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The role of Sirt3 on astrocyte activation following status epilepticus injury

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The role of Sirt3 on astrocyte activation following status epilepticus injury

Directed by Professor Chul Hoon Kim

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Science

JING ZHU

December 2023

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December, 2023

Jing Zhu

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<TABLE OF CONTENTS>

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YONSEI UNIVERSITY

연세대학교
YONSEI UNIVERSITY

LIST OF FIGURES

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ABSTRACT

The role of Sirt3 on astrocyte activation following status epilepticus injury

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The Graduate School, Yonsei University

(Directed by Professor Chul Hoon Kim)

Hyperactivation of astrocytes is a hallmark of epilepsy. Sirtuin3 (Sirt3) is a nicotinamide adenine dinucleotide enzyme that contributes to aging, cancer, and neurodegenerative diseases. Recent studies have demonstrated that Sirt3 exerts anti-inflammatory effects in several neuropathophysiological disorders. However, the functional role of Sirt3 in astrocyte activation after status epilepticus (SE) remains unclear. In this study, *Sirt3* wild-type (WT) and knockout (KO) mice were used to investigate the effects of Sirt3 on astrocyte activation and inflammation following SE. After SE, astrocytes were activated and Sirt3 expression was obviously enhanced in the hippocampus. Interestingly, intense Sirt3 expression was detected in activated astrocytes following SE. Pharmacological upregulation of Sirt3 by adjudin treatment alleviated SE-induced astrocyte activation. The underlying mechanisms involved the reduction of NF-κB activity and interleukin-1β (IL1β) expression, which may be mediated through the Notch1 signaling pathway. By contrast, Sirt3 deficiency exacerbated astrocyte activation, resulting in increased NF-κB activity and IL1β expression through the activation of the Notch1 signaling pathway following SE injury. Considering these findings, Sirt3 may play a regulatory role in astrocyte activation by affecting the Notch1/NF-κB signaling pathway, which contributes to the inflammatory

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response after SE. Therefore, therapies targeting Sirt3 may provide effective insights in limiting neuroinflammation following SE.

Keywords: sirt3, astrocyte, neuroinflammation, notch1 signaling, status epilepticus

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The role of Sirt3 on astrocyte activation following status epilepticus injury

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Directed by Professor Chul Hoon Kim

I. INTRODUCTION

Epilepsy is one of the most common neurological disorders characterized by recurrent and unprovoked seizures, affecting more than 70 million people worldwide. Temporal lobe epilepsy (TLE) is the most common type of focal epilepsy, which is associated with hippocampal sclerosis and causes cognitive impairment. Status epilepticus (SE) is a serious life-threatening medical emergency characterized by high mortality and morbidity that lead to the development of epilepsy [1]. It is defined as a seizure that lasts longer than five minutes, or two or more seizures without recovery of consciousness between them. In clinical settings, SE requires immediate treatment with antiepileptic drugs such as benzodiazepines and phenytoin, but at least 30% of patients develop drug resistance [2]. Therefore, there is a need to find new therapeutic strategies for the treatment of epilepsy.

There are several pathophysiological events such as reactive gliosis, neuroinflammation, and neuronal cell death have been observed in the hippocampus of

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TLE patients and epilepsy mouse models [3]. Astrocytes, as one of the most abundant and important cells in the central nervous system, provide structural and metabolic support for neurons. They also play important roles in regulating of ion homeostasis and the concentration of various neurotransmissions. Astrocyte activation has been implicated in both the development and progression of epilepsy. Epileptic seizures trigger astrocyte activation, which could release several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL1β), and IL-6, which can exasperate epileptic damage. On the other hand, activated astrocytes increase glutamate release, and compromise the function of the blood-brain barrier to further release inflammatory factors. As a result, these processes lead to neuronal hyperexcitation and promote SE [4]. Therefore, attenuation of astrocyte activation after SE could provide therapeutic meaning and identification of potential targets is needed to be researched.

Sirtuin3 (Sirt3) is a member of the sirtuin family of nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylases located mainly in the mitochondria, which plays a crucial role in regulating cellular energy metabolism. Emerging evidence has shown that Sirt3 possesses anti-oxidant and anti-inflammatory effects in several disease including cancer and neurodegenerative disorders [5, 6, 7]. Previous studies have shown that Sirt3 mediates mitochondrial metabolism in several neurodegenerative diseases [8], and also exerts neuroprotective effects by interacting with MnSOD in an in vitro epilepsy model [9]. In addition, Sirt3 expression was upregulated in all three major glial cell types including astrocytes, microglia and oligodendrocytes following hypoxia [10]. However, less is known about the role of Sirt3 in astrocyte activation in the context of prolonged seizure activity. Therefore, the aim of the present study was to investigate the effect of Sirt3 in the context of astrocyte activation and the associated molecular mechanism in the hippocampus after pilocarpine-induced SE.

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II. MATERIALS AND METHODS

1. Animals

All procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at Yonsei University Health System and performed in accordance with National Institute of Health guideline for the Care and Use of Laboratory Animals. Adult male C57BL/6 mice (8 weeks old, Orientbio, Gyeonggi, Korea), Sirt3 wile-type (WT) and knock-out (KO) mice were used in this study. 4-6 week old male mice extract genomic DNA from their tows for genetic identification for *Sirt3* WT and KO mice. The Primer sequences of PCR were used: F1; 5′-gagatccatcagcttctgtg-3′, R1; 5′-ccctcaatcacaaatgtcgg-3′, F2; 5′-gggagcactctcatactcta- 3′, R2; 5′-ttactgctgcctaacgttcc-3′. Primers F1 and R1 are located within intron 4 and amplify the wild-type allele (450 bp). Primers F2 and R2 are located within intron 1, and the combination of primers F2 and R1 amplifies the deleted allele (486 bp) as described (Figure 6A) [11]. All mice are housed under a 12-h light/dark cycle with food and water ad libitum.

2. Pilocarpine-induced SE

Adult male mouse weighing 20-25 g were administered scopolamine methyl nitrate intraperitoneally (i.p.) at a dose of 1 mg/kg to inhibit the peripheral cholinergic properties. After 30 min, SE was induced by pilocarpine hydrochloride (325 mg/kg, i.p.; Sigma), as previously described [12]. Control mice received an injection with saline. Seizure behavior were classified according to the Racine scale: 1. facial movements; 2. head nodding; 3. forelimb clonus; 4. rearing; 5. rearing and falling [13]. Only mice that developed SE were used for this study. Then, diazepam (5 mg/kg) was injected 2h after SE onset to terminate the seizure activity. After 3 days, mice brain was harvested and processed for further analysis.

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3. Experimental design

To investigate the role of Sirt3 on astrocyte activation after SE, the experimental group was divided into the two phases: pharmacological activation of Sirt3 and genetic ablation of Sirt3. In phase 1, adjudin (Ad), a potential Sirt3 activator, was dissolved in 10% DMSO, 40% PEG400 and 5% Tween-80 with saline and administered at a dosage 50 mg/kg/day, starting 1h after seizure termination and continued until 3 days, as previously described [14]. Equally, DAPT, a Notch1 signaling inhibitor, was injected at a dosage of 10 mg/kg/day for 3 days starting 1h after termination. The animals were divided 4 groups: 1) Sham (n = 4), 2) SE3d-Veh (n = 4), 3) SE3d-Ad (n = 4), 4) SE3d-DAPT (n = 4). In phase 2, I used *Sirt3* knockout condition mouse compared with *Sirt3* WT mouse. The animals were divided 4 groups: 1) Sham-*Sirt3* WT (n = 4), 2) Sham-*Sirt3* KO (n = 4), 3) SE3d-*Sirt3* WT (n = 4), 4) SE3d-*Sirt3* KO (n = 4).

4. Brain tissue preparation

Mice were anesthetized with an intraperitoneal injection of urethane in saline and transcardially perfused with saline followed by 4% paraformaldehyde. After perfusion, the brain was removed and post-fixed in the same fixative for 24h at 4°C. The fixed brain was dehydrated by 30% sucrose in 0.1 M PB for 3 days and frozen rapidly. Then, serial coronal sections (30 μm) were collected between bregma – 1.46 mm and -2.30 mm using a cryomicrotome (Leica Microsystems, Wetzlar, Germany). Three sections from each brain were randomly selected for histological examination.

5. Immunofluorescence assay

Immunofluorescence staining was performed as previously described [12]. Briefly, the

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selected three sections were washed 3 times with 0.01 M phosphate-buffered saline (PBS, pH 7.4), and then blocked by 5% bovine serum albumin (BSA) in PBS for 1h at room temperature. The brain tissue was immersed in an antibody solution overnight at 4°C. The primary antibodies used in this study were as follows: anti-GFAP, anti-activated Notch1 (1:200; Abcam, Cambridge, MA, USA), anti-IL1β (1:100, Cell Signaling Technology, Beverly, MA, USA) and anti-Sirt3 (1:100, Abcam). Next, the tissue was washed 3 times in PBS for 5 min and immersed with Cy3-conjugated IgG (1:400; Jackson ImmunoResearch; West Grove, PA, USA) or FITC-conjugated IgG antibody for 2h. The sections were counterstained with DAPI and observed by a fluorescence microscope (Carl Zeiss).

6. Western blot analysis

Western blot analysis was performed on hippocampus obtain 3 days after SE. As previously described [12], isolated hippocampus was homogenized with lysis buffer and centrifuged at 4 °C for 15 min at 14,000 × g. Only the supernatants were obtained, and the protein concentration was determined by using a bicinchoninic acid assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples were separated in SDS-polyacrylamide gels using gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA) using an electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween-20 for 1h and then incubated with a primary antibody at 4 °C overnight. The following antibodies were used: anti-GFAP (1:1000, Millipore), anti-Sirt3 (1:1000, Abcam), anti-IL1β (1:1000, Cell Signaling Technology), anti-activated Notch1 (1:500, Abcam), anti-Notch1 (1:1000, Cell Signaling Technology), anti-phospho-NF-κB (1:1000, Cell Signaling Technology), anti-NF-κB (1:1000, Cell Signaling Technology) and anti-β-actin (1:4000, Santa Cruz Biotechnology, Dallas, TX, USA). After washing,

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the membranes were incubated for 1h using anti-rabbit IgG and anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase (HRP). The membranes were reacted with ECL western blotting detection reagents (Amersham Biosciences; Piscataway, NJ, USA), and the bands intensities were visualized with an ImageQuant LAS 4000 (Fuji Film, Japan), and quantified by a Computer Imaging Device.

7. Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism version 9 (GraphPad Software, Inc., San Diego, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons, and Student's t-test for two-group analysis. Two-way ANOVA followed by Tukey's post-hoc test was used for multiple groups. Statistical significance was set at $p < 0.05$ for all analyses. Statistical tests and parameters are described in the figure legends.

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III. RESULTS

1. Increased Sirt3 expression detected in GFAP following SE, and pharmacologically upregulated Sirt3 decreased SE-induced astrocyte activation

Astrocyte activation is one of the hallmarks of epilepsy. To examine whether Sirt3 expression changed in astrocyte activation in epilepsy, I used a well-established pilocarpine mouse model of temporal lobe epilepsy. By immunohistochemistry, Sirt3 was detected in small amounts consistent with the low expression level of GFAP in the Sham group, whereas SE induction significantly increased the expression levels of Sirt3 and GFAP in the hippocampus (Figure 1A). Notably, Sirt3-positive cells merged with GFAP-positive cells (Figure 1A). In line with the immunochemistry results, the protein levels of Sirt3 and GFAP were increased in the SE-Veh group compared with the sham group (Figure 1B, C, D).

To explore the effect of Sirt3 on astrocyte activation following SE, I pharmacologically upregulated Sirt3 by using adjudin treatment. Injection of adjudin resulted in a significant increase in Sirt3 expression and a decrease in the GFAP expression after SE in both immunochemistry and western blot assay (Figure 1). These results indicated that activation of Sirt3 results in astrocyte activation after SE, and pharmacological Sirt3 upregulation attenuated SE-induced astrocyte activation.

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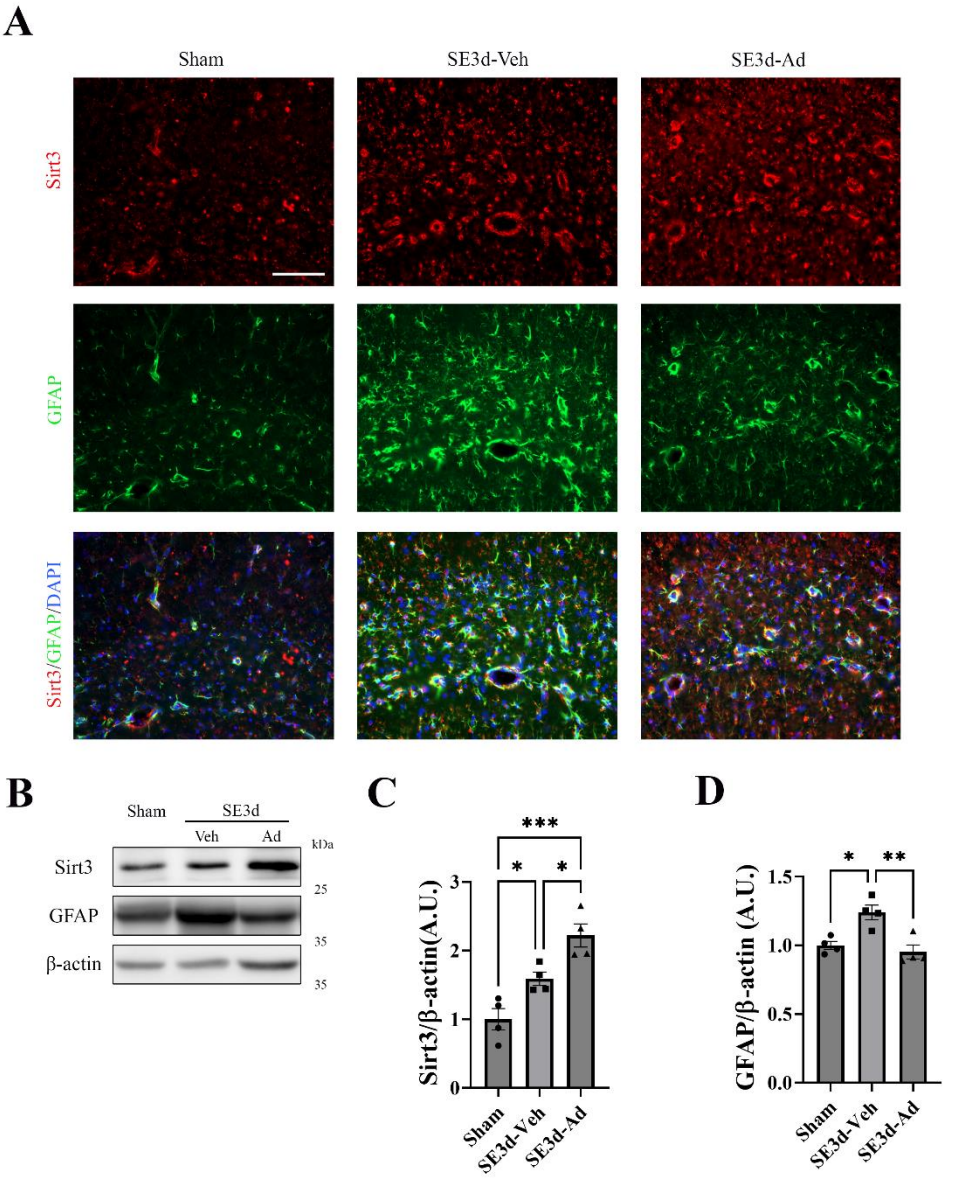

Figure 1. Increased Sirt3 expression detected in GFAP following SE, and pharmacologically upregulated Sirt3 decreased SE-induced astrocyte activation. A) Representative images showing Sirt3 and GFAP expression in the hippocampus.

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Adjudin (Ad), a Sirt3 activator, administrated after SE. Scale bar, 100 μm. B) Western blots of Sirt3 and GFAP among three groups. C, D) Quantification of western blots of Sirt3 and GFAP (mean ± SEM; n = 4 mice for each group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA with Tukey's post-hoc test).

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2. Upregulated Sirt3 inhibited the Notch1 signaling activation involved in SE

Previous studies revealed that activation of Notch1 signaling mediated astrocyte activation in several inflammatory pathological diseases [15, 16]. In addition, Sirt3 regulated astrocyte activation through Notch1 signaling in response to ischemic stroke [17]. Therefore, I tested the effect of upregulated Sirt3 on the activation of Notch1 signaling to investigate the molecular mechanism by which Sirt3 might be involved in SE. The immunostaining intensity of NICD (Notch1 intracellular domain), an activated form of the Notch1 signaling, was increased in the SE-Veh group compared with the Sham group (Figure 2A). Sirt3 activation caused a significant decrease in NICD intensity levels after SE (Figure 2A). Consistent with the immunostaining results, western blot also showed the protein level of NICD was increased in the SE-Veh group as compared with the Sham group. However, administration of adjudin markedly reduced the protein level of NICD, compared to the vehicle-treated SE group (Figure 2B, C). Furthermore, I found that the protein level of Notch1 was increased following SE, whereas adjudin treatment decreased SE-induced Notch1 expression (Figure 2B, D). These results suggest that upregulated Sirt3 might suppress SE-induced Notch1 signaling activation in the hippocampus.

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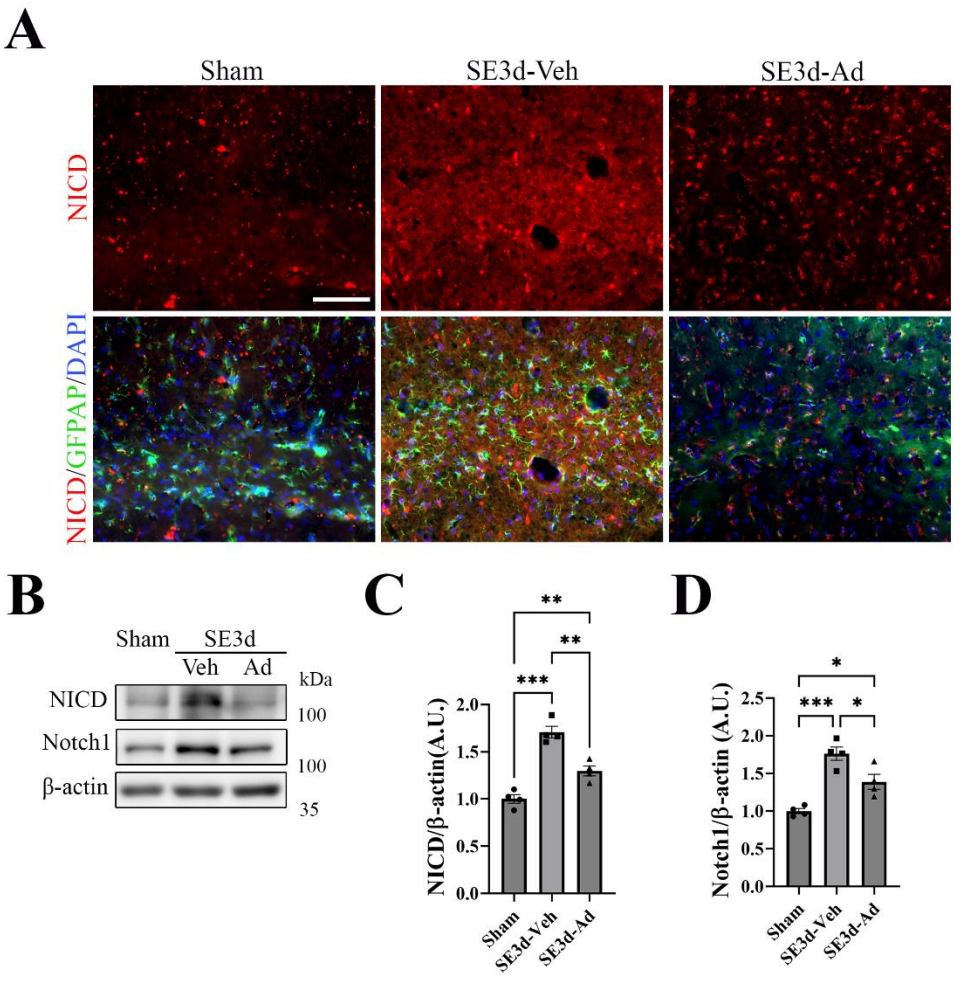

Figure 2. Upregulated Sirt3 inhibited the Notch1 signaling activation involved in SE. A) Representative immunohistochemistry images of NICD (Notch1 intracellular domain), an activated form of the Notch1 signaling, and GFAP expression in the hippocampus. Scale bar, 100 μm. B) Western blots of NICD and Notch1 levels among three groups. C, D) Quantification of western blots of NICD and Notch1 (mean ± SEM; n = 4 mice for each group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with Tukey's post-hoc test).

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3. Upregulated Sirt3 alleviated SE-induced inflammatory response

One of the major functions of NF-κB is to regulate inflammatory response and the secretion of cytokines such as IL1β [18]. In addition, activation of Notch1 signaling induced inflammation process through the NF-κB pathway [19]. Thus, to investigate whether Sirt3 affects the SE-induced inflammatory response, I performed NF-κB activation and IL1β expression in the hippocampus. Both in immunohistochemistry and western blot assays, the expression level of IL1β was enhanced in the SE-Veh group compared with the Sham group, while adjudin treatment decreased SE-induced IL1β expression (Figure 3A, B, E). In addition, the protein expression levels of p-NF-κB and NF-κB were increased after SE, however, adjudin treatment abolished the above change of p-NF-κB (Figure 3B, C, D). These results suggest that upregulation of Sirt3 alleviated the SE-induced inflammatory response following SE.

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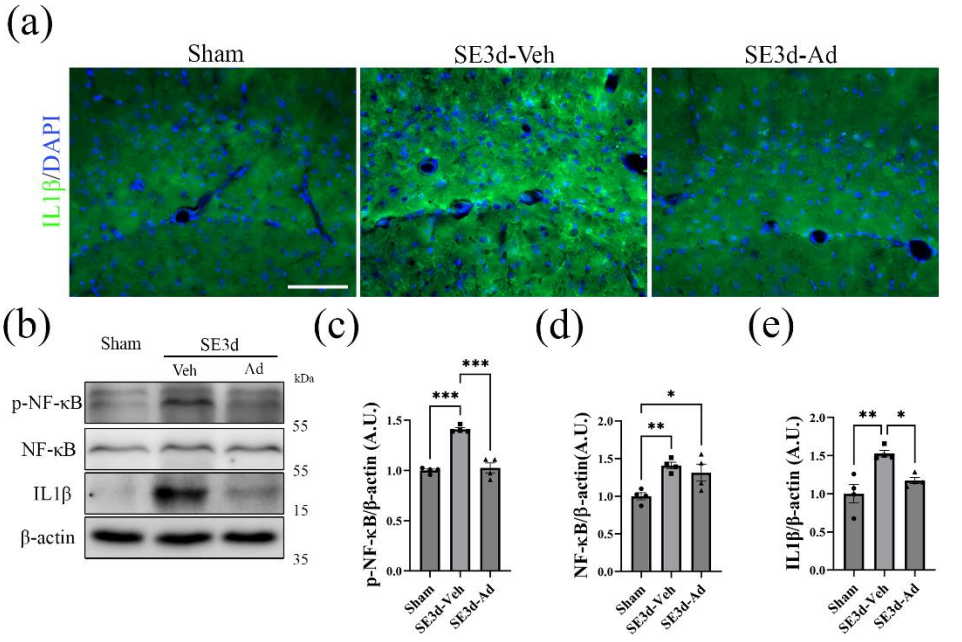

Figure 3. Upregulated Sirt3 alleviated SE-induced inflammatory response. A) Representative immunohistochemistry images of IL1β in the hippocampus. Scale bar, 100 μm. B) Western blots of IL1β, p-NF-κB and NF-κB levels in different groups. C, D, E) Quantification of relative protein expressions by western blots (mean ± SEM; n = 4 mice for each group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA with Tukey's post-hoc test).

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4. Administration of Notch1 inhibitor decreased astrocyte activation after SE without influencing Sirt3 expression

To evaluate the underlying mechanism of Sirt3 on astrocyte activation following SE, I further examined the effect of DAPT, an inhibitor of Notch1. In the immunohistochemistry studies, upregulation of Sirt3 inhibited astrocyte activation, and administration of DAPT also reduced SE-induced astrocyte activation and did not alter Sirt3 expression (Figure 4A). In line with the above effect of upregulated Sirt3 on astrocyte activation, western blot analysis also showed the injection of DAPT markedly reduced GFAP expression after SE, while there was no effect on Sirt3 expression (Figure 4B, C, D).

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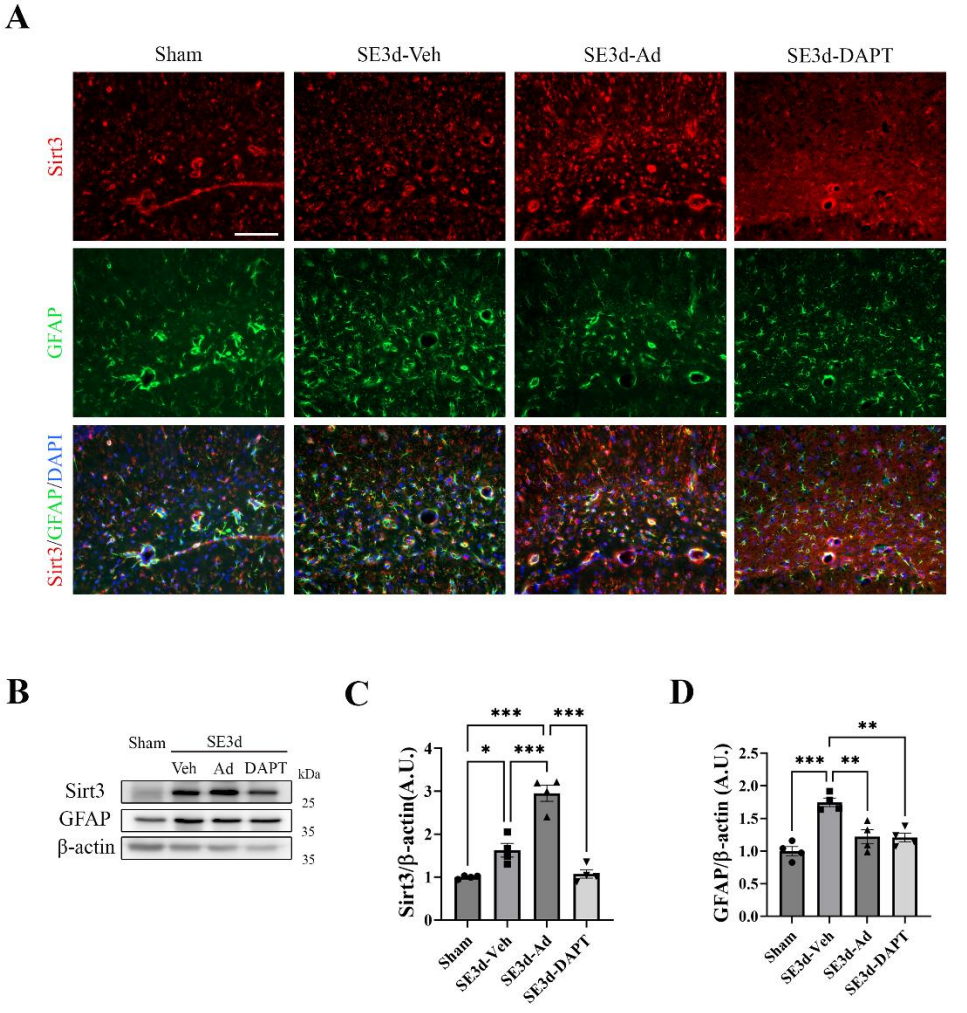

Figure 4. Administration of Notch1 inhibitor decreased astrocyte activation after SE without influencing Sirt3 expression. A) Representative immunostaining of Sirt3 and GFAP in the hippocampus among different groups. DAPT, an inhibitor of Notch1, administrated after SE. Scale bar, 100 μm. B) Western blots of Sirt3 and GFAP among four groups. C, D) Quantification of western blots of Sirt3 and GFAP (mean ± SEM; n

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= 4 mice for each group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with Tukey's post-hoc test).

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5. Administration of Notch1 inhibitor alleviated SE-induced inflammatory response

I further investigated the inhibition of Notch1 signaling affected SE-induced inflammatory response. DAPT treatment showed a significant decrease in NICD expression both in immunostaining and western blot assays compared with the SE-Veh group, similar to the results of adjudin administration (Figure 5A, B, C). In addition, SE-induced protein level of Notch1 was decreased by DAPT treatment (Figure 5B, D). Western blot analysis showed that DAPT treatment prevented the increased protein levels of Notch1 caused by SE and decreased the activity of NF-κB, which is an important inflammatory process, and also reduced the expression of IL1β, a proinflammatory cytokine, showed similar effects to Sirt3 upregulation (Figure 5B, E, F, G). Taken together, these data indicated that upregulation of Sirt3 inhibited Notch1 signaling activation that may contribute to astrocyte activation following SE.

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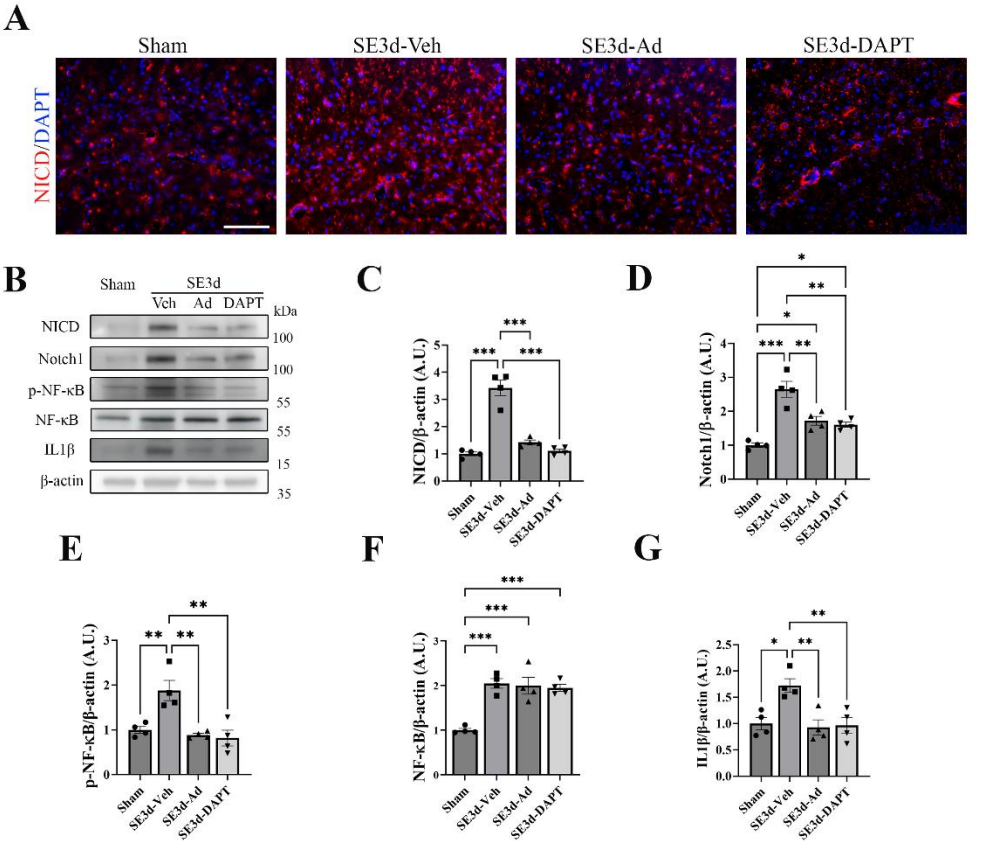

Figure 5. Administration of Notch1 inhibitor alleviated SE-induced inflammatory response. A) Representative immunohistochemistry images of NICD expression in the hippocampus. Scale bar, 100 μm. B) Western blots of NICD, Notch1, p-NF-κB, NF-κB and IL1β levels among different groups C-G) Quantification of western blots of NICD, Notch1, p-NF-κB, NF-κB and IL1β (mean ± SEM; n = 4 mice for each group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by one-way ANOVA with Tukey's post-hoc test).

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6. Sirt3 deficiency enhanced astrocyte activation and promoted Notch1 signaling activation after SE

As our results showed that Sirt3 upregulation inhibited SE-induced astrocyte activation, I examined whether *Sirt3* deficiency reversed the above change under SE conditions by using *Sirt3* KO and WT mice. First, *Sirt3* KO and WT mice were verified by genotyping of the genomic DNA extracted from tows (Figure 6B). In the sham-operated groups, there was no significant difference in the expression of GFAP and NICD between the *Sirt3* WT and KO groups. Under SE-induced condition, the *Sirt3* KO group revealed a significant increase in the expression of GFAP and NICD compared with the *Sirt3* WT group (Figure 6C). Western blot also showed that the expression of GFAP and NICD in *Sirt3* KO mice increased more than in WT mice under SE conditions while no significant changes were found under the sham conditions (Figure 6D, E, F). In addition, the protein level of Notch1 were increased after SE compared with the Sham groups, however, there was no significant difference between WT and KO groups under both sham and SE conditions (Figure 6D, G). These results indicated that *Sirt3* deficiency enhanced SE-induced astrocyte activation and promoted activation of Notch1 signaling.

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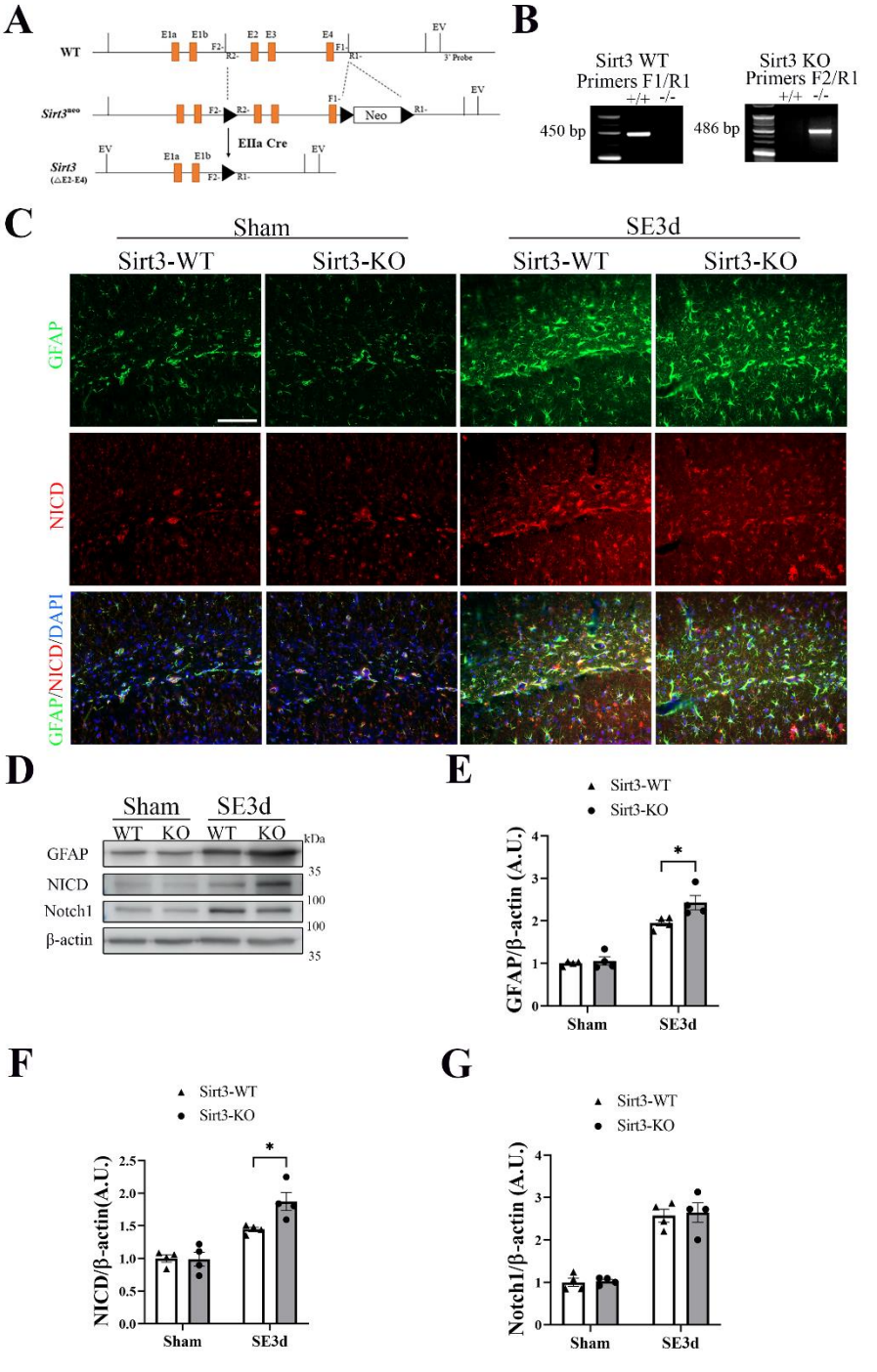

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Figure 6. *Sirt3* deficiency enhanced astrocyte activation and promoted Notch1 signaling activation after SE. A) Schematic of *Sirt3* gene-targeting strategy. Arrowhead, loxp sites; Neo, Neomycin gene; F1, R1, F2, and R2, positions of primers for genotyping; EV, EcoRV restriction site. Mice heterozygous for ploxPneo insertion ($Sirt3^{+/neo}$) were crossed with mice carrying a EIIa-Cre transgene to deleted exons 2–4 of *Sirt3*. B) Validation of *Sirt3* mutant mouse by genotyping. C) Representative images of GFAP and NICD expression between *Sirt3* WT and KO mice under control and SE conditions. Scale bar, 100 μm. D) Western blots analyzing protein levels of GFAP, NICD and Notch1. E, F, G) Quantification of protein expression by western blots (mean ± SEM; n = 4 mice for each group; $*p < 0.05$ by two-way ANOVA followed with Tukey's post-hoc test).

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7. *Sirt3* deficiency increased SE-induced inflammatory response

Next, I investigated whether *Sirt3* deficiency affected the SE-induced inflammatory response. After 3 days of SE induction, the intensity of IL1β in the hippocampus was increased compared with the sham-operated groups, while it was more enhanced in the *Sirt3* KO group both in IHC and western blot assay (Figure 7A, B, E). On the other hand, *Sirt3* deficiency caused an increase in NF-κB activity after SE, while there was no difference in the expression of IL1β and NF-κB activity between *Sirt3* WT and KO mice under the sham condition (Figure 7B, C, D). These results showed that *Sirt3* deficiency exacerbated the inflammatory response after SE.

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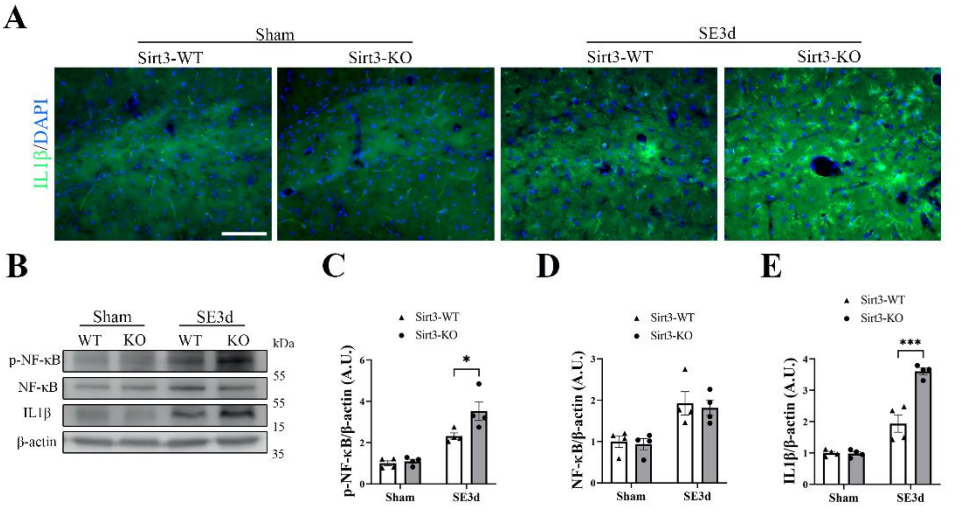

Figure 7. *Sirt3* deficiency increased inflammatory response following SE. A) Representative images of IL1β in the hippocampus. Scale bar, 100 μm. B) Western blots of p-NF-κB, NF-κB and IL1β levels in different groups. C, D, E) Quantification of relative protein expressions by western blots (mean ± SEM; n = 4 mice for each group; $*p < 0.05$, $***p < 0.001$ by two-way ANOVA followed with Tukey's post-hoc test).

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8. SE induction exacerbated astrocyte activation, activated the Notch1/NF-κB pathway, and increased IL1β expression, and these effects were reversed by the Notch1 inhibitor in *Sirt3* knockout mice

To further confirm whether *Sirt3* deficiency was involved in SE-induced Notch1 signaling activation, I observed the effects of DAPT in *Sirt3* KO mice. Western blot analysis showed that the protein levels of GFAP, NICD, Notch1, p-NF-κB, NF-κB and IL1β were increased respectively in the SE-Veh group compared with the sham group, and administration of DAPT significantly decreased the expression of GFAP, NICD, Notch1, p-NF-κB and IL1β (Figure 8). These results indicated that *Sirt3* deficiency further enhanced Notch1 signaling activation, which exacerbated the SE-induced inflammatory response.

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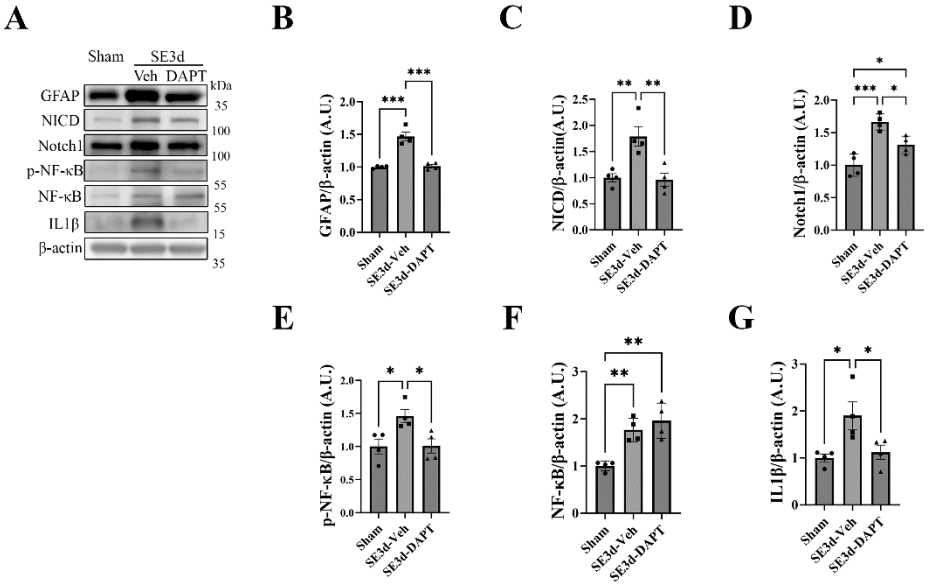

Figure 8. Inhibition of Notch1 signaling reduced astrocyte activation and inflammatory response after SE in *Sirt3* deficiency mice. A) Western blots of GFAP, NICD, Notch1, IL1β, p-NF-κB and NF-κB levels in each group under *Sirt3* deficiency condition. B-G) Quantification of western blots of GFAP, NICD, Notch1, IL1β, p-NF-κB and NF-κB (mean ± SEM; n = 4 mice for each group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 by one-way ANOVA with Tukey's post-hoc test).

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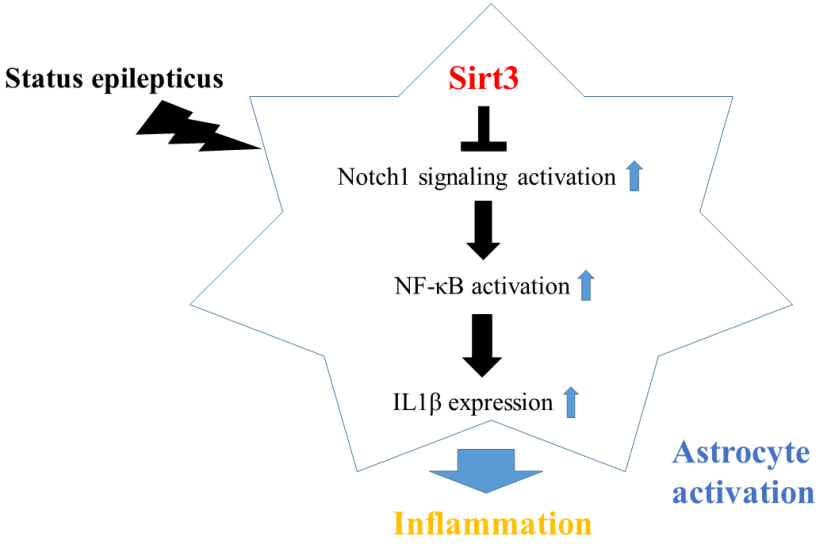

Figure 9. Schematic diagram representing the mechanism of Sirt3 on astrocyte activation following SE. Sirt3 mediates astrocyte activation through the Notch1/NF-κB pathway to contribute to the inflammatory response after SE injury.

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IV. DISCUSSION

In the current study, I investigated the effects of Sirt3 on astrogliosis and neuroinflammation in a pilocarpine-induced mouse model of epilepsy. In particular, the results demonstrated that the increase in the expression of Sirt3 due to SE was expressed in activated astrocytes and that this phenomenon was found in the pilocarpine-induced mouse model. In addition, pharmacologically upregulated Sirt3 by adjudin treatment alleviated astrocyte activation. Mechanistically, upregulation of Sirt3 reduced the NF-κB activity and IL1β expression, which might be mediated through the Notch1 signaling pathway. In contrast, *Sirt3* deficiency exacerbated astrocyte activation and increased the NF-κB activity and IL1β expression via activation of Notch1 signaling after SE. Taken together, this study demonstrates that Sirt3 regulates astrocyte activation through the Notch1/NF-κB pathway to contribute to the inflammatory response after SE injury (Figure 9).

In recent years, there has been growing interest in the potential role of Sirt3 in neuroinflammation, which plays a critical role in various central nervous system (CNS) disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. In addition, Sirt3 overexpression protects against postoperative cognitive dysfunction and ameliorates the anesthesia/surgery-induced mitochondrial oxidative stress response, microglia activation and neuroinflammation [20]. Besides, Sirt3 regulates the Foxo3a-mediated antioxidant pathway in microglia and ischemic stroke-induced glial scar in astrocytes [17, 21]. It has been reported that Sirt3 deficiency upregulates neuroinflammation, and overexpression of Sirt3 plays a protective role against ischemic stroke through regulation of HIF-1α/VEGF signaling in astrocytes [22]. Despite emerging evidence show that Sirt3 expressed in glial cells to possess antioxidant and anti-inflammatory functions, the relationship between Sirt3 and astrocyte in epilepsy is still poorly understood. To the best of our knowledge, this study reveals for the first time

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that the increased changing pattern of Sirt3 expression was accompanied by astrocyte expression triggered by SE both in human and a mouse model of SE. It is well established from a variety of studies that adjudin has anti-cancer, anti-inflammation, and antioxidant and anti-aging effects [23]. In addition, adjudin has been reported to reduce lipopolysaccharide (LPS)-induced inflammation by inhibition of the NF-κB pathway [24]. In particular, adjudin as a potential Sirt3 activator inhibits ROS production and reduces apoptotic cells [25], and attenuates stroke-induced neuroinflammation through acting on astrocyte activation [17]. Previous studies have shown that adjudin effectively evaluates Sirt3 expression to reduce oxidative stress and inflammation in pathological conditions [24, 26]. Similarly, in the present study, I found that upregulation of Sirt3 by adjudin decreased SE-induced astrocyte activation while *Sirt3* deficiency reversed the above changes. According to these results, I propose that Sirt3 may mediate astrocyte activation following SE.

Notch signaling, a cell-surface receptor, is well known for its role in regulating several events during central nervous system development, including neuronal cell differentiation, neurite development, and gliogenesis. In addition, Notch signaling plays an important role in hippocampal synaptic plasticity in the adult brain [27]. Ample studies have shown that Notch1 signaling mediates astrocyte activation in different pathological conditions [15, 16, 28]. Moreover, astrocytes regulate epilepsy-induced angiogenesis through the Notch1 signaling pathway, and upregulated Sirt3 inhibits Notch1 signaling to reduce astrogliosis following stoke [17, 29]. In particular, SE induction increases levels of NICD, the activated form of Notch1 signaling, and its activation further promotes epileptic seizures [30], and Sirt3 suppresses inflammation by acting on the Notch1 signaling pathway [17, 31]. Previous study showed that Sirt1, a member of the sirtuin family, associates with NICD and functions as a NICD deacetylase, which opposes the acetylation-induced NICD stabilization in endothelial cells [32]. In addition, Sirt1 has been reported to participate in macrophage differentiation, and inhibit Notch1 signaling through NICD deacetylation in macrophages and thus alleviate inflammation

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[33, 34]. Sirt1-mediated deacetylation of NICD is required for NICD-mediated anti-apoptotic activity, and Sirt1 agonist inhibits LPS-induced acute lung injury through deacetylation and degradation of NICD [35, 36]. Likewise, the results in the present study showed that Sirt3 upregulation effectively reduced SE-induced NICD level, and the NICD expression was more pronounced in the *Sirt3* deficient condition following SE. However, less is known about how Sirt3 acts on Notch1 in epilepsy. This study show that Sirt3 negatively regulates NICD, but it is unclear whether it directly interacts to deacetylate it. Therefore, the relationship between Sirt3 and Notch1 and the related mechanism is needed to be further investigated. Based on these findings, I suggest that Sirt3 mediates the Notch signaling activity under SE conditions.

In epilepsy, activation of the NF-κB pathway is upregulated in neurons, glial cells and endothelial cells due to neuronal loss, glial cell proliferation and blood-brain barrier dysfunction [37]. As inflammation is one of the key features of epilepsy, seizures could induce the release of inflammatory factors which could activate the NF-κB pathway, and these activated NF-κB signaling promotes their transcription to lead to inflammatory processes [38, 39]. In particular, activation of the NF-κB signaling pathway is attenuated by DAPT, an inhibitor of Notch1 pathway, and Notch1 signaling augments the activity of the NF-κB pathway through its nuclear retention [40, 41]. Moreover, Notch1 signaling interacts with the NF-κB pathway to mediate the inflammatory response in several brain injuries such as hypoxia and ischemic stroke [16, 42, 43]. Notch1 signaling has reported to enhanced NF- κB activity in LPS-induced macrophage activation, and the inhibition of Notch signaling by *RBP-J* knockout macrophages results in the inactivation of the NF-κB pathway [33, 44]. The production and release of IL1β are tightly regulated at the transcriptional level, and the NF-κB pathway is a major regulator of IL1β expression. In the nucleus, the activated NF-κB binds to the promoter region of the IL1β gene, promoting its transcription and subsequent production of the IL1β protein [18]. Consistent with previous reports, the current study showed that NF-κB activation and the level of IL1β were inhibited by Sirt3 upregulation after SE, while

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Sirt3 deficiency exacerbated the NF-κB activity and the expression of IL1β. As shown in previous studies, the activity of NF-κB is decreased due to the Notch1 inhibitor in other brain diseases [16, 42], such a relationship is also expected in epilepsy. Here, I applied the Notch1 inhibitor DAPT to mimic the reduction in the Notch1 signaling activation observed in WT mice and *Sirt3* deficiency mice. This study suggests that reduced Notch1 activity is mainly responsible for the NF-κB activation in *Sirt3* KO mice after SE.

Nevertheless, there are some limitations in this study. First, I focused on whether Sirt3 regulated SE-induced astrocyte activation, not other glial cells like microglia. Considering that other studies have reported Sirt3 acts on microglia to perform antioxidant and anti-inflammatory effects, it is worth to further investigate the effect of Sirt3 on microglia in epilepsy. Second, I observed the outcomes at postoperative 3 days, therefore, the role of Sirt3 in the chronic phase of epilepsy requires further validation. Additionally, I observed the role of Sirt3 on astrocyte activation in pilocarpine induced-SE model however, other epilepsy models also need to be investigated.

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V. CONCLUSION

In summary, the results uncover the role of Sirt3 in reactive astrogliosis in epilepsy. Sirt3 was involved in SE-induced inflammation by regulating astrocyte activation via the Notch1 signaling pathway and further mediating the release of IL1β, a pro-inflammatory cytokine induced by the transcription factor NF-κB. Taken together, Sirt3 could be a viable and potential therapeutic target for the treatment of epilepsy.

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ABSTRACT (IN KOREAN)

뇌전증지속상태 손상 후 성상세포 활성화에 대한 Sirt3의 역할

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주경

성상교세포의 과활성화는 뇌전증의 특징 중 하나이다. 시르투인 3(Sirt3)는 노화, 암 및 신경 퇴행성 질환에 기여하는 니코틴아미드 아데닌 디뉴클레오티드 효소이다. 최근 연구에 따르면 Sirt3 는 여러 신경 병리 생리학적 장애에서 항염증 효과를 발휘하는 것으로 나타났다. 그러나 뇌전증지속상태 후 성상교세포 활성화에서 Sirt3 의 기능적 역할은 아직 명확하지 않다. 이 연구에서는 Sirt3 야생형 및 결핍(KO) 마우스를 사용하여 뇌전증지속상태 후 성상세포 활성화 및 염증에 대한 Sirt3 의 영향을 조사했다. 뇌전증지속상태 후 성상교세포가 활성화되고 해마에서 Sirt3 발현이 분명히 증가했다. 흥미롭게도 뇌전증지속상태 후 활성화된 성상교세포에서 강렬한 Sirt3 발현이 검출되었다. Adjudin 처리에 의한 Sirt3 의 약리학적 상향 조절은 뇌전증지속상태에 의한 성상교세포 활성화를 완화시켰다. 근본적인 메커니즘은 NF-κB 활성과 인터루킨-1β(IL1β) 발현의 감소를 포함하며, 이는 Notch1 신호 경로를 통해 매개될 수 있다. 대조적으로, Sirt3 결핍은

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성상교세포 활성화를 악화시켜 뇌전증지속상태 손상 후 Notch1 신호 경로의 활성화를 통해 NF-κB 활성과 IL1β 발현을 증가시켰다. 이러한 결과를 고려할 때, Sirt3 는 뇌전증지속상태 후 염증 반응에 기여하는 Notch1/NF-κB 신호 경로에 영향을 미쳐 성상세포 활성화에 조절 역할을 할 수 있다. 따라서 Sirt3 를 표적으로 하는 치료법은 신경세포를 제한하는 데 효과적인 통찰력을 제공할 수 있다.

핵심되는 말: 시르투인 3, 성상교세포, 신경염증, Notch1 신호, 뇌전증지속상태

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PUBLICATION LIST

1. Cho I, Jeong KH, Zhu J, Choi YH, Cho KH, Heo K, et al. Sirtuin3 Protected Against Neuronal Damage and Cycled into Nucleus in Status Epilepticus Model. Mol Neurobiol. 2019 Jul;56(7):4894-4903.

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