivity\textsuperscript{71} (Figure 5B). In summary, our data suggest, although speculative currently and with the caveat that Srf mutants were hyperactive already without TBI (Figure 5A and see above), a role of SRF as a potential molecular link between TBI and ADHD.

### 4.3 SRF is a novel upstream gene regulator for TBI-associated induction of neuronal activity related genes

Neuronal activity results in IEG upregulation.\textsuperscript{23} For instance, \textit{cFos} is frequently used as surrogate markers to identify neurons activated by neurotransmitters in physiological but also pathologic conditions.\textsuperscript{23}

In this study, SRF was identified as upstream regulator of several IEGs including \textit{Egr1}, \textit{Egr2}, \textit{Egr3}, \textit{Npas4}, \textit{Arc}, and \textit{Atf3} but also other target genes, \textit{Ptgs2} and \textit{Nptx2}, so far not typically annotated as IEGs (Figures 6 and 7). Of note, \textit{Ptgs2} encoding a prostaglandin synthase (COX2) mediates the brain injury related neuroinflammation.\textsuperscript{72} \textit{Ptgs2} induction was compromised in Srf mutants (Figure 6), however no obvious changes in neuroinflammation in wt versus Srf mutant TBI-affected brains were observed (Figure 3). Interestingly, COX2 downregulation also affects locomotor activity in rodents. Thus, similar to SRF-dependent COX2 mRNA downregulation (Figure 5), pharmacological COX2 inhibition, correlated with increased locomotor activity in a rat posttraumatic stress disorder (PTSD) model.\textsuperscript{73}

In summary, SRF deletion resulted in failure to induce an entire gene set of neuronal activity encoding genes 1 hour after TBI. This 1 hour timepoint after TBI fits well with the proposed rise in neuronal activity triggered by TBI early after the impact.\textsuperscript{10} These SRF regulated genes fall into two classes, i) genes encoding TFs (\textit{Egr1}, \textit{Egr2}, \textit{Egr3}, \textit{Atf3}, \textit{Npas4}) thereby eliciting a second gene expression wave and ii) putative “effector” genes with direct impact on cellular processes such as \textit{Nptx2} and \textit{Arc}. For the TF encoding SRF target genes we noted that Fos family members were largely SRF independently regulated during TBI (Figures 6 and 7), a finding in agreement with previous reports.\textsuperscript{37,52} In contrast, during LTP and seizure propagation \textit{cFos} was regulated in an SRF-dependent manner.\textsuperscript{34,38,39} Thus, \textit{cFos} and family members appear as facilitative SRF target genes and other TFs such as CREB might have a stronger impact.\textsuperscript{60}

In contrast, \textit{Egr1}, \textit{Egr2}, \textit{Egr3}, and also \textit{Npas4} appear to be more constitutively SRF dependent for a wide range of in vivo stimuli.\textsuperscript{34,52}

How might SRF-dependent gene expression during TBI be connected to the hyperactivity phenotype observed in Srf mutant mice? In one possible scenario, TBI results in an immediate increase in neuronal activity in wt brains. This activates SRF and in turn results in a first gene expression wave of TF encoding IEGs such as \textit{Egr1/2/3}, \textit{Atf3} and \textit{Npas4} and “effector” genes such as \textit{Nptx2} and \textit{Ptgs2}. \textit{Npas4} was reported to trigger a delayed gene response promoting inhibition onto excitatory neurons while inducing excitation onto inhibitory neurons.\textsuperscript{74,75} Similarly, \textit{Nptx2} is involved in enhancing feedforward inhibition on neuronal networks.\textsuperscript{61}

Overall, although not shown in this study, these SRF target genes could enhance mechanisms of neuronal inhibition during TBI and thereby limit hyperactivity related behavior. In keeping with this, mouse mutants of several of these IEGs including \textit{Egr3}, \textit{Nptx2}, \textit{Ptgs2}, and \textit{Npas4} also induce hyperactivity\textsuperscript{35-59} suggesting a function for the wt proteins in restricting hyperactive behavior.

Upon SRF deficiency, a “composite” IEG mouse mutant phenotype would be induced, affecting expression of several IEGs (Figures 6 and 7). Such impaired induction of, for example, \textit{Npas4} and \textit{Nptx2} would fail to elevate neuronal inhibition after TBI, thereby shifting the balance from inhibitory to excitatory neuronal transmission which might facilitate together with failure to upregulate hyperactivity repressing IEGs such as \textit{Egr3}, \textit{Nptx2}, \textit{Ptgs2}, and \textit{Npas4} (see above) development of a hyperactive phenotype. Of note, the
hippocampal IEG response after TBI was in general stronger compared to the cortex (Figure 6) suggesting a stronger hippocampus contribution to post-TBI behavioral phenotypes such as hyperactivity observed in this study. In support, a previous study in Srf mutant mice emphasized the importance of the hippocampus over the cortex for habituation in the OF.40

Further studies will have to address this mechanism in more detail, but our data provide a first molecular link between TBI and hyperactivity related behaviors via a signaling cascade involving SRF and neuronal activity encoding genes.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

P. Förstner designed research, performed research, and analyzed data. B. Knöll designed research, analyzed data, and wrote the paper.

REFERENCES

1. Stocchetti N, Zanier ER. Chronic impact of traumatic brain injury on outcome and quality of life: a narrative review. Crit Care. 2016;20:148.
2. Bruns J Jr, Hauser WA. The epidemiology of traumatic brain injury: a review. Epilepsia. 2003;44(Suppl 10):2-10.
3. Vos BC, Nieuwenhuijsen K, Sluiter JK. Consequences of traumatic brain injury in professional American football players. Clinical Journal of Sport Medicine. 2018;28:91-99.
4. Verellen RM, Cavazos JE. Post-traumatic epilepsy: an overview. Therapy. 2010;7:527-531.
5. Rogers JM, Read CA. Psychiatric comorbidity following traumatic brain injury. Brain Inj. 2007;21:1321-1333.
6. Adeyemo BO, Biederman J, Zafonte R, et al. Mild traumatic brain injury and ADHD: a systematic review of the literature and meta-analysis. J Atten Disord. 2014;18:576-584.
7. Schumann J, Alexandrovich GA, Biegon A, Yaka R. Inhibition of NR2B phosphorylation restores alterations in NMDA receptor expression and improves functional recovery following traumatic brain injury in mice. J Neurotrauma. 2008;25:945-957.
8. Ping X, Jin X. Transition from initial hypoactivity to hyperactivity in cortical layer V pyramidal neurons after traumatic brain injury in vivo. J Neurotrauma. 2016;33:354-361.
9. Patel TP, Ventre SC, Geddes-Klein D, Singh PK, Meaney DF. Single-neuron NMDA receptor phenotype influences neuronal rewiring and reintegration following traumatic injury. J Neurosci. 2014;34:4200-4213.
10. Biegon A, Fry PA, Paden CM, Alexandrovich A, Tsenter J, Shohami E. Dynamic changes in N-methyl-D-aspartate receptors after closed head injury in mice: implications for treatment of neurological and cognitive deficits. Proc Natl Acad Sci U S A. 2004;101:5117-5122.
11. Almeida-Suhett CP, Prager EM, Pidoplichko V, et al. GABAergic interneuronal loss and reduced inhibitory synaptic transmission in the hippocampal CA1 region after mild traumatic brain injury. Exp Neurol. 2015;273:11-23.
12. Almeida-Suhett CP, Prager EM, Pidoplichko V, et al. Reduced GABAergic inhibition in the basolateral amygdala and the development of anxiety-like behaviors after mild traumatic brain injury. PLoS ONE. 2014;9:e102627.
13. Yap EL, Greenberg ME. Activity-regulated transcription: bridging the gap between neural activity and behavior. Neuron. 2018;100:330-348.
14. Benito E, Barco A. The neuronal activity-driven transcriptome. Mol Neurobiol. 2015;51:1071-1088.
15. Titus DJ, Wilson NM, Freund JE, et al. Chronic cognitive dysfunction after traumatic brain injury is improved with a phosphodiesterase 4B inhibitor. J Neurosci. 2016;36:7095-7108.
16. Sen T, Gupta R, Kaiser H, Sen N. Activation of PERK elicits memory impairment through inactivation of CREB and down-regulation of PSD95 after traumatic brain injury. J Neurosci. 2017;37:5900-5911.
17. Hu B, Liu C, Bramlett H, et al. Changes in trkB-ERK1/2-CREB/Elk-1 pathways in hippocampal mossy fiber organization after traumatic brain injury. J Cereb Blood Flow Metab. 2004;24:934-943.
18. Dash PK, Moore AN, Dixon CE. Spatial memory deficits, increased phosphorylation of the transcription factor CREB, and induction of the AP-1 complex following experimental brain injury. J Neurosci. 1995;15:2030-2039.
19. Pardo L, Schluter A, Valor LM, et al. Targeted activation of CREB in reactive astrocytes is neuroprotective in focal acute cortical injury. Glia. 2016;64:853-874.
20. Olson EN, Nordheim A. Linking actin dynamics and gene transcription to drive cellular motile functions. Nat Rev Mol Cell Biol. 2010;11:353-365.
21. Knoll B, Nordheim A. Functional versatility of transcription factors in the nervous system: the SRF paradigm. Trends Neurosci. 2009;32:432-442.
22. Xia Z, Dudek H, Miranti CK, Greenberg ME. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci. 1996;16:5425-5436.
23. Minatohara K, Akiyoshi M, Okuno H. Role of immediate-early genes in synaptic plasticity and neuronal ensembles underlying the memory trace. Front Mol Neurosci. 2015;8:78.
24. Kim S, Kim H, Um JW. Synapse development organized by neuronal activity-regulated immediate-early genes. Exp Mol Med. 2018;50:11.
25. Gallo FT, Katche C, Morici JF, Medina JH, Weisstaub NV. Immediate early genes, memory and psychiatric disorders: focus on c-Fos, Egr1 and Arc. Front Behav Neurosci. 2018;12:79.
26. Whitfield PC, Pickard JD. Expression of the immediate early genes c-Fos and c-Jun after head injury in man. Neurol Res. 2000;22:138-144.
27. Michael DB, Byers DM, Irwin LN. Gene expression following traumatic brain injury in humans: analysis by microarray. J Clin Neurosci. 2005;12:284-290.
28. Natale JE, Ahmed F, Cernak I, Stoica B, Faden AI. Gene expression profile changes are commonly modulated across models and species after traumatic brain injury. J Neurotrauma. 2003;20:907-927.

29. Greer JE, McGinn MJ, Povlishock JT. Diffuse traumatic axonal injury in the mouse induces atrophy, c-Jun activation, and axonal outgrowth in the axotomized neuronal population. J Neurosci. 2011;31:5089-5105.

30. Greer JE, Hanell A, McGinn MJ, Povlishock JT. Mild traumatic brain injury in the mouse induces axotomy primarily within the axon initial segment. Acta Neuropathol. 2013;126:59-74.

31. Dutcher SA, Underwood BD, Walker PD, Diaz FG, Michael DB. Patterns of immediate early gene mRNA expression following rodent and human traumatic brain injury. Neurol Res. 1999;21:234-242.

32. Chandrasekar A, Aksan B, Heuvel FO, et al. Neuroprotective effect of acute ethanol intoxication in TBI is associated to the hierarchical modulation of early transcriptional responses. Exp Neurol. 2018;302:34-45.

33. Zimprich A, Mroz G, Meyer zu Beckendorf C, et al. Serum Response Factor (SRF) ablation interferes with acute stress-associated immediate and long-term coping mechanisms. Mol Neurobiol. 2017;54:8242-8262.

34. Losing P, Niturad CE, Harrer M, et al. SRF modulates seizure occurrence, activity induced gene transcription and hippocampal circuit reorganization in the mouse pilocarpine epilepsy model. Mol Brain. 2017;10:30.

35. Forstner P, Rehman R, Anastasiadou S, et al. Neuroinflammation after traumatic brain injury is enhanced in activating transcription factor 3 mutant mice. J Neurotrauma. 2018;35:2317-2329.

36. Flierl MA, Stahel PF, Beauchamp KM, Morgan SJ, Smith WR, Forstner P, Rehman R, Anastasiadou S, et al. Neuroinflammation affects immediate and long-term coping mechanisms. Mol Neurobiol. 2017;54:8242-8262.

37. Parkitna JR, Bilbao A, Rieker C, et al. Loss of the serum response factor in the dopamine system leads to hyperactivity. FASEB J. 2010;24:2427-2435.

38. Kuzniweska B, Nader K, Dabrowski M, Kaczmarek L, Kalita K. Adult deletion of SRF increases epileptogenesis and decreases activity-induced gene expression. Mol Neurobiol. 2016;53:1478-1493.

39. Ramanan N, Shen Y, Sarsfield S, et al. SRF mediates activity-induced gene expression and synaptic plasticity but not neuronal viability. Nat Neurosci. 2005;8:759-767.

40. Etkin A, Alarcon JM, Weisberg SP, et al. A role in learning for SRF: cAMP response element-binding protein is a primary hub of activity-driven neuronal gene expression. J Neurosci. 2006;50:127-143.

41. Kuzniweska B, Nader K, Dabrowski M, Kaczmarek L, Kalita K. Adult deletion of SRF increases epileptogenesis and decreases activity-induced gene expression. Mol Neurobiol. 2016;53:1478-1493.

42. Stern S, Haverkamp S, Sinske D, et al. The transcription factor serum response factor stimulates axon regeneration through cytoplasmic localization and cofillin interaction. J Neurosci. 2013;33:18836-18848.

43. Stern S, Sinske D, Knoll B. Serum response factor modulates neuron survival during peripheral axon injury. J Neuroinflammation. 2012;9:78.

44. Anastasiadou S, Liebenem S, Sinske D, et al. Neuronal expression of the transcription factor serum response factor modulates myelination in a mouse multiple sclerosis model. Glia. 2015;63:958-976.

45. Olde Heuvel F, Holl S, Chandrasekar A, et al. STAT6 mediates the effect of ethanol on neuroinflammatory response in TBI. Brain Behav Immun. 2019;81:228-246.

46. Anastasiadou S, Knoll B. The multiple sclerosis drug fingolimod (FTY720) stimulates neuronal gene expression, axonal growth and regeneration. Exp Neurol. 2016;279:243-260.

47. Mettang M, Reichel SN, Lattke M, et al. IKK2/NF-kappaB signaling protects neurons after traumatic brain injury. FASEB J. 2018;32:1916-1932.

48. Philippar U, Schratt G, Dieterich C, et al. The SRF target gene Fhl2 antagonizes Rho/MLC-dependent activation of SRF. Mol Cell. 2004;16:867-880.

49. Needham EJ, Helmy A, Zanier ER, Jones JL, Coles AJ, Menon DK. The immunological response to traumatic brain injury. J Neuroimmunol. 2019;332:112-125.

50. Mokalled MH, Johnson A, Kim Y, Oh J, Olson EN. Myocardin-related transcription factors regulate the Cdk5/Pcatpr1 kinase cascade to control neurite outgrowth, neuronal migration and brain development. Development. 2010;137:2365-2374.

51. Alberti S, Krause SM, Kretz O, et al. Neuronal migration in the murine rostral migratory stream requires serum response factor. Proc Natl Acad Sci U S A. 2005;102:6148-6153.

52. Zimprich A, Garrett L, Deussing JM, et al. A robust and reliable non-invasive test for stress responsivity in mice. Front Behav Neurosci. 2014;8:125.

53. Schaukowitch K, Reese AL, Kim SK, et al. An intrinsic transcriptional program underlying synaptic scaling during activity suppression. Cell Rep. 2017;18:1512-1526.

54. Guerci A, Lahoute C, Hebrard S, et al. Srf-dependent paracrine signals produced by myofibers control satellite cell-mediated skeletal muscle hypertrophy. Cell Metab. 2012;15:25-37.

55. Wong CT, Bestard-Lorigados I, Crawford DA. Autism-related behaviors in the cyclooxygenase-2-deficient mouse model. Genes Brain Behav. 2019;18:e12506.

56. Pacchioni AM, Vallone J, Worley PF, Kalivas PW. Neuronal peroxisome proliferator-activated receptor gamma modulates cocaine-induced neuroadaptations. J Pharmacol Exp Ther. 2009;328:183-192.

57. Ohtsuki YN, Ohtsuki YH, Hokama M, et al. FosB is essential for the enhancement of stress tolerance and antagonizes locomotor sensitization by DeltaFosB. Biol Psychiatry. 2011;70:487-495.

58. Gallitano-Mendel A, Izumi Y, Tokuda K, et al. The immediate early gene early growth response gene 3 mediates adaptation to stress and novelty. Neuroscience. 2007;148:633-643.

59. Couteller L, Beraki S, Ardestani PM, Saw NL, Shamloo M. Npas4: a neuronal transcription factor with a key role in social and cognitive functions relevant to developmental disorders. PLoS ONE. 2012;7:e46604.

60. Benito E, Valor LM, Jimenez-Minchan M, Huber W, Barco A. cAMP response element-binding protein is a primary hub of activity-driven neuronal gene expression. J Neurosci. 2011;31:18237-18250.

61. Pelkey KA, Barksdale E, Craig MT, et al. Pentraxins coordinate excitatory synaptic maturation and circuit integration of parvalbumin interneurons. Neuron. 2015;85:1257-1272.

62. Shohami E, Biegon A. Novel approach to the role of NMDA receptors in traumatic brain injury. CNS Neurol Disord Drug Targets. 2014;13:567-573.

63. Dorsett CR, McGuire JL, DePasquale EA, Gardner AE, Floyd CL, McCormum Smith RE. Glutamate neurotransmission in Rodent Models of Traumatic Brain Injury. J Neurotrauma. 2017;34:263-272.

64. Tomasevic G, Ragupathi R, Scherbel U, Wieloch T, McIntosh TK. Deletion of the p53 tumor suppressor gene improves...
neuromotor function but does not attenuate regional neuronal cell loss following experimental brain trauma in mice. J Neurosci Res. 2010;88:3414-3423.

65. Neher MD, Rich MC, Keene CN, et al. Deficiency of complement receptors CR2/CR1 in C2(-/-) mice reduces the extent of secondary brain damage after closed head injury. J Neuroinflammation. 2014;11:95.

66. Sullivan PG, Bruce-Keller AJ, Rabchevsky AG, et al. Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury. J Neurosci. 1999;19:6248-6256.

67. Hanell A, Clausen F, Bjork M, et al. Genetic deletion and pharmacological inhibition of Nogo-66 receptor impairs cognitive outcome after traumatic brain injury in mice. J Neurotrauma. 2010;27:1297-1309.

68. Thomsen GM, Vit JP, Lamb A, et al. Acute traumatic brain injury does not exacerbate amyotrophic lateral sclerosis in the SOD1 (G93A). Rat Model. 2015:eNeuro:2.

69. Nader K, Krysiak A, Beroun A, et al. Loss of serum response factor in mature neurons in the dentate gyrus alters the morphology of dendritic spines and hippocampus-dependent behavioral tasks. Brain Struct Funct. 2019.

70. Zimmermann AM, Jene T, Wolf M, et al. Attention-deficit/hyperactivity disorder-like phenotype in a mouse model with impaired actin dynamics. Biol Psychiatri. 2015;78:95-106.

71. Mychasiuk R, Hehar H, Esser MJ. A mild traumatic brain injury (mTBI) induces secondary attention-deficit hyperactivity disorder-like symptomology in young rats. Behav Brain Res. 2015;286:285-292.

72. Aid S, Bosetti F. Targeting cyclooxygenases-1 and -2 in neuroinflammation: Therapeutic implications. Biochimie. 2011;93:46-51.

73. Wang M, Duan F, Wu J, et al. Effect of cyclooxygenase2 inhibition on the development of posttraumatic stress disorder in rats. Mol Med Rep. 2018;17:4925-4932.

74. Spiegel I, Mardinly AR, Gabel HW, et al. Npas4 regulates excitatory-inhibitory balance within neural circuits through cell-type-specific gene programs. Cell. 2014;157:1216-1229.

75. Lin Y, Bloodgood BL, Hauser JL, et al. Activity-dependent regulation of inhibitory synapse development by Npas4. Nature. 2008;455:1198-1204.

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