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Research Paper

# Nitric Oxide-induced Activation of the Type 1 Ryanodine Receptor Is Critical for Epileptic Seizure-induced Neuronal Cell Death

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ABSTRACT

Status epilepticus (SE) is a life-threatening emergency that can cause neurodegeneration with debilitating neurological disorders. However, the mechanism by which convulsive SE results in neurodegeneration is not fully understood. It has been shown that epileptic seizures produce markedly increased levels of nitric oxide (NO) in the brain, and that NO induces $Ca^{2+}$ release from the endoplasmic reticulum *via* the type 1 ryanodine receptor (RyR1), which occurs through *S*-nitrosylation of the intracellular $Ca^{2+}$ release channel. Here, we show that through genetic silencing of NO-induced activation of the RyR1 intracellular $Ca^{2+}$ release channel, neurons were rescued from seizure-dependent cell death. Furthermore, dantrolene, an inhibitor of RyR1, was protective against neurodegeneration caused by SE. These results demonstrate that NO-induced $Ca^{2+}$ release *via* RyR is involved in SE-induced neurodegeneration, and provide a rationale for the use of RyR1 inhibitors for the prevention of brain damage following SE.



## 1. Introduction

Epilepsy is caused by a wide variety of insults to the brain as well as by congenital abnormalities in ionic channels (Berkovic et al., 2006; Jentsch et al., 2004; Meisler and Kearney, 2005). Status epilepticus (SE) is a neurological emergency and results in brain damage that increases the risk of recurrent seizures and debilitating neuronal abnormalities including death (Chang and Lowenstein, 2003; Goldberg and Coulter, 2013). Its high morbidity and mortality makes SE one of the most significant neurological disorders in terms of high social costs (Betjemann and Lowenstein, 2015). Therefore, understanding of the pathophysiology of SE-induced brain damage is required for the treatment of the neurological emergency. During epileptic seizures, NO formation has been observed to increase as a result of $Ca^{2+}$-dependent activation of NO synthase (NOS) in neurons (Mülsch et al., 1994). This increase in NO levels has been implicated in seizure-induced neuronal cell loss based on the finding that neurodegeneration is attenuated in neuronal NOS (nNOS)-deficient mice (Parathath et al., 2007). Thus NO is strongly implicated as a neurodegenerative factor in epileptic states. However, the molecular mechanisms by which NO exerts its role in SE-induced neurodegeneration requires clarification. Thus, rational treatment for the prevention of brain damage associated with SE has yet to be explored.

In addition to the cyclic guanosine monophosphate-dependent protein kinase pathway, NO is known to regulate the function of target proteins through *S*-nitrosylation of cysteine residues (Hess et al., 2005; Jaffrey et al., 2001). One such target is the type 1 ryanodine receptor (RyR1), which is the $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) channel in the endoplasmic reticulum (ER). NO induces the opening of the RyR1 channel through *S*-nitrosylation (Aghdasi et al., 1997; Eu et al., 2000; Sun et al., 2001). The activity of RyR1 due to this activation mechanism has been implicated in $Ca^{2+}$ leakage from skeletal muscle $Ca^{2+}$ stores that has been attributed to certain pathological conditions (Bellinger et al., 2009; Durham et al., 2008). A single cysteine residue at 3635 (C3635)

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in rabbit RyR1 is responsible for sensitizing the skeletal muscle $Ca^{2+}$ release channel to NO (Sun et al., 2001). An alanine substitution for C3635 (C3635A) of RyR1 expressed in human embryonic kidney (HEK) 293 cells has been shown to reduce *S*-nitrosylation levels and abolish the regulation of the skeletal muscle $Ca^{2+}$-release channel by physiological concentrations of NO (Sun et al., 2001). In the brain, NO induces $Ca^{2+}$ release from the ER through *S*-nitrosylation of RyR1, which results in an increased concentration of intracellular $Ca^{2+}$ in neurons (Kakizawa et al., 2012). $Ca^{2+}$ release *via* RyR1 has also been implicated in NO-induced neuronal cell death, as shown by studies in which cell death is significantly milder in cultured neurons taken from RyR1-deficient mice than in controls (Kakizawa et al., 2012). These studies raise the possibility that NO-induced $Ca^{2+}$ release (NICR) is involved in certain pathological states in the brain; however, the pathophysiological role of NICR remains to be established. Furthermore, pathophysiological significance of NICR *in vivo* has not been examined.

In this study, we examined whether NICR *via S*-nitrosylated RyR1 is involved in SE-induced neurodegeneration. In order to study the role of NICR *in vivo*, we generated a knock-in mouse line, in which the essential cysteine residue at 3636 of mouse RyR1 (corresponding to cysteine 3635 in humans and rabbits) was replaced by alanine (*Ryr1*$^{C3636A}$) to prevent its *S*-nitrosylation. We show that NICR was indeed silenced in neurons from *Ryr1*$^{C3636A}$ knock-in mice, which allowed us to examine the role of NICR in a kainic acid (KA)-model of temporal lobe epilepsy. Here we provide evidence that NICR exacerbates neurodegeneration in the hippocampus following KA-induced seizures, suggesting that RyR1 is a promising therapeutic target candidate to ameliorate the neurodegenerative effect of SE.

## 2. Materials and Methods

### 2.1. Chemicals

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), D(+)-glucose, ethanol, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), magnesium chloride, *N*-ethylmaleimide (NEM), Nonidet P 40 (NP-40), potassium permanganate, sodium chloride, sodium dodecylsulfate (SDS) and tris(hydroxymethyl)aminomethane were purchased from Nacalai Tesque (Kyoto, Japan). Calcium chloride dehydrates, dantrolene sodium salt, L-glutamic acid, β-mercaptoethanol, neocuproine and TritonX-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, acetone, caffeine, dithiothreitol (DTT) and potassium chloride were purchased from Wako Pure Chemicals (Osaka, Japan). Calcein-AM, 4′-6-diamidino-2-phenylindole (DAPI) solution, ethylenediaminetetraacetic acid (EDTA), Hoechst33342, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7), 3-morpholinosydnonimine (SIN-1), were purchased from Dojindo (Kumamoto, Japan). Kainic acid (KA), (5*S*,10*R*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) and *N*-methyl-D-aspartate (NMDA) were purchased from Tocris Bioscience (Bristol, UK).

### 2.2. Animals

All animal-related procedures were in accordance with the guidelines of the University of Tokyo. Mice were housed in a transparent plastic cage, fed with food and water *ad libitum*, and kept under controlled lighting conditions (12 h-light/12 h-dark) in specific pathogen-free conditions in the University of Tokyo animal facility. The maximum number of adult mice in a cage was 7. The *Ryr1*$^{+/C3636A}$ knock-in mouse was generated at UNITECH (Kashiwa, Japan). Briefly, the targeting vector contained genomic DNA encompassing exon 74 of the mouse *Ryr1* gene with a nucleotide change encoding for the mutation at codon 3636 (TGT → GCT, C3636A). The targeting vector was transfected into embryonic stem (ES) cells, and targeted ES clones were screened and confirmed by Southern analysis. The correctly targeted ES cells were injected into blastocysts to generate chimeric mice. Mice carrying the targeted allele were crossed with Tg-Cre transgenic mice to remove the floxed neomycin cassette and produce heterozygous *Ryr1*$^{+/C3636A}$ mice. Crossing of heterozygous *Ryr1*$^{+/C3636A}$ mice yielded homozygous *Ryr1*$^{C3636A/C3636A}$ mice, designated as *Ryr1*$^{C3636A}$ mice.

The primer sequences for genotyping were as follows: forward, 5′-GCTTAAGGACTGGACATAGAGCTAA-3′; and reverse, 5′-CTGAATATGTGGATATGGGTATAGG-3′. PCR was conducted using *Thermococcus kodakaraensis* (KOD-FX) DNA polymerase (Toyobo, Osaka, Japan) with the following amplification cycle: 94 °C for 1 min followed by 40 cycles of 94 °C for 10 s and 68 °C for 1 min. The 471 and/or 361 bp bands were detected by 2.5% (*w*/*v*) agarose gel electrophoresis stained with ethidium bromide.

### 2.3. Preparation of Cerebral Neuronal Culture

Neurons were prepared from the cerebral cortices of mice fetuses (postnatal day 0) based on a modification of a previously described procedure (Kakizawa et al., 2012; Kanemaru et al., 2007). Briefly, minced cerebral cortices were treated with 1.0% (*w*/*v*) trypsin and 0.1% (*w*/*v*) Deoxyribonulease I (Sigma-Aldrich) in $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline (PBS) (Takara, Shiga, Japan) for 5 min at room temperature (RT). Cells were washed with Neurobasal-A medium supplemented with 5% (*v*/v) fetal bovine serum (FBS), penicillin (100 units $mL^{-1}$), streptomycin (100 units $mL^{-1}$), B-27 supplement, and 2 mM L-glutamine (Gibco, ThermoFisher Scientific, Grand Island, NY, USA) and dissociated by triturating with a fire-polished Pasteur pipette in $Ca^{2+}$/$Mg^{2+}$-free PBS containing 0.05% (*w*/*v*) Deoxyribonulease I and 0.03% (*w*/*v*) trypsin inhibitor (Sigma-Aldrich). Dispersed cells were plated at $1.0 \times 10^5$ cells $cm^{-2}$ on glass slide coated with poly-L-lysine and laminin (Sigma-Aldrich). Cells were then cultured at 37 °C under a humidified atmosphere containing 5% $CO_2$. The medium was changed every 2 d by replacing half of the old medium with fresh FBS-free medium. Cells cultured for 7–10 d were used for experiments.

### 2.4. Preparation of Skeletal Muscle Primary Culture

Primary cultured myoblasts from newborn mice were prepared based on a modification of a previously described procedure (Rando and Blau, 1994). Briefly, the forelimbs and hindlimbs were removed and bones dissected away. The muscle was cut into small fragments and enzymatically dissociated with collagenase (from *Clostridium histolyticum*, 2.5 mg $mL^{-1}$, Wako Pure Chemicals) at 37 °C for 15 min. The fragments were passed through 40-μm cell strainer and the suspension subjected to low-speed centrifugation. The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (*v*/v) FBS, penicillin (100 units $mL^{-1}$), streptomycin (100 units $mL^{-1}$), 2 mM L-glutamine and 10 ng $mL^{-1}$ recombinant human fibroblast growth factor (FGF)-basic (Gibco). Myoblasts were differentiated into myotubes with DMEM containing 2% horse serum.

### 2.5. Intracellular $Ca^{2+}$ Imaging

$Ca^{2+}$ imaging was carried out based on a modification of the previously described procedure (Kakizawa et al., 2012). Briefly, neurons and myocytes were loaded with 5 μM Fura-2 acetoxymethyl ester (Fura-2AM) (Molecular Probes, ThermoFisher Scientific, Eugene, OR, USA) for 30 min in HEPES-buffered saline (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5.6 mM glucose, pH adjusted to 7.4 with NaOH). Fluorescence images were acquired at 510 nm using an inverted microscope (IX81, Olympus, Tokyo, Japan) with a UApo/340 40× (numerical aperture (NA) 1.35; Olympus) and a cooled CCD camera (EM-CCD C9100, Hamamatsu Photonics, Shizuoka, Japan) at a rate of one frame every 3 s. Excitation wavelengths were 340 and

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