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Spatio-temporal and cell type-specific expression of Nrf2 following an acute epileptic seizure in rats

Sereen Sandouka[1#], Aseel Saadi[1#], Rhoda Olowe[1], Prince Kumar Singh[1], Tawfeeq Shekh-Ahmad[1*]

[1]The Institute for Drug Research, The School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel – 91120.

[#]S.S. and A.S. contributed equally to this work.

*Correspondence: Tawfeeq Shekh-Ahmad, The Institute for Drug Research, The School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel – 91120. E-mail: Tawfeeq.Shekh-Ahmad@mail.huji.ac.il

Abstract

The modulation of Nrf2 activity has been reported to be implicated in the pathology of various neurological disorders, including epilepsy. Previous studies have demonstrated that Nrf2 is activated in the post-status epilepticus rat model, however, the spatio-temporal, as well as cell type-specific expression of Nrf2 following brief epileptic seizures remains unclear. Here, we evaluated how an acute epileptic seizure affected the expression of Nrf2 and its downstream genes in the cortex and the hippocampus up to 1-week following the induced seizure.

We found that after a pentylenetetrazol-induced seizure, Nrf2 significantly increased at 24 h at the mRNA level and 3 to 6 h at the protein level in the cortex. In the hippocampus, the Nrf2 mRNA level peaked at 3 h after the seizure, and no significant changes were observed in the protein level. Interestingly, the mRNA level of Nrf2 downstream genes peaked at 3-6 h after seizure in both the cortex and the hippocampus. A significant increase in the expression of Nrf2 was observed in the neuronal population of CA1 and CA3 regions of the hippocampus, as well as in the cortex. Moreover, we observed no change in the co-localization of Nrf2 with astrocytes neither in the cortex nor in CA1 and CA3.

Our results revealed that following a brief acute epileptic seizure, the expression of Nrf2 and its downstream genes is transiently increased and peaked at early timepoints after seizure predominantly in the hippocampus, and this expression is restricted to the neuronal population.

Keywords: Nrf2, Oxidative stress, Antioxidants, Pentylenetetrazol, Epilepsy, Seizure.

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Abbreviations: Cat-2 = Catalase 2; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GCLC-1 = Glutamate cysteine ligase catalytic; HO-1 = Haem oxygenase 1 gene; NQO1 = NAD(P)H: quinone oxidoreductase 1; Nrf2 = Nuclear factor erythroid 2-like 2; OS = Oxidative Stress; PTZ = Pentylenetetrazol; ROS = Reactive Oxygen Species; SE = Status Epilepticus; Srxn = Sulfiredoxin 1 homolog (Saccharomyces cerevisiae). Keap1= Kelch-like ECH-associated protein 1.

1. Introduction

Epilepsy is a prevalent, chronic, and progressive neurological disorder that is often associated with cognitive impairment, imposing a substantial impact on the quality of life of patients and imposing significant challenges for their caregivers and healthcare providers[1, 2]. Although a significant number of anti-seizure medications (ASMs) are currently being licensed for the treatment of epileptic seizures, over 30% of all epileptic patients are resistant to these ASMs and continued to experience spontaneous seizures[3]. Moreover, most of these epilepsies arise as a result of brain insults such as traumatic brain injuries (TBI)[4], prolonged seizures *i.e.*, status epilepticus (SE), or stroke[5]. Various studies confirmed that oxidative stress (OS) and overproduction of reactive oxygen species (ROS) are crucial players in acute neurological injuries such as TBI[6], and stroke[7], as well as in neurodegenerative disorders like Parkinson's[8], and Alzheimer's disease[9, 10].

Recently, Nrf2 has emerged as a therapeutic target for various diseases, including neurological disorders[11]. Under unstimulated or resting conditions, Nrf2 is sequestered within the cytosol by Kelch-like ECH-associated protein 1 (Keap1) and is then degraded by proteasomal degradation[12]. Upon stimulation by oxidation or other disturbance in cell homeostasis, Nrf2 translocates to the nucleus and binds to antioxidant response element (ARE) in the promoter regions of crucial genes, (e.g., heme-oxygenase 1; HO-1 and NAD(P)H: quinone oxidoreductase-1; NQO1) [13-15], responsible for detoxification, antioxidant activity, and anti-inflammatory responses, resulting in an orchestrated protective responses[16]. Kraft *et al.* 2006 reported that Nrf2 knockout mice are more susceptible to kainate toxicity, and showed high frequency and duration of seizure, hippocampal neuronal damage, and mortality[17]. Later on, Wang *et al.*, confirmed that oxidative stress is responsible for kindled seizure in rats and showed that following kindling seizure expression of Nrf2 and associated genes significantly increased in the hippocampus, suggesting the activation of the Nrf2-ARE signaling cascade in the hippocampus following seizure[18]. Mazzuferi *et al. 2013*, reported

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that the Nrf2 pathway is overexpressed in humans and mice[19]. In their study, they observed a 2-fold increase in Nrf2 mRNA expression in human hippocampal tissue from patients with TLE[19]. In mice, Nrf2 levels were increased progressively after SE, reached a peak at 72 hours, and then declined[19]. Moreover, similar expression patterns were also observed for 3 Nrf2-regulated genes[19]. Several previous studies suggested that the observation that Nrf2 is repressed in cultured neurons and that activation in cultured cells is restricted to astrocytes, may not reflect the situation in neurodegenerative animal models[20].

These studies strongly supported the hypothesis that activation of the Nrf2 pathway may exert a protective role in epilepsy. Indeed, activation of Nrf2 has been shown to be protective against OS in various pathologies[12, 21]. In epilepsy animal models, Nrf2 has been shown to increase in kindled animals, and overexpressing Nrf2 showed a neuroprotective effect in chemical kindling in rats and SE in mice[18, 19, 22]. Recently, we have demonstrated that pharmacological activation of Nrf2 can suppress the development of epilepsy in the rat temporal lobe epilepsy (TLE) model when given alone or in combination with an exogenous antioxidant[23-25]. We also showed that overactivation of Nrf2 following SE significantly decreased the neuronal cell death in rats[22].

Interestingly, changes in the expression of Nrf2 has been reported in a number of different cell types in brain of epileptic animals[19, 26-28]. Although in cell culture, Nrf2 is more often expressed in astrocytes, neuronal Nrf2 activation is apparent in humans and mice[20]. The activation of Nrf2 has been previously studied in different cell types in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease in human brain and animal models of neurodegeneration[20].

Nevertheless, the spatial, temporal and cell type-specific expression of Nrf2 following acute epileptic seizure has not been investigated yet. In this study, we studied the spatio-temporal and cell type-specific distribution of Nrf2 following acute seizure induced by the common convulsant agent pentylenetetrazol (PTZ). Here, we demonstrated that the expression of Nrf2 increased significantly at mRNA level at 24 h and 3 to 6 h at the protein level in the cortex after the PTZ-induced seizure. In the hippocampus, the mRNA level of Nrf2 significantly increase and peaked at 3 h after seizure while there are no significant changes detected in the protein level. However, we also determined the expression of Nrf2 target genes and found that expression at mRNA level significantly increase at 3 to 6 h after seizure in both cortex and hippocampus of PTZ induced seizure rats. Our immunohistochemical investigations confirmed that after epileptic seizure expression of Nrf2 predominantly increased in the CA3 and CA1 regions of the hippocampus and interestingly, this expression is predominantly restricted to the

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neuronal population of the CA3 region of the hippocampus. However, we didn't observe any significant changes in the cortex.

2. Materials and methods

2.1.Experimental epilepsy models

2.1.1. Animals

All animal experiments were performed according to Association for Assessment and Accreditation of Laboratory Animal Care International and authorized by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem (Approval No.: MD-20-16254-5). This study conducted on male and female Sprague-Dawley (SD) rats (150-220 gm), a strain of the Hebrew University of Jerusalem (received from Harlan, Jerusalem, Israel). All animals were housed individually with free access to food and water and under regulated environmental conditions (23±1 °C; 50–60% humidity; 12-h light/dark cycle). All animals were acclimatized to animal house for at least 3-7 days prior to experimental use.

2.1.2. Induction of acute seizure

To induce acute seizure, naïve SD rats were administered pentylenetetrazol (PTZ) (Sigma, St. Louis, MO, USA) dissolved in 0.9% saline, administered subcutaneously at a dose of 90 mg/kg. After PTZ injection, rats were placed in their home cages and seizure behavior was recorded until convulsions stopped and rats were fully recovered. The severity of seizures was measured according to modified Racine's scale [29, 30]. Rats successfully achieved stage 5 generalized seizure were sacrificed at respective timepoint *i.e.*, 3, 6, 24, 48, and 72 hrs as well as 1 week post seizure onset and brain samples were recovered, flash frozen and stored at -80^{o}C for further investigation.

2.2.Real Time-Polymerase Chain Reaction

Name	Accession number	Forward	Reverse
Nrf2	NM_031789	GCAACTCCAGAAGGAACAGG	GGAATGGCTCTCTGCCAAAAGC
GAPDH	NM_017008	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

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NQO1	NM_017000	GTTTGCCTGGCTTGCTTTCA	ACAGCCGTGGCAGAACTATC
HO-1	NM_012580	ACAGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG
GCLC-1	NM_012815	GAGTAGAGTTCCGACCAAT	GCTCCTGTGCCACTTTCA
Srxn1	NM_001047858	AATAGTGAGGTCACCAGCTTC	CAGACAGTATGAGTCCTGGTTG
Cat-2	NM_012520	CCTGACATGGTCTGGGACTT	CAAGTTTTTGATGCCCTGGT

Quantitative real-time PCR was utilized to demonstrate the expression of Nrf2 and its downstream target genes including NQO1 [NAD(P)H: quinone oxidoreductase 1], HO-1 (Heme Oxygenase 1), Srxn (Sulfiredoxin), GCLC1 (Glutamate-Cysteine Ligase Catalytic Subunit), and CAT-2 (Catalase). Primers for selected genes were designed with Primer express software 3.0 (Applied biosystem) by Gen-Bank sequences as listed in Table 1.

Total RNA, from rat cortex and hippocampal tissue, were extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) followed by purity and concentration assessment achieved by using Nanodrop spectrophotometer (Nanodrop Technologies, Thermo, Waltham, MA, USA) spectrophotometry) and 260:280 were 1.8-2.0. Complementary DNA (cDNA) were synthesized from 1000 ng of extracted RNA from each sample using oligo-dT_{15} primers and GoScript Reverse Transcription System (Promega). Expression of above selected redox-relevant genes was determined by using SYBER Green (PerfeCTa SYBR Green FastMix, Quantabio) and RT-PCR instrument (CFX-Bio-Rad). The master mix was prepared for 15 µl per reaction (7.5 µl of SYBR Green, 3 µl of primer (500nM each), 1.5 µl of RNase free water and 3 µl of cDNA template) and prepared reaction were subject to real-time PCR reaction cycles. Reaction cycles consist of initial incubation for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 5s, at 60°C for 15s and a final step of 5s at 65°C and 30s at 95°C. Each reaction was performed in duplicates. For each mRNA, gene expression was corrected by the mRNA GAPDH subunit content in each sample. The results were estimated and represented with respect to the sham group, normalized to 1, and Pfaffl *et al*., 2001[31] method was used to determine relative expression of each gene.

2.3.Western blot analysis

Rats were deeply anaesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) and their brains were collected rapidly, cortex and hippocampus were dissected-out following by washing with chilled PBS and tissue samples were subjected to homogenization and lysis using RIPA buffer (Thermo Scientific) with freshly added protease inhibitor PI-(III) cocktail (Sigma). Protein concentration was quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific). An equal amount of protein sample *i.e.,* 25 µg was loaded onto each well of 10-

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12% SDS-PAGE. Resolved proteins were transferred to the nitrocellulose membrane (Bio-rad, Hercules, CA, USA) followed by blocking either with 5% skim-milk for Nrf2 and NQO1 or 5% BSA for β actin. Blocked membranes were incubated overnight at 4°C with primary antibodies against Nrf2 (1:1000, ab31163), NQO1 (1:1000, ab34173) and β actin (1:2000, ab8226). Overnight incubated membranes were subject to three washing, 10 min each with 0.1% Tween-20 in PBS (PBST) followed by 2 h of dark incubation with HRP conjugated secondary antibodies at room temperature. Secondary antibody incubated membranes were washed three times, 10 min each under dark condition. Immunological complexes were visualized with electrochemiluminescence (ECL, Bio-Rad) and band intensities were analyzed by Image Lab software (Bio-Rad, Hercules, CA, USA). Protein band densities were normalized against β-actin band densities values, and the average of the sham group was normalized to 1. The values of all other timepoints band densities were expressed with respect to sham group.

2.4. Immunohistochemistry and microscopy

Animals were sacrificed at 24 hrs following PTZ seizure. Rats were terminally anesthetized with sodium pentobarbital (CTS chemical industries) and transcardially perfused first with chilled (4° C) heparinized phosphate buffer saline (PBS) then by 4% paraformaldehyde (PFA, Sigma) in chilled PBS. Brain samples were extracted and kept overnight in 4% PFA-PBS solution at 4°C then subsequently transferred to 10-20-30% sucrose gradient for 3-4 days until brains sank. Following cryoprotection brain samples were fixed with O.C.T. compound (Scigen) and stored at -80°C. For each brain, 20 µm coronal sections selected from hippocampus were cut using cryostat (Leica CM1950) at -20°C and fixed on poly-L-lysine coated slides (Thermo Fisher Scientific) and allowed for air dry at room temperature for 2 h. Brain sections were circled with water repellent pen (Dako pen, Agilent) followed by permeabilization with 0.2% Triton X-100 (Sigma) in PBS for 30 min, followed by blocking with 7% goat serum (Sigma) for 2-3 h then three time washing, 10 min each with PBS. Sections were incubated overnight at 4°C with a rabbit primary antibody against Nrf2 (1:100, ab31163), mouse primary antibody against NeuN (1:500, ab104224) and chicken primary antibody against GFAP (1:1000, ab4674) in the solution of PBS, 0.1% Triton X-100 and 1% BSA. Followed by three washes with PBS, 10 min each; sections were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:500; ab150117, Abcam), Alexa Fluor-568 goat anti-rabbit secondary antibody (1:500; ab 175695, Abcam) and Alexa Fluor-647 goat anti-chicken (1:500; ab150175, Abcam) for 2 h at room temperature under dark condition. The sections were washed three times with PBS, 10 min each and mounted with Vectashield

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and 4', 6-diamidino-2-phenylindole (DAPI) mounting medium (Vector Labs). Images were captured with 20x and 40x objective of Nikon confocal A1R microscope at resolution of 1024×1024. Image analysis was performed using ImageJ software in a manual cell counting image-based tool. NeuN+ and GFAP+ cells were counted, by investigators blinded to treatment, on 2-3 sections from each animal, and 2-3 images of each ROI. Cell densities were normalized to sham animals as percentage of Nrf2+/NeuN+; Nrf2+/GFAP+ per area (mm^2).

2.5. Statistical analysis

All statistical analysis were performed using GraphPad Prism v9.3.1 software (GraphPad, USA). All data acquisition and analysis were performed blindly. Data were analyzed using Student's t-test or ordinary One-way analysis of variance (ANOVA) followed by Dunnett *post-hoc* test. All data are expressed as mean ± standard error of the mean (SEM). Statistical significance was defined as a value of $p < 0.05$. The number of animals utilized are specified in the respective figure's legend.

3. Results

3.1. Nrf2 expression is progressively increase in the cortex following PTZ- induced seizure

To demonstrate the spatial and temporal expression of Nrf2 in the brain following acute epileptic seizure, we evaluated the expression of Nrf2, as well as its target genes in the cortex and the hippocampus extracted from PTZ-induced seizure rats (n=6) following 3, 6, 24, 48, and 72 hrs as well as 1 week after seizure, along with sham animals (n=6 rats/time point) (Figure 1). In the cortex, the expression of Nrf2 at the mRNA level was progressively increased and peaked at 24 h after seizure, and then return to basal expression for up to 1-week after seizure (Figure 2A). At the protein levels, the expression of Nrf2 followed a similar pattern of initial increase, peaked at 3-6 h after seizure, then return to baseline levels (Figure 2C-D). Further, we measured the expression of NQO1, a prototypic Nrf2 target enzyme[32]. Interestingly, the expression of NQO1 did not follow a similar expression pattern of its key regulator Nrf2, and there were no significant changes observed at any timepoint following seizure neither at the mRNA level nor at the protein level (Figure 2B, C, and E).

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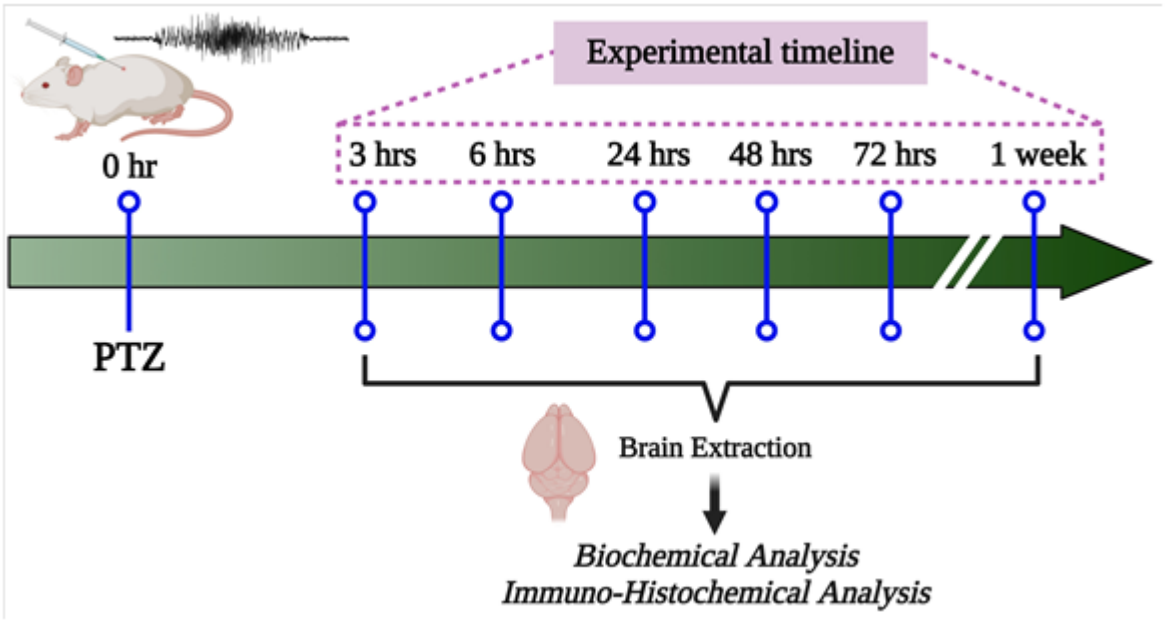

Figure 1: Schematic illustration of experimental design showing induction of PTZ-induced acute seizure following 3, 6, 24, 48, and 72 hrs as well as 1 week of post-seizure brain extraction and performing biochemical and immunohistochemical analysis.

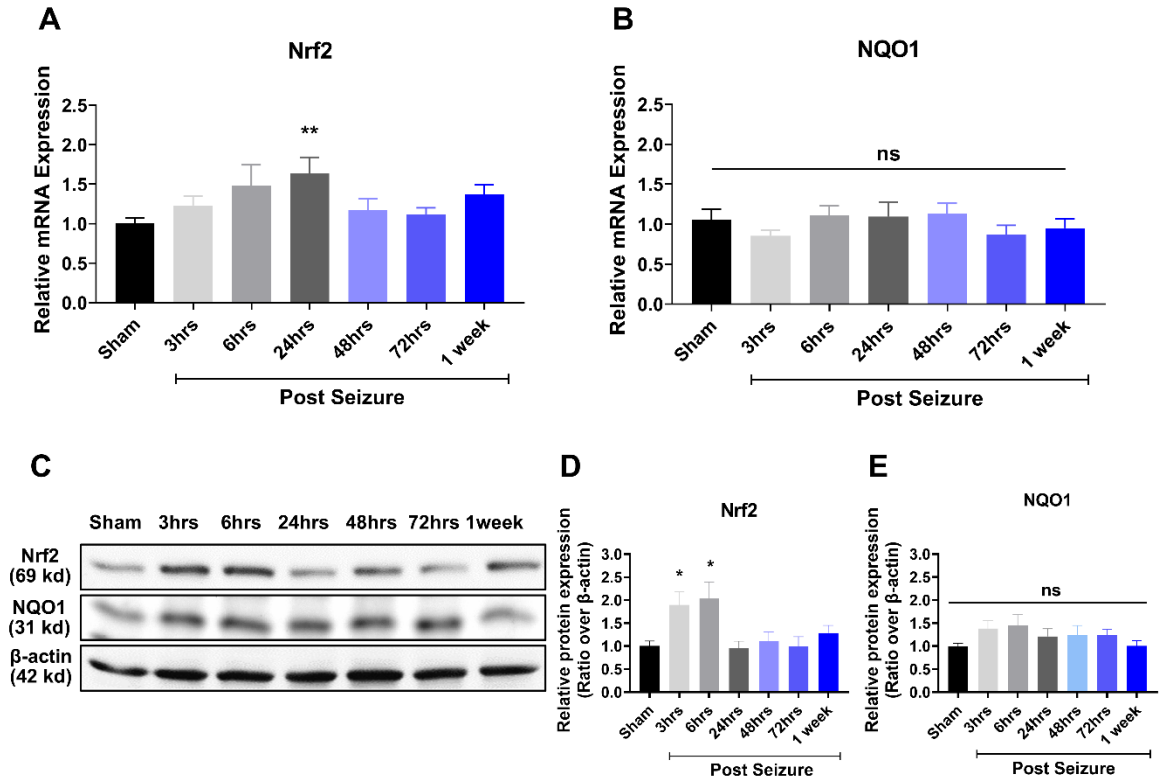

Figure 2. Nrf2 is transiently activated in the cortex after acute seizure. The temporal expression of Nrf2 (A) and NQO1 (B), at mRNA level using RT-PCR as well as the Nrf2 and NQO1 expression at the protein level using western blot analysis for Nrf2 and NQO1 (C) in the cortex following PTZ-induced acute seizure. Relative protein quantification of each time points of all blots (n=6) for Nrf2 (D) and NQO1 (E). Results are expressed as relative

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protein expression and reported as mean±SEM. **P<0.05, **P<0.01, analyzed by one-way ANOVA followed by Dunnett* post-hoc *test.*

3.2.Nrf2 expression is increased early after PTZ-induced seizure in the hippocampus

Next, we asked whether the Nrf2 pathway may exert a regional-specific expression following acute seizure. We therefore measure the expression of Nrf2 and NQO1 in the hippocampi of rats following PTZ-induced seizure. We found that the Nrf2 mRNA levels increased significantly 3 h after seizure then returned to basal levels for up to 1-week afterwards (Figure 3A). However, the expression of Nrf2 at the protein level showed no significant changes at all timepoints (Figure 3C-D). Unlike the expression of NQO-1 in the cortex, the mRNA levels of NQO1 were initially increased at 3 h after seizure (Figure 3B; p=0.031 vs sham group), then return to normal levels followed by an additional peak at 1-week after seizure (Figure 3 B; p=0.037 vs sham group). At the protein levels, NQO1 was progressively increased, and peaked at 72 h after PTZ-induced seizure (Figure 3C, E; p=0.034 vs sham group).

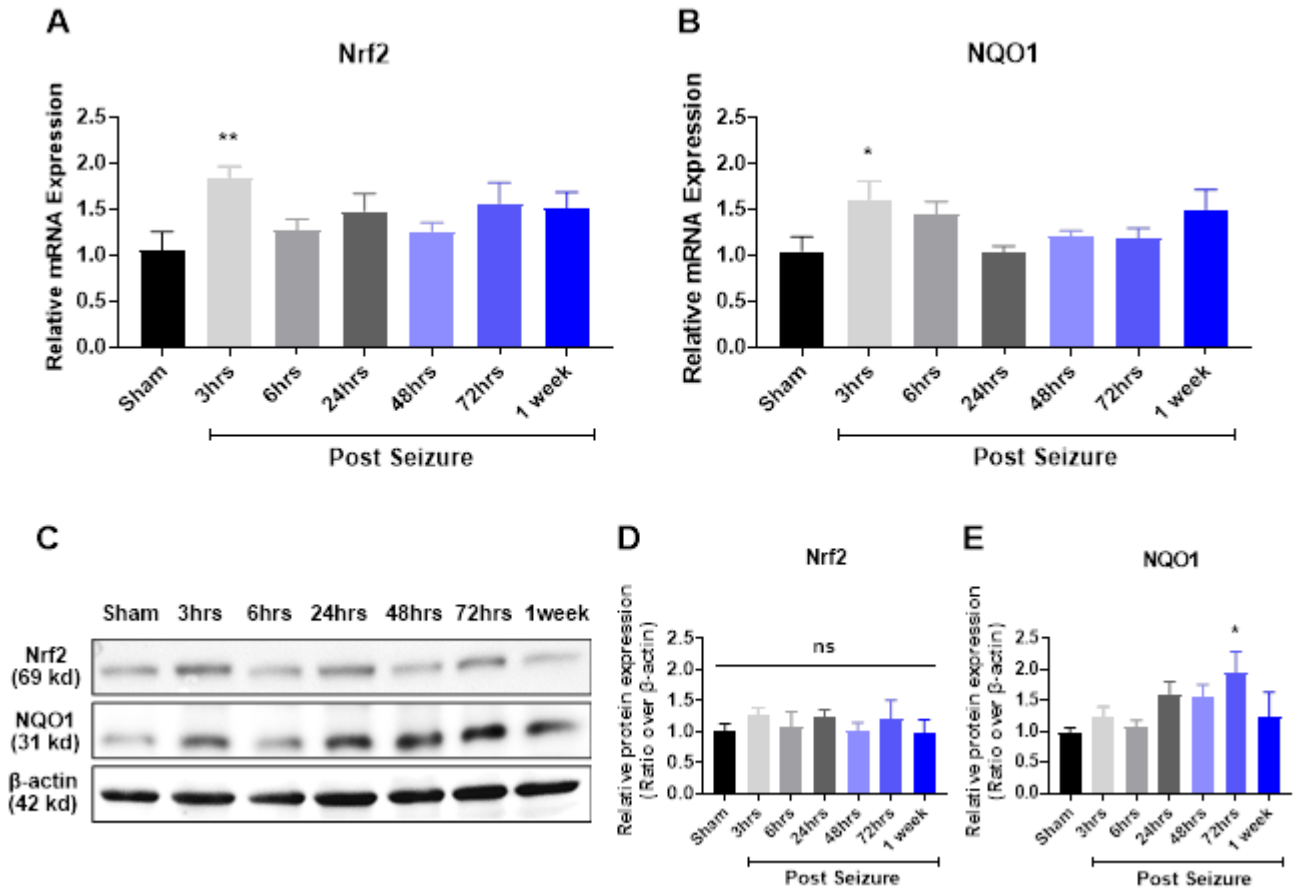

Figure 3. Nrf2 and NQO1 are activated early in the hippocampus after acute seizure. The temporal expression of Nrf2 (A) and NQO1 (B), at mRNA level using RT-PCR as well as Nrf2 and NQO1 expression at the protein level using western blot analysis for Nrf2 and NQO1 (C) in the hippocampus following PTZ-induced acute seizure (n=6). Relative protein quantification of each time points of all blots (n=6) for Nrf2 (D) and NQO1

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(E). Results are expressed as relative protein expression and reported as mean±SEM. *$P<0.05$, **$P<0.01$, *analyzed by one-way ANOVA followed by Dunnett* post-hoc *test.*

3.3.Nrf2 target genes are transiently expressed in the cortex and the hippocampus after PTZ-induced seizure

In response to stimuli such as oxidative stress, Nrf2 is known to activate a wide battery of antioxidant and detoxification genes[33]. We therefore asked whether PTZ-induced seizure will evoke any change in the expression of 4 key Nrf2 target genes in the cortex or in the hippocampus. We measured the mRNA levels of HO-1 (Haem oxygenase 1), Srxn (Sulfiredoxin 1 homolog), GCLC1 (Glutamate cysteine ligase catalytic), and CAT-2 (Catalase 2). In the cortex, HO-1 was initially increased (peaked at 3 h) then progressively decreased to normal levels within 24 h after seizure (Figure 4A). A similar behavior was observed also in the hippocampus, however to a greater increase in the hippocampus compared to the cortex (270% vs. 190%, respectively; Figure, 4A, E). The expression of Srxn was also significantly increased in the cortex at 3 and 6 h after seizure, as well as in the hippocampus at 3 h after seizure (Figure 4B, F). While the expression of GCLC1 showed an expression pattern similar to that of HO-1 and Srxn in the cortex (Figure 4C), its expression in the hippocampus was significantly increased at 3 h after seizure, then immediately decreased to basal level and followed by a progressive increase up to 1-week after seizure (Figure 4G). The expression of CAT-2 peaked at 24 h in the cortex (Figure 4D), while in the hippocampus there was a peak expression at 3 h after seizure, then a decrease within 24 h followed by a progressive increase that plateaued at 72 h after seizure and lasted for an additional 1 week after seizure (Figure 4H).

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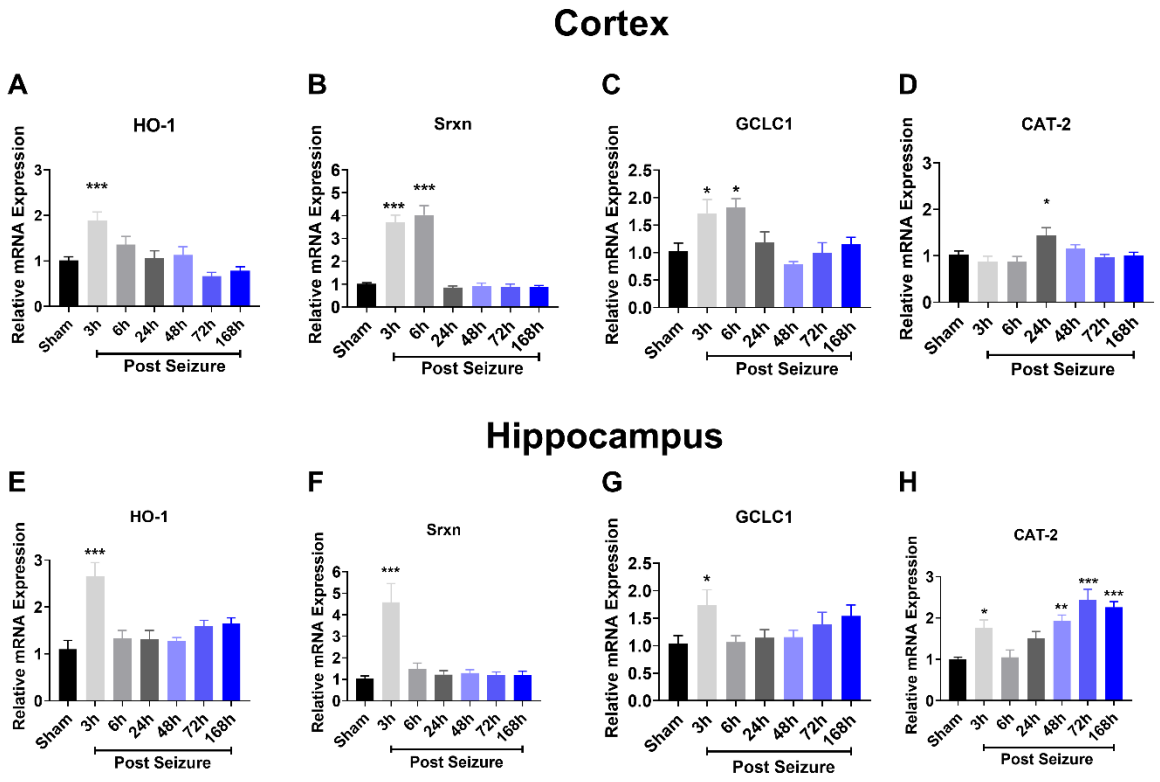

Figure 4. The temporal expression of Nrf2 target genes after acute seizure in the cortex and hippocampus. The temporal expression of Nrf2 downstream target genes: (A) HO-1 (B), Srxn (C), GCLC1 (D), and CAT-2, at mRNA level using RT-PCR in the cortex following PTZ-induced acute seizure (n=6). The temporal expression of Nrf2 downstream target genes: (E) HO-1 (F), Srxn (G), GCLC1 (H), and CAT-2, at mRNA level using RT-PCR in the hippocampus following PTZ-induced acute seizure (n=6). Results are expressed as relative mRNA expression and reported as mean±SEM. **P<0.05, **P<0.01, ***P<0.001 analyzed by one-way ANOVA followed by Dunnett* post-hoc *test.*

3.4.Expression of Nrf2 is predominant in hippocampi-neurons following PTZ-induced seizure

In a variety of animal models of neurological diseases, it was shown that activation of Nrf2 mediate gene expression mainly in astrocytes[34-39], and only a weak expression was reported in neurons[40, 41]. We therefore asked if epileptic acute seizure can modulate the cell type-specific expression of Nrf2. Using immunohistochemical analysis, we studied the expression of Nrf2 in neurons and astrocytes (utilizing neuronal marker, NeuN, and the astrocytic marker, GFAP) in the cortex as well as in the CA1 and CA3 regions of the hippocampus, 24 h after PTZ-induced seizure.

In the cortex, we observed a 2-fold increase in the percentage of neurons expressing Nrf2 (normalized to Sham group; p=0.038 vs. Sham, Student's t-test; Figure 5A-B), while in the astrocytes, there was no change in the percentage of cell expressing Nrf2 after PTZ-induced seizure (Figure 6A-B; p=0.800 vs. Sham, Student's t-test). In both CA3 and CA1 regions of

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the hippocampus, the percentage of neurons expressing Nrf2 was increase by 240% and 460%, respectively (Figure 5C-F). Interestingly, this was not the case in the astrocytes, and no change was observed neither in CA3 nor in CA1 (Figure 6 C-F).

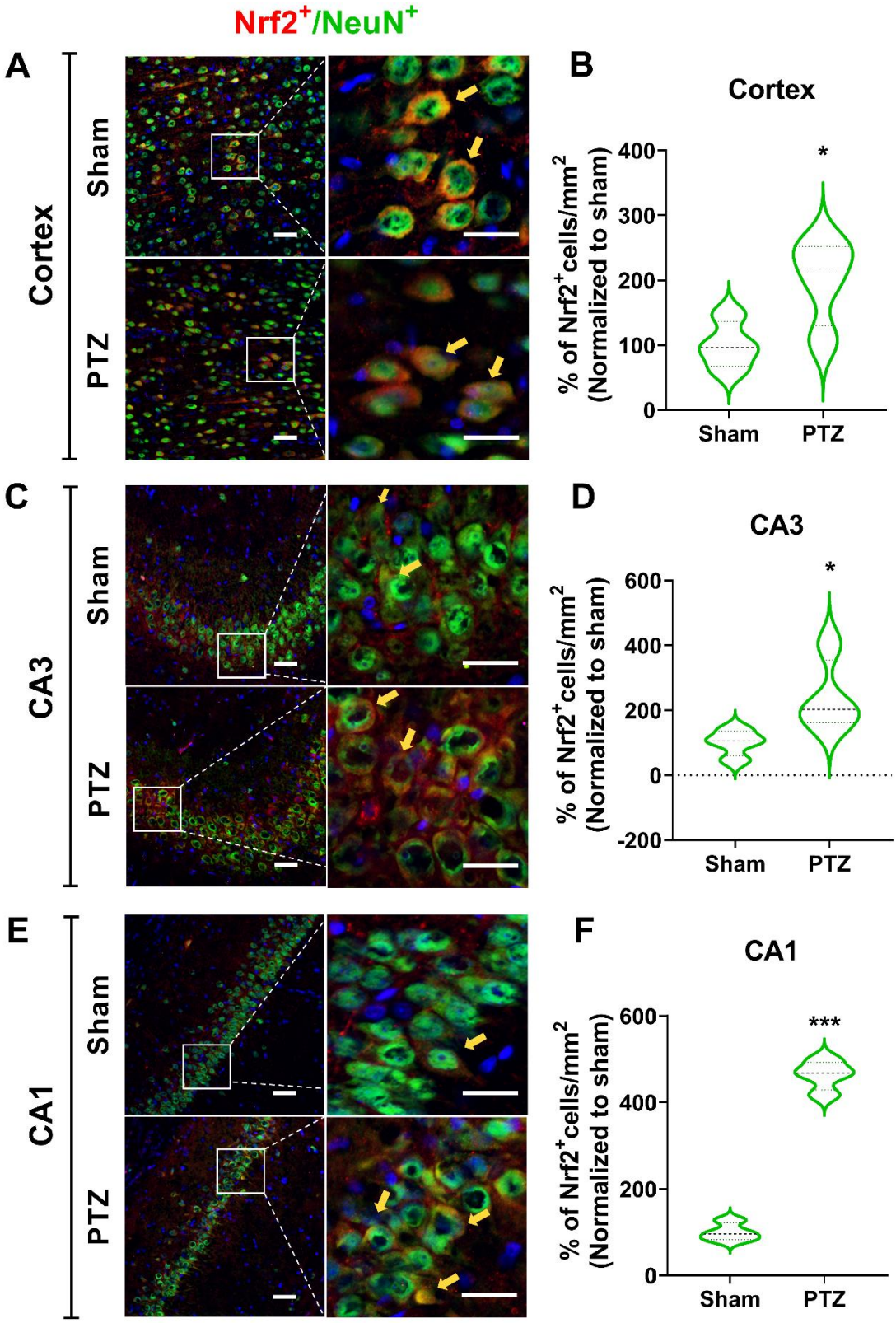

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Figure 5. Nrf2 expression in the neuronal population of cortex and hippocampus after acute seizure. A, C, E: Representative confocal microscopy images of the cortex (A), CA3 (C), and CA1 (E) regions in the hippocampus of intact (sham-operated) and PTZ-induced acute seizure animals (n=4), illustrating colocalization of Nrf2 (red) with NeuN (green), and DAPI (blue). Scale bar = 50 µm (main picture) and 15 µm (zoomed view). B, D, F: Corresponding quantification of the number of Nrf2+/NeuN+ cells in 1 mm^2 of tissue, expressed as changes in percentage of Nrf2+/NeuN+ cells, normalized to the sham group. Results were expressed as mean ± SEM. **P< 0.05, and ***P < 0.001 compared to sham group by Student's t-test.*

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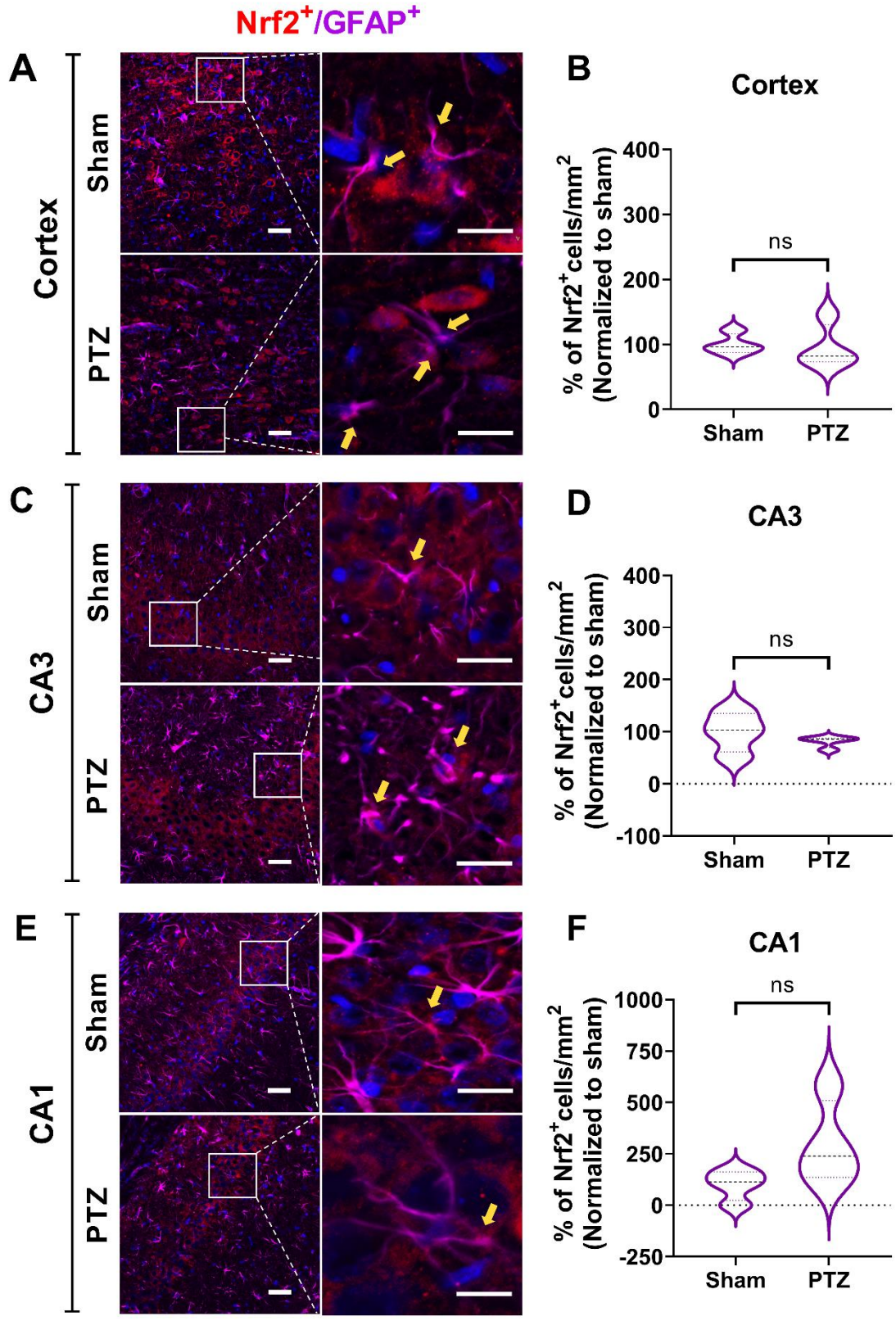

Figure 6. Nrf2 expression in the astrocyte's population of cortex and hippocampus after acute seizure. A, C, E: Representative confocal microscopy images of the cortex (A), CA3 (C), and CA1 (E) regions in the hippocampus of intact (sham-operated) and PTZ-induced acute seizure animals (n=4), illustrating colocalization of Nrf2 (red) with GFAP (magenta), and DAPI (blue). Scale bar = 50 µm (main picture) and 15 µm (zoomed

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view). B, D, F: Corresponding quantification of the number of Nrf2+/GFAP+ cells in 1 mm^2 of tissue, expressed as changes in percentage of Nrf2+/GFAP+ cells, normalized to the sham group. Results were expressed as mean ± SEM. **P< 0.05, and ***P < 0.001 compared to sham group by Student's t-test.*

4. Discussion and Conclusion

In recent years, accumulating evidence established that elevated level of ROS and induction of oxidative stress (OS) play a crucial role in the progression of brain injuries, prolonged seizure, stroke, and many other neurological conditions[6-10, 42]. The transcription factor, nuclear factor erythroid 2-related factor 2, (Nrf2), an endogenous regulator of antioxidant defense[16], has been recently emerged as a master regulator of endogenous defense against OS, thus draws the researcher's focus to utilize it as a therapeutic target in a variety of diseases. Previous studies have demonstrated that Nrf2 is activated in the brain in various animal models of neurological diseases such as TBI[43, 44], neurodegenerative diseases such as Alzheimer and Parkinson disease[45], multiple sclerosis[46, 47] and in epilepsy[19].

In their work, Mazzuferi *et al* evaluated the temporal changes in the expression of Nrf2 in the hippocampus after pilocarpine-induced SE in mice and demonstrated dynamic changes within days to weeks post SE in the expression of Nrf2 and its target genes[19]. However, time-dependent changes in the expression of Nrf2 following an acute epileptic seizure remain unclear. Moreover, region-dependent and/or cell-type specific Nrf2 expression following acute seizure has not been investigated before. In our study, we asked how, if at all, an acute seizure modulate the Nrf2 activity, and whether different brain regions and cell-types respond differently to it. We found that Nrf2 mRNA levels in the cortex are increased progressively and peaked at 24 h post seizure (Figure 2A), while in the hippocampus a significant increase was observed only 3 h post seizure (Figure 3A).

The activity of Nrf2 was subsequently confirmed by its downstream target genes expression, NQO1 (Nrf2: Quinone oxidoreductase 1), HO-1 (heme oxygenase-1), Srxn (Sulfiredoxin), GCLC1 (Glutamate-Cysteine Ligase Catalytic), and CAT-2 (Catalase 2)[48-51].

NQO1 is a prototypic downstream gene of Nrf2 and considered as readout parameter to evaluate its activity[32]. In the cortex, we did not observe any significant changes neither in the mRNA (Figure 2B) nor in the protein levels (Figure 2C, E). In the hippocampus, however, we found a slight increase in the NQO1 mRNA levels early after seizure (Figure 3B), and a progressive increase in the protein levels that reach significant at 72 h after seizure (Figure 3C, E).

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Interestingly, we have previously demonstrated a particular higher increase in the GSH levels in the hippocampus compared to the cortex following kainate-induced SE in rats, suggesting a regional-specific activation of the Nrf2 pathway[24]. Following Nrf2 activation using Omaveloxolone, a potent Nrf2 activator approved recently by FDA for Friedreich's Ataxia[24, 52, 53], GSH levels were increased by ~1.8 fold in the cortex and ~ 3 folds in the hippocampus, suggesting a regional-specific activation of the Nrf2 pathway following seizures[24].

Neurons have been reported to be more vulnerable to oxidative insults than astrocytes, partly due to weaker antioxidant defenses and lower levels of glutathione and antioxidant enzymes activity[54-56]. In several neurodegenerative diseases, it has been reported that Nrf2 is expressed mainly in astrocytes but not in neurons[34-41]. Moreover, it is well documented that Nrf2 activation is not observed in pure primary neuronal cultures without astrocytes, and that activation of Nrf2 in neurons is highly dependent on the abundance of astrocytes[54]. In addition, a specific Nrf2 inhibition in astrocytes resulted in abolished protection against oxidative stress even in mixed neuron and astrocyte cultures[54, 57].

To the best of our knowledge, the cell-type specific expression of Nrf2 has not been reported before following acute seizures. We therefore asked how acute epileptic seizure affects the cell-type specific expression, if at all. We have used specific neuronal and astrocytic markers to estimate the colocalization of Nrf2+ cells with NeuN+ and GFAP+ cells in the cortex and the hippocampus, at peak-time expression of Nrf2 after seizure. Interestingly, a robust increase in the expression of Nrf2 was detected in the neurons after PTZ-induced seizure, while no change was detected in the astrocytes. Moreover, while a trend of increase in the Nrf2 expression was observed in the cortex, a more profound effect was found in the CA1 and CA3 regions of the hippocampus, suggesting that Nrf2 pathway might have a regional specific expression, in addition to the cell-type specific response to a stimulus such as an epileptic seizure. This observation is in agreement with our previous report on increased Nrf2 activation in the hippocampus than cortex following kainic acid-induced SE[24].

In response to oxidative stress, Nrf2 has been identified to activate a battery of antioxidant and detoxification genes[33]. It was previously reported that kainic acid-induced SE altered the expression of >250 Nrf2-mediated genes[58]. We therefore looked at the expression of several Nrf2-regulated genes after PTZ-induced seizures, including NQO1, HO-1, Srxn, GCLC1, and CAT-2 [48-51]. Interestingly, the expression of Nrf2 prototypic target gene NQO1 did not show any significant increase neither at the mRNA level (Figure 2B) nor at the protein level (Figure 2C, E). In our previous report, we observed that upon induction of Nrf2 using sulforaphane following KA-SE the expression of Nrf2 downstream target gene NQO1 is much

lower with respect to Nrf2[22] possibly suggesting that significant induction of NQO1 requires prolonged activation of Nrf2. However, the expression of HO-1 at the mRNA level significantly increases at 3 h following PTZ seizure (Figure 2C). Similar to that, we previously showed that upon Nrf2 induction, expression of HO-1 at mRNA level also increased significantly[22]. We also observed that the expression of other Nrf2 downstream target genes significantly increased at early time point, confirming a profound activation of the Nrf2 pathway mainly at early times after seizure.

The results from our study reveal that the Nrf2 pathway response to a stimulus such as a brief epileptic seizure in a time-, regional and cell-type dependent manner. The restricted expression of Nrf2 in neurons suggest that in different cells, Nrf2 mediates different pathophysiological roles, and is affected by the role of this specific cell-type in the pathophysiology. These data indicate that the neuroprotective roles of Nrf2 against seizure are primarily mediated by neurons, targeting of which might be a potential strategy for attenuating seizure-mediated oxidative stress damage.

Conflict of interest

All authors declared that they have no competing interest in connection with this manuscript.

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Author contributions

SS: Investigation, Formal analysis, Writing-original draft; AS: Investigation, Analysis, Manuscript review, and editing; RO: Analysis, Manuscript review; PKS: Writing-review and editing; TSA: Conceptualization, Writing-original draft, Review and Editing, Funding acquisition, and Supervision.

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Institutional Review Board Statement

Animal experiments were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International and approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem (protocol code MD-20-16254-5 and date of approval 13 July 2020).

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