Direct Ryanodine Receptor-2 Knockout in Primary Afferent Fibers Modestly Affects Neurological Recovery after Contusive Spinal Cord Injury

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Abstract

Neuronal ryanodine receptors (RyR) release calcium from internal stores and play a key role in synaptic plasticity, learning, and memory. Dysregulation of RyR function contributes to neurodegeneration and negatively impacts neurological recovery after spinal cord injury (SCI). However, the individual role of RyR isoforms and the underlying mechanisms remain poorly understood. To determine whether RyR2 plays a direct role in axonal fate and functional recovery after SCI, we bred Advillin-Cre: tdTomato (Ai9) reporter mice with “floxed” RyR2 mice to directly knock out (KO) RyR2 function in dorsal root ganglion neurons and their spinal projections. Adult 6- to 8-week-old RyR2KO and littermate controls were subjected to a contusive SCI and their dorsal column axons were imaged in vivo using two-photon excitation microscopy. We found that direct RyR2KO in dorsal column primary afferents did not significantly alter secondary axonal degeneration after SCI. We next assessed behavioral recovery after SCI and found that direct RyR2KO in primary afferents worsened open-field locomotor scores (Basso Mouse Scale subscore) compared to littermate controls. However, both TreadScan™ gait analysis and overground kinematic gait analysis tests revealed subtle, but no fundamental, differences in gait patterns between the two groups after SCI. Subsequent removal of spared afferent fibers using a dorsal column crush revealed similar outcomes in both groups. Analysis of primary afferents at the lumbar (L3–L5) level similarly revealed no noticeable differences between groups. Together, our results support a modest contribution of dorsal column primary afferent RyR2 in neurological recovery after SCI.

Keywords: axon degeneration; ryanodine receptor; secondary degeneration; spinal cord injury
Introduction

Neurological recovery after spinal cord injury (SCI) is dependent on the location and extent of mechanical impact, delayed loss of spared tissue that succumbs to secondary degeneration, and neuroplasticity of remaining circuitry. Known mediators of secondary degeneration include vascular changes, neuroinflammation, edema, biochemical changes, and loss of ionic homeostasis. With regard to the latter, it is well known that pathological Ca\(^{2+}\) overload in white matter contributes to secondary degeneration; however, clinically viable and efficacious approaches to prevent Ca\(^{2+}\) overload remain elusive.

Several extracellular routes of Ca\(^{2+}\) influx in axons have been implicated in secondary degeneration, including voltage-gated Ca\(^{2+}\) channels, reversal of Na\(^{+}/Ca\(^{2+}\) exchange, ionotropic and metabotropic glutamate receptors, and mechanopore formation. Despite initial promising results, targeting many of the above “outside-in” sources of Ca\(^{2+}\) have proven to be ineffective in animal models of SCI and after a clinical trial.

We and others have shown that “inside-out” sources of Ca\(^{2+}\), that is, those that reside within the axon, can also contribute to axonal injury. In support, the axoplasmic reticulum, the major Ca\(^{2+}\) store in axons, releases Ca\(^{2+}\) through ryanodine receptors (RyR) and IP\(_3\)R\(_1\), and both have been implicated in white matter injury. RyR are Ca\(^{2+}\) release channels that amplify cytosolic Ca\(^{2+}\) levels by releasing Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores and are expressed in most tissues, including the central nervous system (CNS). There are three known isoforms (RyR1, -2, and -3), and RyR1 and -2 mediate excitation-contraction coupling in skeletal and cardiac muscle, respectively. All three RyR isoforms are expressed in the CNS and are localized to neuronal soma, axons, dendrites, and spines and glial cells. Loss-of-function studies have implicated RyRs in synaptic plasticity, learning and memory, pain, and neurodegeneration.

Globally targeting RyR has been shown to be axo-protective after traumatic and ischemic injury to spinal cord white matter ex vivo and after contusive SCI in vivo. RyR2 and RyR3 messenger RNA are elevated in dorsal root ganglions (DRGs) and at the lesion site, as well as localized to dystrophic axons and end-bulbs acutely after SCI. Further, RyR2 protein expression has been shown to increase at 1, 14, and 28 days at the lesion site post-SCI. Gain-of-function mutations of RyR2 worsen secondary axonal degeneration after SCI and systemic knockdown of RyR2 improves recovery after SCI, suggesting that RyR2 plays an important role in SCI pathophysiology. However, the relative contributions of each RyR isoform and whether they play a direct role in axonal pathology after SCI remain unknown.

To determine whether RyR2 plays a direct role in secondary axonal degeneration after SCI, we generated RyR2 knockout (KO) mice directly in sensory neurons by breeding “floxed” RyR2 KO mice with Advillin-Cre: tdTomato+ reporter mice (Avil-Cre:tdTom\(^{0/0}\),Ryr2\(^{0/0}\)). Mice received a contusive SCI and intravital microscopy was used to observe dynamic changes in tdTomato axons in real time, and behavioral tests were performed to assess functional recovery. We report that direct RyR2 KO in DRG neurons did not prevent secondary axonal degeneration of the dorsal column projecting axons. Although RyR2 KO in primary afferents worsened functional recovery, as assessed using Basso Mouse Scale, detailed gait analysis using TreadScan™ and overground kinematics revealed that their overall gait patterns were not different.

Methods

Subjects

All experiments were approved by the University of Louisville Institutional Animal Care Committee, adhering to the Guide for the Care and Use of Laboratory Animals. Adult 6- to 8-week-old male and female Advillin (Avil)-Cre+:(Ai9) tdTom\(^{0/0}\),Ryr2\(^{0/0}\) and Avil-Cre−:tdTom\(^{0/0}\),Ryr2\(^{0/0}\) transgenic mice were established and bred in-house. The Avil-Cre−:tdTom\(^{0/0}\) reporter mouse line was obtained from Dr. Jeffrey C. Petruska, and the Ryr2\(^{0/0}\) mouse line was a kind gift from Dr. S.R. Wayne Chen. Both strains have been previously used and described in detail here. Because of the Advillin promoter driving Cre recombinase, tdTomato (strain #007909; The Jackson Laboratory, Bar Harbor, ME) is expressed and RyR2 knocked out only in Advillin-expressing cells. In the context of thoracic SCI, only DRG neurons that project their axons to and within the spinal cord will be tdTomato positive and lack functional RyR2. Genotyping confirmed the presence or absence of Cre, Ai9 (tdTomato), and Ryr2 floxed alleles in offspring.

A total of 32 adult 6- to 8-week-old male and female mice (16 Ryr2KO and 16 wild-type [WT] mice) were used in this study and equally divided between the
in vivo imaging and behavioral experiments. Two RyR2 WT mice were excluded from the imaging study because of damage to the spinal cord during laminectomy and baseline imaging. Two RyR2 KO mice were excluded from the 7-day imaging sessions because of damage to the spinal cord while removing scar tissue that formed over the laminectomy site between the 1- and 7-day imaging sessions. Two mice were eliminated from the behavior study; 1 mouse did not recover from the contusive SCI surgery, and 1 mouse died before the 1-week behavioral testing, possibly because of bladder complications.

Surgery
Mice were anesthetized with isoflurane and received saline and buprenorphine to manage pain. Body temperature was maintained at 37°C using a heating pad. Soothe lubricant eye ointment (Valeant Pharmaceuticals North America LLC, Bridgewater, NJ) was used to protect the eyes during surgery. A custom spinal cord clamp was used to firmly hold the vertebral column as previously described. A laminectomy was performed at T12 to allow for baseline recordings of tdTomato-positive axons within the superficial dorsal columns, and then mice received a 30-kdyn contusive SCI (Infinite Horizon Impactor Device, Precision Systems and Instrumentation LLC, Lexington, KY) with a dwell time of 0 sec. The location and mild injury for imaging experiments was chosen to limit breathing and motion artifacts and allow enough surviving dorsal column axons to assess secondary axonal degeneration respectively.

Mice were then repositioned under the microscope to collect four-dimensional two-photon excitation (2PE) images at 1 and 7 days after injury. Post-surgery care included lactated Ringer’s solution (2 cc, subcutaneous [s.c.]) given within 20 min after surgery, and gentamycin (Gentafuse; 5 mg/kg, s.c.) given within 20 min after surgery, and once-daily thereafter for 5 days to prevent infection. Mice also received buprenorphine once before surgery, and twice per day for 48 h to manage pain. After the 7-day image was collected, animals were euthanized, perfused with PBS followed by 4% PFA, and spinal cords fixed in 4% PFA overnight.

Microscopy
An A1·MP+ multi-photon confocal microscope (Nikon Instruments Inc., Melville, NY), equipped with a 25×1.1 numerical-aperture water immersion lens, was used for 2PE imaging of the spinal cord in vivo and images collected using Nikon Elements software. For the in vivo imaging experiments, the laser was tuned to 950 nm to excite tdTomato and 2PE images were collected at baseline and at the epicenter of the lesion site at 1 and 7 days after injury.

For assessment of post-mortem expression patterns of spinal tdTomato-positive fibers, confocal microscopy, using a 561-nm single-photon laser source, was used to capture tdTomato-positive afferent fiber expression patterns from transverse spinal cord sections that were collected at the lumbar level (L3–L5) of the spinal cord after the behavioral studies. These images were subsequently denoised to remove the shot-noise component from images using Denoise.ai on Nikon’s Nis-Elements Advanced Research imaging software.

Image analysis
All images were analyzed using the imaging processing software, ImageJ or Fuji (National Institutes of Health, Bethesda, MD), by experimenters’ blind to genotype. For the in vivo imaging study, four-dimensional imaging data sets were collected, and axonal survival was quantified by drawing a perpendicular line (ImageJ; NIH) between the dorsal roots and dorsal vein at the epicenter of the lesion. All axons that touched the line were counted and divided by the length of the line and normalized to the pre-injury baseline axonal count. Axon survival was calculated as fractional
survival. \( N = 6–8 \) per group was assessed, and mean ± standard deviation (SD) is reported (Fig. 2). Axonal spheroids were identified as axonal swellings with a diameter >3 times the mean axonal diameter at baseline. Retraction (End) bulbs were further identified if they had an axon connected only at one end. Spheroid and end-bulb counts were normalized to the visible area of the image to allow comparison between animals and quantified per mm². \( N = 6–8 \) animals per group were assessed, and mean ± SD is reported (Fig. 2).

**Basso Mouse Scale and Basso Mouse Scale subscore**

Hindlimb locomotor scores were assessed using the BMS, a 10-point scale ranging from 0 to 9 (no limb movement to normal locomotor function). Assessments were performed at pre-injury, 1 day, 3 days, and weekly post-injury for 6 weeks and then again 2 weeks post-dorsal crush by two trained observers who were blind to genotype. BMS subscores were also calculated when BMS hindlimb assessments reached a score of ≥5 as detailed here.\(^{40}\)

**TreadScan gait analysis**

Treadmill walking was assessed (Single Lane Gait Analysis Treadmill; Columbus Instruments, Columbus, OH) at pre-injury baseline, post-injury weekly for 6 weeks, and 2 weeks post-dorsal crush, as previously described.\(^{41}\) Briefly, as animals walked on the treadmill belt, a high-speed digital video camera recorded each pass from the ventral view. Digital video images were recorded at 100 frames/s and analyzed using TreadScan (Clever Sys Inc, Reston, VA). Acceptable passes included walking in the middle of the treadmill, stepping with few lateral deviations, and without pauses.

**Overground kinematics**

Hindlimb kinematic analysis was performed as described previously.\(^{42–44}\) Animals walked freely inside a Plexiglass walkway (length × width × height: 155 × 10 × 24 cm) while high-speed (100 frames/s) videos were acquired from a ventral view using custom NI LabVIEW Software. Videos were then digitized using MaxTRAQ software (Innovision Systems, Colombiaville, MI), as previously described,\(^{42,43}\) by digitizing each paw as it was in contact with the ground for each pass. Coordinates from MaxTRAQ were then analyzed using a custom MS Excel add-in,\(^{42,43}\) which calculates all the gait measures. A minimum of three passes were analyzed that met the following criteria: continuous walking that started before the first step in the video; a relatively straight trajectory without hesitations; and was visually representative of animals’ overall walking capabilities based on BMS scores.

**Horizontal ladder**

The horizontal ladder test was performed pre-injury and biweekly (weeks 2, 4, and 6 post-injury and 2 weeks post-dorsal crush), using the Columbus Instruments Sensor and RS-232 Mini Counter (2.5 mm rungs spaced 3.5 cm apart; Columbus Instruments, Columbus, OH). Briefly, each animal underwent five stepping trials per session and an autocounter quantified the total number of footfalls. Only animals with a higher degree of weight-supported stepping ability were tested on the ladder post-injury (i.e., a bilateral BMS scores of at least 5 equaling frequent or consistent plantar stepping on both the left and right sides). Acceptable crossings could be obtained from either direction and were defined as those performed at a steady pace in which there was little or no hesitation or stopping and limbs were not dragged on the rungs. The five trials were averaged for each animal.

**Euthanasia, tissue processing, and image analysis**

After the final imaging session and final behavior testing, mice were given pentobarbital for euthanasia and transcardially perfused with PBS, then 4% PFA. The entire brain and spinal cord were dissected and post-fixed in 4% PFA. Spinal cords were then transferred to 30% sucrose for cryoprotection for several days, and then the L1, L2, and L3–L5 segments were isolated, embedded in Tissue-Tek® O.C.T. compound, and rapidly frozen on dry ice. Transverse 20-µm sections were collected on SuperFrost™ slides for assessment of primary afferent projections and terminations within the gray matter. Images of L3–L5 sections from both groups were imaged and visually compared (blind to treatment) for notable differences in termination patterns within the intermediate gray zone and ventral horns \( N = 7 \) per group).

**Statistical analysis**

Statistical analysis for all imaging experiments were performed using SigmaPlot software (Version 11; Systat Software Inc., San Jose, CA). We determined previously that an \( n = 6 \) per group was sufficient to determine a significant difference in secondary degeneration.\(^ {10,14} \) For axonal survival, axonal spheroids, and end-bulbs, data are presented as mean ± SD, and group differences were assessed using a \( t \)-test. For the
behavioral assessment groups, we determined that a group size of 8 was sufficient to achieve a desired power of 0.80, with a difference in means of 1.5, SD of 1.0, and alpha 0.05. Behavioral experimental data were analyzed using a repeated-measures analysis of variance (ANOVA) followed by Bonferroni’s post hoc t-tests. Kinematic TreadScan and over-ground (except regularity index [RI] and coordinated pattern index [CPI]) outcome measures represent percentage of baseline values. Differences with a p value <0.05 were considered significant. Behavior assessments were analyzed using SPSS (v25; SPSS, Inc., Chicago, IL).

**Results**

To determine a direct role for RyR2 in primary afferent secondary axonal degeneration and contributions to functional recovery after SCI, we generated a Ryr2 loss-of-function transgenic mouse line. Mice bearing Ryr2 “floxed” alleles were crossed with mice expressing Cre recombinase under the Advillin promoter and floxed Ai9 tdTomato reporter mouse line (Avil-Cre:Ai9fl/fl). The resulting offspring were genotyped and Avil-Cre:Ai9fl/fl:Ryr2KO mice and WT littermate controls (Avil-Cre:Ai9fl/fl:Ryr2wt/wt) were used for all subsequent experiments. Thus, in the context of SCI, RyR2 KO is restricted to Advillin-expressing

**FIG. 1.** Effects of direct RyR2 KO in primary afferents on secondary axonal degeneration after contusive SCI. Representative maximum intensity projections of gracile fasciculus axons from advil-Cre:tdtomfl/fl:Ryr2wt/wt (referred to as RyR2WT; panels A–C) and advil-Cre:tdtomfl/fl:Ryr20/0 (referred to as RyR2KO; panels D–F) before and after a mild 30-kdyn T12 contusive SCI. All images were recorded in vivo using two-photon excitation microscopy. Axons (tdTomato+; gray) are shown in all images at baseline (A,D,G), 24 h (B,E,H,I), and 7 days after SCI (C,F). In baseline conditions, axons appear normal and linearly arranged with few signs of degeneration (A,D,G) regardless of genotype. In contrast, at 24 h after SCI, axonal spheroids (defined as axonal swellings with a diameter ≥3 times the mean axonal diameter at baseline; arrows) and end-bulbs (axonal swellings with diameters ≥3 times than their parent axon but had an axon connected at only one end; arrowheads) are present and represent the major axonal pathology at this time. By 7 days after injury, fewer axonal spheroids and end-bulbs are present regardless of genotype (C,F). Higher magnification images of axonal spheroids (arrows) and end-bulbs (arrowheads) are shown for RyR2WT mice (H; dashed rectangle region from panel B) and RyR2KO mice (I; dashed rectangle region from panel E), revealing very similar amounts of axonal degeneration between the groups. Scale bar: 100 µm in panels A–F; 20 µm in panels G–I. KO, knockout; SCI, spinal cord injury; WT, wild type.
DRG neurons and their primary afferent axons, which terminate throughout the spinal cord gray matter and form the ascending dorsal columns.37,45

Direct RyR2 knockout in dorsal column axons does not prevent axonal spheroid and end-bulb formation after contusive spinal cord injury

As shown in Figure 1, in vivo imaging of superficial tdTomato⁺ dorsal column fibers revealed their linear arrangement without any apparent signs of axonal injury (i.e., axonal spheroids or end-bulbs) in baseline (laminectomy-only) conditions (Fig. 1A,D,G). In addition, there was no significant difference (t-test, \( t = -0.56, df = 12, p = 0.59 \)) in the number of axonal spheroids between RyR2KO and WT littermate controls in baseline conditions (9.34 ± 12.51, mean ± SD; RyR2KO, \( n = 8 \) vs. 14.31 ± 18.92, RyR2WT, \( n = 6 \)). Similarly, there was no significant difference (t-test, \( t = 0.15, df = 12, p = 0.89 \)) in the number of axonal end-bulbs between groups in baseline conditions (2.67 ± 4.96, mean ± SD; RyR2KO, \( n = 8 \) vs. 2.32 ± 3.30, RyR2WT, \( n = 6 \)). Importantly, these data reveal that the laminectomy to expose the spinal cord does not induce any overt damage to white matter.

In contrast, at 1 day after a T12, 30-kdyn contusive SCI, many tdTomato-positive axons formed axonal spheroids and end-bulbs, resulting in significant loss of axons (Fig. 1B,E,H,I). By 7 days after SCI, fewer axonal spheroids and end-bulbs were evident in both RyR2KO and WT animals (Fig. 1C,F). Despite a trend for a reduction in acute axonal spheroid formation between RyR2KO (204.48 ± 175.49, mean ± SD, \( n = 8 \)) and RyR2 WT (425.05 ± 281.17, \( n = 6 \)) controls, there was no significant difference (t-test, \( t = -1.81, df = 12, p = 0.10 \)) in both axonal spheroid formation (Fig. 2A) and end-bulb formation (RyR2 KO; 29.16 ± 12.40, mean ± SD versus RyR2 WT; 29.48 ± 13.78, t-test, \( t = -0.046, df = 12, p = 0.96, n = 6–8 \) per group) at 1 day after SCI (Fig. 2B).

The same animals were then reimaged at 7 days post-SCI. Although, there were significantly less axonal spheroids (1 day RyR2 WT; 425.05 ± 281.18 vs. 7 days RyR2 WT; 130.57 ± 112.72, t-test, \( t = 2.38, df = 10, p = 0.02 \), \( n = 6–8 \) per group) at 1 day after SCI (Fig. 2B).
at 7 days compared to 1 day after SCI, there was no significant difference between RyR2KO and RyR2WT controls (Figs. 1C,F and 2A). Similarly, there was no significant difference in end-bulb formation between groups at 7 days after SCI (Fig. 2B).

Direct RyR2 knockout in dorsal column axons does not prevent axonal loss after spinal cord injury

We next assessed axonal survival between RyR2 KO and RyR2 WT mice post-SCI. At both 24 h (RyR2 KO; percent axonal survival: 37.0 ± 16.0, mean ± SD vs. RyR2 WT: 28.1 ± 12.3, t-test, t = 1.08, df = 12, p = 0.30, n = 6–8 per group) and 7 days (RyR2 KO; 36.4 ± 22.3 vs. RyR2 WT; 24.7 ± 14.1, t-test, t = 1.08, df = 10, p = 0.30, n = 6 per group) after SCI, there was no significant difference in axonal survival between groups (Fig. 2C). There was also no significant difference between axonal loss for both groups of mice between 24 h and 7 days after SCI, suggestive that the spared tdTomato-positive axons within these groups remain stable during this period (Fig. 2C). Collectively, these data suggest that secondary degeneration of advillin-expressing DRG primary afferents post-SCI occurs in both the presence and absence of RyR2.

**FIG. 3.** Direct RyR2 KO in primary afferents worsens open-field locomotor recovery after contusive SCI. Functional recovery was assessed after a T9, 50-kdyn IH contusion SCI for 42 days and then 14 days after a dorsal column (DC) crush injury using BMS (A) and BMS subscores (B). (A) BMS analysis of hindlimb locomotion revealed no significant group differences between RyR2KO and RyR2WT animals. However, the pattern of locomotor recovery is significantly different over time for the two groups. Data are expressed as mean ± SD. RM ANOVA (Group, F = 0.200, df = 1, 92, p = 0.20; Time, F = 5.80, df = 9, 23, p = 0.001; Group vs. Time, F = 2.6, df = 9, 23, p = 0.031; n = 7 per group). (B) BMS subscore analysis of hindlimb locomotion revealed a significant worsening of BMS subscores for RyR2KO versus RyR2WT animals from 28 to 42 days after a contusive SCI that also remained significant 14 days after DC crush. Data are expressed as mean ± SD. RM ANOVA (Group, F = 32.8, df = 1, 75, p < 0.001; Time, F = 18.9, df = 6, 23, p < 0.001; Group vs. Time, F = 1.6, df = 6, 23, p = 0.20; n = 7 per group). Group differences were determined using a Bonferroni post hoc t-test; *p < 0.05, **p < 0.01; n = 7 per group. BMS, Basso Mouse Scale; KO, knockout; RM ANOVA, repeated-measures analysis of variance; SCI, spinal cord injury; SD, standard deviation; WT, wild type.

Direct RyR2 knockout in Advillin-expressing dorsal root ganglion spinal projections worsens open-field locomotor recovery after spinal cord injury

The RyR2 isoform has been implicated in neuronal activity-induced Ca²⁺ release and synaptic plasticity and is localized to the soma, dendrites, spines, and axons. We hypothesized that loss of RyR2 function in primary afferents may alter sensory input required for normal coordinated stepping movements. To test this hypothesis, we assessed locomotor recovery of RyR2 KO and RyR2 WT mice in an open-field environment after a T12, 30-kdyn contusive SCI as used for the imaging studies. However, we were unable to detect clear functional deficits between contused and uninjured (laminectomy only) mice using standard BMS and BMS subscore assessments, possibly attributable to the mild nature of the injury (data not shown).
We then subjected mice to a standard moderate T9/10, 50-kdyn contusive SCI that reproducibly induces hindlimb paralysis that spontaneously, but only partially, recovers after SCI. 8,40,48 Although there were no significant group differences between BMS scores from baseline to 6 weeks after moderate SCI (Fig. 3A), the pattern of the locomotor recovery was different over time for the two groups (repeated-measures two-way ANOVA; group non-significant; Time, p < 0.001; Group by Time interaction, p < 0.05). However, when we tabulated BMS subscores that discriminate changes in fine details of locomotor recovery (stepping frequency, forelimb-hindlimb coordination, paw placement, and trunk stability), RyR2 KO mice had significantly worse recovery (p < 0.001) than RyR2 WT mice from 28 to 42 days (d28–42; p < 0.05) after contusive SCI (Fig. 3B). These data suggest that RyR2 expression in primary afferents may contribute to spontaneous functional recovery after SCI.

To test this hypothesis, we subjected these mice to a superficial (0.5-mm depth) dorsal column complete crush injury to determine whether RyR2 loss of function impacted residual and/or sprouting of primary afferents within the dorsal columns that may have contributed to the observed locomotor deficits between RyR2 KO and RyR2 WT mice. However, elimination of RyR2 function in these superficial dorsal column primary afferents resulted in similar BMS scores at 2 weeks after crush (i.e., 8 weeks after contusive SCI) compared to 6 weeks after contusive SCI (Fig. 3B). Interestingly, there remained a significant difference (p < 0.05, t = 2.82) in BMS subscores between RyR2KO (4.29 ± 0.76; mean ± SD) and RyR2WT (6.33 ± 1.75) groups at 2 weeks after a superficial dorsal column crush injury, which nearly mirrored the significant differences between groups at 6 weeks after contusion SCI. This suggests that the residual primary afferent fibers and other superficial fibers within the dorsal columns traversing the lesion site from either cohort were likely not involved in the observed functional differences between RyR2KO and RyR2WT animals.

**Direct RyR2 KO in Advillin-expressing dorsal root ganglion spinal projections modestly affects gait after a contusive spinal cord injury**

Concurrently with open-field observations, we performed more in-depth quantitative assessments of gait dynamics using TreadScan analysis, overground kinematics, and a horizontal ladder test. Overall, gait patterns were very similar between RyR2KO and their WT littermate controls for both swing time and stride length, with only modest differences observed at a single time point (compare TreadScan [Fig. 4A–D] and overground [Fig. 5A–D]). Interlimb coordination was also assessed using the RI and CPI. Both calculate the percentage of normal step-sequence patterns out of the total number of paw placements; however, the latter includes both dorsal and plantar foot placements. Though we did observe significant differences using TreadScan analysis between the RyR2KO and RyR2WT groups with indices of limb coordination, that is, the RI (p < 0.05) and CPI (p < 0.05), at 7 days after contusive SCI, the groups recovered similarly from 2 to 6 weeks after SCI (Fig. 4E,F). These differences in interlimb coordination were not supported using overground kinematic analysis (Fig. 5E,F). Further, we did not detect any significant differences between groups using the horizontal ladder test (42 days, RyR2KO 3.8 ± 3.3, mean ± SD footfalls vs. RyR2WT 2.6 ± 0.9, F = 1.8, df = 1.16, p = 0.20). Similarly, although we observed some significant differences between groups after a dorsal column crush injury, these differences were scattered across different measures and unsupported by other related outcomes, for example, TreadScan RI and CPI versus overground RI and CPI (compare Figs. 4E,F and 5E,F). Taken together, these patterns were consistent with observations from previous studies that showed that the lesion site from either cohort were likely not involved in the observed functional differences between RyR2KO and RyR2WT animals.

**FIG. 4.** TreadScan analysis of gait after SCI reveals that direct RyR2 KO in primary afferents modestly affects coordinated stepping. TreadScan assessment of gait parameters swing time (panel A, rear right [Rt.] paw; panel B, rear left [Lt.] paw) and stride length (panel C, rear Rt. paw; panel D, rear Lt. paw) reveals no significant group differences between RyR2KO and RyR2WT animals after contusive SCI and after a DC crush. Data are expressed as percent baseline; mean ± SD, n = 7 per group. TreadScan assessment of coordination using the regularity index (RI) (E) and coordinated pattern index (CPI) (F) shows that RyR2 KO significantly improved RI and CPI at 7 days after contusive SCI and again at 14 days after a DC crush injury. RI: RM ANOVA (F = 5.6, df = 1, 71, p = 0.020); Bonferroni’s post hoc t-test, *p < 0.050. CPI: RM ANOVA (F = 7.1, df = 1, 69, p = 0.010); Bonferroni’s post hoc t-test, *p < 0.050, n = 7 per group. DC, dorsal column; KO, knockout; RM ANOVA, repeated-measures analysis of variance; SCI, spinal cord injury; SD, standard deviation; WT, wild type.
together, these data do not support a functionally significant role for primary afferent expression of RyR2 in gait recovery after SCI.

**Direct RyR2 knockout in Advillin-expressing dorsal root ganglion neurons does not alter spinal projections and termination patterns within the L3–L5 level of the spinal cord**

To compliment the behavioral studies, we assessed tdTomato+ primary afferent axons and their terminations in intermediate and ventral gray matter at the level of the L3–L5 spinal cord in RyR2WT and RyR2KO animals. As expected, the majority of tdTomato+ afferent axons were present within the ascending dorsal columns and absent within the underlying cortical spinal tract (Fig. 6). Most of these axonal projections terminated in the superficial layers of the gray matter (i.e., lamina I and II), with a decreasing number of projections and terminations in the deeper lamina. In agreement with the gait analysis, there were no notable differences in termination patterns between groups (Fig. 6A,B).

**Discussion**

Collectively, our results do not implicate RyR2-mediated intra-axonal Ca2+ release in primary afferent fibers as an important contributor to delayed primary afferent degenerative phenomena after contusive SCI. In support, axonal spheroid and end-bulb formation and axonal survival were similar between both groups from 1 to 7 days after SCI. Despite a significant worsening of BMS subscores in RyR2 KO mice versus RyR2 WT controls, both TreadScan and overground kinematic analyses failed to detect a pattern of meaningful, robust, and lasting gait differences between groups. These data suggest that RyR2 expression in primary afferents does not play a major role in spontaneous recovery of gait after SCI.

There are several possibilities underlying why, in the current study, we failed to show an axoprotective effect by knocking out RyR2 directly in axons *in vivo*. Among the possibilities are: 1) Other RyR isoforms (i.e., RyR1 and/or -3) may play a more important role than RyR2 in axonal injury; 2) compensation through other RyR isoforms and/or IP3R may have masked the effect of RyR2 KO; 3) other routes of Ca2+ release and/or entry may play a larger role; and 4) indirect effects of RyR2 inhibition.

Given that previous studies have shown that targeting all RyR isoforms with ryanodine and other RyR antagonists are protective across a broad spectrum of white matter injury models (see Introduction), we cannot rule out the possibility that other RyR isoforms contributed to axonal loss. In addition, given that targeting other inside-out sources of Ca2+ (e.g., IP3R9,14,49 as well as mitochondria release of Ca2+49) protects white matter, these additional sources could have played a role. As detailed in the Introduction, several sources of outside-in Ca2+ are well known to contribute to white matter injury and that, together with inside-out sources, may lead to toxic levels of intra-axonal Ca2+ accumulation. Given the complexity and overlap of Ca2+ sources that are involved in white matter injury, further studies using combinatorial targeting strategies will likely be necessary to rescue white matter from secondary degeneration.

To the best of our knowledge, this is the first study to directly knock out an RyR in neurons and their spinal projections. We therefore cannot exclude the possibility that indirect effects of RyR inhibition, rather than direct effects on axons *per se*, may underlie the

**FIG. 5.** Overground kinematic analysis of gait after SCI reveals that direct RyR2 KO in primary afferents modestly alters swing time and stride length. Overground kinematic assessment of swing time reveals that there was no significant difference between groups up to 42 days after a contusive SCI. There was a significant difference between groups for swing time for the rear Rt. paw (panel A, RM ANOVA, F = 8.0, df = 1, 76, p = 0.010; Bonferroni’s post hoc t-test, *p < 0.050) and rear Lt. paw (panel B, F = 4.8, df = 1, 80, p = 0.032: Bonferroni’s post hoc t-test, *p < 0.050) at 14 days after DC crush injury. Data are expressed as percent baseline; mean ± SD, n = 7 per group. (C) Rear Rt. stride length, N.S. (D) There was a significant difference for stride length between groups for the rear Lt. paw at 28 days post-contusive SCI and 14 days post-DC crush (both p < 0.050, Bonferroni’s post hoc t-test). Data are expressed as percent baseline; mean ± SD, n = 7. There was no significant difference for both RI and CPI between RyR2KO and RyR2WT animals; mean ± SD, n = 7 per group. CPI, coordinated pattern index; DC, dorsal column; KO, knockout; N.S., non-significant; RI, regularity index; RM ANOVA, repeated-measures analysis of variance; SCI, spinal cord injury; SD, standard deviation; WT, wild type.
OVERGROUND

A  Swing time (% BL)

Rear Rt.

DC crush

0 7 14 21 28 35 42 49 56
Days post-SCI

B  Swing time (% BL)

Rear Lt.

DC crush

0 7 14 21 28 35 42 49 56
Days post-SCI

C  Stride length (% BL)

Rear Rt.

DC crush

0 7 14 21 28 35 42 49 56
Days post-SCI

D  Stride length (% BL)

Rear Lt.

DC crush

0 7 14 21 28 35 42 49 56
Days post-SCI

E  RI

DC crush

BL 7 14 21 28 35 42 49 56
Days post-SCI

F  CPI

DC crush

BL 7 14 21 28 35 42 49 56
Days post-SCI
protection of RyR inhibition reported in previous studies. In support of this, most of the previous studies used 

**FIG. 6.** Direct RyR2 KO in DRG primary afferents does not notably alter their projection and termination patterns within the L3–L5 spinal cord gray matter. (A, B) Representative 10× confocal images of transverse spinal cord sections from the L3–L5 level of the spinal cord, revealing the projection and termination patterns of primary afferent fibers (tdTomato+; magenta) from RyR2WT (Ai,Ai,Aii) and RyR2KO (Bi,Bi,Bii) animals post-SCI. As expected and based on their known role in sensation, the majority of DRG primary afferents are clearly visible within the dorsal columns (DC) and their terminations within the dorsal horn (DH) with a tapering of terminations within the intermediate gray region and ventral horns. Higher magnification images of the intermediate gray region (Ai,Bi) and ventral horn (Aii,Bii) are indicated in hashed boxes in panels A and B and were captured using confocal microscopy with a water-dipped 20× objective. Termination patterns between groups were very similar at each location assessed (n=7 per group). Primary afferent fibers (tdTomato; magenta; autofluorescence; green to indicate cell bodies). Scale bar: 100 μm. CC, central canal; DRG, dorsal root ganglion; KO, knockout; SCI, spinal cord injury; WT, wild type.
functional changes, overall, it is difficult to ascribe these changes to the initial rationale for the study. One potential explanation is that RyR2 KO involved an unrecognized sensory phenotype that, in turn, exercised a subtle impact on stepping that would require a much deeper investigation to uncover. This concept is supported by previous work implicating RyR2 in pain syndromes; however, because we did not assess pain and nociception in the current study, this remains speculation.

Another potential limitation of our study was the use of a non-inducible transgenic mouse line that may unavoidably have acquired developmental and/or compensatory mechanisms of neuron-specific RyR2 KO beyond our control. Even though the Advil-Cre driver mouse line has been previously assessed and no developmental or behavioral deficits were evident, we detected minor, but significant, differences in gait at baseline between groups of mice. Although we normalized post-SCI outcomes to baseline, it is possible that the observed differences in gait detected post-SCI may reflect inherent underlying differences at baseline between RyR2WT and RyR2KO mice. Further work using an inducible-Advil-Cre may help avoid potential confounding developmental/compensatory effects. Last, we did not evaluate possible differences in synaptic function between WT and RyR2 KO animals that may have contributed to the subtle differences in gait. Collectively, this leads us to conclude that there were neither neuroprotective effects on axons nor real functionally significant outcomes attributable to direct RyR2KO in DRG primary afferents.

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Authors’ Contributions
B.C.O.: investigation, formal analysis, revision. J.R.M.: investigation, revision. D.A.B.: investigation, formal analysis, revision; S.A.: investigation, revision; D.S.K.M.: interpretation of data for the study, and revision of the article; D.P.S.: conception and design of the study, analysis, and interpretation of data for the study, drafting and revision of the article, funding acquisition.

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Abbreviations Used

| Abbreviation | Description |
|--------------|-------------|
| 2PE | two-photon excitation |
| ANOVA | analysis of variance |
| BMS | Basso Mouse Scale |
| CNS | central nervous system |
| CP | coordinated pattern index |
| DRG | dorsal root ganglion |
| KO | knockout |
| PBS | phosphate-buffered saline |
| PFA | paraformaldehyde |
| RI | regularity index |
| RyR | ryanodine receptors |
| SCI | spinal cord injury |
| SD | standard deviation |
| WT | wild type |