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Original Article

# Neuronal conditional knockout of *NRSF* decreases vulnerability to seizures induced by pentylenetetrazol in mice

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**Neuron restrictive silencer factor (NRSF), also known as repressor element-1 silencing transcription factor, has been reported to modulate neuronal excitability and acts as endogenous anticonvulsant in kainic acid-induced or kindling-evoked seizure activity. However, whether NRSF functions in pentylenetetrazol (PTZ)-induced seizure activity has never been studied. To investigate the role of endogenous NRSF in the epileptogenesis induced by PTZ, in our experiment, *NRSF* neuronal conditional knockout mice (*NRSF* cKO) were adopted, in which *NRSF* was specifically deleted in neurons by the Cre-loxP system. Seizure threshold for PTZ, including the dose-response convulsions and the threshold dose, was compared between *NRSF* cKO and control mice. The threshold dose of PTZ that induced clonic and tonic seizures was significantly higher in *NRSF* cKO mice compared with the control. Similarly, the median lethal dose ($LD_{50}$) of PTZ in *NRSF* cKO mice was also considerably higher than that of the control mice. These results revealed that *NRSF* cKO mice are of higher resistance to convulsions induced by PTZ. Our work first demonstrated the function of NRSF in PTZ-induced seizure and provided new evidence for differential pathways in diverse types of seizure.**

*Keywords* neuron restrictive silencer factor; conditional knockout; pentylenetetrazol; seizure

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## Introduction

Neuron restrictive silencer factor (NRSF) plays an important role in embryogenesis and neurogenesis by spatial and temporal control of neuronal genes expression, such as *STMN2*, *SCN2A*, and *CHRM4*, by binding a specific consensus 21-bp *cis*-element called NRSE/RE1 located in target genes and recruiting different cofactors, such as CoREST, mSin3A, histone deacetylases, and methylases [1–10]. Previous findings showed that *NRSF* mRNA is expressed abundantly in non-neuronal and undifferentiated neuronal stem/progenitors cells, but decreased severely during neural development [10]. However, though it is downregulated during neurogenesis, NRSF has been detected in differentiated mature neurons in the adult central nervous system, and dynamically regulated in many neurological diseases such as epilepsy, global ischemia, Huntington's disease, Parkinson's disease, and Down's syndrome [10–19]. Different *NRSF* transcripts have been found in adult brain due to alternative splicing [11]. Among them, the full-length NRSF is widely expressed in all types of cells in mouse brain while a truncated form, REST4 (repressor element-1 silencing transcription 4), exists mainly in neurons and may block the repressor activity of NRSF by forming hetero-oligomers [11,20,21].

Previous studies demonstrated that full-length NRSF and the truncated REST4 are differentially regulated in the hippocampal and cortical neurons following kainic acid stimulus [11,19]. Increasing evidence suggested that the expressions of neuronal-specific genes in epilepsy, such as *BDNF* (brain-derived neurotrophic factor), *GluR2*, and *PPT-A* (preprotachykinin-A gene), are regulated by NRSF and REST4, indicating their important roles in epileptogenesis [10,13,19]. In mice, targeted mutation of *NRSF* caused embryonic lethality, and thus cannot be used to study NRSF function in postnatal stages [22]. Hence, to evaluate the role of NRSF in epileptogenesis, we constructed an *NRSF* gene conditional knockout (cKO) mouse model in which the *NRSF* gene was specifically disrupted in excitatory neurons of the postnatal mouse forebrain [14]. In the kindling model, *NRSF* cKO mice exhibited dramatically

accelerated seizure progression and prolonged after discharge duration compared with control mice [14]. This result suggested that NRSF functions as an intrinsic repressor of epileptogenesis. However, experimental data we got recently from pentylentetrasole (PTZ)-induced seizure in neuronal *NRSF* gene knockout mice (*NRSF* cKO) gave different results.

PTZ has been used to study seizure phenomenon in many researches, especially for evaluation of antiepileptic effects of drugs [23–25]. The recorded behavioral symptoms are from partial to generalized convulsions, sometimes followed by death of animals [26]. We compared the behavioral indicators of PTZ-induced seizure model, such as latency to the first myoclonic twitch, to clonic convulsions for at least 5 s, to tonic hindlimb extension, and the $LD_{50}$ (lethal dose) value. Our study demonstrated that conditional deletion of *NRSF* in neurons attenuates the susceptibility to PTZ, and promotes animal survival in PTZ-induced seizure model.

## Materials and Methods

### Animals

*NRSF/REST* neuronal cKO mice (*NRSF* cKO) were generated in our laboratory by crossing $NRSF^{flox/flox}$ mice (in which two loxP elements were introduced into the mouse genome flanking exon 2 of the *NRSF* locus) with neuron-specific enolase (NSE)-Cre transgenic mice (hereafter referred to as 'Cre', in which Cre recombinase is expressed exclusively in neurons) [13]. NSE-Cre mice served as controls. Mice had access to food and water *ad libitum* except during tests. The behavioral experiments were always conducted during the light phase of the cycle. In all experiments, the investigators were blind to the genotype of mice. All procedures used in the present study were approved by the Institutional Animals Care and Use Committee of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Induction and analysis of PTZ-induced acute seizures

PTZ was purchased from Sigma Chemical Company (St Louis, USA), dissolved in saline and administered intraperitoneally. Male mice aged 2–4 months were injected with PTZ at a dosage of 50, 60, 65, or 75 mg/kg. Seizure activity was videotaped during an observation period of 3 h. The times of seizure onset, clonic convulsions, and tonic hindlimb extension were recorded. The $LD_{50}$ value (median lethal dose, causing death of 50% of the test animals) was also calculated.

### Reverse transcription and quantitative real-time PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Two micrograms of RNA per sample was reverse transcribed to cDNA by using moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA) according to the manufacturer's instruction. Quantitative real-time polymerase chain reaction (PCR) was performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA) with SYBR-Green I as fluorescent dye according to the previous study [27]. The primers used in the real-time PCR were: *GAPDH* forward primer 5′-AGTCAAGGCCGAGAATGGGAAG-3′; *GAPDH* reverse primer 5′-AAGCAGTTGGTGGTGCAGGATG-3′; *NRSF* forward primer 5′-CACCTGCGAGCTGGCGAGAAC-3′; *NRSF* reverse primer 5′-CACATTTAAATGGCTTCTCACCTG-3′; *REST4* forward primer 5′-CTGCACGTACACGACGGTCAGCGAG-3′; and *REST4* reverse primer 5′-ACATTTAAATGGCTTCTCACCCAAC-3′.

### Statistic analysis

Data were analyzed by one-way analysis of variance followed by Bonferroni posthoc analysis and represented as the mean ± SE. Statistical software was OriginPro 7.0 (OriginLab, Northampton, USA). In every case, the acceptance level for statistical significance was $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

## Results

### Neuronal conditional knockout of NRSF decreased vulnerability to seizures induced by PTZ in mice

To investigate whether NRSF functions in the development of PTZ-induced seizure activity, in the present study, *NRSF* cKO mice were adopted, in which *NRSF* gene was conditionally knocked out in the neurons (see 'Materials and Methods' section) [13]. The efficiency and specificity of NRSF deletion in the brains of *NRSF* cKO mice have been confirmed in our previous work [13]. *NRSF* cKO mice could survive into adulthood with normal body weight, locomotor activity (data not shown).

*NRSF* cKO and control mice received an injection of PTZ (60, 65, or 75 mg/kg) and were immediately placed in individual Plexiglas boxes for observation. The presence or absence of convulsions following injection was videotaped during an observation period of 3 h. The behavioral indicators of seizure activity were as follows: (i) the first myoclonic twitch, (ii) clonic convulsions for at least 5 s, (iii) tonic hindlimb extension, and (iv) death. The time from the start of injection to the onset of each behavioral indicator was recorded.

Incidence of seizures increased in a dose-dependent manner in both genotypes following injection of PTZ

**Table 1 Dose–response of the convulsant effects by PTZ in *NRSF* cKO and control mice[a] ($n = 7–16$)**

| Groups | Control (60 mg/kg) | cKO (60 mg/kg) | Control (65 mg/kg) | cKO (65 mg/kg) | Control (75 mg/kg) | cKO (75 mg/kg) |
|---|---|---|---|---|---|---|
| Clonic[b] | 6 (85.7%) | 7 (77.8%) | 7 (100%) | 8 (80%) | 12 (100%) | 13 (81.3%) |
| Tonic[c] | 5 (71.4%) | 0 | 6 (85.7%) | 0 | 12 (100%) | 9 (56.3%) |
| Death[d] | 4 (57.1%) | 0 | 6 (85.7%) | 0 | 12 (100%) | 9 (56.3%) |
| Total | 7 | 9 | 7 | 10 | 12 | 16 |

[a]Expressed as the percentage of animals displaying seizures in response to PTZ injection.
[b]The percentage of clonic convulsions for at least 5 s.
[c]The percentage of tonic hindlimb extension.
[d]The percentage of death.

(Table 1). However, when the percentages of animals exhibiting clonic convulsions and tonic hindlimb extension were considered for each dose of PTZ, we found that the percentages of PTZ-induced convulsions and death were significantly decreased in *NRSF* cKO mice when compared with the control mice (Table 1). This result demonstrated that neuronal conditional deletion of NRSF in mice attenuates the susceptibility to PTZ, and promotes animal survival in this specific seizure model.

### Neuronal conditional deletion of NRSF-alleviated epileptiform convulsions induced by PTZ in mice

Convulsions induced by PTZ are dramatically attenuated in *NRSF* cKO mice, especially in terms of latency to tonic hindlimb extension, latency to the first myoclonic twitch, and latency to clonic convulsions (**Fig. 1**). The dose of PTZ necessary to induce tonic hindlimb extension was higher in *NRSF* cKO as compared with the control mice [**Fig. 1(A)**]. In detail, at the dose of 60 or 65 mg/kg PTZ, none of the *NRSF* cKO mice developed tonic hindlimb extension during whole experimental period, while five of seven control mice displayed tonic hindlimb extension in the first 30 min at the dose of 60 mg/kg PTZ, and six of seven control mice showed tonic hindlimb extension in the first 12 min at the dose of 65 mg/kg PTZ. Only higher dose of PTZ (75 mg/kg) induced tonic hindlimb extension in 9 of the 16 *NRSF* cKO mice while all control mice experienced the tonic hindlimb extension, the latency between the two groups was similar [**Fig. 1(A)**]. These results show that, neuronal conditional deletion of *NRSF* in mice leads to insensitivity to PTZ, and higher concentration of PTZ is required for *NRSF* cKO mice to elicit tonic hindlimb extension.

We also compared the latency with the first myoclonic twitch and to clonic convulsions stimulated by PTZ injection between *NRSF* cKO and control mice [**Fig. 1(B,C)**]. Latency to the first myoclonic twitch at the dose of 65 mg/kg PTZ was $108.6 \pm 9.53$ and $65.23 \pm 4.06$ s, respectively, in *NRSF* cKO and control mice, but the delayed myoclonic twitch could not be detected at the dosage of 60 or 75 mg/kg [**Fig. 1(B)**]. Similarly, the latency to clonic convulsions was prolonged in *NRSF* cKO mice at the dose of 65 mg/kg PTZ when compared with the control mice, but at the dose of 60 or 75 mg/kg, these two groups of mice showed no difference [**Fig. 1(C)**]. Taken together, this result demonstrated that *NRSF* cKO mice displayed higher resistance to convulsions engendered by PTZ.

### Death rate of *NRSF* cKO mice by PTZ stimulation was far lower than that of control mice

Further, we calculated the lethal rate caused by different PTZ stimuli. The doses of PTZ in this experiment were 50, 60, 65, and 75 mg/kg. At 50 mg/kg PTZ, none of the *NRSF* cKO mice died while 3 of 11 control mice died; at 60 mg/kg PTZ, *NRSF* cKO could survive while 4 of 7 control mice died; at 65 mg/kg PTZ, still none of the *NRSF* cKO mice died while 6 of 7 control mice died; finally at 75 mg/kg PTZ, 9 of the 16 *NRSF* cKO mice died while all the control mice died [**Fig. 2(A)**].

Based on the above data, we calculated the $LD_{50}$ value (median lethal dose) of PTZ in *NRSF* cKO and control mice. The $LD_{50}$ value of *NRSF* cKO mice was significantly higher than that of control mice ($74.13 \pm 6.31$ vs. $55.68 \pm 5.31$ mg/kg) [**Fig. 2(B)**]. These data demonstrated that neuronal cKO of NRSF promotes animal survival in the PTZ-induced seizure model and leads to insensitivity to chemoconvulsant PTZ.

### The expressions of *NRSF* and *REST4* are upregulated in PTZ-induced seizure model in mice

The $LD_{50}$ value of PTZ in wild-type mice is $55.68 \pm 5.31$ mg/kg, so we used a dose of 60 mg/kg PTZ for intraperitoneal injection and examined the relative expression level of *NRSF* gene in mouse hippocampus by quantitative PCR (qPCR). qPCR analysis of *NRSF* and *REST4* expression was carried out at 3, 6, 12, 18, and 24 h after administration of PTZ, and normalized to the values from saline controls. We found that both expressions of *NRSF* and *REST4* displayed dynamic changes (**Fig. 3**). Three hours after PTZ stimulation, there was a dramatic and transient

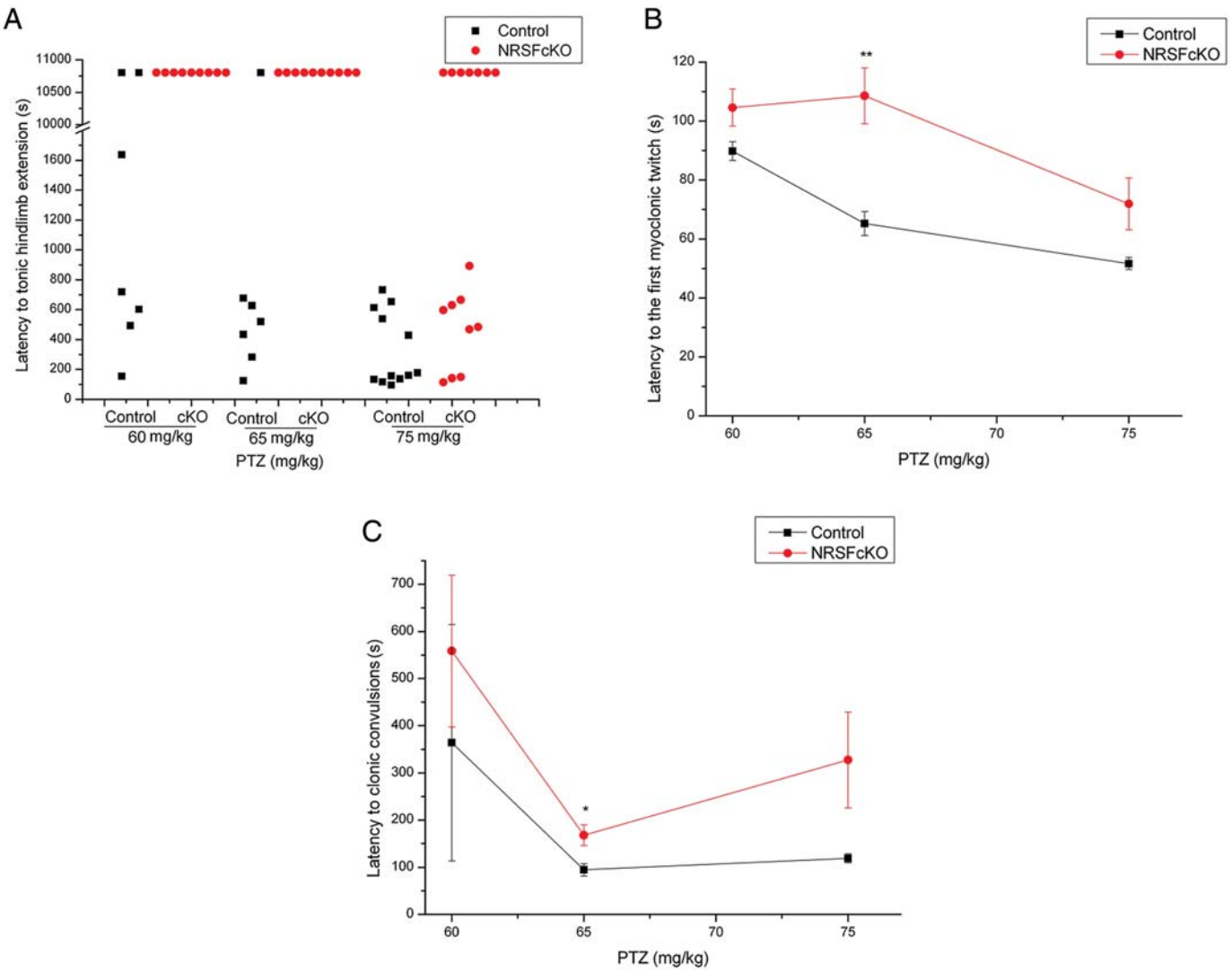


**Figure 1 Latency to convulsant effects of different doses of PTZ (60, 65, or 75 mg/kg) in *NRSF* cKO and control mice** Latency to (A) tonic hindlimb extension; (B) the first myoclonic twitch; and (C) clonic convulsions. $^{*}P < 0.05$, $^{**}P < 0.01$, and $n = 7–16$.

about 2-fold increase of *REST4* compared with the saline controls. However, interestingly, *NRSF* expression was fairly constant at the same time point, with a more modest 1.3 fold but statistically significant increase. This result suggests that *NRSF* and *REST4* are differentially regulated in PTZ-induced seizure, which is consistent with reports by Spencer [19]. Moreover, *REST4* was more tightly regulated than *NRSF*, which confirms previous findings in other epilepsy model [11,13,19]. These findings demonstrate that NRSF and REST4 are of great importance in the development of PTZ-induced seizure. And further researches about functions of NRSF/REST4 in PTZ-induced epileptogenesis are required.

## Discussion

In this paper, we used *NRSF* neuronal cKO mice to study the role of NRSF in PTZ-induced epileptogenesis. We found that, comparing with the control mice, the onset of the tonic hindlimb extension induced by PTZ administration is delayed in *NRSF* cKO mice. Also the death rate of *NRSF* cKO mice resulted from PTZ treatment is significantly lower. And the $LD_{50}$ values are $74.13 \pm 6.31$ and $55.68 \pm 5.31$ mg/kg in *NRSF* cKO and control mice, respectively. These data demonstrated that deletion of *NRSF* in neuronal cells attenuates susceptibility to PTZ-induced seizure in mice and promotes animal survival after PTZ stimuli. The result indicated that NRSF functions as an intrinsic mediator of PTZ-induced seizure activity in the adult brain.

There is an apparent inconsistency between this finding and our previous report that conditional deletion of *NRSF* accelerates epileptogenesis [14]. The discrepancy may be caused by the different seizure models. PTZ is a tetrazol derivative that has been shown to have convulsant actions in mice presumably by impairing gamma-aminobutyric

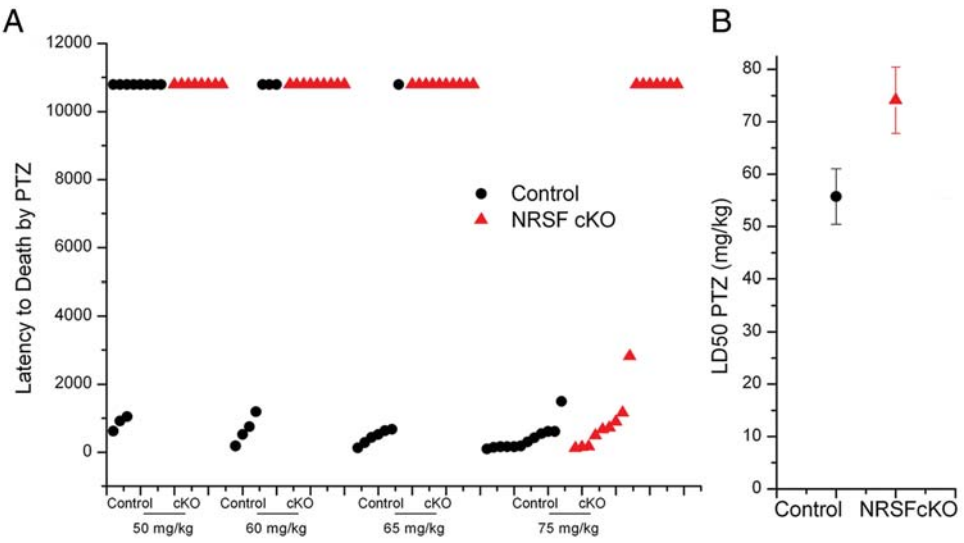


**Figure 2 Latency to death after PTZ injection (50, 60, 65, or 75 mg/kg) and $LD_{50}$ value (medial lethal dose) of PTZ in *NRSF* cKO and control mice** Black circle and red triangle represent control and *NRSF* cKO mice, respectively. Each dot represents an individual mouse. (A) Latency to death, *x*-axis showed the genotype and doses used and *y*-axis showed the latency to death; (B) $LD_{50}$ value (medial lethal dose) of PTZ in control and *NRSF* cKO mice, which is the dosage causing the death of 50% of the test animals. *x*-axis showed the genotype and *y*-axis showed the dose of PTZ.

acid (GABA)-mediated inhibition through an action at the GABA receptors (GABARs) [28–31], while electrical amygdala kindling is a model of synaptic plasticity that produces functional and structural alterations in brain which leads to permanent increases in seizure susceptibility [32,33]. Therefore, different neurochemical pathways could be responsible for the generation of seizure activity depending on chemical or electrical kindling. Another reason is that *NRSF* gene was deleted in different cell types in the two studies. In this paper, *NRSF* was disrupted in all neuronal cells, while in our previous work, *NRSF* was found to be only deleted in excitatory neurons of the mouse forebrain. The results indicated that NRSF may have different functions depending on the stimuli of seizure development and the locations of neurons.

It has been proposed that different isoforms of NRSF can regulate distinct patterns of gene expression and, at least in certain seizures, may function antagonistically [19,34]. Therefore, it is important to define whether differential regulation of these isoforms exists in PTZ-induced seizure. Our study found that the expressions of NRSF and REST4 are significantly upregulated by PTZ in the brains of adult wild-type mice (**Fig. 3**). Since the alleviation of the PTZ-induced epileptic seizure in *NRSF* cKO mice may be achieved by alteration of seizure-related gene expression, we checked the expression of seizure-related genes which are regulated by NRSF in different genotypes of mice, such as BDNF, tropomyosin-related kinase B, PPT-A, *N*-methyl-D-aspartate receptor subunit type I (NR1), and GABAR2 [10,12,19,35,36]. However, we could not find significant differences in their expressions between *NRSF* cKO and control mice (data not shown).

In light of the fact that we observed significant differences in NRSF and REST4 expression at baseline between *NRSF* cKO and control mouse brains, we propose that differential expression and localization of NRSF isoforms during epilepsy could have profound consequences for the individuals, and might modulate the susceptibility of the brain to different chemoconvulsants [19]. Loss of NRSF and REST4 in neurons might disrupt intrinsic homeostatic mechanisms of excitatory and inhibitory circuits. Our work suggested that NRSF may play complex and diverse functions in epileptogenesis.

NRSF expression has been found reduced in Down's syndrome (DS) [18], and chronic systemic treatment of DS model mice with PTZ can ameliorate the hippocampus-dependent cognitive deficits [37]. Our study here provides a link between these two separate findings that administration of PTZ can partially restore NRSF levels in DS mice hippocampal neurons and improve their cognitive deficiency.

Taken together, NRSF could be an important potential contributor to a variety of neurodegenerative diseases. Much more detailed analysis would have to be carried out for the contribution of NRSF in seizure and DS pathology, which will provide important rationales for novel therapeutic approaches.

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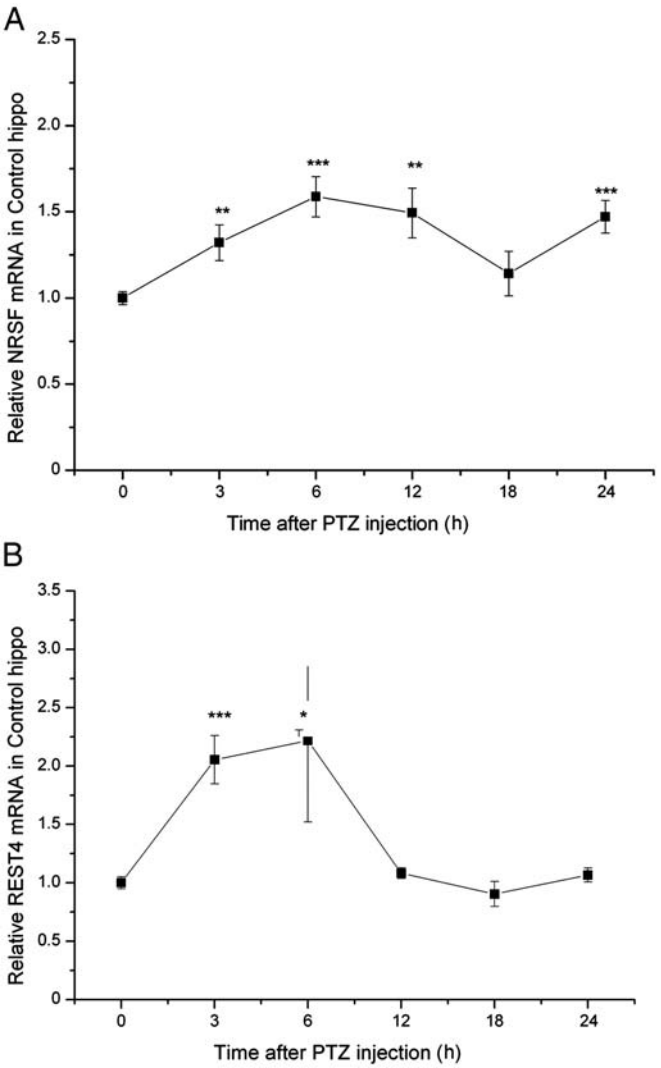


**Figure 3 The expressions of *NRSF* and *REST4* were upregulated in animal model of PTZ-induced epilepsy** qPCR analysis of relative *NRSF* and *REST4* mRNA levels (normalized to *GAPDH* mRNA level) in the hippocampus (hippo) of wild-type mice at different time point after 60 mg/kg PTZ injection. (A) *NRSF* mRNA expression in hippocampus and (B) *REST4* mRNA expression in hippocampus. Bars represent means ± SE from three independent experiments, $n \geq 4$ per group in each experiment. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

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