Cerebral Cortex Advance Access published April 4, 2011

Cerebral Cortex
doi:10.1093/cercor/bhq284

Conditional Deletion of NRSF in Forebrain Neurons Accelerates Epileptogenesis in the Kindling Model

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Neuron-restrictive silencer factor (NRSF), also known as repressor element-1 silencing transcription factor, is a transcriptional repressor that plays important roles in embryonic development and neurogenesis. Recent findings show that NRSF is upregulated after seizures activity however, the link between NRSF and epileptogenesis remains poorly understood. To investigate the role of NRSF in epilepsy, we employed a Cre–loxp system to specifically delete NRSF in excitatory neurons of the postnatal mouse forebrain. In the kindling model of epileptogenesis, conditional NRSF knockout (NRSF-cKO) mice exhibited dramatically accelerated seizure progression and prolonged afterdischarge duration compared with control mice. Moreover, seizures activity–induced mossy fiber sprouting was enhanced in the NRSF-cKO mice. The degree of upregulation of Fibroblast growth factor 14 and Brain-derived neurotrophic factor (BDNF) following kainic acid-induced status epilepticus was significantly increased in the cortex of NRSF-cKO mice compared with control mice. Furthermore, the derepression of BDNF was associated by activation of PLCγ and PI(3)K signaling pathways. These findings indicate that NRSF functions as an intrinsic repressor of limbic epileptogenesis.

Keywords: BDNF, epilepsy, kainic acid, kindling, NRSF

Introduction

Neuron-restrictive silencing factor (NRSF), also known as repressor element-1 silencing transcription factor (REST), is a Krüppel-type zinc-finger transcription factor that plays an important role in embryogenesis and neurogenesis (Chong et al. 1995; Schoenherr and Anderson 1995; Chen et al. 1998; Ballas et al. 2005). NRSF binds to a 21-nt DNA sequence called neuronal restrictive silencing element (NRSE) or repressor element-1(RE1). In the mouse and human genomes, NRSE sequences reside in a large number of genes, especially those encoding nervous system constituents, such as ion channels, neurotransmitter receptors, and synaptic transmission factors (Bruce et al. 2004). NRSF binds to the corepressors CoREST (Andres et al. 1999) and mSin3A (Grimes et al. 2000), which in turn recruit histone modifiers such as HDAC1/2 that modify chromatin structure (You et al. 2001). During neurogenesis, downregulated expression of NRSF leads to enhanced transcription of its target genes that are required for neuronal differentiation (Ballas et al. 2005).

Although NRSF is downregulated in neural development, it can be upregulated by seizures activity (Palm et al. 1998) and by global ischemic stimulation (Calderone et al. 2003) in mature neurons. The molecular consequence and functional significance of these events remain largely unknown. Spencer demonstrated that kainic acid (KA)-induced seizures activity upregulates NRSF expression, causing a corollary downregulation of NRSF target gene expression (Spencer et al. 2006). BDNF and TrkB have been demonstrated to play important roles in epileptogenesis. Kindling-induced epilepsy development was significantly delayed by deletion of BDNF and was completely blocked by deletion of TrkB (Kokaia et al. 1995; He et al. 2004). NRSF repression complexes deacetylate and demethylate histones, thereby led to condensed chromatin structure, and to repressed transcription of target genes such as *bdnf* (Zuccato et al. 2003) and *trkb* (Garriga-Canut et al. 2006). These findings raise the possibility that NRSF may play an important role in the modulation of the development of epilepsy.

There are several animal models with which to study the underlying mechanisms of epileptogenesis. The kindling model, by mimicking the "seizures begets seizures" phenomenon (Mello and Covolan 1996), is widely used for reliable and reproducible quantification of epilepsy progression. In this model, repetitive administration of an initially subconvulsive electrical stimulus results in progressive intensification of behavioral seizures and prolongation of afterdischarges (ADs). When combined with refined techniques to monitor seizures activity, electrophysiological and behavioral paradigms, the kindling model is a powerful tool for discriminating an animal's susceptibility to epilepsy (Morimoto et al. 2004).

In this study, we coupled the kindling model of epileptogenesis with electrophysiological and behavioral monitoring of seizures activity to study the regulatory role of NRSF in epilepsy. To this end, we generated a conditional knockout mouse (NRSF-cKO) to delete NRSF specifically in excitatory neurons of the postnatal mouse forebrain. We found that kindling development was significantly accelerated and AD duration was dramatically extended in NRSF-cKO mice. Moreover, we also observed exaggerated mossy fiber sprouting in kindled NRSF-cKO mice. Further examination of NRSF target genes revealed that Fibroblast growth factor 14 (*Fgf14*) and *Bdnf* were significantly increased in NRSF-cKO mice both in messenger RNA (mRNA) and in protein level, and these changes also were associated with the enhanced activation of PLCγ and Akt signaling.

Materials and Methods

Generation of NRSF-cKO Mice

The floxed NRSF allele was produced by introducing loxP elements into the mouse genome flanking the first coding exon (exon 2) of the NRSF locus. Recombinant ES cells were injected into C57/BL6

blastocysts to produce chimeras. Chimeras were then crossed to C57/BL6 mice to produce mice heterozygous for the floxed NRSF allele ($NRSF^{flox/+}$). These mice were then backcrossed with C57/BL6 mice for at least 3 generations. CamKIIα-iCre mice (C57/BL6 background) were obtained from Prof. Günther Schütz (Casanova et al. 2001) and crossed with homozygous $NRSF^{flox/flox}$ mice to generate *CamKIIα-iCre;*$NRSF^{flox/+}$ offspring. Progeny were intercrossed to generate *CamKIIα-iCre;*$NRSF^{flox/flox}$ (cKO) mice. In all experiments, *CamKIIα-iCre;*$NRSF^{+/+}$, $NRSF^{flox/+}$, and $NRSF^{flox/flox}$ mice served as controls. Mice were housed in a barrier facility. Two weeks before experimentation, mice were transferred into a standard air- and temperature-conditioned environment on a 12-h day/night cycle. All experimental procedures were approved by the animal care and use committee of the Institute of Neuroscience, Chinese Academy of Sciences.

In Situ Hybridization

Three-month-old mice were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS). Whole brains were removed and placed into 4% PFA in Diethylpyrocarbonate (DEPC)-PBS for 12–16 h at 4 °C and then dehydrated in a sucrose gradient (15%, 30%, dissolved in DEPC-PBS). Brains were cut into 20 μm coronal sections and mounted onto RNase-free, poly-D-lysine-coated slides (stored at –20 °C; Fisher Scientific). A 744 bp *NRSF* cDNA fragment (exon 2) was cloned into the pGEM-T Easy Vector. Plasmids were then linearized for in vitro RNA transcription of antisense and sense DIG-labeled RNA probes using a SP6/T7 transcription kit (Roche). Hybridization was performed following standard protocols (Ma et al. 1998); anti-DIG-AP, Fab fragments, and NBT/BCIP (Boehringer Mannheim) were used for detection. A sense probe did not show any staining on brain slides. Images were captured with a Nikon microscope using Neurolucida software.

Reverse Transcription Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction

The hippocampus or forebrain cortex was removed from both sides of the brain, and total RNA was extracted using Trizol reagent (Invitrogen). RNA (3 μg) was used for reverse transcription (RT) with M-MLV reverse transcriptase (Invitrogen). RT products were subjected to polymerase chain reaction (PCR) amplification. Quantitative real-time PCR was performed using SYBR Ex taq premix (Takara) in a thermocycler (prism 7000, ABI). Primers used were described as in Table 1.

Western Blot

The hippocampus or forebrain cortex from both sides of the brain was collected, homogenized, and centrifuged. Whole protein extract was further processed for immunoblot analysis and probed against NRSF using anti-NRSF antibody (1:2000; Santa Cruz, sc-25398), anti-Pan Nav (1:1000; Alomone Labs, ASC-003), anti-L1cam (1:1000; Abcam, ab24345), anti-GluR2 (1:1000; Millipore, AB1506), anti-NR2B (1:1000; Millipore, 06-600), anti-FGF14 (1:500; Santa Cruz, sc-16814), anti-GABRγ2 (1:1000; Millipore, 06-868), anti-BDNF (1:500; Santa Cruz, sc-546), anti-TrkB (1:1000; Santa Cruz, sc-12), anti-phospho-ERK (1:5000; Cell Signaling, #9101), anti-ERK (1:5000; Cell Signaling, #9102), anti-PLCγ (1:200; Santa Cruz, sc-81), anti-phospho-PLCγ (1:100; Santa Cruz, sc-22141-R), anti-Akt (1:1000; Cell Signaling, #9272), anti-phospho-Akt (1:500; Cell Signaling, #4058s), and anti-GAPDH (1:2000; KangChen Biotech). Band intensities were quantified using Image-Quant software. In experiments measuring NRSF targets expression, all data were normalized to the level of GAPDH and compared with saline-treated control mice.

Table 1
Primers used for quantitative real-time PCR

Genes	Primers
NRSF-F	5′-gcgaactcacacaggagaacg-3′
NRSF-R	5′-ggtcacttcatgctgattagagg-3′
Nav-F	5′-ctgcatggaggtcgcaggcc-3′
Nav-R	5′-gttgttcatttcgttatcgtcatctgtg-3′
L1cam-F	5′-ctgtttggcaagccagattt-3′
L1cam-R	5′-ctggtgatcagggctcactt-3′
TrKB-F	5′-ggactttgggatgtcccgagatgtgt-3′
TrKB-R	5′-aacacaacgcccaggctccaga-3′
NR2B-F	5′-tcttctattggcagttccgacattg-3′
NR2B-R	5′-atcacggattggcgctcctcta-3′
Glur2-F	5′-atgcggttaacctcgcagtactaaaa-3′
Glur2-R	5′-ggtcttttccttggaatcacctcc-3′
Gabrg2-F	5′-tcagctctgattgagtttgccaca-3′
Gabrg2-R	5′-acgcttttgccatcccacgca-3′
FGF14-F	5′-gttgccatccagggagtga-3′
FGF14-R	5′-gcctgtacagcatggatgagt-3′
BDNF-F	5′-cagtattagcgagtgggtca-3′
BDNF-R	5′-gcagccttccttggtgta-3′
Gapdh-F	5′-ggttgtctcctgcgacttca-3′
Gapdh-R	5′-ccaccaccctgttgctgtag-3′

Immunohistochemistry

Mice under deep anesthesia were transcardially perfused with ice-cold 4% PFA followed by postfixation and cryoprotected with sucrose. Coronal brain sections were cut on a cryostat (–23 °C) at 30 μm. Slices were washed, incubated in 1% Triton X-100 in PBS for 5 min, and blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Next, slices were incubated with anti-NeuN (1:1000; Millipore, MAB377) and anti-REST (1:500; Millipore, 07-579) antibodies at 4 °C for 36 h and washed 3–6 times with PBS. Slices were then incubated with Alexa-488 (goat anti-rabbit) or Alexa-546 (donkey anti-mouse) conjugated secondary antibodies overnight at 4 °C (1:2000; Invitrogen). All antibodies were diluted in 5% BSA. Images were acquired with fluorescent confocal microscopes (Nikon A1R). Hoechst 33342 (Beyotime) staining was used to identify cell nuclei.

Drug-Induced Status Epilepticus

Kainic Acid

KA (20 mg/kg), a specific agonist of ionotropic glutamate receptors (kainate receptors; Ben-Ari et al. 1979), was injected intraperitoneally (i.p.) into control and NRSF-cKO mice. Animals were observed continuously for behavioral seizures for 2 h after injection.

Pilocarpine

Pilocarpine (300 mg/kg), a nonselective muscarinic receptor agonist (Spalding et al. 2002), was injected i.p. into control and NRSF-cKO mice after scopolamine (1 mg/kg) pretreatment. Diazepam (2 mg/kg i.p.) was administered 1 h after the onset of status epilepticus (SE).

Kindling Procedure

Mice aged 3–6 months were anesthetized with 5% chloral hydrate (300 mg/kg). Stainless bipolar electrodes for stimulating and recording were stereotaxically implanted into the right amygdala (1.2 mm posterior to the bregma, 2.8 mm lateral to the midline, and 4.9 mm below the dura). Four screws were drilled into the skull without piercing the dura. After allowing at least 7 days for recovery, the AD threshold (ADT) was determined by applying one second train of 1-ms rectangular pulse at a frequency of 60 Hz. The first train was begin at 50 μA; subsequent trains were delivered after 2 min rest periods, with the current increased by 10 μA each interval and were administered until an AD lasting at least 3 s was elicited. The behavioral progression of kindling-induced seizures was scored according to Racine's standard classification. Fully kindled was defined as the occurrence of 3 consecutive class 4 or 5 seizures (He et al. 2004). Animals that required an AD threshold larger than 400 μA, and animals that displayed stage 5 seizures during the first threshold determination were excluded. Experiments were carried out in a double-blind manner.

Timm Staining

Timm staining was performed on fully kindled mice that had been allowed to rest for at least 2 months. Timm staining was performed according to previously described standard protocols (Qiu et al. 2008). The extent of sprouting was assessed by calculating Timm index scores, determined by dividing the total area covered by Timm granules by the

total length of the dentate gyrus (DG; Sutula et al. 1996; Sprengel et al. 1998). For each animal, the mean Timm index score was calculated based on 3 sections.

Statistical Analysis

AD thresholds and durations, seizures stages, protein expression, and mRNA expression were compared using unpaired *t*-tests. The number of stimulations required to elicit stage 3 or stage 5 seizures was compared using one-way analysis of variance and Dunnett post hoc analysis. For all analyses, *P* values of less than 0.05 were considered significant. All data are presented as mean ± standard error of the mean (s.e.m.).

Results

NRSF Expression Is Upregulated in Animal Models of Epilepsy

To measure the dynamics of *NRSF* expression during development, we first examined the relative level of *NRSF* expression at different developmental time points by quantitative real-time PCR. *NRSF* expression has been shown to be downregulated in neural progenitors as they differentiate into neurons (Ballas et al. 2005; Sun et al. 2005). Consistently, we found that in the postnatal and adult mouse brain, *NRSF* mRNA expression decreased to about 10% of the level measured at embryonic 12.5 (Supplementary Fig. 1). In the adult hippocampus, however, NRSF has also been reported to be upregulated following seizures activity or ischemia (Palm et al. 1998; Calderone et al. 2003; Spencer et al. 2006). Thus, we next examined the change in *NRSF* expression after different types of stimulations.

Using in situ hybridization, we found that the levels of *NRSF* mRNA significantly increased in most forebrain neurons after kindling- and pilocarpine-induced seizures activity (Fig. 1*A*). Using semiquantitative PCR, we found that *NRSF* mRNA was significantly increased in the hippocampus and forebrain cortex 24 h after injection of KA (Fig. 1*B,C*). Consistent with the observed changes in mRNA levels, protein immunoblot analysis revealed that NRSF protein levels were also increased in the hippocampus and cortex after KA stimulation (Fig. 1*D,E*). These results confirm previous findings and suggest that upregulation of NRSF might play a role in seizures development (Palm et al. 1998; Calderone et al. 2003; Spencer et al. 2006).

NRSF Is Specifically Deleted in the Adult Mouse Forebrain of CamKIIα-iCre;NRSF$^{flox/flox}$ Mice

To explore the significance of NRSF upregulation following seizures activity, we generated an *NRSF* flox mouse line (*NRSF^{flox}*) that contained 2 loxp elements within the introns flanking the first coding exon (exon 2). Homozygous *NRSF$^{flox/flox}$* mice were then crossed to mice carrying a *CamKIIα-iCre* transgene (referred to herein as "Cre"; see Materials and Methods), in which Cre recombinase is expressed in excitatory neurons of the postnatal forebrain (Fig. 2*A*; Casanova et al. 2001) to generate *CamKIIα-iCre;NRSF$^{flox/flox}$* (NRSF-cKO) mice. NRSF-cKO mice could survive into adulthood with normal body weight, locomotor activity, and fertility. The efficiency of the *NRSF* deletion in NRSF-cKO mice was confirmed by Southern blot (Fig. 2*B*). Furthermore, 3 pairs of primers targeting different fragments of the *NRSF* locus were used for PCR-based genotype confirmation (Fig. 2*C*). In situ hybridization also revealed that total *NRSF* mRNA expression was markedly reduced in NRSF-cKO mouse forebrains (Fig. 2*D*).

In contrast to control mice, seizures activity did not cause an upregulation of *NRSF* mRNA in NRSF-cKO mice, as shown by in situ hybridization (Fig. 2*D*), indicating that activity-dependent upregulation of *NRSF* in the forebrain occurs predominantly in neurons. These results were further confirmed by real-time PCR, which revealed that *NRSF* mRNA rapidly increases after KA injection in control mice but not in NRSF-cKO mice (Fig. 2*E*). In accordance with this finding, NRSF protein was virtually undetectable by fluorescent immunohistochemistry in the NRSF-cKO cortex, both before and after KA injection (Fig. 3*F,H,N,P*). This was in contrast to the control forebrain cortex, where the level of NRSF protein was greatly increased in NeuN-labeled neurons after KA injection (Fig. 3*E,G,M,O*). These results demonstrate that NRSF is specifically deleted in excitatory neurons of the postnatal forebrain, and that seizures-induced upregulation of NRSF expression occurs predominantly in neurons.

Kindling Epileptogenesis Is Accelerated in NRSF-cKO Mice

To investigate the role of NRSF in epilepsy, we first compared KA-induced seizures development in NRSF-cKO mice with that of control mice. However, the high number of deaths that occurred during SE using this method led us to instead use the kindling model of epileptogenesis. In the kindling method, a twice-daily stimulation at the individually determined AD threshold current is administered, leading to gradual progression of behavioral seizures.

Prior to kindling stimulation, we tested whether the deletion of *NRSF*, or the presence of *CamKII-iCre*, or the *loxP* inserts had an effect on basal responses to initial electrical simulation. We did not observe any significant difference in the initial AD

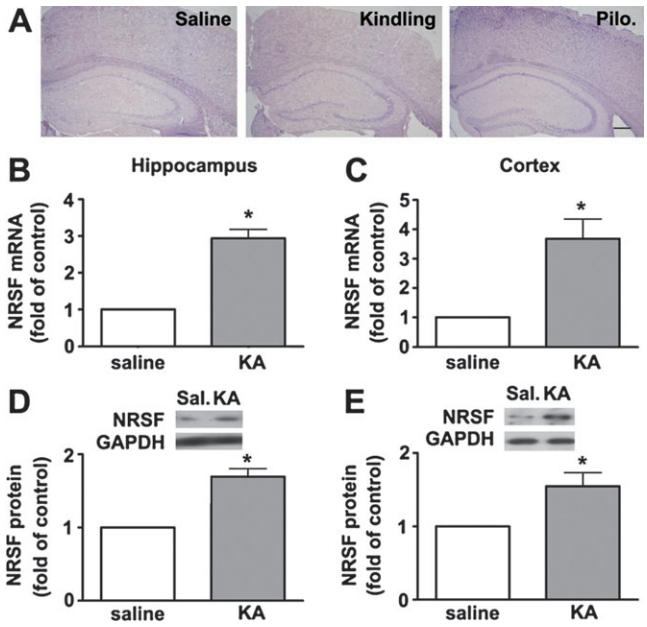

Figure 1. NRSF expression was upregulated in animal model of epilepsy. (*A*) mRNA expression of NRSF in forebrain is detected by in situ hybridization after kindled (mice sacrificed at 24 h after last stimulation) and pilocarpine-induced SE (mice sacrificed at 24 h after SE onset). Scale bar: 250 μm. (*B*) mRNA expression of NRSF in hippocampus is detected by real-time PCR at 24 h after KA treatment (*$P < 0.05$). (C) NRSF mRNA in forebrain cortex is detected by real-time PCR at 24 h after KA treatment (*$P < 0.05$). (*D,E*) Immunoblot results revealed NRSF protein increased in hippocampus (*D*) and forebrain cortex (*E*) after KA-induced SE (24 h after SE onset). *$P < 0.05$, $n = 4$, data presented as mean ± s.e.m.

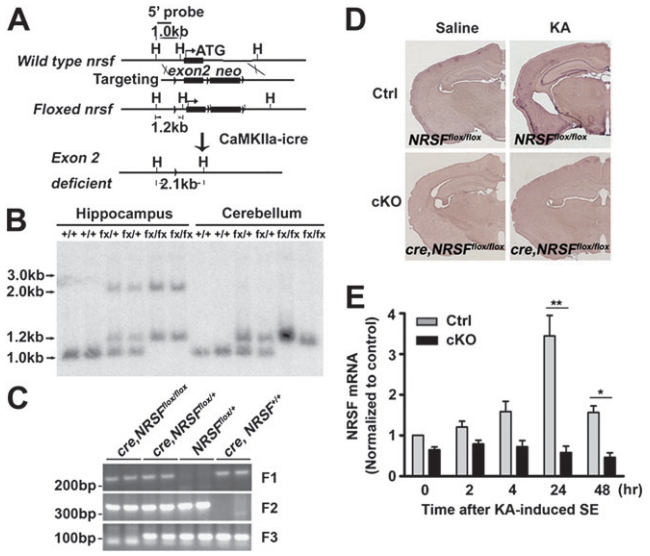

Figure 2. NRSF was specifically deleted in NRSF conditional knockout mice. (*A*) Diagram showing strategy to generate *NRSF* conditional knockout mice. Loxp elements were introduced into genomic DNA flanking NRSF exon 2 (that contains the initial codon and encodes the zinc-finger domains) to generate the *NRSF* ^{flox} allele. When crossed to *CaMKII*α-*iCre* mice, exon 2 of the floxed allele is excised. H: *Hind*III; *neo*: neomycin gene. (*B*) Southern blot of genomic DNA from the hippocampus and cerebellum of 1-month-old *CaMKII*α-*iCre*, *NRSF*$^{+/+}$ (+/+); *CaMKII*α-*iCre*, *NRSF* $^{flox/+}$ (fx/+); and *CaMKII*α-*iCre*, *NRSF* $^{flox/flox}$ (fx/fx) mice. Hippocampal or cerebellar DNA extracts were digested with *Hind*III and probed with the 5′ probe shown in (*A*). (*C*) Genotyping result from 3 pairs of primers: F1, identified Cre gene, >200 bp; F2, identified the insertion of floxed sequence, >300,bp; and F3, identified the wild type fragment without floxed insertion, >100 bp. (*D*) In situ hybridization results of *NRSF* $^{flox/flox}$ and *NRSF*-cKO mice using an exon 2-specific probe of *NRSF* 24 h after injection of saline or KA. (*E*) Real-time PCR result of mRNA quantification of NRSF from hippocampus of control and NRSF-cKO mice after KA-induced SE. "0" indicated no KA injection. *$P < 0.05$, **$P < 0.01$, data presented as mean ± s.e.m. ($n = 4$).

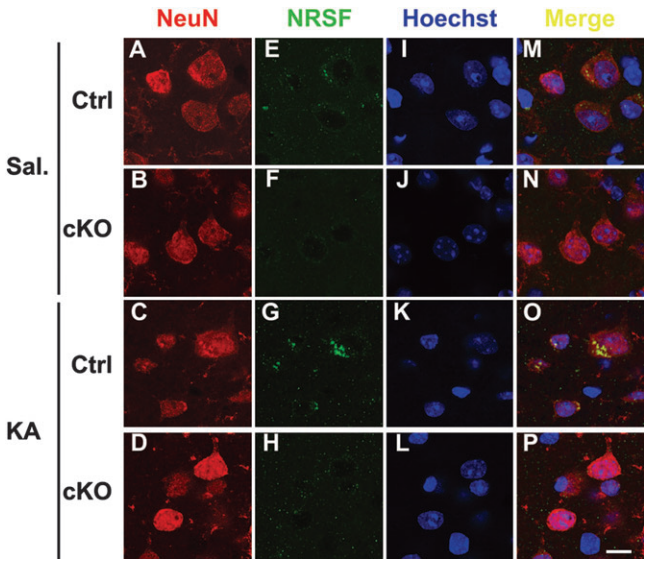

Figure 3. Fluorescent immunohistochemistry results of NRSF protein (green) and neuronal marker NeuN (red) in parietal cortex of basal (saline) and after seizures (KA). (*A–D*) NeuN (red) expresses in control and cKO mice under treatment of saline and KA. (*E–H*) NRSF protein (green) expresses in control and NRSF -cKO mice. NRSF expresses at low level in basal but increased at KA 24 h of control mice (*E*,*G*). Increased NRSF expression is absent in NRSF-cKO mice under treatment KA (*F*,*H*). (I-L)Hoechst staining (blue) in control and cKO mice under treatment of saline and KA. (*M–P*) Merged staining of NeuN, NRSF, and Hoechst. Scale bar: 10 μm.

threshold value among the 3 groups (*cre;NRSF*$^{+/+}$, 156.6 ± 16.9 μA; *NRSF*$^{flox/flox}$, 190.8 ± 15.5 μA; and *cre;NRSF*$^{flox/flox}$, 147.2 ± 14.5 μA; $P > 0.05$; Fig. 4*E*), indicating that neither the transgenic insertions nor the removal of *NRSF* change baseline sensitivity to kindling stimulation. However, we found that epilepsy progression was significantly accelerated in NRSF-cKO mice compared with controls. It required 9.4 ± 1.2 stimulations in *cre;NRSF*$^{+/+}$ mice and 8.7 ± 0.6 in *NRSF*$^{flox/flox}$ mice to elicit the first stage 3 seizures, whereas in NRSF-cKO mice it required only 3.7 ± 0.5 ($P < 0.001$ compared with control mice). Similarly, it required significantly fewer stimulations to elicit the first stage 5 seizures in NRSF-cKO mice (*cre;NRSF*$^{+/+}$, 11.8 ± 1.2; *NRSF*$^{flox/flox}$, 10.7 ± 0.7; and *cre;NRSF*$^{flox/flox}$, 6.0 ± 0.5; $P < 0.001$; Fig. 4*A*,*B*). Moreover, NRSF-cKO mice also exhibited prolonged AD durations from the eighth stimulation until the end of testing ($P < 0.01$; Fig. 4*C*,*D*,*F*). Together, these results demonstrate that conditional deletion of *NRSF* results in accelerated epilepsy progression and intensified seizures activity during kindling.

Mossy Fiber Sprouting Is Exaggerated in Fully Kindled NRSF-cKO Mice

A mossy fiber is a bundle of granule cell axons from the DG that innervates hippocampal CA3 area neurons. The sprouting of recurrent excitatory collaterals on granule cell axons in the molecular layer is an anatomical consequence of repeated seizures activity and head trauma and is also well correlated with kindling development (Represa et al. 1989; Sutula et al. 1989). To investigate whether mossy fiber sprouting was altered in NRSF-cKO mice after kindling, we performed Timm staining on control and NRSF-cKO mice. The extent of Timm staining in the DG can be scored, thus yielding a semiquantitative measure of mossy fiber sprouting (Sutula et al. 1996; Sprengel et al. 1998). Consistent with a previous study (Cavazos et al. 1991), the Timm index score of control mice after kindling was 2-fold higher than in sham-stimulated control mice ($P < 0.05$; Fig. 5*A*,*B*). Intriguingly, Timm staining revealed greatly exaggerated mossy fiber sprouting in kindled NRSF-cKO mice, representing a 7-fold increase above the score for sham-stimulated NRSF-cKO mice ($P < 0.001$; Fig. 5*B*).

Derepression of FGF14 and BDNF after NRSF Deletion

Previous studies have identified many genes related to synaptic transmission and plasticity that are transcriptionally regulated by NRSF (Bruce et al. 2004; Johnson et al. 2007). We hypothesized that NRSF target genes would be derepressed in adult forebrain neurons of NRSF-cKO mice. To test this, we screened the expression of several NRSF target genes in the hippocampus and forebrain cortex of sham-stimulated (saline) and KA-stimulated NRSF-cKO mice, focusing in the genes involved in synaptic transmission, axon guidance, and neuronal excitability. Compared with KA-treated control mice, we found that mRNA levels of 2 target genes, *fgf14* and *bdnf*, were significantly upregulated in the cortex of KA-stimulated cKO mice ($P < 0.05$; $n = 5$; Fig. 6*A*). The expression of all other target genes tested (*Scn2a1*, *L1cam*, *Gria2*, *Grin2b*, *Gabrg2*, and *Ntrk2*) was not significantly different between control and cKO mice (Fig. 6*D*). Western blot analysis of target gene expression in the cortex yielded consistent results; no significant differences in protein expression were observed, except for increased levels of FGF14 and BDNF in NRSF-cKO

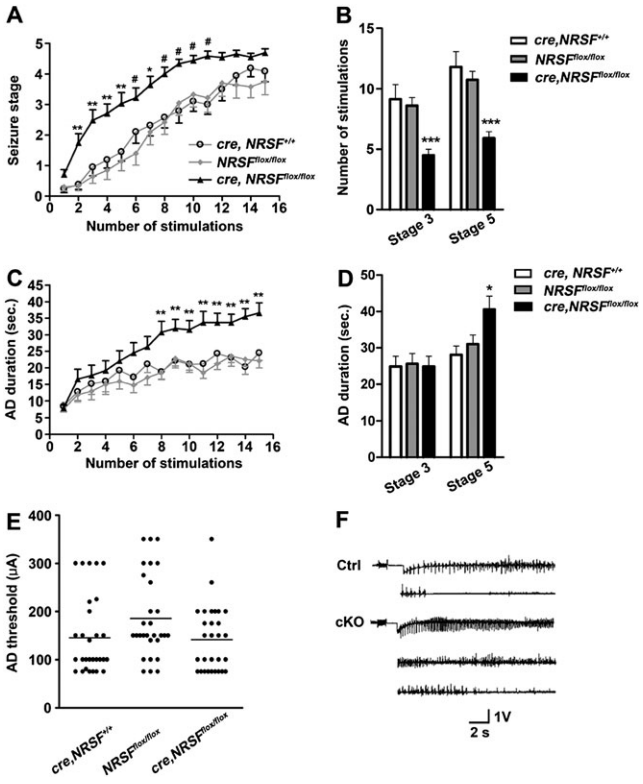

Figure 4. Forebrain removal of NRSF accelerated progression of kindling. (*A*) Mean seizures stage accompanying the twice-daily stimulus at the individual ADT level in NRSF-cKO mice and 2 groups of controls. $*P < 0.05$, $**P < 0.01$, $\#P < 0.001$. (*B*) Comparison of the number of stimulations required to elicit the first stage 3 and stage 5 seizures, $***P < 0.001$. (*C*) AD duration of each stimulus during kindling, $**P < 0.01$. (*D*) Comparison of the AD duration of the first stage 3 and stage 5 seizures, $*P < 0.05$. (*E*) Individual initial ADTs of NRSF-cKO mice and 2 groups of controls. (*F*) Typical electroencephalogram of an AD duration accompanying the first stage 5 seizures (truncated stimulus artifact). For above data, *cre,NRSF* $^{flox/flox}$ ($n = 29$), *cre,NRSF*$^{+/+}$ ($n = 31$), and *NRSF* $^{flox/flox}$ ($n = 29$), all data presented as mean ± s.e.m.

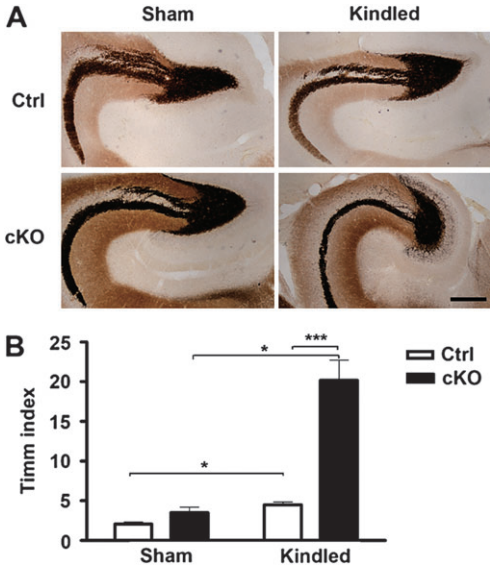

Figure 5. Mossy fiber sprouting has been exaggerated in kindled cKO mice. (**A**) the view of hippocampal horizontal section of Timm staining in sham operated and kindled control and cKO mice. Scale bar: 250 μm. (*B*) Statistic of Timm granule of DG inner molecular layer in both sham and kindled control and cKO mice ($n = 4$–8 for each group; $*P < 0.05$; $***P < 0.001$).

mice 24 h after KA treatment (Fig. 6*B,C,E,F*). In the hippocampus, BDNF was the only NRSF target gene to show significantly increased expression in both mRNA and protein levels in KA-treated cKO mice compared with KA-treated control mice (Supplementary Fig. 2*A–C*). In the sham-stimulated group, no significant expression changes were found between control and NRSF-cKO mice.

Increased PLCγ and PI(3)K Signaling in NRSF-cKO Mice

Deletion of BDNF in the hippocampus led to retarded kindling-induced progression of epilepsy, while conditional deletion of the BDNF receptor TrkB in the same region abolishes it completely (He et al. 2004), indicating that the BDNF signaling pathway plays an important role in epileptogenesis. Thus, the increased BDNF expression that we observed in the hippocampus and cortex of NRSF-cKO mice following seizures activity might be critical to the accelerated rate of kindling development in these mice. To determine which signaling cascades downstream of BDNF-TrkB are affected by NRSF-dependent inhibition of seizures activity, we examined Extracellular-signal-regulated kinases (ERK), PLCγ, and Akt phosphorylation in sham- and KA-induced mice. Twenty-four hours after the onset of KA-induced seizures in control mice, we observed increased phosphorylation of ERK, PLCγ, and Akt in the forebrain cortex (Fig. 7*A–C*), consistent with the dramatic increases in BDNF protein levels (Fig. 6*B,C*) and TrkB phosphorylation at this time point after KA stimulation (Binder et al. 1999; He et al. 2004). In NRSF-cKO mice, the increase of PLCγ and Akt phosphorylation 24 h after KA treatment was significantly enhanced compared with control mice, whereas ERK phosphorylation remained similar to control (Fig. 7*A–C*). Recently, He et al. (2010) reported that TrkB-mediated activation of PLCγ was a critical molecular event in kindling-induced epileptogenesis. These results indicate that loss of NRSF results in increased activation of BDNF downstream signaling PLCγ and Akt, which may contribute to accelerated epilepsy progression.

Discussion

The induction of NRSF after seizures or ischemic insult suggests a potential role of NRSF in pathological conditions (Palm et al. 1998; Calderone et al. 2003; Spencer et al. 2006). In this study, we found that NRSF functions as an intrinsic moderator of epilepsy progression and seizures activity. This conclusion is supported by several results. First, we confirmed that NRSF is selectively upregulated in excitatory forebrain neurons in response to seizures activity induced by kindling or KA. Second, conditional knockout of NRSF in forebrain neurons accelerated epilepsy progression and potentiated kindling-induced seizures. Third, loss of NRSF resulted in exaggerated mossy fiber sprouting from granule cell axons. Finally, loss of NRSF in the forebrain cortex derepressed activity-dependent expression of the NRSF target genes *Fgf14* and *Bdnf* and enhanced the activation of PLCγ and PI(3)K signaling.

Mice lacking NRSF die during embryonic development (Chen et al. 1998) and thus cannot be used to study NRSF function in postnatal stages. Here, using a Cre/loxP conditional knockout strategy to delete NRSF specifically in postnatal excitatory neurons of the mouse forebrain, we tested the development of kindling-induced epileptogenesis in the absence of postseizure upregulation of NRSF. Kindling is a sensitive animal model for discriminating factors that influence susceptibility to the

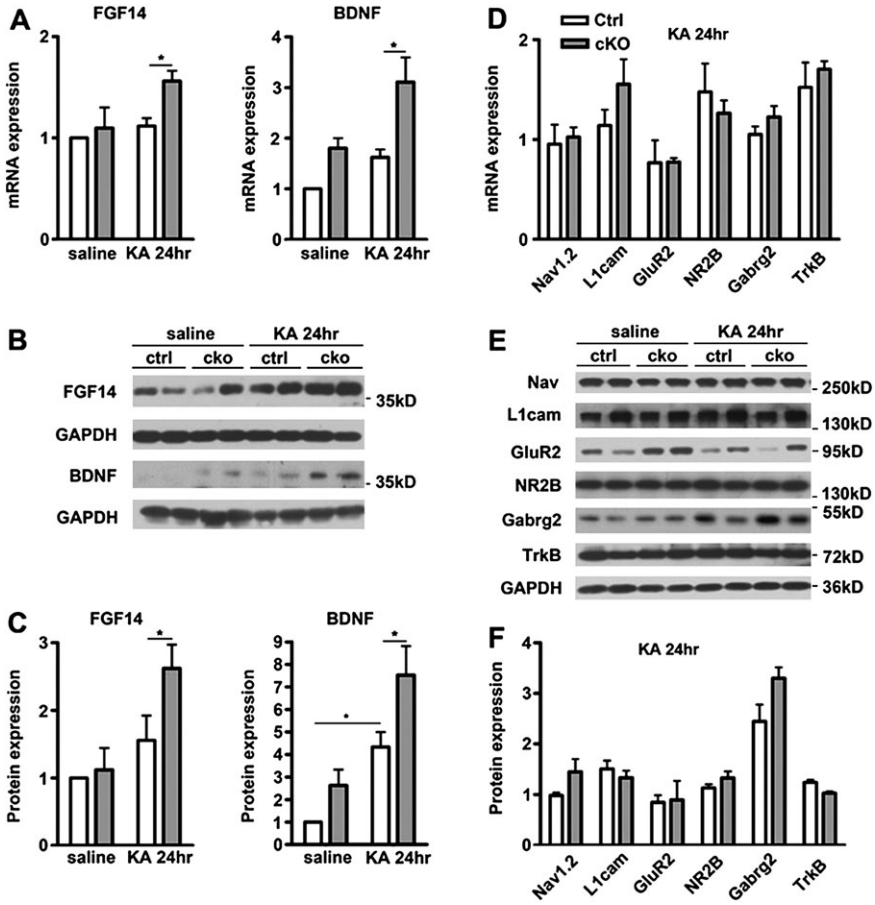

Figure 6. The mRNA and protein expression of NRSF target genes is shown in control and NRSF-cKO mice at basal and after KA-induced seizures. (*A*) Quantitative PCR results of FGF14 and BDNF mRNA extracted from forebrain cortex. (*B*) Protein expression of FGF14 and BDNF in cortex of control and cKO mice. (*C*) Quantification of immunoblot results of FGF14 and BDNF in cortex. (*D*). Quantitative PCR revealed no significant different of mRNA expression for NRSF target gene in cortex after KA treatment (24 h after SE onset): *Scn2a1* (Nav1.2), *L1cam*, *Gria2* (GluR2), *Grin2a* (NR2B), *Gabrg2*, and *Ntrk2* (trkB). Data normalized to the value of control mice with saline treated. (*E*) Immunoblot results of NRSF target genes in cortex. (*F*) Statistic results of immunoblot results from 24 h KA-induced SE. $^*P < 0.05$, data presented as mean ± s.e.m. ($n = 4$–5).

development of epilepsy. We found that conditional deletion of NRSF in postnatal forebrain neurons resulted in accelerated kindling-induced epileptogenesis. This finding is especially interesting in the context of 2 other findings. First, in NRSF-cKO mice, baseline AD intensity—both threshold and duration of the AD—was comparable with control mice. Second, during the development of kindling, AD durations in NRSF-cKO were significantly prolonged from the 8th to 15th stimulations (Fig. 4*C*).

In light of the fact that we did not observe any significant differences between NRSF-cKO and control forebrains in NRSF expression at baseline and at early time points after a KA-induced seizures event (Fig. 2*D*,*E* and Fig. 3*E*,*F*), we propose that the upregulation of NRSF might mitigate susceptibility of the brain to uncontrolled network activity. Loss of NRSF might disrupt intrinsic homeostatic mechanisms of excitatory and inhibitory circuits and results in elevated network excitability in response to excitatory insults. This conclusion is supported by the recent finding that glycolysis inhibitor-2-deoxygen-glucose inhibits epileptogenesis by strengthening the transcriptional repression activity of the NRSF–CtBP complex (Garriga-Canut et al. 2006). Our work provides the first direct in vivo evidence that NRSF suppresses the progression of epilepsy.

Based on results of large-scale Chip-seq screens of genes with canonical and noncanonical NRSF DNA binding sites (NRSEs; Johnson et al. 2007; Otto et al. 2007), we measured mRNA and protein levels of a selection of neuronal activity-relevant NRSF target genes, including Fgf14, Gabrg2 (Johnson et al. 2007), Scn2a1, L1cam, Gria2, Grin2a, Ntrk2, and Bdnf (Bruce et al. 2004) in NRSF-cKO mice. Interestingly, we found that deleting NRSF in the forebrain did not cause a change in the expression of most of these genes at baseline or a change in the expression dynamics of these genes following seizures. The only exceptions were the small molecule growth factors BDNF and FGF14. Twenty-four hours after KA-induced seizures, both factors were increased to a significantly greater level in the forebrain cortex of NRSF-cKO mice than in control mice (Fig. 6). The selective derepression of NRSF-dependent target genes in response to KA-induced seizures suggests that BDNF and FGF14 are both critical to the acceleration of kindling-induced epileptogenesis in NRSF-cKO mice. Previous studies have demonstrated that BDNF activity is important for the development of epilepsy. Heterozygotes of BDNF mutant mice show suppressed kindling development (Kokaia et al. 1995). Deletion of BDNF impaired whereas conditional deletion of its receptor TrKB abolished kindling development (He et al. 2004). These data revealed the critical role of BDNF signaling in kindling-induced epileptogenesis. Our findings further suggest that FGF14 might be an important regulator of neuronal

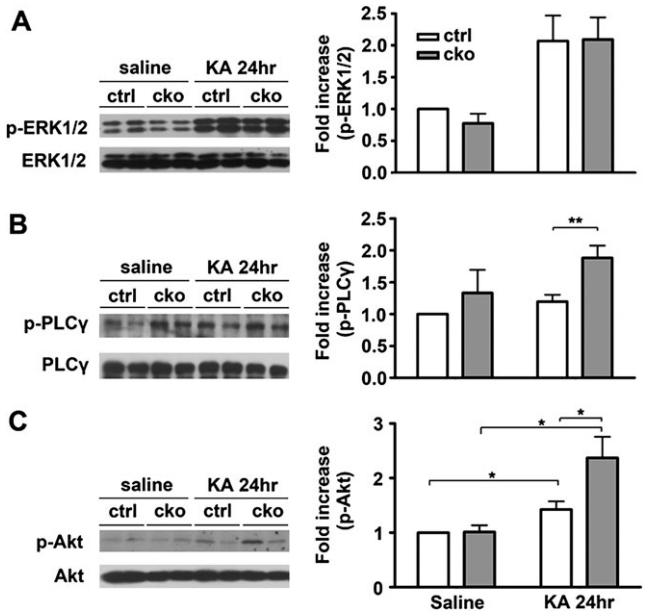

Figure 7. Increased PLCγ and PI(3)K signaling after NRSF removal. (*A*) Immunoblot results of phospho-ERK at basal and after seizures. Right penal shows the statistic results of ERK phosphorylation. Data compared with total ERK and normalized to the value of saline-treated control mice. (*B*,*C*) Immunoblot results of phospho-PLCγ (*B*) and phospho-Akt (*C*) at basal and after seizures. Right penal shows the statistic results. Data compared with total PLCγ (*B*), total Akt (*C*), and normalized to the value of saline-treated control mice. Cell lysate extract from forebrain cortex. $^*P < 0.05$, data presented as mean ± s.e.m. ($n = 4$–5).

excitability in response to KA or kindling. FGF14 expression in cultured neuroblastoma cells has been reported to regulate the functional properties of sodium channels (Laezza et al. 2009), and mice lacking FGF14 show spatial learning defects and impairment of theta burst-induced LTP (Wozniak et al. 2007). These results indicate that FGF14 functions in regulating neuronal excitability and may thus participate in regulating epilepsy development.

In order to investigate, whether increased BDNF expression in NRSF-cKO mice leads to a concomitant increase in downstream signaling activity, we compared ERK, PLCγ, and Akt phosphorylation in response to KA-induced seizures. We found that PLCγ and Akt phosphorylation was significantly enhanced in NRSF-cKO mice, indicating that the absence of NRSF results in hyperactivation of specific intracellular signaling cascades downstream of BDNF in excitatory forebrain neurons, and this may be responsible in part for the sensitization of the forebrain to seizures-inducing stimuli.

Funding

National Basic Research Program of China (2011CBA00407); The National Natural Science Foundation of China (31021063, 30925016); and SSSTC program (GJHZ0902 to Z.-Q.X.).

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

Notes

We are grateful to Prof. Günther Schütz for providing CaMKIIα-icre transgenic mice, Dr Gail Mandel for providing the human REST/NRSF construct, and Dr David Anderson for the NRSF antibody. *Conflict of Interest* : None declared.

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