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Research article

Differential expression of ryanodine receptor isoforms after spinal cord injury

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ABSTRACT

Ryanodine receptors (RyRs) are highly conductive intracellular Ca^{2+} release channels and are widely expressed in many tissues, including the central nervous system. RyRs have been implicated in intracellular Ca^{2+} overload which can drive secondary damage following traumatic injury to the spinal cord (SCI), but the spatiotemporal expression of the three isoforms of RyRs (RyR1-3) after SCI remains unknown. Here, we analyzed the gene and protein expression of RyR isoforms in the murine lumbar dorsal root ganglion (DRG) and the spinal cord lesion site at 1, 2 and 7 d after a mild contusion SCI. Quantitative RT PCR analysis revealed that RyR3 was significantly increased in lumbar DRGs and at the lesion site at 1 and 2 d post contusion compared to sham (laminectomy only) controls. Additionally, RyR2 expression was increased at 1 d post injury within the lesion site. RyR2 and -3 protein expression was localized to lumbar DRG neurons and their spinal projections within the lesion site acutely after SCI. In contrast, RyR1 expression within the DRG and lesion site remained unaltered following trauma. Our study shows that SCI initiates acute differential expression of RyR isoforms in DRG and spinal cord.

1. Introduction

Spinal cord injury (SCI) inflicts damage to ascending and descending axons and thereby contributes to neurological impairment. Although the precise mechanisms of secondary axonal injury are not completely understood, intra-axonal Ca^{2+} overload is thought to play a major role [1–3]. Extracellular sources of Ca^{2+} influx in axons include voltage-gated Ca^{2+} [4–7], transmitter-operated channels [8–11], reversal of Na^{+}-Ca^{2+} exchange [12], and diffusion through mechanopores [13]. However, most attempts to target extracellular-mediated Ca^{2+} entry in animal models of SCI [14–18] and clinical trials have failed [19]. Alternatively, other sources of pathological Ca^{2+} release may play an important role in axonal loss following SCI, as has been shown for ischemic axons [7].

Indeed, major intracellular sources of Ca^{2+} implicated in axonal injury include mitochondria [20,21], and the ER (or axoplasmic reticulum in axons) [7,22–24]. With regards to the latter, Ca^{2+} can be mobilized into the cytoplasm from intracellular Ca^{2+} stores by two main types of receptors through differential signaling pathways: RyRs and inositol 1,4,5-trisphosphate receptor (IP_3R). RyR exist in three isoforms in mammals (RyR1-3) and RyR1 and RyR2 are well known for their role in mobilizing Ca^{2+} during excitation-contraction coupling in skeletal and cardiac muscle respectively [25,26]. All three RyR isoforms are differentially expressed in the CNS and have been implicated in diverse functions such as synaptic plasticity, vesicle fusion and synaptic release of transmitters, pain, and growth cone dynamics [27–31].

Although it is well established that Ca^{2+} toxicity occurs in neurons in several pathological disorders, and Ca^{2+} release from intracellular stores is clearly involved in these processes [32–35,22,23,36,37], little is known about the role and potential expression changes of the different RyRs isoforms after SCI. One purpose of this study was to establish whether different time points after injury result in a differential transcriptional response of RyRs. Accordingly, in the present work we examined the gene and protein expression of RyRs in DRGs and spinal cord of mice at 1, 2 and 7 d after a contusion-induced SCI.

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2. Materials and methods

2.1. General animal care and surgical procedures

All animal procedures were performed in strict accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, *Guide for the Care and Use of Laboratory Animals* 8th edition [45], and with the approval of the University of Louisville Institutional Animal Care and Use Committee. Adult 6–8 week old heterozygous *Cx3cr1*$^{GFP/+}$: *Thy1*$^{YFP+}$ double transgenic mice bred in house to visualize microglia and axons simultaneously were used for all experiments. For surgeries, mice were anesthetized with ketamine/xylazine (100 mg/kg, Hospira Inc., Lake Forest, IL and 10 mg/kg, Akorn Inc., Lake Forest, IL, respectively). Body temperature was maintained at 37 °C throughout the surgeries that typically lasted 20–30 min. Artificial tear ointment (Akorn Inc.) was used to protect the eyes during surgery. After skin incision and muscle separation, a laminectomy at T13 was performed to expose the spinal cord followed by a mild 30 kdyne contusion injury using the Infinite Horizon Impactor Device (Precision Systems and Instrumentation, LLC, Lexington, KY). After producing the contusion, the wound was closed in layers and the skin incision was stapled. Following surgery, mice were given lactated ringer's solution (2cc, sc) to replenish electrolytes and fluids, and gentamycin (Gentafuse; 0.1 ml, sc., every day for 5 days) to prevent infection. Mice were monitored daily and housed together throughout the experiment. All surgical procedures except for the SCI were as described above for the laminectomy-only (sham) mice. All assessments in SCI as well as sham animals were also compared to naïve mice (no surgical procedure was applied except for the extraction of DRGs and spinal cord at the established time points).

2.2. RT-qPCR analysis

For tissue collection, mice were euthanized with an overdose of ketamine/xylazine. Four to six DRGs from the lumbar region (L4-6) were removed bilaterally, pooled together, and placed in 100% Trizol Reagent (Thermo Fisher Scientific, Waltham, MA) overnight at −80 °C. Then approximately 0.5 cm of the spinal cord (centered at the lesion site) was also placed in 100% Trizol. For sham and naïve groups, spinal cord from the lumbar region (using the dorsal roots as reference) was also collected. Tissue was homogenized on ice in 0.5 mL Trizol and RNA was isolated using Trizol/chloroform extraction method as previously described [38]. RNA concentration and purity was determined using a Nanodrop 2000 (Thermo Fisher Scientific), and 500 ng of RNA was reverse transcribed using High capacity Reverse Transcriptase Kit (Applied Biosystems). All cDNA were diluted 50X with DEPC- treated water before using as a template for Quantitative PCR (q-PCR). cDNA were added to Taqman Gene Expression master mix (Thermo Fisher Scientific) along with Taqman primers (IDT) for ryanodine receptor 1, 2 and 3 (with FAM fluorophore) separately, duplexed with GAPDH (with HEX fluorophore) as internal control. Q-PCR was performed using Corbett Rotor Gene 6000 (Qiagen). Finally, ryanodine receptors and GAPDH expression levels were analyzed using ΔΔct method ($2^{-\Delta\Delta ct}$). Primer sequences for the genes analyzed are as follows:

RyR1: CTGAGCTGAATGAATACAACGC (forward primer 5′-3′); CCATGAGCCTTTCTAGCACTG (reverse primer 5′-3′),

RyR2: GGTGGATGTGGAAAAGTGGA (forward primer 5′-3′); CTGTAGGAATGGCGTAGCAA (reverse primer 5′-3′);

RyR3: ACCCACTTCACCAGATCATTC (forward primer 5′-3′); TCTTCTTCATCCTCACCACTCT (reverse primer 5′-3′);

GAPDH: AATGGTGAAGGTCGGTGTG (forward primer 5′-3′); GTGGAGTCATACTGGAACATGTAG (reverse primer 5′-3′).

2.3. Immunofluorescence and microscopy

At 1, 2, and 7 d after SCI, sham and naïve mice (n = 3 naive; n = 4 sham and SCI) were euthanized as above, and perfused with ice-cold 1x PBS, followed by perfusion fixation with 4% paraformaldehyde (PFA) in PBS. The spinal cords and lumbar DRGs (L4-6) were isolated and post-fixed in 4% PFA overnight, and subsequently cryoprotected in 30% sucrose solution. DRG and spinal cords were embedded in OCT compound (Sakura Finetek, Japan), frozen, and cryosectioned at a thickness of 25 μm for spinal cord and 14 μm for DRGs using a cryostat (Leica CM3050S; Leica Biosystems Inc., Buffalo Grove, IL) and collected on superfrost slides. For staining, representative sections from the spinal cord injury site and lumbar DRGs caudal to the lesion site (L4-6) from injured or control mice were thawed at room temperature, washed in TBS, and blocked (1x Tris-buffered saline, 0.5% bovine serum albumin, 0.1% Triton and 5% normal goat serum), and incubated with the following antibodies overnight at 4 °C: RyR2 (1:500, SAB4502707, rabbit anti-ryanodine receptor 2, (Sigma-Aldrich, St. Louis, MO)) and RyR3 (1:4000, AB9082, rabbit Anti-Ryanodine receptor 3, (Millipore, Temecula, CA)). Slides were then washed in TBS and incubated with secondary antibody (1:5000, A-21245, Alexa Fluor 647 goat anti-Rabbit IgG (Thermo Fisher)) for 1 h at room temperature. Slides were then washed and mounted with cover glass (Warner Instruments, Hamden, CT). Primary antibody specificity was assessed using sections from cardiac muscle and cerebellum for RyR2 and RyR3 respectively (data not shown), western blot images provided by the distributor, and peer-reviewed publications. Secondary antibody specificity was determined by omitting the primary antibody and revealed no nonspecific signal (data not shown). Detection controls, i.e. the inclusion of tissue sections without label, were used to rule out endogenous signal such as autofluorescence using identical microscope settings and confirmed with a spectral detector. As expected no spectral overlap was detected between YFP and Alexa647 using appropriate emission filters. Images were captured using a Nikon A1R Multiphoton microscope with spectral and confocal capabilities equipped with a digital camera.

3. Statistics

All treatment groups were compared and graphs produced using Microsoft Excel. Differences between multiple groups were analyzed by one-way ANOVA test followed by the Tukey's method. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the StatPlus software (version 6, AnalystSoft Inc., Walnut, CA).

4. Results

4.1. Differential RyR isoform gene expression within the DRG following SCI

RyR2 protein expression has been shown to increase at 1, 14 and 28 d at the lesion site following SCI [34]. However, whether SCI induces changes in protein and gene expression of RyR1 and -3 and the cell types that express RyRs after SCI remains largely unknown. We therefore first investigated transcriptional responses of RyRs in DRG neurons that project their axons within the dorsal columns of the spinal cord and are thereby directly injured following contusion SCI. RyR3 expression increased approximately 8- and 4-fold in lumbar DRGs caudal to the lesion site at 1 and 2 d post contusion in comparison to sham (laminectomy only) and naïve controls (n = 4 per treatment group, $P < 0.01$) (Fig. 1). At 7 d, the increase was only significant when compared to naïve mice. RyR2 expression was significantly increased at 1 d after injury versus naïve group (n = 4 per treatment group, $P < 0.05$). In contrast, no differences in RyR1 expression were detected between injured and control animals from 1 d to 7 d after SCI (Fig. 1).

4.2. Differential RyR isoform expression within the lesion site following SCI

We next examined expression of the RyR isoforms in the lesion site

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