Molecular Neurobiology (2019) 56:4381–4394
https://doi.org/10.1007/s12035-018-1384-2

Aberrant Amygdala-Dependent Cued Fear Memory in Na^+/Ca^{2+} Exchanger 1 Heterozygous Mice

Shigeki Moriguchi[1] · Satomi Kita[2,3] · Ryo Inagaki[1] · Yasushi Yabuki[1] · Yuzuru Sasaki[1] · Shun Ishikawa[1] · Hiroyuki Sakagami[4] · Takahiro Iwamoto[2] · Kohji Fukunaga[1]

Received: 12 April 2018 / Accepted: 8 October 2018 / Published online: 15 October 2018

Abstract

Na^+/Ca^{2+} exchangers (NCXs) are mainly expressed in the plasma membrane and exchange one Ca^{2+} for three Na^+, depending on the electrochemical gradients across the plasma membrane. NCXs have three isoforms, NCX1–3, encoded by distinct genes in mammals. Here, we report that heterozygous mice lacking NCX1 ($NCX1^{+/-}$) exhibit impaired amygdala-dependent cued fear memory. $NCX1^{+/-}$ mice showed significant impairment in fear-related behaviors measured with the elevated-plus maze, light-dark, open-field, and marble-burying tasks. In addition, $NCX1^{+/-}$ mice showed abnormality in cued fear memory but not in contextual fear memory in a fear-conditioning task. In immunohistochemical analyses, $NCX1^{+/-}$ mice had significantly increased number of c-Fos-positive cells in the lateral amygdala (LA) but not in the central amygdala following fear-related tone stimuli. c-Fos expression peaked at 1 h. In concordance with the aberrant fear-related behaviors in $NCX1^{+/-}$ mice, enhanced long-term potentiation was also observed in the LA of these mice. Furthermore, enhancement of CaMKII or CaMKIV activity in the LA was observed in $NCX1^{+/-}$ mice by immunoblot analyses. In contrast, $CaMKII^{+/-}$ but not $CaMKIV^{-/-}$ mice insufficiently exhibited tone-induced cued fear memory and there was no increase in the number of c-Fos-positive cells in the LA. Altogether, the increased CaMKII activity and consequent c-Fos expression likely account for the dysregulation of amygdala-dependent cued fear memory in $NCX1^{+/-}$ mice.

Keywords Na^+/Ca^{2+} exchanger · Amygdala-dependent cued fear memory · CaMKII · c-Fos expression · Lateral amygdala

Abbreviations

AD	Alzheimer's disease
BDNF	Brain-derived neurotrophic factor
CeA	Central amygdala
CaN	Calcineurin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CREB	cAMP-responsive element binding protein
ERK	Extracellular-signal-regulated kinase
fEPSPs	Field excitatory post-synaptic potentials
LA	Lateral amygdala
LTP	Long-term potentiation
NCXs	Na^+/Ca^{2+} exchangers
NCX1–3 heterozygous	$NCX1–3^{+/-}$
PKA	protein kinase A
WT	Wild-type

✉ Shigeki Moriguchi
shigeki@m.tohoku.ac.jp

✉ Takahiro Iwamoto
tiwamoto@fukuoka-u.ac.jp

[1] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-Aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan

[2] Department of Pharmacology, Faculty of Medicine, Fukuoka University, 7-45-1, Nanakuma, Jonan-ku, Fukuoka, Fukuoka 814-0180, Japan

[3] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan

[4] Department of Anatomy, Kitasato University School of Medicine, Sagamihara, Japan

Introduction

Chronic stress is strongly implicated in the progression of numerous psychiatric disorders, such as post-traumatic stress disorder and anxiety disorder, characterized by severe fear-

related memory formation [1–4]. Both the hippocampus and amygdala are implicated in major neuroanatomical stress circuits. Chronic stress leads to aberrant brain plasticity, and the alteration of brain plasticity results in functional and structural changes in both the hippocampus and amygdala [5, 6]. Chronic stress may lead to decrease in dendritic branches, mossy-fiber synapses, and adult neurogenesis in the hippocampus [7–9] and to increased dendritic arborization and spine density in the amygdala [10, 11].

Pavlovian fear conditioning is one way to study chronic stress models in rodents. Phillips and LeDoux previously reported that hippocampal lesions may disturb the conditioning of fear responses to contextual memory, whereas amygdala lesions may also disturb the conditioning of fear responses to both contextual and tone (cued) memory [12]. In rodents, impaired hippocampal long-term potentiation (LTP) was accompanied by conditioned fear stress (five trains at 1 Hz each composed of eight pulses at 400 Hz) [13]; however, increased LTP-like changes in synaptic strength in the lateral amygdala (LA) occurred following conditioned fear stress [14, 15]. The synaptic circuit of conditioned fear stress to contextual and tone cues is complicated.

Alteration in intracellular Ca^{2+} homeostasis is essential for proper physiological function in neurons. Na^+/Ca^{2+} exchangers (NCXs), which belong to a family of neuronal plasma-membrane proteins, play a role in the maintenance of intracellular Ca^{2+} homeostasis. Under physiological conditions, in neurons, NCXs extrude Ca^{2+} from the cytosolic to the extracellular space depending on the electrochemical gradients across the plasma membrane in forward mode [16]. However, NCXs regulate Ca^{2+} influx into neurons in reverse mode under pathological conditions [17–19]. NCXs occur in three isoforms, NCX1, NCX2, and NCX3 [20, 21], and all NCXs are abundantly distributed in numerous brain regions [22, 23]. Recently, mice lacking specific NCXs were established, and the phenotype of brain function in NCX-mutant mice was reported. In memory-related behaviors, NCX2 homozygous ($NCX2^{-/-}$) mice exhibited improved memory-related performance [24]; however, NCX3 homozygous ($NCX3^{-/-}$) mice displayed impaired memory-related performance [25]. We also previously reported that memory impairment seen in NCX2- and NCX3-heterozygous ($NCX2^{+/-}$ and $NCX3^{+/-}$) mice reflected dysregulated hippocampal calcium/calmodulin-dependent protein kinase II (CaMKII) activity [26]. By contrast, NCX1 heterozygous mice did not exhibit memory impairment in our previously report [26]. In contrast, $NCX2^{-/-}$ and $NCX3^{-/-}$ mice exhibited impaired contextual fear-related behaviors, and $NCX3^{-/-}$ but not $NCX2^{-/-}$ mice also exhibited disordered cued fear-related behaviors in a fear-conditioning task [24, 25]. Thus, NCXs are critical in intracellular Ca^{2+} mobilization and neuronal plasticity in the brain of mammals. However, the association between NCX1 proteins and cued fear memory remains unclear.

In this study, we first report that heterozygous mice lacking NCX1 ($NCX1^{+/-}$) exhibited impaired amygdala-dependent cued fear memory but not hippocampal-dependent contextual fear memory in a fear-conditioning task. Increased CaMKII activity and in turn c-Fos expression in the LA likely account for the dysregulation of amygdala-dependent cued fear memory in $NCX1^{+/-}$ mice.

Materials and Methods

Animals

$NCX1–3^{+/-}$ Mice NCX1 and NCX2 heterozygous ($NCX1^{+/-}$ and $NCX2^{+/-}$) mice were produced as reported previously [27–29]. NCX3 heterozygous ($NCX3^{+/-}$) mice were generated as follows: a clone containing 13 kb of the murine NCX3 gene was isolated from the C57BL/6 mouse genomic sequence. The targeting vector was constructed by replacing the 1.9 kb MunI-MunI fragment containing exon 2 of the NCX3 gene with a Neo cassette. The diphtheria toxin-A fragment was ligated to the 3′ position of the targeting vector for negative selection. The targeted ES clones were verified by Southern blot analysis and used in the generation of germline chimera. $NCX1–3^{+/-}$ and wild-type (WT) male mice (8–10 weeks of age) bred on a C57BL/6J genetic background for more than nine generations were used in experiments. Mice were housed in cages with free access to food and water at a constant temperature (23 ± 1 °C) and humidity (55 ± 5%) with a 12-h light/dark cycle (09:00–21:00 h).

$CaMKII\alpha^{+/-}$ Mice $CaMKII\alpha^{+/-}$ mice (strain B6; 129P2-CaMKII2a/J) were obtained from Jackson Laboratory (Bar Harbour, ME, USA). Here, we used heterozygous CaMKIIα mice bred for more than 20 generations on a C57BL/6J background and continuously backcrossed on that strain thereafter. Mice were housed one per cage after weaning. Details relevant to mouse construction are provided by Yamasaki et al. [30]. $CaMKII\alpha^{+/-}$ mice (male) 8–10 weeks of age were used in experiments.

$CaMKIV^{-/-}$ Mice Calcium/calmodulin-dependent protein kinase IV $(CaMKIV)^{-/-}$ mice were provided from Prof. Hiroyuki Sakagami at Kitasato University. To disrupt the CaMKIV gene, the exon containing the initiation codon was replaced with a neomycin resistance cassette. Southern blot analysis using a 3′ external probe confirmed homologous recombination in CCE embryonic stem (ES) cells derived from the 129/SvJ mouse strain. A detailed protocol is provided by Takao et al. [31]. $CaMKIV^{-/-}$ mice (male) 8–10 weeks of age were used in experiments.

Behavioral Analyses

In the following behavioral analyses, evaluators were blinded to treatment conditions.

(1). *Elevated-plus maze task:* A detailed protocol of this task has been reported [32]. The elevated plus maze task measures anxiety behavior in mice and employs an apparatus consisting of a central 6 × 6 cm platform and four arms radiating in plus-sign form from the platform: two open and two enclosed arms, all 30 cm long, 6 cm wide, and 15 cm high with nontransparent side and end walls. The maze is elevated 70 cm above the floor level. At the beginning of a 5-min test session, mice are placed at the center facing an enclosed arm and the following parameters are scored: (1) the number of total, open, and closed arm entries and (2) time spent in different parts of the maze (open and closed arms and central platform). For the test, time spent in open and closed arm is recorded, with longer periods spent in closed arms associated with increased anxiety behavior.

(2). *Marble-burying task:* A detailed protocol of this task has been reported [32]. This task is based on the tendency of mice to exhibit increased digging behavior in high anxiety states. A cage is filled approximately 5–10 cm deep with wood chips lightly tamped down to create a flat surface. Glass marbles, evenly spaced approximately 4 cm apart, are placed on the surface. Animals are then placed in the cage for 30 min, and at the end of the test, the number of marbles covered by bedding is measured.

(3). *Light-dark task:* A detailed protocol of this task has been reported [32]. This task is based on the tendency of mice to prefer a dark room in states of high anxiety. The light/dark box apparatus consists of a wooden box (48 × 24 × 27 cm) divided into two equal-size compartments by a barrier containing a doorway (10 cm height × 10 cm width). One compartment is painted black and covered with a lid, and the other is painted white and illuminated with a 60-W light bulb positioned 40 cm above the upper edge of the box. Mice are initially placed in the dark compartment, and the time to enter the light compartment is recorded.

(4). *Open-field task:* A detailed protocol of this task has been reported [32]. This task is based on the tendency of mice to stay close to a wall in states of high anxiety. Mice are placed in a Plexiglas box (60 × 60 × 60 cm) for 10 min, and their movements are recorded with an overhead video camera. A circle with a 15-cm radius marks the center of the test floor, and the time spent in that zone is recorded.

(5). *Fear-conditioning task:* The mice are placed individually in a conditioning chamber (25 × 25 × 25 cm) and the floor is constructed of stainless steel rods, and the rods in the chamber are connected to an electronic stimulator (Nihon Kohden, Tokyo, Japan). The conditioned stimulus (CS) was a 3-kHz tone (60 dB) produced by a frequency generator. Electric shock (of 0.8-mA intensity for 2 s) was used as the unconditioned stimulus (US). During the acquisition phase (day 1), mice were placed in the conditioned chamber for 2 min and then exposed to tones for 30 s (1-min intervals, three trains). A foot shock was delivered at the offset of each tone. Mice were removed from the chamber 1 min after the last foot shock. During the test phase (day 2), mice were separated into two groups. One group was placed in the same chamber for 4 min without exposure to tones (contextual fear conditioning), and other group was placed in a different chamber for 5 min with exposure to tones (cued fear conditioning) as in the acquisition phase. We measured the time spent freezing in the acquisition phase for 5 min.

Electrophysiology

Protocols were similar to those described previously [33]. Mice were anesthetized with sevoflurane and killed by decapitation. Transverse amygdala slices (400 μm thick, coronal slices) prepared by a vibratome (Microslicer DTK-1000) were incubated for 2 h in continuously oxygenized (95% O2, 5% CO2) artificial cerebrospinal fluid at 23 °C. After a 2-h recovery period, the slices were transferred to an interface-type recording chamber and perfused at a flow rate of 2 mL/min with artificial cerebrospinal fluid at 34 °C. Field excitatory post-synaptic potentials (fEPSPs) were evoked by a test stimulus (0.05 Hz) through a bipolar stimulating electrode placed on the fibers from the cortical input using a glass electrode filled with 3 M NaCl. High-frequency stimulation of 100 Hz for 1 s was applied twice with a 10-s interval, and test stimulation was continued for the indicated periods. A bipolar stimulating electrode was placed on the capsula externa, and potentials were recorded from the LA.

Biochemical Analysis

Immunoblotting was performed as described [34], using the following antibodies: anti-NCX1 (1:1000, [35]), anti-NCX2 (1:1000, [35]), anti-NCX3 (1:1000, [35]), anti-phospho CaMKII (1:5000, [36]), anti-CaMKII (1:5000, [37]), anti-phospho-CaMKIV (Thr-196) (1:2000, Abcam, Cambridge, MA, USA), anti-phospho-MAP kinase (di-phosphorylated extracellular-signal-regulated kinase (ERK) 1/2) (1:2000, Sigma-Aldrich, St. Louis, MO, USA), anti-phospho-GluA1

(Ser-831) (1:1000, Millipore, Billerica, MA, USA), anti-phospho-synapsin I (Ser-603) (1:2000, Millipore), anti-phospho- cAMP-responsive element binding protein (CREB) (Ser-133) (1:1000, Millipore), anti-brain-derived neurotrophic factor (BDNF) (1:1000, Santa Cruz Biotechnology Inc., Dallas, TX, USA), and anti-β-tubulin (1:5000, Sigma-Aldrich). Bound antibodies were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) and analyzed semiquantitatively using the National Institutes of Health ImageJ program.

Immunohistochemistry

Immunohistochemistry was performed as described in Moriguchi et al. [34]. For double immunofluorescence staining, sections were incubated with a mixture of anti-NCX1, anti-NCX2, or anti-NCX3 polyclonal antibodies (rabbit, 1:500 each, Iwamoto et al. [35]) with MAP (mouse, 1:200 each, Millipore), synaptophysin (guinea pig, 1:200 each, Syn-GP-Af300, Frontier Institute, Ishikari, Japan), anti-PSD-95 (guinea pig, 1:200 each, PSD-95-GP-Af660, Frontier Institute), and GFAP (mouse, 1:400 each, Millipore) antibodies. After thorough washing in phosphate buffer saline (PBS), sections were incubated for 3 h in Alexa 405-labeled anti-chicken, Alexa 488-labeled anti-rat IgG, or Alexa 594-labeled anti-mouse IgG. After several PBS washes, sections were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent images were analyzed using a confocal laser scanning microscope (Nikon EZ-C1, Nikon, Tokyo, Japan; JSM700, Zeiss, Thornwood, NY, USA).

A mouse monoclonal c-Fos antibody (1:50; Santa Cruz Biotechnology) was used as a primary antibody. The sections were incubated with secondary antibodies, including Alexa 594 anti-goat IgG (1:500; Invitrogen, Carlsbad, CA, USA). Visualization of c-Fos immunoreactivity following diaminobenzidine (DAB) staining was performed using the VECTASTAIN ABC kit (Vector Laboratories). Immunofluorescent images were analyzed using a confocal laser scanning microscope (LSM700, Zeiss). The numbers of c-Fos-positive cells were counted in the central amygdala (CeA) and LA.

Measurement of BDNF mRNA

Total LA RNA was extracted using TRI reagent (Sigma-Aldrich) and the RNeasy Plus Mini Kit protocol (QIAGEN, Tokyo, Japan). Reverse transcription was performed as previously described [32]. Real-time PCR was performed in 48-well PCR plates (Mini Opticon Real-Time PCR System; Bio-Rad Laboratories, Hercules, CA) using the iQ SYBR Green Supermix 2X (Bio-Rad Laboratories). The primer sequences were as follows: mouse BDNF exon I, 5′-CCTGCATCTGTTGGGGAGAC-3′ and 5′- GCCTTGTC CGTGGACGTTTA-3′; mouse BDNF exon IV, 5′-CAGA GCAGCTGCCTTGATGTT-3′ and 5′- GCCTTGTC CGTGGACGTTTA-3′; mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TGTGTCCGTCGTGG ATCTGA-3′ and 5′- CACCACCTTCTTGATGTCAT CATAC-3′.

Statistical Analysis

Data are expressed as means±SEM. Statistical analysis was performed using Prism 6 (Graphpad software, San Diego, CA, USA). Comparisons between two experimental groups were performed using the unpaired Student's *t* test. Statistical significance for differences among groups was tested by one-way or two-way analysis of variance, followed by a post-hoc Bonferroni's multiple comparison test between the control and other groups. Asterisks ($*P < 0.05$, $**P < 0.01$) denote statistical significance in graphs.

Results

NCX1$^{+/-}$ Mice-Enhanced Anxiety-like Behaviors

We first measured the protein levels of NCX1–3 in the LA of NCX1–3$^{+/-}$ mice (Fig. 1a, b), as NCX1 null and NCX2 null mice exhibit embryonic lethality [28]. As expected, when we assessed NCX1–3 protein levels in respective heterozygotes, they were approximately half those seen in WT mice (Fig. 1a, b). Therefore, we further tested the effects of anxiety-like behaviors in NCX1–3$^{+/-}$ mice (Fig. 1c–g). In the elevated-plus maze task, NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice spent significantly less time in the open arms than did WT mice (number: WT mice 4.5 ± 0.5; NCX1$^{+/-}$ mice 13.0 ± 0.9, $n = 6$ each, time: WT mice 33.3 ± 2.4 s; NCX1$^{+/-}$ mice 15.0 ± 2.1 s, $n = 6$ each) (Fig. 1c, d). We next measured the number of buried marbles in the marble-buried task. NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice showed an increase in the number of buried marbles compared to WT mice (WT 12.1 ± 1.3; NCX1$^{+/-}$ mice 16.0 ± 0.7, $n = 6$–8 each) (Fig. 1e). Similarly, NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice showed an increase in the latency time to enter the open compartment in the light-dark task (WT mice 36.8 ± 6.5 s; NCX1$^{+/-}$ mice 72.0 ± 16.6 s, $n = 6$–8 each) (Fig. 1f). We found that NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice spent significantly less time in the center circle than did WT mice in the open-field task (WT mice 114.0 ± 12.4 s; NCX1$^{+/-}$ mice 37.5 ± 8.7 s, $n = 6$–8 each) (Fig. 1g).

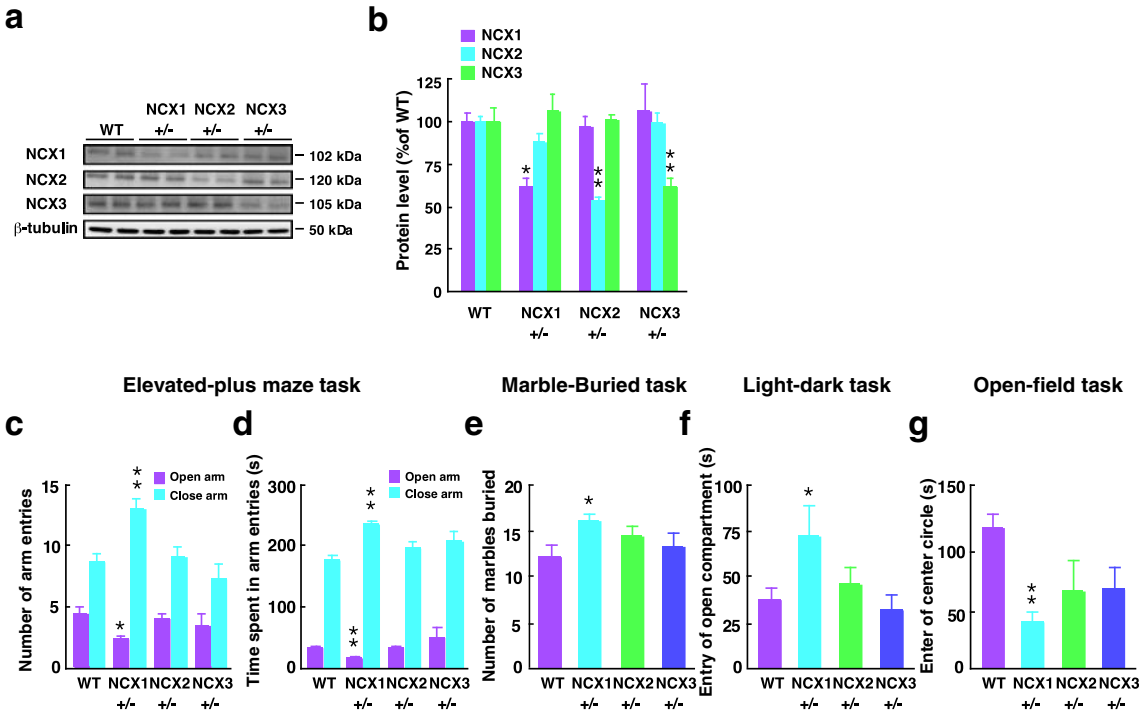

Fig. 1 NCX1$^{+/-}$ mice exhibit an increase in anxiety-like behaviors. **a** Representative images of immunoblots of the lateral amygdala (LA) probed with NCX1–3 and β-tubulin antibodies. **b** Quantitative analyses of NCX1–3 protein levels in the LA of NCX1–3$^{+/-}$ mice. **c**, **d** Representative activity traces in the elevated-plus maze task. Total arm entries (**c**) or total time spent (**d**) in the open and closed arms by NCX1–3$^{+/-}$ mice compared to WT mice ($n = 6$ in each group). **e** The numbers of marbles buried in the marble-burying task by NCX1–3$^{+/-}$ mice compared to WT mice ($n = 6$ in each group). **f** Latency times to enter the open compartment in the light-dark task by NCX1–3$^{+/-}$ mice compared to WT mice ($n = 6$ in each group). **g** Representative activity traces in the open-field task. Total time spent in the open compartment by NCX1–3$^{+/-}$ mice compared to WT mice ($n = 6$ in each group). Error bars indicate the SEM $*p < 0.05$; $**p < 0.01$ versus WT mice. NCXs, Na^+/Ca^{2+} exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$; WT, wild-type

NCX1$^{+/-}$ Mice Enhanced Cued Fear Memory and Increased c-Fos Expression in the LA

The Pavlovian fear conditioning task is the most common model of aversive memory in rodents, used to study fear-related behaviors based on both hippocampal-dependent contextual fear memory and amygdala-dependent tone (cued) fear memory [12]. We first examined the effect of contextual and cued fear memory in NCX1–3$^{+/-}$ mice compared to WT mice. NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice had significantly enhanced cued fear memory compared to WT mice (WT mice 75.6 ± 6.1 s, $n = 5$; NCX1$^{\pm}$ mice 135.2 ± 8.7 s, $n = 5$) (Fig. 2a). NCX2–3$^{+/-}$ mice also had significantly decreased contextual fear memory compared to WT mice (WT mice 72.8 ± 7.1 s, $n = 5$; NCX2$^{+/-}$ mice 42.4 ± 5.4 s, $n = 5$; NCX3$^{+/-}$ mice 43.6 ± 6.4 s, $n = 5$) (Fig. 2a).

We next performed immunohistochemical analyses of the protein expression levels of c-Fos as a marker of neural excitation in both the LA and the CeA. NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice showed significant increase in the number of c-Fos-positive cells in the LA but not in the CeA compared to WT mice at 1, 2, and 6 h after tone stimulation (NCX1$^{+/-}$ mice: 1 h 43.3 ± 1.7; 2 h 37.6 ± 1.7; 6 h 22.8 ± 3.2, $n = 8$ each) (Fig. 2c, d).

NCX$^{+/-}$ Mice Further Enhanced Long-term Potentiation in the LA

To confirm the role of synaptic plasticity in the LA, we examined LTP induced by high-frequency stimulation (HFS; 100 Hz, 2 trains) of the cortico-amygdala pathway in the LA. In WT mice, HFS caused a stable and long-lasting increase in fEPSPs (1 min 157.9 ± 3.1% of baseline; 60 min 133.9 ± 5.2% of baseline, $n = 5$), with LTP in the LA of NCX1$^{+/-}$ mice significantly further enhanced (1 min 179.9 ± 7.3% of baseline; 60 min 154.5 ± 6.0% of baseline, $n = 5$) (Fig. 3a–d).

NCX1$^{+/-}$ Mice Increased Autophosphorylation of CaMKIIα (Thr-286) and CaMKIV (Thr-196), GluA1 (Ser-831), and CREB (Ser-133) Phosphorylation After Tone Stimulation in the LA

To address the mechanisms underlying the LTP enhancement and aberrant behaviors related to cued fear memory in NCX1$^{+/-}$ mice, we tested autophosphorylation of CaMKIIα (Thr-286), CaMKIV (Thr-196) phosphorylation, and ERK (Thr-202/Tyr-204) phosphorylation in the LA extracts prepared before and

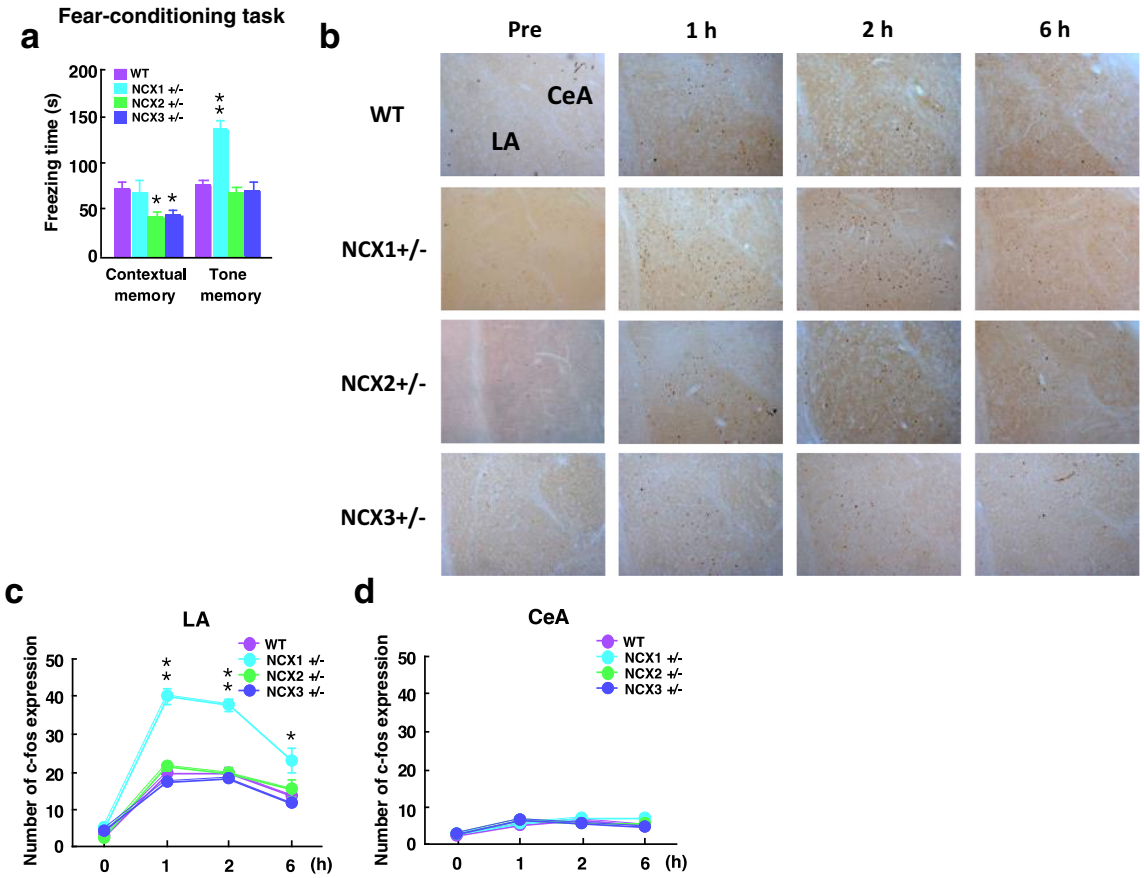

Fig. 2 NCX1$^{+/-}$ mice had enhanced cued fear memory and increased c-Fos expression in the lateral amygdala (LA). **a** Freezing times in contextual and tone-induced memory tests following Pavlovian fear conditioning. NCX1$^{+/-}$ mice showed significantly increased freezing times in the tone-induced, but not contextual-induced, fear memory compared to WT mice ($n = 5$ in each group). By contrast, NCX2–3$^{+/-}$ mice exhibited significantly decreased freezing times in the contextual-induced, but not tone-induced, fear memory compared to WT mice ($n = 5$ in each group). **b** Representative images of c-Fos immunostaining of the LA and central amygdala (CeA). **c**, **d** Expression levels of c-Fos in the LA (**c**) and CeA (**d**) at 1–6 h after tone stimulation ($n = 8$ in each group). Error bars indicate the SEM $^{*}p < 0.05$, $^{**}p < 0.01$ versus WT mice. NCXs, Na+/Ca2+ exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$; WT, wild-type

after tone stimulation in NCX1–3$^{+/-}$ mice compared to WT mice. Before tone stimulation, NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice exhibited slight but significant increase in CaMKIIα (Thr-286) autophosphorylation in the LA compared to WT mice (NCX1$^{+/-}$ mice 122.4 ± 2.9%, $n = 6$) (Fig. 4a, b). At 15 or 60 min after tone stimulation, the significantly further increased in NCX1$^{+/-}$ mice compared to WT mice (NCX1$^{+/-}$ mice 156.6 ± 1.8% (15 min); 152.7 ± 1.4% (60 min), $n = 6$ each) (Fig. 4a, b). In addition, NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice exhibited increased CaMKIV (Thr-196) phosphorylation at 60 min after tone stimulation compared to WT mice (NCX1$^{+/-}$ mice 169.0 ± 4.1%; $n = 6$ each), although it did increase in both compared to the pre-stimulation levels (Fig. 4a, c). By contrast, the difference in ERK (Thr-202/Tyr-204) phosphorylation in the LA was not significant between WT and NCX1–3$^{+/-}$ mice at 60 min after tone stimulation, although it did increase in both compared to the pre-stimulation levels (Fig. 4a, d). Similar with CaMKII autophosphorylation in the LA of NCX1$^{+/-}$ mice, NCX1$^{+/-}$, but not NCX2–3$^{+/-}$, mice exhibited slightly but significantly increased GluA1 (Ser-831) and CREB (Ser-133) phosphorylation in the LA compared to WT mice (GluA1 (Ser-831), NCX1$^{+/-}$ mice 126.7 ± 5.0%; CREB (Ser-133), NCX1$^{+/-}$ mice 123.3 ± 3.9%, $n = 6$ each) (Fig. 4a–g). CaMKII autophosphorylation in the LA of NCX1$^{+/-}$ mice closely paralleled phosphorylation of GluA1 (Ser-831) and CREB (Ser-133) as a downstream post-synaptic target of CaMKII (GluA1 (Ser-831): NCX1$^{+/-}$ mice 170.4 ± 5.5% (15 min), 158.2 ± 8.8% (60 min); CREB (Ser-133): NCX1$^{+/-}$ mice 130.4 ± 6.8% (15 min), 157.6 ± 8.4% (60 min), $n = 6$ each) but not synapsin I (Ser-603) as a downstream pre-synaptic target of CaMKII (Fig. 4a, e–g).

NCX1$^{+/-}$ Mice Increased the Protein and mRNA Levels of BDNF After Tone Stimulation in the LA

We further compared the protein and mRNA levels of BDNF, a major target of CREB transcriptional regulation, 60 min after tone stimulation in the LA between WT and NCX1–3$^{+/}$

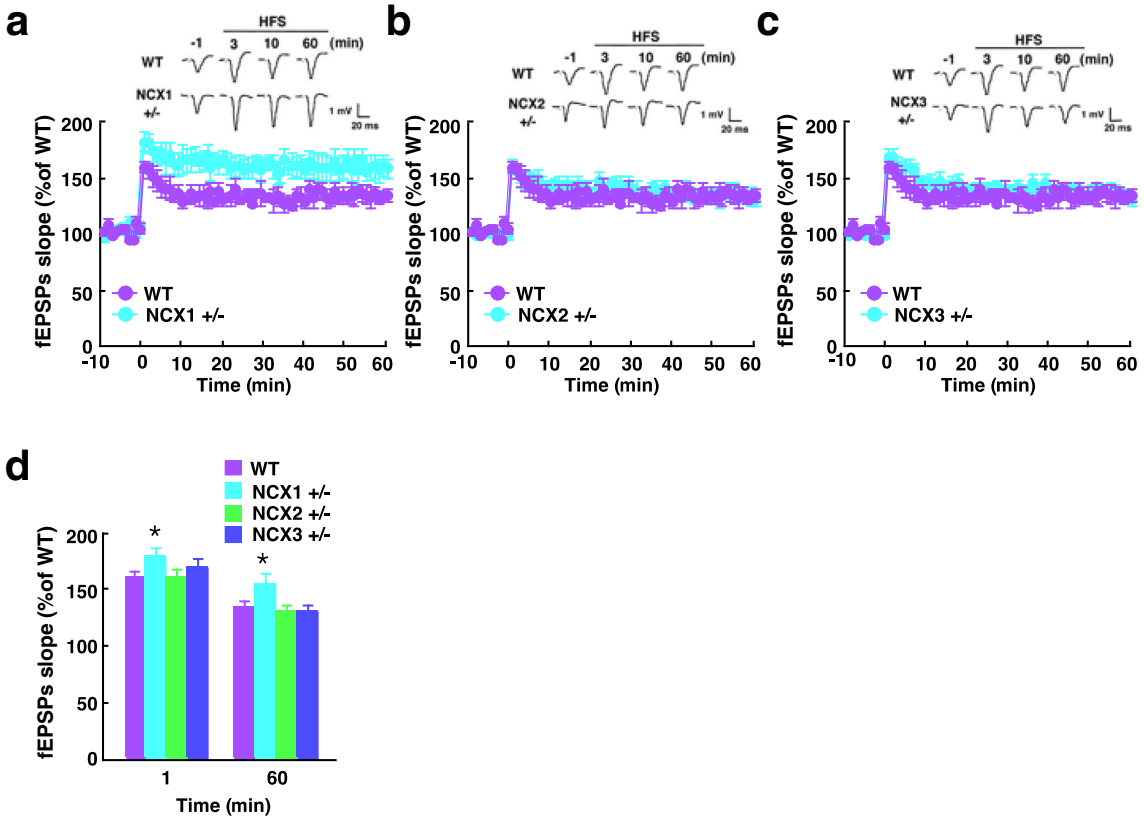

Fig. 3 NCX1$^{+/-}$ mice exhibit enhanced long-term potentiation (LTP) in the lateral amygdala (LA). **a**–**c** Changes in the fEPSP slope following high-frequency stimulation (HFS) of the LA were enhanced in NCX1$^{+/-}$ mice relative to WT mice. **d** Quantitative analyses of the fEPSP slopes shown in (**a**–**c**) at 1 min (post-tetanic potential [PTP]) and 60 min (LTP; $n = 5$ in each group). Error bars indicate the SEM $^{*}p < 0.05$ versus WT mice. fEPSP, field excitatory post-synaptic potential; LA: NCXs, Na^{+}/Ca^{2+} exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$; WT, wild-type

$^{-}$ mice. Because CaMKII and CaMKIV activities are required to induce mRNA levels of BDNF expression containing exon I and IV [38, 39]. Tone stimulation resulted in increased BDNF protein and mRNA levels in the LA of NCX1$^{+/-}$, but not of NCX2–3$^{+/-}$, mice compared to WT mice (protein 159.0 ± 1.6%, $n = 5$; exon I 175.1 ± 12.4%, $n = 9$; exon IV 170.27 ± 11.7%, $n = 9$; Fig. 5a–d).

Localization of NCX Proteins in Both Neurons and Glia Cells in the LA

We next performed immunohistochemistry to define the localization of the LA NCX1–3 proteins. All three NCX1–3 proteins were predominantly expressed in dendritic spines of the LA neurons, where they colocalized with the post-synaptic marker PSD-95 (Fig. 6a–c). In addition, both NCX1 and NCX2 proteins partially colocalized with the pre-synaptic marker synaptophysin in LA neurons (Fig. 6a–c). In contrast, although both NCX1 and NCX3 were expressed in the cell bodies of LA neurons, NCX2 was not observed in the cell bodies of LA neurons, where it colocalized with the cell body marker MAP2 (Fig. 6a–c). Moreover, NCX3 was only weakly expressed in GFAP-positive astrocytes of LA neurons (Fig. 6c).

CaMKIIα$^{+/-}$ Mice Lost Cued Fear Memory and Decreased c-Fos and BNDF Expressions in the LA

Since NCX1$^{+/-}$ mice showed increased CaMKII and CaMKIV activities after tone stimulation in the LA, we further confirmed the effect of cued fear memory in both CaMKIIα$^{+/-}$ and CaMKIV$^{-/-}$ mice compared to WT mice. CaMKIIα$^{+/-}$, but not CaMKIV$^{-/-}$, mice lost cued fear memory after tone stimulation compared to WT mice (CaMKIIα$^{+/-}$ mice 31.9 ± 3.3 s, $n = 6$) (Fig. 7a). Similarly to the behavioral analysis, CaMKIIα$^{+/-}$, but not CaMKIV$^{-/-}$, mice had significantly decreased number of c-Fos-positive cells in the LA compared to WT mice at 1 h after tone stimulation (CaMKIIα$^{+/-}$ mice 9.7 ± 1.2, $n = 6$) (Fig. 7b, c). Finally, we analyzed the protein levels of BDNF in the LA of both CaMKIIα$^{+/-}$ and CaMKIV$^{-/-}$ mice compared with WT mice. CaMKIIα$^{+/-}$, but not CaMKIV$^{-/-}$, mice had significantly decreased BDNF protein levels in the LA 1 h after tone stimulation compared to WT mice ($n = 6$) (Fig. 7d, e).

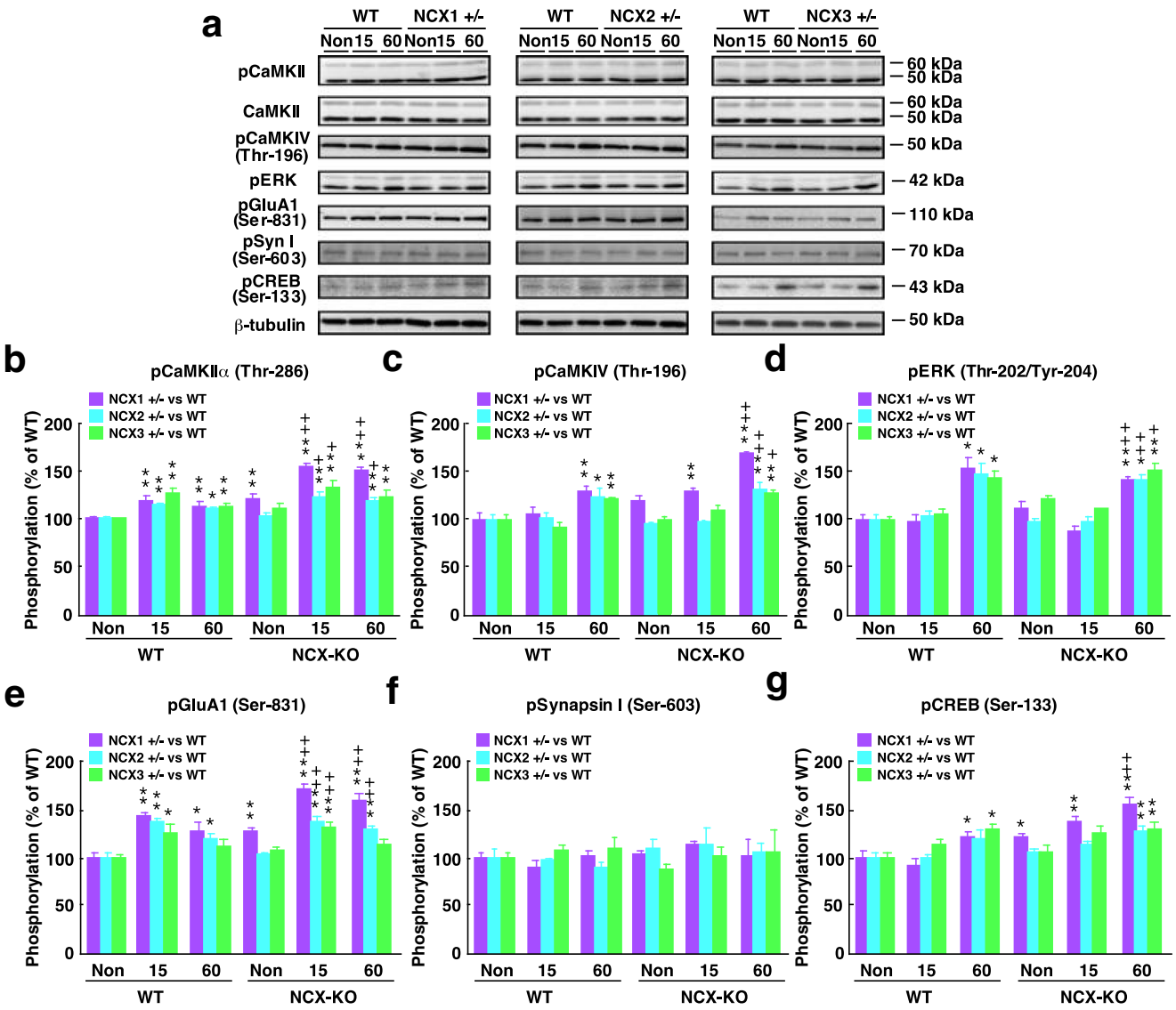

Fig. 4 NCX1$^{+/-}$ mice exhibit increased Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV activities in the lateral amygdala (LA) at 15 min and 60 min after tone stimulation. **a** Representative images of immunoblots probed with antibodies against autophosphorylated CaMKIIα (Thr-286), CaMKII, phosphorylated CaMKIV (Thr-196), CaMKIV, phosphorylated ERK (Thr-202/Tyr-204), phosphorylated GluA1 (Ser-831), phosphorylated Synapsin I (Ser-603), phosphorylated CREB (Ser-133), and β-tubulin. **b** Quantitative analyses of the data shown in (**a**) ($n = 6$ in each group). Error bars indicate the SEM $*p < 0.05$; $**p < 0.01$ versus WT mice. $+p < 0.05$; $++p < 0.01$ versus NCX1–3$^{+/-}$ mice at matching time points after tone stimulation. ERK, extracellular-signal-regulated kinase; WT, wild-type

Discussion

Observations reported here strongly suggest that NCX1$^{+/-}$ mice exhibit abnormality in amygdala-dependent cued fear memory via increased CaMKII activity in the LA (Fig. 8). Maren and Fanselow [40] previously reported that lesions in the nucleus of the LA cause aberrant fear memory. In addition, cued fear memory evoked an increase in synaptic strength in the LA based on LTP-like changes following fear conditioning [14], and HFS-evoked LTP in the LA underlies the acquisition and consolidation of fear memory [15, 41]. In the present study, we also observed that LTP in the cortico-LA pathway was markedly enhanced in NCX1$^{+/-}$ mice compared to WT mice. These results showed that regulation of NCX1 protein is critical for amygdala-dependent cued fear memory and synaptic plasticity via CaMKII activity in the LA.

By contrast, NCX2$^{+/-}$ and NCX3$^{+/-}$ mice also exhibited decreased hippocampal-dependent contextual fear memory (Fig. 1). In line with these results, we previously reported that NCX2$^{+/-}$ and NCX3$^{+/-}$, but not NCX1$^{+/-}$, mice showed impaired hippocampal LTP and memory-related behaviors via dysregulated hippocampal CaMKII activity [26]. In the hippocampus, all NCX proteins (1–3) partially localized with both the pre-synaptic terminal and post-synaptic density, and

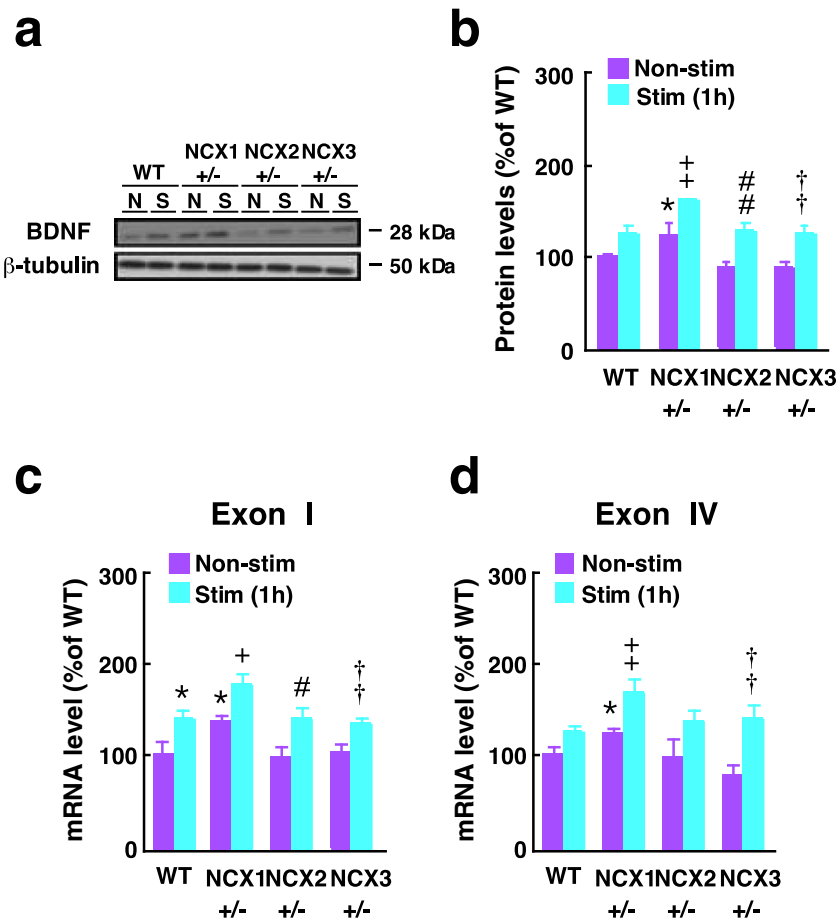

Fig. 5 NCX1$^{+/-}$ mice exhibit increased BDNF expression levels in the lateral amygdala (LA) at 1 h after tone stimulation. **a** Representative images of immunoblots probed with antibodies against BDNF and β-tubulin. **b** Quantitative analyses of the data shown in (**a**) ($n = 4$ in each group). **c**–**d** Quantitative analyses of BDNF mRNA containing exons I (**c**) and IV (**d**) ($n = 9$ in each group). Error bars indicate the SEM *$p < 0.05$ versus WT mice; +$p < 0.05$; ++$p < 0.01$ versus NCX1$^{+/-}$ mice; #$p < 0.05$; ##$p < 0.01$ versus NCX2$^{+/-}$ mice; ††$p < 0.01$ versus NCX3$^{+/-}$ mice at matching time points after tone stimulation. BDNF, brain-derived neurotrophic factor; NCXs, Na^+/Ca^{2+} exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$

there was no difference in the localization of NCX proteins (1–3). In fact, NCX2$^{+/-}$ mice exhibited decreased synaptic density, whereas the ratio of perforated synapses and the size of spines increased in NCX3$^{+/-}$ mice [26]. In the present

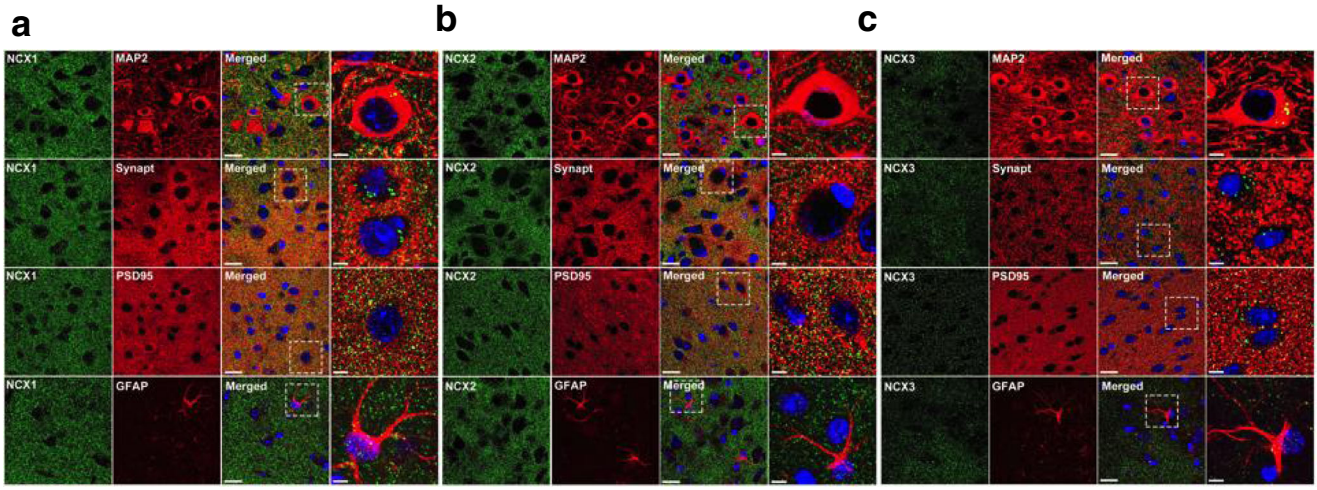

Fig. 6 NCX1, but not NCX2–3, predominantly localizes in lateral amygdala (LA) neurons. **a**–**c** Confocal microscopy images showing double-staining of LA slices for NCX1 (**a**), NCX2 (**b**), or NCX3 (**c**) (green); MAP2, synaptophysin, PSD95 or GFAP (red); and merged images. All three NCX proteins (1–3) were predominantly expressed in dendritic spines of LA neurons (colocalized with PSD95). In addition, both NCX1 and NCX2 partially colocalized with the pre-synaptic terminals (colocalized with synaptophysin). By contrast, although both NCX1 and NCX3 were expressed in the cell bodies of LA neurons, NCX2 is not observed in the cell bodies of LA neurons (colocalized with MAP2). Moreover, NCX3 was only weakly expressed in GFAP-positive astrocyte of LA neurons. Scale bars, 20 μm at low magnification and 5 μm in high-magnification images. NCXs. Na^+/Ca^{2+} exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$

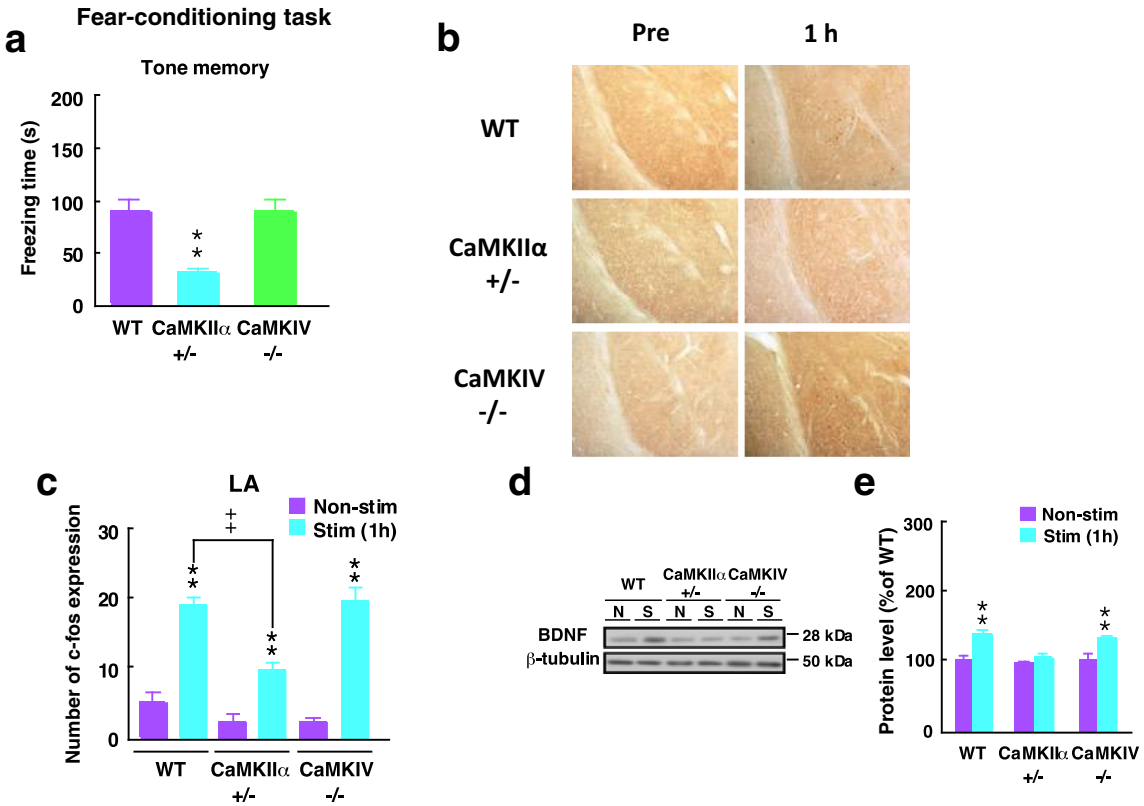

Fig. 7 CaMKIIα$^{+/-}$ mice lost cued fear memory and had increased c-Fos and BDNF expression in the lateral amygdala (LA). **a** Freezing times in tone-induced memory tests following Pavlovian fear conditioning. CaMKIIα$^{+/-}$ mice, but not CaMKIV$^{-/-}$ mice, showed significantly decreased freezing times in tone-induced fear memory compared to WT mice ($n = 6$ in each group). **b** Representative images of c-Fos immunostaining of the LA and central amygdala (CeA). **c** Expression levels of c-Fos in the LA at 1 h after tone stimulation ($n = 6$ in each group). CaMKIIα$^{+/-}$, but not CaMKIV$^{-/-}$, mice showed significantly decreased c-Fos expression levels in the LA at 1 h after tone stimulation. **d** Representative images of immunoblots probed with antibodies against BDNF and β-tubulin. **e** Quantitative analyses of the data shown in (**d**) ($n = 6$ in each group). Error bars indicate the SEM $^{**}p < 0.01$ versus WT mice. CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIV, calcium/calmodulin-dependent protein kinase IV; WT, wild-type

study, NCX proteins (1–3) exhibited different localization in the LA. In particular, the NCX1 protein predominantly localized in both the pre-synaptic terminal and post-synaptic density, whereas the NCX2 and NCX3 proteins partly disappeared from the pre-synaptic terminal or astrocytes of LA neurons. Thus, the differences in the localization of NCX proteins (1–3) are associated with amygdala-dependent cued fear memory.

In previous reports of key signaling mechanisms for LTP induction in the cortico-LA pathways, LTP induction in the LA enhanced post-synaptic Ca^{2+} mobilization via both the N-methyl-D-aspartate receptor and L-type Ca^{2+} channel in the post-synaptic density [42, 43]. We found that the expression of aberrant amygdala-dependent cued fear memory and enhanced synaptic plasticity in the LA were associated with increased CaMKIIα (Thr-286) autophosphorylation in the LA of NCX1$^{+/-}$ mice. Chen et al. [44] reported that CaMKIIα mutant mice showed attenuated fear memory, whereas Hasegawa et al. [45] also reported that overexpressing CaMKIIα transgenic mice exhibited enhanced anxiety-like behaviors. In the preset study, we also observed that CaMKIIα$^{+/-}$ mice lost cued fear memory and had decreased c-Fos and BDNF expression levels in the LA (Fig. 7). We previously reported that NCX2$^{+/-}$ mice exhibit memory deficits via decreased CaMKII and increased calcineurin (CaN) activities in the hippocampus [26]. By contrast, NCX3$^{+/-}$ mice also exhibit memory deficits via increased CaMKII activity. However, CaN activity did not change in the hippocampus [26]. Thus, we speculate that the levels of CaN activity in the LA may be critical for the increased CaMKII activity in the LA of NCX1$^{+/-}$ mice.

In contrast, we also found that CaMKIV (Thr-196) phosphorylation was increased after tone stimulation in the LA of NCX1$^{+/-}$ mice (Fig. 4). Ho et al. [46] reported that CaMKIV$^{-/-}$ mice showed impaired hippocampal LTP through CREB (Ser-133) phosphorylation in CA1. However, Takao et al. [31] reported that CaMKIV$^{-/-}$ mice exhibited normal contextual or cured fear memory. Similarly to previous reports, CaMKIV$^{-/-}$ mice showed normal cured fear memory and c-Fos and BDNF expression levels in the LA (Fig. 7). In addition, ERK (Thr-202/Tyr-204) phosphorylation also regulates CREB (Ser-133) phosphorylation [47]; however, ERK (Thr-

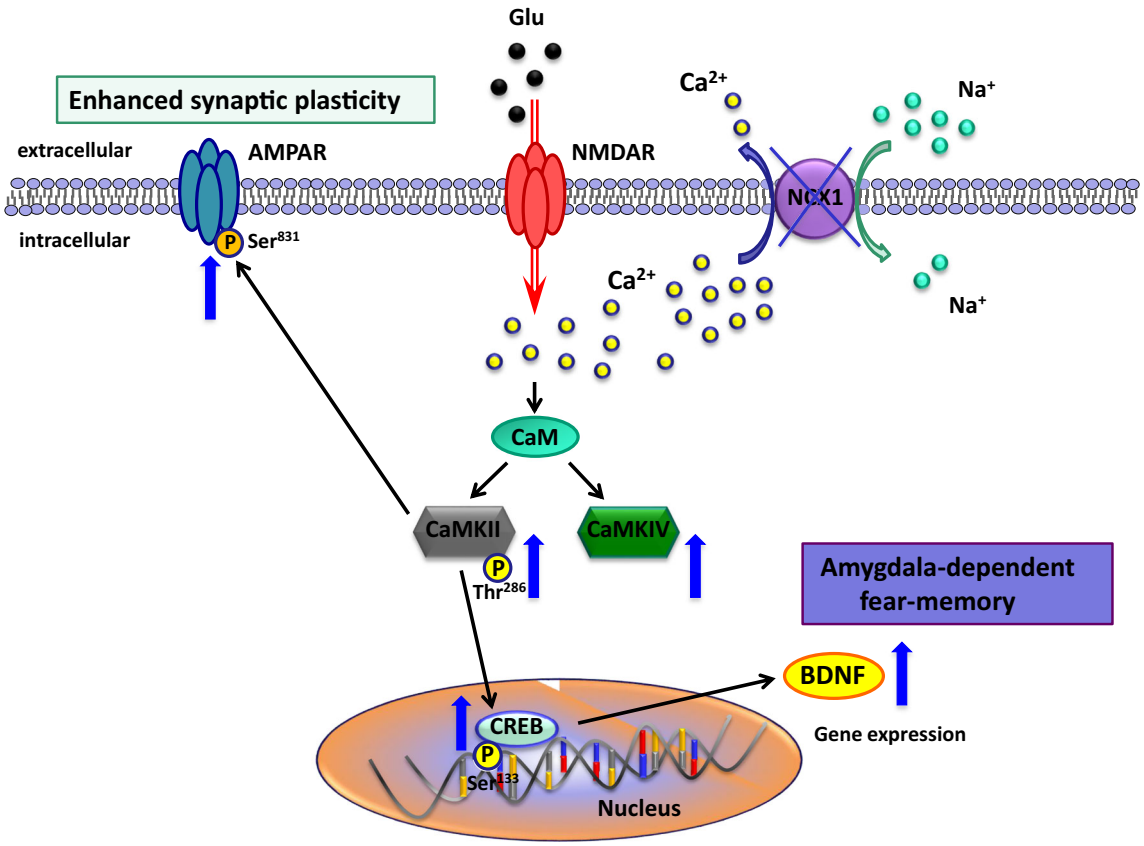

Fig. 8 The proposed model of the mechanisms underlying aberrant cued fear memory in NCX1$^{+/-}$ mice. CaMKII activity enhanced in the LA of NCX1$^{+/-}$ mice. As a result, CREB (Ser-133) phosphorylation and BDNF expression increased. In addition, CaMKII upregulation increased GluA1 (Ser-831) phosphorylation. Upregulation of CaMKII activity promoted the injured cued fear memory seen in NCX1$^{+/-}$ mice. BDNF, brain-derived neurotrophic factor; CeA, central amygdala; CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CREB, cAMP-responsive element binding protein; NCXs, Na^+/Ca^{2+} exchangers; NCX1–3 heterozygous, NCX1–3$^{+/-}$

202/Tyr-204) phosphorylation was unchanged in the LA of NCX1$^{+/-}$ mice (Fig. 4). These results suggest that both CaMKIV (Thr-196) and ERK (Thr-202/Tyr-204) phosphorylation after tone stimulation is not concomitant with abnormal cued fear memory in NCX1$^{+/-}$ mice. By contrast, Huang et al. previously reported that protein kinase A (PKA) is critical for the expression of a persistent phase of LTP via CREB (Ser-133) phosphorylation in the LA [48]. Thus, other protein kinases such as PKA are also important for CREB (Ser-133) phosphorylation in the LA.

Interestingly, we confirmed increased CREB (Ser-133) phosphorylation and BDNF mRNA or protein levels after tone stimulation in the LA of NCX1$^{+/-}$ mice (Figs. 4 and 5). BDNF gene expression is regulated by CREB phosphorylation [49], and BDNF plays a critical role in the acquisition and consolidation of amygdala-dependent fear memory [50, 51]. In fact, BDNF heterozygous null mice show impaired fear memory and LTP in the cortico-LA synapses [52]. In addition, BNDF is critical for the regulation of synaptic strength and plasticity in the LA [53–55]. Furthermore, the expression levels of BDNF increased in the amygdala of cued fear memory [56, 57]. Recently, we demonstrated that CaMKII is required for CREB (Ser-133) phosphorylation and BDNF expression in the CA1 of the hippocampus in CaMKIV$^{-/-}$ mice [58]. Moreover, protein kinase A was also essential for CREB (Ser-133) phosphorylation [59]. These data indicate that CaMKII is partially essential for CREB activity and BDNF expression in the LA of NCX1$^{+/-}$ mice.

Recently, Sokolow et al. [60] reported that protein and mRNA levels of NCX3 significantly decreased in the brain of patients with Alzheimer's disease (AD). We previously reported that protein or mRNA expression levels of NCX proteins (2–3) decreased in the CA1 of both APP23 and APP-KI mice [26]. Thus, NCX2$^{+/-}$ and NCX3$^{+/-}$ mice exhibited cognitive deficits resembling the cardinal symptoms of AD and NCX1$^{+/-}$ mice showed impaired cued fear memory and anxiety-like behaviors resembling behavioral and psychological symptoms of dementia in the present study. We suggest that dysfunction of NCX proteins is associated with the pathological mechanism of AD.

In conclusion, NCX1$^{+/-}$ mice exhibit enhanced amygdala-dependent cued fear memory. This enhancement is mediated

by increased synaptic plasticity in the LA. Increased CREB/BDNF pathway stimulated by CaMKII activation, which in turn results in the changes in synaptic plasticity observed in $NCX1^{+/-}$ mice.

Acknowledgements We would like to thank Profs. Issei Komuro and Tsuyoshi Miyakawa for providing the $NCX1^{+/-}$ and $CaMKII\alpha^{+/-}$ mice, respectively.

Authors' Contribution S.M., R.I., Y.Y., Y.S., and S.I. performed the experiments. S.K. and T.I. generated the $NCX2^{+/-}$ and $NCX3^{+/-}$ mouse lines and NCX antibodies, and provided experimental advice. S.H. provided the $CaMKIV^{-/-}$ mice. S.M. and K.F. wrote the manuscript and designed the study.

Funding Information This work financially was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health and Welfare of Japan (KAKENHI 22390109 to K.F., 20790398 to S.M., 16K08565 to S.K., 17K08610 to T.I.), the Smoking Research Foundation (to S.M.), and Mochida Memorial Foundation for Medical and Pharmaceutical Research (to S.M), and Takeda Science Foundation (S.M.).

Compliance with Ethical Standards

Ethical Approval All animal protocols were approved by the Committee of Animal Experiments at Tohoku University.

Conflict of Interest The authors declare that they have no competing interests.

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