Neuroscience 313 (2016) 110–121

ANALYSIS OF THE PROTECTIVE EFFECTS OF A NEURONAL Cav2.1 CALCIUM CHANNEL IN BRAIN INJURY

T. Y. KIM, T. YOSHIMOTO, Y. AOYAMA, K. NIIMI AND E. TAKAHASHI*

Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Abstract—We previously reported that *rolling Nagoya* mice carrying a mutation in the α_1 subunit of the Cav2.1 channel protective from ischemia- and kainate-induced neuronal damage. However, the protective effect of this mutation and its relationship to brain injury recovery have not been examined. To examine the relationship between Cav2.1 channel function and brain injury, we induced cryogenic brain damage in homozygous *rolling Nagoya* (*rol/rol*), control wild-type (+/+), ω-agatoxin IVA-pretreated +/+ (ω-aga +/+), and ω-agatoxin IVA-post-treated +/+ (ω-aga-post-treated +/+) mice. We measured the lesion area, blood brain–barrier permeability and performed immunohistochemistry and western blot analysis. The lesions of *rol/rol* and ω-aga +/+ mice were significantly smaller than those observed in +/+ mice at both day 1 and day 7 after injury. Similar results were shown in blood–brain barrier permeability. We observed more reactive astrogliosis in +/+ mice than in *rol/rol* or ω-aga +/+ mice. *rol/rol* and ω-aga +/+ mice had fewer degenerating cells due to cryogenic injury than did +/+ mice at both day 1 and day 7. ω-Aga-post-treated +/+ mice 24 h after injury were sacrificed on day 7. The lesions were smaller in ω-aga-post-treated +/+ mice than those in vehicle-treated +/+ mice. We also examined phosphorylated p38 (pp38) at the injured site. ω-Aga-post-treated +/+ mouse brain slices showed weak pp38 signal; vehicle-treated +/+ mouse brain slices were pp38-positive. These findings demonstrate that the mutant Cav2.1 channel exerts a protective effect against cryogenic brain injury in *rolling Nagoya* mice. Our results indicate that inhibitors of the Cav2.1-dependent p38 signaling cascade would be useful as therapeutic agents in the treatment of brain injury.

Key words: Cav2.1 channel, cryogenic brain injury, ω-agatoxin IVA, rolling Nagoya mice, phosphorylated p38.

INTRODUCTION

Neuronal voltage-gated Ca^{2+} channels (VGCCs), including Cav2.1 (P/Q-type), Cav2.2 (N-type), and Cav2.3 (R-type) channels, are located on presynaptic terminals. These channels mediate a number of neuronal functions, including neuronal excitation, neurite outgrowth, synaptogenesis, neurotransmitter release, neuronal survival, differentiation and plasticity, and regulate gene expression (Evans and Zamponi, 2006; Jarvis and Zamponi, 2007; Catterall and Few, 2008; Simms and Zamponi, 2014).

VGCCs are comprised of α_1, β, $\alpha_2\delta$, and γ subunits. The α_1 subunit forms an ion-conducting pore, which contains a voltage sensor and drug/toxin binding sites, and contributes to elevated intracellular Ca^{2+} concentration (Rama et al., 2014). Subunit composition determines the calcium channel subtype (Catterall et al., 2005). Thus, alterations in the Ca^{2+} current through the α_1 subunit likely induce neuronal and circuit dysfunction.

Mutations in the Cav2.1α1 subunit (Cav2.1α1) gene have been found in humans and mice (Pietrobon, 2005). The *rolling Nagoya* mouse has a mutation in Cav2.1α1. Ca^{2+} current amplitude of homozygous *rolling Nagoya* mutants (*rol/rol*) is 40% lower than that of wild-type controls (Mori et al., 2000). *rol/rol* mice are characterized by a severe ataxic gait (Oda, 1973). Heterozygous *rolling Nagoya* (*rol/+*) mice exhibit altered emotional behavior (Takahashi et al., 2011) and learning deficiency (Takahashi and Niimi, 2009; Takahashi et al., 2009, 2010). We also reported that the Cav2.1α1 mutation in *rolling Nagoya* mice had a protective effect against ischemia- and kainate-induced neuronal death. In ischemic neuronal injury, *rol/rol* mice had a smaller infarct area compared with that of +/+ mice (Tian et al., 2013). In another study, kainate-injected *rol/+* mice displayed less severe seizures and fewer degenerating cells compared with +/+ mice (Kim et al., 2014). These results suggest that the Cav2.1α1 mutation results in a protective effect against neuronal damage. However, the role of the Cav2.1 channel in brain injury has not been examined.

Regardless of age, brain injury is a serious health concern following falls or motor vehicle- or sports-related accidents. VGCC inhibitors have been considered potential therapeutic agents (Gurkoff et al., 2013). Administration of Cav1 channel and Cav2.2 channel antagonists after brain injury reduced cell death and improved cellular function. The use of Verapamil, a Cav1 channel antagonist, resulted in significant amelioration of cortical injuries. Another Cav1 channel antagonist,

*Corresponding author. Tel: +81-48-467-5871; fax: +81-48-467-9692.
E-mail address: etakahashi@brain.riken.jp (E. Takahashi).
Abbreviations: ω-aga, ω-agatoxin; BBB, blood–brain barrier; EB, Evans Blue; IHC, immunohistochemistry; pp38, phosphorylated p38; VGCCs, voltage-gated Ca^{2+} channels.

http://dx.doi.org/10.1016/j.neuroscience.2015.11.035
0306-4522/

nimodipine, resulted in improved initial clinical trial outcomes. Ziconotide, the synthetic version of a Cav2.2 channel antagonist, also exerted a neuroprotective effect (Gurkoff et al., 2013). However, the therapeutic effects of Cav2.1 channel antagonists have not been studied. ω-Agatoxin IVA (ω-aga) is a toxin isolated from the venom of the funnel web spider, *Agelenopsis aperta*, and has been used as a Cav2.1 channel antagonist (Mintz et al., 1992). It is a dose-dependent gating modifier, as it induces a channel activation shift toward more depolarized potentials (Ogura et al., 1998; Pringos et al., 2011). ω-Aga is highly selective for the Cav2.1 channel and works in a dose-dependent manner; therefore, it is important for research on Cav2.1 channels (Pringos et al., 2011). In this study, we used a cryogenic method to model brain injury, which can be induced experimentally (Murakami et al., 1999; Nag, 2002). Mechanical damage by cryogenic injury breaks down the blood–brain barrier (BBB), increases tight junction permeability, in addition to excitotoxicity, involving astrocytes and neurons (Murakami et al., 1999; Lozano et al., 2015). Evans Blue (EB) was used to evaluate changes in BBB permeability, as it leaks through damaged BBB (Murakami et al., 1997; Kakinuma et al., 1998). Abnormal release of neurotransmitters, including glutamate, induces excitotoxicity, which is independent of BBB integrity (Unterberg et al., 2004).

In the present study, we aimed to investigate the role of Cav2.1 channel in cryogenic brain injury. Thus, we examined cryogenic injury-induced cortical brain damage in *rol/rol*, wild-type (+/+), and ω-aga-pretreated +/+ (ω-aga +/+) mice. We also investig ated the possible therapeutic role of the Cav2.1 channel in brain injury by post treating +/+ mice with ω-aga one day after the cryogenic procedure.

EXPERIMENTAL PROCEDURES

Animals

This research was conducted in accordance with the Declaration of Helsinki and was approved by the Animal Experiments Committee of the RIKEN Brain Science Institute. All animals were cared for and treated humanely in accordance with the Institutional Guidelines for Experiments using Animals (Approval ID: No. H26-2-204). Twelve-week-old male *rol/rol* and twelve-week-old male +/+ mice with a C57BL/6 genetic background (Tian et al., 2013) were used. Ten *rol/rol*, ω-aga +/+, +/+, ω-aga-post-treated +/+ and vehicle-treated +/+ mice were used in each experiment. All the mice were housed in groups of three under environmental conditions of 21 ± 2 °C, humidity of 55 ± 10%, lighting of 350 lux (at bench level) and a 12:12 light:dark cycle with lights on at 0800 h and off at 2000 h. Animals were housed in microisolation cages (MBS7115RHMV, 19.1 × 29.2 × 12.7 cm, Allentown, NJ, USA) with bedding (TEK-FRESH, Harlan Teklad, Madison, WI, USA) and given access to irradiated food (5058 PicoLab Mouse Diet 20; LabDiet, MO, USA) and filtered water *ad libitum*. The health status of animals was monitored twice daily.

Cryogenic model preparation

Animals were anesthetized with a mixture of medetomidine, midazolam, and butorphanol dissolved in saline (0.3 mg/kg body weight [b.w.] medetomidine, 4.0 mg/kg b.w. midazoram, 5.0 mg/kg b.w. butorphanol, intraperitoneal) (Kirihara et al., 2013). An incision was made along the midline of the scalp to expose the skull. Cortical lesions were produced using a liquid nitrogen-cooled metal rod (tip diameter: 2 mm) placed on the left parietal bone (coordinates from the bregma: 1.5 mm posterior, 1.5 mm lateral) for 30 s. The skin incision was closed with a surgical clip. The mice were sacrificed on day 1 or day 7 after cryogenic injury, similar to a previously published protocol (Shi et al., 2012).

Determination of lesion size

Lesion size was measured by 2% 2,3,5-triphenyltetrazolium chloride staining (TTC; Sigma–Aldrich), as described previously (Raslan et al., 2010). Deeply anesthetized mice were intracardially perfused with 0.9% NaCl. The brain was removed and cooled in ice-cold saline for 5 min. The damaged brain area was then dissected into 1-mm-thick coronal slices using a brain matrix (Stainless Steel Brain Matrix; Harvard Apparatus, MA, USA). Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride in phosphate-buffered saline (PBS) at 37 °C for 30 min and stored in 10% neutral-buffered formalin for visualization. Since the damaged brain is fragile, we also considered torn-off areas as damaged areas. Lesion size was measured using ImageJ v1.48 software.

Determination of BBB permeability

The extravasation level of EB was measured by fluorophotospectrometry. EB solution (2% in saline, 4 mg/kg) was administered intraperitoneally after cryogenic injury, and on day 6. After 24 h, mice were transcardially perfused, and the damaged cortex areas were removed, homogenized in 1 mL 50% trichloroacetic acid (TCA), and centrifuged as described previously (Wang et al., 2014). The supernatant was collected and absorbance was measured spectrofluorometrically at 600 nm with a Du 730 Life Science Spectrophotometer (Beckman Coulter, CA, USA). EB leakage of each sample was quantified using a standard curve and expressed as μg per gram of wet tissue. The whole brain was photographed with a digital camera (Tough TG-610; Olympus, Tokyo, Japan). The damaged brain area was then dissected into 1-mm-thick coronal slices using a brain matrix (Stainless Steel Brain Matrix; Harvard Apparatus, MA, USA).

Drug application

ω-Aga (Smartox Biotechnology, Saint-Martin-d'Hères, France) was dissolved in AfCS solution (1× phosphate-buffered saline, 0,1% bovine serum albumin). Under anesthesia, 2 μL of 1 ng/μL ω-aga solution or vehicle (AfCS solution) was injected into the lateral ventricle 20 min before the cryogenic injury procedure